William B. Coleman
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Editors

The Molecular Basis of Human Cancer

Second Edition

Springer

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ISBN 978-1-934115-18-3 ISBN 978-1-59745-458-2 (eBook)
DOI 10.1007/978-1-59745-458-2

Library of Congress Control Number: 2016953141

Printed on acid-free paper

This Humana Press imprint is published by Springer Nature
The registered company is Springer Science+Business Media LLC
The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

The information contained in this textbook describes the ever-expanding field of cancer biology and our contemporary understanding of the pathology, pathogenesis, and pathophysiology of the diverse collection of diseases representing human cancer. Hence, this work represents the culmination of innumerable small successes that emerged from the ceaseless pursuit of new knowledge by countless clinical, experimental, and translational cancer biologists working around the world on all aspects of human cancer. Their ingenuity and hard work have dramatically advanced the field of cancer pathobiology over time, and particularly during the last 25 years. This book is a tribute to the dedication, diligence, and perseverance of individual scientists who contributed to the advancement of our understanding of the molecular basis of human cancer, especially graduate students, laboratory technicians, and postdoctoral fellows, whose efforts are so frequently taken for granted, whose accomplishments are so often unrecognized, and whose contributions are so quickly forgotten.

We especially dedicate this book to the loving memory of Chris Helms Austin who passed away far too young on August 17, 2014 after a long battle with breast cancer. Chris was cherished by her family and friends, and was a gifted teacher who made significant impressions on her students, their parents, and her colleagues. She did so much good in her short lifetime and that goodness continues through the lives of her students and everyone else she touched. Chris' journey through breast cancer showed us the realities associated with a cancer diagnosis, treatment, and disease progression. Her obstinate optimism and resolve in the face of an unrelenting disease provides a lesson and example for all of us on how to attack life's challenges no matter how insurmountable the odds of success.

We also remember people we have known and loved, that taught us through example about dignity, positivity, strength, courage, and tenacity in the fight against cancer – Samuel Apostola, Dr. Bobby G. Bell, Jeffery A. Bell, Dr. Sharon Ricketts Betz, Linwood Braswell, Bobbie Coleman Clark, William B. Coleman, Jr., Anne Griffin Clawson, Jewell T. Coleman, George G. Gerding, Evelyn B. Hadden, Shayne Snyder Hall, Dr. Eugene F. Hamer, Jerry S. Harris, Effie H. Helms, Larry Hendley, Joel C. Herren, Jean G. Herren, Kathleen M. Jackson, Jerry W. Kirkman, Gloria Morin, John Panu, Dr. Kathleen Rao,

Dr. Rhonda Simper Ronan, Alexandria Rucho, Peter Rucho, Beverly Clark Tice, Ruth E. Trull, Josephine Caccavallo Vasquez, and W. Kenneth Weatherman. This book is also dedicated to the cancer survivors and those who continue to live with cancer, for their bravery and determination, for the inspiration that they provide, and for reminding us that there is far too much left to be done to rest on our accomplishments.

We also dedicate The Molecular Basis of Human Cancer – Second Edition to the many people that have played crucial roles in our successes. We thank our many scientific colleagues, past and present, for their camaraderie, collegiality, and support. We especially thank our scientific mentors for their example of dedication to research excellence. We are truly thankful for the positive working relationships and friendships that we have with our faculty colleagues, for the mentoring we received from our elders and for the opportunity to mentor those that follow us. We also thank our undergraduate students, graduate students, and postdoctoral fellows for teaching us more than we might have taught them. We thank our parents for believing in higher education, for encouragement through the years, and for helping make dreams into reality. We thank our brothers and sisters, and extended families, for the many years of love, friendship, and tolerance. We thank our wives, Monty and Nancy, for their unqualified love, unselfish support of our endeavors, understanding of our work ethic, and appreciation for what we do. Lastly, we give special thanks to our children, Tess, Sophie, Pete, and Zoe. Their achievements and successes as young adults are a greater source of pride for us than our own accomplishments. As when they were children, we thank them for providing an unwavering bright spot in our lives, for their unbridled enthusiasm and boundless energy, and for giving us a million reasons to take an occasional day off from work just to have fun.

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Preface

The practice of medical oncology has been in a period of sustained significant positive change over the last two decades that is primarily due to advances in the basic science of cancer biology. In recent years, developments in molecular biology techniques have substantially increased our ability to detect and characterize genetic defects in human cells, resulting in significant increases in our understanding of the normal molecular mechanisms controlling cellular proliferation and differentiation. The advancement of our comprehension of these basic molecular mechanisms has been paralleled by comparable increases in our understanding of the molecular basis of the processes involved in neoplastic transformation and tumorigenesis. Information gleaned from studies conducted in basic molecular research laboratories is being applied with unprecedented speed to the development of new molecular tests for cancer detection, diagnosis, and prediction of clinical outcomes, as well as to the development of new strategies for cancer prevention and treatment through therapies that target specific molecular pathways in the cancer cell. Basic scientists, clinical scientists, and physicians have a need for a source of information on the current state-of-the-art of the molecular biology of human neoplastic diseases. In this Second Edition of *The Molecular Basis of Human Cancer* we attempt to provide such a source of current information, as well as providing a look to the future of the discipline and the potential impact of scientific advances on the practice of medical oncology. This book is directed primarily to advanced graduate students and medical students, postdoctoral trainees, and established investigators having basic research interests in the molecular basis of human neoplastic disease. However, this book is also well suited for the non-expert with similar interests since it provides a broad overview of general themes in the molecular biology of cancer. To be sure, our understanding of the many processes of neoplasia and their molecular basis is far from complete, but numerous areas of thematic or conceptual consensus have developed. We have made an effort to integrate accepted principles with broader theoretic concepts in an attempt to present a current and comprehensive view of the molecular basis of human cancer. We hope that this book will accomplish its purpose of providing students and researchers who already possess strong but diverse basic science backgrounds with unifying concepts, so as to stimulate new research aimed at furthering our understanding of the array of diseases that represent human cancer.

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Cancer Epidemiology: Incidence and Etiology of Human Neoplasms

1

William B. Coleman and Gregory J. Tsongalis

1.1 Introduction

Cancer does not represent a single disease. Rather, cancer is a myriad collection of diseases with as many different manifestations as there are tissues and cell types in the human body, involving innumerable endogenous or exogenous carcinogenic agents, and various etiological mechanisms. What all of these disease states share in common are certain biological properties of the cells that compose the cancer, including unregulated (clonal) cellular growth, impaired cellular differentiation, invasiveness, and metastatic potential. It is now recognized that cancer, in its simplest form, is a genetic disease, or more precisely, a disease of abnormal gene expression. Recent research efforts have revealed that different forms of cancer share common molecular mechanisms governing uncontrolled cellular proliferation, involving loss, mutation, or dysregulation of genes that positively and negatively regulate cell proliferation, migration, and differentiation (generally classified as proto-oncogenes and tumor suppressor genes). Essential to any discussion of the molecular mechanisms that govern disease pathogenesis for specific cancers is an appreciation for the distribution of these diseases among world populations, with consideration of specific risk factors and etiologic agents involved in disease causation. This introduction will describe cancer incidence and mortality for the major forms of human cancer, and will briefly review some of the known risk factors and/or causes of these cancers for specific at-risk populations.

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1.2 Cancer Incidence and Mortality

Cancer is an important public health concern in the USA and worldwide. Due to the lack of nationwide cancer registries for all countries, the exact numbers of the various forms of cancer occurring in the world populations are unknown. Nevertheless, estimations of cancer incidence and mortality are generated on an annual basis by several domestic and world organizations. Estimations of cancer incidence and mortality for the USA are provided annually by the American Cancer Society (ACS—www.cancer.org) and the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program (http://seer.cancer.gov/data/). Global cancer statistics are provided by the International Agency for Research on Cancer (IARC—http://globocan.iarc.fr/), the World Health Organization (WHO—http://www.who.int/en/), and Cancer Research UK (http://info.cancerresearchuk.org/cancerstats/world/). Monitoring of long-range trends in cancer incidence and mortality among different populations is important for investigations of cancer etiology. Given the long latency for formation of a clinically detectable neoplasm (up to 20–30 years) following initiation of the carcinogenic process (exposure to carcinogenic agent), current trends in cancer incidence probably reflect exposures that occurred many years (and possibly decades) before. Thus, correlative analysis of current trends in cancer incidence with recent trends in occupational, habitual, and environmental exposures to known or suspect carcinogens can provide clues to cancer etiology. Other factors that influence cancer incidence include the size and average age of the affected population. The average age at the time of cancer diagnosis for all tumor sites is approximately 65 years [1, 2]. As a higher percentage of the population reaches age 60, the general incidence of cancer will increase proportionally. Thus, as the life expectancy of the human population increases due to reductions in other causes of premature death (due to infectious and cardiovascular diseases), the average risk of developing cancer will increase.

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_1

1.3 Cancer Incidence and Mortality in the USA

1.3.1 General Trends in Cancer Incidence

The American Cancer Society estimates that 1,658,370 new cases of invasive cancer were diagnosed in the USA in 2015 [3]. This number of new cancer cases reflects 848,200 male cancer cases (51 %) and 810,170 female cancer cases (49 %). The estimate of total new cases of invasive cancer does not include carcinoma in situ occurring at any site other than in the urinary bladder, and does not include basal and squamous cell carcinomas of the skin. In fact, basal and squamous cell carcinomas of the skin represent the most frequently occurring neoplasms in the USA, with an estimated occurrence of >1 million total cases in 2015 [3]. Likewise, carcinoma in situ represents a significant number of new cancer cases in 2015 with 60,290 newly diagnosed breast carcinomas in situ and 63,440 new cases of melanoma carcinoma in situ [3].

Estimated site-specific cancer incidence for both sexes combined is shown in Fig. 1.1. Cancers of the reproductive organs represent the largest group of newly diagnosed cancers in 2015 with 329,330 new cases [3]. This group of cancers includes prostate (220,800 new cases), uterine corpus (54,870 new cases), ovary (21,290 new cases), and uterine cervix (12,900 new cases), in addition to other organs of the genital system (vulva, vagina, and other female genital organs; testis, penis, and other male genital organs). The next most frequently occurring cancers originated in the digestive tract (291,150 new cases), respiratory system (240,390 new cases), and breast (234,190 new cases). The majority of digestive system cancers involved colon (93,090 new cases), rectum (39,610 new cases), pancreas (48,960 new cases), stomach (24,590 new cases), liver and intrahepatic bile duct (35,660 new cases), and esophagus (16,980 new cases), in addition to the other digestive system organs (small intestine, gallbladder, and others). Most new cases of cancer involving the respiratory system affected the lung and bronchus (221,200 new cases), with the remaining cases affecting the larynx or other components of the respiratory system. Other sites with significant cancer burden include the urinary system (138,710 new cases), lymphomas (80,900 new cases), melanoma of the skin (80,100 new cases), leukemias (54,270 new cases), and the oral cavity and pharynx (45,780 new cases).

Estimated cancer incidence by cancer site for males and females are shown in Fig. 1.2. Among men, cancers of the prostate, respiratory system (lung and bronchus), and digestive system (colon and rectum) occur most frequently. Together, these cancers account for 61 % of all cancers diagnosed in men. Prostate is the leading site, accounting for 220,800 new cases and 26 % of cancers diagnosed in men (Fig. 1.3). Among women, cancers of the breast, respiratory system (lung and bronchus), and digestive system (colon and rectum) occur most frequently. Cancers at these sites

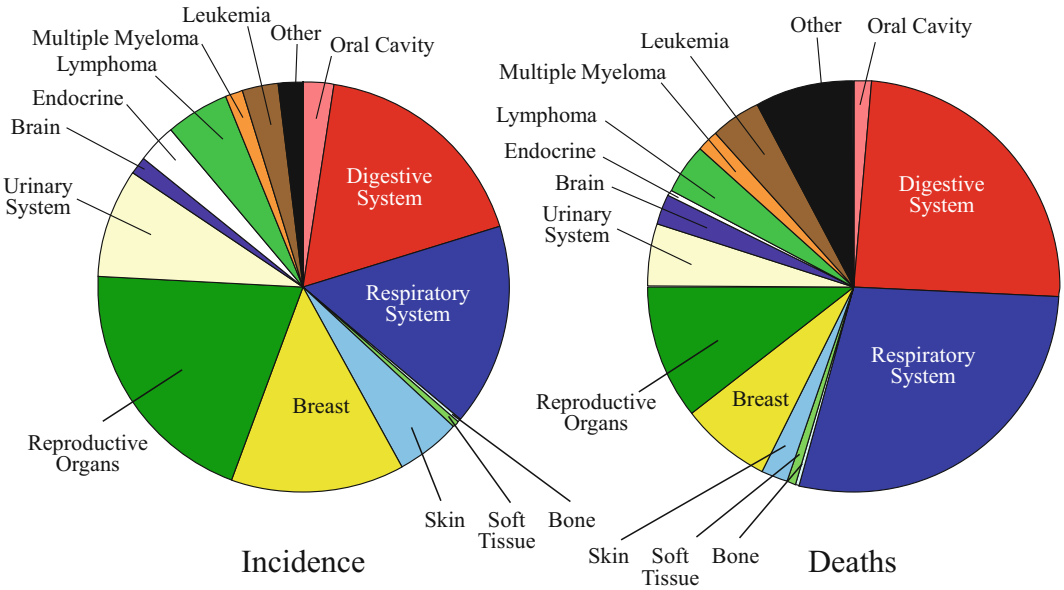

Fig. 1.1 Cancer incidence and mortality by site for both sexes (USA, 2015). The relative contributions of the major forms of cancer to overall cancer incidence and cancer-related mortality (both sexes combined) were calculated from data provided by Siegel et al. [3]. Cancers of the reproductive organs include those affecting the prostate, uterine corpus, ovary, uterine cervix, vulva, vagina, testis, penis, and other organs of the male and female genital systems. Cancers of the digestive system include those affecting esophagus, stomach, small intestine, colon, rectum, anus, liver, gallbladder, pancreas, and other digestive organs. Cancers of the respiratory system include those affecting lung, bronchus, larynx, and other respiratory organs.

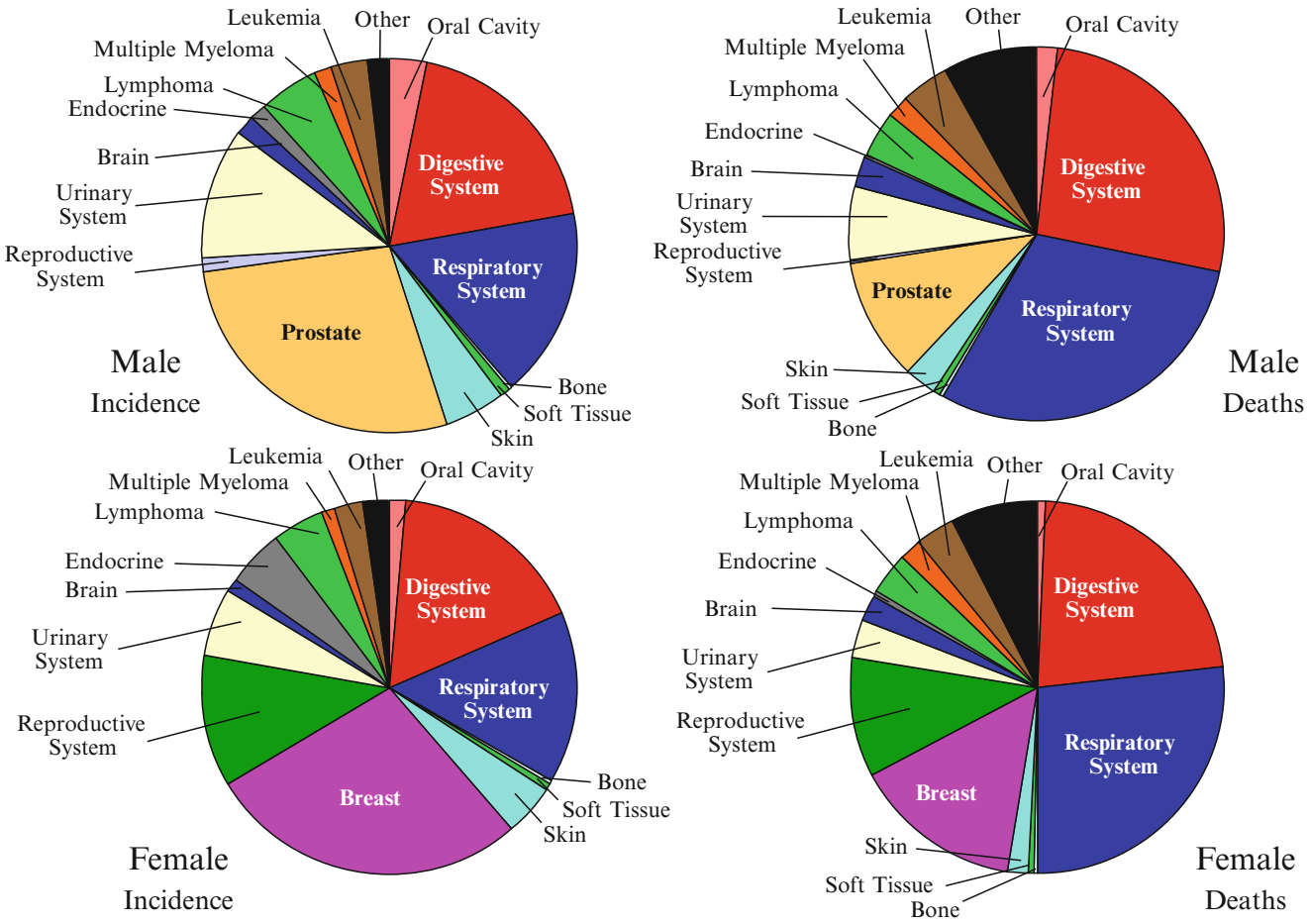

Fig. 1.2 Cancer incidence and mortality by site (USA, 2015). The relative contributions of the major forms of cancer to overall cancer incidence and cancer-related mortality for males and females were calculated from data provided by Siegel et al. [3]. Cancers of the male reproductive organs include testis, penis, and other organs of the male genital system. Cancers of the female reproductive organs include those affecting the uterine corpus, ovary, uterine cervix, vulva, vagina, and other organs of the female genital systems. Cancers of the digestive system include those affecting esophagus, stomach, small intestine, colon, rectum, anus, liver, gallbladder, pancreas, and other digestive organs. Cancers of the respiratory system include those affecting lung, bronchus, larynx, and other respiratory organs.

combine to account for 58 % of all cancers diagnosed in women. Breast is the leading site for cancers affecting women, accounting for 231,840 new cases and 29 % of all cancers diagnosed in women (Fig. 1.3).

1.3.2 General Trends in Cancer Mortality in the USA

Mortality attributable to invasive cancers produced 589,430 cancer deaths in 2015. This reflects 312,150 male cancer deaths (53 % of total) and 277,280 female cancer deaths (47 % of total). Estimated numbers of cancer deaths by site for both sexes are shown in Fig. 1.1. The leading cause of cancer death involves tumors of the respiratory system (162,460 deaths), the majority of which are neoplasms of the lung and bronchus (158,040 deaths). The second leading cause of cancer deaths involve tumors of the digestive system (149,300 deaths), most of which are tumors of the colorectum (49,700 deaths), pancreas (40,560 deaths), stomach (10,720 deaths), liver and intrahepatic bile duct (24,550 deaths), and esophagus (15,590 deaths). Together, cancers of the respiratory and digestive systems account for 53 % of cancer-associated death.

Trends in cancer mortality among men and women mirror in large part cancer incidence (Fig. 1.2). Cancers of the prostate, lung and bronchus, and colorectum represent the three leading sites for cancer incidence and cancer mortality among men (Fig. 1.3). In a similar fashion, cancers of the breast, lung and bronchus, and colorectum represent the leading sites for cancer incidence and mortality among women (Fig. 1.3). While cancers of the prostate and breast represent the leading sites for new cancer diagnoses among men and women (respectively), the majority of cancer deaths in both sexes are related to cancers of the lung and bronchus (Fig. 1.3). Cancers of the lung and bronchus are responsible

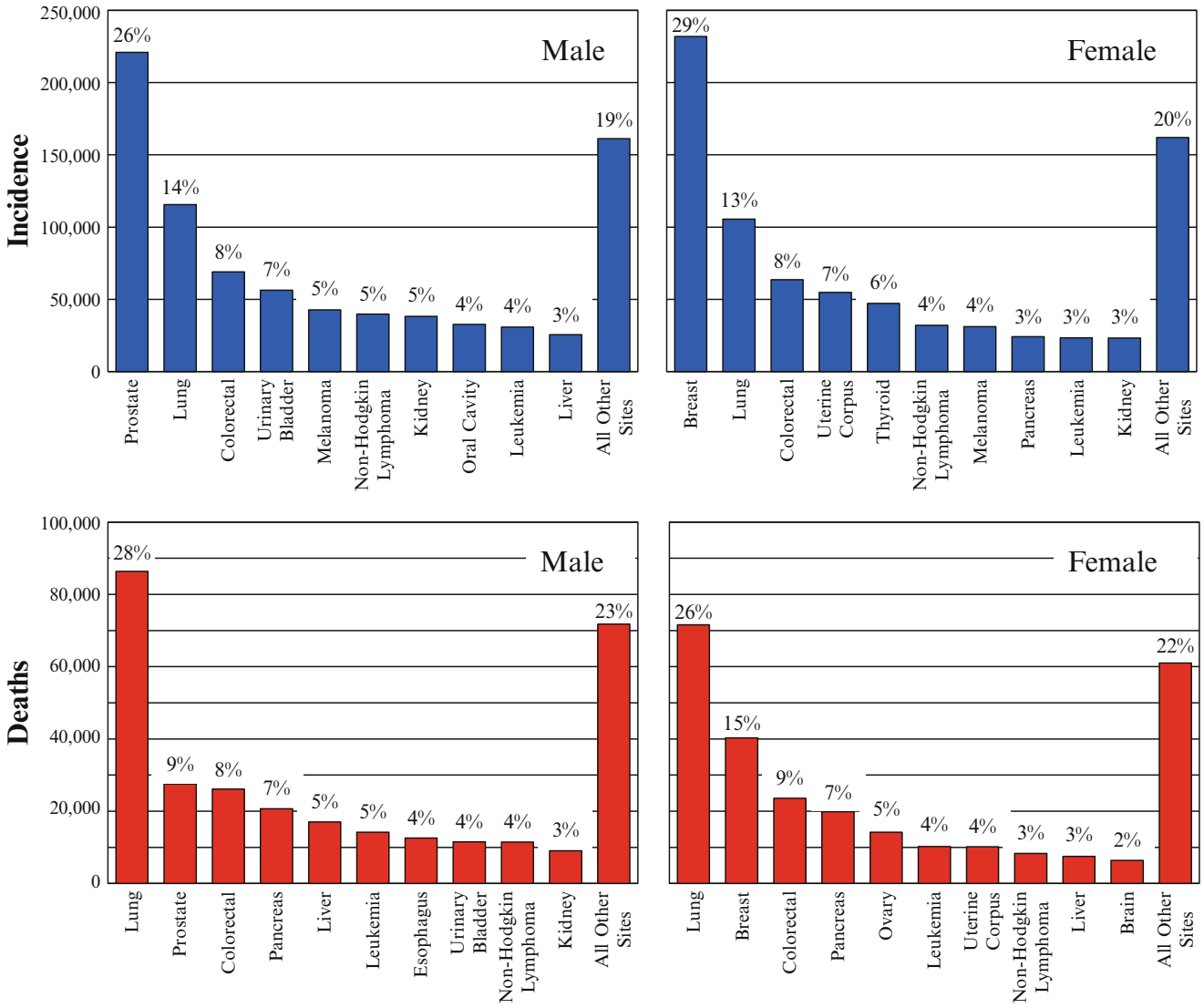

Fig. 1.3 Cancer incidence and mortality by leading site (USA, 2015). The numbers of cancers (and percentage of total cancers) and numbers of cancer-related deaths (and percentages of cancer-related deaths) for the leading sites for males and females were calculated from data provided by Seigel et al. [3]. The numbers provided for lung include tumors of the lung and bronchus, and numbers for colorectal cancer include tumors of the colon and rectum.

for 28 % of all cancer deaths among men and 26 % of all cancer deaths among women (Fig. 1.3). The age-adjusted death rate for lung cancer among men increased dramatically during the six decades between 1930 and 1990, while the death rates for other cancers (like prostate and colorectal) remained relatively stable (Fig. 1.4). However, since 1990, the age-adjusted death rate for lung cancer among men has decreased, although it remains very high compared to all other cancers. The lung cancer death rate for women increased in an equally dramatic fashion since about 1960, becoming the leading cause of female cancer death in the mid-1980s after surpassing the death rate for breast cancer (Fig. 1.4).

1.4 Global Cancer Incidence and Mortality

1.4.1 Current Trends in Cancer Incidence and Mortality Worldwide

The IARC estimates that 12,667,500 new cancer cases were diagnosed worldwide in 2008 [4]. This number of new cases represents 6,629,100 male cancer cases (52 %) and 6,038,400 female cancer cases (48 %). Mortality attributed to cancer for the same year produced 7,571,500 deaths worldwide [4]. This reflects 4,225,700 male cancer deaths (56 %) and

3,345,800 female cancer deaths (44 %). The leading sites for cancer incidence worldwide in 2008 included cancers of the lung (1,609,000 new cases), breast (1,383,500 new cases), colorectum (1,233,700 new cases), stomach (989,600 new cases), and prostate (903,500 new cases; Fig. 1.5). The leading sites for cancer mortality worldwide in 2008 included cancers of the lung (1,378,400 deaths), stomach (738,000 deaths), liver (695,900 deaths), colorectum (608,700 deaths), and breast (458,400 deaths; Fig. 1.5). As can be seen, lung cancer accounted for the most new cancer cases and the most cancer deaths among men and women combined during this period of time (Fig. 1.5). The leading sites for cancer incidence among males worldwide included cancers of the lung (1,095,200 new cases), prostate (903,500 new cases), colorectum (663,600 new cases), stomach (640,600 new cases), and liver (522,400 new cases). Combined, cancers at these five sites account for nearly 48 % of all cancer cases among men [4]. The leading causes of cancer death among men included tumors of the lung (951,000 deaths), liver (478,300 deaths), stomach (464,400 deaths), colorectum (320,600 deaths), and esophagus (276,100 deaths). Deaths from these cancers account for 59 % of all male cancer deaths [4]. The leading sites for cancer incidence among females included breast (1,383,500 new cases), colorectum (570,100 new cases), cervix uteri (529,800 new cases), lung (513,600 new cases), and stomach (349,000 new cases). The leading causes of cancer death among females directly mirrors the leading causes of cancer incidence: breast (458,400 deaths), lung (427,400 deaths), colorectum (288,100 deaths), cervix uteri (275,100 deaths), and stomach (230,000 deaths). Combined, these five cancer sites accounted for approximately 55 % of all female cancer cases and 51 % of female cancer deaths [4].

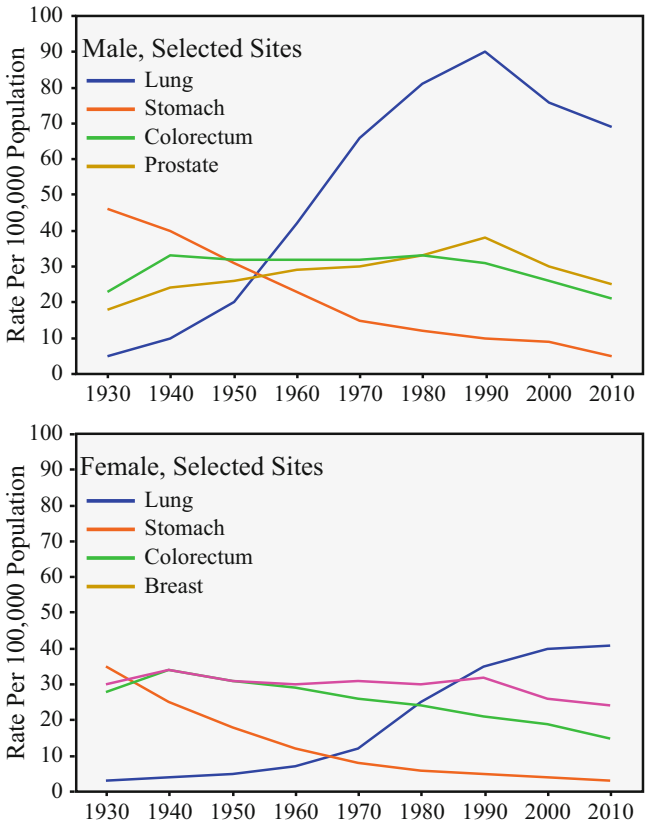

Fig. 1.4 Age-adjusted cancer mortality by site. The age-adjusted death rates for males and females for selected sites were adapted from the data provided by Seigel et al. [3]. Death rates are per 100,000 population and are age-adjusted to the 2000 standard population of the USA.

1.4.2 Geographic Differences in Cancer Incidence and Mortality

Cancer incidence and mortality differs between developed and developing countries [4]. In 2008, developed countries accounted for 43.9 % of new cancers (5,560,000 cases) and 36.3 % of cancer deaths (2,751,400 deaths), whereas developing countries accounted for 56.1 % of new cancers (7,107,600 cases) and 63.7 % of cancer deaths (4,820,100 deaths). The leading sites for cancer occurrence among men from developed countries include prostate (648,400 new cases), lung (482,600 new cases), and colorectum

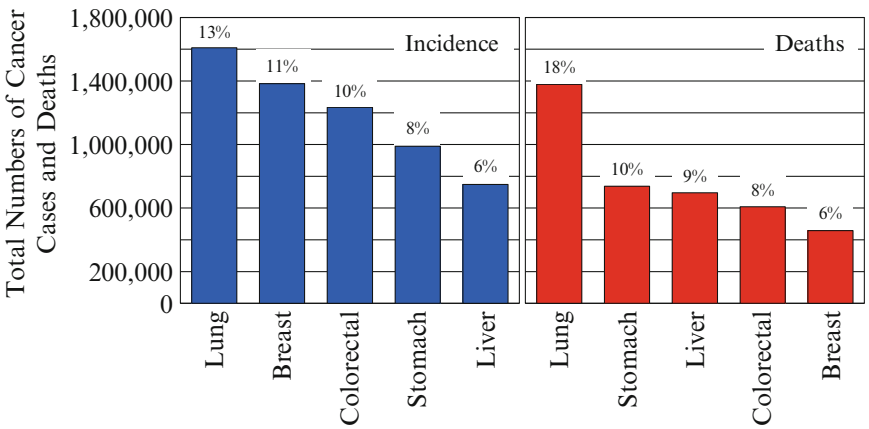

Fig. 1.5 Worldwide cancer incidence and mortality by leading site. The numbers of cancers (and percentage of total cancers) and numbers of cancer-related deaths (and percentages of cancer-related deaths) for the leading sites worldwide were calculated from data provided by Jemal et al. [4]. The numbers provided for lung include tumors of the lung and bronchus and the numbers for colorectal cancer include tumors of the colon and rectum.

(389,700 new cases). In contrast, the leading sites for cancer occurrence among men from developing countries include lung (612,500 new cases), stomach (466,900 new cases), and liver (440,700 new cases). The incidence of prostate cancer and liver cancer provide excellent examples of differences in incidence between men from developed and developing countries. In 2008, prostate cancer affected 648,400 men in developed countries (ranked first for cancer incidence in this cohort) compared to 255,000 men in developing countries (ranked sixth for cancer incidence in this cohort). Likewise, in 2008, liver cancer affected 440,700 men in developing countries (ranked third for cancer incidence in this cohort) compared to 81,700 men in developed countries (ranked tenth for cancer incidence in this cohort). While incidence rates vary, lung cancer represents the most frequent cause of cancer death among men from both developed and developing countries. Among women, breast cancer is the most frequent site of cancer in both developed (692,200 new cases) and developing countries (691,300 new cases) in 2008. Likewise, lung cancer occurs frequently in both groups—241,700 new cases in developed countries (ranked third for cancer incidence) and 272,000 new cases in developing countries (ranked third for cancer incidence). However, significant differences in cancer incidence among women from developed and developing countries can be seen for cancer of the cervix. Women from developing countries develop cervical cancer frequently (453,300 new cases, ranked second for cancer incidence), while women in developed countries develop cervical cancer less often (76,500 new cases, ranked tenth for cancer incidence).

New cancer incidence and cancer-related mortality can differ tremendously from world area to world area, country to country, and even from region to region within a single country. The leading world areas for new cancer cases includes Eastern Asia/China (17.3 % of new cases), North America (14.9 % of new cases), South Central Asia (11.6 % of new cases), Eastern Europe (10.4 % of new cases), and Western Europe (8.3 % of new cases). Collectively, Asia accounted for approximately 40 % of all new cancer cases worldwide in 1990 [5]. Recognizing the significant contribution of the population density of China and other regions of Asia to these numbers of cancers, it is appropriate to consider the cancer burden of these countries after correction for population. The numbers of cases/deaths and the incidence/mortality rates for these world regions in 1999 are given in Fig. 1.6. It is evident from the data contained in Fig. 1.6 that there is a marked disparity between total numbers of cancer cases/deaths and the incidence/mortality rate for specific world regions [5]. In 2012, men from Australia/New Zealand exhibit the highest cancer incidence rate worldwide (365 cases per 100,000 population), followed closely by men from North America (344 cases per 100,000 population) and

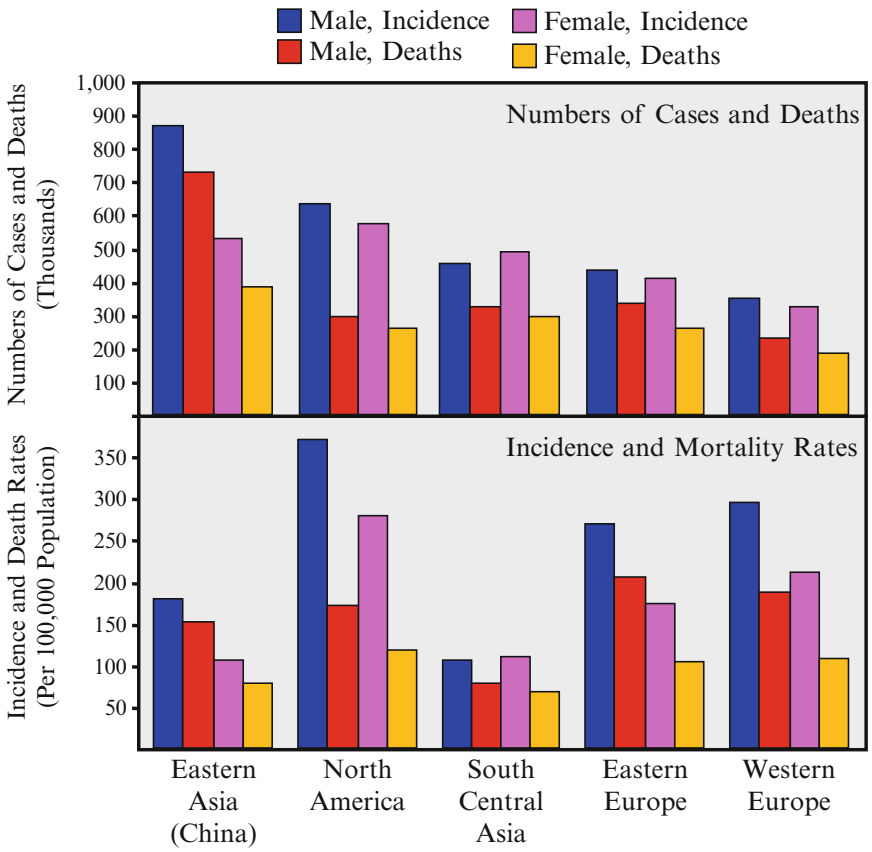

Fig. 1.6 Worldwide cancer incidence and mortality for both sexes by world region. The total numbers of cancers and cancer-related deaths, and the total incidence of cancer and cancer-related mortality rates for selected world regions were calculated from data provided by Parkin et al. [5].

Western Europe (344 cases per 100,000 population), while the lowest cancer incidence rate is found among men from Western Africa (79 cases per 100,000 population) [6]. While demonstrating the highest cancer incidence rate worldwide, the male populations of Australia/New Zealand, North America, and Western Europe rank 13th, 9th, and 5th (respectively) for cancer mortality rate, possibly reflecting the relative quality and availability of healthcare and treatment options among the various world regions [6]. The highest cancer mortality rate for men is found in Central/Eastern Europe (173 deaths per 100,000 population), followed by Eastern Asia (159 deaths per 100,000 population), Southern Europe (138 deaths per 100,000 population), Southern Africa (137 deaths per 100,000 population), and Western Europe (131 deaths per 100,000 population), while the lowest mortality rate is found among men from West Africa (69 deaths per 100,000 population) [6]. The North American female population shows the highest cancer incidence rate worldwide (295 cases per 100,000 population), followed by Australia/New Zealand (278 cases per 100,000 population), Northern Europe (264 cases per 100,000 population), and Western Europe (264 cases per 100,000 population), while the lowest incidence rate is found among women from South/Central Asia (103 cases per 100,000 population) [6]. The highest mortality rate for females worldwide is found in Melanesia (119 deaths per 100,000 population), followed by Eastern Africa (111 deaths per 100,000 population), Southern Africa (99 deaths per 100,000 population), Northern Europe (94 deaths per 100,000 population), and Polynesia (93 deaths per 100,000 population), while the lowest mortality rate is found among women from Micronesia (56 deaths per 100,000 population) [6].

1.5 Population Factors Contributing to Cancer Incidence and Mortality

1.5.1 Age-Dependence of Cancer Incidence and Mortality

Cancer is predominantly a disease of old age. Most malignant neoplasms are diagnosed in patients over the age of 65, making age the most important risk factor for development of many types of cancer [7, 8]. The age-specific incidence and death rates for cancers of the prostate, breast (female), lung (both sexes combined), and colorectum (both sexes combined) for the period of 1992–2012 [2] are shown in Fig. 1.7. The trends depicted in this figure clearly show that

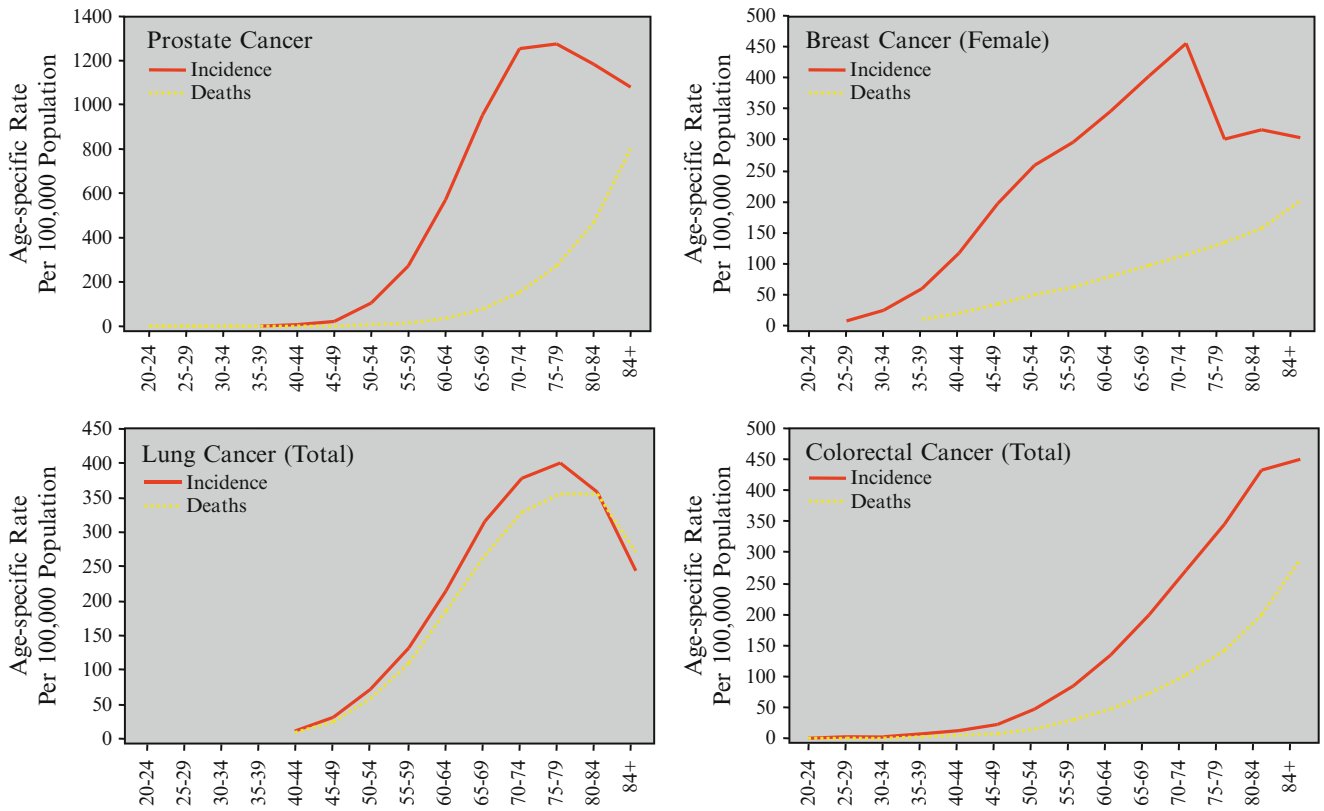

Fig. 1.7 Age-specific incidence and mortality rates for selected sites, 1992–2012. The age-specific rates for breast cancer incidence and mortality are for females only. The age-specific rates for lung cancer and colorectal cancer are combined for both sexes. These data were adapted from Howlander et al. [2]. Rates are per 100,000 population and are age-adjusted to the 2000 standard population of the USA.

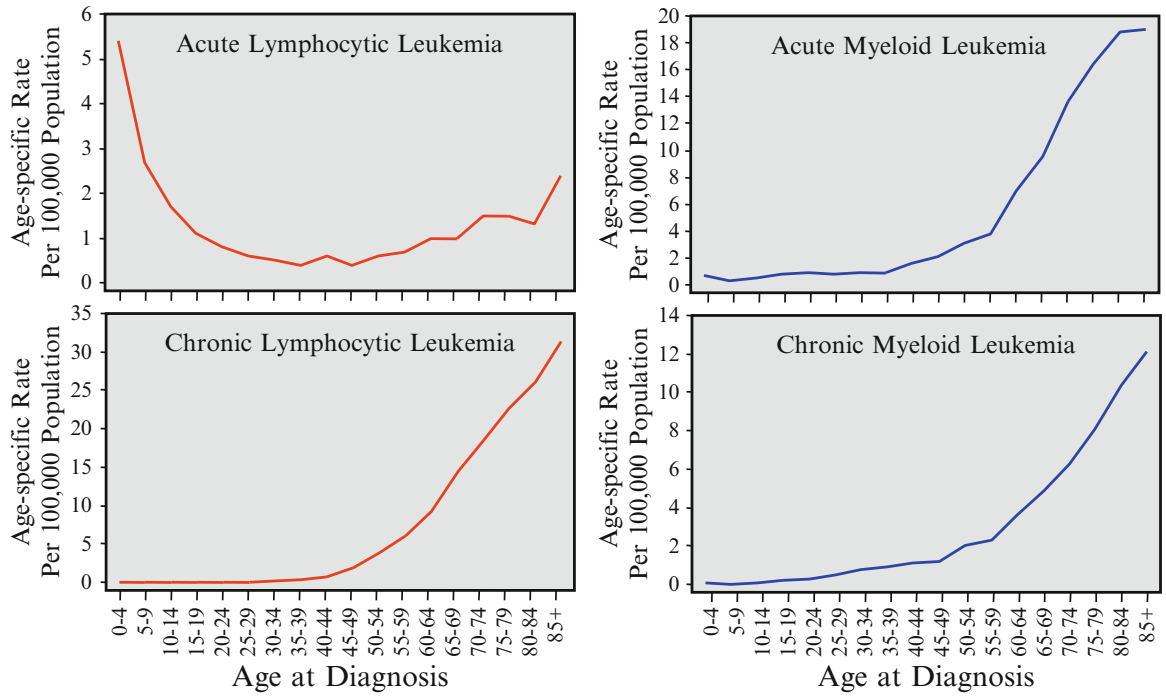

Fig. 1.8 Age-specific incidence rates for acute and chronic leukemias, 1992–2012. The age-specific rates for incidence and mortality for the major forms of leukemia are combined for both sexes. These data were adapted from Howlander et al. [2]. Rates are per 100,000 population and are age-adjusted to the 2000 standard population of the USA.

the majority of each of these cancer types occur in individuals of advanced age. In the case of prostate cancer, 86 % of all cases occur in men over the age of 65, and 99.5 % occur in men over the age of 50. Likewise, 97 % of prostate cancer deaths occur in men over the age of 65 (Fig. 1.7). In contrast, female breast cancer occurs much more frequently in younger individuals. Nonetheless, 63 % of cases occur in women over the age of 65, and 88 % of cases occur in women over the age of 50 (Fig. 1.7). A notable exception to this relationship between advanced age and cancer incidence involves some forms of leukemia and other cancers of childhood. Acute lymphocytic leukemia (ALL) occurs with a bimodal distribution, with highest incidence among individuals less than 20 years of age, and a second peak of increased incidence among individuals of advanced age (Fig. 1.8). The majority of ALL cases are diagnosed in children, with 40 % of cases diagnosed in children under the age of 15, and 45 % of cases occurring in individuals under the age of 20. Despite the prevalence of this disease in childhood, a significant number of adults are affected. In fact, 32 % of ALL cases are diagnosed in individuals over the age of 65 years of age. In contrast to ALL, the other major forms of leukemia demonstrate the usual pattern of age-dependence observed with solid tumors, with large numbers of cases in older segments of the population (Fig. 1.8). Among 54,270 new cases of leukemia in 2015, 88 % (48,020 new cases) represent forms of leukemia that primarily affect older individuals (acute myeloid leukemia, chronic myeloid leukemia, or chronic lymphocytic leukemia), with the remainder (6250 new cases) reflecting childhood ALL.

1.5.2 Cancer Incidence and Mortality by Race and Ethnicity

Cancer incidence and mortality can vary tremendously with race and ethnicity [9]. In the USA, African Americans and Caucasians are more likely to develop cancer than individuals of other races or ethnicities (Fig. 1.9). African Americans demonstrated a cancer incidence for all sites combined of approximately 443 cases per 100,000 population, and Caucasians exhibited a cancer incidence rate of 403 cases per 100,000 population. In contrast, American Indians showed the lowest cancer incidence among populations of the USA with 153 cases per 100,000 population for all sites combined. Mortality due to cancer also differs among patients depending upon their race or ethnicity. Similar to the cancer incidence rates, mortality due to cancer is higher among African Americans (223 per 100,000 population) and Caucasians (167 deaths per 100,000 population) than other populations, including Asian/Pacific Islanders, American Indians, and Hispanics (Fig. 1.9). For both cancer incidence and mortality, racial and ethnic variations for all sites combined differ from those for individual cancer sites. African

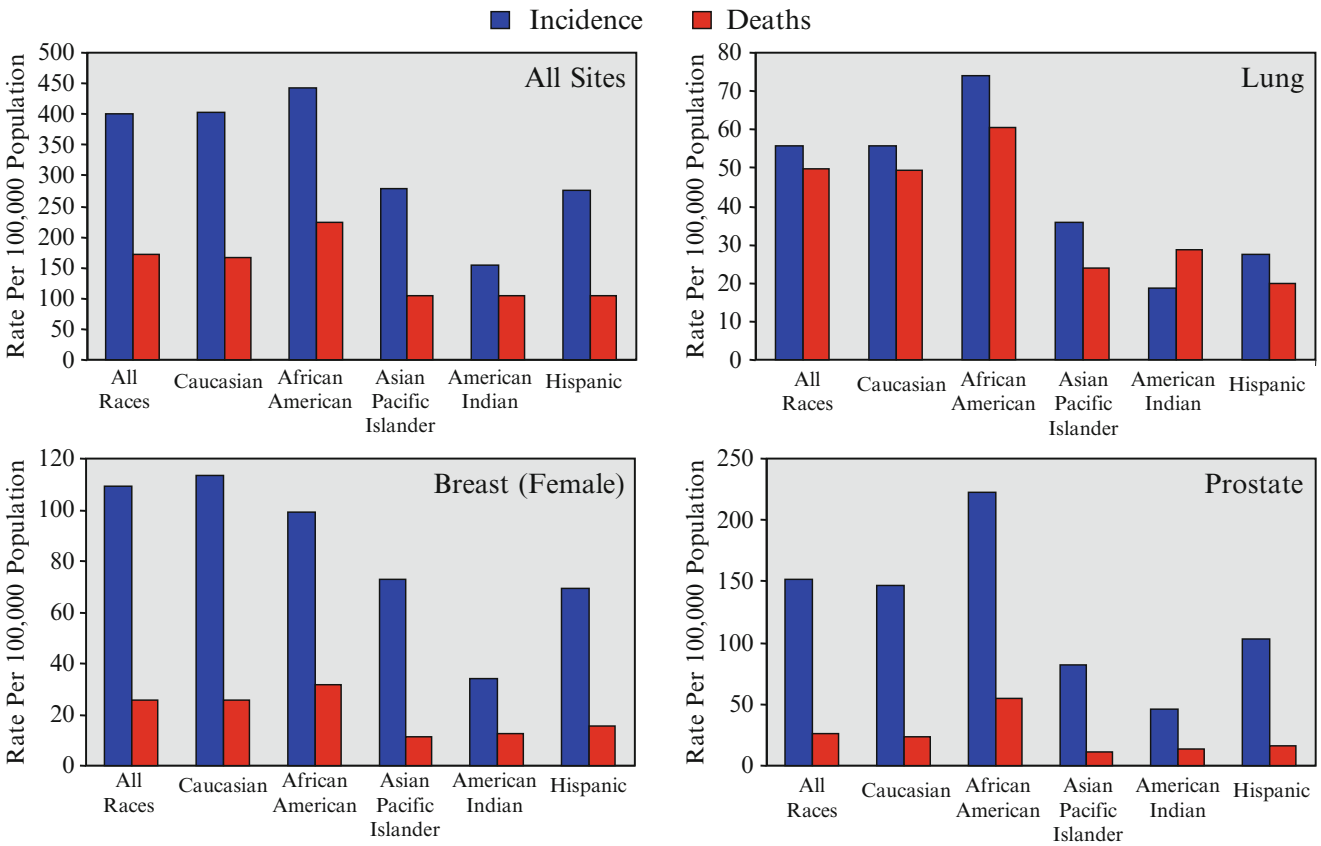

Fig. 1.9 Cancer incidence and mortality by race and ethnicity (USA, 1990–1996). The age-specific rates for cancer incidence and mortality for all races and select ethnicities residing in the USA are given for all cancer sites and select organ-specific cancer sites. The rates for all sites and lung cancer are for both sexes combined. These data were adapted from Reis et al. [9]. Rates are per 100,000 population and are age-adjusted to the 1970 standard population of the USA.

Americans and Caucasians demonstrate an excess of cancer incidence compared to the general population for a number of primary sites. African Americans exhibit high incidence rates for cancers of the prostate (223 cases per 100,000 population), lung (74 cases per 100,000 population for both sexes combined), colorectum (50 cases per 100,000 population), pancreas (13 cases per 100,000 population), oral cavity and pharynx (13 cases per 100,000), stomach (12 cases per 100,000 population), cervix uteri (12 cases per 100,000 population), and esophagus (8 cases per 100,000 population). The cancer incidence rates for lung, prostate, pancreas, and esophagus among African Americans are 33 %, 47 %, 50 %, and 115 % (respectively) higher than those rates for the general population. Caucasians exhibit high incidence rates for cancers of the breast (113 cases per 100,000 population), uterine corpus (23 cases per 100,000 population), urinary bladder (18 cases per 100,000), ovary (16 per 100,000 population), and melanoma (14 cases per 100,000 population). The excess of melanoma in the Caucasian population compared with populations possessing darker skin pigmentation is clearly shown in Fig. 1.10. The Asian/Pacific Islander population exhibit high rates of liver cancer (11 cases per 100,000 population) and stomach cancer (15 cases per 100,000 population), which are 173 % and 97 % (respectively) higher than the rates for the general population (Fig. 1.10). The Hispanic population demonstrates high rates of incidence for cancers of the cervix uteri (16 cases per 100,000 population) and stomach (11 cases per 100,000 population). These rates are 78 % and 36 % (respectively) higher than rates for these cancers in the general population (Fig. 1.10).

1.6 Trends in Cancer Incidence and Mortality for Specific Sites

1.6.1 Lung Cancer

Approximately 221,200 new cases of lung cancer were diagnosed in the USA in 2015, with 115,610 new cases among men and 105,590 new cases among women, and 13 % of all invasive cancer diagnoses [3]. The relative lung cancer incidence for all races and genders was estimated to be 56 cancers per 100,000 population in 2012 [2]. The relative lung

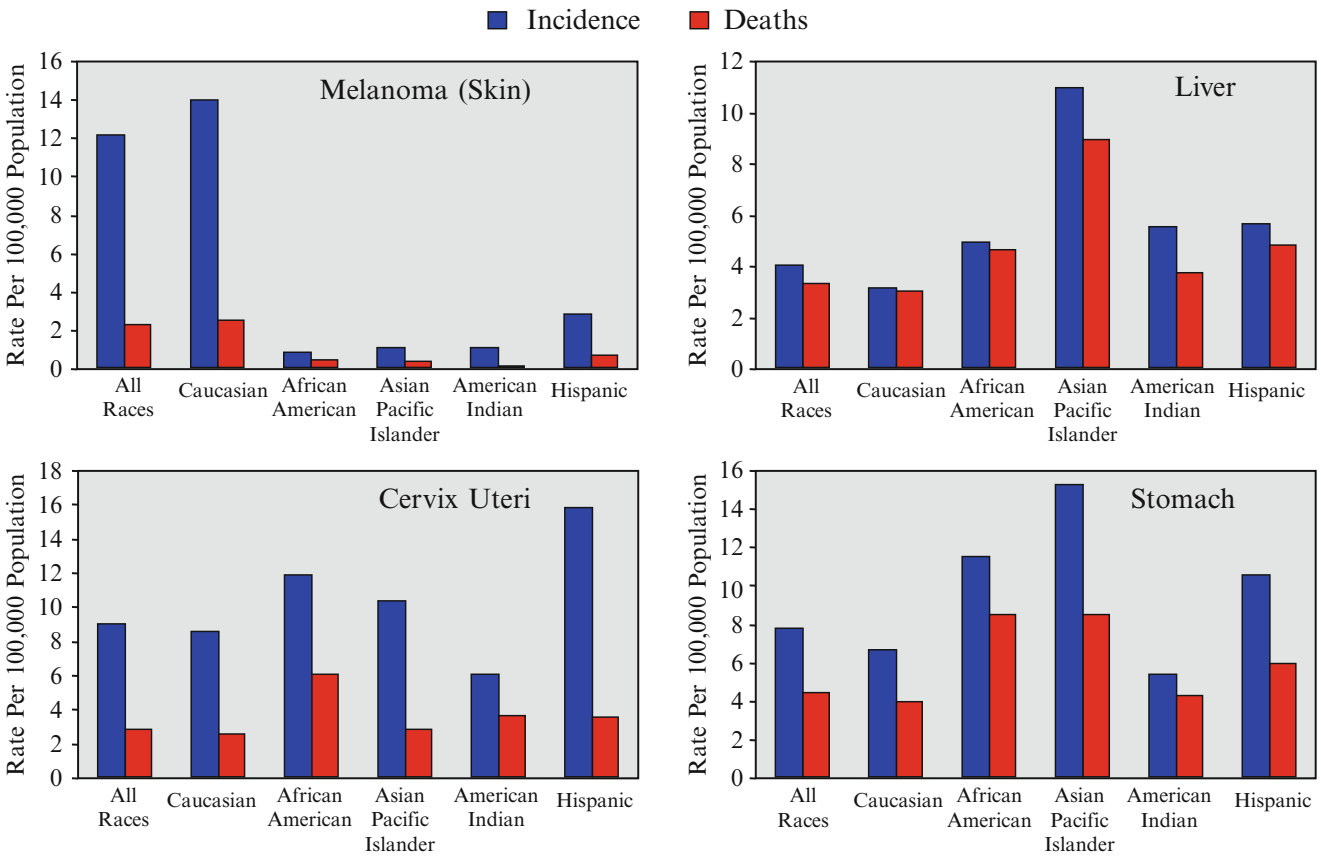

Fig. 1.10 Cancer incidence and mortality by race and ethnicity (USA, 1990–1996). The age-specific rates for cancer incidence and mortality for all races and select ethnicities residing in the USA are given for all cancer sites and select organ-specific cancer sites. The rates for melanoma, and cancers of the liver and stomach are for both sexes combined. These data were adapted from Reis et al. [9]. Rates are per 100,000 population and are age-adjusted to the 1970 standard population of the USA.

cancer incidence for men (all races) was estimated to be 65 cancers per 100,000 population in 2012, down from the all-time high of 102 cases per 100,000 population in 1984 [2]. African-American men exhibit a higher incidence of lung cancer (90 cancers per 100,000 population in 2012) compared to Caucasian-American men (64 cancers per 100,000 population in 2012). However, lung cancer incidence has declined among both groups from their all-time highs (159 cancers per 100,000 population for African-American men in 1984, and 101 cancers per 100,000 population for Caucasian-American men in 1987). The relative lung cancer incidence rate among women increased to 54 cancers per 100,000 population in 2007, but has been declining since that time, reaching approximately 50 cases per 100,000 population in 2012 [2]. Cancer of the lung and bronchus accounted for an estimated 158,040 deaths in 2015, which represents 27 % of all cancer deaths [3]. Furthermore, lung cancer is the leading cause of cancer deaths among men (86,380 deaths, 28 % of cancer deaths) and women (71,660 deaths, 26 % of cancer deaths) (Fig. 1.3).

Cancers of the lung and bronchus represent 92 % of all respiratory system cancers [3]. The remainder of respiratory system cancers include tumors of the larnyx and nasal cavities. The majority of lung cancers are histologically classified as either small cell lung carcinoma (SCLC, 13 % of all lung cancers) or non-small-cell lung carcinoma (NSCLC, 83 % of all lung cancers). The NSCLC class includes the morphologic subtypes of squamous cell carcinoma (SCC), adenocarcinoma, and large cell undifferentiated carcinoma. Squamous cell carcinomas (SCC) account for approximately 35 % of lung cancers [10]. This histologic subtype of lung cancer is closely correlated with cigarette smoking and represents the most common type of lung cancer among men. SCCs display varying levels of differentiation, from tumors consisting of well-differentiated keratinized squamous epithelium to tumors consisting of undifferentiated anaplastic cells. Adenocarcinomas have increased in frequency in recent years and now account for nearly 35 % of lung cancers [10]. These tumors grow faster than SCCs and frequently metastasize to the brain. Lung adenocarcinomas can present as well-differentiated tumors consisting of well-differentiated glandular epithelium, or as undifferentiated tumors composed of highly mitotic anaplastic cells. Large cell undifferentiated carcinomas account for approximately 15 % of all

lung cancers [10]. These tumors lack squamous or glandular cell characteristics and are typically composed of large anaplastic cells with frequent mitotic figures. Clinically, these tumors metastasize early and have a poor prognosis. Small cell lung carcinomas (SCLC) make up the majority of the remaining cancers (13%) [3, 10]. These cancers are also associated with smoking history. SCLCs tend to produce a variety of neuroendocrine substances that can cause symptoms related to the biological activity of the hormonal substance. About 10% of SCLCs display a paraneoplastic phenotype related to production of these neuroendocrine effectors [11]. These cancers grow rapidly, metastasize early, and have a very poor prognosis.

The majority of lung cancers are attributable to exposure to known carcinogenic agents, particularly cigarette smoke. Several lines of evidence strongly link cigarette smoking to lung cancer. Smokers have a significantly increased risk (11- to 22-fold) for development of lung cancer compared to non-smokers [12], and cessation of smoking decreases the risk for lung cancer compared to continued smoking [12, 13]. Furthermore, heavy smokers exhibit a greater risk than light smokers, suggesting a dose-response relationship between cigarette consumption and lung cancer risk [12, 13]. Numerous mutagenic and carcinogenic substances have been identified as constituents of the particulate and vapor phases of cigarette smoke, including benzo[*a*]pyrene, dibenza[*a*]anthracene, nickel, cadmium, polonium, urethane, formaldehyde, nitrogen oxides, and nitrosodiethylamine [14]. There is also evidence that smoking combined with certain environmental (or occupational) exposures results in potentiation of lung cancer risk. Urban smokers exhibit a significantly higher incidence of lung cancer than smokers from rural areas, suggesting a possible role for air pollution in development of lung cancer [15]. Occupational exposure to asbestos, bis(chloromethyl) ether, chromium has been associated with increased risk for development of lung cancer [16, 17]. Exposure to the radioactive gas radon has been suggested to increase the risk of lung cancer development. This gas is ubiquitous in the earth's atmosphere, creating the opportunity for exposure of vast numbers of people. However, passive exposure to the background levels of radon found in domestic dwellings and other enclosures are not sufficiently high to increase lung cancer risk appreciably [18, 19]. High level radon exposure has been documented among miners working in uranium, iron, zinc, tin, and fluorspar mines [20, 21]. These workers show an excess of lung cancer (compared to non-miners) that varies depending upon the radon concentration encountered in the ambient air of the specific mine [20, 21].

Therapy for lung cancer varies depending upon the tumor type and other clinical variables (tumor stage, grade, location, and size). Surgery is the preferred treatment choice for SCC and adenocarcinoma, whereas SCLC is generally treated with chemotherapy. In some cases special treatment modalities are employed, such as chemotherapy followed by surgery or surgery followed by radiation treatment. Despite the variety of treatment modalities that can be applied to lung cancer management, the overall survival rates for affected individuals are not good (Fig. 1.11). The average 5-year survival rate for all patients and all stages of disease is only 17% [3, 9, 22].

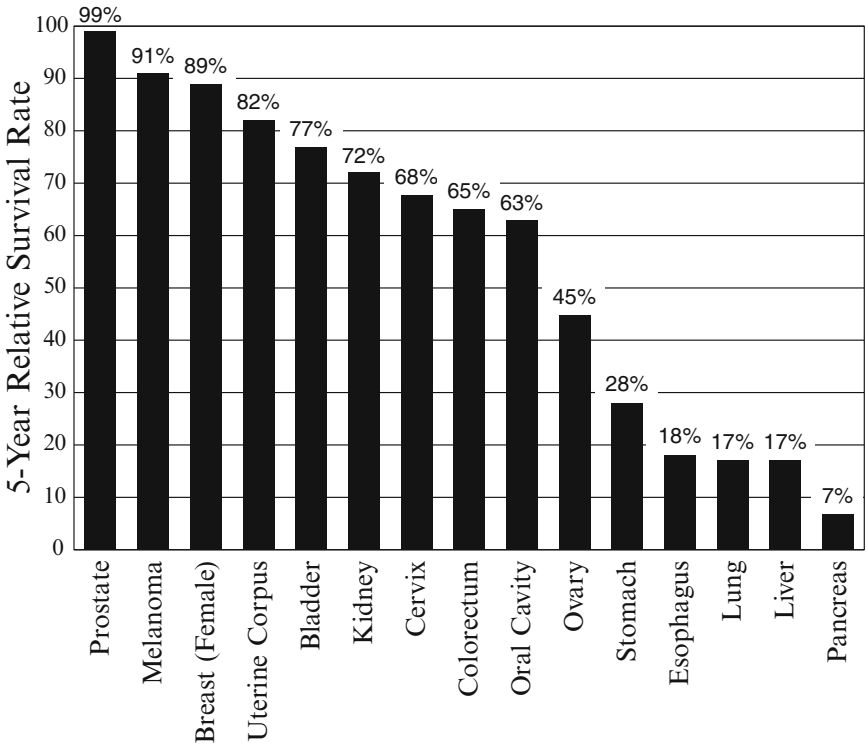

Fig. 1.11 Five-year relative survival rates for invasive cancers (all stages, 2004–2010). The average 5-year survival rates for select invasive cancers (including cancers diagnosed at all stages) among affected individuals residing in the USA for 2004–2010 are shown. These data were adapted from Seigel et al. [3].

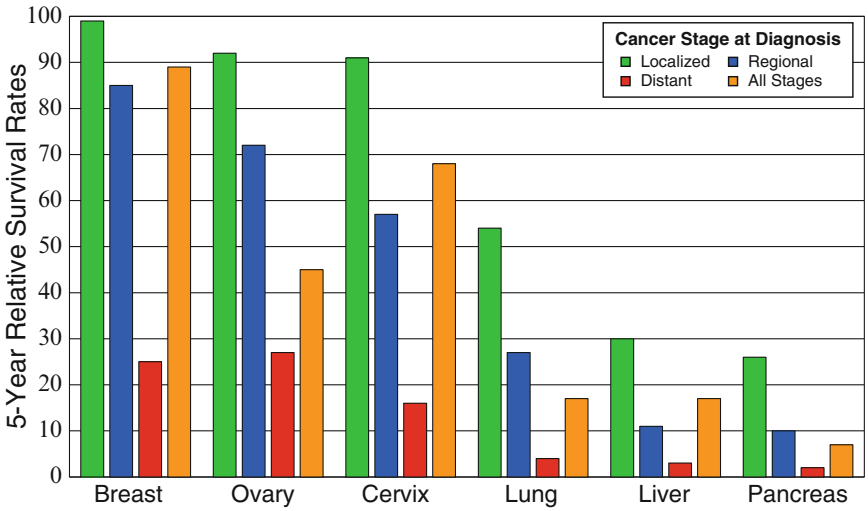

Fig. 1.12 Five-year relative survival rates for selected sites by stage at diagnosis. The percentage of tumors for select invasive cancers according to tumor stage at diagnosis and the corresponding 5-year survival rates among affected individuals residing in the USA for 2004–2010 are shown. These data were adapted from Seigel et al. [3].

The survival rate increases to 54 % if the disease is detected early (localized), but few lung cancers (15 %) are discovered this early (Fig. 1.12). The majority of lung cancer cases are not detected until after the development of regional spread (27 %) or distant metastases (58 %). The 5-year survival rate for patients with regional disease is 27 %, and this drops to 4 % in patients with distant metastasis (Fig. 1.12). The overall poor probability of surviving lung cancer probably reflects the difficulty with early detection of this tumor (or the failure to detect tumors while localized) and the ineffectiveness of traditional therapies (radiation and chemotherapy). However, over the last decade or so, a variety of targeted drugs have received market approval for treating NSCLC [23]. These new drug modalities for early stage or advanced NSCLC include inhibitors of the epidermal growth factor receptor (gefitinib, erlotinib, and afatinib), the anaplastic lymphoma kinase inhibitor crizotinib, and the antivascular endothelial growth factor receptor monoclonal antibody, bevacizumab [23]. In parallel, molecular alterations of the epidermal growth factor receptor (EGFR) and the anaplastic lymphoma kinase (ALK) have been characterized, enabling the development of molecular diagnostics of discrimination of lung cancer patients that are more likely to benefit from specific targeted therapies versus those patients that will not respond to a specific drug [23].

1.6.2 Colorectal Cancer

Approximately 132,700 new cases of colorectal cancer were diagnosed in the USA in 2015 (approximately 8 % of all cancers), with 69,090 new cases among men and 63,610 new cases among women [3]. Colorectal cancer represent 46 % of all digestive system tumors, and is the third leading site for cancer diagnosis among men (8 % of new cases among men) and women (8 % of new cases among women). In 2012, the relative incidence rate for colorectal cancer was 45 cancers per 100,000 population for men (all races) and 35 cancers per 100,000 for women (all races) [2]. However, colorectal cancer incidence rates vary by race (as well as gender). The overall incidence rates for colorectal cancer among Caucasians in 2012 was 39 cancers per 100,000 population compared to 48 cancers per 100,000 population among African-Americans. African-American men exhibit the highest incidence rate for colorectal cancer at 57 cancers per 100,000 population (versus 43 cancers per 100,000 population for Caucasian men), and colorectal cancer incidence rates for African-American women (41 cases per 100,000 population in 2012) exceed that for Caucasian women (34 cases per 100,000 population in 2012) [2]. Colorectal cancer accounted for an estimated 49,700 deaths in 2015, which represents 8 % of all cancer deaths [3]. Colorectal cancer is the third leading cause of death among men and women, accounting for 8 % and 9 % of all cancer deaths, respectively (Fig. 1.3).

Colorectal tumors are often first recognized as a polyp protruding from the wall of the bowel, which may be either hyperplastic (non-dysplastic) or dysplastic (adenomatous). Hyperplastic polyps consist of large numbers of cells with normal morphology that do not have a tendency to become malignant [24]. Adenomatous polyps contain dysplastic cells that fail to show normal intracellular and intercellular organization. Expanding adenomas become progressively more dysplastic and likely to become malignant. The majority of malignant neoplasms of the colon are thought to be derived from benign polyps. The malignant nature of colorectal tumors are defined by their invasiveness. The major histologic type of colorectal cancer is adenocarcinoma, which account for 90–95 % of all colorectal cancers [25, 26], although other rare epithelial tumor types do occur, including squamous cell carcinomas, adenosquamous carcinomas, and undifferentiated carcinomas which contain no glandular structures or features such as mucinous secretions [25, 27].

There are several recognized risk factors for development of colorectal cancer, some of which are genetic or related to benign pathological lesions of the colorectum, and others that are related to lifestyle or environment. Approximately 5–10 % of colorectal cancers are thought to be related to an inherited predisposition. Familial colorectal cancer can arise in presence or absence of polyposis, which is characterized by the occurrence of multiple benign polyps lining the walls of the colon. Several polyposis syndromes have been described, the major form of which is familial adenomatous polyposis (FAP) [28]. The hereditary nonpolyposis colorectal cancer (HNPCC) syndrome predisposes affected individuals to the development of colorectal cancer, as well as tumors at other tissue sites [29, 30]. These two hereditary syndromes account for a large percentage of familial colorectal cancers. Individuals affected by these syndromes carry mutations in one or more genes that function as tumor suppressor genes or that encode critical components of the DNA repair mechanisms that protect the genome from mutation [28]. Patients with inflammatory bowel disease (ulcerative colitis) or Crohn's disease (granulomatous colitis) exhibit an increased risk for development of colorectal cancer [28, 31]. Epidemiologic studies indicate that individuals consuming diets that are high in animal fat and red meat [32–34], or low in fiber [35, 36] are associated with increased risk for development of colorectal cancer. In addition, there is some evidence that alcohol intake and cigarette smoking can increase the risk for colorectal cancer [37, 38].

Treatment for colorectal cancer may include surgery, radiation therapy, chemotherapy, or a combination of these treatment modalities. Chemotherapy alone is not very effective, but some drug combinations that are now employed show promise. In general, survival of colorectal cancer is closely correlated with early detection of localized disease, and the mortality due to this disease has been declining in recent years [3, 39]. The average 5-year relative survival rate for colorectal cancer is 65 % (Fig. 1.11). The relative survival rate increases to 90 % if the cancer is detected early (localized), and remains relatively high (71 % survival) when detected with regional metastasis (Fig. 1.12). In contrast, the 5-year survival is only 13 % when the cancer is detected after development of distant metastases [3, 9]. Most colorectal cancers are now detected while localized (40 % of tumors) or with limited regional spread (37 % of tumors), which contributes to the generally favorable probability of survival for this form of cancer. Several screening methods are available for surveillance of patients that are at high risk for development of colorectal cancer, and some screening strategies are now routinely applied to the general population. These screening methods include digital rectal examination, fecal occult blood testing, and various forms of colonoscopy [40].

1.6.3 Liver Cancer

Hepatocellular carcinoma (HCC) is a relatively rare neoplasm in the USA, with 35,660 new cases diagnosed in 2015 [3]. However, liver cancer occurs at high incidence when the world population is considered. In 2008, there were 748,300 new cases of liver cancer worldwide [3], which represents 5.9 % of all cancers and the fifth leading site for cancer incidence (Fig. 1.5). Deaths attributed to liver cancer for the same year totaled 695,900, which represents 9.2 % of all cancer deaths and the third leading site for cancer mortality (Fig. 1.5). The prevalence of primary liver cancer varies greatly from world region to world region (Fig. 1.13).

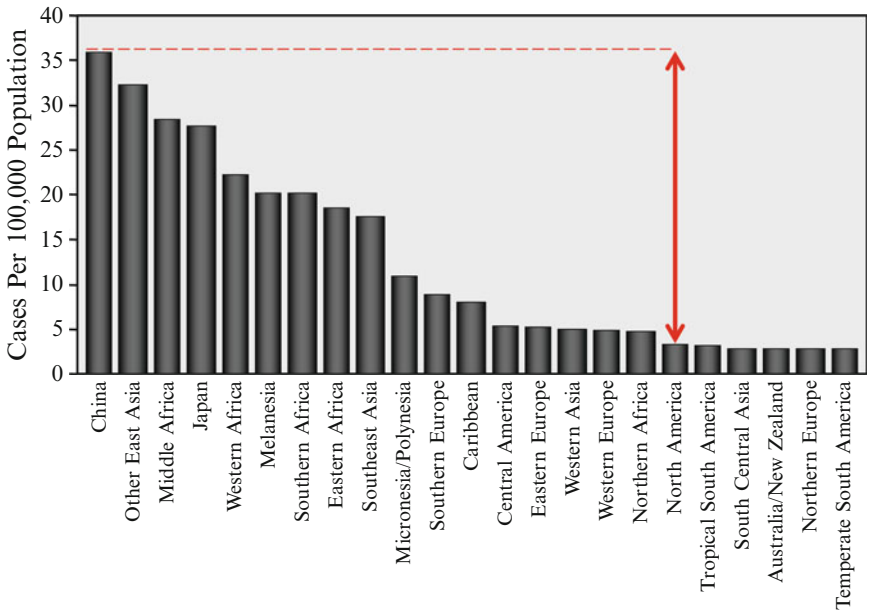

Fig. 1.13 Incidence of liver cancer among men by world region. The primary liver cancer incidence for 1990 for males residing in select world regions is shown. These data were adapted from Parkin et al. [5], and are expressed per 100,000 population.

Approximately 85 % of all liver cancers worldwide occur in regions of Asia and Africa [41, 42]. The highest incidence of liver cancer worldwide is found in China [41, 42], where men exhibit an incidence rate of approximately 36 cases per 100,000 population (Fig. 1.13). In fact, >50 % of liver cancers worldwide occur in China [41, 42]. High rates of liver cancer incidence are found throughout large portions of Asia and Africa, with much lower incidence rates for this cancer found in Europe and the Americas (Fig. 1.13). Early studies called attention to the extremely high incidence of hepatocellular carcinoma among black males in Mozambique [43, 44], which demonstrate the highest incidence worldwide at 113 cases per 100,000 population [45]. In fact, the incidence of this tumor among black Mozambican males aged 25–34 years is over 500-fold higher than the incidence for comparably aged while males in the USA and UK [44, 46]. These statistics strongly suggest that factors related to genetic background and/or environmental exposure contribute significantly to the incidence of this tumor among world populations. Whereas the incidence rate for primary HCC in the USA has remained fairly low compared to other world regions, the occurrence of this neoplasm has been increasing dramatically over the last two decades (Fig. 1.14). As shown in Fig. 1.14, the incidence of primary liver cancer in the USA has increased from approximately 15,000 new cases in 2000 to >35,000 new cases in 2015 (233 % increase in 15 years). Likewise, deaths associated with liver cancer have increased from approximately 15,000 in 2005 to nearly 25,000 in 2015 (159 % increase in 10 years). The likely cause of this dramatic increase in liver cancer incidence in the USA is the increase in HCV infection prevalence. Increased incidence of liver cancer has been observed in a number of developed countries during the last decade [41, 42]. There has also been a dramatic rise in HCC incidence among Japanese men during the last 30 years [47], possibly reflecting some significant change in risk factors or environmental exposures.

Liver cancer affects men more often than women. The ratio of male to female incidence in the USA is approximately 2:1 [2], and worldwide is approximately 2.6:1 [4]. However, in high incidence countries or world regions, the male to female incidence ration can be as high as 8:1 [48, 49]. This consistent observation suggests that sex hormones and/or their receptors may play a significant role in the development of primary liver tumors. Some investigators have suggested that hepatocellular carcinomas overexpress androgen receptors [50], and that androgens are important in the promotion of abnormal liver cell proliferation [51, 52]. Others have suggested that the male predominance of liver cancer is related to the tendency for men to drink and smoke more heavily than women, and are more likely to develop cirrhosis [53].

The etiology of HCC is clearly multifactorial [54]. HCC is usually associated with chronic hepatitis [55–58], and 60–80 % of HCC occurring worldwide develop in cirrhotic livers [55, 58–61], most commonly nonalcoholic posthepatitic cirrhosis [62]. Numerous causative factors have been identified that are suggested to contribute to the development of HCC in humans, including exposure to naturally occurring carcinogens, industrial chemicals, pharmacologic agents, and various pollutants [63, 64]. In addition, viral infection, genetic disease, and life-style factors (like alcohol consumption) contribute to the risk for development of HCC [41, 63, 64]. The most well studied hepatocarcinogen is a natural chemical carcinogen known as aflatoxin B_1 that is produced by the *Aspergillus flavus* mold [41, 65, 66]. This mold grows on rice or other grains (including corn) that are stored without refrigeration in hot and humid parts of the

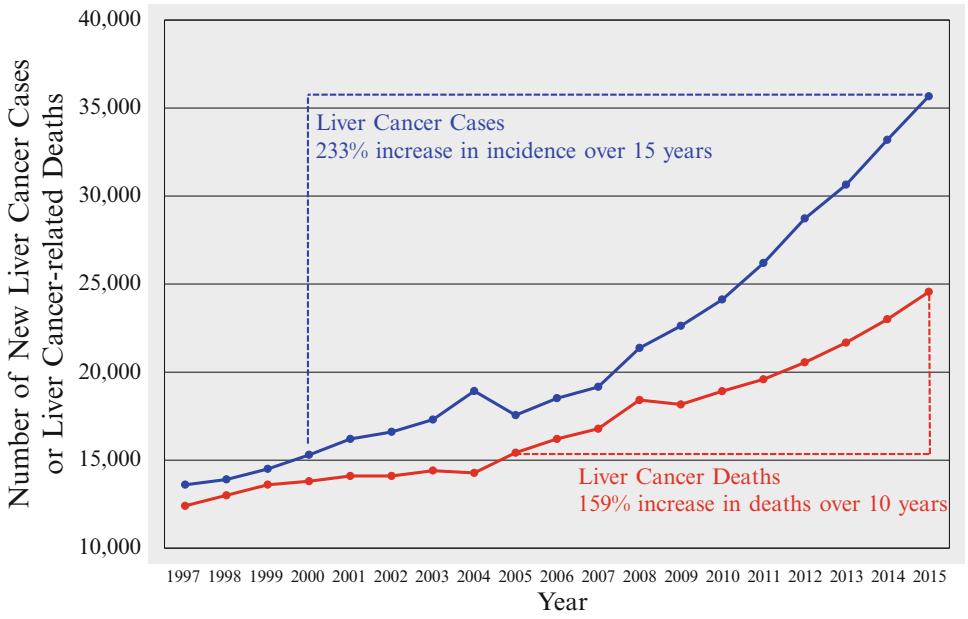

Fig. 1.14 Increasing incidence of primary liver cancer in the USA, 1997–2015. Numbers of new primary liver cancer cases (*blue trend line*) and deaths (*red trend line*) are provided. These data were assembled from the annual cancer statistics reviews for 1997–2015 from the American Cancer Society (www.cancer.org).

world. Ingestion of food that is contaminated with *Aspergillus flavus* mold results in exposure to potentially high levels of aflatoxin B_1 [67]. Aflatoxin B_1 is a potent, direct-acting liver carcinogen in humans, and chronic exposure leads inevitably to development of HCC [66]. Numerous studies have shown a strong correlation between hepatitis B virus (HBV) infection and increased incidence of HCC [45, 68–70]. More recently, an association between chronic HCV infection and HCC has been recognized [70–73]. In certain geographic areas (such as China), large portions of the population are concurrently exposed to aflatoxin B_1 and HBV, which increases their relative risk for development of liver cancer [68]. Pharmacologic exposure to anabolic steroids and estrogens can lead to development of liver cancer [74]. Several genetic diseases that result in liver pathology can increase the risk of development of a liver cancer, including hemochromatosis, hereditary tyrosinemia, glycogen storage disease types 1 and 3, galactosemia, Wilson's disease, and others [75]. Chronic alcohol consumption is associated with an elevated risk for HCC [76–79]. Alcohol is not directly carcinogenic to the liver, rather it is thought that the chronic liver damage produced by sustained alcohol consumption (hepatitis and cirrhosis) may contribute secondarily to liver tumor formation [80]. Other lifestyle factors may also contribute to risk for development of HCC, including tobacco smoking [81, 82]. Several chemicals, complex chemical mixtures, industrial processes, and/or therapeutic agents have been associated with development of HCC in exposed human populations [64]. These include therapeutic exposure to the radioactive compound thorium dioxide (Thoratrast) for the radiological imaging of blood vessels [83, 84], and exposures to certain industrial chemicals, such as vinyl chloride monomer [85].

Treatment of hepatocellular carcinoma may include surgery, chemotherapy, radiotherapy, or some combination of these treatments. The overall 5-year survival rate for HCC is only 5 % (Fig. 1.11). The poor survival rate for HCC primarily reflects both the lack of effective treatment options and the advanced stage of disease at diagnosis. HCC is diagnosed as localized disease in approximately 21 % of cases, with region spread in 23 % of cases, and with distant metastases in 22 % of cases [9]. The 5-year survival for patients diagnosed with distant metastases is only 1.2 %, and survival improves to only 14.7 % when the patient presents with localized disease [9]. Most patients with HCC also have underlying cirrhosis which makes surgical resection of the tumor very difficult. Furthermore, cirrhosis itself is a preneoplastic condition, which opens the possibility for development of additional secondary neoplasms in the unresected tissue after surgery. In fact, the recurrence rate after surgery for HCC was found to be 74 % within 5 years [86]. However, orthotopic liver transplantation can afford a complete cure for HCC if surgery is carried out prior to tumor spread from the liver [87]. While the application of liver transplant in the treatment of HCC is not favored by transplant surgeons, the results emerging from such treatment are very encouraging in some cases. In one study of 17 patients [88], liver transplant was performed to correct metabolic disorders of the liver (such as tyrosinemia) and small HCCs (without invasion or local spread) were incidentally discovered in the resected specimens. Of these patients, 90 % survived >5 years without recurrence of the HCC [88]. Other studies have not produced such favorable results [89], but it appears that certain types of HCC (such as fibrolamellar carcinoma) do very well after transplant, with >50 % survival 5 years after transplant [90]. A number of chemotherapeutic agents and combinations of these agents have been used to treat HCC. However, chemotherapy for HCC is generally not very effective and the response rates are very low, particularly when a single agent is used [91]. Nonetheless, there are occasional reports of dramatic responses using systemic chemotherapy, and some reports of complete remission [92], suggesting that responsiveness of HCC to chemotherapy is totally unpredictable [62]. Radiation therapy for HCC can be effective at reduction of tumor size, but produces a number of serious side effects, including progressive atrophy of the liver parenchyma which leads to fulminant liver failure in some patients [62]. More recent developments in radiation therapy have resulted in treatment modalities that limit collateral damage to the liver and surrounding tissues but are effective at reducing tumor burden [93].

1.6.4 Skin Cancer

There are several major forms of skin cancer, including basal cell carcinoma, squamous cell carcinoma, and malignant melanoma [94]. Non-melanoma skin cancer represents the most frequently occurring tumor-type in the USA, with several million total cases in 2015 [3]. These types of skin cancer tend to be slow growing, minimally invasive, not readily metastatic, and usually curable (given appropriate treatment). Thus, these forms of skin cancer are not typically included in cancer statistics for incidence, mortality, and survival rates (and these cancers are not reported to cancer registries making it difficult to quantify the magnitude of the disease). Nonetheless, the common occurrence of these tumors among the human population suggests that it is an important group of diseases to consider. Basal cell carcinoma accounts for most cases of non-melanoma skin cancer and is the most frequently occurring form of skin cancer. Squamous cell carcinoma is the second-most common form of skin cancer. Basal cell carcinoma accounts for at least 75 % of non-melanoma skin cancers diagnosed each year in the USA, while squamous cell carcinomas account for approximately 20 % [95]. In contrast to the other forms of

skin cancer, malignant melanoma of the skin is an aggressive and invasive cancer that can metastasize to many tissue locations. The incidence of melanoma has been rising about 4 % per year in the USA. In 2015, there were 73,870 new cases of malignant melanoma, representing 5 % of all male cancers (42,670 new cases) and 4 % of all female cancers (31,200 new cases) (Fig. 1.3). Mortality due to malignant melanoma accounted for 9940 deaths in 2015 [2].

Basal cell carcinoma is a malignant neoplasm of the basal cells of the epidermis and this tumor occurs predominately on sun-damaged skin. The carcinogenic agent that accounts for the neoplastic transformation of the basal cells is ultraviolet (UV) radiation. Thus, sun bathing and sun tanning using artificial UV light sources represent significant lifestyle risk factors for development of these tumors. Basal cell carcinoma is now diagnosed in some people at very young ages (second or third decade of life) reflecting increased exposures to UV irradiation early in life. The incidence of this tumor increases with increasing age and increasing exposure to sunlight. Some researchers have suggested that the increasing frequencies of skin cancer can be partially attributed to depletion of the ozone layer of the earth's atmosphere [96], which filters out (thereby reducing) some of the UV light produced by the sun. Squamous cell carcinoma is a malignant neoplasm of the keratinizing cells of the epidermis. It tends to occur later in life and is diagnosed more frequently in men than in women. Like basal cell carcinoma, extensive exposure to UV irradiation is the most important risk factor for development of this neoplasm. When left untreated, squamous cell carcinoma can metastasize to regional lymph nodes and/or distant sites. Development of malignant melanoma occurs most frequently in fair-skinned individuals and is associated to some extent with exposure to UV irradiation. This accounts for the observation that Caucasians develop malignant melanoma at a much higher rate than individuals of other races and ethnicity's (Fig. 1.10).

The preferred treatment of skin cancer is surgery. In fact, many, if not most, cases of basal cell carcinoma and squamous cell carcinoma are treated with minor surgery in the setting of the physician's office. In the case of malignant melanoma, surgery is employed for localized disease, with radiotherapy used in the palliative treatment of metastasis to the central nervous system and bone. Chemotherapy is employed for metastatic disease, but clinical responsiveness is limited to 15–20 % of patients [97]. More recently, targeted therapies and immunotherapy has been employed to treat advanced melanoma. The 5-year survival rate for malignant melanoma is 91 % (Fig. 1.11), reflecting the high rate of diagnosis (82 %) of localized disease where survival is high (98 %) [3, 9]. The 5-year survival of this disease drops precipitously with increasing spread of the cancer. Patients diagnosed with region disease exhibit a 5-year survival of 63 % and this drops to 16 % with distant metastasis [3, 9].

1.6.5 Prostate Cancer

An estimated 220,800 new cases of prostate cancer were diagnosed in the USA in 2015 [3], representing approximately 150 cases per 100,000 population (Fig. 1.9), and 26 % of all cancers in men (Fig. 1.3). The incidence rate for prostate cancer has been increasing in the last several decades at a rate of approximately 4 % per year, and this increase is paralleled by an increase in the mortality rate (Fig. 1.4). These increases are probably related to the increasing average age of the male population, increased reporting, and increased screening of older men [98]. Detection of prostate cancer can be achieved through the application of the digital rectal examination, screening based upon detection of the prostate specific antigen (PSA) in serum, and using ultrasonography. Elevations of PSA can be detected in both benign prostatic hypertrophy and in prostate cancer, but levels of this protein in the serum are markedly elevated in cancer. While the serum PSA assay is extremely sensitive, it lacks specificity which limits its usefulness as a definitive diagnostic for prostate cancer. However, when used in combination with the digital rectal exam and ultrasonography, PSA increases the ability to detect occult prostate cancer [99].

Cancer of the prostate provides a dramatic example of the age-dependent cancer development (Fig. 1.7). Cancers at this site occur with negligible frequency in men that are less than 55 years of age, and the vast majority of cases (56 %) occur in men over the age of 65 (Fig. 1.7). Almost all prostate cancer cases (97 %) occur in men older than age 50 [3]. In addition, prostate cancer occurs with greatly varied frequency among men of different races and ethnicity (Fig. 1.9). In the USA, African-American men exhibit a significantly higher incidence of this cancer than Caucasian men, whereas American Indians demonstrate the lowest incidence of all groups (Fig. 1.9). The reason for these dramatic differences in prostate cancer incidence are not readily apparent. However, differences in the levels of circulating testosterone among men from these different groups has been suggested as one factor contributing to the observed variations in prostate cancer occurrence. The incidence of prostate cancer also varies widely from world region to world region, with the highest incidence among men living in North America (Fig. 1.15). It is notable that the incidence of prostate cancer among men from the various regions of Africa are significantly lower than the rates for African-American men living in the USA.

Treatment for prostate cancer includes surgical removal of the prostate, radiation therapy for locally invasive tumors, and hormone therapy. Strategies for hormone therapy in the treatment of prostate cancer include both administration of estrogenic hormones (such as diethylstilbesterol) or ablation of androgenic hormones through surgical or chemical castration methods. The 5-year survival rate for prostate cancer has

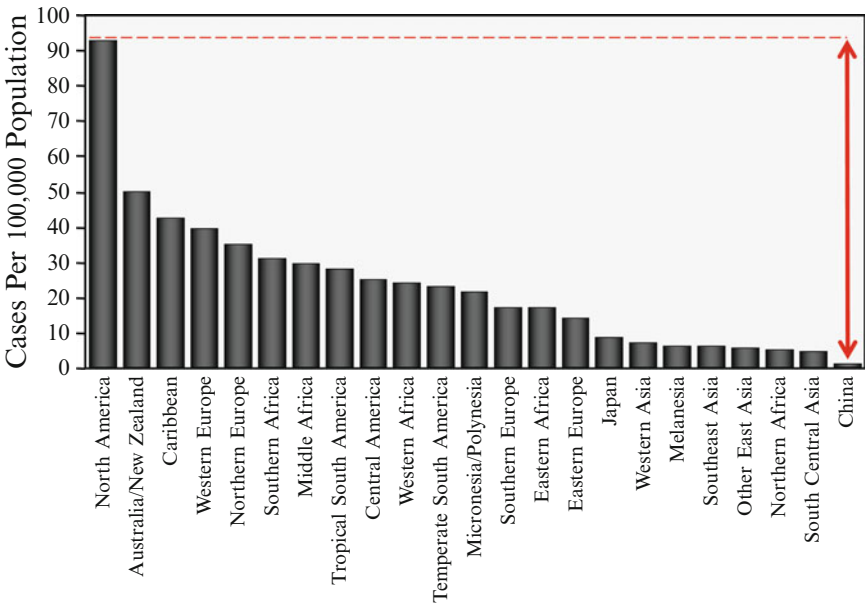

Fig. 1.15 Incidence of prostate cancer by world region. The incidence of prostate cancer for 1990 for individuals residing in select world regions is shown. These data were adapted from Parkin et al. [5], and are expressed per 100,000 population.

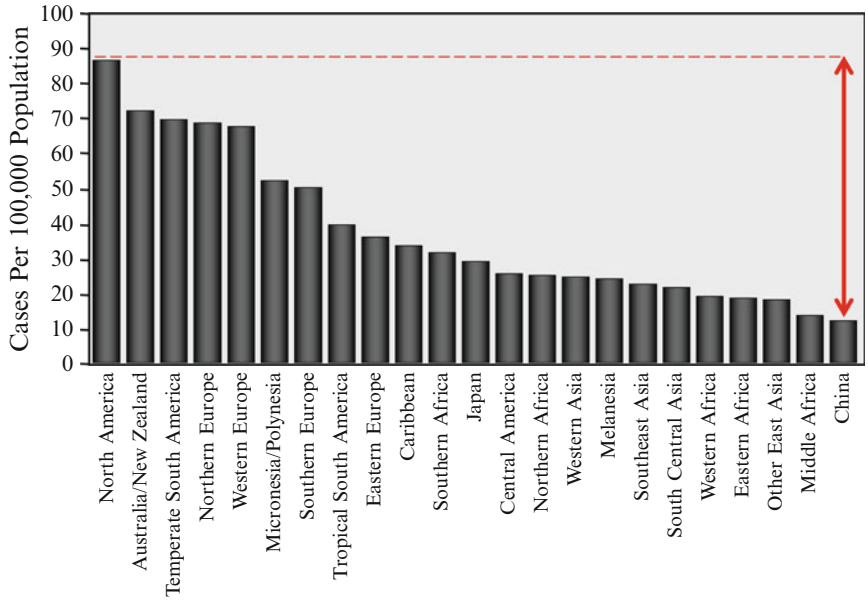

Fig. 1.16 Incidence of breast cancer among women by world region. The incidence of breast cancer for 1990 for individuals residing in select world regions is shown. These data were adapted from Parkin et al. [5], and are expressed per 100,000 population.

increased from 50 % in the years 1963–1965 to 93 % in the years 1989–1995 to 99 % in the years 2004–2010 [3, 9]. This dramatic improvement in survival can be attributed to advances in early detection of this cancer. Currently, approximately 79 % of prostate cancers are detected while they are still localized with minimal regional spread, and the 5-year survival among this group >99 % [9]. While the overall survival of prostate cancer is excellent (Fig. 1.11), early diagnosis and treatment are essential. This point is highlighted by the observation that the 5-year survival among prostate cancer patients that have distant metastasis at the time of diagnosis drops to 9 % [9].

1.6.6 Breast Cancer

An estimated 231,840 new cases of breast cancer among women were diagnosed in the USA in 2015 [3], accounting for 29 % of all female cancers diagnosed (Fig. 1.3). Breast cancer occurs more frequently among women in North America than among women from other parts of the world, while women in Asia have the lowest occurrence of breast cancer worldwide (Fig. 1.16). During the 1980s the number of new cases of breast cancer among women each year rose at a rate of about 4 % per year [9]. The incidence of breast cancer among women (all races) in the USA in the mid-1970s

was approximately 100 cancers per 100,000 population, and rose to 142 cancers per 100,000 population by 1999 [2]. In 1999, the incidence rate for breast cancer among Caucasian women peaked at 147 cases per 100,000 population and has since declined [2]. In contrast, the incidence rate for breast cancer among African-American women has increased steadily since 1976 when it was 86 cases per 100,000 population [2]. Since 1999, the incidence rate for breast cancer among women (all races) has declined somewhat and is now apparently stabilized at approximately 130 cases per 100,000 women [2]. The relative incidence of breast cancer is similar between Caucasian women (132 cases per 100,000 population in 2012) and African-American women (132 cases per 100,000 population in 2012) [2]. The increases in breast cancer incidence that were observed in the 1980s and 1990s have been suggested to reflect increased early diagnosis as mammography screening became an established standard for surveillance of the general female population. Evidence supporting this suggestion includes the fact that the average primary breast tumor at diagnosis is of smaller size and earlier stage than those diagnosed more than a decade ago. The 5-year survival for breast cancer patients diagnosed in the 1975 was approximately 75 %, compared to approximately 85 % for patients diagnosed in 1990 [9], and 89 % for patients diagnosed from 2004–2010 [3]. Nonetheless, the overall mortality rate for breast cancer has not changed substantially during this same period (Fig. 1.4), suggesting that earlier diagnosis has not significantly impacted patient outcome for this cancer. These observations suggest that the earlier diagnosis of smaller (and lower stage) cancers has impacted on the 5-year survival, without affecting the overall survival for breast cancer patients. In fact, it has been documented that breast cancer can recur after long periods of time, well after 5-years from the initial diagnosis. There are also differences in mortality rates associated with breast cancer depending upon the race of the patient. In 2012, the breast cancer-associated death rate for all women (all races) was 21 deaths per 100,000 population, which is closely mirrored by the breast cancer-associated death rate for Caucasian women (21 deaths per 100,000 population in 2012, down from the high of 33 deaths per 100,000 population in 1977). In contrast, African-American women exhibit higher death rates from breast cancer—29 deaths per 100,000 population in 2012 (down from 38 deaths per 100,000 population in 1995). An estimated 40,290 women died as a result of breast cancer during 2015 [2], making breast cancer the second leading cause of cancer death among women, accounting for 15 % of all cancer deaths (Fig. 1.3).

Risk factors for development of breast cancer include, advancing age (over 50 years of age), early age at menarche, late age at menopause, first childbirth after age of 35, nulliparity, family history of breast cancer, obesity, dietary factors (such as high-fat diet), and exposure to high dose radiation to the chest before age 35 [100–104]. In addition, fibrocystic breast disease is recognized as an established risk factor for breast cancer, especially when accompanied by cellular proliferation and atypia [105]. Epidemiologic evidence has consistently pointed to family history as a strong and independent predictor of breast cancer risk. A substantial amount of research has led to the discovery of several breast cancer susceptibility genes, including *BRCA1*, *BRCA2*, and *p53* [106], which may account for the majority of inherited breast cancers. It has been estimated that 5–10 % of breast cancers occurring in the USA each year are related to genetic predisposition [107]. Despite the recognition of multiple genetic and environmental risk factors for development of breast cancer, approximately 50 % of affected women have no identifiable risk factors other than being female and aging [108].

Treatment of breast cancer includes surgery, radiation therapy, chemotherapy, and hormone-modification therapy. Overall 5-year survival rates for breast cancer are very good—89 % irrespective of stage at diagnosis (Fig. 1.11). Localized disease can very often be cured by partial mastectomy (lumpectomy) alone or in combination with localized radiation treatment. Hence, survival of early stage (localized) breast cancer is 99 % (Fig. 1.12). Total mastectomy remains a treatment of choice for more extensive localized disease, but breast-conserving surgery has been used with increasing frequency in recent years [109]. Outcome data indicate that patients that undergo more conservative surgery with radiation therapy demonstrate survival rates that are similar to patients that are treated with total mastectomy for localized disease [110]. Five year survival rates for breast cancer patients with regional disease is 85 % (Fig. 1.12). Patients presenting with metastatic spread to regional lymph nodes are typically treated with chemotherapy, and the 5-year survival rate drops to 25 % (Fig. 1.12). The antiestrogenic drug tamoxifen is effective when used as a single agent or when used in combination with other chemotherapeutic agents in patients that are postmenopausal, have positive regional lymph node involvement, and whose tumors express estrogen or progesterone receptors [111]. Patients that receive adjuvant chemotherapy respond better to regimes that employ multiple drugs in combination. Commonly employed combinations include cyclophosphomide, methotrexate, and 5-fluorouracil (CMF), adriamycin plus cyclophosphomide, adriamycin followed by CMF, or one of these combinations with the addition of tamoxifen [109]. The decision to use adjuvant chemotherapy depends upon several variables (patient's age and general health), including the patient's estimated risk for recurrence. Typically, patients with a risk for recurrence of less than 10–15 % are spared adjuvant chemotherapy, while all others are expected to benefit from the adjuvant treatment [109].

1.6.7 Ovarian Cancer

An estimated 21,290 new cases of ovarian carcinoma were diagnosed in the USA in 2015 [3], accounting for 3 % of all female cancers (Fig. 1.3). The majority of these cancers are diagnosed in postmenopausal women. Recognized risk factors for development of ovarian cancer include advancing age (>60 years-old), infertility, use of fertility drugs (such as clomiphene), history of breast cancer and other genetic predispositions, as well as some lifestyle and dietary factors [112–119]. Pregnancy decreases the risk for ovarian cancer and multiple pregnancies increase the protective effect [120]. In addition, the use of oral contraceptives in nulliparous women reduces the risk for ovarian cancer to that for parous women [121].

Treatment for ovarian cancer typically involves surgical removal of the ovaries, the uterus, and fallopian tubes. This is usually accomplished as part of a comprehensive staging laparotomy, after which the clinical findings and histologic evaluation of the tumor are used to select appropriate postoperative therapy. A subset of patients (stage IA or IB, with well-differentiated or moderately well-differentiated tumors) exhibit excellent long-term disease-free survival in the absence of adjuvant therapy [122]. Several options exist for treatment of early-stage ovarian cancer with unfavorable prognosis, including radiation (external beam radiotherapy or intraperitoneal radioisotope) and chemotherapy. However, it is a matter of controversy whether treatment should be immediate or delayed until the disease begins to progress. The generally accepted therapy for advanced ovarian cancer is surgery followed by chemotherapy. The standard chemotherapy combination consist of paclitaxel and one of several platinum compounds (such as cisplatin or carboplatin).

Ovarian cancer accounted for 14,180 deaths in 2015 [3], making this the leading cause of death among the cancers of the female reproductive tract (Fig. 1.3). Contributing to the significant mortality associated with this cancer is the fact that there are no obvious symptoms in affected patients until late in the disease. This is reflected in the poor 5-year survival rate among patients with distant metastases at the time of diagnosis (27 %) (Fig. 1.12). In contrast, the 5-year survival rates for patients diagnosed with localized disease or regional disease are 92 % and 72 %, respectively (Fig. 1.12). However, most patients are diagnosed after development of distant metastases; 34 % of cases are diagnosed as localized/regional disease, while 60 % of cases present with distant metastases [9]. The overall 5-year survival rate for ovarian cancer is 45 % (Fig. 1.11). Successful screening for ovarian cancer would be expected to decrease mortality by increasing the percentage of affected individuals that are diagnosed early in the progression of the disease. However, the currently available screening techniques (ovarian palpation, transvaginal ultrasonography, and serum CA125 measurements) lack sufficient specificity and sensitivity to allow for routine screening [123].

1.6.8 Leukemia

Approximately 54,270 new cases of leukemia were diagnosed in the USA in 2015 [2]. These new cases were divided between myeloid (51 %) and lymphocytic (38 %) forms of the disease, with chronic forms of the disease representing 39 % of all leukemias versus acute disease representing 50 % of all leukemias [3]. The major types of leukemia include acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). AML represents the most common form of leukemia (20,830 new cases), followed by CLL (14,620 new cases), CML (6660 new cases), and ALL (6250 new cases). Rarer forms of leukemia include monocytic, basophilic, eosinophilic, and erythroid leukemia (combining for approximately 11 % of all leukemias). Although it is commonly regarded as a childhood cancer, leukemia affects more adults than children on an annual basis [3, 9]. Development of leukemia has been linked to certain environmental and genetic risk factors, including exposure to radiation (such as atomic bomb radiation), toxic chemicals (such as benzene), previous exposures to chemotherapeutic agents (mutagenic drugs), Down's syndrome, genetic disorders associated with chromosomal instability (Fanconi's anemia, Bloom syndrome, ataxia telangiectasia), and some viral infections [124–129]. Cigarette smoking has also been linked causally to development of leukemia, particularly in patients of advancing age [130, 131]. Exposure to electromagnetic radiation or fields has been suggested as a risk factor for development of leukemia [132], but a causal relationship has not been firmly established [129].

Acute leukemia is relatively rare, representing approximately 1.6 % of all cancers [3]. However, the acute leukemias are the leading cause of cancer-related mortality in the USA for persons less than 35 years of age [133, 134]. In 1993, leukemias accounted for 3.9 % of total cancer-related deaths among men in the USA [135]. However, leukemias were responsible for approximately 37 % of cancer-related deaths among men less than 15 years of age, and approximately 22 % of cancer-related deaths among men less than 35 years of age [135]. Likewise, in 1997, leukemia was responsible for approximately 33 % of all cancer-related deaths (both sexes combined) among individuals <20 years of age [136]. Figure 1.8 shows the age-specific incidence rates for the major forms of leukemia. ALL occurs with an incidence of >5 cases per 100,000 population for children that are less than 4 years-old, declining with increasing age to approximately one case per 100,000 population in individuals over 15 years of age [9]. In fact, ALL accounts for

the majority (approximately 76 %) of childhood leukemias (individuals <20 years-old). In contrast, AML is rare in individuals less than 40 years of age, but increases with advancing age from approximately 1.6 cases per 100,000 population at age 40 to >13 cases per 100,000 at age 70 [9]. Likewise, CML occurs very rarely in individuals less than 40 (≤1 case per 100,000 population), but increase to >6 cases per 100,000 population in individuals over age 70 [9]. The incidence of CLL is strictly age-dependent, occurring only rarely in individuals less than 45 years of age, increasing with advancing age to six cases per 100,000 population by age 65 years, and to >31 cases per 100,000 population in individuals over 85 years [6]. As can be seen in Fig. 1.8, >90 % of all leukemias occur in individuals >20 years of age [3].

Conventional treatment of leukemia involves aggressive chemotherapy. Post-remission therapy can involve bone marrow ablation (through high dose chemotherapy or whole body radiation) with allogeneic bone marrow transplant. A variety of drugs and drug combinations have been evaluated for treatment efficacy in various forms of leukemia [137, 138]. Appropriate drug regimens are chosen based upon various diagnostic and prognostic factors, including the nature of chromosomal rearrangements in the leukemic clone [139]. Improvements in chemotherapeutic drugs and drug regimens for leukemia have dramatically improved treatment success rates for several forms of leukemia. Whereas childhood ALL was nearly uniformly fatal in the 1950s, today the majority (90–95 %) of affected children achieve complete remission, and long-term survival (5-year survival) for patients diagnosed at <45 years of age is approximately 69 % [9]. The overall 5-year survival rate for patients with CLL is approximately 71 %, but is somewhat lower (58 %) among older patients (diagnosed at ≥75 years of age). In contrast, the overall 5-year survival rates for AML (approximately 15 %) and CML (approximately 32 %) remain dismally low despite improvements in treatments during the last 25 years [9]. With the exception of CLL, the long-term survival of older leukemia patients is dramatically lower than that of younger patients. In fact, most adult patients with acute leukemia ultimately succumb to their disease [140]. The 5-year survival of patients older than 65 years of age is approximately 6 % for ALL, compared to 63 % for patients younger than 65 years of age [9]. Likewise, the 5-year survival of patients older than 65 years of age is approximately 3 % for AML, compared to 25 % for patients younger than 65 years of age, and 35 % for patients younger than 45 years of age [9].

1.6.9 Lymphoma

Approximately 80,900 new cases of lymphoma were diagnosed in the USA in 2015 [3]. The majority of lymphomas are classified as non-Hodgkin lymphoma (71,850 new cases), representing 89 % of all lymphomas diagnosed [3], and the remaining cases are classified as Hodgkin lymphoma. Non-Hodgkin lymphoma is a broad category consisting of several distinct lymphoid neoplasms [141], 85 % of which are B-cell lymphomas (including follicular lymphoma, diffuse large B-cell lymphoma, mantle cell lymphoma, and several others), and 15 % of which are T-cell lymphomas (including peripheral T-cell lymphoma, anaplastic large-cell lymphoma, and several others). Risk factors associated with non-Hodgkin lymphoma include immunodeficiency associated with congenital diseases (such as ataxia telangiectasia, severe combined immunodeficiency, and X-linked lymphoproliferative disorder), acquired immunodeficiency (related to HIV infection, bone marrow transplant, or organ transplant with iatrogenic immunosuppression), various autoimmune disorders, infectious agents (Epstein-Barr virus, HTLV-1, and others), and chemical and physical agents (diphenylhydantoin, certain herbicides, radiation) [141]. Hodgkin lymphoma is a unique form of neoplasm consisting of small numbers of putative neoplastic cells (known as Reed-Sternberg cells) in an inflammatory background [142]. Most immunophenotypic and genetic data suggest that Reed-Sternberg cells represent some form of altered B-cell [142], while other evidence supports the suggestion that these cells represent a novel lymphoid cell type [143]. Risk factors for development of Hodgkin lymphoma have not been definitively characterized. However, increased incidence of this disease is associated with HIV infection, other immunodeficiency syndromes (such as ataxia telangiectasia), autoimmune disorders (such as rheumatoid arthritis), certain genetic factors, and viral infections (such as Epstein Barr virus) [144–147].

Therapeutic approaches for the treatment of non-Hodgkin lymphoma are based upon a number of factors, including the specific lymphoid neoplasm, cancer stage, prognostic factors, and the physiologic status of the patient. Treatment options include radiotherapy alone, single-agent chemotherapy, or combination chemotherapy (mild or aggressive). The majority of patients with aggressive forms of non-Hodgkin lymphoma require aggressive combination chemotherapy, often with additional radiotherapy. Treatment of Hodgkin lymphoma involves radiation therapy, radiation and chemotherapy, or chemotherapy alone. The results from a large number of randomized trials suggest some advantages of combine modality radiation/chemotherapy in the treatment of Hodgkin lymphoma over radiation alone [148]. However, there was no significant difference in the overall survival of patients between these approaches to treatment [148, 149]. Chemotherapy is effective against Hodgkin lymphoma. However, the use of drugs in combination is essential to effect complete and lasting remission of the disease [150–152].

Approximately 20,940 deaths were attributed to lymphoma in 2015, including 1150 deaths related to Hodgkin lymphoma and 19,790 deaths related to non-Hodgkin lymphoma [3].

Lymphoma affects more men than women on an annual basis, and non-Hodgkin lymphoma represents a leading site for cancer-related mortality among men. Non-Hodgkin lymphoma is the leading site for cancer-related mortality among men 20–39 years of age, accounting for 13 % of cancer deaths [136]. Furthermore, non-Hodgkin lymphoma accounts for significant numbers of cancer-related deaths among men <20 years-old (7 % of cancer deaths), and 40–79 years-old (5 % of cancer deaths). The overall 5-year survival rate for non-Hodgkin lymphoma is 72 % [2]. In general terms, long-term survival among non-Hodgkin's lymphoma patients does not differ with age. Despite an overall 5-year survival of 88 % (all races/genders) [2], there is a clear age-related difference in long-term survival among patients with Hodgkin lymphoma. Hodgkin lymphoma patients under the age of 65 display a 5-year survival rate of nearly 87 %, whereas patients older than 65 have a 5-year survival rate of only 45 %, and patients older than 75 exhibit a 5-year survival of only 31 % [9].

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Essential Concepts and Techniques in Molecular Biology

2

Claudine L. Lefferts and Joel A. Lefferts

2.1 Biology of Nucleic Acids

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are macromolecules that convey genetic information. Both DNA and RNA are made up of nucleotides, molecules that in turn are composed of a nitrogenous base, a sugar, and one or more phosphate groups. There are five nitrogenous bases found in nucleic acids: adenine, guanine, cytosine, thymine, and uracil. Adenine, guanine, and cytosine are found in both DNA and RNA; thymine is found only in DNA and uracil is found only in RNA. A second difference between DNA and RNA is the sugars that are incorporated into the nucleotides. The sugar in the nucleotides of DNA is deoxyribose, RNA nucleotides contain ribose. Finally, DNA molecules are double-stranded, while RNA molecules are usually single-stranded.

2.1.1 Composition and Structure of DNA

DNA is formed by the linear polymerization of nucleotides. The four nitrogenous bases found in DNA are either purines (adenine or guanine) or pyrimidines (cytosine or thymine), and the backbone of the DNA polymer is formed by linkage of these bases via deoxyribose and phosphate groups (Fig. 2.1). The informational content of DNA is governed by the sequential arrangement and primary structure of the nucleotide polymer. The DNA strand is polar, with no nucleotide attached to the 5′ position of the deoxyribose at one end (referred to as the 5′ end), and no nucleotide attached to the 3′ hydroxyl group at the other end (referred to as the 3′ end).

The DNA within the eukaryotic nucleus is arranged in a double-stranded helix composed of two strands of opposing polarity. The helix is stabilized by the formation of hydrogen bonds between complementary bases (A-T and G-C), by pi bonding that occurs when the bases are stacked together, and by the association of proteins [1, 2]. In eukaryotic cells, most of the DNA is in the B-form, a right-handed helix with bases on the inside where they are protected from damage by oxidating or alkylating agents. The Z-form of DNA occurs when a left-handed helix is formed, and is usually associated with portions of the DNA that are highly methylated and are not transcribed actively. Enzymatic reactions within the nucleus are responsible for conversion of DNA from the B-form to the Z-form and vice versa [3].

The DNA comprising the human genome is segmented into 46 discrete structural units, termed chromosomes. The DNA in each eukaryotic cell must be compressed to fit within the nucleus, which is only about 10 μm in diameter. Chromosomal DNA is condensed by the formation of nucleosomes, which consist of a group of small basic proteins (histones) with 160–180 base pairs of DNA wrapped around them [4]. Formation of nucleosomes is not sequence-dependent and it occurs in mammalian, bacterial, and viral DNA [5]. Nucleosomes are wound into a left-handed helix for further condensation of the DNA, and higher orders of structure include supercoils and/or rosettes [2]. Ultra-condensed DNA (heterochromatin) is inactive metabolically, and is found primarily in the periphery of the nucleus, while less condensed DNA (euchromatin) is readily accessible by transcription machinery and is located in the center of the nucleus [6].

2.1.2 Gene Organization

The majority of genes that are transcribed into mRNA and translated into cellular proteins exist as two copies in the nucleus of each cell, one maternal and one paternal copy. Some genes are present at a high copy number (100–250 copies) within the genome, including the genes that encode transfer RNA (tRNA), ribosomal RNA (rRNA), and the histone proteins [7]. These tandemly repeated genes are present

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_2

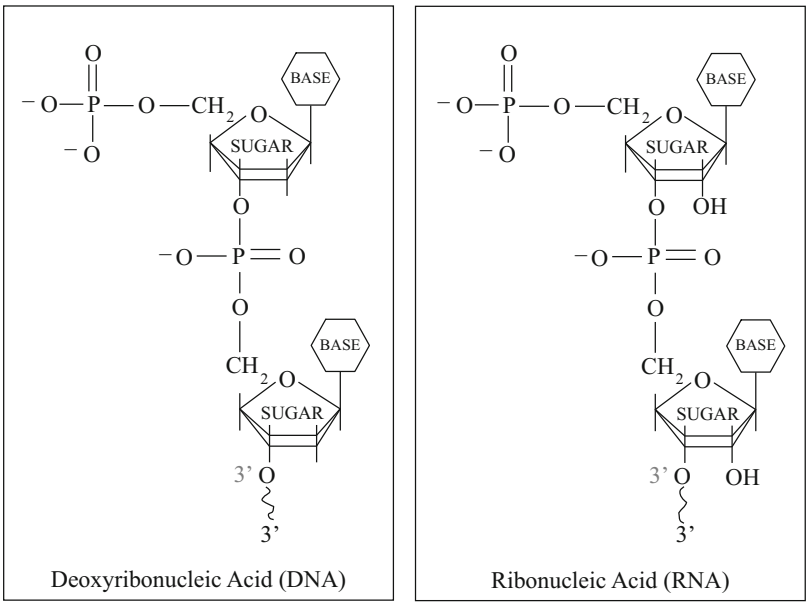

Fig. 2.1 Chemical structures of DNA and RNA.

on several chromosomes and associate in the nucleus to form a nucleolus [8, 9]. Highly repetitive sequences with thousands of copies, called satellite DNAs, are found at the telomeric ends of chromosomes and around the centromeres [10]. It is likely that the centromeric sequences play a role in the establishment and maintenance of chromosome structure. Telomeric repeats are involved in completing replication of chromosome ends [11], and it has been demonstrated that the length of the telomeric repeat sequences decreases with life-span of cultured human cells [12]. The evidence of telomeric shortening in normal cells along with observations that immortalized or transformed cells display limited telomere degeneration has led to the hypothesis that telomeric shortening is involved in the cellular aging process [12]. Other repetitive sequences, such as the *Alu* sequences, are found throughout chromosomes; their function is largely unknown, but a role in regulation of gene function has been proposed [13].

Simple polymorphic repetitive elements composed of dinucleotide or trinucleotide repeats are present in the human genome and have been associated with cancer and other diseases such as myotonic dystrophy, Fragile X syndrome, Huntington disease, and spinocerebellar ataxia [13, 14]. Symptomatic problems arise when the affected DNA is inappropriately methylated and inactivated (as in Fragile X syndrome) [15], or when the repeats cause detrimental changes in the encoded protein (as in Huntington disease) [16].

2.1.3 DNA Replication and Cell Division

In order for the DNA in a cell to be replicated prior to cell division it must be single-stranded. This is accomplished by an enzyme (helicase), which denatures the DNA and allows DNA-binding proteins to associate with the DNA and prevent reformation of the DNA helix [17]. A small strand of RNA 10–20 nucleotides in length acts as a primer, initiating synthesis of new complementary strands of DNA from multiple replication starting points. Deoxynucleotide triphosphates (dNTPs) are added to the primer by DNA polymerase, the RNA primers are removed, the gaps are filled in with dNTPs by DNA polymerase, and the nucleotide strands are joined by DNA ligase. The enzymatic action of topoisomerase removes twists generated during denaturation of the helix and allows the helix to re-form [18]. DNA replication is complete when the telomerase enzyme has added the nucleotide repeats to the telomeres at the 5′ end of the DNA strands.

As a new strand of DNA is synthesized, dNTPs are selected based on hydrogen bonding to complementary dNTPs in the template strand, which results in an error rate of 1 in 10^4–10^5 [2]. Eukaryotic cells employ a proofreading mechanism that removes mispaired dNTPs in the strand before the next dNTP is added, which decreases the error rate to 1 in 10^6–10^7 [2, 19]. Prior to cell division, another error correction system recognizes and repairs mismatched nucleotides and decreases the error rate further to 1 in 10^8–10^9 [2, 19]. Several inherited disorders are due to dysfunctional DNA damage-repair, including ataxia telangiectasia, Fanconi anemia, and xeroderma pigmentosum [20].

The DNA within the nucleus of a eukaryotic cell can be replicated completely in about 8 h, during the S phase of the cell cycle. Resting cells (G_0) receive a mitotic stimulus, which causes transition into the G_1 phase, where the cell prepares for DNA synthesis (S phase). The G_2 phase occurs after replication but before division, and mitosis (M) involves actual nuclear and cellular division. The cell cycle is pivotal in cellular and organismal homeostasis, so it is tightly controlled by phosphorylation and dephosphorylation of kinases and cyclins, and by two major checkpoints [21,

22]. The first checkpoint occurs between G_1 and S, and can prevent cells with damaged or unrepaired DNA from entering S phase. The second checkpoint occurs between G_2 and M, and can prevent the initiation of mitosis [21].

2.1.4 Structure and Composition of RNA

RNA, or ribonucleic acid, is a linear polymer of ribonucleotides linked by 5′–3′ phosphodiester bonds (Fig. 2.1). RNA differs from DNA in that the sugar group of RNA is ribose, rather than deoxyribose, thymine is replaced by uracil (U) as one of the four bases, and RNA molecules are usually single-stranded. The extra hydroxyl group present on the ribose causes RNA to be more susceptible to degradation by nucleases than DNA. Single-stranded RNA molecules form complex secondary structures, such as hairpin stems and loops, via Watson–Crick base pairing between adenine and uracil, and between guanine and cytosine.

RNA molecules are classified by function and cellular location, and there are three major forms of RNA in eukaryotic cells. Ribosomal RNA (rRNA) is the most stable and most abundant RNA. rRNA is highly methylated, and complexes with proteins to form ribosomes upon which proteins are synthesized [23]. In eukaryotic cells, two major species of rRNA are present, 28S and 18S, as well as two minor species of 5.8S and 5S. Transfer RNA (tRNA) is responsible for carrying the amino acid residues that are added to a growing protein chain during protein synthesis. All tRNAs form secondary structures consisting of four stems and three loops, and many bases found in tRNA are modified by methylation, ethylation, thiolation, and acetylation [24, 25]. Messenger RNA (mRNA) mediates gene expression by carrying coding information from the DNA to the ribosomes, where the mRNA molecule is translated into protein. Messenger RNA is the most heterogeneous type of RNA, and also has the shortest half-life.

Many other RNA species exist in eukaryotic cells. Heterogeneous nuclear RNAs (hnRNA) are the precursors to mature mRNA [26]. Small nuclear RNAs (snRNAs) associate with proteins to form small nuclear ribonucleoprotein particles, which participate in RNA processing [27]. Small interfering RNAs (siRNAs) and microRNAs are double-stranded RNAs 21–25 nucleotides in length involved in regulation of gene expression at the translational level [28].

2.1.5 Transcription of RNA

Even simple eukaryotic organisms, such as yeast, contain a large number of genes (~2000), and higher eukaryotes, such as mammals, have ~20,000–25,000 protein encoding genes [29]. Clearly, the proteins encoded by all genes are not expressed simultaneously at any given time. Transfer of genetic information from DNA to protein begins with synthesis of RNA molecules from a DNA template by RNA polymerase, a process termed transcription. The RNA polymerase holoenzyme works processively, building an RNA chain with ribonucleoside triphosphates (ATP, GTP, CTP, UTP) [30]. Initiation of transcription involves association of the transcription machinery (RNA polymerase and transcription factors) with the DNA template and the synthesis of a small ribonucleotide primer from which the RNA strand will be polymerized [31]. Initiation of transcription is not random, but occurs at specific sequences called promoters that are located at the 5′-end of genes. Every gene initiates transcription independently at its own promoter, therefore the efficiency of the process varies greatly depending on the strength of the promoter. Once RNA polymerase binds to a promoter, the DNA helix is opened and an RNA primer is synthesized. Elongation occurs as the RNA polymerase moves along the DNA strand, opening the DNA helix and conducting DNA-directed RNA synthesis until the gene is transcribed [30]. Termination of transcription is poorly understood in eukaryotes, but takes place at sites that include a stretch of Ts on the non-template strand of the gene [32].

2.1.6 RNA Processing

The majority of eukaryotic RNAs require extensive modifications before they attain their mature structure and function. RNA strands may be modified by (1) the removal of RNA sequences, (2) the addition of RNA sequences, or (3) the covalent modification of specific bases. The long, relatively unstable mRNA precursor strand (hnRNA) is synthesized and remains in the nucleus where it is subjected to several stability-enhancing processes. With the exception of mitochondrial mRNAs, the 5′-ends of eukaryotic mRNA precursor molecules are capped, which involves removal of the terminal phosphate group of the 5′-nucleoside triphosphate and subsequent linkage of the 5′-diphosphate group to a GTP molecule [33]. The cap structure is covalently modified by methylation of the newly added guanine. The hnRNA is also modified at the 3′ end by the addition of a poly-A tail, a string of 50–250 adenine residues. The poly-A tail serves to extend the life of the mRNA by protecting the 3′-end of the molecule from 3′-exonucleases, and may also act as a translational enhancer [34]. The mRNA molecule is stabilized further by the association of a ~70 kDa protein with the poly-A tail [35].

The majority of protein-encoding eukaryotic genes contain intervening sequences, termed introns, which do not encode any portion of the protein. These introns, which may be between 65 and 10,000 nucleotides in length, are maintained during transcription of the hnRNA molecule, resulting in production of a long hnRNA that must be modified in order to become a continuous template for synthesis of the encoded protein. Maturation of the hnRNA requires a splicing event in which the introns are removed in conjunction with the joining

of the coding sequences, termed exons. Three short consensus sequences are necessary for splicing to occur; two are found at the intron–exon boundary at both the 5′ and 3′ of the intron and the third is found within the intron near the 3′-end [36]. The consensus sequences mark splice sites and act as targets for the spliceosome, a large multisubunit protein complex comprised of 45 proteins and thousands of snRNA [36]. The spliceosome catalyzes removal of introns and rejoining of exons, ultimately resulting in the formation of a protein-encoding mRNA. Some genes encode for more than one protein, which is accomplished by alternative splicing of the primary hnRNA transcript. One mechanism of alternative splicing involves removal of one or more exons during splicing when the spliceosome ignores one or more intron–exon boundaries [37]. Alternative transcripts may also be generated by the use of a secondary polyadenylation site [38].

RNA processing is not limited to mRNA. Eukaryotic tRNAs are modified posttranscriptionally, as are eukaryotic rRNAs [39]. Introns present within rRNAs are classified as Group I or Group II introns. Group I introns are removed as linear molecules by a self-splicing mechanism that requires magnesium and guanosine as cofactors [40]. Group II introns are also self-splicing, are removed as a lariat structure, and require spermidine as a cofactor [41].

2.2 Basic Molecular Analysis and Interpretation

Investigations into the molecular mechanisms of disease depend on the analysis of cellular and often times microbial DNA and RNA to identify and characterize the genes involved. Target genes can be identified, localized to specific chromosomes, amplified by cloning, and subjected to sequence analysis. DNA analyses are used practically in the identification of individuals for forensic or parentage testing, detection of gene mutations, amplifications, and deletions, associated with disease and RNA analyses to characterize gene expression in various forms of human cancer.

2.2.1 Isolation of Nucleic Acids

In theory, nucleic acids can be isolated from any tissue that contains nucleated cells. Common starting material for nucleic acid isolations include blood and other body fluids, cultured cells, buccal swabs, and a variety of fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE) tissue specimens. Although isolation may need to be optimized for any particular tissue type or sample and application, there are basic steps in the extraction process that are universal: the cells must be lysed, proteins and cellular debris must be removed, and the nucleic acid must be made available in solution.

FFPE tissue is the most common specimen type used when interrogating solid tumors for somatic changes in the clinical setting. FFPE tissue is readily available following tumor resection or biopsy, is easily stored at room temperature and the tissue being used for molecular analysis can be viewed microscopically to determine the precise histological diagnosis and to determine the adequacy of the specimen (percent tumor cells, exclude excessive necrosis, etc.). Nucleic acid derived from FFPE tissue, however, can be very problematic because the fixation process degrades nucleic acid and creates cross-links between nucleic acids and proteins. Molecular analysis of FFPE tissue, therefore, requires special attention during isolation and the design of downstream molecular applications. For example, the majority of an FFPE DNA sample is fragmented to 100-200 bp and will not work at all in most assays designed for high quality DNA. Assays specifically designed to detect these smaller fragments must be used in when FFPE-derived DNA is needed.

Before nucleic acids can be separated from the rest of the cellular contents, the cells must be lysed. This is often accomplished in some type of lysis buffer that can contain agents such as proteinase K and sodium dodecyl sulfate which are needed to digest structural proteins and to disrupt the cellular and nuclear membranes. Some samples such as solid tissues often require additional steps first, such as mincing, homogenization, or freezing the sample in liquid nitrogen followed by pulverization with a mortar and pestle to obtain a complete lysis. After the sample has been adequately lysed, the protein component in the sample must be removed. This can be accomplished in several ways, including: (1) adding a phenol–chloroform–isoamyl alcohol mixture to the sample which contains an organic phase and an aqueous phase whereby proteins and lipids dissolve into the organic phase and nucleic acids are retained in the aqueous phase of the mixture; (2) precipitation of proteins; (3) absorbance of nucleic acids to a column or magnetic bead and washing away proteins.

After the DNA has been separated from the rest of the other cell components, it must be transferred to a suitable buffer such as tris–EDTA (TE) or deionized water. This can be accomplished by adding ethanol or isopropanol which causes the DNA to precipitate out of solution. The DNA can then be pelleted by centrifugation, and resuspended in deionized water or a buffer. Alternatively, nucleic acids can be eluted directly from columns or magnetic beads in some of the more automated methods.

Due to the time involved and the toxicity and waste disposal issues associated with phenol, many laboratories choose other isolation protocols that remove proteins by other means. After lysis of the cells a protein precipitation solution can be added followed by centrifugation to separate the DNA from the cellular proteins. DNA can then be further purified by the use of silica gel particles in suspension or bound in a column. These silica columns can be purchased from numerous companies and are available in a wide range of formats and sizes, including the popular spin columns which rely on centrifugation to move solutions through the column. DNA binds to the silica gel as it passes through the column, allowing unwanted

salts and contaminants to wash through. After the DNA is bound to the column it can be washed and then eluted off the column with water or a low salt buffer such as TE. Similar methods have been developed using magnetic beads capable of binding DNA in solution. DNA can be purified by binding it to these beads in a tube. When these tubes are placed next to a magnet, the beads can be held in place on the side of the tube while the original solution and contaminants are removed. This process can then be repeated with wash solutions and finally an elution buffer that is often heated to facilitate removal of the DNA from the beads.

Although these alternative methods can be faster, they are often more expensive and can result in lower yield and slightly lower quality DNA. However, this is not generally a problem for most molecular techniques commonly used today. Another improvement on traditional nucleic acid isolation methods has been the introduction of automated isolation systems which can greatly reduce the hands-on time needed for isolating DNA. Once loaded, these instruments can go through the entire DNA isolation procedure, often using some form of magnetic separation. Although these automated systems are much more expensive than manual methods, the time they save will often make these systems a valuable investment for laboratories that process larger numbers of samples.

RNA isolation methods are similar to those for DNA. However, when isolating RNA extra care must be taken to avoid RNA degradation due to its susceptibility to RNA-digesting enzymes (RNases). To avoid RNase digestion of the sample, it is often necessary to work quickly and to work in a clean area. Care should be taken to ensure that all solutions purchased for use in these extractions have been designated as "RNase-free" or treated with special chemicals such as diethylpyrocarbonate (DEPC) that inactivate RNases.

2.2.2 Use of Enzymes in Molecular Biological Techniques

DNA or RNA isolation techniques only produce the starting material needed for molecular evaluation of nucleic acid. Almost every method used to examine DNA or RNA relies heavily on the use of enzymes, often isolated from various microbial species. These enzymes can be exploited to perform various tasks such as selectively cutting DNA or RNA into smaller pieces or digesting them completely, modifying the ends of DNA strands to remove or add phosphate groups, ligating two strands of DNA together to form one, and replicating nucleic acids.

2.2.2.1 Nucleases

Nucleases make up a diverse group of enzymes that are able to cleave or digest nucleic acids. This group of enzymes can be divided into various classifications based on the type of nucleic acid they use as substrates and the way in which they digest. Exonucleases begin digesting nucleic acids at the ends while endonucleases make internal cuts in a nucleic acid molecule. Restriction endonucleases make up a very large group of enzymes found in bacteria that recognize very specific sequences of DNA, often six base pairs in length. For example, the restriction endonuclease EcoRI (isolated from *E. coli*) recognizes the sequence GAATTC and makes a single cut within this recognition site. If the sequence of a particular piece of DNA is unknown, observing the digestion patterns of various restriction enzymes can provide valuable information about its sequence. For example, if a particular mutation occurs within the recognition sequence of a specific restriction enzyme, that enzyme may not cut when the mutation is present.

When complete digestion of DNA or RNA is desired, for example when DNA is being isolated and contaminating RNA is not desirable, RNase can be used to selectively digest the RNA, leaving a pure population of DNA. In the same way, DNases can be used to digest unwanted DNA molecules in an isolation of RNA.

2.2.2.2 DNA Ligases, Kinases, and Phosphatases

In addition to digesting DNA, enzymes are also available which facilitate the joining or ligation of two or more DNA fragments. Enzymes with this capability are called DNA ligases and commercially, T4 DNA ligase is widely available and most often used in molecular laboratories. Since DNA ligases work by creating a phosphodiester bond between the 5′ phosphate and 3′ hydroxyl termini of two DNA strands, the presence of the 5′ phosphate is essential. DNA phosphatases, such as calf intestinal phosphatase or shrimp alkaline phosphatase, remove the phosphate group on the 5′ end of a DNA strand, preventing ligation; a DNA kinase, T4 polynucleotide kinase, can restore the 5′ phosphate in order to allow ligation.

2.2.2.3 Polymerases

DNA and RNA polymerases are enzymes that synthesize new strands of DNA or RNA, normally using a preexisting strand of DNA (or RNA) as a template. Reverse transcriptase is another polymerase that is unique in its ability to use RNA as a template for synthesizing DNA. DNA polymerases are essential for the polymerase chain reaction (PCR), a technique described below that has revolutionized molecular biology.

2.2.3 Electrophoretic Separation of Nucleic Acids

Gel electrophoresis of DNA is widely used in procedures such as Southern blotting and PCR in which separation or sizing of a population of DNA fragments is an essential step in the analytical process. In some cases, a significant amount of information can be gleaned from relatively simple electrophoretic procedures that take advantage of the various properties of DNA molecules (i.e., charge, size, and conformation).

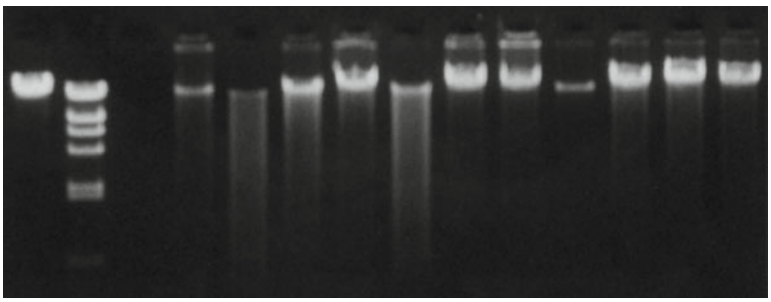

Fig. 2.2 Agarose gel electrophoresis of human genomic DNA.

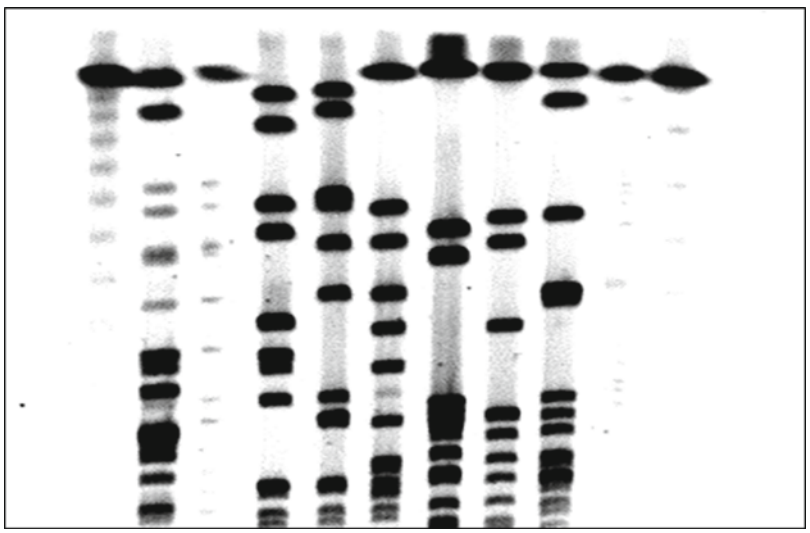

Fig. 2.3 Pulse field gel electrophoresis for strain typing of *Staphylococcus aureus.*

Gels employed in electrophoretic techniques are generally composed of agarose or polyacrylamide, which act as a matrix through which DNA (or RNA) will travel when an electric current is passed through the gel. Generally, larger fragments of DNA will migrate more slowly through the gel and smaller fragments will migrate more quickly. DNA of a given size will travel through the gel at the same rate and form a band that is visualized by staining the gel with a dye such as ethidium bromide, which binds to DNA and fluoresces when viewed under UV light (Fig. 2.2). Agarose gels are useful in most traditional molecular techniques for visualizing DNA greater than 100 base pairs. However, when smaller DNA fragments are to be analyzed, special agarose formulations or polyacrylamide gels are needed for proper resolution and visualization.

When larger fragments of DNA need to be resolved, a technique called pulsed-field gel electrophoresis can be utilized. Conventional gel electrophoresis techniques are not useful for separation of extremely long pieces of DNA, because the constant current eventually unravels the DNA strands completely so that they travel, end first, through the gel at a rate that is independent of their length. Pulsed-field gel electrophoresis (PFGE) overcomes this challenge by periodically switching the orientation of the electric fields with respect to the gel, thus preventing the DNA strands from losing secondary structure and allowing long strands to be size-differentiated [42]. PFGE is often used in the identification of pathogens [43], i.e., differentiating between strains of bacteria (Fig. 2.3). Other applications include chromosomal length polymorphism analysis, and large-scale restriction and deletion mapping in DNA that is hundreds or thousands of kb in length. The effectiveness of PFGE is dependent on high-integrity starting material, and DNA that is degraded or sheared will not yield informative results. High quality DNA is often generated by embedding the cells of interest in agarose plugs, lysing cell membranes with detergent, and removing proteins enzymatically, leaving intact DNA which can be digested by restriction enzymes in situ and easily loaded into a gel apparatus [42, 44]. In a typical analysis, PFGE will produce a pattern of DNA fragments that range in size from 10 to 800 kb.

Electrophoresis of RNA to check the quality of a sample or for blotting techniques is typically accomplished by electrophoresis through a 1–2 % agarose gel containing formaldehyde, which maintains the denatured state of the RNA strands. When secondary structure of an RNA molecule is to be investigated, samples are subjected to non-denaturing polyacrylamide gel electrophoresis.

2.2.4 Denaturation and Hybridization

The majority of techniques used in molecular analyses require single-stranded nucleic acids as starting material. Prior to hybridization with a complementary nucleotide sequence such as an oligonucleotide primer or a nucleic acid probe, double-stranded DNAs and single-stranded RNAs must be denatured to generate single strands and eliminate secondary structure. Denaturation of nucleic acids is rapid, and can be induced by various conditions, including extremes of pH (pH < 4 or pH > 10), hydrogen-bond disrupting agents (such as urea or formamide), or heat [45]. Heat is the most commonly used means of disrupting the hydrogen bonds that occur between complementary strands of double-stranded DNA to produce single-stranded DNA. The melting temperature (T_m) of a specific double-stranded DNA sequence is reached when the disruption of the hydrogen bonding in a population of DNA causes 50 % of the DNA to become single-stranded. Since more hydrogen bonding occurs between G and C as compared to A and T, the T_m will be greater for DNA containing a larger percentage of G and C nucleotides. The denaturation process is easily monitored by spectrophotometry, since the absorbance of the DNA at 260 nm increases as denaturation progresses [45].

Hybridization of nucleic acid strands is a relatively slow process and the rate is governed by the relative concentration of strands with complementary sequences and by the temperature. When two complementary strands are aligned properly, hydrogen bonds form between the opposing complementary bases and the strands are joined. Target nucleic acid sequences can hybridize to a complementary DNA (cDNA) or RNA strand, or to other complementary sequences such as oligonucleotide primers or nucleic acid probes. Hybridization between two complementary RNA molecules is strongest, followed by RNA–DNA hybrids, and DNA–DNA hybrids [46].

2.2.5 Concepts and Applications of Southern Blotting

Southern blotting is a technique that relies heavily on the concepts of denaturation and hybridization of DNA. Southern blotting has been used in clinical or forensic settings to identify individuals, determine relatedness, and to detect genes associated with genetic abnormalities or viral infections. Southern blot analysis is also used in basic scientific research to confirm the presence of an exogenous gene, evaluate gene copy number, or to identify genetic aberrations in models of disease. Although this technique has been replaced to a large extent by newer, faster techniques, it still has its place in a molecular laboratory.

The first step in successful Southern blotting is to obtain DNA that is reasonably intact (Fig. 2.4). DNA that has been degraded by excessive exposure to the elements or mishandling will not produce a good quality Southern blot because it cannot be fragmented uniformly prior to the blotting procedure. The test DNA must be fragmented with restriction enzymes, which cut the double strands of DNA at multiple sequence-specific sites, creating a set of fragments of specific sizes which represent the regions of DNA between restriction sites. The fragmented DNAs are size-fractionated via agarose gel electrophoresis and are subsequently denatured, which enables them to later be hybridized to complementary nucleic acid probes. The DNA from the gel is transferred to a solid support such as a nylon or nitrocellulose membrane via capillary action or electrophoretic transfer and is bound permanently to the membrane by brief UV crosslinking or by prolonged incubation at 80 °C. Blots at this stage may be stored for later use or may be probed immediately. For detailed protocols, the reader is referred to other sources [46, 47].

Interpretation of the Southern blot is easy when the question is whether or not a gene is present in a particular sample, as long as appropriate positive and negative controls are included (a sample of DNA known to be positive and a sample of DNA known to be negative). The presence of multiple copies of a gene indicates gene amplification, which may occur in oncogenes during cancer development [48, 49]. Amplifications are obvious on a Southern blot as a band or bands that are more intense than the normal single-copy control; numerical values that reflect intensity may be assigned to bands using a densitometer. Structural aberrations in a gene of interest can be detected by Southern blotting, including the insertion or deletion of nucleotides or gene rearrangements (Fig. 2.4). When nucleotides are mutated, inserted, or deleted, the ladder of fragments produced may be abnormal due to the obliteration of restriction sites, the generation of novel restriction sites, or alterations in fragment size due to an increase or decrease in the number of nucleotides between restriction sites. The majority of these aberrations are apparent on Southern blots as abnormal banding patterns. The Southern blot remains a useful and reliable way to obtain definitive data on gene structure.

2.2.6 Polymerase Chain Reaction (PCR)

The development of PCR has increased the speed and accuracy of DNA analysis, and has resulted in the rapid development of new and creative techniques for detecting, replicating, and modifying DNA. Since it was described originally [52, 53], PCR has evolved to encompass an enormous array of specific applications. This section will cover the basic concepts of PCR and several applications that are useful in molecular analyses of cancer. For a complete technical description of PCR techniques, the reader is referred to a more detailed source [50].

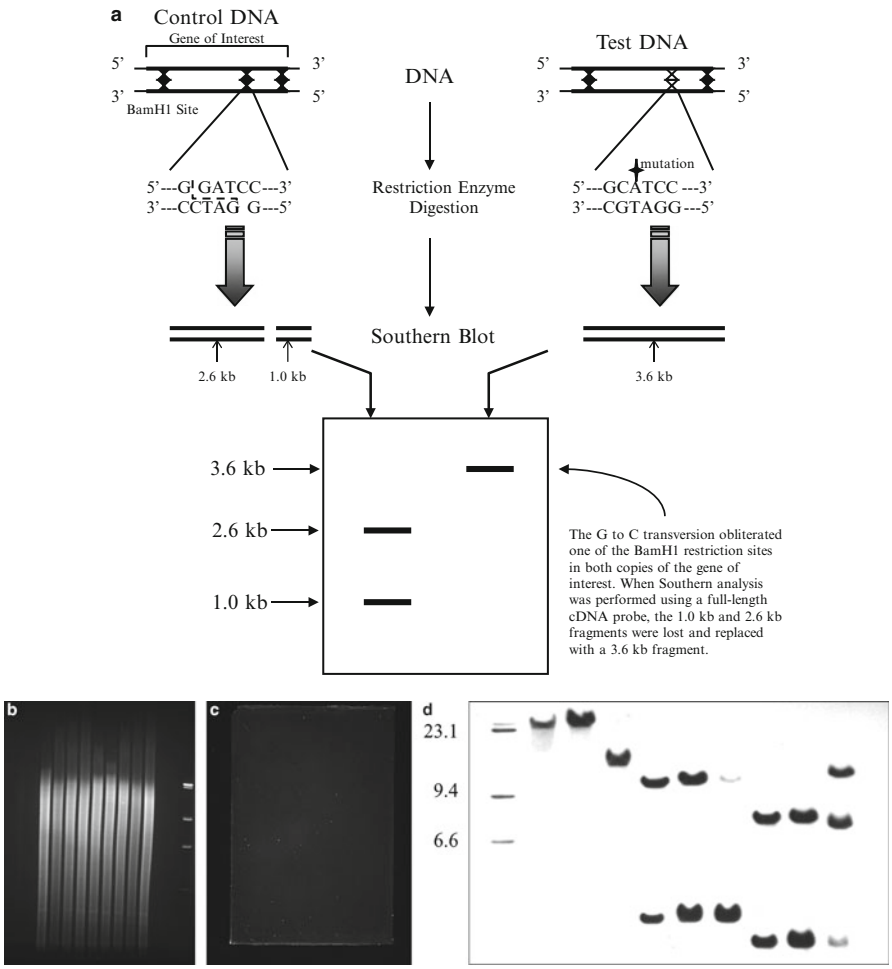

Fig. 2.4 Southern blotting. Variations in DNA sequences that alter restriction endonuclease recognition sites can be detected by Southern blotting (**a**). After digesting genomic DNA with restriction endonucleases, the DNA is subjected to agarose gel electrophoresis and then transferred to a membrane. The agarose gel can be imaged before (**b**) and after (**c**) the transfer has taken place to confirm adequate amounts of digested DNA and proper transfer of the DNA from the gel to the membrane. The membrane is then incubated with radiolabeled or chemiluminescent DNA probes which hybridize to specific DNA fragments. Exposing the membrane to film allows for visualization and sizing of these fragments (**d**).

2.2.6.1 Principles of PCR

Every PCR reaction must contain several key components: a small amount of target DNA to be used as a template; a thermostable polymerase, such as *Taq* polymerase that is not denatured at high temperatures; a pair of single-stranded DNA oligonucleotide primers; and deoxynucleotides (dNTPs), the building blocks for the DNA to be amplified. The DNA may be genomic DNA isolated directly from experimental or patient material, or it may be cDNA that has been synthesized from an RNA template by reverse transcriptase. The target for any PCR reaction is dictated by the specific oligonucleotide primers used. Two primers are designed to anneal to sites at either end of the region of interest, on opposite template strands (Fig. 2.5). The primers are extended in the 5′–3′ direction by DNA polymerase to yield overlapping copies of the original template. PCR is a cyclic process, consisting of three steps: denaturation of template (94 °C), annealing of primers (temperature is sequence dependent; often between 50 and 60 °C), and extension of primers (72 °C). The three steps are repeated, with each cycle resulting in amplification of the target sequence. By the end of the third cycle, a new double-stranded molecule is formed in which the 5′- and 3′-ends coincide exactly with the primers [51, 52]. The copies that are produced of this targeted DNA sequence are referred to as the amplicon. These double-stranded molecules accumulate exponentially during subsequent cycles of PCR, so that the majority of products are of a defined size and are seen clearly as a sharp band upon electrophoretic separation. Due to the incredible sensitivity of PCR, even a miniscule amount of DNA can be amplified, which makes it a powerful tool but also mandates that precautions are taken to avoid introduction of contaminating DNA which could result in misinterpretation.

2.2.6.2 Design of Primers for PCR

When constructing primers for PCR, it is important to keep in mind a few basic concepts. Primer length can influence target specificity and efficiency of hybridization. As a general guideline, primers should be 20–30 nucleotides in length. Whenever possible, both primers should be the same length because primer length is considered when calculating an appropriate annealing temperature. The base composition of the primers is also important, since annealing temperature is governed in part by the percent G+C content of the primers. Ideally, G+C content is between 40–60 %, and the percent G+C should be the same in any primer pair. A simple formula can be used to

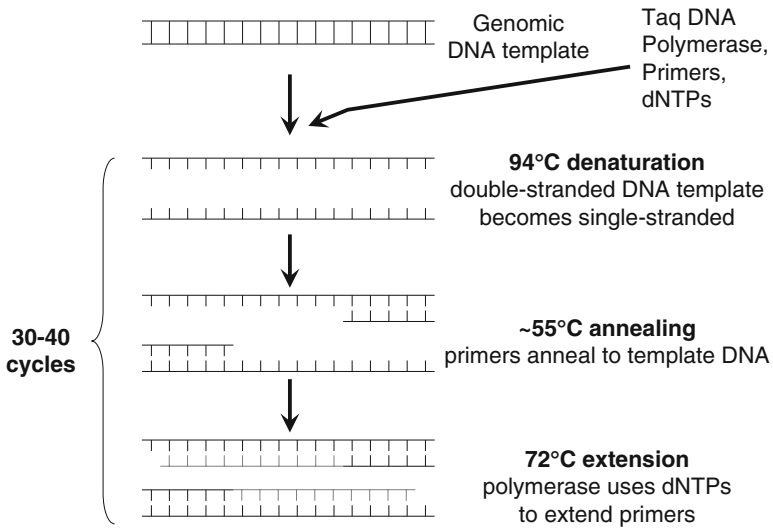

Fig. 2.5 The polymerase chain reaction (PCR). Specific sequences of DNA are amplified by PCR using pairs of oligonucleotide primers. Genomic DNA, primers, *Taq* DNA polymerase, and deoxynucleotides (dNTPs) are combined in a suitable buffer and subjected to repeated cycles of alternating temperatures which denature the template DNA, allow for primer annealing to the template and finally extend the primer to create a new strand of DNA.

calculate an appropriate annealing temperature for any given primer: $T_m = 69.3 + 0.41(\%G + C) - (650/L)$, where L = primer length in bases [53]. Repetitive or palindromic sequences should be avoided in a primer to avoid self-hybridization, and primer pairs should not contain sequences complementary to each other to avoid primer dimers.

2.2.6.3 The Role of Polymerase in PCR

A DNA polymerase enzyme is essential for the primer extension step of PCR. Early PCR experiments employed the Klenow fragment of *E. coli* DNA polymerase I, but this enzyme is heat labile and must be replenished with each amplification cycle. The developments of thermostable DNA polymerase and commercially available thermal cyclers have greatly improved the efficacy of PCR methodology. *Taq* DNA polymerase was isolated from *Thermus aquaticus*, and is characterized by its 5′–3′ exonuclease activity, thermostability, and optimum performance at 70–80 °C [54, 55]. Temperature, pH, and concentration of Mg^{++} influence the activity of *Taq* polymerase. Lower divalent cation (Mg^{++}) concentrations decrease the rate of dissociation of enzyme from template by stabilizing the enzyme-nucleic acid interaction [56]. The optimum pH for a given PCR reaction will be between 8.0 and 10.0 (usually ~8.3), but must be determined empirically. While *Taq* DNA polymerase is ideal for routine PCR, there are many other DNA polymerases with unique qualities which make them useful for special PCR applications such as amplification of long stretches of DNA or high-fidelity amplification [56].

2.2.6.4 Detection of PCR Products

Once the PCR is complete, the products must be analyzed and interpreted. Amplification products of routine PCR reactions can be separated by standard agarose gel electrophoresis and visualized by staining with ethidium bromide or other DNA dye. When finer resolution is needed, such as in the analysis of very small (<100 bp) products, polyacrylamide gel electrophoresis is standard. In addition, newer technologies such as bead arrays, microarrays, and capillary electrophoresis can be used to detect PCR products (Fig. 2.6).

2.2.6.5 Contamination Issues

Due to the extreme sensitivity of PCR, care must be taken to avoid exposing PCR reagents, set-up areas, and equipment to DNA that is not intended to be a part of the PCR reaction, especially amplicons from previous PCR runs. Unwanted false-positive results can occur if issues of contamination are not addressed. One laboratory practice that is often used to avoid this type of contamination issue is designating separate pre- and post-PCR work areas. After a PCR run is complete, all handling of the amplicon, such as for gel electrophoresis, is performed in an isolated work area with equipment that is designated for post-PCR use only. Pipettes, pipette tips, and buffers, for example, used in post-PCR handling should not be outside of the area designated for post-PCR work. Gloves used in this area should also be discarded before returning to other work areas in the lab. Additionally, the use of filtered pipette tips, especially in the pre-PCR work area, can help in eliminating PCR contamination issues.

2.2.7 Modifications and Improvements of PCR

Many variations to the original PCR procedure have been developed to improve its utility, ease, sensitivity, and specificity. Many companies now offer PCR master mixes which contain a DNA polymerase and dNTPs in buffers optimized to amplify most target sequences. This often eliminates the need for exten-

sive optimization of PCR components and cycling conditions and simplifies PCR set-up by only requiring the addition of a DNA template and primers to the master mix. In order to eliminate some types of PCR contamination, some master mixes are available that contain the enzyme uracil N-glycosylase (UNG) and the nucleotide dUTP along with the normal dNTPs needed for PCR. If a PCR is contaminated by an amplicon from a previous PCR made with dUTP, a pre-amplification incubation will degrade the contaminating amplicon, preventing it from being used as a template in the current PCR.

2.2.7.1 Hot Start PCR

Hot start PCR was developed to reduce background from nonspecific amplification by preventing polymerization of new DNA during the set-up and the initial phase of the reaction when nonspecific binding may occur between primers and other DNAs in the mixture [57, 58]. Hot start may be achieved by limiting the initial Mg^{++}, dNTP, or enzyme concentration, or by separating the components with a barrier, such as wax beads that melt as the mixture is heated. Alternatively, hot start polymerases bound by antibodies or

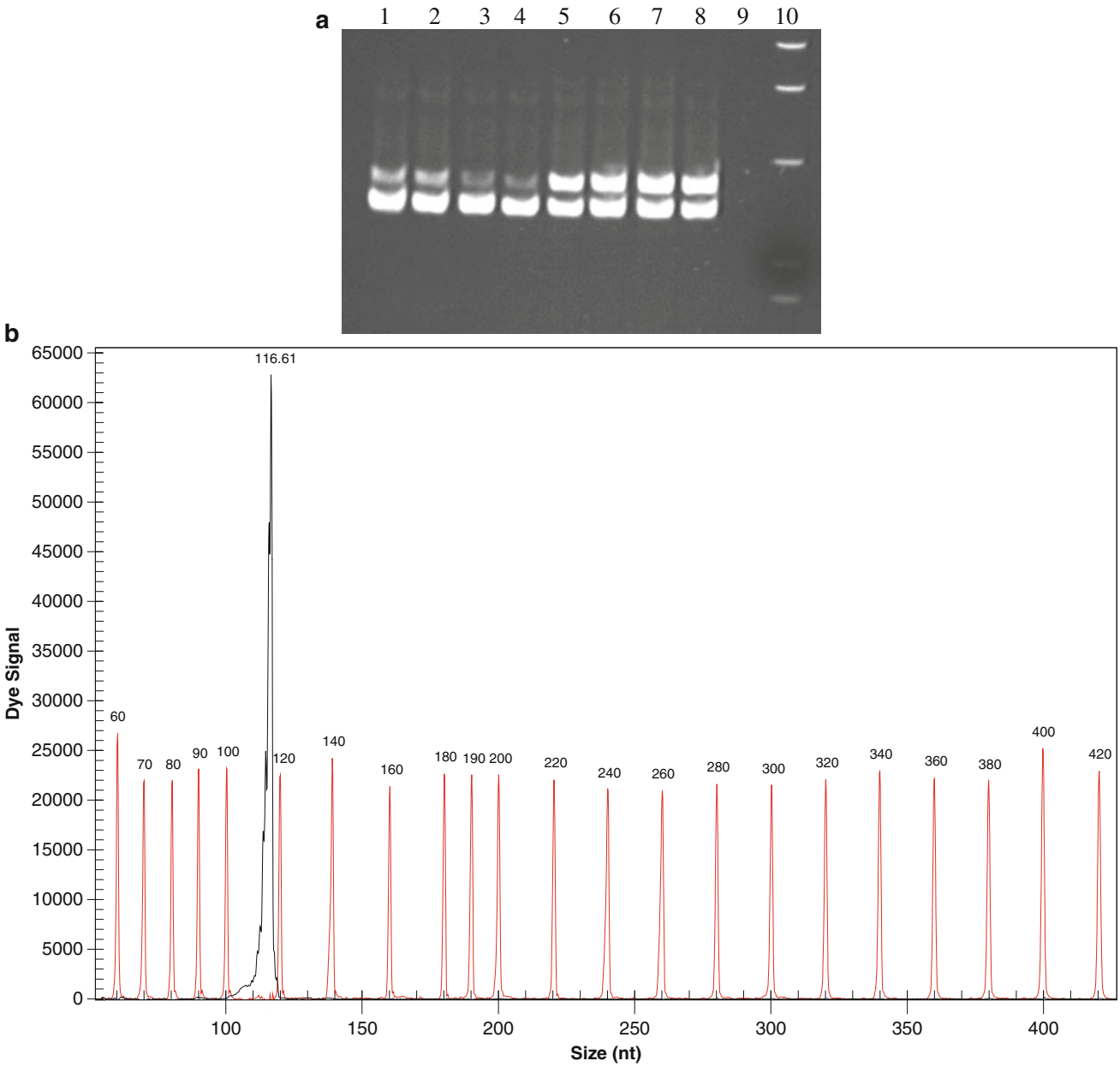

Fig. 2.6 Methods for analysis of DNA amplified by PCR. Gel electrophoresis separates DNA fragments based on size since smaller DNA fragments migrate at a faster rate through the gel (**a**). When DNA of an unknown size is run alongside a "ladder" containing DNA fragments of known sizes, the length of the unknown DNA can be estimated. More precise sizing of DNA can be made when using capillary electrohoresis (**b**), in which fluorescently labeled DNA (*black peak*) is moved through thin capillary tubing along with a DNA sizing standard (*red peaks*) to obtain higher resolution than possible with standard gel electrophoresis. Alternatively PCR-amplified DNA can be analyzed based on sequence due to its ability to hybridize with probes in microarrays and bead arrays (**c**).

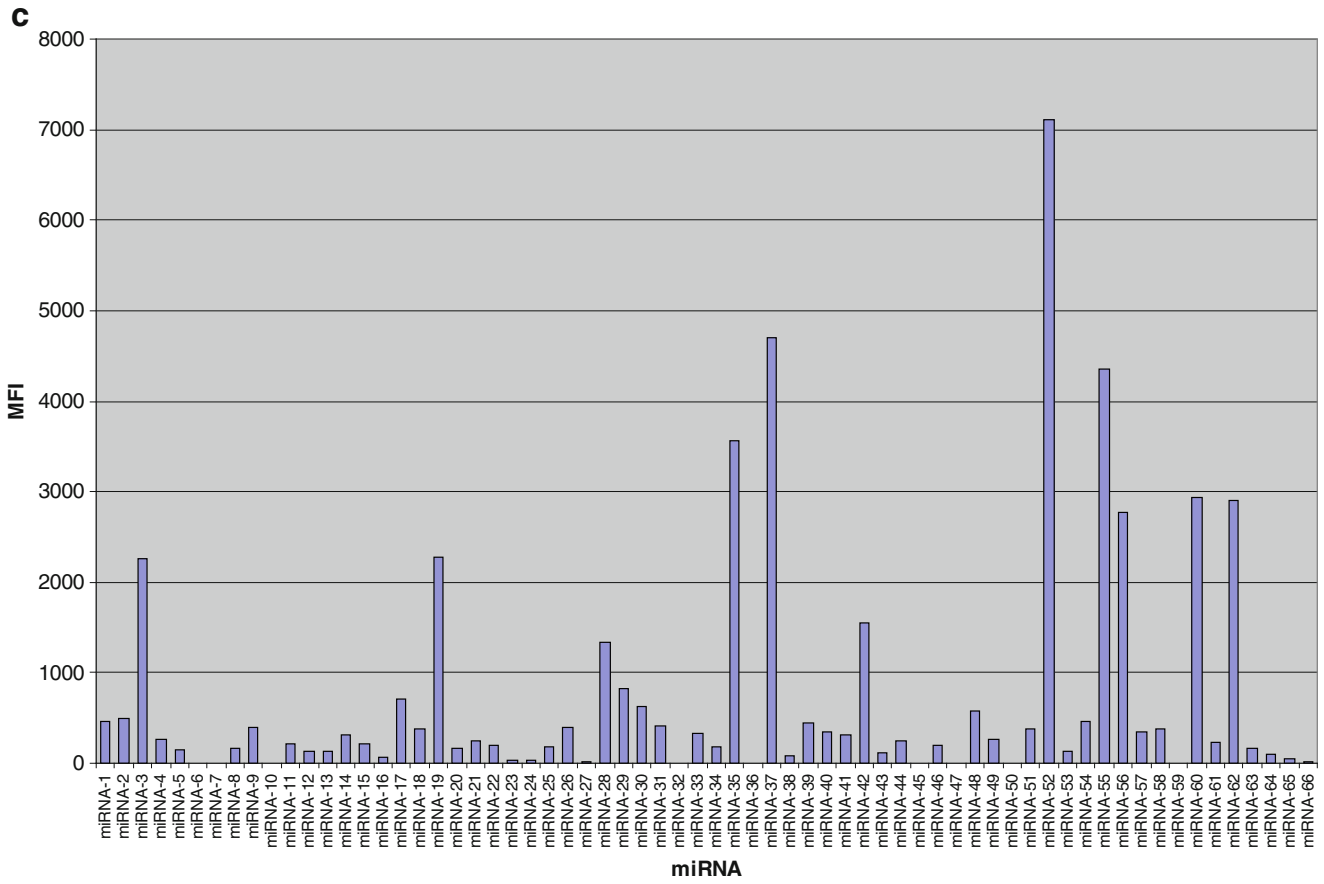

Fig. 2.6 (continued)

other molecules are enzymatically inactive until a high temperature incubation (immediately preceding normal PCR cycling) activates the polymerase, thereby preventing premature or nonspecific DNA amplification in the time between mixing the components of the reaction and the PCR cycling.

2.2.7.2 Touch-Down PCR

Touch-down PCR was developed to enhance amplification of the desired target sequences while reducing amplification of artifacts [59, 60]. The initial cycle begins with an annealing temperature that is greater than the expected T_m of the primer and the annealing temperature is lowered progressively with each cycle. As a result, the desired amplicon will accumulate preferentially while the amplification of undesired products is minimal.

2.2.7.3 Nested PCR

Performing nested PCR can increase both the sensitivity and specificity of amplification [52]. The amplification product(s) generated in the first PCR reaction are used as the template for a second PCR reaction, in which primers are used that are internal, or nested, within the first primer pair. Nonspecific products that are produced within the first round of PCR are not likely to contain sequences complementary to the nested primers, so that spurious products are eliminated during the second round of PCR. Extremely rare target sequences can be detected using nested PCR, since the first round of PCR effectively amplifies the specific template for the second round of PCR. Due to the sensitive nature of nested PCR, special care must be taken to avoid contamination.

2.2.7.4 Long-Distance PCR

It is possible to amplify sequences as large as 50 kb using long-distance PCR (LD-PCR) [61]. One step toward successful LD-PCR is the use of thermostable, long-life polymerases that are capable of generating long strands of cDNA. The first LD-PCR was accomplished by using a 5′-endonuclease-deficient, N-terminal deleted variant of *Taq* DNA polymerase in combination with Pfu DNA polymerase in a 180:1 ratio [62]. Many special DNA polymerases capable of performing well in LD-PCR are now available commercially. Other prerequisites for successful LD-PCR are high quality DNA for use as template, and carefully constructed primers with matching melting temperatures.

2.2.7.5 Reverse Transcriptase PCR (RT-PCR)

Specific RNA sequences can also be detected using PCR. An enzymatic reaction using reverse transcriptase creates a complementary DNA (cDNA) copy of the RNA, which can then be amplified using standard PCR conditions. RT-PCR is

a useful tool for detecting the presence or absence of mRNAs for specific genes and can be performed quantitatively or semiquantitatively to detect variations in gene expression between samples.

2.2.7.6 Multiplex PCR

Often it is desirable to amplify more than one target sequence at a time. The use of more than one pair of PCR primers in a single reaction is often possible, although these multiplexed reactions become increasingly difficult to optimize with increasing numbers of amplicons. Designing primers with similar melting temperatures helps ensure successful multiplexed PCRs and using Mg^{++} concentrations that are higher than normal PCRs is usually necessary.

2.2.7.7 Quantitative PCR

Quantitative PCR provides a quick and simple alternative to Southern blot analysis for the evaluation of gene copy number or gene expression levels [63]. The underlying premise for quantitative PCR is that the accumulation of amplified products occurs exponentially and follows a predictable curve. The overall profile of product accumulation throughout the course of a reaction may be reproducible enough to extrapolate the amount of starting material. Accurate quantification requires that the analysis be done during the exponential (sloped) part of the amplification curve, and not during the plateau phase when the DNA amplification rate has leveled off. Accurate quantitative PCR experiments using traditional (endpoint) PCR methods must include control template fragments, which may be synthesized or may be isolated from other sources. The control fragments should have priming sites and secondary structure that is identical to the test DNA, but should be sufficiently different in size that they can be discriminated upon electrophoretic separation. In a typical quantitative PCR reaction, replicate tubes are prepared with a fixed concentration of test DNA; then known quantities of control DNA are added in a range of concentrations, PCR is conducted, and the samples are subjected to gel electrophoresis. When one template is in excess, it will yield a greater abundance of PCR product; but when the concentration of both the control and test templates are equal, amplification will occur at equal rates, ultimately producing two bands of equal intensity on the gel. Quantitative approaches to PCR are useful when careful attention is paid to experimental reproducibility [63, 64]. Although this method can achieve quantitative results, it is rather tedious and has been replaced in most situations by one of the most useful modifications of the basic PCR method: real-time PCR.

2.2.8 Real-Time PCR

Previously, DNA amplified by PCR was analyzed in a post-PCR process. Newer technologies allow users to observe the amplification of DNA as each cycle occurs, in real-time. Real-time PCR eliminates the need for post-amplification analysis, often saving hours of analytical time and minimizing the risk of contamination. Real-time PCR also increases the ease with which quantitative results can be obtained and thus be applied to gene dosage and expression analysis. For this reason the term "quantitative PCR" (qPCR) has become synonymous with real-time PCR.

Real-time PCR is made possible by the use of fluorescent signals which increase as the target sequence is amplified. This can be accomplished in several ways, but the two main methods include the addition of SYBR Green (or similar dyes that emit fluorescence in the presence of double-stranded DNA) to a standard PCR and using fluorescent probes specific for sequences between PCR primers.

SYBR Green is a dye that fluoresces only in the presence of double-stranded DNA. As the amount of amplicon increases during each PCR cycle, the amount of fluorescence also increases. Measuring the amount of fluorescence at each PCR cycle makes it possible to graph the amplification that occurs during the PCR (PCR cycle number on the *X*-axis and fluorescence on the *Y*-axis) showing the extent of amplification over time (Fig. 2.7) [65]. Since any double-stranded DNA will be detected by SYBR Green, precautions must be taken to ensure any increase in fluorescence represents the desired amplicon from the target sequence. In standard PCR this is accomplished using gel electrophoresis to make sure the length of the amplicon matches the expected size and that no nonspecific PCR products appear on the gel.

A second and slightly more complex method for detecting amplification in real-time PCR uses oligonucleotide probes that are fluorescently labeled. The exact design of these fluorescently labeled probes can vary but they all contain a sequence complementary to a region of the target sequence between the forward and reverse primers. In one example of a fluorescent probe-based real-time PCR chemistry, 5′ hydrolysis probes or TaqMan® probes an oligonucleotide probe is designed with a fluorescent molecule such as FAM bound on one end and a quencher molecule on the other end. The fluorescent signal is usually not detected due the close proximity of the quencher. During the annealing step of each PCR cycle this probe anneals to the target sequence along with the primers (Fig. 2.8). During the extension stage of each PCR cycle, the 5′–3′ exonuclease activity of *Taq* polymerase digests the probe, separating the quencher dye from the fluorescent dye, which results in a detectable increase in fluorescence [66, 67]. This fluorescence can be used to create an amplification curve similar to the one produced when SYBR Green is used.

2.3 Microarray Technology

DNA and RNA microarrays have greatly expanded the amount of data that can be obtained in a single experiment, often using the same basic principles of hybridization as Southern blotting.

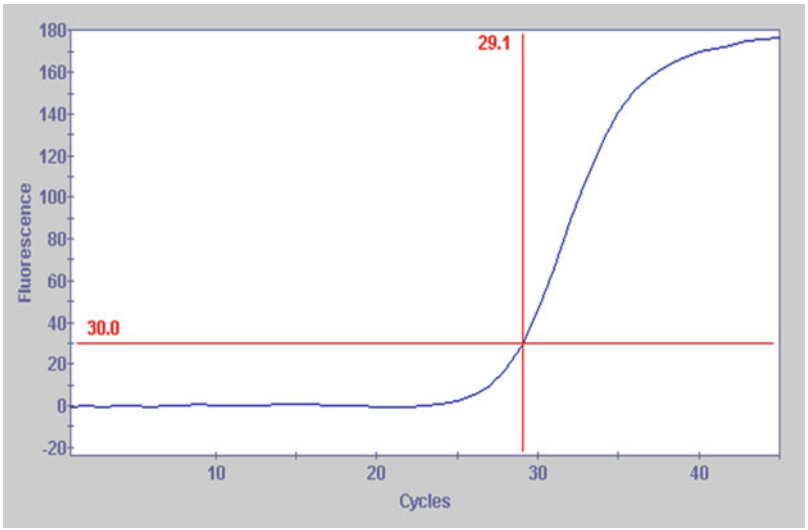

Fig. 2.7 Real-time PCR. Fluorescent detection of DNA being amplified by PCR can be in "real-time" by measuring the fluorescence emitted at each cycle. This fluorescence can be produced by adding a dye such as SYBR Green to the PCR buffer which fluoresces when bound to double-stranded DNA. Alternatively, fluorescence can be produced by adding sequence-specific probes that can be fluorescently labeled in various ways to produce fluorescence at specific points during each PCR cycle. The exact or relative amount of starting template can be inferred by determining when the amplification curve (*blue*) crosses over a certain threshold (30.0) of fluorescence. The cycle when this occurs is designated as the cycle threshold value or Ct (29.1).

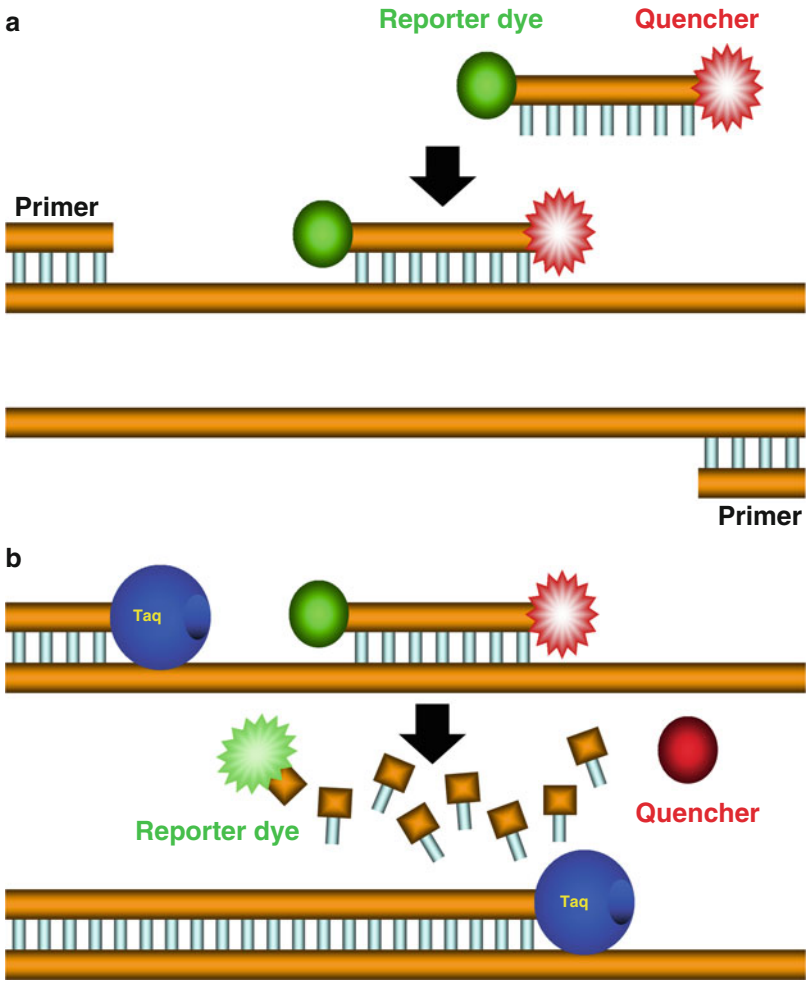

Fig. 2.8 Real-time PCR using hydrolysis (TaqMan) probes. PCR primers are used to amplify target DNA as in standard PCR. During the annealing step of each PCR cycle a fluorescently labeled oligonucleotide probe, complementary to one strand of the template DNA, anneals along with the primers (**a**). The TaqMan probe contains one detection dye (shown in *red*) whose fluorescence is absorbed by another quencher dye (shown in *green*) when the probe is intact. During the extension step of each PCR cycle (**b**) the TaqMan probes are digested. This physical separation of the dye from the quencher allows for increased fluorescence during the extension step of each PCR cycle.

Microarray designs vary greatly among manufacturers but in the more traditional arrays contain many thousands or millions of unique DNA probe sequences that appear as separate dots or features on a fixed surface. Each of these features contains a unique nucleotide sequence that represents a specific gene, chromosomal location or other nucleic acid sequence being interrogated. Labeled nucleic acids, prepared from the samples being analyzed, are incubated on the surface of the microarray to allow hybridization between the microarray probes and the sample-derived nucleic acid. A washing step is then used to remove any labeled sample that is not hybridized to reduce background signaling on the chip when scanned.

Numerous formats and applications for microarrays exist but three main microarray applications predominate in both the research and clinical laboratory setting: gene expression arrays, chromosome arrays (CMA), and genotyping arrays (Fig. 2.9). These three applications differ in the design of the microarrays and in the type of nucleic acid being interrogated [68–77]. In gene expression microarray applications, mRNA or cDNA is applied to a microarray containing features for large groups of genes, often all known genes expressed in a particular organism. The levels of gene expression can be determined by the intensity of the signals at each spot. Microarray results using mRNA isolated from two or more groups of cells or tissue can

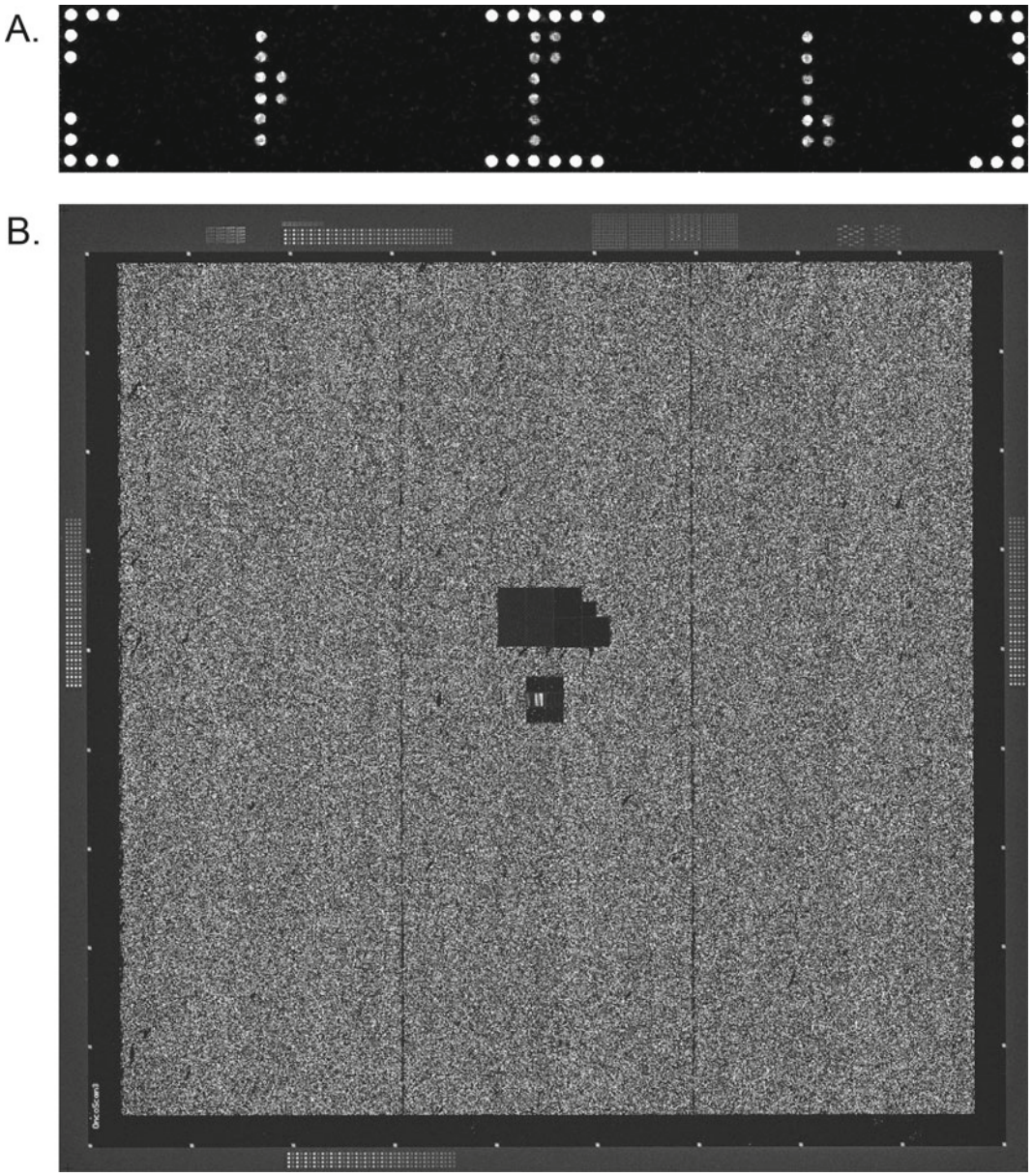

Fig. 2.9 Microarray technology. Genomic DNA or PCR-amplified DNA can be detected by hybridizing with probes that have been attached to specific locations on the surface of a microarray. Interrogation of a few or up to a few million different sequences in a DNA sample can be determined simultaneously. Genotyping of three loci is performed with a low-density array developed by Nanosphere which can use PCR-amplified or non-amplified genomic DNA (**A**). High-density arrays such as the OncoScan microarray (Affymetrix, Inc.) can detect changes in copy number and loss of heterozygosity by targeting hundreds of thousands of single nucleotide polymorphism (SNPs) across the genome (**B**).

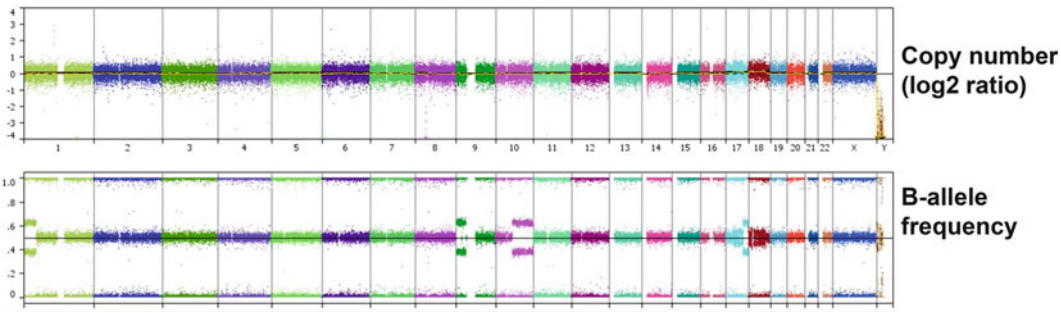

Fig. 2.10 SNP-based microarrays. Genomic DNA derived from formalin-fixed, paraffin-embedded tumor tissue can be used to detect changes in copy number and loss of heterozygosity. In this example, changes in copy number are not detected but regions of copy neutral LOH can be observed within chromosomes 1, 9, 10 and 17. This example shows microarray data from OncoScan FFPE Assay kit (Affymetrix, Inc) analyzed with using Nexus software.

be compared to look for up- or down-regulation of each gene represented on the microarray [68, 69]. This is often used to compare normal and diseased tissue, varying grades of tumors, or samples treated and untreated with a drug or other agent in the research setting. In the clinical setting, gene expression microarrays are used with metastatic tumor in which the site of the primary tumor is unknown despite the available imaging and pathological findings. The expression profile of a tumor with an unknown primary is compared to known expression profile signatures of tumors in which the site of the primary tumor is known. Clinical expression profile tests may be helpful in this setting for predicting the site of origin and choosing the most appropriate form of therapy [70, 71]. In addition to mRNA from protein-coding genes, expression microarrays can also measure the expression of a large number of regulatory noncoding microRNAs or miRNAs.

While some microarrays provide information about the presence, absence and abundance of a particular target, genotyping microarrays are able to differentiate between two alleles at a given locus. This type of technology allows for the genotyping of many thousands or millions of single nucleotide variants (SNVs), also called single nucleotide polymorphisms (SNPs), in a given sample for a relatively small cost. The methodology varies between platforms but in some cases can be as simple as having two nearly identical oligonucleotide probes for each locus being genotyped. The sequence of each probe will differ near the middle of the probe at the position of the SNV with the probe at one feature annealing to one allele and the probe at another feature annealing to the other allele. Signal at one or both of these features indicates that a sample is homozygous or heterozygous for an allele with respect to each loci represented on the microarray. High-density genotyping arrays have been used frequently in genome-wide association studies (GWAS) but lower density genotyping arrays are more common in the clinical setting to evaluate a smaller number of clinically relevant loci [72, 73].

Genotyping microarrays can be designed to examine genomic DNA or select PCR-amplified targets for any number of single nucleotide polymorphisms, variants or mutations [72]. In the clinical laboratory, these SNP microarrays have been used as a tool to create low to high density multiplexed genotyping assays [73, 74].

Chromosome microarrays (CMA) detect copy number variations (CNVs), such as deletions, duplications and gene amplifications in genomic DNA samples. These changes in copy number include trisomies or monosomies of entire chromosomes or smaller CNVs affecting only one arm of a chromosome or smaller regions of chromosomes. The dense coverage across each chromosome offered in many chromosome microarrays allows for detection of CNVs down to the range of 10–100 kb, which would be too small for detection by traditional cytogenetic techniques [75–77]. Depending on the design of the microarray used, CMAs can fall into one of two main categories. Copy number microarrays, commonly called array comparative genomic hybridization (aCGH), previously used BAC probes and now shorter oligonucleotide sequences to compare signal intensities across the genome of an unknown or patient same to that of a normal control genomic DNA sample. Alternatively, CMAs based on high-density SNP arrays similar to the high-density genotyping microarrays described above detect both copy number variations and long contiguous stretches of homozygosity. These stretches of homozygosity may suggest consanguinity, uniparental disomy (UPD) or in cancer, copy neutral loss of heterozygosity (cnLOH) (Fig. 2.10).

2.4 Sequencing Technologies

2.4.1 Sanger Sequencing

For many years DNA sequencing has been an essential tool in both the research and clinical laboratory. Until recently, DNA sequencing has been performed almost exclusively by Sanger sequencing technology in which target DNA, amplified by PCR or cloning into a bacterial vector, is sequenced by the extension of a single oligonucleotide primer by a DNA polymerase in the presence of both dNTPs and labeled dideoxynucleotide triphosphates (ddNTPs). The addition of

a ddNTP blocks the addition of any other dNTPs or ddNTPs, creating a somewhat random assortment of single-stranded DNA molecules of various lengths, each labeled with a fluorescent molecule corresponding to the ddNTP added on the 3′ end (A, T, C, or G). These DNA fragments are separated, most commonly by capillary electrophoresis, to create an electropherogram from which the DNA sequence following the sequencing primer can be determined [78].

The sequencing of individual human genes and later, the entire human genome, led to the identification of countless sequence variants with pathological significance in the fields of genetics, hematology, and oncology. Likewise, sequencing portions of or entire genomes of bacterial and viral pathogens provided valuable biological information regarding infectious disease processes and also lead to the advent of molecular infectious disease testing. Clinical sequencing of bacterial 16S rRNA genes aids in identification of unknown organisms and sequencing of HIV genes from patient specimens can identify the presence of mutations causing resistance to antiviral therapies [79, 80]. Information obtained from sequencing also makes it possible to design clinical molecular tests to monitor viral loads in patients infected with HIV, HCV, BKV, and others and to quickly detect organisms that are difficult to grow and identify in culture.

Although traditional DNA sequencing is an invaluable tool in molecular diagnostics, it does have its limitations. A single sequencing reaction can produce a read length of approximately 600 nucleotides and often requires another sequencing reaction of the opposite DNA strand (bidirectional sequencing) for confirmation. When the sequencing of large stretches of DNA is required, the costs and time involved can be limiting. The analytic sensitivity of traditional sequencing methods can also be problematic in some situations. Sanger sequencing can easily detect two alleles when they are present at nearly equal frequencies as in genomic DNA samples that is heterozygous at a given locus. In some situations, however, an allele or sequence may be present at levels below 10–20 % of the total targeted DNA sequence which may not be detectable by Sanger sequence [81]. For example, tumor specimens submitted for somatic mutation testing often contain a significant amount of non-tumor cells. Germline DNA from these cells can dilute the signal from a low-level somatic mutation to the point of where the signal from the mutation is indistinguishable from background noise.

2.4.2 Next-Generation Sequencing

Newer DNA sequencing technologies, normally referred to as next-generation sequencing, have been developed by several companies that can produce massively parallel sequencing data to overcome some of these limitations of more traditional DNA sequencing [82]. Instead of the individual reads of amplified DNA segments obtained by Sanger sequencing, next-generation sequencing can produce large numbers of shorter reads across an entire gene, group of genes, or an entire genome with each segment of DNA being sequenced tens, hundreds, or thousands of times. These features provide an increased ability to detect low-level variations and also make sequencing across large stretches of DNA more feasible. The cost of sequencing an entire genome is currently too high to make the regular use of whole genome sequencing a reality in the clinical setting but more targeted approaches to these next-generation sequencing technologies are becoming more feasible. Assays capable of sequencing an entire gene or group of genes related to a specific genetic syndrome or cancer have quickly infiltrated clinical laboratories in recent years. In coming years this approach to large-scale sequencing may replace current multiplexed genotyping methods used to screen patients for large numbers of mutations such as those in the *CFTR* gene responsible for cystic fibrosis and the often tedious, large-scale approaches to whole gene sequencing to detect mutations that can predispose carriers to cancers [83]. The coverage or read-depth can also be used to detect low-level somatic mutations found in various tumor types. Although the expenses involved in next-generation sequencing are still quite high the technology is becoming more and more affordable everyday and the typical cost per nucleotide sequenced for laboratories using these technologies on a regular basis is much lower than traditional Sanger sequencing [84].

Although the chemistry and workflow can vary greatly between next-generation sequencing platforms and even between different applications on the same platform, there are a number basic steps that are common to most next-generation sequencing. After isolating DNA from a specimen, the sample must be processed in such a way to enrich for the desired target sequences and to modify the DNA to make it suitable for sequencing. For example, in clinical testing of cancer specimens, this target enrichment may be performed using PCR to specifically amplify exons of cancer genes known to be hotspots for clinically relevant mutations. After PCR amplification various adapter oligonucleotide sequences can be attached to the ends of the amplified DNA. These adapters often include molecular barcodes to uniquely identify sequences obtained from a specific sample. This barcoding allows for pooling of multiple samples in a single sequencing reaction. If PCR-based enrichment is not used, the addition of adapters can be used along with various capture methods to enrich for the desired sequences. Capture-based enrichment is typically preferred when sequencing larger numbers of target sequencing, as is needed in whole exome sequencing [85, 86]. Regardless of the method used, these prepared libraries DNA to be sequenced can be pulled together and processed on next-generation sequencing platform [87]. After the sequencing is complete, the raw sequence data must be submitted to a bioinformatics pipeline to align the sequence reads to a reference sequence, make variant calls and then filter out benign variants and rank the remaining variants based on clinical significance. This pipeline may vary greatly based on platform, application,

types of variants expected and the preference of a particular lab. Much of this pipeline may be automated but manual review and interpretation is always needed to make the final calls and interpretations. Various software programs and algorithms can be used for this alignment and variant calling and care must be taken to select a suitable component of an analysis pipeline. Compared to more focused sequencing, one of the biggest challenges to successfully implementing clinical next-generation sequencing is the interpretation of variants and dealing with a larger number of variants of unknown significance. This becomes more of an issue as one moves from gene panels to whole exome to whole genome sequencing. In some situations where sequencing a larger number of exons or genes may be technically and financially viable, restricting the targeted sequences of an assay may be desired to reduce the possibility of uncertain findings [88-91].

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Cancer Genomics

3

Jonathan R. Pollack

3.1 Cancer Genomics

3.1.1 Genetics Versus Genomics

Cancer is at heart a genetic disease, resulting from the accumulation of somatic mutations (and epigenetic alterations) in key genes controlling processes like cell proliferation and cell death, that together drive cancer development and progression. Over the past three decades, cancer researchers have made enormous progress identifying and characterizing important cancer genes, like the oncogenes *MYC*, *KRAS*, and *ERBB2*, and the tumor suppressor genes *RB1* and *TP53*. For most of that time, cancer geneticists have studied one or a small number of genes at a time.

If cancer genetics is the genetic study of cancer genes, cancer genomics is the study of whole cancer genomes. Increasingly, newer technologies like DNA microarrays have enabled researchers to study thousands of genes simultaneously, essentially characterizing genetic or gene expression variation across whole cancer genomes. As shall be seen, genomic-scale or global analyses are much more than the sum of their parts. In addition, several overarching considerations emerge: (1) Discussions of cancer genomics often emphasize emerging technologies. However, the real novelty and power of genomic approaches is that they provide an entirely new looking glass onto cancer through the exploration of high-dimensional (i.e., many genes, many samples) datasets, revealing patterns of genes with biological meaning and clinical relevance. (2) Genomics studies are exploratory investigations, and as such have been disparaged as descriptive, non-hypothesis-driven, or perhaps at worst fishing expeditions. However, a steady stream of paradigm-shifting discoveries has silenced most critics, and such hypothesis generating research is now increasingly recognized as an important complement to traditional hypothesis-driven research. (3) Analyzing and critically evaluating genomics data requires knowledge and facility in basic statistical and biocomputational methods. Cancer research, like other fields in biological sciences, is becoming increasingly quantitative, and the cancer researcher at any level of training benefits from a solid biostatistical foundation. (4) Cancer researchers have long benefited from sharing reagents like cell lines, antibodies, recombinant DNA clones, and even DNA sequences submitted and curated in public databases like Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Cancer researchers now benefit as well from the public availability of well-annotated genomics datasets. Depositing genomics data in public repositories is increasingly a requirement for journal publication, and regardless should be viewed as a community responsibility, especially for publicly funded research.

3.1.2 Spectrum of Genomic Aberrations

The immediate objective of cancer genomics is to catalog the genetic and epigenetic aberrations of cancer genomes. Different methods are used to assay different types of aberrations, and it is therefore useful to enumerate the major classes. DNA sequence changes can result in mutant or truncated proteins which can activate oncogenes or inactivate tumor suppressors. Chromosome translocations can result in aberrantly expressed oncogenes, or create gene fusions and chimeric oncoproteins. DNA copy number alterations (CNAs) include unbalanced chromosome number (i.e., aneuploidy), unbalanced chromosome rearrangements, and focal DNA amplifications and deletions, all of which alter the dosage and potentially expression of affected cancer genes. Loss of heterozygosity (LOH) refers to the absence of one parent's alleles along a chromosome segment, caused by deletion or from a copy-number neutral event like mitotic recombination, and can result in loss of the wild type allele of a tumor suppressor. In addition to these genetic aberrations,

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_3

cancer cells amass epigenetic changes—DNA modifications that are heritable through cell divisions, but do not involve mutations in the DNA itself. Such epigenetic changes include principally DNA methylation and chromatin modifications such as histone acetylation and methylation. Finally, all the above mentioned genetic and epigenetic aberrations result in the altered expression of genes and proteins. Measuring gene expression can therefore provide an integrative readout of the genetic and epigenetic changes in tumor cells.

3.1.3 Goals of Cancer Genomics

While the catalog of aberrations is the currency of cancer genomics research, the broader goals are multifold. Some studies aim to improve our basic understanding of cancer, for example by discovering new cancer genes, or defining genes and pathways underlying processes like cancer metastasis. Other studies are more clinically oriented. For example, gene-expression patterns (also called signatures) are sought to refine cancer diagnosis and classification, which guide selection of therapy. Likewise, gene-expression signatures are sought to improve prognostication, to predict response to specific therapies, and to monitor therapeutic efficacy. Genomics approaches can also speed cancer drug development, for example identifying new drug targets and mechanisms of action, and anticipating off-target effects, facilitating the ultimate goal of personalized medicine and tumor-tailored therapies.

3.2 DNA Microarrays for Expression Profiling

3.2.1 DNA Microarray Platforms

The workhorse for cancer genomics research over the past 10–12 years has been the DNA microarray. DNA microarray technology arose in the 1990s, building on two concurrent advances. First, large-scale sequencing efforts, preceding the Human Genome Project and targeting cDNAs (the expressed complement of genes), provided DNA sequences and physical clone sets for thousands of human genes. Second, new microfabrication methods permitted the manufacture of microarrays, comprising thousands of DNA sequence features (functioning as hybridization probes) ordered on the surface of a slide or chip as a high density array of rows and columns.

Two different DNA microarray platforms emerged. Patrick Brown and colleagues at Stanford University developed cDNA microarrays, in which PCR-amplified cDNA clones (0.5–2 kb in length and each representing a different gene) are robotically spotted onto a glass microscope slide [1]. This relatively low cost spotting technology was adopted at many academic centers. Meanwhile, Stephen Fodor and colleagues at Affymetrix (Santa Clara, CA) adapted photolithography methods from the semiconductor microchip industry to manufacture GeneChips [2, 3]. In this process, a series of light masks is used to direct the highly parallel in situ synthesis of DNA oligonucleotide probes on a silicon wafer. The oligonucleotide probes are 20–25 nucleotides in length, and typically multiple different probes are used to report on each gene.

While there were many potential uses for DNA microarrays, profiling gene expression became the predominant application in the early years. The power of the technology derives from the ability to measure, in the case of gene expression, mRNA levels across thousands of genes simultaneously. Microarray technology continues to evolve, and there are now many different in-house and commercial options. One general trend has been the increased use of oligonucleotide probes, eliminating the need to track recombinant DNA clone sets. Another trend has been the increased number of probe features (along with feature density), permitting the assay of additional genes, transcript variants or genetic loci.

3.2.2 Labeling and Hybridization

Protocols for profiling gene expression differ among microarray platforms. Experiments using spotted cDNA (or oligonucleotide) microarrays are typically carried out as two-color hybridizations (Fig. 3.1). Two different RNA samples are labeled with two different fluorescent dyes (e.g., Cy5 and Cy3), typically using reverse transcriptase to incorporate fluorophore-conjugated nucleotides in a first-strand cDNA synthesis reaction. The labeled nucleic acid target molecules are then combined and hybridized to the microarray, where they bind their cognate DNA sequence probes by Watson–Crick base pairing. Following washing to remove unbound nucleic acid, the microarray is imaged using a fluorescence scanner. For each gene represented on the microarray, the ratio of fluorescence represents the relative level of that gene's expression (i.e., transcript abundance, a dynamic balance between synthesis and degradation) between the two samples. Two-color hybridizations permit robust target quantification in spite of potential variation in hybridization conditions, since any effect on the ratio numerator will be canceled by a similar effect on the denominator.

Experiments using Affymetrix GeneChip arrays, in contrast, are carried out as one-color hybridizations (Fig. 3.1). A single RNA sample is labeled by cDNA synthesis, using an oligo(dT) primer with a T7 RNA polymerase promoter site,

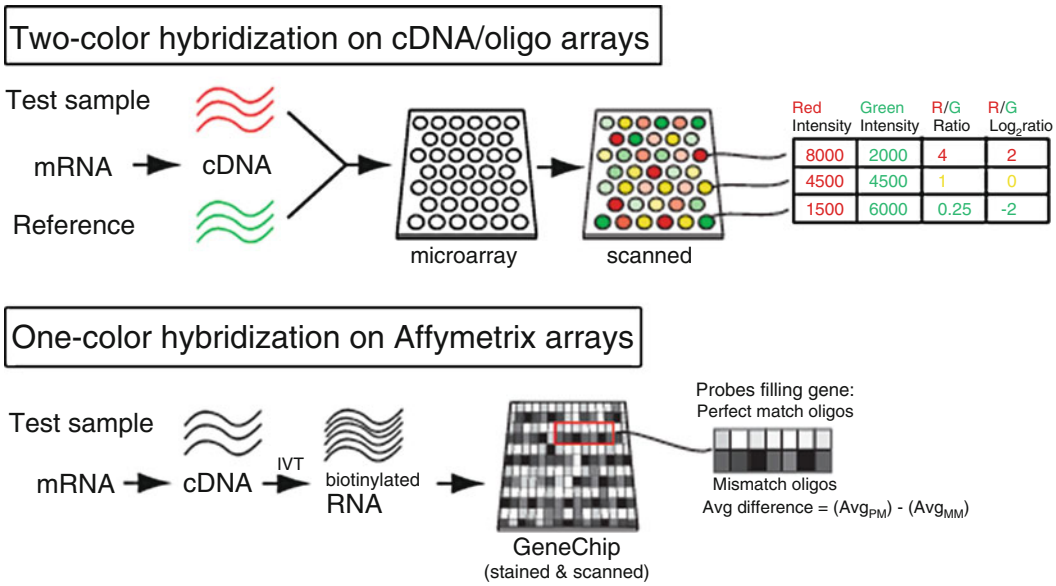

Fig. 3.1 Expression profiling schema. A*bove*, schematic depiction of two-color hybridization on cDNA or oligonucleotide microarrays. RNA isolated from test and reference samples is labeled with two different fluors (here *pseudocolored red* and *green*), then co-hybridized to the array. For each gene on the array, the ratio of red/green fluorescence (typically reported in $\log_2$ scale) reflects its relative transcript abundance in the test compared to reference sample. *Below*, schematic depiction of one-color hybridization on Affymetrix GeneChip oligonucleotide arrays. RNA is biotin-labeled by in vitro transcription (IVT), then hybridized to the array. Multiple different probes report on each gene, where expression levels can be represented by the difference in average staining intensity between perfect match (PM) and control mismatch (MM) probes.

followed by in vitro transcription in the presence of biotinylated nucleotides. The resultant biotinylated antisense RNA targets are then fragmented and hybridized to the microarray, where they bind to their cognate sense oligonucleotide probes. Following washing, a streptavidin–phycoerythrin fluorophore conjugate is used to stain bound target which is then visualized by fluorescence scanning. For each gene represented on the array, the fluorescence intensity reflects that gene's level of expression in the labeled sample.

3.2.3 Experimental Design Considerations

For experiments comparing test and reference samples, a two-color microarray platform provides a more direct comparison. Examples of such studies include treatment (e.g., drug or siRNA) versus control, tumor versus matched normal, or time course experiments where the zero time point is a natural reference. For studies profiling collections of tumors there is no such natural reference, but a universal reference RNA, e.g., comprising pooled RNA from a set of tumor-derived cell lines, can be used [4]. Since all tumor samples are hybridized against the same reference RNA, tumor gene-expression ratios share the same denominator and can be compared amongst one another [5]. For tumor profiling studies, a single-color microarray platform obviates the need for a reference RNA.

Like any other assay, microarray results need to be reproducible. Technical replicates, where the same sample is labeled and hybridized again, can be used to verify measurements. Dye-swap experiments, where for two-color hybridizations the fluorophores used to label the test and reference sample are interchanged, serve as a control for possible dye-labeling bias. Biological replicates, in which the experimental samples are themselves recreated, and then labeled and hybridized, provide the greatest measure of reproducibility, controlling for biological as well as technical variation [6]. However, for exploratory profiling studies, assaying additional cancer samples may generate greater depth of information than assaying fewer samples, but in replicate. Microarray results, at least for key genes, are often verified using independent assays. Such technical validation might include Northern blot or quantitative reverse-transcription PCR (qPCR). Using a second, different microarray platform can serve to verify measurements for many genes at once.

A goal of expression profiling studies is often to identify genes differentially expressed between two groups. Because such studies are exploratory, it is difficult to estimate the number of samples needed. Analysis of existing datasets indicates that at least five samples from each group are needed [7]. However, reason would dictate that the more similar the sample groups are to one another, the larger the number of samples would need to be profiled to identify significant expression differences.

3.2.4 Specimen Considerations

Common epithelial cancer types are heterogeneous at the tissue level, composed of varying fractions of neoplastic cells, normal epithelial cells, and tumor stroma, including fibroblasts, endothelial cells, and various immune cells. Even different parts of the same cancer may vary in these cell fractions. Studies using undissected cancer tissues should be interpreted with this cellular heterogeneity in mind. Microdissection techniques, including laser capture microdissection [8], can be used to obtain and profile specific tissue compartments like cancer epithelium. Alternatively, computational methods can be used for in silico dissection of whole cancers, where for example specific gene-expression patterns are attributed to cell types by comparison to expression patterns of cognate cultured cells [9], or by correlating patterns with cell fractions observed histologically [10].

Conventional RNA labeling methods require microgram quantities of input RNA. However, microdissected specimens typically yield only nanogram amounts. Protocols are available to amplify the input RNA. A common method of linear amplification, better preserving relative transcript abundances, makes use of in vitro transcription [11]. Alternatively, signal amplification can be used to augment the hybridization signal from low amounts of input RNA [12]. While fresh or freshly frozen specimens provide the highest quality RNA for microarray analysis, some success has been reported in extracting usable RNA from formalin-fixed paraffin-embedded (FFPE) specimens [13, 14], which are more readily available from pathology archives.

3.3 Analysis of Expression Profiling Data

3.3.1 Data Processing

After imaging the hybridized microarray, feature extraction (spot finding) software is used to locate DNA features on the microarray, and to associate annotated probe features with fluorescence intensity measurements. For two-color hybridizations on spotted DNA microarrays, background fluorescence intensity surrounding spot features is typically subtracted out to better estimate specific hybridization signal. For each gene spot, fluorescence intensities for the two colors are next converted to a single fluorescence ratio, which describes the relative expression of the corresponding gene between the two samples. By converting fluorescence intensities to ratios, information on the absolute level of expression is lost, but might be inaccurate anyway due to variation in the amount of spotted DNA probe. Fluorescence ratios are then converted to log ratios, most often using $\log_2$ values. In log space, increased and decreased expression is mathematically symmetric with opposite signs (while in linear space decreased expression is compressed within ratios between 0 and 1).

For Affymetrix GeneChip arrays, where a set of DNA oligonucleotide probes is used to report on each gene, fluorescence intensities are calculated as the mean intensity for the probe set. Nonspecific hybridization, calculated from the mean intensity of a matched set of oligonucleotide probes containing a single central nucleotide mismatch, can be subtracted out to yield an average difference. While fluorescence intensities bear some relation to absolute expression levels, target amplification is inherent in the labeling protocol and can bias transcript representation. Therefore, log intensities are typically converted to ratios as described further below.

Once fluorescence signals are extracted, the data need to be normalized to compensate for any differences in the amount of input RNA, or in the labeling or detection efficiency occurring either between two samples on the same array, or between samples on different arrays [15]. For two-color microarray hybridizations, global normalization entails scaling intensities such that the sum of all gene intensities for the two fluorescence channels is set equal (which is equivalent to scaling the average log ratio to zero). Lowess normalization can also correct for signal intensity-dependent dye biases [15]. Another approach is to use the so-called housekeeping genes, those whose expression is most invariant across samples, to normalize ratios between different samples. Likewise for Affymetrix arrays, normalization between different arrays can be accomplished by scaling intensities globally or by using housekeeping genes.

In addition to array normalization, another common data transformation is mean centering genes across a set of array hybridizations. This transformation is often applied to two-color microarray datasets such as tumor profiling studies where a universal reference RNA is used. For each gene in each sample, the log fluorescence ratio is mean centered by subtracting that gene's average log fluorescence ratio for the sample set. The result is that fluorescence ratios reflect gene expression levels in relation to the sample set average, rather than to the arbitrary universal reference. For Affymetrix datasets, log intensities for each gene are often converted to log pseudo-ratios by subtracting the average log intensity for that gene in the sample set, which in effect centers genes.

The quality of a microarray hybridization can be assessed using external spike-in controls (e.g., bacterial transcripts, with cognate probes on the array), where observed and expected fluorescence ratios can be compared. In addition, replicate features on the microarray can provide a measure of intra-array reproducibility indicative of performance. Ultimately, raw and processed microarray data files, along with annotations compliant with minimal information about a microarray experiment (MIAME) standards [16], can be deposited into a public repository like GEO [17] or ArrayExpress [18].

3.3.2 Visualizing Data

Whichever microarray platform is used, the end result of a set of array hybridizations can be summarized in a table (or matrix) of gene expression values (typically ratios) (Fig. 3.2). Each row of the table corresponds to a different gene probe on the array, each column corresponds to a different arrayed sample, and each entry in the table represents the expression level of a particular gene for a particular sample. To more readily discern patterns in the data, these numeric tables are typically visualized as colorimetric tables, or heatmaps, where expression levels are represented with a color scale (Fig. 3.2). Common colorimetric representations include red/green (or red/blue) for increased and decreased expression, respectively. Several software tools are available to process, visualize and/or perform basic analyses of microarray data, from academic (Stanford Microarray Database [19]), government (BRB-ArrayTools [20]), and commercial sources (GeneSpring, SpotFire, and Oncomine [21, 22]).

3.3.3 Analyzing Data

Microarray data are often filtered prior to analysis. For example, genes whose expression varies little across the sample set can be excluded from subsequent analysis, using ratio-fold or standard deviation cutoffs. Such data filtering can increase the subsequent yield of significant genes (by reducing the total number of genes tested), though also represents a source of variable findings among different microarray studies.

Analysis of microarray data can be broadly divided into two approaches, supervised and unsupervised [23, 24]. Supervised methods make up-front use of specimen

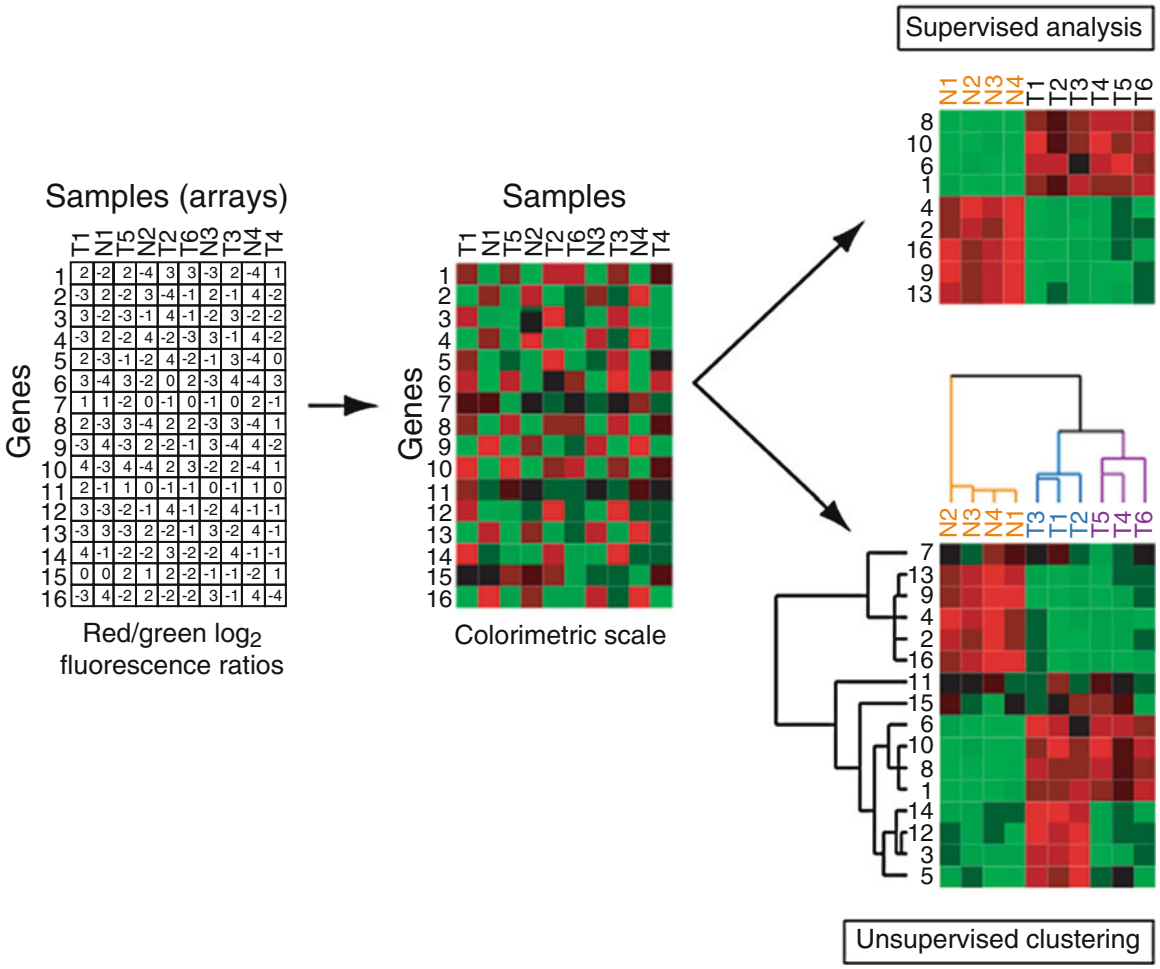

Fig. 3.2 Microarray data analysis. *Left*, the starting point of microarray analysis is a matrix of gene expression ratios, where each row is a different gene and each column is a different sample (array). *Center*, viewing ratios in colorimetric scale facilitates recognition of expression patterns. *Above right*, analysis supervised on the tumor (T) versus normal (N) distinction identifies the subset of genes with significant differences in expression, here ranked by *t*-statistic. *Below right*, hierarchical clustering analysis of the same input data matrix reveals the tumor-normal distinction, but also suggests the presence of two tumor subclasses (highlighted by *blue* and *purple dendrogram branches*).

information, for example to identify genes differentially expressed between two different specimen classes such as cancer and normal (Fig. 3.2). Various metrics can be used, like ratio-fold difference, or the Student's *t*-statistic which highlights genes with a large between-class difference in average expression compared to within-class expression variance. While metrics like the *t*-statistic provide an associated *P*-value, it is important to correct for multiple hypothesis (gene) testing (discussed more below). The number of truly (and falsely) significant genes can be estimated by comparing the number of genes observed (at any given threshold) in the real data to the median number observed in many samplings of randomly permuted data, generated for example by shuffling the sample class (e.g., cancer versus normal) labels. Significance Analysis of Microarrays (SAM) is a commonly used analysis software tool that provides such false discovery rate estimates [25].

Genes differentially expressed between sample classes can provide a basis to classify new samples. There are different methods for classifying samples, including weighted voting [26] and Prediction Analysis of Microarrays (PAM), based on nearest shrunken centroids [27]. Though the specifics differ, the principle is to determine whether the expression of classifier genes in a new sample better matches that of one or the other known sample classes used to develop (or train) the classifier. The new sample is assigned to the class with the better match, and the confidence of that assignment can be summarized as the strength of the prediction for one class over the other. The overall performance of a classifier can be estimated in the training set (where classes are known) by leave-one-out cross-validation (LOOCV), wherein one sample is omitted and a classifier is trained on the remaining samples and tested by predicting the class of the omitted sample. The process is repeated leaving a different sample out each time, and performance is then summarized by the overall classification accuracy on all samples. Classifier performance should also be validated by analyzing a separate test sample set that is independent of the training set.

In contrast to supervised approaches, unsupervised methods organize data agnostic to information about the samples, and are therefore useful for discovering previously unknown relationships in the data. Hierarchical clustering analysis is one such widely used unsupervised method [28] (Fig. 3.2). In hierarchical clustering, an iterative agglomerative algorithm is used to reorder the rows (genes) and columns (arrayed samples) of the expression matrix such that genes with similar vectors of expression across the samples (e.g., by Pearson correlation) are clustered (or grouped) together, and, independently, samples with similar vectors of expression across the genes are clustered together. Analogous to phylogenetic analysis, dendrograms (or trees) display the hierarchical relationships among genes and among samples, and the reordered heatmap highlights prominent patterns of gene expression. Information about the samples and genes can then be overlaid on the dendrograms to assist in interpreting patterns of expression. Such clustered heatmaps have been likened to molecular portraits [9], and provide a powerful new approach to observe, describe, and understand the molecular variation within cancer specimens, and in particular to discover previously unrecognized cancer subtypes.

Another useful way to organize and view microarray data, and to interpret expression patterns identified from supervised or unsupervised analysis, is pathway (or network) analysis. Gene ontology (GO) terms categorize genes by cellular component, biological process and molecular function [29]. Likewise, database resources like BioCarta and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [30] place genes in biological pathways (e.g., metabolic and signal transduction pathways) [31]. Finding statistical enrichment of specific GO terms or cellular pathways can suggest biological meaning for a group of genes of interest. Another related approach is gene set enrichment analysis (GSEA), which evaluates enrichment for each of several hundred curated gene sets by determining their overrepresentation at the top of a list of genes rank ordered by their expression distinction between two sample classes [32]. The gene sets include groupings from biological pathway databases, as well as groups with shared expression (from published microarray studies), promoter regulatory motifs, or cytogenetic location. Related methods can be used to assess enrichment of interactive networks of genes [Ingenuity Pathways Analysis—http://www.ingenuity.com/] or molecular concepts [33].

3.3.4 Common Pitfalls in Data Analysis

In profiling across many genes and specimens, the resultant high-dimensional microarray datasets have brought new statistical challenges [6, 34]. A common pitfall is reporting significant differentially expressed genes without having corrected for multiple gene testing. In large datasets, almost any particular expression pattern sought can be found, but may not be statistically meaningful. For example, in identifying genes differentially expressed between cancer and normal tissue from a dataset of 10,000 genes, about 500 genes would be expected to have individual *P*-values of less than 0.05 (the standard threshold of statistical significance) just by chance! True statistical significance is best assessed by comparison of observed findings to those from randomly permuted data.

Another common pitfall is over-fitting the data, where data models (like classifiers or outcome predictors) are developed and then tested on the exact same samples, rather than on an independent sample set. The result is an inflated estimate of test performance. An additional common shortcoming is the use of insufficiently large sample sets in both training and

validation phases of analysis. Early microarray studies using inappropriate statistical methods likely contributed to overly inflated expectations of the technology.

3.4 Expression Profiling—Applications

3.4.1 Cancer Classification

A major goal of many DNA microarray studies is cancer classification. Cancers are classified principally based on their tissue of origin and histology, and sometimes with the aid of ancillary tests like immunohistochemistry, flow cytometry, and cytogenetics. Classification schemes provide important information for prognostication and for the selection of optimal therapies. Much as the pathologist uses histologic patterns to classify cancers, DNA microarrays describe patterns of molecular variation, hitherto unrecognized, having the potential to improve cancer classification.

In an early landmark study, Golub et al. [26] described the computational framework for applying DNA microarrays to the problem of cancer classification. Using supervised methods, the investigators identified genes differentially expressed between two classes of leukemia, acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL). Statistically significant differences could be defined as those occurring above what was expected by chance, estimated by comparison to randomly permuted data. Using the top-most significant genes, a classifier could be developed that accurately predicted the diagnosis of new cases. While AML and ALL are in reality readily distinguishable by existing cytochemical staining and flow cytometry techniques, the concepts developed, and many variations on the original computational methods, are applicable to more challenging classification problems.

One such classification problem currently receiving attention is that of metastatic cancers of unknown primary (CUP), which account for up to 5 % of newly diagnosed cancers [35]. Despite subsequent immunohistochemical staining and imaging studies (like computed tomography), the anatomic site and tissue of origin remains undetermined in many cases, where knowing that information is important for selecting the optimal treatment regimen [36]. DNA microarrays have been used to define cancer type-specific patterns of gene expression, which have been shown to classify new cancer cases (where clinical truth is known) with ~80–90 % accuracy [37–40].

3.4.2 Cancer Class Discovery

Another important application of DNA microarrays is the discovery of new, previously unrecognized tumor classes, as first reported by Alizadeh et al. [41]. By unsupervised cluster analysis of variably expressed genes, these investigators identified two subtypes of diffuse large B-cell lymphoma (DLBCL) with distinct expression patterns. One pattern shared similarities with normal germinal center B cells, while the other with activated B cells. The latter DLBCL subtype was also associated with constitutive NFκB activity and less favorable prognosis [41, 42]. Therefore, while indistinguishable by histology, expression profiling nonetheless suggested a refined classification of DLBCL that might improve outcome prediction and possibly selection of therapies. Indeed, *BCL6* gene expression, a surrogate indicator of the germinal center B cell-like subtype, has since been shown to predict survival independently of the currently used International Prognostic Index score [43].

Microarray analysis of breast cancer has also identified multiple tumor subclasses, refining the existing classification [9, 44]. Estrogen receptor (ER)-positive breast tumors could be subdivided into two luminal subtypes (called so because of shared expression markers with the luminal layer of normal breast epithelium), luminal A and B, with the latter associated with higher proliferation rates and less favorable outcome. ER-negative tumors included those with *ERBB2* (Her2/neu) amplification as well as a previously underappreciated basal-like subtype (with shared expression markers of the basal/myoepithelial layer of normal breast epithelial) with poor prognosis. Similar microarray studies have identified tumor subclasses within other tumor types as well [45].

3.4.3 Outcome Prediction

Microarray analysis has also been applied to directly define gene signatures for prognostication and for prediction of response to therapies. In a seminal study, van't Veer et al. [46] compared gene-expression profiles of breast cancers from women who either did or did not develop distant metastases within 5 years of follow-up. Supervised analysis defined a 70-gene signature that could predict disease-free and overall survival in an independent cohort of breast cancer patients [47], outperforming current prognostic indices based on clinical and histological parameters such as the St. Galen and NIH consensus criteria [48]. The poor-prognosis signature might therefore improve the selection of patients who would benefit from adjuvant therapy. Prognostic and predictive signatures have been proposed for other cancer types as well, including a 133-gene prognostic signature in AML [49], independently validated [50] and with potential utility in risk-stratification for cases with normal cytogenetics.

3.4.4 Biological Insight

Expression profiling has made many other significant contributions to our understanding of cancer biology. For example, Ramaswamy et al. [51] explored gene expression differences

between unmatched primary and metastatic adenocarcinomas of diverse tumor types. The investigators defined a 17-gene signature of metastasis which, unexpectedly, was also expressed in a subset of primary tumors where its presence predicted metastasis and poor clinical outcome. Importantly, this study challenged the existing paradigm that metastases arise from rare cells in the primary tumor that have acquired additional genetic alterations, suggesting rather that the propensity to metastasize characterizes the bulk population of tumor cells, and therefore by inference is determined early in tumor development.

Another seminal contribution of microarray analysis was the relatively recent discovery of recurrent gene fusions in prostate cancer. By analyzing outlier values of gene expression in microarray datasets, Tomlins et al. [52] identified elevated expression of *ERG* and *ETV1* (oncogenic ETS family transcriptional factors) in subsets of prostate cancer. Further characterization revealed chromosome rearrangement and gene fusion, resulting in the promoter of the androgen-regulated gene *TMPRSS2* driving overexpression of *ERG* or *ETV1*. This finding provides novel insight into androgen-dependent prostate tumorigenesis, and challenges the longstanding assumption that recurrent chromosomal rearrangements, frequent in hematologic and mesenchymal malignancies, are rare in common epithelial tumor types. Indeed, this discovery has reinvigorated the search for recurrent rearrangements in epithelial tumors.

It is worth noting that in both the above examples, the key discoveries emerged from exploratory rather than hypothesis-driven investigations, underscoring the importance of exploratory research. In addition, both studies benefited enormously from the public availability of clinically annotated microarray datasets.

3.4.5 Therapeutic Targets

Microarray studies have also aimed to identify new targets for cancer therapy. For example, in a study of acute leukemias Armstrong et al. [53] found that ALL cases with rearrangements of the *MLL* (mixed lineage leukemia) gene exhibited patterns of gene expression distinct from other ALL (and AML) cases, and in particular noted high-level expression of the *FLT3* receptor tyrosine kinase. Further studies validated FLT3 as a therapeutic target in MLL, where a small molecule inhibitor of FLT3 was shown active in a mouse model of the disease [54].

Looking beyond individual genes, a promising strategy to discover new treatments has been to connect gene-expression signatures of specific disease states to gene-expression signatures of cultured human cells perturbed with various bioactive small molecules, a compilation called the Connectivity Map [55]. For example, Wei et al. [56] identified that among 164 different drug-associated expression profiles, the profile of the mTOR inhibitor rapamycin significantly (by GSEA) overlapped with the expression profile of glucocorticoid-sensitive (as compared to resistant) ALL. This finding suggested that rapamycin might revert glucocorticoid resistance, which was subsequently verified in cultured ALL cells, and is now being evaluated in clinical trials (facilitated because rapamycin is already an FDA-approved drug).

3.5 Genomic Profiling

3.5.1 Array-Based Comparative Genomic Hybridization

In addition to profiling RNA transcripts, profiling DNA aberrations in cancer genomes has emerged as a major application of DNA microarray technology. One such method is array-based comparative genomic hybridization (aCGH), derived from the cytogenetic method CGH [57] and used to delineate genomic DNA copy number alterations (CNAs). In aCGH, test (tumor) and reference (normal) genomic DNAs are labeled with two different fluors, then compared by hybridization onto microarrays comprising DNA probes of defined human (or mouse, etc.) genome map position, such as large genomic clones (e.g., bacterial artificial chromosomes; BACs) [58, 59], genes (cDNAs) [60], or oligonucleotides [61–63] (Fig. 3.3). For each probe present on the microarray, the ratio of fluorescence represents the relative copy number of that locus in the tumor compared to normal sample.

In contrast to expression profiling, analysis of aCGH data emphasizes genome position information. Tumor/normal log fluorescence ratios are first normalized for each array so that the average log ratio is set to zero. As such, CNAs are defined relative to the average copy number for the sample (which may well vary from diploid). Normalized tumor/normal ratios are then mapped onto an ordered representation of the normal genome sequence, where DNA gains and losses are identified as ratio peaks and valleys (Fig. 3.3). Note, this method does not reveal the actual location of CNAs in the cancer genome, where it is known for example that DNA amplification can occur in situ on the chromosome (homogeneously staining regions) or elsewhere in the genome, even extra-chromosomally (double minutes).

Because genomic DNA comprises a more complex mixture of DNA sequences compared to the subset of expressed genes (transcripts), aCGH presents additional technical challenges compared to expression profiling. Further, accurate quantification of very small ratio-fold changes, like single-copy tumor DNA gains and losses in

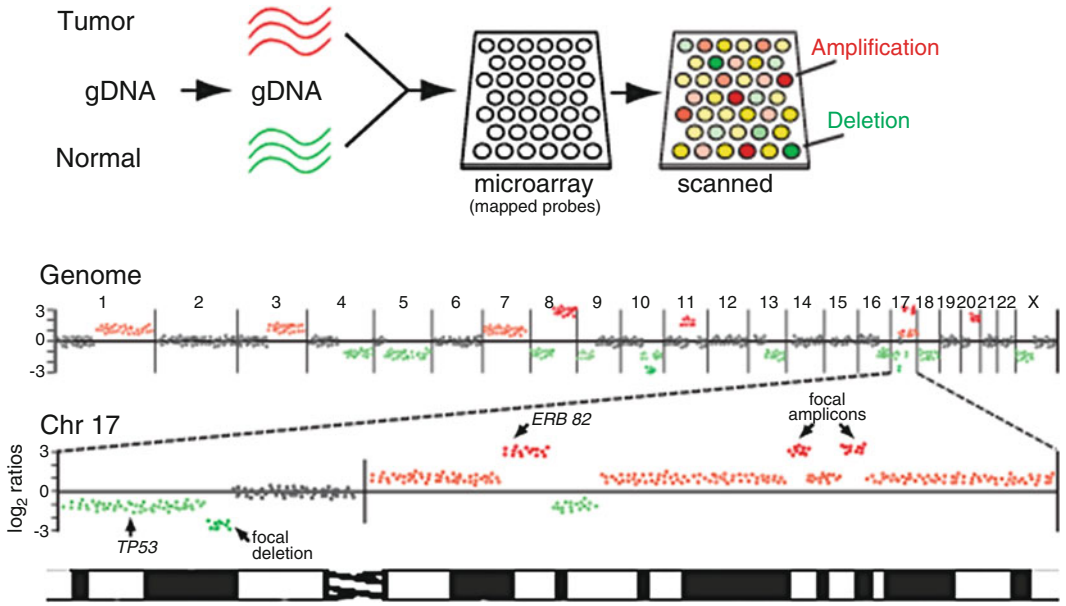

Fig. 3.3 Genomic profiling by array CGH. *Above*, schematic depiction of array-based comparative genomic hybridization (aCGH). Genomic DNA isolated from tumor and normal reference is labeled with two different fluors (here *pseudocolored red* and *green*), then co-hybridized to the array. For each gene on the array, the ratio of red/green fluorescence reflects its relative DNA copy number in the tumor compared to normal reference. *Below*, tumor/normal $\log_2$ ratios (here, for a hypothetical breast cancer sample) are plotted according to reference genome position, shown for the whole genome and for an enlarged view of chromosome 17. *Red* and *green ratios* indicate DNA gain and loss, respectively, some corresponding to known cancer genes (e.g., *ERBB2*, *TP53*), while other focal amplicons and deletions pinpoint novel cancer genes.

admixtures of normal DNA stromal contamination, is key to localizing and discovering new cancer genes. The sensitivity of detecting low amplitude and focal aberrations is dependent on many factors, including the performance characteristics of the array platform and the density of probe coverage across the genome, with the trend moving towards arrays with 100,000 or more oligonucleotide probes. Measurement accuracy can be improved by averaging ratios across neighboring probes, though at the expense of spatial resolution. Statistical algorithms (and corresponding software tools) are available to call gains and losses in aCGH data [64, 65] and to identify loci recurrently gained or lost across tumor samples [66, 67]. Such recurrently aberrant loci are more likely to harbor cancer genes whose altered copy number and expression provides selective growth advantage, rather than represent secondary inconsequential alterations resulting from genomic instability.

While the above discussion centered on somatic CNAs, germ line DNA copy number variants (CNVs) are increasingly recognized as a source of hereditable variation [68]. CGH arrays can be used to identify CNVs, some of which might be associated with increased cancer risk, or represent preferred sites of tumor genome rearrangement. In defining somatic CNAs, the use of normal reference DNA matched from the same individual can assist in discriminating between somatic CNAs and germ line CNVs.

3.5.2 Single Nucleotide Polymorphism (SNP) Arrays

DNA sequence changes are a major source of heritable variation, and measuring DNA sequence variation was a major motivation for developing DNA microarray technology. Single nucleotide polymorphisms (SNPs) are present on average every 300 bp in the human genome [69], and several million SNPs have been characterized [70]. Affymetrix SNP arrays [71] comprise oligonucleotide probes spanning tens of thousands of SNPs, with separate probes matching the major (i.e., more frequent) and minor SNP alleles (A and B), and can be used to type alleles by DNA hybridization. In brief, target genomic DNA is digested with a restriction endonuclease, and then PCR amplified (using ligated universal adapters) and biotin-labeled for hybridization. Array probes are designed to detect SNPs predicted to reside on restriction fragments within the preferred PCR size range (~0.25–2 kb), and the PCR step serves to reduce target complexity, thereby improving assay performance. SNP genotypes (AA, AB, or BB) are called based on relative hybridization intensities for allele-specific probes. Illumina BeadChip arrays provide a distinct approach with comparable performance for SNP genotyping [72].

SNP arrays can be used to genotype SNPs for whole-genome scan genetic linkage and association studies [73],

for example to discover loci conferring cancer risk [74]. SNP arrays can also be used to identify somatic DNA aberrations in tumors, including both CNAs, by scoring hybridization intensity, and LOH, by scoring allelic loss (at informative heterozygous loci) along chromosome segments [75, 76]. Because LOH can result in loss of genetic information in the absence of deletion, SNP arrays can provide additional information useful for mapping tumor suppressors.

3.5.3 Genomic Profiling—Applications

The major application of genomic profiling by CGH (or SNP) arrays has been the discovery of new cancer genes. CGH on microarrays affords orders of magnitude higher mapping resolution compared to prior cytogenetic methods, and has revealed hitherto unappreciated complexities of CNAs in cancer genomes [60, 77]. In various tumor types, recurrent CNAs have been identified that do not contain known cancer genes, and therefore presumably pinpoint new cancer genes. Functionally validated cancer genes discovered by genomic profiling include *PPM1D* (at 17q23.2), a negative regulator of TP53 amplified in breast cancer [78], and *MITF* (3p14.1), a master transcriptional regulator of melanocytes amplified in melanomas [79]. Indeed, the discovery of *MITF* amplification suggested the concept of lineage-dependency oncogenes, where the aberrant expression of genes with key roles in normal cell lineage proliferation or survival is required for tumor cell survival in certain genetic contexts [80]. Another such example is *NKX2-1* (*TITF1*), a transcriptional regulator of normal lung development recently found amplified by genomic profiling of lung cancers [81–83].

With the commercial availability of high-density CGH and SNP array platforms, genomic profiling is increasingly utilized, and has been a featured technology of NCI's The Cancer Genome Atlas (TCGA) project [84] to systematically characterize cancer genomes. Beyond single genes, patterns of CNA, analogous to signatures of gene expression, have been proposed for cancer classification and outcome prediction [85–88].

3.5.4 Data Integration

While expression and genomic profiling each provides important information, integrating data from both these methods can reveal additional insight. For example, while many genes exhibit elevated expression in cancer, the subset that is also highly amplified is enriched for key genes driving tumorigenesis (Fig. 3.4); such an integrative analysis is therefore valuable for cancer gene discovery. Integrative

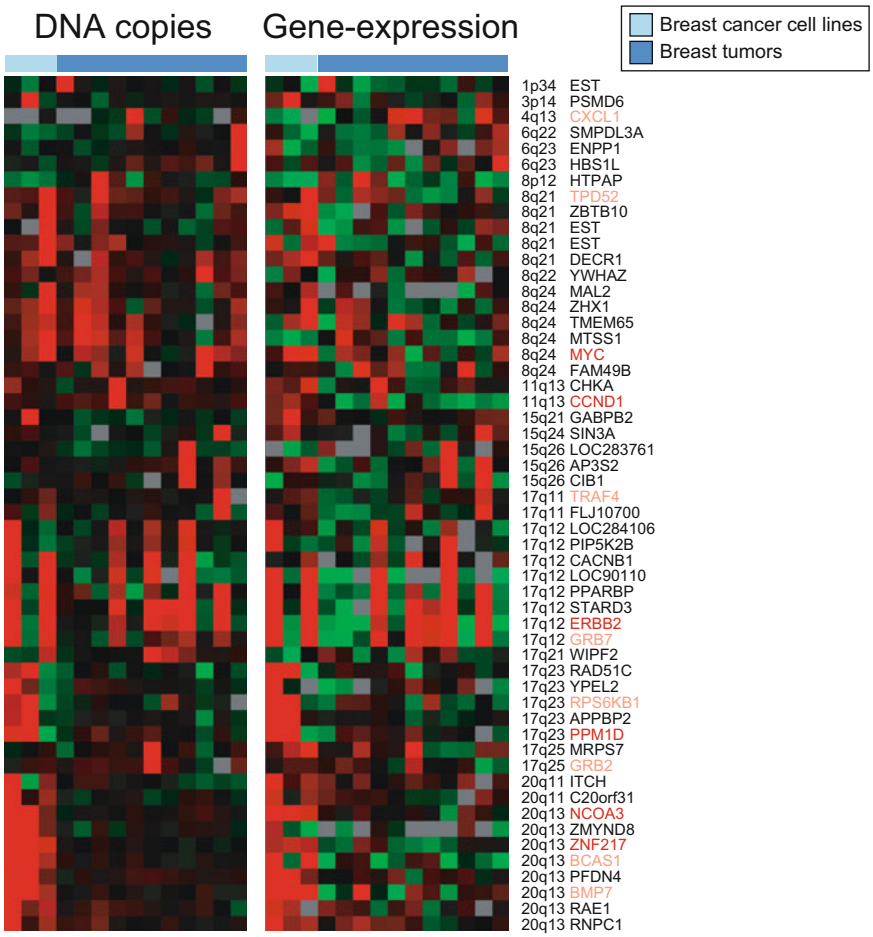

Fig. 3.4 Data integration. Shown is an integrative analysis of aCGH and expression profiling data. Gene copy number (heatmap, *left*) and expression (*right*) are depicted for the subset of genes identified by microarray to be both highly amplified and overexpressed (a characteristic of oncogenes) in breast cancer cell lines or tumors. Samples are ordered identically in both panels; genes are ordered by chromosome location. *Red* indicates amplification (*left*) or overexpression (*right*). This subset of amplified overexpressed genes is a rich source for breast cancer gene discovery; known putative (*pink text*) and bona fide oncogenes (*red text*) are highlighted, the latter including *MYC*, *CCND1*, and *ERBB2*. Data abstracted from Pollack et al. [89].

analysis of DNA copy number and expression has also uncovered a significant impact of aneuploidy (chromosome copy number imbalances) on gene expression patterns [89, 90]. This finding, observed in many tumor types, suggests that aneuploidy might contribute to cancer progression through the altered expression of many (possibly even hundreds of) genes. Integrative analysis has also revealed that breast cancer subtypes are associated with distinct patterns of CNA, with luminal-B tumors characterized by high-level DNA amplifications and basal-like tumors by low-amplitude segmental gains and losses [88, 91]. This finding suggests that breast cancers arise not only via different oncogenic pathways but also by distinct underlying mechanisms of genomic instability.

There is an increasing interest in integrating data across diverse platforms, not just gene expression and copy number, but additional layers of genomic-scale data (like epigenetic regulation), protein expression (and modification and activity), protein interactions, and phenotypes, some generated from model organisms [92–94]. Such analyses have been dubbed integromics [95] or systems approaches. Finally, integrating data may reveal interplay between the germ line and tumor genomes, for example in predisposition to specific cancer subtypes (e.g., *BRCA1* mutation carriers develop basal-like breast cancers [96]) or in determining response to specific anticancer therapy (i.e., pharmacogenomics) [97].

3.6 Other Microarray Platforms

3.6.1 DNA Methylation Arrays

The DNA microarray platform has been adapted to other applications as well (Fig. 3.5), including defining patterns of 5-methylcytosine DNA methylation. Cancer genomes exhibit global hypomethylation, but focal hypermethylation upstream of tumor suppressors (often within CpG islands) associated with transcriptional silencing [98]. Several microarray-based approaches have been developed to assay DNA methylation, useful for locating new tumor suppressor genes. Transcriptional profiling following treatment of cells with the DNA methyltransferase inhibitor 5-aza-deoxycytosine can reveal methylation-silenced genes, but the method is limited to cultured cells and does not distinguish methylated genes from secondary transcriptional changes. One approach to directly assay DNA methylation, by Differential Methylation Hybridization (DMH) [99], takes advantage of methylation-sensitive restriction endonucleases. Genomic DNA from tumor and matched normal are separately restriction endonuclease digested, ligated to universal PCR linkers, and then further digested with a methylation-sensitive restriction endonuclease. DNA fragments methylated in tumor (but not normal) are protected from methylation-sensitive digestion, and therefore produce PCR products that can be detected by two-color hybridization (cancer versus normal) to a microarray containing probe sequences from the upstream regions (CpG islands) of genes.

In a second approach, Methylated DNA Immunoprecipitation (MeDIP) [100], cancer-derived DNA is fragmented and then immunoprecipitated using an antibody specific for 5-methylcytosine. Immunoprecipitated and reference input DNA are differentially labeled and co-hybridized to a microarray containing upstream sequence probes, where increased ratios indicate methylated DNA. A third approach makes use of bisulfite modification, whereby unmethylated but not methylated cytosines are converted to thymine by chemical treatment followed by PCR. DNA methylation is then detected by differential hybridization to methylation-specific oligonucleotide (MSO) microarrays, with probes designed to discriminate between the unmethylated (converted) and methylated (unconverted) DNA sequences [101].

3.6.2 Protein–DNA Interactions by ChIP on Chip

Another application of DNA microarrays is determining genome-wide the physical positioning of DNA-associated proteins, including chromatin proteins and transcription factors (to identify transcription targets), by chromatin-immunoprecipitation followed by microarray (chip) hybridization, or ChIP on Chip [102, 103]. First, cultured cells are treated with formaldehyde to reversibly crosslink proteins to DNA. Next, cell lysate DNA is fragmented, and the protein of interest (along with bound DNA) is immunoprecipitated using an antibody specific to the native protein, or to an epitope tag (where the corresponding protein has been tagged). The DNA is then separated, PCR-amplified, labeled and hybridized to a microarray containing upstream or tiling DNA sequence probes. Protein–DNA interactions are identified by increased hybridization signal compared to an appropriate reference, for example input DNA or control mock-immunoprecipitated input DNA.

3.6.3 Gene Function by Transfected Cell Microarrays

DNA microarrays have also been applied to assess gene function. In one approach, cultured cells are overlaid onto a spotted DNA microarray comprising full length genes (cDNAs) cloned into mammalian expression vectors [104]. Cells overlaying DNA spots take up the DNA by reverse transfection, such that each cell cluster expresses a different transfected gene. Gene functions are then inferred by resultant

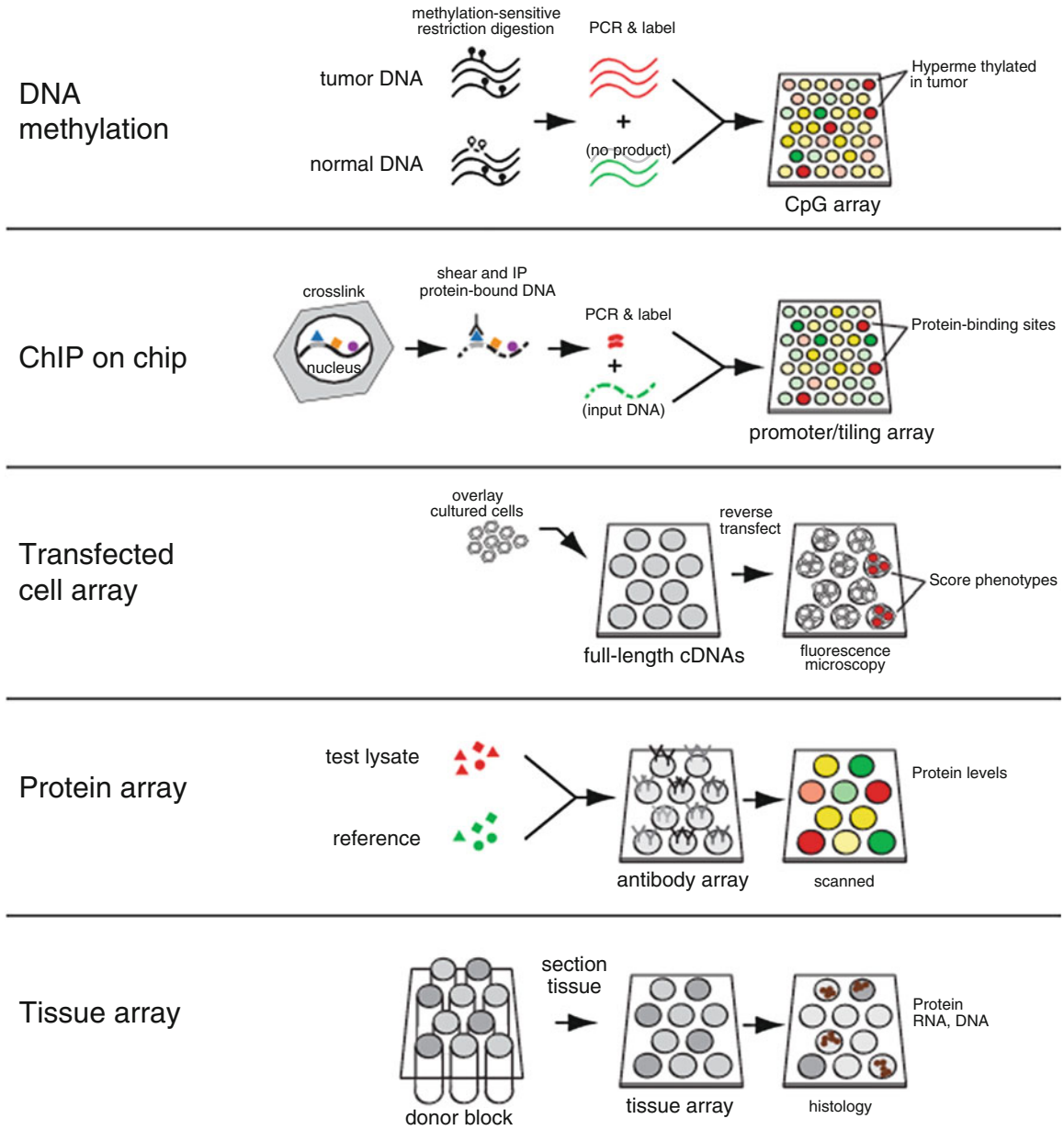

Fig. 3.5 Other array platforms and applications. Shown schematically are representative microarray-format applications for profiling DNA methylation by Differential Methylation Hybridization (*filled circles* indicate methylated CpG sites); protein–DNA interactions by ChIP on chip; gene function by transfected cell array; protein levels by antibody array; and high-throughput histology by tissue array. See main text for additional detail.

molecular and cellular phenotypes, scored for example by fluorescence microscopy. An analogous approach uses microarrays of small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) effecting gene knock down to screen for loss of function phenotypes [105, 106].

3.6.4 Proteomic Arrays

The previously described applications utilize microarrays containing DNA probes. Other applications make use of arrays containing other classes of molecules. For example, arrays of spotted antibodies can be used for the highly parallel measurement of protein levels (or phosphorylated-state) in body fluids or cell lysates [107, 108]. Conversely, spotted proteins can be used to identify protein interactions [109] or, as antigens, to define the antitumor humoral immune response [110]. While for DNA arrays binding by Watson–Crick base pairing is relatively uniform across an array, a challenge for protein arrays is the considerable variation in binding kinetics and affinities for different protein-protein interactions.

3.6.5 Tissue Microarrays

With a DNA microarray, a single sample is profiled across many genes. The converse approach is implemented with tissue microarrays (TMAs) [111], where a single gene is profiled across many samples. TMAs comprise up to several hundred different tissue pieces (each 0.6–2 μM in diameter), not individually spotted but rather sectioned from a donor block (assembled as an array of cylindrical tissue core biopsies) onto a glass microscope slide. TMAs permit the highly parallel histologic analysis of gene expression by RNA in situ hybridization (RISH) or immunohistochemistry (IHC), or copy number determination by fluorescence in situ hybridization (FISH). TMAs, which also provide information on cellular localization of expression, markedly speed the evaluation and validation of initial DNA microarray discoveries across larger patient cohorts. Because they are typically built from formalin-fixed paraffin-embedded (FFPE) specimens often linked to long-term clinical follow-up, TMAs are particularly useful for retrospective evaluation of prognostic markers.

3.7 Clinical Applications

3.7.1 Biomarkers and Signatures

Current diagnostic applications resulting from microarray discoveries rely mainly on methods already in common use in histopathology and molecular pathology laboratories, such as IHC or RT-PCR. For example, *AMACR* (alpha-methylacyl-CoA racemase) was identified by microarray analysis to be more highly expressed in prostate cancer compared to normal prostate [112–114]. IHC analysis of AMACR expression is now in routine use in some pathology centers to evaluate difficult biopsy cases for the presence of prostate cancer.

Multigene tests derived from microarray data are also being evaluated for clinical use. A 70-gene breast cancer prognostic signature, described above and assayed by DNA microarray analysis of freshly frozen specimens (Agendia's MammaPrint), has been FDA-cleared for the prediction of breast cancer recurrence for node-negative tumors [115]. Similarly, a 21-gene signature (16 cancer-related and five reference genes), derived in part from microarray studies and assayed by Q-RT-PCR using FFPE specimens (Genomic Health's Onco*type* DX), predicts risk of tumor recurrence in ER-positive, node-negative breast cancers [116]. Such tests, in conjunction with other clinical and laboratory information, might be used to select patients who are likely to benefit from adjuvant chemotherapy. Another interesting group of multigene signatures with potential prognostic utility reports on key biological features of tumors, like metastasis [51], wound response [117], stemness [118], stroma [119], oncogenic pathways [120], hypoxia [121], chromosomal instability [122], and invasiveness [123].

3.7.2 Microarray Platform for Clinical Diagnosis

Many challenges remain in adopting DNA microarrays as a commonplace platform for diagnostic testing [124]. Early concerns with expression profiling centered on discordances of microarray findings among different research laboratories. Gene-expression signatures ostensibly reporting on the same biological or clinical parameter often shared few genes in common. Such discrepancies are likely attributable in large part to differences in specimen cohorts, array platforms (and probes), protocols, and analysis methods. More recently, investigators have shown high reproducibility of findings when standard operating procedures are followed, and improved inter-platform concordance with careful matching of probe annotations between platforms [125, 126]. In addition, further scrutiny has revealed that the disparate gene-signatures might nonetheless reflect the same underlying biology [32], or provide comparable clinical utility [127]. Microarray-based clinical tests are likely to emerge where (1) microarrays provide additional clinical information or outperform standard histopathologic markers, (2) many genes provide more information than one or a few, (3) adequate performance characteristics are demonstrated, (4) testing impacts a patient management decision, (5) there has been appropriate validation of clinical utility, and (6) testing is cost worthy.

3.8 DNA Sequencing Technologies

3.8.1 Conventional Dideoxy Sequencing

DNA sequence point mutations represent a major class of cancer genome aberrations only recently surveyable by genomic-scale approaches. For the past three decades, Sanger dideoxy DNA sequencing has been the preferred methodology, and advances in sequencing chemistry and automation have led to increased read lengths, accuracy and throughput, culminating in the completion of the draft human genome sequence in 2001 [128]. Subsequent directed resequencing efforts focusing on oncogene candidates in cancer genomes have led to the discovery of novel mutations, including activating mutations of *BRAF* (in the MAP kinase pathway) in two-thirds of melanomas [129], and of *PIK3CA* in approximately 30–40 % of colorectal [130] and breast cancers [131, 132]. Larger scale sequencing efforts have identified other candidate cancer genes [133, 134], though a

Fig. 3.6 Serial Analysis of Gene Expression (SAGE). Shown is a schematic depiction of the SAGE method. Captured cDNAs are digested with an anchoring endonuclease followed by a tagging endonuclease, releasing DNA sequence tags that uniquely identify transcripts. Counting of tags by sequencing tag concatamers provides quantitative information on transcript abundances.

remaining challenge in the field is distinguishing pathogenic driver mutations from noncontributory passenger mutations consequent to genomic instability.

3.8.2 Serial Analysis of Gene Expression

Sanger sequencing has also been used for census applications, where counting DNA sequence tags permits a quantitative characterization of complex nucleic acids populations. Serial Analysis of Gene Expression (SAGE) is one such application developed to quantify transcripts (Fig. 3.6) [135]. In SAGE, mRNA transcripts from a specimen are converted to cDNA using a biotinylated oligo(dT) primer, then digested with a frequent cutting (anchoring) restriction endonuclease where the 3'-most cDNA portion is captured on streptavidin beads. Bound cDNAs are then digested with a tagging type IIS restriction endonuclease which recognizes a site in a linker ligated to the 5′ end but cleaves 14 bp downstream. Resultant released short sequence tags are then dimerized, PCR amplified, serially concatenated (to increase throughput), cloned and dideoxy sequenced. The resultant 9–10 bp sequence tags (or up to 22 bp using a type III restriction endonuclease [136]), located at a defined position within the transcripts (between the anchoring and tagging sites) uniquely identify transcripts, and therefore counting tags provides a quantitative cataloging of expressed genes. Potential advantages of SAGE (over DNA microarray-based expression profiling methods) are that it provides absolute quantification as well as information on previously uncharacterized transcripts. A major disadvantage has been the substantial effort of SAGE library construction and the resultant low sample throughput.

3.8.3 Next-Generation DNA Sequencing Methods

In the past 3–4 years, the so-called next-generation (next-gen) DNA sequencing technologies have been introduced that provide orders of magnitude increased sequencing throughput capability, and have revolutionized cancer genomics (much as DNA microarrays had a decade earlier). Most of these technologies rely on cyclic array sequencing methods, where DNAs to be sequenced are immobilized as clonal features on an ordered or random array (Fig. 3.7) [137]. Each cycle, the identity of a single base position is interrogated at each feature on the array using an enzymatic sequencing chemistry and read out by imaging luminescence or fluorescence. By carrying out multiple cycles, contiguous DNA sequences are simultaneous decoded for each of the up to tens of millions of arrayed DNA features. This massively parallel sequencing combined with low reagent volumes (shared across the array) translates to enormously increased throughput and concomitant per base pair (bp) cost reduction.

While the technology is still evolving, several commercial platforms are in use, differing mainly in the method for generating DNA features, the sequencing chemistry, and the resultant sequence read lengths, throughput, and error types [138, 139]. Both the 454/Roche [140] and Applied Biosystems (ABI/SOLiD) [141] platforms use compartmentalized water-in-oil emulsion PCR to generate clonal polymerase colonies (polonies), where multiple copies of each DNA sequencing template are captured on micron-scale beads (Fig. 3.7). Thousands to millions of beads, each with a different DNA template, are then either orderly self-assembled on an etched fiber optic bundle (454/Roche) or immobilized on a glass surface as a disordered array (ABI/SOLiD). The 454/Roche platform uses a DNA polymerase-based sequencing-by-synthesis pyrosequencing method (with real-time luminescence detection) to interrogate bases, and achieves long sequencing read lengths (~400 bp), though errors can occur at long homopolymer stretches. The ABI/SOLiD system uses a sequencing-by-ligation chemistry with fluorescence detection to query bases, and achieves significantly higher sequencing capacity (by increased feature density) though with shorter read lengths (35–75 bp).

The Solexa/Illumina platform generates DNA features by bridge PCR, where universal PCR primers are immobilized on a glass slide such that resultant PCR-amplified sequencing templates form locally tethered clonal features (Fig. 3.7) [138]. Bases are interrogated using a polymerase-based single nucleotide extension method with reversible fluorescent terminators, yielding high sequencing capacity (comparable to ABI/SOLiD) with modest read length (36–150 nts). For all these platforms, throughput can be doubled by sequencing both ends of DNA templates. Such paired-end sequencing can also facilitate sequence alignment (and in particular de novo assembly), which is more challenging for short reads.

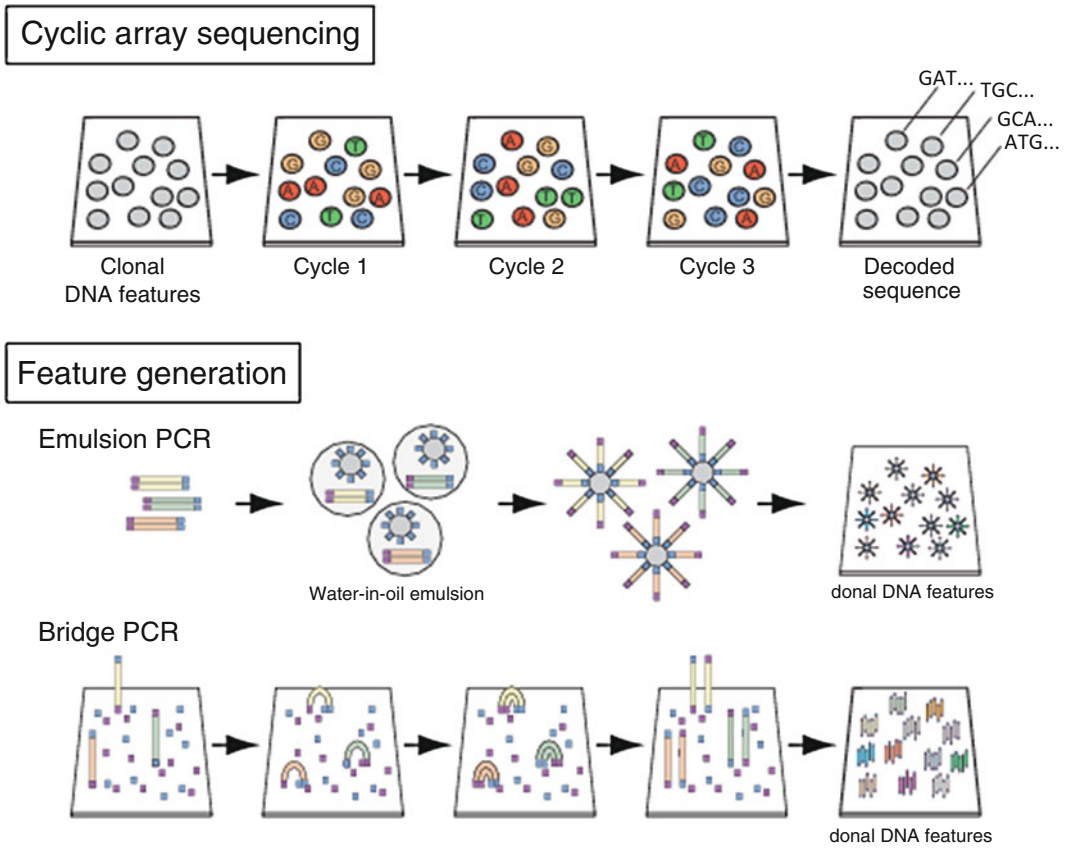

Fig. 3.7 Next-generation DNA sequencing. *Above*, schematic depiction of next-gen cyclic array sequencing. Arrayed DNAs are clonal features each comprising multiple PCR-generated copies of a different DNA sequencing template. Each cycle (only three cycles shown), enzymatic sequencing chemistry interrogates a subsequent base pair position, and multiple cycles permit the decoding of DNA sequence stretches. DNA sequences are decoded across millions of features in parallel, providing ultra-high throughput capability. *Below*, schematic depiction of two different methods of generating clonal DNA features for next-gen sequencing. In emulsion PCR, individual DNA sequencing templates (along with primer-coated capture beads) are sequestered within micelles formed by water-in-oil emulsion. Resultant clonally amplified beads are subsequently arrayed on a solid surface for sequencing. In bridge PCR, individual sequencing templates are captured on a primer-coated surface, such that resultant PCR-amplified products remain locally tethered as clonal features.

Recent and emerging technologic advances include single molecule DNA sequencing (rather than sequencing PCR-amplified features), for example as developed by Helicos BioSystems [142] and Pacific Biosciences [143]. With the continued rapid pace of technologic advances, a stated goal [144] to sequence a human genome (or cancer genome) at a cost of less than $1000 is within sight.

3.9 Next-Gen Sequencing Applications

3.9.1 Census Applications

For practical concerns of throughput and cost, next-gen sequencing technologies were first widely adopted in census applications of lower-complexity (compared to whole cancer genomes) nucleic acid mixtures [145, 146]. For example, in RNA-seq, analogous to SAGE, transcripts (or small RNAs) are profiled by next-gen sequencing of cDNAs (typically prepared by first fragmenting and then reverse transcribing RNA) [147, 148]. Resultant sequence reads are mapped to the reference genome or transcriptome using alignment software (e.g., ELAND, MAQ, Bowtie) (Fig. 3.8) [139, 149]. Transcript counts are then normalized with respect to the kilobase size of the transcript (to compensate for larger transcripts having more sequence reads), as well as the total number (of million) of mapped reads (which can vary among samples), often expressed as reads per kilobase per million (RPKM) [149]. The resultant dataset can then be analyzed and visualized as described for DNA microarrays.

Compared to DNA microarrays, RNA-seq is more quantitative (providing a digital count rather than an analog read-out by hybridization), covers a larger dynamic range, provides greater coverage (not limited to what is printed on a microarray), and yields single base-pair resolution. These latter advantages make it particularly suited to the discovery of novel transcripts, and the delineation of transcript variants (from reads spanning splice junctions). RNA-seq has similarly found

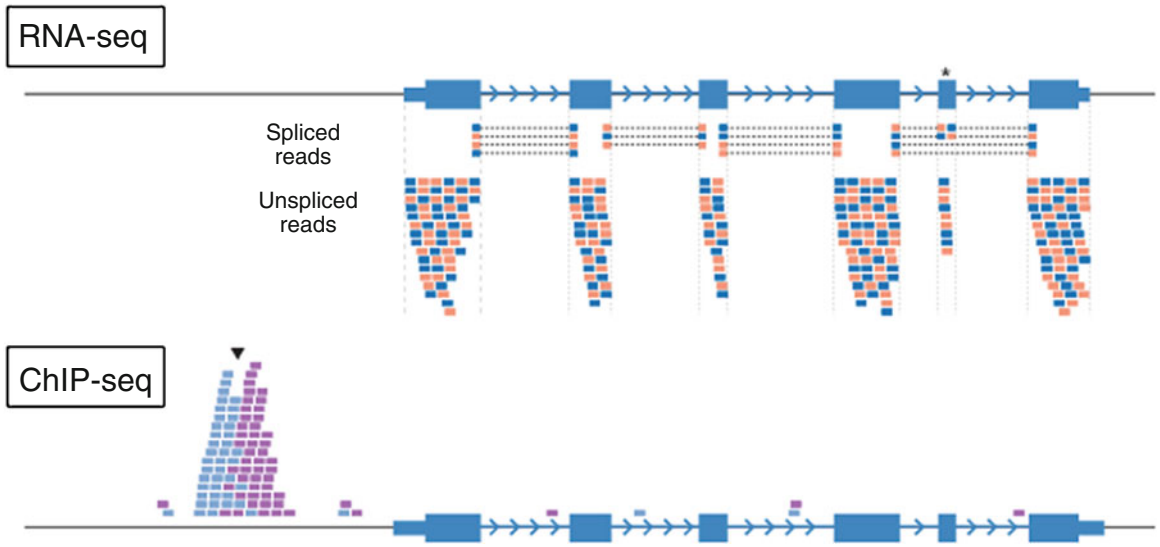

Fig. 3.8 Next-gen sequencing census applications. *Above*, schematic depiction of RNA-seq reads aligned to a gene-coding segment of the genome (exon structure shown). Sequence reads are denoted by *small blue* (aligned to + strand) and orange (aligned to − strand) rectangles. Most reads align to single exons, while some span exon splice junctions, defining transcript variants (including here, an alternatively spliced exon denoted by asterisk). *Below*, schematic depiction of ChIP-seq reads aligned to the same gene-coding region. Sequence reads are denoted by small blue (aligned to + strand) and purple (aligned to − strand) rectangles. A region of increased read density upstream of the gene defines a protein-binding peak. Note, single-end reads of ChIP-enriched fragments result in shifted peaks for + and − strand reads, where an inferred central summit (*arrowhead*) identities the protein binding site.

utility in the search for oncogenic fusion transcripts, as chimeric sequence reads [150] or paired-end reads mapping to two different genes [151]. Short-read RNAseq is also well suited for analysis of the fragmented RNA typical of formalin-fixed paraffin-embedded specimens [152]. Potential obstacles to sequence-based approaches include the availability of sequencing instruments, the infrastructure for data management and analysis, and the still generally higher reagent costs. However, with improving infrastructure and analysis tools, and ever-declining costs, next-gen sequencing is poised to replace DNA microarrays for transcript profiling as well as other applications. Nonetheless, the shared fundamentals of experimental design and dataset analysis remain valid.

Another census-based application now in wide use is ChIP-seq, the enumeration of protein interactions on DNA by next-gen sequencing of chromatin-IP enriched DNAs [153, 154]. Resultant sequence reads are aligned to the reference genome, and then binding peaks (regions of increased read density) called in relation to background (often determined by sequencing non-IP'd input DNA) (Fig. 3.8) [149]. Noted advantages, compared to microarray-based ChIP on chip, include the higher (single bp) mapping resolution and whole genome coverage [155]. DNA methylation can also be surveyed by next-gen sequencing, for example in Methyl-seq by comparing reads for the small DNA fragments generated by paired methylation sensitive and insensitive restriction endonucleases [156, 157].

Similar to aCGH, DNA copy number alterations can be enumerated by next-gen technology, by sequencing tumor genomic DNA, and then aligning and simply counting reads (e.g., per 10 kb window) across the reference genome. Further, by sequencing the paired ends of genomic DNA fragments, structural rearrangements can be identified, including balanced rearrangements otherwise invisible to aCGH (Fig. 3.9). For example, paired end reads mapping (on the reference genome) to greater or less than the expected distances apart (i.e., as compared to the known size of the fragments sequenced) are indicative of DNA deletions or insertions, respectively. Paired ends in reverse of the expected orientation identify inversions, and paired ends mapping to different chromosomes reveal chromosomal translocations. Proof-of-principle for this approach was first shown by conventional Sanger sequencing of paired ends from cloned BAC libraries derived from cancer cell lines [158]. More recently, next-gen sequencing of small (e.g., 0.5–3 kb) genomic DNA fragments has revealed the detailed landscape of structural alterations in lung and breast cancer genomes [159, 160].

3.9.2 Surveying DNA Mutations

In a remarkable feat, Parsons et al. [161] Sanger-sequenced essentially all protein-coding genes from 22 glioblastoma multiforme samples, where they discovered somatic mutations of *IDH1* (isocitrate dehydrogenase) in 12 % of cases. The mutations have since been shown to alter enzymatic activity [162], suggesting IDH1 as a tractable target for cancer therapy. This finding portended the value of screening

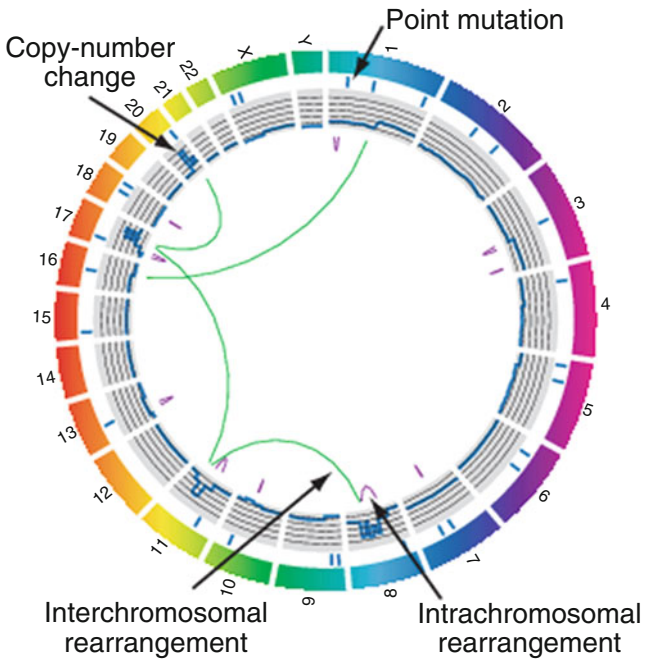

Fig. 3.9 Landscape of the cancer genome. Schematic Circos plot illustrating somatic alterations in a single cancer genome. Concentric tracks (from outside to in) depict reference genome (chromosome) position, point mutations, DNA copy number alterations (from counting sequence reads), and intrachromosomal (*purple*) and interchromosomal (*green*) rearrangements (from discordant paired-end reads).

cancer genomes for DNA mutations, an undertaking that is now facilitated enormously by next-gen technology.

Many early efforts (motivated in part by costs) have focused on sequencing transcriptomes or targeted exomes [163] (the 1.5 % of the genome that codes for proteins). The first whole cancer genome sequence (an AML case) was described in 2008 [164], and with decreasing costs many more cancer genomes are being reported. Challenges of short-read sequencing are in large part bioinformatic, and include accurate assessment of error rate, alignment and de novo assembly, and calling of polymorphisms and somatic mutations [139]. Oversampling (e.g., 30-fold redundant sequence coverage) can mitigate sequencing errors, and facilitate assembly (as does paired-end sequencing). Sequencing matched normal DNA can assist in distinguishing somatic mutations from personal germ line variants (that might not be represented in SNP databases). Distinguishing oncogenic driver mutations from nonsynonymous passenger mutations remains problematic, though a gene recurrently mutated at a significant frequency implies a driving role. Ultimately, functional studies are required to verify oncogenic activity. Despite the many challenges, early successes of next-gen mutation discovery include *ARID1A*, identified by transcriptome sequencing and mutated in ~50 % of clear cell ovarian cancers [165], and *DNMT3A*, found by whole-genome sequencing and mutated in ~20 % of AML cases [166].

Beyond discovery of cancer genes, surveying somatic mutations can also reveal the carcinogenic exposures and forces shaping the landscape of cancer genomes [167]. For example, next-gen sequencing of a small cell lung cancer genome uncovered mutation signatures reflective of tobacco exposure [168]. Likewise, analysis of a melanoma genome revealed a mutational signature of ultraviolet light exposure, a known risk factor for melanoma [169].

Concerted efforts to fully sequence the genomes of diverse cancer types are now underway, organized by the International Cancer Genome Consortium (ICGC) [170], an umbrella organization that includes the Wellcome Trust Sanger Institute's Cancer Genome Project, and coordinates with the NCI's TCGA research network. An up-to-date *c*atalog *o*f *s*omatic *m*utations *i*n *c*ancer is accessible at Sanger Institute's COSMIC database [171]. Finally, a growing number of actionable mutations/aberrations (i.e., that impact clinical decisions including choice of therapy) in cancer genomes [172], coupled with decreasing sequencing costs, suggests the possible utility of next-gen sequencing for clinical diagnosis in the not so distant future.

3.10 Conclusions

The past decade has witnessed an explosion of genomics technologies, ushered in by DNA microarrays, and providing for the first time global views of cancer genomes and the altered complement of transcripts and proteins. Genomics approaches have yielded new insights into cancer pathogenesis, and suggest strategies for improved cancer diagnosis, prognostication and patient management. Genomics technologies continue to advance, most recently in the arena of next-gen DNA sequencing. As cancer genomics data accumulate in the public domain, bioinformatics approaches are becoming increasingly important in integrating data from different platforms and disparate sources to derive biological meaning and clinical insight. Cancer researchers knowledgeable in both wet bench and computational analysis are best prepared to leverage the potential of cancer genomics.

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Cancer Cytogenetics

4

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4.1 The Development of Cancer Cytogenetics as a Scientific Discipline

The term *chromosome* was coined by the German pathologist Wilhelm Waldeyer in 1888. At that time, chromosomes just appeared as *stained bodies*, and their biological relevance had not been recognized. It was probably David von Hansemann who first speculated about the fact that the number of chromatin segments and their irregular distribution to daughter cells could have something to do with cancer. He summarized this concept in a study published in 1890 with the title "*Über asymmetrische Zellteilung in Epithelkrebsen und deren biologische Bedeutung*" (Translated: *On asymmetrical cell division in epithelial cancers and its biological relevance*) [1]. In this report he wrote that "*Chromatin plays a crucial role for the inheritance of specific cellular features, and it is in particular the number of segments that carries important biological significance which is proven through the constant number in different tissues and species*." He continues to state that "*The asymmetric divisions do not follow a specific rule regarding the numbers of chromatin segments. i.e., in one and the same tumor we can observe cells with a different numbers of segments*." This phenomenon is "*…in many cases consistent with an anaplasia, because the results of tumorigenesis are cells with increased growth potential…*". These cells "*…frequently can be found far away from the initial tumor and can become the origin of identical tumors*." Clearly, von Hansemann was on the right track [2]. However, it was the zoologist Theodor Boveri of the University of Würzburg (Fig. 4.1a), who injected his chromosome theory of heredity into the cancer problem and with that generated the theoretical scaffold for cancer cytogenetics to develop as a scientific discipline [3–5] (Fig. 4.1b and c). Boveri did not work with cancer tissues or cancer cells. He instead studied ascaris larvae or sea urchin eggs, which, when fertilized with two sperms begun to show centrosome aberrations and abnormal mitoses. Boveri hypothesized that chromosomes should have continuity throughout the cell cycle, and in fact, individuality. This implies that chromosomes are unique. He then postulated that chromosome segregation errors would ensue as a consequence of centrosome abnormalities, leading to daughter cells that in terms of the genetic make up would be different from each other. This imbalance could cause cancer. He wrote: "*To assume the presence of definitive chromosomes which inhibit division, would harmonize best with my fundamental idea. If their inhibitory action were temporarily overcome by external stimuli, then cell division would follow. Cells of tumors with unlimited growth would arise if those "inhibiting chromosomes" were eliminated.*" And he continues: "*On the other hand, the assumption of the existence of chromosomes which promote division, might satisfy this postulate. On this assumption, cell-division would take place when the action of these chromatin parts, which are as a rule too weak, should be strengthened by a stimulus; and the unlimited tendency to rapid proliferation in malignant tumors cells would be deduced from a permanent predominance of the chromosomes that promote division*." Arguably, what he proposed is consistent with the interpretation that chromosomal aberrations would activate specific oncogenes when gained, or impair the function of tumor suppressors when lost, as a consequence of genomic copy number changes. We now know that this is correct [6–8].

Progress in the years to follow was slow. This was probably not attributable to a lack of interest or due to an a priori rejection of Boveri's hypotheses, but due to insurmountable challenges in the analysis of chromosomes. The analysis of tissue sections per se was exceedingly difficult, fixation and

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_4

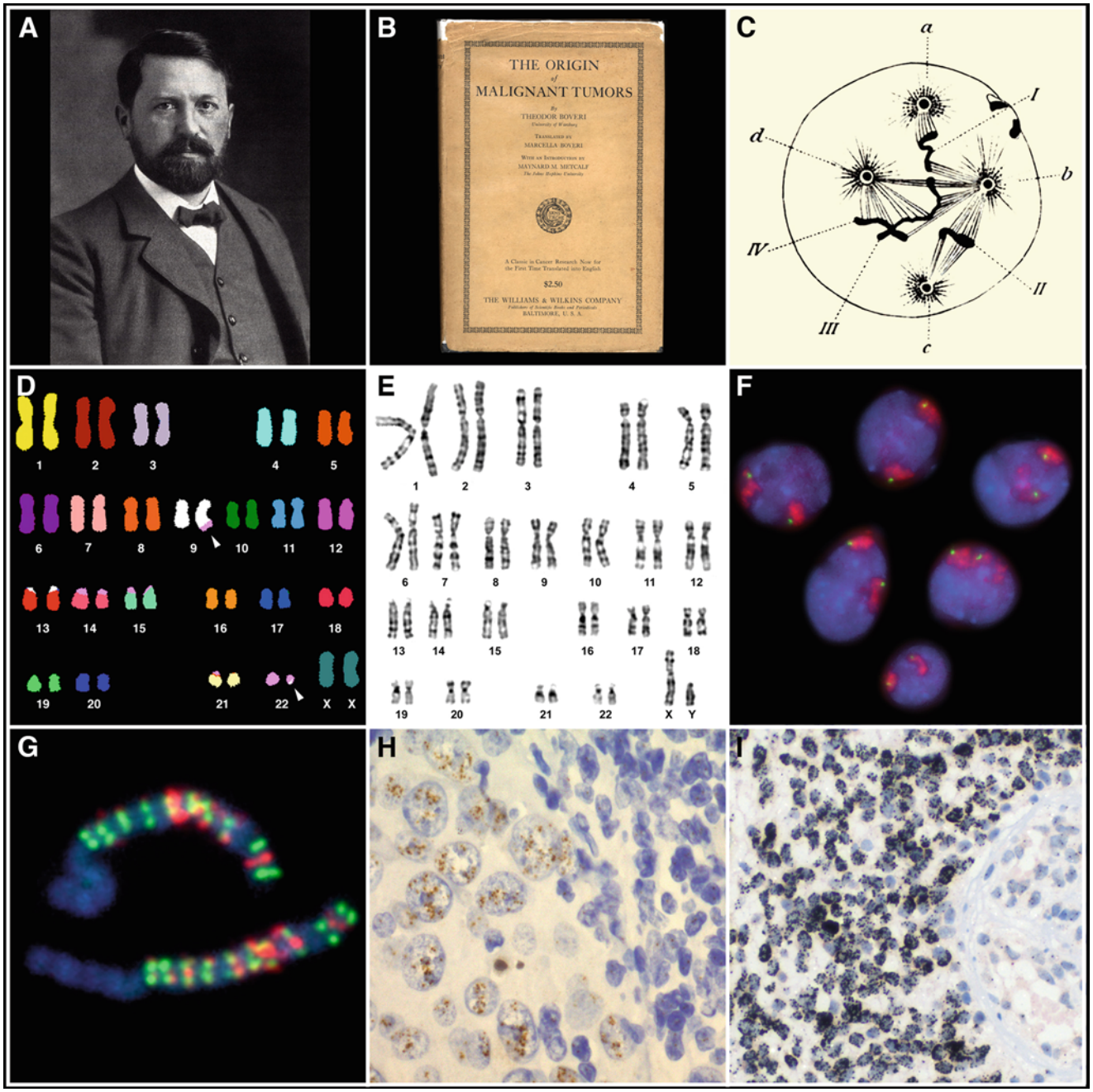

Fig. 4.1 Early chromosome analysis and molecular cytogenetics. (**a**) Photograph of Theodor Boveri (1862–1915). Boveri was a professor of zoology in Würzburg, Germany. Combining his theory that chromosomes carry genetic information with observations he made when studying cell division in ascaris larvae and double fertilized sea urchin eggs, Boveri postulated that cancer is a disease of the chromosomes. These studies were published in 1914 with the title "*Zur Frage der Entstehung maligner Tumoren.*" (**b**) Cover of the English edition of Boveri's book, which appeared in 1929. His wife and former student Marcella Boveri translated the text. (**c**) Additional centrosomes and aberrant mitoses in ascaris. These observations led Boveri to conclude that tumorigenesis ensues as a consequence of the loss of *inhibiting* and the gain of *promoting* chromosomes. (**d**) SKY analysis of chromosomes from a patient with chronic myelogenous leukemia. The balanced translocation between chromosomes 9 and 22, t(9;22), the so-called Philadelphia chromosome, is readily visible (*arrowheads*). The consequence of this translocation is the generation of a fusion protein with increased tyrosine kinase activity, generated by the juxtaposition of the genes *BCR* and *ABL*. Note that all other chromosomes are normal. (**e**) G-banded, normal male human karyotype. Chromosome banding revolutionized the discipline of cancer cytogenetics by providing the possibility to identify nonrandom, cancer specific chromosomal aberrations. (**f**) Dual color hybridization targeting chromosome 8 (*red*) and the *MYC* oncogene (*green*), which resides on chromosome band 8q24. Note that chromosomes occupy specific territories in the interphase nucleus. The *MYC* oncogene, expressed in the colon cancer cell line DLD1, is contained in the territory. Note that most of the territories are in the periphery of the nucleus, consistent with the fact that chromosome 8 is gene poor. (**g**) Dual color high-resolution mapping of multiple BAC clones on the long arm of chromosome 6. These clones are part of the CCAP collection, which was established to systematically integrate the cytogenetic and sequence maps of the human genome by FISH mapping sequenced BAC clones (http://cgap.nci.nih.gov/Chromosomes/CCAP). (**h**) Chromogenic in situ hybridization (CISH) of a breast cancer sample with probes detecting the *ERBB2* oncogene. The brown precipitates indicate high-level amplification in cancer cells. The adjacent lymphocytes are negative. The slide was stained with hematoxylin (*blue*). (**i**) Silver-enhanced in situ hybridization (SISH) of a malignant brain tumor (glioblastoma multiforme) showing amplification of the gene encoding the epithelial growth factor receptor, *EGFR*. The pronounced black stain can be analyzed automatically. Cells of vascular proliferation (right side of the slide) are negative for this gene amplification. The slide was stained with hematoxylin (*blue*).

preparation techniques were insufficient, and the preparation of metaphases slides was impossible, to name just a few hurdles for a systematic cytogenetic approach to the analysis of chromosomal abnormalities. The inability to identify consistent cancer-specific cytogenetic changes did not help to promote the field, and it is very conceivable that the subsequent frustration, which was mainly attributable to shortcomings in analytical technologies, supported the prevalent interpretation that cytogenetic abnormalities were present in cancer, but were merely disease-correlated, but not the cause of this disease [9, 10]. The fact that the correct number of human chromosomes was only established 4 years after the structure of the double helix was deciphered attests to the problems that cytogeneticists faced [11, 12] (the reader may consult the enlightening publication by Kottler, who discussed some additional shortcomings in cytogenetics at that time [13]).

After establishment of the normal human chromosome count, the field of human cytogenetics exploded. In particular, numerical chromosomal aberrations associated with congenital abnormalities were reported in short order [14–18]. However, the identification of recurrent aberrations in cancer cells came later. In 1960, Nowell and Hungerford described a consistent marker chromosome (termed the Philadelphia chromosome) in patients with chronic myelogenous leukemia (CML) [19]. This description was undeniably facilitated by the discovery that lymphocyte cultures can be stimulated with phytohemagglutinin (PHA) by the same authors [20]. For the first time, cancer was associated with a specific genetic abnormality that is visible through a microscope. The importance of this discovery cannot be overestimated. The consistency with which the Philadelphia chromosome was observed in CML all but proved that its presence reflected the molecular lesion accounting for the development of this hematological malignancy. Yet technical hurdles again prevented a thorough characterization of this marker chromosome because the structural integrity of chromosomes could not be analyzed in detail due to the lack of discernible features along individual chromosomes. This changed with the introduction of chromosome banding techniques [21, 22] (Fig. 4.1d). This revolution also facilitated the identification of intrachromosomal changes, and most importantly, the description of the reciprocal character of the Philadelphia chromosome—a balanced translocation involving chromosomes 9 and 22 [23] (Fig. 4.1e). Many more specific reciprocal translocation were subsequently identified, the translocation t(8;14) in Burkitt's lymphoma among them [24, 25]. Similar consistent aberrations were found in mouse models of human cancer, such as those characterized in murine plasma cell tumors [26, 27]. Together, these findings prompted the development of the hypothesis that the sites of the consistently observed breakpoints pointed to the location of cancer-promoting genes, which would be activated as a consequence of such translocations [28]. In fact, this was the case. The human *MYC* oncogene is juxtaposed to the immunoglobulin heavy chain gene via the translocation t(8;14) in Burkitt's lymphoma [29, 30], and an equivalent situation occurs in mouse plasma cell tumors on the respective orthologous chromosomes [26, 31]. The functional consequence is the deregulation of MYC expression by an active promoter of *IGH* in hematological cells, which is sufficient for malignant transformation. The Philadelphia chromosome represents a second mechanism for the translocation-induced activation of an oncogene—the generation of a fusion transcript with constitutively active tyrosine kinase—again sufficient and the cause for the emergence of leukemic cells.

The paradigm of translocation-induced oncogene activation and tumorigenesis was discovered based on cytogenetic analyses [32]. The importance of these discoveries cannot be overstated—not only does the detection of such aberrations serve as a diagnostic and prognostic tool and can be used for monitoring the efficacy of treatments, but it also identifies specific molecular targets for therapeutic intervention. For instance, BCR-ABL is the target for Gleevec in the case of CML [33]. The comprehensive analysis of chromosomal aberrations in solid cancers remained more complicated and had to await the development of molecular cytogenetic techniques that we review in the following sections.

4.2 The Emergence of Molecular Cytogenetics

The development of chromosome banding techniques is likely the single most important technical development in cytogenetics. Chromosome banding was first developed by Torbjörn Caspersson and Lore Zech in Stockholm using quinacrine mustard fluorescence and was immediately applied to establish karyotypes of hamster, human, and mouse chromosomes. As described above, the application for the detection of chromosomal abnormalities followed in short sequence. A conference held in Paris in 1971 published an official version of the human karyogram and established a map of human chromosomes. With the introduction of Giemsa banding by Seabright in 1971 [34], which omitted the requirement for fluorescence microscopy and generated permanent banding patterns, the method quickly entered laboratories around the world, and for decades to come was the most frequently performed genetic test (Fig. 4.1d). Besides its application in cancer, chromosome banding has become the method of choice for the detection of constitutional chromosomal abnormalities, both in the postnatal and prenatal settings. While the knowledge gained through the systematic application of chromosome banding techniques is vast, the technique has limitations. First, the resolution is low. Second, chromosome banding can only be applied to fresh tissues from which the cells can be cultured and arrested

in metaphase (or prometaphase when higher resolution was desired). Third, the interpretation of banded karyotypes requires a skill set that not everyone can muster. These limitations combine to account for the fact that considerably more karyotypes from hematological malignancies have been published than from solid cancers [35, 36]. Most solid cancers are difficult to culture, and the morphology of metaphase chromosomes is often poor. In addition, the sometimes enormous number of cytogenetic abnormalities in solid cancers prevented a comprehensive analysis of all aberrations, and cytogenetic correlates of oncogene amplification, such as double minute chromosomes (dmin) and homogeneously staining regions (hsr), could be described, yet their chromosome of origin remained elusive. Most of these shortcomings were overcome by the marriage of classical cytogenetic chromosome analysis with independently developed molecular techniques (DNA cloning and hybridization).

4.2.1 In Situ Hybridization

The first relevant achievement was the successful in situ hybridization with radioactively labeled nucleic acids by Joe Gall and Mary Pardue in 1969 [37–39]. This was followed by the first in situ hybridization with fluorescently tagged probes by Rudkin and Stollar in 1977 [40]. Clearly, omitting radioactivity had intrinsic advantages, such as increased resolution, shorter detection times, and the possibility to increase the number of simultaneously detectable targets. Rudkin and Stollar used indirect immunofluorescence for the visualization of RNA/DNA hybrids. Another very important development for the success of molecular cytogenetics was the development of covalent labeling of nucleotides with haptens, the first of which, and still one of the most widely used, was biotin [41]. These hapten-conjugated nucleotide analogs can be incorporated enzymatically into DNA molecules, hybridized, and visualized with suitable detection systems, such as avidin conjugated fluorochromes or antibodies against other haptens, such as digoxygenin or dinitrophenol. Their advantage lies in the fact that their use increases the multiplicity and sensitivity of in situ hybridization experiments, increasing experimental versatility. More recently, nucleotide analogs directly coupled with fluorochromes have become available as well. The authors of this chapter purposely abstain from providing details of protocols for in situ hybridization experiments. Such protocols can be found in great detail and abundance for instance in the following references [42–45] and websites (http://www.riedlab.nci.nih.gov/).

Using a hybridization format for cytogenetic analyses obviously brought with it the possibility of designing DNA probes that were suitable to answer the specific experimental question asked. Among the first probes used for fluorescence in situ hybridization (FISH) were probes that targeted the centromeric or paracentromeric heterochromatin of specific chromosomes. Such probes readily allowed chromosome enumeration in metaphase preparations, but even more importantly enabled the concept of interphase cytogenetics. This meant that numerical chromosomal aberrations could be detected in nondividing cells, throughout all stages of the cell cycle, opening the door for pathologists to use cytogenetics to augment the morphology-based diagnosis of samples in the clinical laboratory. The concept and the term *interphase cytogenetics* were first conceived and published in a paper by Cremer and colleagues, who used a radioactively and nonradioactively labeled probe to detect the number of chromosome 18 in fibroblasts [46]. The concept of interphase cytogenetics proved to be very useful for the detection of aneuploidies in prenatal diagnostics, where numerical chromosomal abnormalities of chromosomes 13, 18, and 21 play a major role for constitutional syndromes, which previously could be diagnosed only after in vitro culture of amniotic fluid cells [47]. Interphase FISH is now widely used in the screening for chromosomal disorders in prenatal diagnostics.

4.2.1.1 Chromosome Painting

A second set of probes that became immensely useful in the diagnosis of chromosomal disorders were probes that allowed delineation of entire chromosomes. Chromosome painting probes were first reported for the human Y chromosome by Christoph Cremer, Joe Gray, and colleagues at the University of Freiburg and the Los Alamos National Laboratory [48]. Two groups, one with Thomas Cremer and David Ward at Yale University, and the other with Dan Pinkel and Joe Gray at the University of California in San Francisco, developed methods for painting specific chromosomes based on suppression hybridization [49–51]. Initially, such painting probes were cloned in phage or plasmid vectors [52], which resulted in acceptable signal to noise ratios. However, chromosome painting probes were soon prepared by either flow-sorting or by microdissection of individual chromosomes [53, 54]. Both techniques require amplification of the isolated nucleic acids using universal DNA amplification [55], but the signal to noise ratio is superior to that of the cloned chromosome libraries. Suppression of repetitive sequences is necessary to assure chromosome specific hybridization, which is achieved by including unlabeled DNA (either whole genomic DNA or repetitive sequence enriched Cot1-DNA) in the hybridization mixture. Chromosome painting probes are now routinely used to verify suspected aberrations in the clinical cytogenetic or in the cancer cytogenetic laboratory. Probes already labeled with different fluorescent dyes are commercially available from a variety of commercial suppliers. Chromosome painting probes have proven to be valuable experimental tools above and beyond their applications in diagnostics, for instance in

the study of the three dimensional (3D) architecture of the interphase nucleus [56]. FISH with painting probes to interphase nuclei has unambiguously confirmed classical studies using UV-microbeam experiments [57] showing that chromosomes maintain their integrity throughout all stages of the cell cycle and are arranged as domains, or territories. Now we also know that chromosomes are not randomly distributed in the 3D nuclear space. Several groups have generated solid evidence that gene-rich chromosomes tend to be found more towards the interior compartment, whereas gene-poor chromosomes are positioned more towards the nuclear periphery [58, 59]. This organization appears to also be maintained to a certain degree in cancer cells, and in cells which contain artificially introduced chromosomes [60–62]. Chromosome painting has therefore become instrumental in attempts to decipher how the architecture of the nucleus affects transcriptional regulation in physiological states as well as in cancer, and how the dedifferentiation of cancer cells affects or is affected by the organization of chromosome territories. In combination with probes for specific genes, chromosome painting probes can be used to investigate whether the transcriptional state of a specific gene is reflected by the relative position in its territory [63, 64] (Fig. 4.1f).

4.2.1.2 Gene-Specific Probes

With the improvements in microscopic techniques and the availability of more and better probe labeling methods and fluorochromes, the versatility of FISH experiments also greatly increased [65]. These developments were paralleled by progress of the Genome Project and the availability of bacterial artificial chromosome (BAC) clones for essentially every gene. Historically, some of the first gene specific probes were cloned into lambda phage vectors and used for the visualization of specific chromosomal breakpoints associated with different malignancies, including CML and Burkitt's lymphoma. For instance, the groups of Pinkel and Gray first succeeded in visualizing the *BCR-ABL* fusion in malignant cells from a patient with CML in interphase cells using a two-color hybridization format which resulted in a co-localization event on metaphase chromosomes and in interphase nuclei in which the Philadelphia chromosome was present [66]. Ried and colleagues developed a three-color approach that included a chromosome 8 painting probe together with probes specific for the *MYC* oncogene and probes for the *IGH* locus to achieve specific metaphase and interphase detection of the translocation t(8;14) in cell lines derived from a patient with Burkitt's lymphoma [67]. The usefulness of this probe set was also explored for the detection of minimal residual disease [68]. The obvious advantage of being able to visualize specific chromosomal translocations directly in nondividing cells led the community of hematological oncologists to embrace the use of FISH very quickly. Today, specific FISH probes for a plethora of chromosomal translocations, inversions, deletions, and amplifications are available from a variety of suppliers. An appealing feature of FISH is that it can be combined with immunohistochemistry or specific pertinent stains. This allows identification of cancer-causing chromosomal aberrations in tissues that maintain the phenotypical appearance that a diagnostic pathologists is used to [69–71].

FISH probes are not restricted to specific genes known to be the target of chromosomal aberrations. The National Cancer Institute has established a resource that systematically integrates the cytogenetic and sequence maps of the human genome. This was achieved by mapping a set of more than 1300 sequence-verified BAC clones to high-resolution banded chromosomes. Any suspected cytogenetic abnormality can now be confirmed and characterized with these tools, and the molecular events at sites of chromosomal aberrations pursued to the very sequence [72–75] (Fig. 4.1g).

4.2.1.3 Molecular Cytogenetics for the Pathologist

While many molecular techniques never became part of routine pathology, FISH was implemented relatively soon after its development. This was due to the fact that the protocol was fairly simple, that tissue morphology was conserved, the method was affordable, and it was able to detect aneuploidy and structural chromosomal rearrangements, both highly relevant for carcinogenesis. Applying in situ hybridization with appropriate probes augments the morphological assessment and can contribute to diagnosis, differential diagnosis, and to prognostication. Using sequence-specific probes, virtually all known translocations can be identified. Next, we review two of the main nonfluorescent in situ hybridization formats used in routine pathology laboratories. In subsequent sections we summarize some of the major applications of in situ hybridization in pathology, with an emphasis on solid cancers.

4.2.1.4 Chromogenic In Situ Hybridization

Evaluation of FISH slides has some drawbacks since it requires an epifluorescence microscope, the analysis needs to be performed in a dark environment, and the signals eventually fade. To overcome these disadvantages, an alternative has been developed—chromogenic in situ hybridization (CISH). CISH is based on the same molecular principles as FISH but uses chromogens instead of fluorescent dyes to visualize the hybridized probes. A comparison of features between FISH and CISH can be found in Table 4.1. The best staining results are obtained with indirect labelling protocols using either biotin or digoxigenin as haptens. The digoxigenin-labeled DNA probe is detected by using anti-digoxigenin antibodies, while the biotin labeled probe is visualized with avidin conjugated with enzymes such as alkaline phosphatase (AP) or horseradish peroxidase (HRP). These enzymes produce, in a secondary reaction, a stable precipitate of chromogens that are generated by the enzymatic conversion

Table 4.1 Overview of commonly used in situ hybridization systems in the pathology laboratory

	FISH	CISH	SISH
Automation	Semi	Semi	Full
Specific equipment	Yes (epifluorescence microscope)	No (standard bright field microscopy)	No (standard bright field microscopy)
Assay time	Overnight	Overnight	6 h
Scoring time	Time consuming, needs trained personnel and expertise	Fast interpretation of the results	Fast interpretation of the results
Morphology conservation	Reduced due to fluorescence	Normal "IHC" morphology	Normal "IHC" morphology
Signal quality	Good	Sometimes faint	good
Multiple probes simultaneously	Yes, direct	Yes, indirect	Not yet
Concordance with FISH	–	Good	Good
Image Analysis	Yes, but not routinely	Yes, but not routinely	Yes, with fast results
Processing time	1 day	0.5 days	1 h
Archivability	No	Long term	Long term

from soluble states. The sections are subsequently counterstained with hematoxylin and can be evaluated with bright field microscopy. Most of the current CISH applications are based on a single-color detection method, which does not allow simultaneous illustration of a reference probe on the same slide [76–78]. This limitation was overcome by the development of dual-color and triple-color in situ hybridization protocols [79–81]. Recent publications attest to the robustness and reproducibility of such protocols. The concordance with FISH based experiments is excellent [82, 83] (Fig. 4.1h).

4.2.1.5 Silver-Enhanced In Situ Hybridization

CISH overcomes some of the limitations of FISH, but has not yet found broad acceptance in pathology laboratories. The reasons for this might be the requirement for manual processing, overnight hybridization, and the need for two separate detection steps (one for the gene of interest and one for the reference probe). Furthermore, some CISH samples are difficult to evaluate because the signal can be weak. This led to the development of a fully automated in situ hybridization protocol, producing a stable and discrete chromogenic reaction product—silver in situ hybridization (SISH) [83]. SISH is fast and the experiment is analyzed by standard light microscopy. The technology applies an enzyme-linked probe, which in turn accumulates silver particles, forming a dense, punctuated, high-resolution black signal, which is easily recognizable. It provides a permanent record, with no fading or photobleaching, and its sensitivity allows visualization of single genes. The SISH technique is performed as an automated protocol and completed within one working day (about 6 h). Several studies have verified a very high concordance rate between SISH and FISH, proving clinical applicability [84–88] (Fig. 4.1i). A comparison of features between FISH and SISH can be found in Table 4.1.

4.2.1.6 Hybridization With Genomes

Chromosome banding, albeit with limited resolution, allows the survey of an entire genome. This feature is critical if the investigator has no a priori knowledge of the aberrations to be identified or characterized. The previously described molecular cytogenetic methods lack this characteristic and were therefore most useful to confirm, specifically and in nondividing cells or tissue sections, the presence of suspected aberrations. The research community therefore attempted to combine the advantages of both and developed genome-screening tools based on fluorescence in situ hybridization.

4.2.1.7 Comparative Genomic Hybridization (CGH)

The first such concept was realized with the introduction of comparative genomic hybridization (CGH) by Kallioniemi and colleagues at the University of San Francisco in California and by the group of Thomas Cremer in Heidelberg. Using a CCD camera (or other digital imaging device) fluorescent intensities can be measured quantitatively, that is the measured intensity is proportional to the amount of hybridized fluorescently labeled probe. If sequences in a test genome labeled with a green fluorochrome are more abundant than in a red-labeled reference genome, this difference should become visible upon hybridization to normal metaphase chromosomes as reflected by increased fluorescent signal. Conversely, losses of genetic material in the test genome would result in reduced green intensity. In papers published in 1992 and 1993 both groups presented convincing proof-of-principle experiments [89–91] by mapping genes amplified in cell lines and primary cancer, and by showing that by hybridizing DNA from individuals with constitutional chromosomal abnormalities such numerical imbalances can be identified. CGH has become one of the most important techniques in the study of cancer genomes,

and essentially all major cancers and cell lines derived from them have been studied in great detail. Several databases now contain comprehensive catalogues of chromosomal imbalances, and several reviews summarize the many primary publications [92–94].

As apparent from the experimental concept, screening the genome for imbalances using CGH does not require the preparation of metaphase chromosomes from cancer samples for banding analysis. This feature was particularly useful for the analysis of solid cancers of epithelial origin (carcinomas), because cell culture of such samples is often difficult, and only for some carcinomas could cell lines be established in sufficient quantities. Karyotyping was further impeded because the morphology of metaphase chromosomes was poor, and in general the karyotypes revealed more aberrations compared to spreads prepared from hematological malignancies [36]. The fact that only cancer DNA was required for genome screening had another significant advantage—it opened the door to apply cytogenetic analysis to formalin fixed and paraffin embedded samples [95–97]. This feature allowed accessing the richly stocked pathology archives to perform retrospective analyses and to compare genomic aberration profiles with clinically relevant parameters, such as outcome and response to therapy. Moreover, it greatly facilitated the analyses of premalignant lesions. It is eminently difficult to establish cell lines for cytogenetic analysis from bona fide precursor lesions of carcinomas, and the earlier the lesions are situated in the sequential progression events to malignant, invasive disease the more normal cells are present. This problem could be overcome by dissecting morphologically identified premalignant lesions from surrounding normal tissue and extracting DNA exclusively from morphologically suspicious lesions [98, 99]. Of course, the earlier such lesions are detected the smaller they usually are, and not always is the quantity of DNA recovered sufficient for standard CGH experiments (which is in the range of a few micrograms). This limitation was overcome by the development of protocols for the sequence independent universal amplification of DNA using degenerate primers followed by cycles of PCR [100]. In essence, these combinations of methodologies enabled characterization of the sequential genomic imbalances in the progression of solid cancers. When combined with micro-dissection or macro-dissection of morphologically characterized lesions one could then establish a phenotype/genotype correlation and provide insights into the dynamics of genomic aberrations during tumorigenesis [99, 101]. Through improvements in protocols for microdissection, genome amplification, and CGH,)genomic aberrations profiles could be established from only a few, and possibly from single cells, with considerable implications for the study of cancer heterogeneity, clonal composition, tumor dormancy, identification of disseminated cancer cells, and for the identification of the cell of origin of metastatic lesions [102–105].

CGH does not require the analysis of aberrant chromosomes, yet even the correct identification of DAPI banded normal chromosomes requires a certain cytogenetic skill set and prevented automation and high-throughput. Furthermore, the resolution with which chromosomal gains and losses could be detected is limited by the resolution of the target metaphase chromosomes (in the megabase range), and was dependent on the copy number change. High-level copy number amplifications could be more readily detected than subtle deletions (but the mapping resolution along the axis of chromosomes of course remained the same). Accordingly, the molecular cytogenetic community quickly realized that these drawbacks could be overcome if metaphase chromosomes are replaced by specific nucleic acids arrayed on a suitable medium as the hybridization target [106, 107]. The analysis of CGH experiments on such a format does not require identification of banded chromosomes, increases the resolution essentially to the length of the blotted nucleic acid sequences, allows automation, and with the genome sequence available enables investigators to seamlessly identify the nature of the genes affected by copy number imbalances. Initially the concept of array-based CGH was realized using BAC clones immobilized on glass slides. While suitable for detecting gene-specific copy number variations, improvements in this technology now utilize defined oligonucleotide sequences, either synthesized or printed onto slides, or attached to beads, to provide remarkably robust and reproducible tools for the high-resolution mapping of genomic imbalances in test genomes. Many of these arrays not only allow detection of copy number changes, but also identification of sequence polymorphisms, to provide information on allelic imbalances (loss of heterozygosity). The application of aCGH to cancer samples of the same entities as analyzed by CGH)confirmed the predominance of recurrent, cancer-specific chromosomal gains and losses (Fig. 4.2a). However, aCGH is particularly useful for the high-resolution mapping of amplified regions, and for the detection of subtle deletions (Fig. 4.2b). Furthermore, aCGH shows that chromosomal breakpoints in carcinomas (here specifically colorectal carcinomas) map to the site of copy number variants (CNVs) in the human genome [108] (Fig. 4.2c). Only high-resolution platforms enabled the detection of such unrecognized plasticity of the human genome in cancer cells.

4.2.1.8 Spectral Karyotyping (SKY) and M-FISH

An alternative avenue to pursue hybridization-based analysis of entire genomes was taken by increasing the multiplicity (the number of simultaneously discernible targets of chromosome painting experiments) with the ultimate goal of visualizing all human chromosomes in different colors. Serious attempts to increase multiplicity were pioneered in Leiden, The Netherlands, by realizing the concept of differentially labeling chromosome painting probes and dissecting

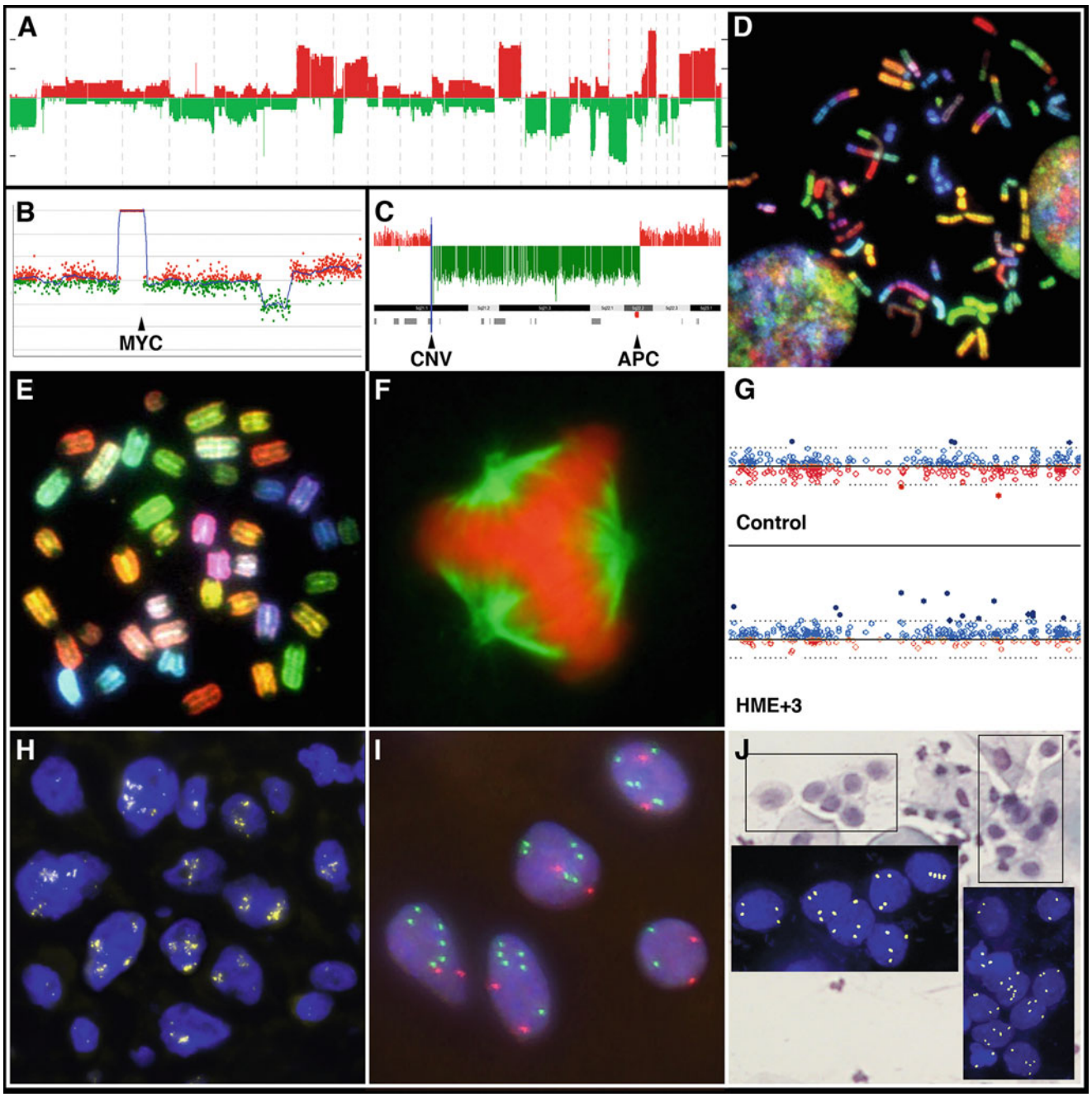

Fig. 4.2 Methods and applications of molecular cytogenetics. (**a**) aCGH analysis of colorectal carcinomas. Shown is a map of chromosomal gains and losses over the entire genome. The *vertical dotted lines* demarcate individual chromosomes. The distribution of genomic imbalances is strictly conserved and specific for this cancer entity. For instance, gain of chromosome 13 occurs only in CRC, but not in other cancers of epithelial origin. CRC is defined by gains of chromosomes 7, 8q, 13, and 20q, and losses that map to chromosomes 8p, 14, 15, 17p, and 18q. This CRC-specific distribution of whole and partial chromosomal gains and losses is maintained in local and distant metastases, and in cell lines derived from primary cancers. (**b**) High-resolution mapping of an amplicon in CRC. In this case, the *MYC* oncogene is part of a localized high level amplification on 8q24, as indicated. aCGH also identified a small deletion (*green dots*) and a copy number gain (*red dots*) towards the telomere of the chromosome. (**c**) Structural variants in the human genome are prone to cause cancer initiating chromosomal aberrations. In this case, the deletion of the *APC* tumor suppressor gene, which is frequently inactivated in CRC, was likely triggered by recombination events at the site of a copy number variant (CNV). Possibly, replicative stress at these sites can induce double strand breaks which can result in chromosomal aberrations. (**d**) SKY analysis of the most malignant brain tumor, glioblastoma multiforme. The poor prognosis of this tumor is paralleled by the enormous amount of genomic instability, accentuated by the fact that most chromosomes are involved in rearrangements. Yet chromosome territories are clearly demarcated in the interphase nuclei (incomplete in the lower left and middle right section of the image). (**e**) SKY analysis of mouse chromosomes. The image shows normal murine chromosomes. SKY was particularly helpful in validating mouse models of human cancer, because it allowed a rapid assessment of chromosomal instability in cells prepared from a great

the fluorescence with specific optical filters and digital imaging devices [109–111]. The approach increased the number of simultaneously visible targets beyond the numbers of utilized fluorochromes. This approach was extended to the detection of up to seven targets, including centromere-specific probes, chromosome painting probes, and cosmid clones along chromosome 5 [112] and was applied to the simultaneous detection of aneuploidies of human chromosomes 13, 18, 21, X, and Y directly in uncultured cells from amniotic fluid [47]. These efforts culminated in the development of spectral karyotyping (SKY) and M-FISH (Fig. 4.2d).

Both techniques) allow simultaneous discernment of all human chromosomes with chromosome specific hues. M-FISH was based on continuous improvements in optical filter technologies, chromosome painting probes and more sensitive imaging equipment. Sequential capture of combinatorially labeled painting probes with narrow band pass optical filters was used to generate composite images that displayed all chromosomes separately. In contrast, SKY used an imaging approach that had not been previously used in cytogenetics—after labeling and hybridization of combinatorially labeled flow-sorted chromosomes, images were acquired in a single exposure using an epifluorescence microscope to which an interferometer and a CCD camera were attached. This combination allowed reconstruction of the spectrum of individual pixels over the entire CCD chip, and hence assignment of spectral information to all points of the image. Specifically developed software then allowed for seamless assignment of pseudo-colors to each pixel, and with that for automated karyotyping based on whole genome hybridization. The first publications reporting the respective approaches were published in 1996 [113, 114]. From then to now, numerous publications followed that attest to the usefulness of SKY and M-FISH with respect to cancer cytogenetics. SKY proved useful for the identification of hidden chromosomal aberrations in hematological malignancies [115], including the characterization of the widely used leukemia cell line HL60 [116]. Other examples pertain to the detection of translocations in multiple myeloma [117, 118], the identification of novel translocations in patients with acute myelogenous leukemia [119], and the identification of mechanisms of drug resistance in acute leukocytic leukemia [120]. Regarding cytogenetic analysis of solid cancers, SKY was instrumental in identifying jumping translocations as a frequent mechanism for the generation of specific genomic imbalances [121], for elucidating the role of centrosome aberrations related to chromosomal instability in colorectal cancers [122], and for a comprehensive and definitive characterization of the karyotype of the HeLa cervical cancer cell line [123].

SKY was quickly adapted to the analysis of murine karyotypes [124] and proved eminently useful for the characterization and eventual validation of mouse models of human cancer (Fig. 4.2e). Two examples demonstrate this here. A knockout mouse model that was designed to study the consequences of deletions of the gene that causes ataxia telangiectasia in patients showed that homozygous deletion of the mouse homologue results in the development of a hematological malignancy (among other features that recapitulate the human disease phenotype). In fact, SKY analysis of the T-cell lymphomas that developed at early age in *Atm*-deficient mice revealed chromosomal aberrations involving mouse chromosome 14, which carries the T-cell receptor genes. Subsequent high-resolution analysis with BAC clones)for these genes confirmed their involvement, validating this mouse model not only as a model for ataxia telangiectasia, but also for human T-cell derived hematological malignancies [125]. A second example pertains to the development of pro-B-cell lymphomas in mice deficient for the DNA repair genes *Ku70/80*. Invariably, these mice succumbed to tumors and the SKY-based cytogenetic analysis disclosed consistent aberrations involving mouse chromosomes 12 and 15—

Fig. 4.2 (continued) number of mouse models. Mouse chromosome analysis based on banding alone is challenging because all mouse chromosomes are acrocentric and less different in length compared to human chromosomes. (**f**) A tripolar mitosis in cancer cells (note the similarity with Fig. 4.1c). Alpha tubulin (stained in *green*) labels microtubules required for proper chromosome segregation upon attachment to the centromeres of metaphase chromosomes. DNA is stained in *red*. In this case the deletion of the tumor suppressor gene *BRCA1* led to an amplification of centrosomes that caused abnormal mitoses. The DNA content of the daughter cells will not be normal (one of Boveri's hypotheses). (**g**) Consequences of chromosomal aneuploidies on the transcriptome. This figure visualizes the result of a chromosomal trisomy on transcriptional activity of genes on that chromosome. The trisomy was induced using microcell-mediated chromosome transfer. Gene expression profiling demonstrated that the expression levels of most genes are dependent on genomic copy number (*lower profile*). This is consistent with observations in primary cancers or cell lines. (**h**) FISH on tissue sections. Shown is the amplification of the cell cycle regulating gene *CCND1* in a tissue section of a head and neck cancer (this malignant neoplasm was located in the tongue) in highly aneuploid nuclei. Diploid copy numbers of this gene are present in lymphocytes in the lower left corner of the image. (**i**) Extra copy numbers of chromosome 3q are always present in cervical cancer. This figure shows cervical epithelial cells collected at a routinely performed Pap smear. The *arrow* denotes a normal diploid cervical cell, with two copies of the long arm of chromosome 3q probed using the human telomerase gene *TERC* (*green*) and a control probe for the centromere of chromosome 7 (*red*). The cells with an aberrant hybridization pattern all show six copies of *TERC,* which clearly suggests a clonal expansion once the cervical cancer specific aneuploidy was acquired. (**j**) Phenotype/genotype correlation of a high-grade cervical dysplasia (high-grade squamous intraepithelial lesion, or HSIL). This Pap smear was repeatedly assessed as being normal, yet was followed by the diagnosis of an invasive cervical carcinoma. Retrospective analysis of this case clearly demonstrates the potential of molecular cytogenetics in augmenting the morphology-based evaluation of cytological specimens. The presence of four copies of *TERC* is obvious. Again, the emergence of cervical cancer is consistent with a clonal expansion of cells that acquired the gain of 3q.

chromosomes that carry the *Myc* oncogene and the *IgH* locus, respectively. This triggered a targeted analysis and showed that indeed the *Myc* oncogene was juxtaposed to the *Igh* locus, creating a genetic error identical to the one observed in human Burkitt's lymphoma [126]. In subsequent analyses, this particular mouse model has become exceedingly useful for the elucidation of mechanisms of double-strand break repair and the emergence of chromosomal translocations [127].

The above-described examples do not provide a complete review of the cytogenetic literature. This was neither intended nor can it be accomplished within the scope of such a book chapter. Rather, the authors wished to exemplify how cytogenetic based screening for chromosomal aberrations can be successfully used to home in on disease initiating, causal molecular mechanisms of cancer development.

4.3 Chromosomes in Cancer Cells

All cancers have chromosomal aberrations. Despite the fact that such apodictic statements are problematic in general, and even more so when related to biology, it is indeed the case that the vast majority of human cancer shows at least one abnormality in their karyotypes. There are a few exceptions to this rule—in some instances of hematological malignancies chromosome banding analyses revealed no aberrations, and familial colorectal cancers that arise due to deficiencies in the DNA mismatch repair machinery can have a stable and normal karyotype. Regarding the hematological malignancies, the failure to detect chromosomal aberrations might be due to the fact that culture conditions favored cells other than those derived from the cancer, and we know that in mismatch-deficient colorectal cancers the enormous number of point mutations might make the acquisition of cytogenetic abnormalities superfluous. Despite these specific exceptions, cancer is a disease of the chromosomes [5]. The cytogenetic analysis of hematological malignancies (leukemias and lymphomas) reveals recurrent chromosomal aberrations, often manifest as translocations, that strongly suggest that they denote disease-causing and not just correlated, genetic changes. We know today that recurrent chromosomal translocations invariably point to the location of genes whose deregulation causes malignant transformation in tissues of hematological origin. In most cases the mechanism of deregulation adheres to one of the following concepts. In one instance, the chromosomal translocation results in the generation of a fusion gene that encodes an aberrant protein with transforming activity. The Philadelphia chromosome translocation t(9;22) and its consequence, the *BCR-ABL* fusion and resulting increased tyrosine kinase signaling, serve as the paradigm [128]. The second mechanism juxtaposes an oncogene into the vicinity of a gene constitutively active in a certain tissue, hence taking over the transcriptional regulation of that oncogene. This mechanism was first identified in Burkitt's lymphoma and in mouse plasma cell tumors [26, 29, 30]. Here, the chromosomal translocation t(8;14), in human, or the T(12:15), in the mouse, result in transcriptional deregulation of *MYC* due to its regulation by the *IGH* regulatory elements (variant translocations of *MYC* to other immunoglobulin loci are observed as well, albeit with lower frequency). Another prominent example is the deregulation of the apoptopic pathways through upregulation of *BCL2* via the t(8;14) translocation in follicular lymphoma [129], or the inversion of chromosome 14 in T-cell leukemia which results in the deregulation of *TCL1* expression because of fusion with the T-cell receptor locus [130, 131], as well as the translocation t(8;21) of *ETO* and *AML1*, which causes acute myelocytic leukemia [132]. A comprehensive summary of cytogenetic abnormalities can be found in the book ***Cancer Cytogenetics*** by Heim and Mitelman and in the compendium by Avery Sandberg [35, 36], or online at the Cancer Genome Anatomy Project website (http://cgap.nci.nih.gov/Chromosomes/).

4.3.1 Patterns of Chromosomal Aberrations and Genomic Imbalances

The comprehensive cytogenetic analysis of cancers of epithelial origin was more difficult to generate for technical reasons, but also due to the sheer number of chromosomal aberrations found in the carcinomas [36]. This feature, together with the observation of centrosome amplification, irregular mitotic spindles, apolar mitoses, anaphase bridges as a consequence of telomere dysfunction, and chromosome segregation errors led to the perception of a cytogenetic chaos that governs the karyotype of carcinomas (Fig. 4.2f). Compared to the hematological malignancies, it was more difficult to identify a recurrent pattern of chromosomal changes, which, as a result of its consistency, would suggest a disease-causing, and not only a disease-associated, event. While in some solid cancer entities chromosome banding analyses showed nonrandom aberrations, such as the loss of chromosome 3p in lung cancer [133] or gain of chromosome 7 in colorectal cancer (CRC) [134], the dominance of specific genomic imbalances was only established with the introduction of molecular cytogenetic techniques, and in particular CGH. It is now common knowledge that distinct carcinoma entities are defined by a strictly conserved and nonrandom distribution of gains and losses of chromosome arms or entire chromosomes with ensuing specific genomic imbalances. The distribution of these imbalances is cancer-specific and can be specific for discrete cancer stages. For instance, cervical carcinomas invariably carry extra copies of the long arm of chromosome 3. This specific lesion occurs before the transition to invasive disease and can be found as

probably the earliest genome mutation. In contrast, in colorectal cancer chromosome 3 does not appear to be important. However, the landscape of genomic imbalances in CRC is defined by the acquisition of extra copies of chromosomes 7, 8q, 13, and 20 which are accompanied by losses on 8p, 17p, and 18q. Chromosome 7 gains are observed in early adenomas. In general, this distribution of genomic imbalances is maintained in metastases of this disease, and is also conserved in cell lines established from these cancers, even after long periods of in vitro culture [135]. These interpretations would be consistent with a constant selection of clones that contain such imbalances [136]. It should be obvious from these features that these specific patterns of chromosomal imbalances are perfectly suited to distinguish one cancer type from another [8]. This was confirmed in a large dataset by Beroukhim and colleagues [137]. Here, the authors compiled results from aCGH analyses of more than 3000 cases of divergent cancer entities and identified somatic copy number alterations that extended over entire chromosomes or chromosome arms, or were localized. Not surprisingly, cancers could be classified into the lineage of origin, and for those belonging to the same lineage (for instance lung versus CRC) into specific cancer entities, based on the distribution of whole chromosome or chromosome arm specific gains and losses. Interestingly, this classification deteriorated when only those events that presumably are the target of genomic imbalances were used, such as amplification of *MYC* on chromosome 8q. This again attests to the functional relevance of chromosomal aneuploidies in tumorigenesis and suggests that genes other than the most obvious targets are required for malignant transformation, or for the maintenance of the transformed phenotype.

The question then arises as to the consequences of chromosomal aneuploidies on the transcriptome of cancer cells. One could envision several scenarios: (1) chromosomal aneuploidy targets the expression of only one or a few genes on the affected chromosomes, (2) the expression of most if not all genes is affected, and (3) chromosomal aneuploidies are neutral regarding gene expression. This basic question in cancer biology has been unambiguously answered. For instance, Upender and colleagues used microcell-mediated chromosome transfer to generate artificial trisomies in otherwise karyotypically normal cells. Gene expression profiling was then used to query the consequences on the transcriptome, and showed that transcript levels followed genomic copy numbers for the majority of genes [138] (Fig. 4.2g). The same situation has been observed in primary cancers and in cancer cell lines in a variety of different cancer entities [139–146]. This justifies the general conclusion that transcript levels in cancer cells are affected by genomic copy numbers. Therefore, aneuploidy results in a massive deregulation of the cancer transcriptome by changing the expression of hundreds to thousands of genes. One of the basic questions in cancer biology that has not been satisfactorily answered is to what extent the aneuploidy-dependent transcriptional deregulation is necessary for carcinogenesis [147]. One could favor the explanation that aneuploidies are just convenient mechanisms for the transcriptional deregulation of a few target genes, and that their frequency is more a reflection of the ease with which they can be acquired and maintained. In this interpretation, aneuploidy is a passenger rather than a driver. Alternatively, one could postulate that tissue-specific metabolic changes (induced via aneuploidy-dependent transcriptional changes) operate in concert with the activation or inactivation of specific oncogenes and tumor suppressor genes, respectively. However, the answer to this important question remains unknown.

4.3.2 Diagnostic and Prognostic Applications

FISH has become a versatile tool for the detection of chromosomal translocations in hematological malignancies. Increased probe multiplicity, improved protocols and more sensitive imaging equipment, and a large set of sophisticated probe designs have made interphase FISH the method of choice in many diagnostic laboratories. The possibility to combine the visualization of disease causing genetic aberrations with the phenotype of cells further enhances its value. The practical application of these methods has been made easier through the commercial availability of probe sets with a high signal to noise ratio.

The role of FISH for the diagnosis of carcinomas is far less developed, which actually cannot be attributed to the lack of disease-specific aberrations. The gain of chromosome arm 3q in cervical carcinomas is as common as is the Philadelphia chromosome in patients with CML [99]. The reason for the delay in adapting FISH to augment the diagnosis of carcinomas and its precursor lesions might be manifold. However, two reasons appear rather obvious: (1) FISH signals are difficult to enumerate on tissue sections from the surgical pathology laboratory, and (2) the target gene (or more likely, target genes) for the recurrent acquisition of genomic copy number changes in solid cancers are not well understood (whereas the disease-initiating role of chromosomal translocations in hematological malignancies is clear). Despite these hurdles, interphase cytogenetics will become more widely applied in solid cancer diagnostics, certainly in such cases in which the diagnosis or screening does not primarily rely on tissue sections but on cytological preparations. We now discuss several applications pertaining to different carcinomas, some of which can be diagnosed on routinely collected cytological specimens, collected by either aspiration or exfoliative sampling, or on histological sections. One general advantage of interphase cytogenetics is the fact that the detection of genetic alterations is performed

on a single cell basis. This makes the test insensitive to dilution with normal cells, which tends to be a problem (in particular when analyzing precancerous lesions). The single cell nature of interphase cytogenetic analysis also compensates for sampling errors to a certain degree. In the following sections we summarize the most pertinent examples of molecular cytogenetic applications for the detection of chromosomal aneuploidies and gene amplifications in a variety of carcinomas.

4.3.2.1 Bladder Cancer

Like most other carcinomas, bladder cancers carry a distribution of chromosomal aneuploidies that are specific to the disease [148, 149]. Accordingly, a test that targets the centromeres of chromosomes 3, 7, and 17, and a locus specific probe for chromosome band 9p21, termed UroVysion (Abbott Molecular), was developed and applied to voided urine samples from patients with bladder cancer and controls [150, 151]. Compared to other standard tests (including cytology), UroVysion exhibits the highest combined sensitivity and specificity for the detection of bladder cancer. There are also successful (and FDA-cleared) imaging solutions that allow for an automated analysis of FISH signals in urine samples.

4.3.2.2 Lung Cancer

Lung cancer is one of the most common causes of cancer death in the world. As with other cancer entities, early detection significantly improves prognosis. The development of radial computed tomography of high-risk patients for lung cancer development has allowed for an earlier diagnosis of lung cancer. However, the challenge in lung cancer diagnosis using this imaging method relates to its relatively low specificity [152]. Hence, imaging requires follow-up analysis and one possibility is the collection of sputum samples or samples collected by fine needle aspiration for cytological characterization [153]. The phenotypic characterization of exfoliated cells (which lacks tissue context) can be difficult. Therefore, the inclusion of genetic markers for lung cancer should be a reasonable approach of improving diagnosis. The feasibility of such an approach was convincingly demonstrated in several studies over the past few years [153–156].

4.3.2.3 Breast Cancer and ERBB2

With the introduction of trastuzumab (Herceptin®), a humanized monoclonal antibody, the first target-specific therapy against the epidermal growth factor receptor 2 (*ERBB2* or *HER-2/neu*) became available [157]. Before a patient becomes eligible for trastuzumab therapy, the FDA requires determination of whether their cancer overexpresses ERBB2 [158]. The *ERBB2* oncogene is located on chromosome 17 (17q12-21.32) and is a member of the human epidermal growth factor receptor family. ERBB2 overexpression occurs primarily through amplification of the wild-type gene and is clinically correlated with poor outcome, positive lymph node status, high histological grade, high proliferation rate, and negative progesterone and estrogen receptor status [157, 159–164]. Twenty-five to 30 % of all breast cancer cases show an amplification of the *ERBB2* gene [165, 166]. In addition to immunohistochemistry to measure ERBB2 protein levels, the FDA approved a test based on in situ hybridization to identify the *ERBB2* gene amplification status. In situ hybridization is a sensitive and specific method that identifies gene copy numbers either with a single detection probe (mono color FISH) or in combination with a reference probe for the centromere of chromosome 17 (dual color FISH) in which the gene and the centromere are visualized using different fluorochromes. The protocol can be applied on formalin-fixed, paraffin-embedded tissues using standard sections. In dual color FISH, 20 cancer cell nuclei are counted for *ERBB2* and centromere 17 signal number. Subsequently, the *ERBB2*/centromere 17 ratio is calculated by dividing the total number of *ERBB2* signals by the total number of centromere 17 signals. A ratio >2.2 serves as the threshold for ERBB2 positivity and eligibility for trastuzumab treatment while a ratio <1.8 defines non-amplified and not eligible for trastuzumab. If the ratio is between 1.8 and 2.2, additional nuclei are counted by a second observer. If the second observer also achieves a ratio of 1.8–2.2, the cut-off is changed to 2.0. A ratio of >2.0 is therefore considered positive, while a ratio of <2.0 indicates a negative case.

4.3.2.4 Non-small Cell Lung Cancer, Colorectal Cancer, and Glioblastoma, and EGFR

With an increasing number of drugs available for targeting EGFR (the epithelial growth factor receptor), tests that evaluate the expression of this receptor and its gene copy numbers have become a clinical necessity [167]. Unfortunately, precise evaluation guidelines for *EGFR* gene status are still missing [168], and are therefore not comparable to current ERBB2 testing. This is partly due to the fact that the patterns of *EGFR* gene alterations detectable by in situ hybridization are complex and dependent on cancer type (Fig. 4.2h). Several scoring systems for EGFR testing have been proposed for selection of patients with non-small-cell lung cancer to treat with tyrosine kinase inhibitors, but these scoring systems lack broad consensus. In colorectal cancer, patients with high *EGFR* gene copy number in general respond better to therapy with cetuximab or panitumumab (human monoclonal antibodies with specific binding to the EGFR), and one group demonstrated that colon cancers with less than 2.92 signals of *EGFR* per nucleus are less likely to respond to cetuximab [169]. Unfortunately, several other studies have suggested different thresholds [170, 171]. In glioblastoma, the most malignant form of brain tumors with less than 12 months median survival [172], *EGFR* gene amplification occurs in 40–50 % of cases and are present almost invariably as high-level amplification which can be determined easily [173].

Clinical trials are ongoing to establish the predictive value of gene amplification for clinical benefit of treatment with tyrosine kinase inhibitors or antibodies [174]. It appears inevitable that cancer type and the nature of anti-EGFR therapy must be taken into account to develop FISH evaluation guidelines.

4.3.2.5 Cervical Cancer

Despite the possibility that an early diagnosis of cervical cancer could be achieved through the implementation of screening cytology based on the Pap (Papanicolaou) test cervical cancer is still one of the most common malignancies in women world-wide [175]. In populations in which Pap testing is offered the incidence of cervical cancer was greatly reduced. However, Pap test-based screening is associated with specific challenges—the test has low sensitivity and is subjective [176]. The introduction of HPV screening overcame some of these shortcomings because the development of cervical cancer in the absence of high-risk HPV genomes is extremely unlikely [177]. However, HPV testing has a very low specificity because most of the cytologically abnormal lesions are positive for HPV, yet only around 15–20 % of them progress to higher-grade disease and to cervical cancer. The diagnostic challenge is to identify a marker that will be both sensitive and specific.

The analysis of cervical cancer and its precursor lesions had been attempted for decades, and some progress has been made. Even before the chromosome-banding era, Ingrid Granberg in Stockholm [178] and Atkin and colleagues in Great Britain [179], among others [180], attempted to identify recurrent chromosomal aberrations in cervical cancer and its precursor lesions. The situation was complicated by the fact that it was exceedingly difficult to establish cell lines from cervical carcinomas. Accordingly, application of CGH, which does not require preparation of metaphase chromosomes for cytogenetic analysis, was exceedingly useful for the identification of genomic imbalances during tumorigenesis. In a paper by Heselmeyer and colleagues such a phenotype/genotype correlation was realized using a combination of tissue microdissection and comparative genomic hybridization [99, 181]. This analysis revealed that (1) invasive cervical carcinomas are invariably associated with a gain of the long arm of chromosome 3q, and (2) that the gain of 3q already occurs in dysplastic lesions (despite the fact that tissue dissection was employed, the percentage of this aberration was probably underestimated because CGH was performed with pooled cells). In many studies that followed this initial description, the prevalence of 3q amplification in cervical carcinomas was confirmed [182, 183]. This led to the development of a FISH test for this disease [184]. The probe cocktail includes a marker for 3q, which is a BAC clone contig centering around the human telomerase gene *TERC*, and pertinent control probes for the ploidy of the cells (Fig. 4.2i and j). Applications in numerous studies have led to the following conclusions: (1) the detection of genomic copy numbers of 3q allows the diagnosis of cervical cancer, independent of morphology, (2) the gain of 3q occurs before the transition to invasive disease, (3) the gain of 3q can occur in diploid cells, or can develop after the genome has become tetraploid, (4) progression from low-grade disease to higher grades or to invasive carcinomas does require the gain of 3q and progression does not occur without the acquisition of extra copies of 3q, and (5) detection of genomic amplification of 3q has the highest combined sensitivity and specificity compared to all other clinically used biomarkers [185–191]. These results, in conjunction with studies in other cancer entities mentioned above, clearly show that the diagnosis of human carcinomas and its precursor lesions can benefit from the introduction of pertinent genetic markers that should be selected based on the profile of genomic imbalances. In our opinion, these markers should be selected based on the pattern of recurrent genomic imbalances in solid cancers. We think that the inclusion of pertinent genetic markers would overcome the limitations of a cytology-based diagnosis of solid cancers, and could reduce the need for histopathological sections in which the tissue context is maintained so that a morphological diagnosis is easier to make [192].

4.4 Conclusion

The comprehensive molecular analysis of carcinomas has unveiled that the theme of what is often lumped together as an ill-defined chromosomal instability phenotype is in fact a remarkable stability on a different plateau of genomic copy number changes. Overcoming the perception that carcinomas are characterized by a cytogenetic chaos, in which ongoing chromosomal instability contributes to a rearranged genome with no recurrent features will help to promote interphase cytogenetics as a valuable tool for the early detection of emerging cancers, will thereby improve diagnosis, and eventually result in a better prognostication of patients with malignant diseases.

Acknowledgements The authors are grateful to Dr. Hesed Padilla-Nash, Dr. Michael J. Difilippantonio, Dr. Darawalee Wangsa, Dr. Madhvi Upender, and Danny Wangsa for providing figures, Turid Knutsen for helpful comments, and Dr. Reinhard Ebner for critically reading the manuscript. We thank all members of our laboratory for their input and most of all for their dedication to cancer research. We greatly appreciate the help of Buddy Chen with creating the figures.

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Epigenetic Basis of Human Cancer

5

Thomas Mikeska and Alexander Dobrovic

5.1 Introduction to Epigenetics

5.1.1 Epigenetics

Although events that we now understand as epigenetic in nature had already been described in model organisms, the origin of the concept of epigenetics may be considered to date back to 1942 when, in the context of development, Waddington wrote "*between genotype and phenotype lies a whole complex of development processes*" and termed the phenotypic result of these processes, the "*epigenotype*" [1]. The most important role of epigenetics is the normal process of the establishment and maintenance of the cell-to-cell heritable gene expression patterns that occur during development. Alterations of the primary DNA sequence do not underlie these changes and yet characteristic patterns of gene expression can be inherited. Over time, the definition of the term epigenetic has varied [2–5]. A current definition that is used in this chapter is: "*an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence*" [6].

So far, several distinct layers of epigenetic regulation are known including DNA methylation, histone modifications, and organization of the chromatin within the nucleus, although the direct heritability of some of these remain unclear. DNA methylation and histone modifications are probably the best understood epigenetic mechanisms to date. In this chapter, we first give a general introduction to DNA methylation and histone modifications, followed by a review of how they are altered in cancer, and concluding with the implications of these changes for cancer diagnostics and therapy.

5.1.2 DNA Methylation

In mammals, DNA methylation occurs almost exclusively at cytosine residues within CpG dinucleotides (CpGs). The covalent attachment of a methyl group to the fifth carbon of a cytosine residue results in the formation of 5-methylcytosine. The origins of DNA methylation in multicellular organisms remain unclear. DNA methylation probably maintains genomic stability by silencing repetitive elements, such as transposons [7, 8]. Furthermore, DNA methylation is involved in maintenance of chromosomal structure as can be seen in the aberrant pericentromeric regions in individuals lacking methylation at certain satellite DNA sequences as a consequence of ICF syndrome [9–11].

CpGs are unevenly distributed throughout the entire human genome and the majority of them are methylated [12]. However, some CpGs are also found in regions of high CpG density spanning hundreds to thousands of base pairs in length, referred to as CpG islands [13]. About 70 % of human gene promoters are associated with the presence of a canonical CpG island [14–16] and the CpGs in these islands are generally unmethylated. By contrast, CpG islands within actively transcribed genes are generally methylated [16].

The proportion of CpGs outside CpG islands is markedly decreased relative to that expected from the base composition. The depletion is a direct consequence of methylation as 5-methylcytosine can undergo spontaneous deamination to thymine [17]. Although the vast majority of ^{5me}CpG:TpG mismatched lesions are repaired by MBD4, a thymine DNA glycosylase that binds to the mismatches when the cytosine is methylated and can initiate the repair of these lesions [18], a proportion is not repaired [19]. Therefore, methylated CpG

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_5

positions are mutational hot spots and this has led to a depletion of CpGs throughout most of the genome during evolution. Many inherited disease-causing mutations arise at CpG dinucleotides because of this increased mutation rate [20]. Methylated CpGs are also mutational hot spots in cancer [21, 22]. Studies on the TP53 gene have shown that some somatic mutations occur at normally methylated CpGs and thus result from the unrepaired spontaneous deamination of 5-methylcytosine [23].

A small but important subgroup of CpG islands are methylated in normal tissues; genes on the inactive X-chromosome, alleles of imprinted genes, and certain tissue-specific genes. X-chromosome inactivation in females is mediated by the expression of the XIST noncoding RNA and is locked into place by DNA hypermethylation of the promoter region CpG islands [24]. DNA methylation regulates the allele-specific expression of imprinted genes by maintaining DNA methylation marks on only one parental allele [25]. It was initially proposed that DNA methylation might underlie tissue-specific gene expression [26], but this seems to be the exception rather than the rule. Epigenomic analysis will soon clarify how frequent DNA methylation actually is in tissue-specific gene expression.

Although the definition of a CpG island is arbitrary, two algorithms have been widely used to identify CpG islands in genomic DNA sequences [13, 27]. The identification is based on three sequence related criteria: sequence length, C+G content, and ratio of the observed CpGs over the expected CpGs. More sophisticated algorithms and approaches have since been developed [28, 29]. Depending on the methods applied and the stringency of the criteria for defining a CpG island, it is estimated that the human genome contains about 28,000–110,000 CpG islands [15, 30, 31].

It is widely recognized that the presence of DNA methylation in the promoter region of a particular gene or transcript generally causes silencing or transcriptional repression [32]. The presence of DNA methylation may prevent the direct binding of transcription factors [33] or facilitate the binding of methyl-CpG-binding proteins, which bind to methylated DNA through their methyl-CpG-binding domain [34, 35]. Methyl-CpG-binding proteins recruit specific histone deacetylases and histone methyltransferases, which then establish a chromatin structure that is not accessible to the transcriptional machinery. However, there is also considerable evidence that the establishment of a repressive chromatin state can be the initial event leading downstream to DNA methylation which then reinforces the original transcriptional silencing [36, 37].

DNA methylation patterns are established by several DNA (cytosine-5) methyltransferases (DNMTs) [38–40] which utilize *S*-adenosyl-L-methionine (SAM) as the methyl group donor. In humans, four DNMTs are known, DNMT1, DNMT3a, DNMT3b, and DNMT3L. In general, DNMT1 acts as the maintenance DNMT, whereas DNMT3a and DNMT3b are de novo DNMTs [41, 42]. DNMT1 exhibits a strong affinity for hemi-methylated DNA [43] and is responsible for reestablishing preexisting, parental DNA methylation patterns during cell divisions [44] and participates in DNA repair, probably by restoring DNA methylation [45]. The two active de novo DNMTs, DNMT3a and DNMT3b, methylate previously unmethylated CpGs and are responsible for reprogramming the DNA methylation pattern during gametogenesis and early developmental stages [40, 44]. DNMT3L belongs to the DNMT3 family, but does not exhibit DNMT activity. It acts as a regulatory factor [46], which enhances the catalytic activity of DNMT3a and DNMT3b [47]. The human methyltransferase DNMT2 (TRDMT1) has a high sequence and structural homology to DNMTs, but has been identified as a methyltransferase [48], which methylates a cytosine moiety on the aspartic acid tRNA using the same catalytic mechanism as DNMTs [49].

DNA methylation marks can also be removed. This process is called DNA demethylation and can be divided into passive and active DNA demethylation. Passive DNA demethylation takes place in the absence of maintenance DNA methylation by reducing the amount of DNA methylation progressively via DNA replication [50]. Active DNA demethylation (replication independent demethylation) requires the presence of enzymes capable of erasing DNA methylation [50–52]. One suggested mechanism is direct enzymatic removal of the 5-methyl group from 5-methylcytosine (bona fide demethylation), thereby restoring cytosine. The formation of 5-hydroxymethylcytosine by hydrolyzing 5-methylcytosine via the TET family of enzymes plays an important intermediary role [53, 54]. Other presumed mechanisms involve the DNA repair machinery for the entire removal of the 5-methylcytosine base, the nucleoside or the nucleotide (indirect demethylation).

5.1.3 Histone Modifications

Another important component of epigenetics is mediated by the nucleosome. The nucleosome comprises an octamer of two each of the four core histones H2A, H2B, H3, and H4 which is then associated with histone H1. A complex, multidimensional, and interrelated regulation mainly arises from posttranslational, covalent modifications of specific conserved residues of core histone tails [55–57]. Multiple posttranslational histone modifications have been characterized. Acetylation of lysine residues [58] and methylation of lysine (or arginine residues) [58–60] are the best characterized modifications. Other modifications include phosphorylation [61, 62], ubiquitinylation [63, 64], poly-ADP-ribosylation [65], sumoylation [66], biotinylation [67], propionylation, and butyrylation [68].

The different and substrate specific enzymes involved in the addition or removal of certain histone marks are responsible for the composition and combination of the different modifications, which defines the chromatin status. The different histone marks are referred to as the "*histone code*" [69–71]. While most of these modifications are reversible, their type and degree allows the dynamic and subtle regulation of the transcription level of the affected region, and more globally their impact on and regulation of cellular processes [72, 73].

5.1.3.1 Histone Acetylation

Acetylation of histone tails, especially H3 and H4, is associated with transcriptionally active genes [74]. The addition of acetyl groups counteracts the positive charges of the lysine residues of the histone tails and therefore diminishes their electrostatic interaction with the negatively charged DNA backbone. The removal of acetyl groups on the other hand unmasks the positive charges of the histone tails and enables them to interact with the negatively charged phosphate groups of the DNA. This leads to a more condensed chromatin structure.

The addition or the removal of an acetyl group at lysine residues of histone tails is dynamic in nature. These processes are mediated by several families of histone acetyltransferases (HATs) [75] and by several families of histone deacetylases (HDACs) [76–78]. The different HATs can be classified as whether they belong to the GNAT, MYST, or p300/CBP families, and the HDACs are divided into four classes based on their homology to yeast histone deacetylases [79].

5.1.3.2 Histone Methylation

Methylation of the lysine residues of the histone tails is more complex. Different methylation marks code for an active or an inactive chromatin state, depending on the histone, the position of the amino acid on the histone tail and the number of methyl groups at each position [73]. The amino groups of the lysine residues can be methylated with one, two, or three methyl groups. The methylation code is probably not as dynamic as the acetylation process. Histone methylation marks are established by histone methyltransferases (HMTs) [80]. Some of the HMTs belong to the trithorax (e.g., MLL) and polycomb (e.g., EZH2) protein groups which were first identified in *Drosophila melanogaster* [81]. They are responsible either for maintaining (trithorax group of proteins) or repressing (polycomb group of proteins) lineage specific transcriptional activity respectively. Proteins capable of removing methyl groups from the lysine residues include lysine (K)-specific demethylase 1A (KDM1A, LSD1) [82], which specifically demethylates monomethylated or dimethylated H3-K4, and the Jumonji family of proteins, with a range of specific substrate specificities [83–85].

5.1.3.3 Active and Inactive Chromatin

Despite the myriad of possible combinations, a useful generalization is that chromatin regions showing a high level of acetylated histones and trimethylation of H3-K4 [86] as well as methylated H3-K36 [87] and H3-K79 [88] are transcriptionally active [56]. On the other hand, deacetylated histones and dimethylation or trimethylation of H3-K9 [89] and trimethylation of H3-K27 [90] and H4-K20 [91] are associated with a more condensed chromatin structure and an accordingly repressed transcription level [56]. Both DNA methylation and histone modifications define a chromatin environment, which regulates gene transcription. The mechanisms and their role in regulating cellular processes may be different, but an intimate relationship is obvious [15, 92].

5.2 Epigenetic Changes in Cancer

Cancer can be considered as a disease of disordered development manifesting as a disease characterized by altered gene expression patterns. Thus, epigenetic mechanisms are likely to be as important in cancer-specific gene expression as they are in developmental gene expression.

5.2.1 Aberrant DNA Methylation in Cancer

During cancer development, the distribution of DNA methylation becomes markedly altered. Two general phenomena are frequently observed in cancer cells: (1) the overall DNA methylation level is decreased, and (2) de novo DNA methylation of promoter-associated CpG islands occurs. We still need to understand what drives and is responsible for global DNA hypomethylation or the establishment of multiple hypermethylation events. Furthermore, we need to determine whether these events are dependent or independent. The rapid development of new technologies, especially those that allow analysis of DNA methylation changes on a global scale will enable us to reevaluate this.

5.2.1.1 Global DNA Hypomethylation

One of the earliest indications of altered DNA methylation in human cancer was the observation that the overall 5-methylcytosine content of cancer cells was decreased [93, 94]. The genome-wide depletion of 5-methylcytosine in cancer is particularly pronounced in repetitive sequences [95, 96]. Multiple copies of repeats are found throughout the human genome and encompass repeats in constitutive heterochromatin (classical satellites), interspersed repeats (LINEs and SINEs), and long terminal repeats (LTRs) (a good overview is given in ref. [97]). The contribution and consequences of hypomethylated repetitive DNA sequences to carcinogenesis

and cancer progression is far from understood. One may expect that these massive changes have a profound impact and could result in inappropriate recombination or replication events. They may even be responsible for the large scale changes in nuclear organization that can be seen under the light microscope and are a hallmark of cancer.

DNA hypomethylation may move the genome into an unstable state, promoting further cancer evolution, involving both genetic and epigenetic changes [96, 98, 99]. Interestingly, depending on the cancer type and cancer stage, the onset, development, and progression of DNA hypomethylation is different. For example, DNA hypomethylation is associated with early stages of gastric cancer [100] and advanced stages of prostate cancer [101]. DNA hypomethylation can lead to chromosomal instability [102]. Genome-wide studies on gastric and colorectal cancer samples showed a direct link between global DNA hypomethylation and genomic instability [103, 104]. Global DNA hypomethylation destabilized all the chromosomes and was not correlated to a particular kind of chromosomal alteration [104]. On the other hand, a significant correlation between DNA hypomethylation and aberrations on chromosome 8 was reported for prostate cancer [105].

The elevated frequency of some chromosomal abnormalities observed for some cancers might be directly related to DNA methylation changes. Wilms' tumor, a malignant pediatric tumor of the kidney, is frequently less methylated in centromeric alpha satellite sequences on chromosome 1 compared to normal kidney tissue. In addition, the satellite 2 sequences of chromosome 1 and 16 are also hypomethylated. Furthermore, Wilms' tumors show an association between hypomethylation of satellite 2 sequences on chromosome 16 and loss of chromosome 16q [106]. It was also shown that hypomethylation of satellite 2 sequences was often associated with an increased chromosome 1q copy number for hepatocellular carcinoma [107].

5.2.1.2 Locus-Specific DNA Hypomethylation

DNA hypomethylation has also been observed for the normally methylated promoters of individual genes. The list of genes aberrantly regulated by DNA hypomethylation is relatively small, compared to the list of genes reported to be affected by DNA hypermethylation [108].

The cancer/testis antigens are a particularly important example of genes that are methylated in nearly all somatic tissues and that become reactivated in cancer [109, 110]. They can be divided in two subgroups: cancer/testis antigen genes located on the X-chromosome (e.g., *MAGEA1*), and those which are located on autosomes. Cancer/testis antigens are normally expressed in germ cells of the testis and placenta but are not expressed in the vast majority of normal somatic tissues. The cancer/testis antigens encoded on the X-chromosome are associated with CpG islands, which are methylated in normal somatic tissues. However, these cancer/testis antigens are frequently reexpressed in several tumor types and reexpression is associated with the global loss of DNA methylation [111, 112]. Cancer/testis antigens are potential targets in cancer therapy and vaccines are currently being tested in clinical trials [113, 114].

Synuclein-γ (*SNCG*) encodes a member of the neuronal protein family whose expression is normally limited to the peripheral nervous system and the brain [115]. *SNCG* is silenced in normal tissues by DNA methylation. Ectopic SNCG protein expression has been observed for several human cancers [116–121]. The level of *SNCG* expression is determined by the extent of decrease of DNA methylation [122]. *SNCG* hypomethylation is observed in multiple cancers [117, 119–124] and is generally associated with a more advanced and invasive tumor phenotype. Interestingly, loss of methylation of *SNCG* is induced in the hepatoma cell line HepG2 by exposure to aflatoxin B1, a carcinogenic mycotoxin often found on certain crops [120]. This finding establishes a link between environmental factors and their influence on aberrant DNA methylation. It is likely that many more such links will be identified.

Cancer-related loss of DNA methylation can also occur at alleles of imprinted genes. The insulin-like growth factor 2 gene (*IGF2*), in which the maternal allele is usually methylated and silenced, is one of the most frequently deregulated genes in human cancer. DNA hypomethylation results in the aberrant expression of *IGF2* from the maternal allele (loss of imprinting). It has been suggested that loss of imprinting of *IGF2* could be a useful DNA methylation-based biomarker for the early detection of colorectal cancers [125]. Furthermore, loss of imprinting of *IGF2* has been detected in normal colon tissue and peripheral blood and might serve as a biomarker for the identification of individuals with cancer predisposition [126].

DNA hypomethylation not only affects protein-coding genes but also affects non-protein-coding genes. A good example is the X (inactive)-specific transcript (*XIST*) locus, which encodes an untranslated RNA transcript that effects X-chromosome inactivation in females. It is inactive in males in whom the promoter is methylated. The 5′-region of the *XIST* locus can become hypomethylated in testicular germ cell cancers [127] and in prostate cancer [128].

Aberrant expression of normally methylated microRNAs can also arise as a consequence of DNA hypomethylation. As examples, cancer-associated hypomethylation and overexpression has been observed for *let-7a-3* in lung cancer [129], *miR-200a/200b* in pancreatic cancer [130], and *miR-196b* in gastric cancer [131].

5.2.1.3 Promoter-Associated DNA Hypermethylation

The gain of DNA methylation at specific CpG islands is a hallmark of cancer development [32, 102, 132, 133]. There are many targets, especially those genes that are kept in a bivalent chromatin state in embryonic stem cells [134, 135]. Although unmethylated in the stem cell, they are frequently methylated in cancer cells.

The first report of a recurrently methylated promoter region in cancer was for the calcitonin gene (*CALCA*) [136]. However, the turning point that implicated DNA methylation as a driver in carcinogenesis was the report that methylation of the retinoblastoma 1 (*RB1*) tumor suppressor gene was observed in a subset of non-inherited retinoblastomas [137, 138]. Initial studies focused on other tumor suppressor genes including *VHL* [139], *CDKN2A* (*p16*) [140], and *CDKN2B* (*p15*) [141].

Promoter-associated DNA hypermethylation is an alternative mechanism for inactivation of a tumor suppressor gene in Knudson's two hit model [32]. Most of the mutations in these genes are recessive, so both alleles need to be inactivated before the function of the gene is lost. Gene silencing as a consequence of DNA methylation has a similar impact to a genomic deletion or an inactivating mutation. Whereas a genomic deletion or an inactivating mutation is a defined alteration of the primary genomic sequence, inactivation by DNA methylation generally occurs in the absence of sequence change and is in principle reversible.

Many of the driver genes (genes which underlie cause of cancer initiation or progression) of carcinogenesis have been discovered by studies of familial cancer syndromes (Table 5.1). Further demonstration of the critical role of DNA methylation in carcinogenesis is made by the observation that many of these genes, in addition to the *RB1* gene, are methylated in sporadic cancers that often phenocopy the type and subtype of the hereditary cancers in which they were first discovered [142]. Thus, the *MLH1* mismatch repair gene not only underlies much of hereditary non-polyposis colorectal cancer (Lynch syndrome) but is also methylated in many sporadic colorectal cancers [143]. Similarly, the *BRCA1* gene underlies both much of hereditary breast and ovarian cancer and is methylated in sporadic breast and ovarian cancers which often share pathological features with their hereditary counterparts [144]. Furthermore, DNA methylation of these genes is rare or absent in tumor types that are not characteristic of the hereditary cancer [145–147].

A recent interesting discovery is that some of these driver genes may show constitutional DNA methylation—methylation of a given gene may occur in multiple somatic tissues (often in a mosaic pattern)—in normal individuals. The individual is then predisposed to develop characteristic cancers similar to the spectrum seen when this driver gene is mutated in familial cancer syndromes [142].

Besides the hereditary cancer genes, a large variety of genes that affect every facet of the cancer phenotype are subject to DNA methylation (a large list is given in ref. [148]). In addition to protein-coding genes, hypermethylation can occur at CpG islands associated with non-protein-coding genes, such as microRNAs, which are frequently dysregulated in cancer [149]. It has been estimated in genome-wide studies, that each cancer can harbor several hundreds of aberrantly methylated CpG islands [95, 150].

It is remarkable how many of the DNA methylation changes in cancer occur in genes that are plausibly involved in cancer, although this probably reflects the preponderance of candidate genes that have been studied. It is however certain that many of the genes that are reported to be methylated in cancer are not directly involved in the initiation or progression of the cancer as they are tissue-specific genes such as *CALCA* and *MYOD1* that are not expressed in the normal cells from which the cancer arose.

Many of the genes that become methylated in cancer are restricted to certain types of tumors. In some cases, such as GSTP1 in prostate cancer, DNA methylation of the gene is virtually a hallmark of the cancer. However, for most cancer types

Table 5.1 Known hereditary cancer genes that are methylated in cancer

Gene symbol	Pathway	Tumors in which DNA methylation occurs	Familial involvement
BRCA1	DNA repair	Breast, ovarian	Breast, ovarian
CDH1	Cell adhesion	Diffuse gastric, lobular breast	Diffuse gastric, lobular breast
CDKN2A (*p16*)	Cell-cycle	Widespread includes non-Hodgkin's lymphoma, gastric, melanoma	Melanoma
DAPK1	Apoptosis	Chronic lymphocytic leukemia, colon, lung, gastric	Chronic lymphocytic leukemia
LKB1	Signal transduction	Colon, lung	Peutz–Jeghers syndrome
MLH1	DNA repair, apoptosis	Colon, endometrium, ovarian	Lynch syndrome (colon, endometrium, ovarian)
RB1	Cell cycle	Retinoblastoma, breast	Retinoblastoma
VHL	Angiogenesis	Renal, chronic lymphocytic leukemia	von Hippel–Lindau syndrome (renal)

DNA methylation of no one single gene is predominant. However, profiling DNA methylation over a large panel of genes reveals patterns that are characteristic of certain types of cancer [151]. Thus, different cancer types can be discriminated by their distinct DNA methylation pattern (epigenetic fingerprint) [150, 152]. Many research groups are trying to identify panels of genes that best characterize subgroups of each major cancer as biomarkers for that cancer [153].

Other methylated genes may be passenger genes (also known as bystander genes). Passenger genes may become aberrantly methylated because they are part of an epigenetically silenced domain. Also, genes that are methylated in cancer may be methylated in the cell of origin of the tumor and escape demethylation. Other genes may become methylated because of aging related incursions of DNA methylation into normally unmethylated regions which then become further methylated in the altered DNA methylation milieu of the cancer [154].

The extent to which a gene promoter region has to be methylated to result in silencing or decreased gene expression is still unknown. DNA methylation patterns are often heterogeneous and show variable degrees of methylation throughout the region analyzed [155–157]. In addition, the amount of DNA methylation of a given promoter tends to increase with cancer evolution [158, 159]. It has been reported that the *CDKN2B* (*p15*) promoter CpG island needs to be methylated at 30–40 % of the CpG sites for silencing to occur [160]. It is expected that the minimal DNA methylation level is dependent on the gene and its genomic context.

Two mechanisms have been suggested to explain local promoter associated CpG island hypermethylation in cancer [15, 161]. One mechanism is based on random seeding of DNA methylation marks by an aberrant DNA methylation machinery. The second mechanism proposes that certain regions are prone to aberrant DNA methylation, either intrinsically or by a misrouted DNA methylation machinery. This may also explain DNA hypomethylation in these cases. However, neither concept can entirely explain aberrant DNA methylation levels and patterns.

5.2.2 Factors Influencing Methylation Changes in the Epigenome

The accumulation of alterations to the normal epigenome (epimutations) can be a step in cancer development analogous to the accumulation of DNA sequence changes (mutations). The underlying causes of and contributions to alterations to the epigenome remain largely elusive. Nevertheless, there is strong evidence, that the aging process and nutritional history have an impact on DNA methylation as well as changes in histones and chromatin structure [162–164]. These parameters are not easily investigated separately and are usually interwoven with other parameters, such as environmental exposures, which may also influence the epigenome alterations to a certain extent [163, 165, 166]. For example, heavy metals (e.g., nickel and arsenic) [167] as well as toxins (e.g., aflatoxin B1) [120] are well recognized substances capable of altering the epigenome.

Aging is associated with a global loss of DNA methylation as well as a local increase in DNA methylation of usually unmethylated CpG islands in normal tissues [168]. Initially, it was reported that the CpG island-associated with the estrogen receptor (*ER*) gene showed increasing level of DNA methylation in colorectal mucosa samples in an age dependent manner [169]. Subsequently, multiple other genes were shown to display age-related DNA methylation in normal colorectal mucosa [154, 170, 171]. Aberrant expression of DNMT1, DNMT3a, and DNMT3b may be responsible for deregulated DNA methylation patterns in aging cells [172–174]. In cultured human fibroblasts, DNMT1 expression decreases during the aging process and this is probably associated with global DNA hypomethylation [175]. Furthermore, the increased expression of the *DNMT3b* gene may lead to aberrant DNA methylation at single-locus genes [175].

The genetic background can also determine susceptibility to alterations cellular DNA methylation. The one-carbon pathway is involved in the synthesis of the methyl group donor, S-adenosyl-L-methionine (SAM), which is utilized by DNMTs to establish DNA methylation. In particular, variants in two members of this pathway, MTHFR (5,10 methylenetetrahydrofolate reductase) and MS (methionine synthase), influence the 5-methylcytosine content of the cell.

TT homozygous and CT heterozygous individuals of the *MTHFR* variant C677T were shown to cause a genome-wide reduction of DNA methylation level in normal tissue [176–178]. The C to T substitution leads to thermolabile MTHFR protein, with reduced catalytic activity [179]. The AA homozygotes of a second *MTHFR* variant, A1298C, were also associated with a lower content of DNA methylation [176, 180]. This MTHFR protein variant shows also a decreased enzymatic activity [181]. GG homozygotes at the A2756G variant of the *MTR* gene (coding for 5-methyl tetrahydrofolate-homocysteine methyltransferase) had less methylation at tumor suppressor genes in their cancers than the other genotypes [178].

Genetic variation in promoter regions may also alter their propensity to become methylated with time. The T allele of the common germ-line polymorphism c.-56C>T (rs16906252) in the promoter region of the *MGMT* gene is associated with aberrant DNA methylation at this locus in colorectal cancer [182] and malignant mesothelioma [183]. Furthermore, gain of DNA methylation in normal tissues such as colonic mucosa [184] and peripheral blood [185] is

also correlated with the presence of the T allele. So far, the underlying mechanism for this predisposition is unknown. As not every individual with the T allele has detectable methylation, and the amount of methylation is highly variable, *trans*-acting factors must also be involved. Susceptibility for aberrant DNA methylation associated with single nucleotide polymorphisms seems to be widespread and the importance of this in cancer predisposition remains to be fully understood [186, 187].

5.2.3 CpG Island Methylator Phenotype (CIMP)

The CpG island methylator phenotype (CIMP) has been widely used to describe cancers with extensive CpG island methylation. This phenotype was first proposed in 1999 for colorectal cancers [188]. Cancers are scored as CIMP-positive if the majority of a small panel of CpG promoter regions are methylated in a manner analogous to the scoring for microsatellite instability. Analysis of a large panel of CpG islands has subsequently confirmed that CIMP is a distinct phenotype in colorectal cancer [189].

Classifying cancers as CIMP-positive is not always straightforward. Alongside the original gene panel of *CDKN2A*, *MINT1*, *MINT2*, *MINT31*, and *MLH1* [188], several other gene panels have been proposed. Weisenberger et al. [189] chose a panel of *CACNA1G*, *IGF2* (a non-imprinted CpG island at this locus), *NEUROG1*, *RUNX3,* and *SOCS1*, while Ogino et al. recommended a minimal panel of *RUNX3*, *CACNA1G*, *IGF2*, and *MLH1* [190]. In addition to the different gene panels, different methods have been used to detect DNA methylation. Quantitative approaches are superior to qualitative approaches (methylation-specific PCR) as they allow for the identification and subsequent exclusion of genes with low-level DNA methylation, which are probably biologically not significant [191]. Further difficulties in defining CIMP-positive cancers arise by using different CIMP cut off values [192, 193] and different CIMP grading systems [189, 192, 194].

Despite the nonstandardized use of different marker panels and methodologies, CIMP-high colorectal cancers share multiple clinicopathological and molecular characteristics. CIMP-high colorectal cancers are usually proximally located [188, 195–197] and are associated with an older patient age [193, 196]. They often exhibit microsatellite instability as the DNA mismatch repair gene *MLH1* is frequently methylated [193, 195–198]. CIMP cancers that are methylated for *MLH1* have a different profile of genetic changes to those that are not methylated for *MLH1* [195]. They are mainly *TP53* wild-type [193, 195, 197].

CIMP cancers show a very high mutation frequency in the *BRAF* gene (V600E) [189, 191, 194–196, 199, 200]. The occurrence of CIMP and the oncogenic *BRAF* mutation has been linked to the serrated pathway of colorectal carcinogenesis. The *BRAF* mutation and CIMP are observed in over 75 % of sessile serrated adenomas which are precursor lesions associated with the serrated neoplasia pathway [194]. Hereditary non-polyposis colorectal cancers caused by germline mutations in the DNA mismatch repair genes *MLH1*, *MSH2*, *MSH6*, or *PMS2* [201] do not belong to the serrated neoplasia pathway and do not show either CIMP or *BRAF* mutations.

A significant problem in understanding CIMP is that the mechanism underlying the phenotype in colorectal cancer has not been identified. Existing theories encompass a genetic and/or environmental cause [202, 203]. An interesting hypothesis raises the possibility that chemotherapy with DNA damaging agents might drive or select for cancers associated with CIMP [204]. It is also important to note that in melanoma, the *BRAF* V600E mutation does not appear to be associated with high levels of aberrant promoter methylation [205].

Since the first report of CIMP in colorectal cancers, other solid tumors and leukemias have been suggested to be CIMP-positive [205, 206]. It is unclear whether the drivers of CIMP in these cancers are related to the drivers of CIMP in colorectal cancer. In glioma, it has been shown that mutation of a single gene, isocitrate dehydrogenase 1 (*IDH1*), establishes the CIMP phenotype but this is unlikely to drive more than a subset of CIMP-positive colorectal cancers [207].

5.2.4 Histone and Chromatin Changes in Cancer

Repressive histone marks and DNA methylation are often found together [208]. However, the contribution of DNA methylation and histone modifications to transcriptional regulation varies from gene to gene. Whereas for certain genes, DNA methylation plays the predominant role, the expression for other gene loci may depend more on the presence of histone modifications.

Cancer cells also exhibit mechanisms of silencing, which are independent of promoter-associated DNA methylation. The establishment of trimethylated H3-K27 histone marks by the polycomb group protein EZH2, was shown to be the major cause for silencing certain genes, such as *ESR1* and *PGR*, as observed for the PC3 prostate cancer cell line. Most of the genes bearing this histone mark were found not to be associated with the presence of DNA methylation, whereas genes harboring DNA methylation did not show the trimethylated H3-K27 histone modification [209].

Two examples of genes that are silenced without DNA methylation are *CDKN1A* (*p21CIP1/WAF1*) and gelsolin (*GSN*). Expression of the cell cycle kinase inhibitor *CDKN1A* is frequently induced after treatment of various cell lines with

HDAC inhibitors [210]. The treatment renders the *CDKN1A* gene open to transcription, at least in part, by increasing the amount of acetylation of histones H3 and H4 at this locus [211]. Gelsolin, an actin binding protein, is also often upregulated after treatment with HDAC inhibitors in cell lines [212–214]. Altered DNA methylation seems to play only a minor role in *GSN* gene expression [213].

Many of the different enzymes involved in histone-modifying and chromatin-modifying activities have been shown to be deregulated in cancer. Aberrant expression, mutations and translocations are frequently the cause for their malfunction in cancer. Good overviews and the presentation of some examples in greater detail are provided in the literature [215–217].

Chromatin remodeling complexes such the SWI/SNF complex can modulate transcriptional activity and alterations in these complexes are associated with cancer development [218, 219]. *SMARCB1* (*SNF5*/*INI1*) codes for a key component of the SWI/SNF chromatin remodeling complex. This gene is frequently deleted or mutated and its malfunction is thought to be the underlying cause for rhabdoid tumors, a rare pediatric cancer [220]. Interestingly, these cancers do not show genomic instability and it seems that the disruption of the epigenetic machinery can substitute for genetic alterations [221].

5.2.5 Long-Range Epigenetic Silencing (LRES)

The concept of aberrant long-range epigenetic silencing (LRES) originated from genetic studies in model organisms of events such as position effect variegation where de novo silencing occurs along a length of chromatin after it is translocated next to heterochromatin [222]. Studies of gene expression in cell culture systems also revealed cases where adjacent genes underwent coordinate changes in gene expression [223, 224]. An important example of LRES in normal development is the inactive X-chromosome in females which is silenced virtually across its entire length. It shows all the hallmarks of silencing, methylation of promoter region associated CpG islands, heterochromatic appearance and late replication of its DNA. It is considered that the DNA methylation appears after the initial silencing to lock the silent phenotype into place [225].

De novo LRES in cancer was first described in colon cancer [226]. The first chromosomal region found to be silenced by LRES spans 4 Mb and comprises three distinct segments of contiguous CpG islands on chromosome 2q14 associated with cancer-specific DNA hypermethylation of *EN1*, *SCTR*, and *INHBB*. These segments were flanked by CpG islands, which were unmethylated in cancer cells as well as normal cells. Importantly, regardless of the CpG island methylation status, the whole region was associated with the repressive histone mark dimethylated H3-K9, leading to transcriptional suppression of the entire region [226]. Since then, several studies showed LRES in other cancer types. LRES affects large chromosomal regions of approximately 100 kb up to 5 Mb and results in silencing of several contiguous genes. LRES functionally resembles large genetic deletions resulting in silencing of long stretches DNA with the important exception that LRES may be reversible [161].

Another LRES region was found in bladder cancer on chromosome 3p22 and comprises 130 kb [227]. This region encompasses four contiguous genes (*VILL*, *PLCD1*, *DLEC1*, and *ACAA1*) that are coordinately downregulated in cancer cells. Again, the cancer-specific inactivation is characterized by the occurrence of trimethylated H3-K9 in the absence of DNA methylation. This same region is a part of a 1.1 Mb LRES region in the majority of microsatellite-unstable colorectal cancers associated with aberrant *MLH1* DNA methylation [228]. This segment comprises the genes *AB002340*, *EPM2AIP*, *MLH1*, *LRRFIP2*, *GOLGA4*, *ITGA9*, *CTDSPL*, *PLCD1*, and *DLEC1*, most of which show methylated promoter-associated CpG islands and the H3-K9 dimethylation repressive histone mark). Interestingly, *LRRFIP2*, *GOLGA4*, and *CTDSPL* escape aberrant DNA methylation but despite being associated with an active chromatin structure are also transcriptionally repressed in cancer samples.

LRES is not restricted to a particular cancer type and many different regions throughout the genome are subject to LRES [229–231]. Indeed, LRES is now recognized as a common event in cancer, affecting multiple regions throughout the compromised cancer cell genome. Genome-wide studies in prostate cancer [232] and neuroblastoma [233] revealed the presence of 47 and 63 cancer-related LRES clusters, respectively. Interestingly, the neuroblastoma study suggested that LRES regions are more often located at chromosome ends [233].

5.2.6 Is Cancer a Genetic Disease or an Epigenetic Disease?

That cancer is a genetic disease is an often repeated truism. However, our current knowledge of epigenetics must make us raise the question of whether cancer is as much or more of an epigenetic than a genetic disease [234–236]. The most compelling evidence for the latter argument is that at least some cancer cells can be reprogrammed into taking part in normal differentiation despite the presence of activated oncogenes [237]. The number of epigenetic changes in a cancer genome also greatly outnumbers the number of genetic changes in a cancer genome [235, 238] although deciphering which of these changes are critical in cancer development remains a challenge. Sequencing of the entire

genome of one case of acute myeloid leukemia (AML) with a normal karyotype revealed that only very few mutations were present and suggested that the changes driving the carcinogenic process in this case were principally epigenetic [239]. Intriguingly, it was subsequently discovered that one of these mutations was a premature stop codon in the *DNMT3a* gene [240]. Indeed, some of the strongest evidence for an epigenetic basis to cancer comes from the leukemias where one of the fusion partners in a leukemogenic translocation often codes for an epigenetic modifier. In many cases, the translocation serves to maintain the leukemic cells in a stem-like or progenitor-like state. Chief among these fusion partners is the *MLL* gene which is involved in numerous translocations [241]. *MLL* has diverse functions including the establishment of trimethylated H3-K4 histone marks [242].

It is ultimately futile to put genetic and epigenetic mechanisms in opposition to each other as alternate hypotheses for cancer development as they both play pivotal roles and are often interrelated [133, 243]. Silencing of DNA repair genes will establish a mutator phenotype which will accelerate the rate of mutational change and provide tumor evolution with additional raw material [244]. For example, silencing of the promoter of the DNA repair gene *MLH1* results in microsatellite instability which further results in mutations at a variety of genes which have an effect on the cancer cell phenotype [240, 245].

On the other hand, the underlying causes of epigenetic instability or methylator phenotypes such as CIMP may arise from mutations in an as yet unidentified gene. Mutations in oncogenes such as *RAS* have been reported to destabilize the epigenetic machinery and initiate DNA methylation changes in the cell [246, 247]. In FOS-transformed cells, it was noted that DNMT1 protein expression was elevated resulting in a 20 % increase of cellular levels of 5-methylcytosine. DNMT1 seemed moreover to play a fundamental role in FOS transformation as blocking of *DNMT1* gene expression resulted in reversion of transformation [248]. This is consistent with other reports that blocking *DNMT1* gene expression can suppress tumorigenesis [249, 250].

A deregulated histone code and altered chromatin remodeling can also lead to increased mutation rates by altering the accessibility of the DNA repair machinery to the genomic DNA. Proper acetylation of H3-K56 in mammals is important to prevent spontaneous DNA damage probably during DNA replication [251]. Therefore, this histone modification participates in maintaining genomic stability and in an appropriate DNA damage response. In yeast, disrupted H3-K56 acetylation sensitizes the cells to compounds that cause DNA strand breaks during replication [252]. Impaired access to certain damage sites or an impaired DNA damage response will further contribute to the genomic instability of the cell, which further promotes its dysregulation.

Aberrant DNA methylation can also target components of the epigenetic machinery. The *RIZ1* (*PRDM2*) promoter is frequently silenced by DNA methylation in several cancer types [253–257]. RIZ1 is one of the two different proteins encoded by the *RIZ* (Retinoblastoma protein interacting zinc finger gene) gene. RIZ1, which contains a SET related PR domain, acts as a histone methyltransferase, which methylates H3-K9 [258]. RIZ is a common target in cancer as loss of heterozygosity, frameshift mutations and missense mutations also contribute to genetic alterations of the *RIZ* gene [258, 259].

5.3 Epigenetic Changes as Tools for Diagnostics and Targets for Therapy

5.3.1 DNA Methylation-Based Biomarkers

DNA methylation has aroused considerable interest because it generates large numbers of potential biomarkers [260]. Biomarkers fall into several classes. Some are useful because they are present in nearly all cancers of a given type and therefore can be used for disease detection and monitoring. These do not necessarily play a key role in cancer pathogenesis. Others are prognostic biomarkers that predict patient outcome. One of the key advantages of DNA methylation-based biomarkers is that methylation is stable after biopsies are removed. This stability gives it a major advantage over RNA-based expression biomarkers [260].

Methodology is a critical issue when locus-specific DNA methylation is used as a biomarker. DNA methylation assays should be used, which provide a quantitative or at least a semiquantitative result. In many cases, they need to be highly sensitive as well. Although, methylation-specific PCR (MSP) is a widely applied method to detect DNA methylation [261], the results obtained by this method are not quantitative. Methods for quantitative DNA methylation measurement have been based on bisulfite pyrosequencing [262–264], MethyLight [265], or SMART-MSP [266]. A useful semiquantitative approach is given by the methylation-sensitive high-resolution melting (MS-HRM) methodology [267]. It must also be considered that quantitative interpretation can be compromised by the presence of heterogeneous DNA methylation [268].

5.3.2 Early Detection and Monitoring of Cancers After Therapy

The early detection and treatment of most cancer types is considered the best prerequisite for a good outcome. Various body fluids or excretions, such as blood, sputum, saliva,

urine, and stool are relatively noninvasive samples, which can potentially be used for the early detection of cancer. In other cases, ductal lavages or bronchial washes can be used. Because of the relatively low frequency of cancers in the groups being screened, even assays with high specificity are likely to produce more false-positives than cancer cases. Thus, it is important to target such screening to individuals already at high risk of developing a certain type of cancer.

Detection of aberrant promoter DNA methylation of the *SEPT9* gene in plasma samples was shown to be correlated to the presence of colorectal cancer [269, 270] and precancerous colorectal lesions [271]. This epigenetic biomarker is relatively specific for colon cancer which shows *SEPT9* methylation level differences dependent on disease staging.

DNA methylation of the *PITX2* promoter, which regulates the expression of transcript variants A and B was shown to be a predictive epigenetic biomarker in breast cancer. DNA hypermethylation of *PITX2* was associated with an increased risk of recurrence in node-negative, steroid hormone-receptor positive breast cancer patients treated by adjuvant tamoxifen monotherapy after surgery [272, 273]. The presence of *PITX2* methylation was also associated with poor patient outcome for node-negative, steroid hormone-receptor positive breast cancer without any systemic adjuvant therapy and node-positive, estrogen receptor-positive, HER-2/*neu*-negative breast cancer treated by adjuvant anthracycline-based chemotherapy, respectively [274, 275]. Furthermore, methylation of *PITX2* is also a prognostic biomarker in prostate cancer. Increased methylation levels were associated with a higher risk of clinical relapse in patients with prostate cancer after radical prostatectomy [276, 277].

The *GSTP1* gene encodes for the pi class glutathione S-transferase enzyme, which is involved in cellular detoxification processes. The presence of DNA methylation at the *GSTP1* associated CpG island was detected in more than 90% of prostate cancer and about 70% of high-grade prostatic intraepithelial neoplasia [278, 279]. On the other hand, DNA methylation of *GSTP1* is rare in benign biopsies and normal prostate tissue. *GSTP1* methylation appears to be an early event in cancer progression and could be used as a diagnostic biomarker [280, 281]. Furthermore, methylation of *GSTP1* in serum of prostate cancer patients was associated with an elevated risk of clinical relapse after surgery [282].

5.3.3 Targeting Individual Epigenetic Changes—Epigenetics and Personalized Medicine

Biomarkers that predict patient responses to particular therapies are critical to personalized therapy. While considerable attention has been focused on gain and loss of function mutations in oncogenes, epimutations because of their greater frequency are likely to determine or influence therapeutic response.

Currently, cancer is usually treated according to the presumed tissue of origin of the cancer. This approach probably works because similar cancer subsets share similar profiles of genetic and epigenetic changes. Nevertheless, there are intrinsic subtypes within each cancer grouping as has been shown by large-scale gene expression studies [283, 284]. Among cancers that are similar in both clinicopathological features and molecular profiles, there is still heterogeneity when individual genes are examined; each cancer is thus a unique entity. As alterations in the expression or function of many of the individual genes can predict the responses to particular therapies, there will be considerable scope for using this molecular information to guide the treatment of the patient. When epigenetic changes in cancers lead to the silencing of genes and where the gene products are involved in sensitivity or resistance to therapeutics, the DNA methylation status of key genes can act as a predictive biomarker. Thus, the information we can extract from the altered epigenome of a cancer will enable us to understand how to treat the cancer. This has been termed pharmacoepigenomics by analogy to pharmacogenetics.

The DNA repair machinery is repeatedly the target of epigenetic change in cancer [244] as it often also is in cancer predisposition syndromes. This machinery is responsible to repair the multiple forms of damage, which is constantly inflicted upon the genome by endogenous (e.g., replication errors, reactive oxygen species) and exogenous sources (e.g., radiation). Many enzymes and proteins participate directly or indirectly in the important processes to repair and maintain a stable genome. Altering or silencing the gene expression of a given DNA repair gene will result in unrepaired damage setting up a mutator phenotype. The mutator phenotype generates multiple sequence variants. Natural selection then acts on the variants that arise, enabling cancer evolution. However, the repair deficiency is also a therapeutic target in the cancer. This underlies the susceptibility of many cancers to one or other form of chemotherapy. Currently, the best example of a gene whose DNA methylation has predictive value is the *MGMT* gene [285]. Its protein product, *O6*-methylguanine DNA methyltransferase, directly removes alkyl groups from the *O6*-position of guanine residues. If the alkylation damage is left unrepaired, it increases the probability of a G to A transition mutation during replication [286]. Thus, a failure in repairing this damage will lead to an accumulation of mutations over time and constant development of new cell clones in the cancer.

The presence of methylation at the *MGMT* promoter was shown to function as a DNA methylation-based biomarker for the susceptibility of cancers treated therapeutically with alkylating agents [287]. The therapeutic benefit of alkylating agents for cancer patients with a methylated *MGMT*

promoter was clearly demonstrated in the treatment of glioblastoma with temozolomide [288]. The presence of DNA methylation at the *MGMT* promoter was also associated with increased survival in diffuse large B-cell lymphoma (B-DLCL) patients being treated with the alkylating drug cyclophosphamide as part of multidrug regimens [289]. Methylation of the *MGMT* gene is increasingly being used to guide the choice of whether to use alkylating agents in the treatment of glioblastoma patients. This has led to *MGMT* promoter methylation being the first DNA methylation-based biomarker assay that is being adopted in cancer molecular pathology for glioblastoma. However, *MGMT* promoter methylation is not currently used to guide treatment decisions for other tumors such as melanoma and colorectal cancer that also can show methylation at the *MGMT* promoter.

Many assays have been developed to detect DNA methylation at the *MGMT* promoter utilizing different approaches [267, 290, 291]. In addition, several commercial products are available based on quantitative methylation-specific PCR (qMSP), bisulfite pyrosequencing and methylation-sensitive high-resolution melting (MS-HRM). It is critical that quantitative approaches are used as, as previously mentioned, certain individuals have the single nucleotide polymorphism that predisposes to a definite background level of DNA methylation but not one that definitely leads to a methylated cancer [185].

Other DNA repair genes, such as *MLH1* [143, 292], *WRN* [293], as well as members of the BRCA-Fanconi anemia pathway, *BRCA1* [146, 294], *FANCC* [295], *FANCF* [296], and *FANCL* [295], also have been reported to show aberrant DNA methylation in several cancer types. These different alterations render cancer cells susceptible to certain chemotherapeutic drugs either directly [297, 298] or through a synthetic lethal approach [299]. Thus knowledge of which of these genes are methylated may help clinicians to choose the appropriate treatment for an individual cancer patient [300].

5.3.4 Clinical Implications of Chromatin Changes in Cancer

The prognostic value of various histone acetylation and methylation alterations was shown in studies of prostate cancer [301–303] lung cancer [304–306], ovarian cancer [307], pancreatic cancer [307, 308], gastric cancer [309], breast cancer [307, 310], and renal cancer [311]. Interestingly, for some cancer types certain histone modifications were found to act as independent prognostic factors, providing additional nonredundant information to the clinicopathological markers currently in use. This was reported for example for acetylated H3-K18 in breast cancer [310] and renal cancer [311] and for trimethylated H3-K27 in breast, ovarian, and pancreatic cancers [307].

One of the most remarkable examples of the clinical implications of epigenetics in cancer is its role in plasticity of the cancer cell phenotype. A cancer is considered to comprise two populations: a pluripotent stem-cell-like population and a more differentiated population. Sharma et al. showed the existence of a highly drug resistant stem-cell-like population in the PC9 lung cancer cell line [312]. The transition between the stem-cell-like population and the more differentiated population seemed to be mediated by the levels of the lysine-specific demethylase 5A (KDM5A, JARID1A). Such epigenetically determined plasticity has major implications for cancer therapy as no single treatment approach is likely to target both populations of cancer cells.

5.3.5 Targeting Epigenetic Changes Globally

DNA methylation and histone modifications are potentially reversible as was shown by the large scale remodeling of a melanoma nucleus after nuclear transfer into oocytes. An embryonic stem cell line could then be derived from the blastocysts that could give rise to multiple tissues in a chimeric mouse [237]. This raises the possibility, that the epigenetic changes in a cancer cell can be pharmacologically reversed. In an ideal scenario, this should result in the restoration of the epigenetic pattern associated with a certain cell type before the malignant transformation. Two different drug types that have shown some therapeutic benefit are currently in clinical use: demethylating agents and HDAC inhibitors.

Dependent on their chemical structure, demethylating agents can be grouped as nucleoside analogues or non-nucleoside based drugs [313]. The drugs based on nucleoside analogues are cytidine or deoxycytidine analogues and exert their impact in an indirect manner; they need to be incorporated into the genomic DNA and substitute for genomic cytosine. Preceding incorporation, these drugs need to be activated to resemble natural occurring dNTPs and to be capable to participate in strand synthesis. Once they have been incorporated into newly synthesized genomic DNA, they either inhibit the DNMTs irreversibly [314, 315] or form a reversible but stable complex between DNMTs and DNA [316, 317]. As a consequence, the newly synthesized DNA strands show less DNA methylation.

So far, 5-azacytidine (azacitidine, Vidaza) [318] and 5-aza-2′-deoxycytidine (decitabine, Dacogen) [319] have been approved for treatment of myelodysplastic syndromes (MDS) and leukemia by the American Food and Drug Administration (FDA). Another demethylating drug, zebularine, might is also promising in cancer therapy [320, 321].

Concerns in using demethylating drugs address their non-specific character—that the epigenetic status of the whole cancer cell is targeted rather than that of individual genes [322]. Despite the fact that the cancer genome is hypomethylated

overall, epigenetic therapy with demethylating agents is presumed to target hypermethylated promoters. A (potentially) unstable genome caused by global DNA hypomethylation may be further destabilized after drug supply and may face (additional) severe chromosomal damage, which further promotes cancer progression or metastasis. In addition, pathogenic DNA methylation patterns tend to be restored after the removal of the drug by mechanisms which remain unclear [323]. Furthermore, chemotherapeutic opportunities to target epigenetic change at particular genes will be lost. For example, a methylated *MGMT* promoter renders patients susceptible to alkylating drugs. By treating this patient group with demethylating agents, they may lose the opportunity to benefit from this kind of treatment [324].

Nevertheless, treatment with demethylating agents has been demonstrably successful in some cancers notably myelodysplasia where the prolonged treatment with low doses of demethylating agents has led to marked increase in survival with minimal side effects [325–327]. The therapeutic benefit of demethylating agents in the treatment of solid cancers is not yet as evident as for the treatment of hematological malignancies [322, 328], although some promising results have been reported in lung cancer [329]. A prerequisite of successfully using demethylating agents is the ability of cancer cells to proliferate. Many solid cancers harbor cell populations which replicate slowly and therefore may escape drug treatment unless specifically targeted [324, 328].

During cancer onset, development, and progression, some regions in the genome, become aberrantly heterochromatic. Inhibiting the enzymatic function of HDACs will result in increased acetylation of histones. Multiple HDAC inhibitors have been developed in the last years [313, 330, 331].

HDAC inhibitors can induce several anticancer effects and the mechanism(s) by which these compounds exert their inhibitory effect is an active area of research. The affected HDACs are dependent on the drug itself and the inhibitory mode has been investigated in greater detail for some of them. Nevertheless, the mechanism(s) how these drugs exert their antitumor activity on the cellular level is not well understood [331, 332]. Like demethylating agents, HDAC inhibitors have genome-wide effects. Their inhibition also leads to acetylation of non-histone proteins, such as p53 [333] which further complicates estimation of the consequences of their use [334].

To date, several HDAC inhibitors including suberoylanilide hydroxamic acid (SAHA, vorinostat, Zolinza) and Romidepsin (depsipeptide, Istodax) have been approved by the FDA for use in cancer therapy. These drugs have been approved for the treatment of cutaneous T-cell lymphomas (CTCL), where their results can be spectacular [335–338]. Another HDAC inhibitor, valproic acid, approved by the FDA for the treatment of epilepsy and bipolar disorder also shows some promising results in the therapy of hematological malignancies [339] and solid cancers [340].

Combination therapies involving both demethylating drugs and HDAC inhibitors are promising avenues of investigation in clinical trials [328, 341]. They promise to have some of the synergistic effects seen in the combinations of these two drug types in in vitro models [327, 342]. Furthermore, HDAC inhibitors could be combined with other anticancer drugs, which could improve the treatment of hematological malignancies as well as the treatment of solid cancers [341].

5.4 Conclusions

Epigenetic changes are clearly key in the initiation and development of cancer. However, we still are a long way from a complete understanding of the interplay of epigenetic and genetic change in the origin and development of cancers. Understanding the contributions of the environment and nutrition to cancer onset via epigenetic mechanisms, especially in an individual context, is increasingly important. This understanding is being accelerated by the currently developing genome-scale methodologies that will reveal the genetic and epigenetic changes in an individual cancer. Determining cancer therapy will also rely on knowledge of both genetic and epigenetic changes in each case of cancer. No characterization of a given cancer can be said to be complete without a characterization of the epigenome. Thus, the analysis of (aberrant) epigenetic patterns will be a key element in future molecular diagnostics and personalized therapies.

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Cancer Proteomics

6

Jeffrey M. Lake and Timothy D. Veenstra

6.1 The Need to Study Cancer

A tremendous amount of intellectual and fiscal resources is poured into cancer research every year. Although statistics between the years 2000 and 2006 reveal a decrease in cancer incidence rates in the USA of 1.3 % and 0.5 % per year for men and women, respectively, cancer remains a major health problem within the USA and virtually everywhere in the world [1]. The death rates among men for all races in the USA decreased 21 % between 1990 and 2006 with decreases in lung, prostate, and colorectal cancers accounting for nearly 80 % of this total. During the same period, the death rates among women decreased 12.3 %, with decreases in breast and colorectal cancers accounting for approximately 60 % of this total. While these numbers are very encouraging, the following numbers are still staggering—1,665,540 new cancer cases and 585,720 cancer deaths are projected for 2014 (www.cancer.org). This number of new cancer cases is larger than the population of all but six US cities (according to the 2000 census) and the number of deaths is just under the population of Washington, DC. A list of estimated new cancer cases and deaths for 2010 is listed for various cancers in Table 6.1.

The fifth leading cause of cancer deaths among women is ovarian cancer. The death rate for this cancer illustrates the need for better diagnostic tools. It is estimated that during 2014, 21,980 women will be diagnosed with ovarian cancer during and 14,270 will die from this disease (http://www.ovariancancer.org/about-ovarian-cancer/statistics/) [1]. Funding to support ovarian cancer research in the USA has predominantly come from federal agencies, which committed $112 million in the year 2010 (http://www.ovariancancerresearch.thegcf.org/). Other nonprofit organizations have contributed another $16 million, for a total national commitment of at least $128 million to support ovarian cancer research. Overall this funding level represents greater than $9000 for every woman who will die from ovarian cancer. Unfortunately, even at this fiscal commitment, the prognosis for women diagnosed with ovarian cancer remains poor. This disease does not have a reliable early detection or screening test, resulting in more than 60 % of patients being initially diagnosed with stage III or stage IV cancer. At these stages the cancer has already spread beyond the ovaries. A lack of progress in diagnosing and/or treating ovarian cancer is reflected in Table 6.2 (http://www.ovariancancer.org/about-ovarian-cancer/statistics/). Over the first decade of this century, there was little change in the number of women diagnosed with this disease and no significant change in the number of deaths. Overall the ratio of new cases report and deaths shows no significant change over this 10 year period.

Ovarian cancer is typically diagnosed in patients that already show symptoms. The symptoms may include pressure or pain within the pelvis, abdomen, back, or legs, bloating within the abdomen, chronic tiredness, gas, diarrhea, constipation, indigestion, and nausea (http://www.medicinenet.com/ovarian_cancer/article.htm; http://www.mayoclinic.com/health/ovarian-cancer/DS00293/DSECTION=symptoms). Other less common symptoms include unusual vaginal bleeding and urinary frequency. Unfortunately the symptoms of ovarian cancer are nonspecific and are similar to those other more common conditions such as digestive and bladder disorders. Before being diagnosed with ovarian cancer, a woman may be told she has another condition such as irritable bowel syndrome, stress and depression. If the physician suspects ovarian cancer he/she will order additional tests. A physical exam, in which the doctor presses on the abdomen to check for

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_6

Table 6.1 Estimated new 2014 US cancer cases and deaths by gender

	Estimated new cases			Estimated deaths		
	Both genders	Male	Female	Both genders	Male	Female
All sites	1,665,540	855,220	810,320	585,720	310,010	275,710
Oral cavity and pharynx	42,440	30,220	12,220	8390	5730	2660
Digestive system	289,610	162,730	126,880	147,260	84,970	62,290
Respiratory system	242,550	130,000	112,550	163,660	90,280	73,380
Bones and joints	3020	1680	1340	1460	830	630
Soft tissue	12,020	6550	5470	4740	2550	2190
Skin	81,220	46,630	34,590	12,980	8840	4140
Breast	235,030	2360	232,670	40,430	430	40,000
Genital system	338,450	243,460	94,990	59,970	30,180	28,790
Urinary system	141,610	97,420	41,190	30,350	20,610	9740
Eye and orbit	2730	1440	1290	310	130	180
Brain and nervous system	23,380	12,820	10,560	14,320	8090	6230
Endocrine system	65,630	16,600	49,030	2820	1300	1520
Lymphoma	79,990	43,340	36,650	20,170	11,140	9030
Myeloma	24,050	13,500	10,550	11,090	6110	4980
Leukemia	52,380	30,100	22,280	24,090	14,040	10,050
Other	31,430	16,370	15,060	44,680	24,780	19,900

Table 6.2 Yearly ovarian cancer cases and deaths in the USA between 1999 and 2009

Year	Cases	Deaths	Cases/Deaths
2009	21,500	14,600	1.47
2005	19,842	14,787	1.34
2004	20,069	14,716	1.36
2003	20,445	14,657	1.39
2002	19,792	14,682	1.35
2001	19,719	14,414	1.37
2000	19,672	14,060	1.40
1999	19,676	13,627	1.44

tumors or ascites fluid buildup, as well as a fluid examination, may be conducted. A pelvic exam to check the ovaries and nearby organs for lumps or abnormalities in shape or size may be done along with an ultrasound to get a picture of the ovaries and surrounding tissues. The doctor may also order a blood test to measure the levels of mucinous glycoprotein serum cancer antigen 125 (CA125). After all of these tests are conducted, a biopsy may be required to physically remove tissue from the ovary, as well as surrounding fluid, and have them examined by a pathologist. It is easily seen why most women that receive a diagnosis of ovarian cancer are already at an advanced stage. It would be ideal if the circulating CA125 levels could be used to routinely screen women for ovarian cancer at yearly physicals. However, its measurement lacks the necessary sensitivity and specificity (http://www.cancer.gov/cancertopics/factsheet/Detection/tumor-markers). In fact the US Food and Drug Administration (FDA) only approves the use of CA125 for monitoring the response to ovarian cancer treatment and for detecting its post-treatment recurrence. One of the major issues associated with using CA125 measurement to diagnose ovarian cancer is the large number of false positives that it predicts [2]. Obviously CA125, like the other well-known prostate cancer biomarker prostate-specific antigen, lack the sensitivity and specificity required for diagnosing patients.

6.2 Hypothesis Versus Discovery-Driven Studies

One important change in how cancer research is conducted in the *omics*-age is perspective. In previous decades where data collection was often challenging most cancer studies were hypothesis driven. In a hypothesis-driven study, the investigator formulates an educated guess to explain a cause-and-effect relationship. A series of experiments are performed to examine this idea and determine if the hypothesis is correct. In a discovery-driven study, the goal is to collect a specific type of data on as many features as possible and use the data to draw a conclusion or formulate a hypothesis. The success of many discovery-driven studies is how well the data can formulate novel specific hypotheses. The advent of technologies that can gather large amounts of data on thousands of biological molecules in clinical samples has driven cancer research towards discovery-driven studies at an exponential rate. A great example is genome-wide association studies (GWAS) [3]. In GWAS, the genomes of different individuals are sequenced to determine how their genes vary. The most common comparisons made in GWAS studies are between healthy individuals and those with a specific disease condition. The aim is to find regions in the genome that can be associated with specific traits such as diseases.

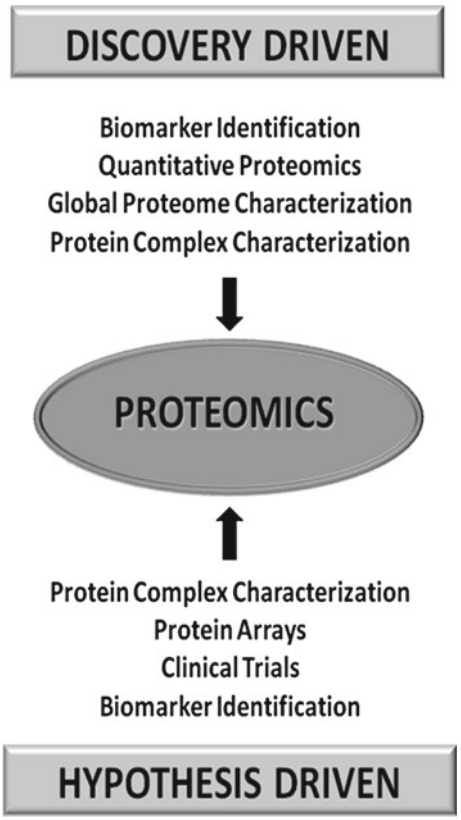


Fig. 6.1 List of discovery-driven and hypothesis-driven studies. In the proteomics era, some studies (such as protein complex characterization and biomarker identification) can be performed using either approach.

In the field of proteomics, the development of high-throughput technologies has enabled discovery-driven studies as never before. As shown in Fig. 6.1, these studies include biomarker discovery, quantitative proteomics, protein complex characterization, and global proteome characterization. Probably the biggest impact to cancer research has been the ability to conduct discovery driven studies to find cancer-specific biomarkers. The greatest challenge in finding biomarkers for specific cancers is having no a priori information as to the identity (or even character) of a protein biomarker for any cancer. It is even impossible to determine which class of protein (e.g., kinase, phosphatase, membrane, or nuclear) a biomarker likely belongs. Therefore, this type of study is often left to a purely unbiased discovery-driven in which samples from healthy and cancer-affected individuals are compared. In addition, the ability to collect large amounts of data has also impacted how hypothesis-driven proteomic experiments are conducted. For example, protein complexes no longer need to be characterized protein by protein, rather the entire complex can be analyzed in a single experiment. The amount of data collected in an experiment does not necessarily indicate if it is hypothesis-driven or discovery-driven. For example, clinical trials that collect a great deal of patient data are primarily hypothesis-driven as specific outcomes are being monitored. This ability to conduct meaningful discovery-driven studies has provided the basis for the large increase in proteomic biomarker discovery, primarily for cancers. Reasonable or not, biomarker discovery projects begin with essentially no information as to the identity of the protein of interest. The only reliable information available is the source of the samples being analyzed. To understand how proteomics is applied to cancer research it is important to gain an understanding of the major technologies used in this field.

6.3 Proteomic Technologies

The previous decade has seen a revolution on how cancer is studied. In times past, cancer research was conducted on a molecule-by-molecule basis, whereas today's experiments aim to simultaneously uncover large numbers of genes, transcripts, proteins, and metabolites that characterize a specific cancer. The primary driver that introduced this new *omics* era was technology development. Massively parallel sequencing methods, such as 454 Sequencing, have made surveying entire genomes for genetic aberrations possible [4]. High-density microarrays allow tens of thousands of transcripts to be characterized in a high-throughput fashion [5]. For proteins and metabolites, advances in chromatography and mass spectrometry (MS) have permitted thousands of these molecules to be characterized in complex clinical samples [6, 7].

In the field of proteomics, gone are the days in which cancer was studied at an individual protein level. While fundamental technologies such as Western blotting, enzyme-linked immunosorbent assays (ELISA), immunoprecipitations (IPs), and other methods will continue to be critical in protein analysis, modern proteomic technologies enable cancer to be studied at pathway, network, and global levels. An excellent example of proteomic technologies changing the way a fundamental experiment is conducted is illustrated by the analysis of protein complexes (Fig. 6.2) [8]. Protein complex isolation has been routinely conducted using IPs. Historically, the extracted material has been separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. To identify a protein interaction, the PVDF membrane is blotted using an antibody directed against a protein hypothesized to interact with the protein targeted in the IP. While this hypothesis-driven strategy has proven fruitful over the years, it provides a limited amount of information and lacks the ability to find truly novel interactions that fall outside the investigators hypotheses.

The proteomics era has brought the ability to conduct more discovery-driven approaches. For example, in the characterization of protein-protein interactions no longer requires a hypothesis to identify individual proteins, rather the entire complex can be characterized using mass spectrometry (MS).

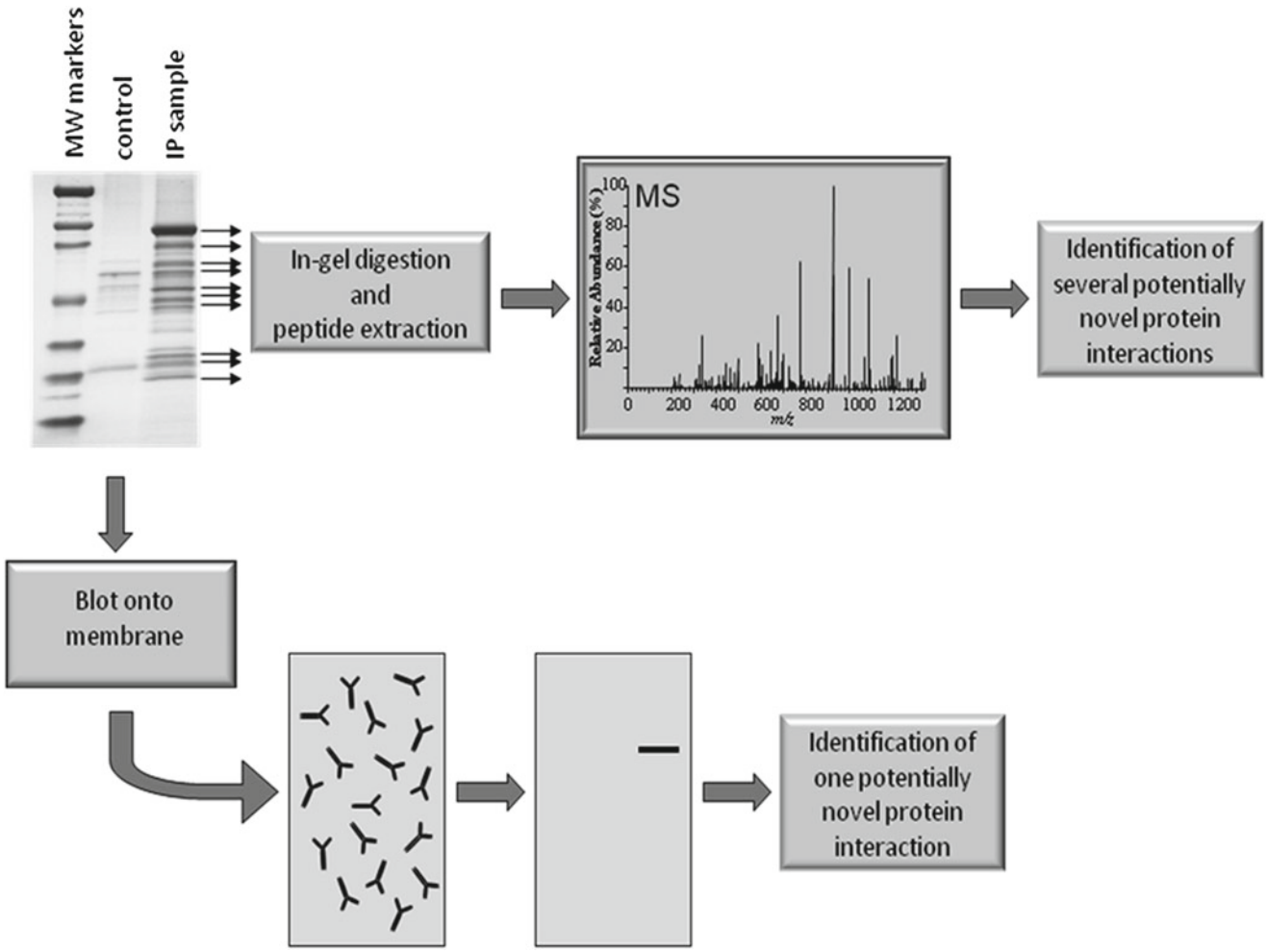


Fig. 6.2 Identifying protein interactions using hypothesis and discovery-driven methods. In a hypothesis-driven approach a protein complex is isolated using a technique such as immunoprecipitation (IP). The complex is separated by gel electrophoresis and the proteins blotted onto a membrane, which is interrogated using an antibody specific for a protein that is hypothesized to interact with the target protein. In the discovery-driven approach the complex is separated using gel electrophoresis, the protein bands are colorimetrically stained, and individually subjected to in-gel digestion. After extracting the peptides from each gel piece, they are individually analyzed using mass spectrometry (MS). While the hypothesis-driven method results in the identification of a single potential protein interaction per experiment, the discovery-driven approach identifies several potentially novel interactions.

The procedure for characterizing protein complexes using MS is straightforward and incorporates many of the steps used in the hypothesis-driven strategy (Fig. 6.2). The isolated protein complex is separated using SDS-PAGE and rather than transferring the proteins to a PVDF membrane, they are stained using a colorimetric reagent such as Coomassie blue or silver stain. The protein bands are excised from the gel and are subjected to in-gel digestion with a proteolytic enzyme (usually trypsin). Peptides are extracted from the individual gel pieces and identified using MS. The identified peptides are then compared to a proteomic or translated genomic database to determine the origin of the peptides. This discovery-driven approach offers a number of key advantages over hypothesis-driven methods. Several interacting proteins can be identified within a single study, no antibodies (save those used to isolate the complex) are required, and truly novel protein interactions can be identified since any testing does not rely on a formulated hypothesis.

6.3.1 Mass Spectrometry

The greatest fundamental advance that proteomics has contributed to cancer research is in the identification of proteins. While having been a mainstay in biochemistry for over a half-century, the technology used for identifying proteins has seen enormous leaps in the past decade. In the 1950s Edman degradation enabled the sequencing of purified proteins [9]. While Edman sequencing is still in use today, it has been largely replaced by MS. The two primary reasons are

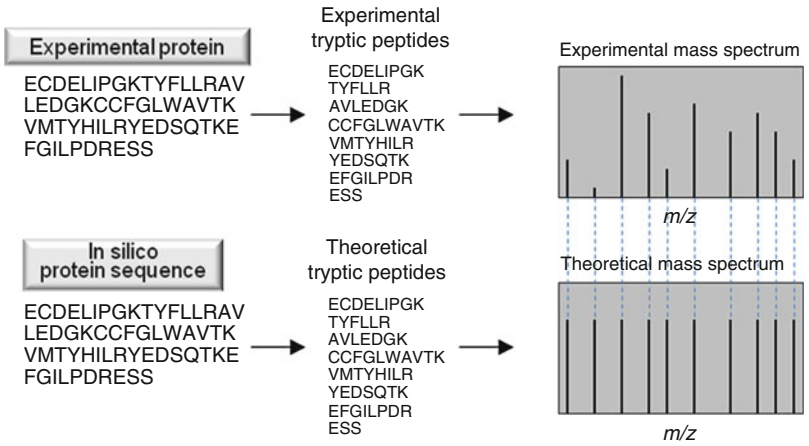


Fig. 6.3 Identification of a protein via peptide mapping. Proteins are digested into peptides (usually tryptic) and their masses are measured using mass spectrometry (MS). The resulting masses are compared to theoretical mass spectra that are generated by an in silico digest of a suitable proteome database. The protein is identified by the correspondence between the list of experimental peptide masses and the in silico digest of each protein within the database.

throughput and the fact that MS does not require a pure sample to confidently identify a protein. Mass spectrometry is able to identify a single highly purified protein or identify thousands of proteins within a complex mixture. The two main methods used to identify proteins by MS are peptide mapping [10] and tandem MS (MS/MS) [11].

To identify a protein using peptide mapping, it is first enzymatically digested into peptide fragments (Fig. 6.3). Trypsin is by far the most commonly used protease for this purpose owing to its specificity and the relative distribution of lysine and arginine residues throughout proteins in the proteome. The masses of the tryptic peptides are measured using MS and this list of experimental masses is compared to theoretical masses of the tryptic peptides that would result from a tryptic in silico digest of proteins present in a protein or genomic database. The protein is identified based on the best match between the experimental peptide masses and the digest of the proteins within the database. The drawback with peptide mapping is that, although it does not require a pure protein, the best identifications come from samples in which the target protein is highly enriched.

When using MS/MS for identification (Fig. 6.4), individual peptides enter the mass spectrometer and are isolated within the instrument and collided with an inert gas (i.e., He, Ar, N_2, etc.). This process, known as collisional induced dissociation (CID) breaks the peptide into fragments that are then sent to detector. Fortunately, the ways in which peptides fragment are fairly well understood with cleavage across the amide bond one of the favored fragmentation pathways. In addition, CID does not cleave every amide bond in every peptide. The fragmentation results in populations of various lengths of residues both from the N-terminus and C-terminus, as well as internally, being recorded for each peptide. Peptides are identified by how well the fragmentation pattern matches the in silico fragmentation patterns calculated from all corresponding peptides within a database. When the sample is primarily comprised of a single protein, the MS/MS spectra of several peptides are used to confidently identify the protein. When very complex mixtures are being analyzed, the MS/MS spectrum of a single peptide can provide evidence for the presence of a specific protein within the sample. However, the rule is that the more peptides identified, the greater the confidence that the protein is present in the sample. The advantage of MS/MS is that it identifies peptides based on sequence information.

When combined with high resolution liquid chromatography (LC), modern mass spectrometers can sequence thousands of peptides per hour. For these types of studies, a complex sample (e.g., biofluid, tissue, or cell lysate) is digested into tryptic peptides. These peptides are fractionated by a selected number of chromatography methods prior to being eluted directly into the mass spectrometer. The most commonly used modes of fractionation involve strong cation exchange followed by reversed phase LC. As they enter the mass spectrometer, the instrument isolates individual peptides based on their intensity and subjects them to CID (Fig. 6.5). The MS/MS spectra are then analyzed against a suitable database turning the raw data into identified peptides. The identified peptides are binned to their specific protein of origin. The seminal example of using MS to identify large numbers of proteins in a biological sample was developed by Dr. John Yates' lab in 2001 [12]. A dual strong cation exchange/reversed-phase LC column was used to separate tryptically digested yeast proteins directly on-line with an ion-trap mass spectrometer. This experiment resulted in the identification of almost 1500 proteins. This multidimensional

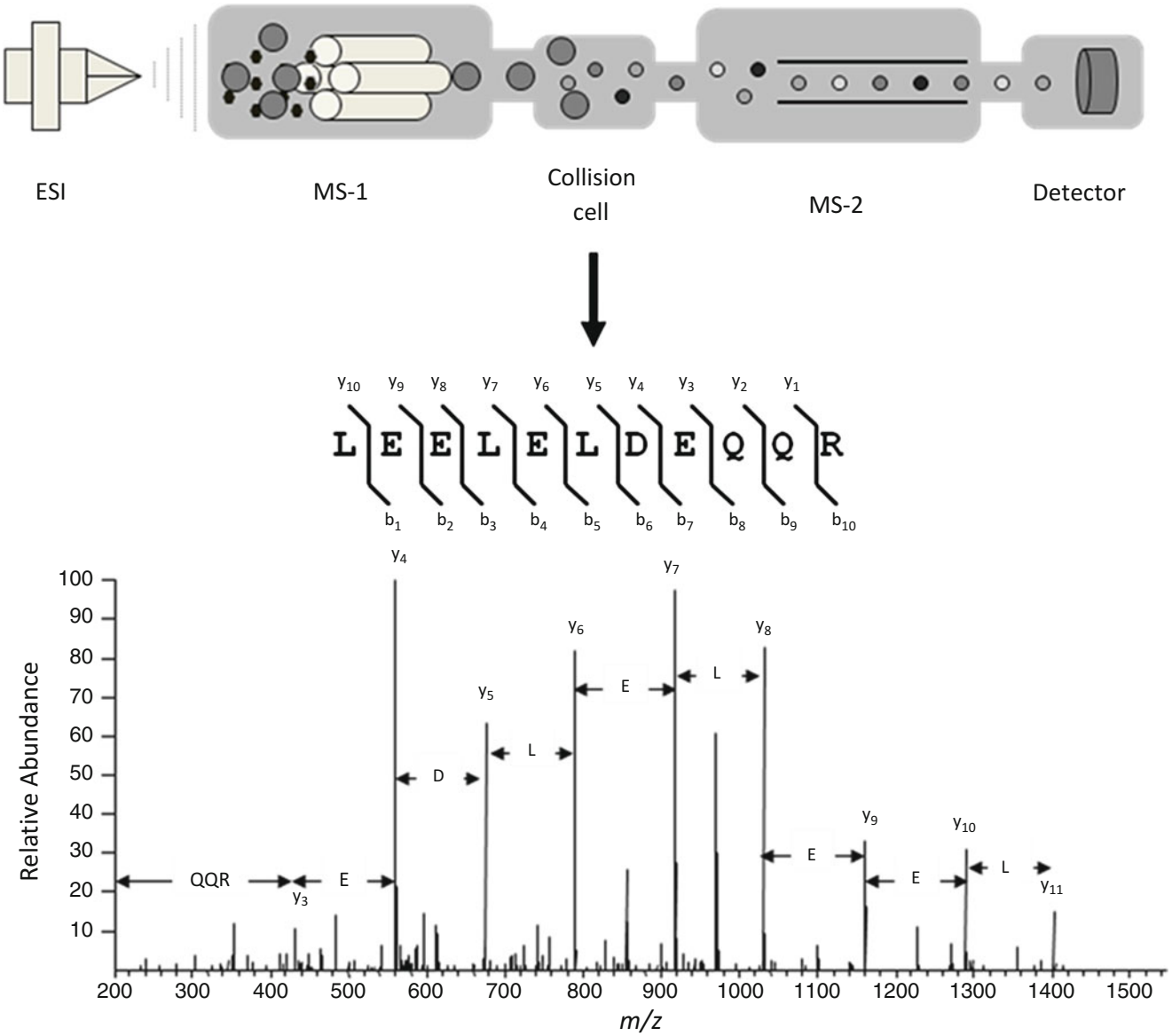


Fig. 6.4 Identification of a protein via tandem mass spectrometry (MS/MS). A specific peptide entering the mass spectrometer (*large grey circle*) is isolated within the instrument and subjected to collisional induced dissociation (CID). The fragments of the peptide produced by CID are then directed onto the detector where the MS/MS spectrum is acquired. The most common fragmentation occurs across the amide bond creating a series of b and y ions. Software programs compare the MS/MS spectra with proteomic databases to determine the sequence of the peptides subjected to CID.

protein identification technology or MudPIT, as it was coined, was subsequently used by different laboratories to interrogate clinical samples with the ultimate goal of discovering biomarkers. This technique, now commonly referred to as *shotgun proteomics*, has been used to tremendously increase our knowledge of the proteome of peripheral body fluids such as serum, plasma, and urine. While today investigators have been able to identify upwards of 4000 proteins within complex samples [13–15], the mass spectrometer is still unable to identify all of the proteins within a complex mixture.

6.3.2 Protein Arrays

While MS has been the primary tool for advancing cancer research into the proteomics era, protein arrays represent a more directed and potentially more powerful tool to perform broad proteome surveys of different cellular systems. Owing to its dominance, this chapter is devoted to the role of MS in cancer proteomics. However, it is worth including a section to make the reader aware of protein array technologies. While MS will continue to play a dominant role in biomarker

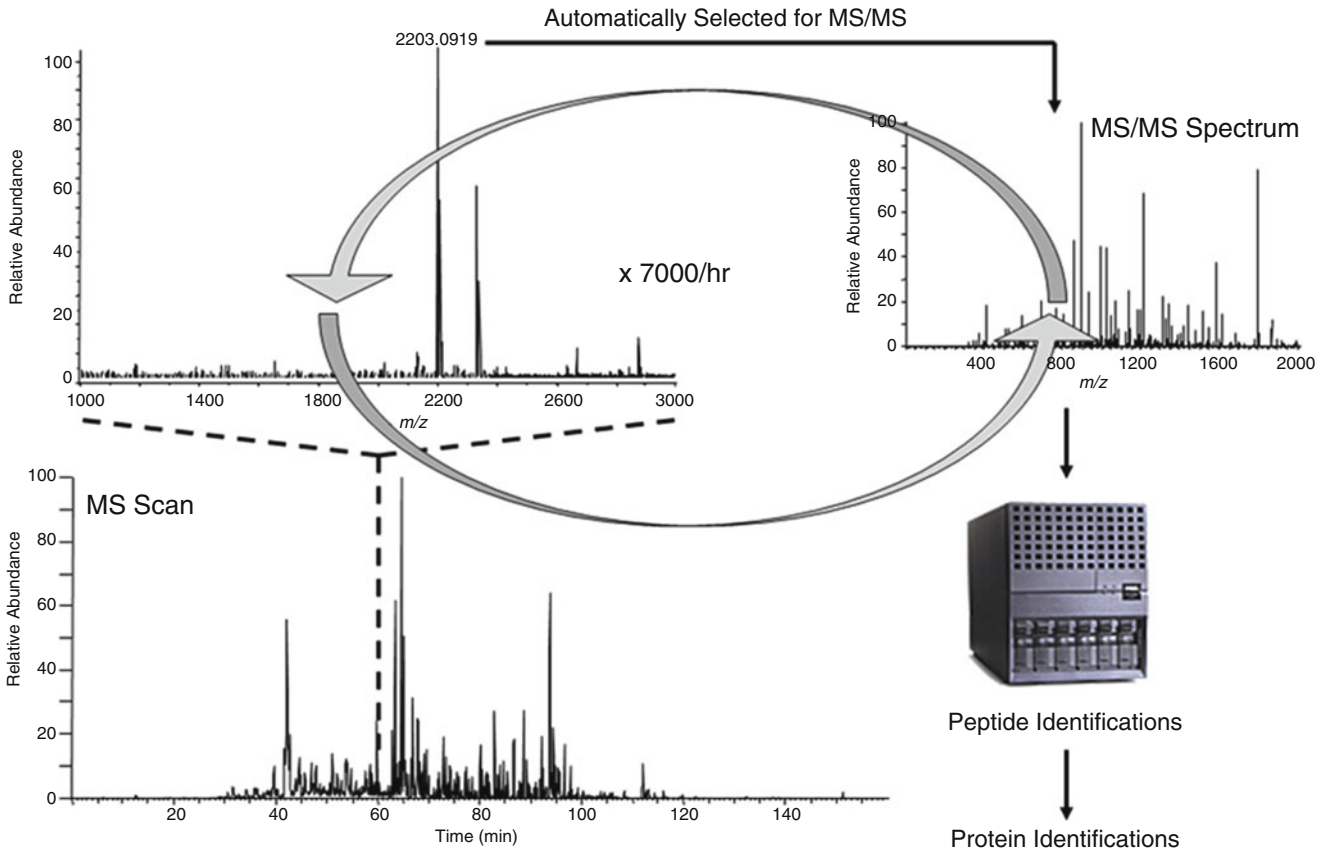


Fig. 6.5 Data-dependent tandem mass spectrometry identification of peptides in complex mixtures. In this method, peptides observed in the MS scan are selected for MS/MS based on their signal intensity. After the MS/MS spectrum of the most intense peptide in the preceding MS scan is acquired, the next most intense peptide signal is selected and so on. After 5–10 peptides are selected the mass spectrometer acquires another MS scan to find new signals from peptides that have eluted into the instrument. This sequence of MS and MS/MS occurs at a rate of approximately 7000 times per hour resulting in the identification of thousands of peptides from complex mixtures.

discovery, protein arrays will be increasingly important in validation studies. Like ELISA's, Western blotting, and immunohistochemistry (IHC), protein arrays make use of affinity reagents (primarily antibodies) to measure changes in proteins present within complex samples. Put simply, protein arrays are essentially an attempt to simultaneously conduct hundreds of microscale IHCs, ELISAs, or Westerns. While protein arrays may seem limited since they target specific proteins (hypothesis-driven), large numbers of different antibodies can be used to provide a comprehensive view of protein changes.

There are currently two types of protein microarrays used in cancer research: (1) forward-phase protein microarrays (FPPAs) [16, 17], and (2) reverse-phase protein microarrays (RPPAs) [18]. The goal of both types of arrays is to map changes in cell pathways and networks in single, high-throughput experiment. The obvious advantage using either type of array is throughput, allowing real-time analysis of multiple patient samples. In FPPAs, antibodies are immobilized onto a solid surface. The antibodies act as bait to capture specific antigens within the sample. As with many immunoaffinity techniques, a secondary antibody is utilized to detect and visualize the antigen. The major disadvantage of FFPAs is the need for two antibodies and the antigen must be in a conformational state capable of binding both unique antibodies.

The second type of protein array is the reverse-phase protein microarray (RPPA). They are called reverse-phase because instead of the antibody, the antigen-containing sample containing the antigens is immobilized to a surface. The types of samples that can be arrayed include cell lysates, laser captured tissues, and tissue cores. The array format allows numerous aliquots of the sample to be arrayed and interrogated with multiple antibodies against unique proteins. The obvious advantage of the RRPA is that a single antibody is required per antigen. The amount of cell lysate obtained from cells acquired using laser capture microdissection (LCM) is plenty for creating an entire RPPA. These arrays typically use on the order of 2 nL of material per spot, allowing hundreds of patient samples to be printed on a single 25 × 75 mm array.

While conventional protein arrays require spotting of cell lysate, investigators have also developed tissue microarrays (TMA) [19–21]. Tissue microarrays are essentially high-throughput devices for conducting multiplexed IHC experiments on tissue sections. Immunohistochemistry is a standard research tool for translational research laboratories as well as being a foundational diagnostic technique in modern clinical pathology. Instead of conducting IHCs on a single tissue, TMAs allow for the concurrent analysis of tens to hundreds of tissue specimen on a single slide. In the construction of a TMA, 0.6–2.0 mm diameter regions are cored from FFPE (formalin-fixed paraffin-embedded) or fresh-frozen tissues and cut into thin sections. The thin slices are then transferred onto a glass slide for automated IHC analysis using specific antibodies. The results can be assessed by an actual pathologist or automatically and then linked to other available clinical data, such as prediction of patient outcome. Beyond the obvious throughput advantage, since several samples are done concurrently TMAs demonstrate a high degree of precision for the analysis of clinically important samples.

6.4 Discovery of Protein Biomarkers

The greatest impact that proteomics could make on cancer research is the discovery of biomarkers. The number of lives that would be saved if more biomarkers could be discovered for cancer would be staggering. The number of deaths due to cancer, in particular, would drop dramatically as biomarkers would open up the possibility of detecting this disease at early stages where modern treatments are more effective. As mentioned earlier, more than 60% of ovarian cancer patients are diagnosed at stage III or IV (http://www.ovariancancer.org/about-ovarian-cancer/statistics/). A biomarker with sufficiently high sensitivity and specificity that could diagnose patients with stage I and II cancer, prior to its spreading beyond the ovaries, would make treatments much more effective. Beyond early detection, biomarkers could have a major impact on treatment and prognosis. Personalized medicine, with the aim of treating patients based on their individual molecular profile, is a major focus in medical science today. To reach the goal of personalized medicine is going to require biomarkers that can be used to evaluate treatment efficacy. These biomarkers would enable the best treatment to be selected quickly eliminating a trial-and-error therapeutic strategy.

The proteomics community has devoted a tremendous amount of time and fiscal resources over the past decade in the search for proteomic biomarkers for cancer. The impetus for this direction stems from the development of high-resolution separation techniques and high-throughput peptide identification ability of modern mass spectrometers. The two primary methods for cancer proteomic biomarker discovery utilize two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or shotgun proteomics [22–24].

6.4.1 Two-Dimensional Polyacrylamide Gel Electrophoresis

The overall strategy for identifying biomarkers using 2D-PAGE is shown in Fig. 6.6. Proteomes are extracted from samples obtained from cancer and normal patients and they are separated on gels based on their isoelectric point (p*I*) and molecular weight (MW). Each sample is usually separated on their own gel followed by Coomassie blue or silver staining. The intensity of the stained protein spots are then compared to their matching spots on all the other gels and those that show a differential abundance between samples obtained from cancer and control (i.e., normal) patients are cored from the gel and identified using peptide mapping or MS/MS.

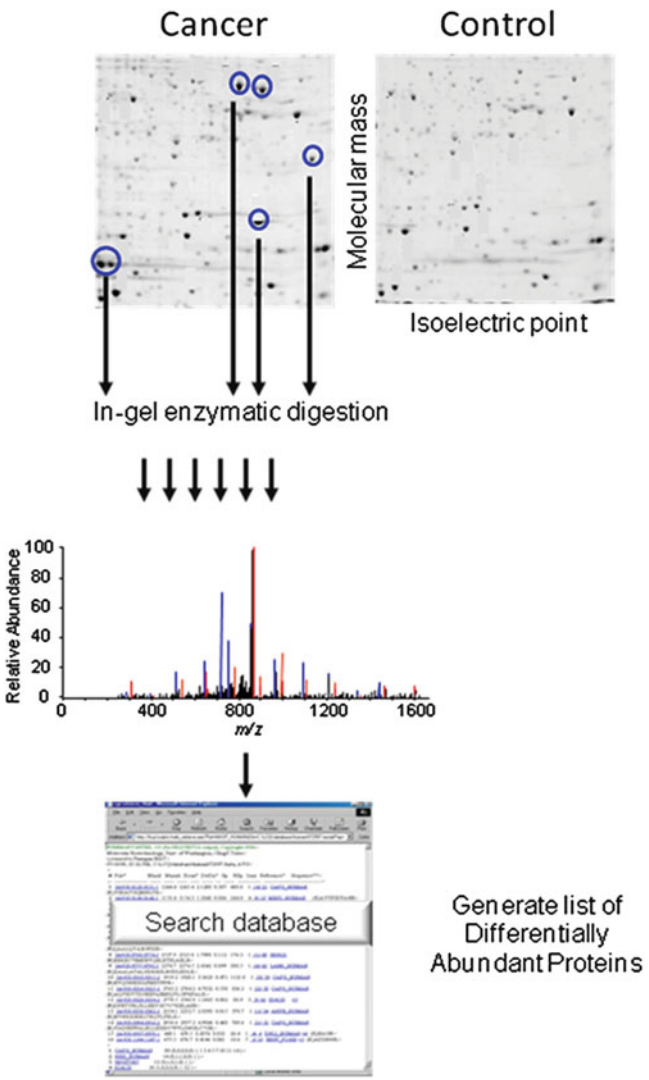


Fig. 6.6 Identification of cancer biomarkers using two dimensional-polyacrylamide gel electrophoresis (2D-PAGE). In this method, proteomes extracted from cancer and control samples are fractionated on individual gels. After staining, protein spots that have a greater intensity compared to their counterpart are cored from the gel, subjected to in-gel digestion and identified using mass spectrometry combined with database searching.

The proteins identified within these spots are then considered putative biomarkers.

The 2D-PAGE strategy has a number of advantages. Only proteins that show a relative abundance difference between the samples being compared require MS identification. The peptides identified for each protein spot can be confidently assigned to that protein as opposed to a potential isoform or another protein that has very high homology. Posttranslationally modified forms of specific proteins, particularly phosphorylation, can be easily recognized via 2D-PAGE as chains of spots across a region of the gel as they will have very similar MWs but different p*I*s.

As with almost every scientific technique, the 2D-PAGE strategy has disadvantages as well. Producing highly reproducible 2D gels can be quite laborious, although automated methods in which several gels are run simultaneously have greatly helped. It also needs to be kept in mind that not every protein on the gel requires MS analysis, saving a lot of labor at the protein identification stage. As far as reproducibility, 2D differential in-gel electrophoresis (2D-DIGE), in which proteomes extracted from different sources are labeled with fluorescent tags, have enabled up to three different samples (as well as an internal control) to be separated on a single gel [25]. This concurrent analysis greatly eliminates irreproducibility. Another disadvantage with 2D-PAGE is the lack of sensitivity. While increasingly sensitive stains can be used to visualize protein spots, ultimately sensitivity is established by the mass spectrometer to identify the spot. Each spot within a gel must be subjected to in-gel digestion and the resultant peptides extracted. The extraction step, in particular, can result in peptide losses and impact overall sensitivity.

6.4.2 Shotgun LC-MS/MS Analysis

While 2D-PAGE has been used, most biomarker discovery studies have been based on shotgun proteomics or slight variations of this method [24]. The basics (and hope) of this strategy are illustrated in Fig. 6.7. A series of samples are collected from disease affected and matched normal patients.

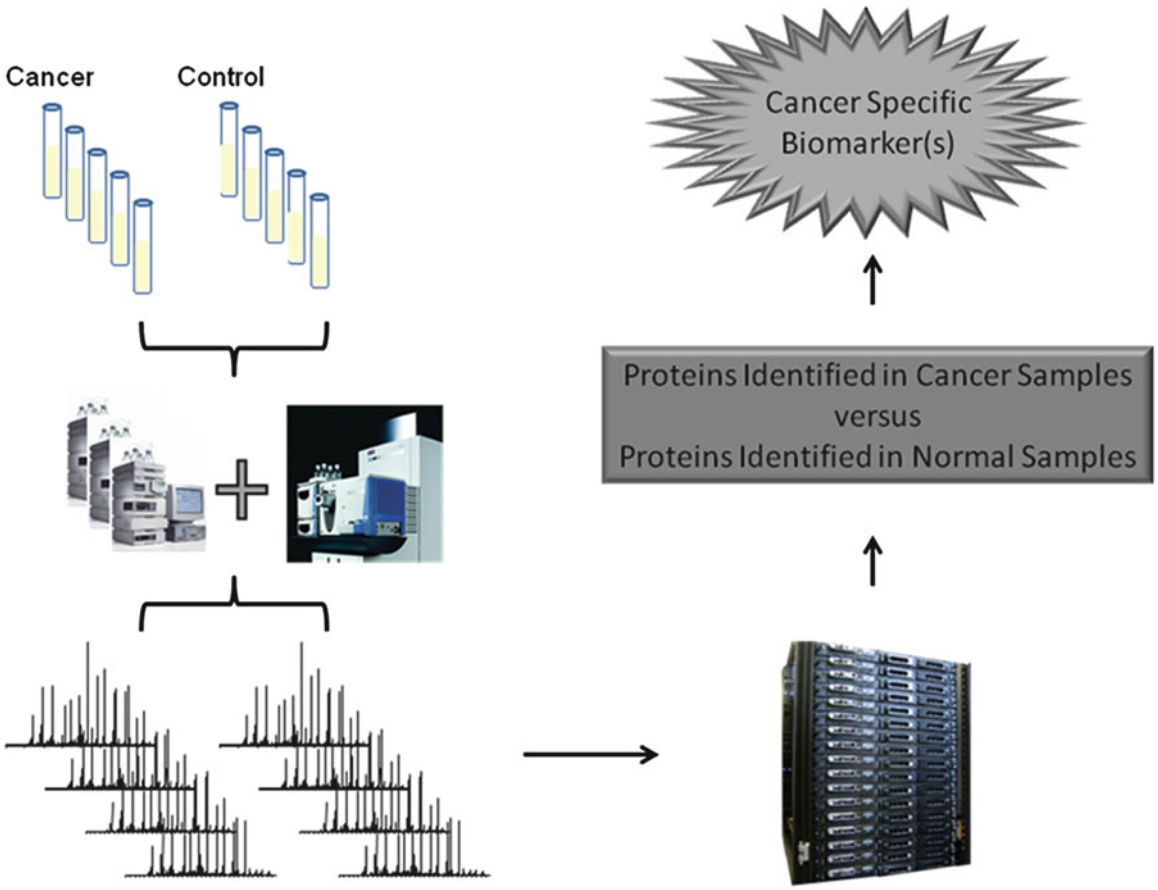


Fig. 6.7 Identification of cancer biomarkers using shotgun proteomic methods. In this method, proteomes extracted from cancer and control patients are digested into peptides. The samples are then separately analyzed using some form of multidimensional fractionation combined with tandem mass spectrometry (MS/MS) to identity as many peptides in each sample as possible. The lists of peptides identified in these samples are then compared to find proteins that have a greater abundance in one of the samples. The hope is that these differences lead to a useful biomarker or a panel of biomarkers.

The proteomes are extracted from each sample and digested into tryptic peptides. If plasma or serum is being analyzed, an immunodepletion step to remove high abundance proteins such as albumin, immunoglobulins, transthyretin, and other abundant proteins must be performed prior to enzymatic digestion or a majority of the peptide identifications observed by MS will originate from these proteins. The tryptic peptides are fractionated prior to being introduced into the mass spectrometer. The aim in fractionation is to elute as few peptides as possible into the mass spectrometer at any time, thereby giving the instrument the opportunity to select as many peptides for identification as possible. While studies have included two, three, and even four dimensions of separation, there is an obvious trade-off between the numbers of fractionation dimensions and throughput. Each LC-MS/MS experiment requires approximately one hour, therefore fractionating a single clinical sample into 20 fractions will require a minimum of 20 h of MS time. If the study contains samples from 20 control and 20 disease-affected patients, the time required will be at least 40 days of constant MS data acquisition. While ultimately the goal is to identify as many proteins as possible from each clinical sample, it is important to balance throughput with number of samples analyzed. For identifying peptides, the mass spectrometer is set up in a data-dependent mode in which the instrument automatically selects peptides to sequence (MS/MS) based on their intensity [26]. The combination of state-of-the-art fractionation and MS allows thousands of peptides within complex samples to be identified. These identified peptides are then correlated to their protein of origin, resulting in a database of proteins observed within each sample and the number of peptides identified for each.

6.4.3 Protein Quantitation

A challenge in conducting shotgun studies for cancer biomarker discovery is how to quantitate differences between peptides within different samples. There are a number of options available with each having their advantages and disadvantages. Isotope-labeling methods [6, 27] such as isotope-coded affinity tags (ICAT), iTRAQ, and O^{16}/O^{18} provide reasonable accuracies. However, these methods only allow at most seven samples (in the case of iTRAQ, the others only permit two) to be directly compared. Biomarker discovery is going to require many more samples than this to be directly compared to gain any sense of confidence in the results even at the discovery phase. To compare large numbers of samples, label-free methods that contrast the number of peptides identified for a specific protein or the actual peak intensities provided by specific peptides in different spectra have been developed.

In one use of label-free quantitation [28], the relative abundances of proteins is determined by the number of peptides identified for each specific protein in different samples. The number of peptides identified for a protein is thought to correlate with its abundance when a sample is analyzed using data-dependent MS/MS. This simple hypothesis is best illustrated by the results obtained in the analysis of undepleted serum. Direct LC-MS/MS analysis of trypsin-digested serum results in the identification of a huge number of albumin peptides, since this protein is present at 40–80 mg/mL. Proteins in the abundance range of ng/mL range (such as a chemokines and cytokines) will probably be identified by one or fewer peptides. To illustrate how relative abundance of a protein is calculated using this method consider an example of two samples—one from a healthy individual and another suffering from ovarian cancer. If nine peptides are identified for CA-125 in serum from the ovarian cancer patient and only two peptides from the other sample, the protein is concluded to be 4.5-fold more abundant in the serum taken from the cancer patient.

This method, known as subtractive proteomics or peptide count, requires minimal sample preparation and allows an unlimited number of samples to be compared. Unfortunately, like most proteomic applications, collecting the data is relatively low-throughput. The method also lacks quantitative precise with the minimum difference that can be reasonably measured being approximately threefold. This lack of accurate quantitation is a major deficiency when attempting to recognize potential biomarkers that are of very low abundance and may be detected by only one or two peptides in the cancer-affected individual. If the protein is not detected in control samples at all, the adage *absence of evidence is not the evidence of absence* applies.

In another label-free method, relative quantitative measurements are based on the ratio of the actual peak area measured for specific peptides in samples being compared [28]. Selected ion chromatograms of the peptides are generated enabling measurement of the peak areas. Proteins of interest are often represented by multiple peptides, therefore the average and standard deviation of the abundance ratio can be calculated for the same protein.

6.5 Sample Types for Biomarker Discovery

A critical decision for designing a proteomic biomarker discovery project is the type of sample to analyze. The choices include tissue (both tumor and stroma), cell scrapings, urine, serum, plasma, cerebrospinal fluid (CSF), saliva, blister fluid, nipple aspirate fluid, breast ductal lavage, and others. The point is there are a lot of different clinical samples to be considered. Unfortunately the guidelines for selection are not well defined and the choice is typically based on what is most available. While the point of this chapter is not to produce

guidelines, a reasonable rule of thumb is to select the sample type that is within closest proximity to the cancer site whenever possible. For example, it may be reasonable to assume that biomarkers for oral cancers will most likely be found within saliva or bladder cancer associated biomarkers found within urine.

6.5.1 Blood

While using the biologic sample that is closest to the cancer site may sound reasonable, in most cases proteomic biomarker discovery studies use serum or plasma [29]. When referring to blood in this chapter, it will be meant to include both serum and plasma. While some studies use plasma and others use serum, there is no definitive study that shows one having an advantage over the other. Approximately 5 L of blood circulates through ~60,000 miles of veins, arteries, and capillaries (http://www.fi.edu/learn/heart/systems/circulation.html) transporting life-sustaining nutrients to cells and carrying waste from cells (http://health.howstuffworks.com/blood.html). Considering the intimacy that blood has with the entire body (no cell is outside of four cell units from the circulatory system) it is not surprising that it contains information concerning the overall pathophysiology of the patient. Essentially everyone has provided a blood sample during a routine physical examination. A variety of different molecules are measured in blood to provide a general assessment of the patient's physical condition. Deviation of these metabolites from a normal concentration range can indicate the presence of a disease.

The question of whether blood is a reasonable source of biomarkers for cancers needs to be addressed. Specific cells within blood contain their own genomes, whereas plasma and serum do not. Many proteins found in blood are believed to arise from those transported out of cells or released by apoptotic or necrotic cells. A comparison of N-linked glycopeptides identified within breast cancer cells, B-lymphocytes and T-lymphocytes, bladder and prostate cancer tissue, and a metastatic liver cancer showed this hypothesis to be accurate. The study demonstrated that extracellular glycoproteins originating from tissues and cells were detected within the blood using current proteomic technology [30]. The study suggests that blood can potentially contain any portion of any protein present within any cell in the body.

Beyond its interaction with cells and tissues, blood is easily acquired from the patient and has a protein concentration on the orders of tens of mg/mL. While on the surface this high protein concentration seems ideal, unfortunately about 99 % of this content is made up of only 22 proteins [31]. The concentration of proteins within blood is estimated to span ten orders of magnitude. This large dynamic range of protein concentration is problematic for MS analysis since the dynamic range of a typical mass spectrometer is only on the order of two orders of magnitude. Therefore, straight LC-MS/MS analysis of serum or plasma will result in the identification of high abundant proteins such as albumin, transferrin, fibrinogen, and immunoglobulins. Low abundance proteins, considered to be the most likely biomarker candidates will rarely be selected for identification.

Two key steps were developed and incorporated to enhance the identification of low abundance proteins in blood. These developments are multidimensional fractionation, such as MudPIT, and immunodepletion of high abundance proteins prior to fractionation [32]. High abundant protein depletion is now a standard technology used in the discovery of biomarkers in blood samples. There are a number of commercially available immunodepletion systems available. One of the earliest, and still quite popular, product is the Agilent multiple affinity removal system (MARS), which immunodepletes albumin, IgG, IgA, transferrin, haptoglobin, and alpha-1-antitrypsin [33]. This column-based product is quite effective in removing these proteins. Subsequently, immunodepletion columns that removed larger numbers of proteins, namely a Top 20 depletion column from Sigma, and the Seppro™ MIXED12 IgY-based affinity LC column, were introduced [34]. Studies have shown that these depletion steps have high reproducibility, an important component when comparing samples from cancer and healthy patients [33].

6.5.2 Urine

The third most popular choice of biofluid, behind plasma and serum, for cancer biomarker discovery is urine. While urine's protein concentration is significantly lower than blood, large amounts of this biofluid can be collected. The completely noninvasive collection procedure allows multiple samples to be easily obtained from a single individual over lengthy time periods. A drawback to its low protein concentration is that urine must be concentrated prior to proteomic analysis. Fortunately, urine does not possess the large dynamic range of protein concentrations that blood does, therefore immunodepletion is not required prior to fractionating and MS analysis. A stark difference between proteomic analysis of urine and blood is in the number of proteins identified. While thousands of proteins can be identified in a typical MS analysis of blood, urine analysis generally only yields a few hundred. Probably owing to its proximity, urine analysis has proven fruitful for the discovery of biomarkers for diseases related to the kidney, bladder, and urinary tract. Indeed, several studies have already found potentially promising biomarkers in urine for conditions such as prostate cancer [35], renal cell carcinoma [36], and bladder cancer [37]. In these studies, a large percentage of proteins originating from lysosomes and

membranes were found showing that urine contains biomarkers for conditions not localized to the kidney, bladder, and urinary tract.

6.5.3 Tissue

Tissue is an obvious choice of sample for the discovery of cancer biomarkers. In a vast majority of cases, the protein(s) that causes a cancer is anticipated to be localized within the cancerous tissue. A useful biomarker does not even have to be the protein(s) responsible for causing cancer formation—the protein could be causally related or simply a bystander whose character (abundance, PTMs, or other) is affected by uncontrolled cell proliferation. Regardless of its role in the cancer, an aberrant protein for a malignant tumor would have the greatest chance of being detected within the cancer tissue itself.

The protein concentration within tissue is in the range of 0.5–1.5 mg/mL of tissue lysate, depending on the source. Unless the tissue is severely infiltrated with blood vessels, immunodepletion of high abundance proteins is not required. These characteristics make tissue a relatively straightforward sample for biomarker discovery. Why then is it not used more to find cancer biomarkers? Simply put the answer is accessibility. It is very difficult to find a cohort of cancerous and matching healthy tissues for conducting a cancer biomarker discovery study and seeking a reasonable number of volunteers for providing such samples is impossible.

Most tissues prepared for cancer research are stored as fresh-frozen or formalin-fixed paraffin-embedded (FFPE) blocks. Thin sections are then cut from these and used for pathological evaluation to determine cancer type and grade. Additional sections may be cut and sent for IHC and fluorescent in situ hybridization (FISH) analysis to measure the levels of specific molecules within the tissue. These tissue blocks have historically only been used sparingly in proteomics biomarker discovery owing to their value. Fortunately, the past 5 years has seen huge breakthroughs in the proteomic analysis of tissue sections. The sensitivity of mass spectrometers made the amount of tissue necessary for analysis quite small. Indeed, studies using as few as d 2–3000 cells have claimed to identify thousands of proteins [13]. While this number is on the low end of the spectrum, reasonable proteome coverage is attainable with 30,000–50,000 cells, a number that can be acquired using techniques such as LCM. Another breakthrough is in the type of tissue samples available for proteomic analysis. Prior to 2005 the only useful source of tissue, either whole or sections, was fresh frozen. Since this time a number of methods have been developed enabling entire proteomes to be extracted from FFPE tissues. These proteomes are extracted in a form making them amenable to MS analysis permitting cancer biomarker discoveries [38]. Previous methods for extracting proteomes from FFPE tissue had proven inadequate owing to the significant covalent cross-linking between biomolecules resulting from formalin treatment [39]. Since FFPE tissues are widely available for almost every cancer imaginable, their availability for cancer biomarker discovery significantly reduces the scarcity of tissue samples intended for these types of studies.

6.5.4 Saliva

Saliva is produced by the parotid, submandibular, sublingual, and other minor salivary glands. Saliva contains proteins, peptides, lipids, minerals, and other small compounds [40]. Salvia is mixed with a variety of components in the oral cavity to form whole saliva. Beyond it role in aiding digestion and speaking, saliva also serves a protective function, through digestion and inhibition of bacterial and viral growth within the oral cavity [41]. Obviously owing to its proximity, saliva is most useful in the detection or oral cancers. There are a number of test procedures for measuring different hormones and drugs in saliva that have been in use since the late 1980s [42]. Saliva collection is obviously quite noninvasive and easy. Since saliva has a protein concentration on the order of a few mg/mL. Saliva, like urine, is not dominated by a few high abundant proteins and does not require immunodepletion prior to downstream MS analysis.

6.5.5 Cerebrospinal Fluid

Cerebrospinal fluid (CSF) is a clear liquid that circulates throughout the central nervous system and is also located between the brain and skull. It is produced from arterial blood by the choroid plexuses of the lateral and fourth ventricles. CSF serves as a mechanical cushion for the brain and transports a variety of molecules including neuroendocrine factors metabolites, toxins, and nutrients to and from cells of the brain and spinal cord. Owing to its direct contact with the extracellular space of the brain, CSF is an ideal biofluid from which to detect biochemical changes in the brain associated with a variety of pathological conditions such as brain tumors, traumatic brain injuries (TBI), inflammatory diseases, infection, neurodegenerative diseases, and hydrocephalus [43]. An adult contains a total volume of about 140 mL of CSF. The protein concentration of CSF of a typical adult is between 150 to 450 mg/L [44]. Studies have shown that the proteome of CSF is significantly different from that of plasma or serum [45]. While the amount of CSF attainable from a patient is sufficient for proteomic biomarker analysis, it is can only be obtained through lumbar puncture, a practice that is not performed as routinely as

blood or urine collection. Therefore, CSF has been used sparingly for biomarker discovery and has been primarily limited to noncancerous conditions such as TBI [46].

6.5.6 Other Biofluids

There are a plethora of other types of fluids that have been analyzed for the purpose of cancer biomarker discovery. These include pancreatic juice [47], tissue interstitial fluid [48], gastric juice [49], nipple aspirate fluid [50], bile [51], and ductal lavage [52]. However, these fluids are not very popular for biomarker discovery studies owing to significant accessibility issues. These fluids could prove useful for the initial discovery phase as long as any useful biomarker was also found to be translated to an accessible biofluid such as serum or urine.

6.6 Moving from Discovery to Validation

With the advent of large-scale shotgun proteomic sequencing, a relatively straightforward strategy was implemented for biomarker discovery. The proteomes extracted from samples obtained from cancer and matched healthy patients are digested into tryptic peptides and then analyzed using multidimensional fractionation combined with MS/MS analysis. The goal is to identify as many peptides within each sample and compare the list of proteins that were observed. The abundance difference between proteins was generally based on the number of peptides identified per protein (the more identified peptides, the more abundant the protein in that sample) or peptide signal intensity. The general belief (or hope) was that potential cancer-specific biomarkers would be obvious when these datasets were compared. Unfortunately, the results were rather surprising (disappointing). Not only were differences found between the datasets, *many* differences were found. Also most of the differences were related to acute-phase response proteins—indicators that the cancer patient is sick but not specific enough to determine the cause.

Why would having too many potential biomarkers be considered a problem? Although it almost sounds paradoxical, having too many potential biomarkers to select from causes a major challenge in deciding how to move forward. The problem is illustrated when considering the steps that a potential biomarker must pass before it can become clinically useful (Fig. 6.8). When potential biomarkers are found in the discovery phase, a more targeted approach is designed to measure these molecules to determine if any pass the qualification stage. Those that pass this stage are specifically measured in a larger number of samples in a verification study. Those that pass this stage are finally measured in a very large number of clinical samples to validate their reliability as diagnostic or prognostic biomarkers.

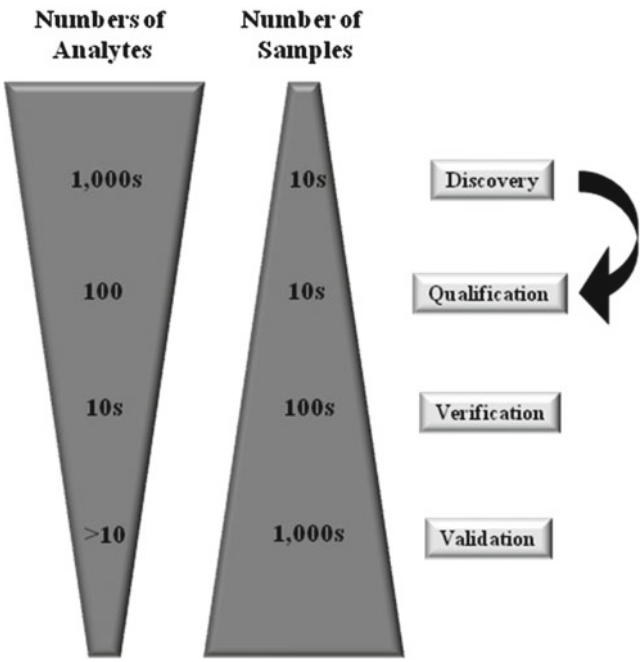


Fig. 6.8 Stages required in the identification of a clinically useful cancer biomarker. As the stages progress through discovery, qualification, verification, and validation the number of analytes measured decreases. However, the number of samples measured increases proportionately. The challenge in finding cancer biomarkers resides in identifying the candidates identified at the discovery phase that have the greatest chance of passing the other three phases.

The problem with the data presented in these shotgun studies is there is little indication which of the numerous potential biomarkers will ultimately pass validation studies. Testing each potential biomarker requires a significant effort and quickly becomes cost prohibitive if too many fail initial qualification. Two recent studies provide a reasonable illustration of the problem. In one study tissue from noncancer diseased kidney, normal kidney, and renal cell carcinoma (RCC) samples were compared [53]. Using a shotgun proteomics approach with isotope labeling for quantitation, 937 proteins were quantitated. A total of 168 under-expressed proteins and 156 over-expressed proteins were identified when the RCC tissue was compared to its normal counterpart. Gene ontology showed that 34 % of the proteins were cytoplasmic, 13 % nuclear, 11 % were localized to the mitochondria, 4 % membranous, and 6 % were extracellular proteins. The authors suggest that the cytoplasmic and membranous proteins are of particular interest since they may have potential for use as cancer markers in biological fluids. In another study, cells from LCM of squamous cell carcinoma of the head and neck and normal oral epithelial FFPE tissue were analyzed using LC-MS/MS and quantitated based on total peptide count [54]. Of the total number of identified proteins, 15 % ($n = 127$) were identified to be unique within cancer cells. This group of potential biomarkers

included those involved in DNA synthesis, metabolism, signaling, and cell structure.

While these studies (amongst countless others) demonstrate that LC-MS/MS is able to discover potential biomarkers, they also illustrate the challenge in determining which of the potential biomarkers should graduate to the qualification phase. Each study had over 100 proteins that showed a significant abundance difference in the proteomes of the cancer and healthy samples. It is impossible to validate all of these potential targets. Beyond having no existing guidelines to determine which potential biomarkers will succeed in passing qualification, it is often impossible to recognize with any certainty those that have the greatest chance of success in being validated. The guidelines should be both data driven (proteins that showed the greatest abundance difference) and biology driven (changes in complement factors are unlikely to be disease specific). As the technology continues to increase in sensitivity and the bioinformatic analysis of raw MS data becomes more sophisticated, this overwhelming problem is only going to get worse.

6.7 Getting Past the Glut of Potential Biomarkers

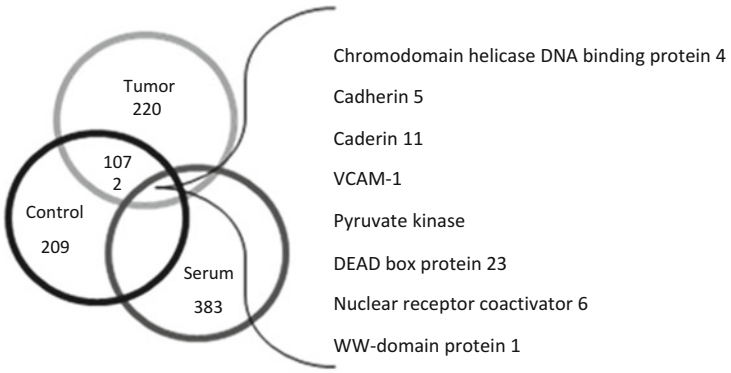
It is becoming obvious that continuing strategies that simply collect large lists of proteins in a particular sample type are not going to be very efficient. New methods that provide ways to filter potential biomarkers based on empirical data are going to be necessary. An additional, concern is the source of the biomarkers. While biomarkers from any source would be helpful to some degree, finding them within biofluids would be invaluable. Biomarkers that could be assayed from accessible biofluids such as urine or blood would have the greatest impact. To illustrate this point, let's consider the case of an individual with an undiagnosed early stage cancer. The concentration of the biomarker would be highest at the site of the cancer making it easier to measure analytically and nearby healthy tissue can be used as a control. Thirdly, once the biomarker is discovered studies to determine how it functions within the context of the cancer can be readily performed. Unfortunately, even if a tissue-specific biomarker was identified, its use would be severely limited as an early diagnostic, since there is no conceivable way that regular biopsies of various tissues can be taken at regular checkups.

Other studies focus on finding cancer biomarkers in biofluids, primarily serum and plasma. Unfortunately the concentration of any biomarker would be much lower in the blood than in the cancer tissue making it more difficult to detect. It is also impossible to determine with absolute certainty the origin of any circulating biomarker. The primary focus on finding blood-based biomarkers is obviously related to this sample's accessibility. Biofluids can be acquired during routine physical examinations giving biomarkers in this context a wide variety of uses (both prognostic and diagnostic).

In one study, blood and cancer tissue from a single patient was analyzed using a shotgun proteomics approach to determine if the advantages afforded by tissue and blood could be realized for finding potentially validatable biomarkers [55]. For this purpose, cancer and adjacent healthy tissue, and preoperational plasma were obtained from a single patient diagnosed with nonmetastatic RCC. The extracted proteomes were digested using trypsin and the resultant peptides identified using MudPIT. Protein abundance differences were determined using total peptide count. The peptide data was analyzed using a filtering scheme with the relative abundance of proteins based on the number of total peptides found for each protein in the cancer, healthy tissue, and preoperative plasma. The proteins that were identified within each of the three clinical samples are shown in the Venn diagram presented in Fig. 6.9a. Over 200 proteins were uniquely identified within the cancer compared to the control tissue. This number of potential biomarkers is much too large to efficiently validate and are limited in their utility since at this point in the analysis, they are only known to exist in the cancer. The data was subsequently filtered to determine which of these cancer-specific proteins were also found within the plasma obtained from the same patient. Not only did the filtering require that they be found in the plasma, but the number of peptides identified for protein had to be higher in cancer tissue than preoperative plasma. Eight proteins passed this data filtering criteria. There were several encouraging characteristics of this protein on this final list. There were no acute phase response, or other commonly known circulating, proteins within this list. Many of the proteins have been associated with some form of cancers, and three specifically (cadherin 11, VCAM-1, and pyruvate kinase) are associated with RCC. These proteins are also localized to the cell membrane, suggesting they can be easily shed into circulation by apoptotic or necrotic cells.

The data for cadherin 5 shows that four and two unique peptides were found for this protein in the cancer and plasma, respectively (Fig. 6.9b). The MS peak assigned to the cadherin 5 peptide, KPLIGTVLAMDPDAAR had a greater intensity in the sample extracted from the cancer compared to plasma (Fig. 6.9c). The primary structure and topography of this protein show that the peptides identified in all the samples reside within an extracellular domain of this transmembrane protein (Fig. 6.9d). The presence of cadherin 5 in the plasma sample was confirmed using Western blotting, while the protein was not observed in plasma obtained from a matched healthy control. Western-blot analysis also confirmed the presence of cadherin-11, DEAD-box protein-23, and pyruvate kinase in the RCC patient plasma, as well as in the blood of four other patients diagnosed with the same cancer. While this study

a



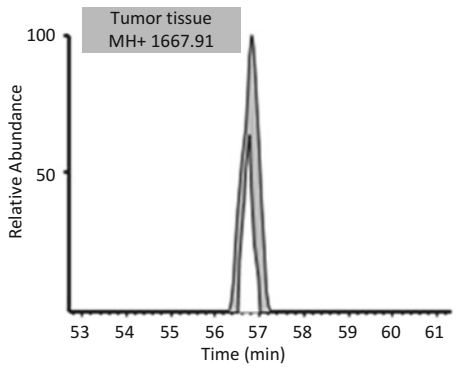
b **RCC tumor tissue**

Protein	MH+	Peptide
Cadherin-5	1105.54	ELDSTGTPTGK
Cadherin-5	966.49	KGDIYNEK
Cadherin-5	1667.91	KPLIGTVLAMDPDAAR
Cadherin-5	1085.54	TSDKGQFFR

RCC pre-op plasma

Protein	MH+	Peptide
Cadherin-5	1105.54	ELDSTGTPTGK
Cadherin-5	1667.91	KPLIGTVLAMDPDAAR

c



d

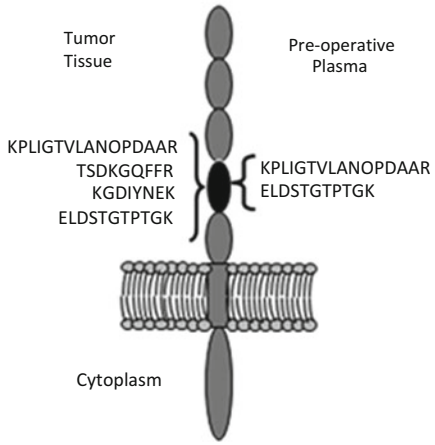


Fig. 6.9 Combined plasma/tissue shotgun proteomic analysis for the identification of potential biomarkers for renal cell carcinoma (RCC). Cancer tissue, normal tissue, and plasma all obtained from a single RCC patient were analyzed using shotgun proteomics. (**a**) After filtering the data, eight proteins were found to be unique to the cancer and plasma, as well as being more abundant in the cancer tissue than the plasma. Cadherin-5 was found to be more abundant in the tumor than the plasma based on both (**b**) peptide count and (**c**) peak intensity. (**d**) These peptides identified for cadherin-5 all originate from an extracellular domain of the protein.

does not definitely solve the problem of finding cancer biomarkers, it does suggest that a combined tissue/biofluid analysis may increase the efficiency of these studies.

6.8 Conclusion

The ability to routinely discover disease-specific biomarkers is the greatest achievement that will emerge in the proteomics era. The impact biomarkers would have on the public health would be enormous. Physicians could diagnose cancer patients at the earliest possible stages when they can be more readily cured. The correct course of treatment could be readily and reliably determined, rather than having to wait for subtle effects that signal disease remission. Unfortunately the rate at which protein biomarkers have been approved by the US FDA has not kept pace with the increase in technology seen in the recent years. While initially progress appears demoralizing, the overall capabilities for the MS analysis of biological samples have seen enormous improvements. Identifying 400 proteins in a milliliter of serum or plasma was considered monumental less than a decade ago. Now laboratories can identify thousands of proteins starting from a few thousand cells. These developments have made discovering proteomic differences within biofluid samples easy. The next challenge will be finding which of these differences leads to validated biomarkers.

Acknowledgements This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

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An Overview of Cancer Genes

7

Ashley G. Rivenbark

7.1 Nature of Genetic Mutations

When functioning properly, oncogenes and tumor suppressor genes prevent the development of cancer. The loss of function of both alleles is required for neoplastic transformation. In recent studies, it has been reported that approximately 80 DNA mutations alter the amino acid sequence in a typical cancer and of these mutations less than 15 are likely to result in the initiation, progression, or maintenance of the tumor [1]. Mutations that are causally involved in the tumorigenesis process and are positively selected for are called *driver* mutations. Whereas, mutations that provide no positive or negative selective advantage to the tumor, but are carried during cell division and expansion, are referred to as *passenger* mutations [1, 2]. Several types of mutations can occur and account for neoplastic transformation such as missense and nonsense mutations, insertions, deletions, duplications, frameshift mutations, and repeat expansions (Table 7.1). Interestingly, for example it has been noted that mutations converting 5′-CpG to 5′-TpG were more frequent in colorectal cancer than in breast cancers [1]. Along with genetic mutations, epigenetic dysregulation of genes also occurs in a wide variety of cancers. Epigenetics is defined as changes in genome function that occur without a change in the DNA sequence. Advances in our understanding of cancer oncogenes and tumor suppressor genes, and the mechanisms of their aberrant regulation, will provide insight to develop novel anticancer approaches and therapeutic strategies.

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7.2 Proto-oncogenes

The discovery of proto-oncogenes is one of the most fundamentally important findings of this century. Oncogenes are activated (frequently mutated) alleles of normally functioning wild-type genes (proto-oncogenes) that function in cell cycle progression or cellular proliferation. Activated or mutated proto-oncogenes promote unregulated cell cycle progression and cell proliferation, leading to cancer development. Proteins encoded by normal cellular proto-oncogenes function in all subcellular compartments including the nucleus, cytoplasm, and cell surface, and exert their function in most intracellular processes by acting as protein kinases, growth factors, growth factor receptors, or membrane associated signal transducers. Mutations in proto-oncogenes alter the normal structure and/or expression pattern, and the resulting oncogene acts in a dominant manner. That is, a mutation in only a single allele is required for activation of the proto-oncogene and/or loss of regulation of the proto-oncogene product. In genetic terms, this is typically referred to as a gain of function mutation.

7.2.1 Viral Oncogenes

The discovery of proto-oncogenes is rooted in the study of mammalian viruses, and in particular, retroviruses. In the earlier years of the twentieth century, radiation, chemicals, and viruses were shown to induce cancer in experimental animals, and later, transform cells in culture. The study of so called tumor viruses advanced quickly compared to studies of radiation-induced and chemical-induced carcinogenesis, for several reasons. Although both chemical carcinogens and radiation are potent inducers of neoplasia, it was found that tumor viruses could more efficiently and reproducibly transform cells in culture and induce tumors in experimental animals. Tumor viruses caused tumors to develop in a matter of days to weeks allowing rapid analysis following infection. Moreover, both radiation and chemical carcinogens act

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_7

Table 7.1 Gene mutations in human cancer

Mutation	Description
Missense	A change in one DNA base pair that results in the substitution of one amino acid
Nonsense	A change in one DNA base pair that results in the substituting of one amino acid that encodes a stop codon
Insertion	Changes in the number of DNA base pairs in a gene by inserting additional DNA base pairs
Deletion	Changes in the number of DNA base pairs in a gene by removing a piece of DNA
Duplication	A piece of DNA that is aberrantly copied one or more times
Frameshift	The addition or loss of DNA base pairs that changes the reading frame
Repeat expansion	Short DNA sequences that are repeated a number of times in a row

randomly on the cellular genome. Examining the cellular genome to determine the carcinogenic effect of these agents at a level of individual genes or DNA segments was a daunting task. In contrast to the mammalian cellular genome, the small genome of the tumor viruses offered a less complex model for identification of specific sequences responsible for induction and progression of tumors, and a more efficient system for elucidation of molecular mechanisms governing neoplastic transformation.

7.2.1.1 DNA Viruses

Some of DNA viruses and one class of RNA viruses (the retroviruses) have been shown to have oncogenic potential. The Shope papilloma virus was one of the first DNA tumor viruses to be described [3]. It causes benign papillomas that can progress to malignant carcinomas in cottontail rabbits. Papillomaviruses, along with other classes of DNA viruses such as the hepatitis B viruses, have the ability to transform cells in their natural host. Most other DNA viruses, including adenoviruses, simian virus 40 (SV40), and polyomaviruses lack transforming ability. In natural hosts, cells infected with these DNA viruses undergo cell death rather than transformation as a consequence of viral replication. However, these later viruses demonstrate their oncogenic potential in heterologous, nonpermissive species in which viruses cannot replicate. Each class of DNA virus has led to remarkable discoveries in proto-oncogenesis [4].

7.2.1.2 RNA Retroviruses

The RNA retroviruses represent the class of tumor viruses that has contributed the most to our understanding of mammalian carcinogenesis. Retroviruses are the only currently known RNA viruses to have oncogenic potential. A feature common to these viruses is the ability to replicate in infected cells via a provirus intermediate. The proviral intermediate is generated through the action of a retroviral enzyme termed reverse transcriptase, which synthesizes a DNA copy of the retroviral RNA genome. RNA to DNA reverse transcription is obligatory for RNA retroviral replication in infected mammalian cells. The DNA transcript of the retroviral genome incorporates into the cellular genome where it replicates along with cellular DNA. The RNA polymerase enzyme of the host cells transcribes the provirus DNA, generating new RNA virions and retroviral mRNAs needed for synthesis of viral proteins. Importantly, unlike most of the DNA viruses, retroviruses are not cytotoxic or cytocidal to the host cells. This reflects the nature of the retroviral lifecycle, where new retroviral particles are released from the cell by budding rather than by cell lysis. Thus, RNA viruses can transform the same cells in which they replicate. The recombination event that occurs between the retroviral DNA (provirus) and host DNA as part of the replication cycle has significant implications for neoplastic transformation and tumor development [5].

The first oncogenic retrovirus discovered was the Rous sarcoma virus (RSV). Peyton Rous inoculated chickens with a chicken sarcoma cellular extract and was able to demonstrate efficient transmission of an agent that propagated tumor growth [6]. Subsequent studies demonstrated that RSV had transforming properties in cultured cells. This was found to be in contrast to another well-studied retrovirus, the avian leukosis virus (ALV). ALV maintained the ability to induce tumors following inoculation in chickens (albeit after months, and not days to weeks compared to RSV), but did not demonstrate the ability to transform cells in culture [4]. The differences in induction efficiency in animals (in vivo activity) and ability to transform cells in culture (in vitro activity) form the basis for dividing retroviruses into two groups: (1) the acutely transforming oncogenic retroviruses, and (2) the weakly oncogenic or non-transforming retroviruses.

7.2.2 Cellular and Retroviral Oncogene Discovery

The differences between the acutely and weakly or non-transforming retroviruses are extremely important and provided clues towards recognition of the first proto-oncogene. In comparing RSV and ALV genomes, RSV was shown to be 1.5 kb greater in size than ALV. This additional segment was correctly postulated to be responsible for the rapid transforming properties of RSV. In 1971, Peter Vogt isolated RSV mutants that had the weakly oncogenic properties of ALV [7]. These weakly oncogenic RSV mutants were approximately the same size as ALV, did not have the ability to transform cultured cells, and did not efficiently induce sarcomas in animals, but maintained retroviral replication capabilities. The missing 1.5 kb sequence in these mutant RSV genomes was subsequently demonstrated to be required not only for initiation but

also for maintenance of neoplastic transformation. Because different RSV mutants were not complimentary and did not lead to neoplastic transformation in cell culture, it was concluded that a single gene could be responsible for both in vitro transformation and in vivo oncogenesis. The first retroviral oncogene was named v-*src* for its sarcoma inducing action. Since then over 30 viral oncogenes have been discovered in over 40 transforming viruses [4, 8, 9].

Similar to the discovery of the first oncogene, the discovery of the origin of retroviral oncogenes had monumental implications. The extra 1.5 kb of nucleic acid in RSV was not necessary for viral replication/growth. It was not clear where the apparently extraneous nucleic acid segment originated. The answer was obtained through the study of retroviral tumors of the very rare animal that developed tumors after being infected by a non-transforming retrovirus. These previously non-transforming retroviruses were found to have incorporated new genetic material in their RNA genome corresponding to a new oncogene which conferred capability for neoplastic transformation. The portion of the proviral genome corresponding to the newly recognized oncogene was used to probe for similar sequences in host cells. This analysis demonstrated that genes possessing the capability for neoplastic transformation were conserved among several different species. This observation suggested that host cell DNA could be incorporated into the genome of a retrovirus during recombination in the infected cell. Further study of the cellular homologues of retroviral oncogenes showed that they are normal cellular genes that encode proteins involved in various aspects of cellular homeostasis, including cell proliferation and differentiation. The normal cellular counterpart of the retroviral oncogenes is referred to as cellular proto-oncogenes. The current paradigm holds that viral oncogenes originate from cellular proto-oncogenes, and that these genes have been altered in a manner which confers the ability to induce cellular neoplastic transformation in infected cells [4, 8]. In like manner, cellular proto-oncogenes can be activated in various ways (point mutation, deletion, amplification, or rearrangement) that result in the synthesis of an oncogenic protein product [4, 8].

The discovery of the ability of genes to induce tumors in animals and humans linked the study of transforming retroviruses with the field of molecular biology of human cancers. However, it is clear that most human cancers are not caused by infection with transforming viruses. Shortly after it was established that specific virus associated genes could cause cellular transformation, alterations in cellular proto-oncogenes were found to be responsible for human tumors. The first instance linking the possibility of a human proto-oncogene with cancer, when retroviral involvement could be eliminated, was reported in 1981 by two groups, who showed that DNA extracted from a human bladder carcinoma cell line (EJ) could induce transformation in an immortalized but non-transformed mouse cell line NIH 3T3 [9, 10]. In 1982, the first human activated proto-oncogenes were isolated and identified from the EJ bladder carcinoma cell line and a human lung carcinoma. These genes were cellular homologs of the Harvey-*ras* and Kirsten-*ras* retroviral oncogenes, both of which had previously been shown to induce rat sarcomas [11]. The discovery of proto-oncogenes solidified the link between genes and cancer, and ushered in an era of genetic discovery focused on identification of genetic abnormalities that contribute to the development of human neoplasms.

7.2.3 Mechanisms of Activation of Cellular Proto-oncogenes

Cellular proto-oncogenes must become activated in order to express oncogenic potential leading to neoplastic transformation. Activation of cellular proto-oncogenes typically involves chromosomal translocation, amplification, or point mutation. The changes that result can be broadly categorized into (1) changes to the structure of a proto-oncogene which result in an abnormal gene product with aberrant function (examples include the *bcr-abl* translocation and c-*ras* point mutations, described below) and (2) changes to the regulation of gene expression resulting in aberrant expression or inappropriate production of the structurally normal growth-promoting protein (examples include translocations involving c-*myc*, amplification involving N-*myc* in neuroblastomas, and some point mutations in c-*ras*).

7.2.3.1 Proto-oncogene Activation Through Chromosomal Translocation

Translocation leading to structural alteration of bcr-abl. Evolving techniques in cytogenetics over the last century have led to increased resolution of individual chromosomes. Abnormalities in chromosomes were known to occur in neoplastic cells from at least 1914, when Boveri noted somatic alterations in the genetic material of sea urchin eggs fertilized by two sperm. The abnormal cells looked similar to tumors, and he hypothesized that cancer might result from cellular aberrations that produced abnormal mitotic figures [12]. However, it was not clear whether chromosomal abnormalities represented primary oncogenic events, or accumulated errors secondary to neoplastic transformation. Initially, the plethora of chromosomal abnormalities favored the latter scenario, as no consistent chromosomal abnormality was identified upon examination of many tumors and similar tumors from different individuals. That changed in 1960 when Nowell and Hungerford described the first reproducible tumor-specific chromosomal aberration in chronic myelogenous leukemia [13]. They observed the presence of a shortened chromosome 22, subsequently named the Philadelphia chromosome after the city in which it was dis-

covered. It was found in cancer cells from over 90 % of patients with chronic myelogenous leukemia (CML). This observation suggested that (1) the abnormality may have imparted some form of growth advantage over other cells and may be causally related to the development of tumors, and (2) other neoplasms may also harbor their own specific chromosomal or genetic aberrations. Since the first recognition of common chromosomal abnormalities in specific human tumors, numerous translocations involving important genes have been characterized (Table 7.2).

Table 7.2 Chromosomal translocation breakpoints and genes

Type	Affected gene	Disease	Rearranging gene
Non-fusions/hematopoietic Tumors			
Basic-helix-loop-helix			
t(8;14)(q24;q32)	c-*myc* (8q24)	BL, BL-ALL	IgH, IgL
t(2;8)(p12;q24)			
t(8;22)(q24;q11)			
t(8;14)(q24;q11)	c-*myc* (8q24)	T-ALL	TCRα
t(8;12)(q24;q22)	c-*myc* (8q24)	B-CLL/ALL	
	BTG (12q22		
t(7;19)(q35;p13)	*lyl1* (19p13)	T-ALL	TCRβ
t(1;14)(p32;q11)	*tal1*/SCL	T-ALL	TCRα
t(7;9)(q35;q34)	*tal2* (9q34)		
LIM proteins			
t(11;14)(p15;q11)	RBTN1/Ttg1 (11p15)	T-ALL	TCRδ
t(11;14)(p13;q11)	RBTN2/Ttg2 (11p13)	T-ALL	TCRδ/α/β
t(7;11)(q35;p13)			
Homeobox protein			
t(10;14)(q24;q11)	*hox11* (10q24)	T-ALL	TCRα/β
t(7;10)(q35;q24)			
Zinc-finger protein			
t(3;14)(q27;q32)	*Laz3*/*bcl6* (3q27)	NHL/DLCL	IgH
t(3;4)(q27;p11)	*Laz3*/*bcl6* (3q27)	NHL	
Others			
t(11;14)(q13;q32)	*bcl1* (*PRAD-1*) (11q13)	B-CLL and others	IgH, IgL
t(14;18)(q32;21)	*bcl2* (18q21)	FL	TCR-Cα
inv14 and t(14;14)(q11;32)	*TCL-1* (14q32.1)	T-CLL	IgH
t(10;14)(q24;q32)	*lyt-10* (10q24)	B lymphoma	IgH
t(14;19)(q32;q13.1)	*bcl3* (19q13.1)	B-CLL	IgH
t(5;14)(q31;q32)	*IL3* (5q31)	Pre-B-ALL	TCRβ
t(7;9)(q34;q34.3)	*tan1* (9q34.3)	T-ALL	TCRα
t(1;7)(p34;q34)	*lck* (1p34)	T-ALL	TCRα
t(X;14)(q28;11)	*C6.1B* (Xq28)	T-PLL	

Type	Affected gene	Protein domain	Fusion protein	Disease
Gene fusions/ hematopoietic tumors				
inv 14(q11;q32)	*TCR*α (14q11)	TCR-Cα	VH-TCR-Cα	T/B-cell lymphoma
	VH (14q32)	lg VH		
t(9;22)(q34;q11)	*CABL* (9q34)	Tyrosine kinase	Serine + tyrosine kinase	CML/ALL
	bcr (22q11)	Serine kinase		
t(1;19)(q23;p13.3)	*PBX1* (1q23)	HD	AD + HD	Pre-B-ALL
	E2A (19p13.3)	AD-bHLH		
t(17;19)(q22;p13)	*HLF* (17q22)	bZIP	AD + bZIP	Pro-B-ALL
	E2A (19p13)	AD-b-HLH		
t(15;17)(q21-q11-22)	*PML* (15q21)	Zinc finger	Zn-finger + RAR DNA	APL
	*RAR*α (17q21)	Retinoic acid receptor-α	and ligand binding	

(continued)

Table 7.2 (continued)

Type	Affected gene	Protein domain	Fusion protein	Disease
t(11;17)(q23;q21.1)	*PLZF* (11q23)	Zinc-finger	Zn-finger + RAR DNA	APL
	*RAR*α (17q21)	Retinoic acid receptor-α	and ligand binding	
t(4;11)(q21;q23)	*mll* (11q23)	A-T hook/Zn-finger	A-T hook + Ser-pro	ALL/PreB-ALL/ANLL
	AF4 (4q21)	Ser-Pro rich		
t(9;11)(q21;q23)	*AF9*/*MLLT3* (9p22)	(Ser-Pro rich)	A-T hook + (Ser-pro)	ALL/PreB-ALL/ANLL
	mll (11q23)	A-T hook/Zn-finger		
t(11;19)(q23;p13)	*mll* (11q23)	A-T hook/Zn-finger	A-T hook + Ser-pro	Pre-B-ALL/
	ENL (19p13)	Ser-Pro rich		T-ALL/ANLL
t(X;11)(q13;q23)	*AFX1* (Xq13)	(Ser-Pro rich)	A-T hook + (Ser-pro)	T-ALL
	mll (11q23)	A-T hook/Zn-finger		
t(1;11)(p32;q23)	*AF1P* (1p32)	Eps-15 homolog	A-T hook + ?	ALL
	mll (11q23)	A-T hook/Zn-finger		
t(6;11)(q27;q23)	*AF6* (6q27)	Myosin homolog	A-T hook + ?	ALL
	mll (11q23)	A-T hook/Zn-finger		
t(11;17)(q23;q21)	*mll* (11q23)	A-T hook/Zn-finger	A-T hook + leucine zipper	AML
	AF17 (17q21)	Cys-rich/leucine zipper		
t(8;21)(q22;q22)	*eto*/*MTG8* (8q22)	Zn-finger	DNA binding +	AML
	aml1/*CBF*α (21q22)	DNA binding Zn-fingers		
		Runt homology		
t(3;21)(q26;q22)	*evi-1* (3q26)	Zn-finger	DNA binding +	CML
	aml1 (21q22)	DNA binding	Zn-fingers	
t(3;21)(q26;q22)	*EAP* (3q26)	Sn Protein	DNA binding +	Myelodysplasia
	aml1 (21q22)	DNA binding	out-of-frame EAP	
t(16;21)(p11;q22)	*FUS* (16p11)	Gln-Ser-Tyr/Gly-rich/	Gln-Ser-Tyr +	Myeloid
		RNA binding	DNA binding	
	erg (21q22)	Ets-like DNA binding		
t(6;9)(p23;q34)	*dek* (6p23)	?	? + ZIP	AML
	can (9q34)	ZIP		
9;9?	*set* (9q34)	?	? + ZIP	AUL
	can (9q34)	ZIP		
t(4;16)(q26;p13)	*IL2* (4q26)	IL2	IL2/TM	T-lymphoma
	BMC (16p13.1)	?/TM domain		
inv(2;2)(p13;p11.2-p14)	*rel* (2p13)	DNA binding-activator	DNA binding + ?	NHL
	NRG (2p11.2-p12)	?		
inv(16)(p13;q22)	Myosin *MYH11* (16p13)		DNA binding?	AML
	*CBF*β (16q22)			
t(5;12)(q33;p13)	*PDGFb* (5q33)	Receptor kinase	Kinase + DNA binding	CMML
	TEL (12p13)	Ets-like DNA binding		
t(2;5)(p23;q35)	*NPM* (5q35)	Nucleolar phosphoprotein	N-terminus NPM	NHL
	ALK (2p23)	Tyrosine kinase	+ kinase	
Gene fusions/solid tumors				
inv10(q11.2;q21)	*ret* (10q11.2)	Tyrosine kinase	Unk + tyrosine kinase	Papillary thyroid
	D10S170 (q21)	Uncharacterized		Carcinoma
t(11;22)(q24;q12)	*fli1* (11q24)	Ets-like DNA binding	Gin-Ser-Tyr + DNA binding	Ewing's sarcoma
	ews (22q12)	Gin-Ser-Tyr/Gly-rich/RNA binding		
t(21;22)(?;q12)	*erg* (21q22)	Ets-like DNA binding	Gin-Ser-Tyr + DNA binding	Ewing's sarcoma
	ews (22q12)	Gin-Ser-Tyr/Gly-rich/RNA binding		

(continued)

Table 7.2 (continued)

Type	Affected gene	Protein domain	Fusion protein	Disease
t(12;22)(q13;q12)	*AFT1* (12q13)	bZIP	Gin-Ser-Try-bZIP	Melanoma
	ews (22q12)	Gin-Ser-Tyr/Gly-rich/RNA binding		
t(12;16)(q13;p11)	*CHOP* (12q13)	(DNA binding?)/ZIP	Gin-Ser-Tyr	Liposarcoma
	FUS (16p11)	Gin-Ser-Tyr/Gly-rich/RNA binding		
t(2;13)(q35;q14)	*PAX3* (2q35)	Paired box/homeodomain	+ (DNA binding?)/ZIP	Rhabdomyosarcoma
	FKHR (13q14)	Forkhead domain		
t(X;18)(p11.2;q11.2)	*SYT* (18q11.2)	None identified	PB/HD + DNA binding	Synovial sarcoma
	SSX (Xp11.2)	None identified		

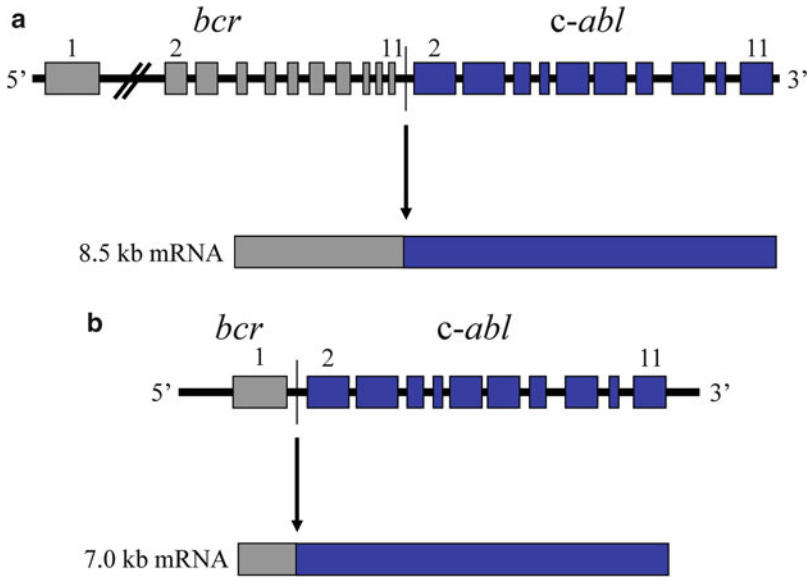

Fig. 7.1 *bcr-abl* translocation in chronic myelogenous leukemia. The c-*abl* proto-oncogene on chromosome 9 is translocated to the breakpoint cluster region (*bcr*) of chromosome 22. The result is a novel tyrosine kinase which functions independently of normal regulatory elements. (**a**) The t(9;22) that is commonly observed in chronic myelogenous leukemia (CML). (**b**) The t(9;22) that is commonly observed in acute lymphocytic leukemia.

Consequent to rapid advances in cytogenetic resolution techniques, Rowley in 1973 [14] found that the Philadelphia chromosome actually resulted from a reciprocal translocation involving the long arms of chromosomes 9 (9q34) and 22 (22q11). Analysis of the affected region on chromosome 9 revealed a proto-oncogene, c-*abl* [15], which when translocated to chromosome 22 generates a fusion gene. The c-*abl* proto-oncogene has 11 exons that encode for a 145 kDa protein with tyrosine kinase activity. The chromosomal breakpoint within the c-*abl* gene consistently involves one of two alternatively spliced exons. Breakpoints along the functional gene on chromosome 22 are clustered near the center in a 6 kb region termed the breakpoint cluster region (*bcr*). Upon translocation, nearly the entire c-*abl* proto-oncogene is placed under *bcr* promoter activity. Transcription and splicing yield a long mRNA transcript encoding a chimeric 210 kDa protein that expresses increased tyrosine kinase activity, likely because it is less responsive to normal regulatory elements (Fig. 7.1). A similar translocation exists in some acute lymphoblastic leukemias, although the breakpoint occurs further upstream in the *bcr* gene which results in a smaller chimeric protein (190 kDa), which has also been shown to have increased tyrosine kinase activity [16–20].

Translocation leading to dysregulation of c-myc. Investigation into the role of the c-*myc* proto-oncogene in neoplastic transformation led to development of a model of proto-oncogene activation based upon insertional mutagenesis. This mechanism of proto-oncogene activation emerged from studies of acutely transforming retroviruses and weakly oncogenic or non-transforming retroviruses. The primary differences between these two classes of retrovirus reflect the amount of time necessary for induction of tumors after infection of cells and the genomic content of their proviral DNA. Acutely transforming retroviruses have oncogenes incorporated into their genome while non-transforming retroviruses do not. Thus, the transformation potential of weakly oncogenic and non-transforming retroviruses depend on insertion adjacent to a cellular proto-oncogene. Although retroviruses insert randomly, in independently derived tumors retroviral sequences were found incorporated into similar chromosomal locations in the host genomic DNA. The site of insertion then became the focus of attention, and

cellular homologs of known retroviral oncogenes and their surrounding sequences were studied intensely. Finally, Hayward and Astrin demonstrated that non-acutely transforming retroviruses insert adjacent to and cause activation of the cellular proto-oncogene c-*myc* [21]. Insertional mutagenesis is based on the ability of proviral DNA to insert into host genomic DNA and cause either activation or inactivation of host genes, independent of expression of retroviral genes (as in the case of acutely transforming viruses). In the case of insertional activation of cellular genes, the proviral DNA may provide a promoter or enhancer for the cellular gene, resulting in an alteration in the normal regulation and expression pattern of the affected gene.

In the early 1980s data on c-*myc* activation by non-acutely transforming retroviruses in chicken lymphomas merged with data accumulating on translocations in Burkitt's lymphoma, a high grade B lymphocyte neoplasm. It was reasoned that if proviral sequences were capable of altering host cellular gene expression to cause tumors, chromosomal alterations that juxtapose promoter or enhancing sequences and cellular proto-oncogenes (through chromosomal translocation) were likely to promote neoplastic transformation. The best studied translocations at the time involved those of Burkitt's lymphoma, in which a portion of the long arm of chromosome 8 is consistently translocated to either chromosome 14, 2, or 22, adjacent to the loci for immunoglobulin heavy chain, k light chain, and λ light chain, respectively. The immunoglobulin loci on chromosomes 14, 2, and 22 were postulated to be good partner candidates to be coupled with and cause activation of a proto-oncogene that was suspected to reside on chromosome 8. Tumor DNA was directly probed for c-*myc* sequences. The gene was detected on chromosome 8, and found to be translocated to chromosomes 14, 2, and 22 in Burkitt's lymphoma and in some plasmacytomas. In plasmacytomas, a form of the c-*myc* proto-oncogene lacking the untranslated exon 1 is involved in the chromosomal translocation (Fig. 7.2). The breakpoints within the c-*myc* gene are more varied in Burkitt's lymphoma. Nonetheless, the translocation results in abnormal (constitutive) expression of a c-*myc* coding sequence identical to its normal allele in both types of tumor. This observation strongly suggested the chromosome translocation-mediated activation of the c-*myc* proto-oncogene as a causal event in human tumorigenesis [4, 22–25].

7.2.3.2 Proto-oncogene Activation Through Gene Amplification

Activation of cellular proto-oncogenes can occur as a consequence of DNA amplification resulting in overexpression of the amplified proto-oncogene, which confers a proliferative advantage to affected cells. Amplified gene segments can be discerned cytogenetically as double minute chromosomes (DMs) and homogeneously staining regions (HSRs). Proto-oncogenes (and other genetic loci) are amplified by repeated DNA replication events that can result in an abnormal homogeneous staining pattern in a karyotypic analysis of affected cells, rather than the familiar chromosomal staining pattern in R-banded or G-banded chromosome spreads. Instead, homogeneously staining regions appear as abnormally extended R-bands or G-bands (Fig. 7.3). The tandem arrays of amplified DNA forming homogeneously staining regions may be excised from the chromosome to form double minutes, which are small chromosomal structures lacking centromeres that do not replicate during cell division. Double minutes may integrate into other chromosomes to create additional stable HSRs able to propagate upon cell division [26, 27].

In the same way that investigation of the c-*myc* proto-oncogene formed the underpinnings of our current understanding of proto-oncogene activation by means of chromosomal translocation, studies of the c-*myc* proto-oncogene led to the unraveling of proto-oncogene activation through gene amplification in human neoplasms [28]. DNA amplification represents

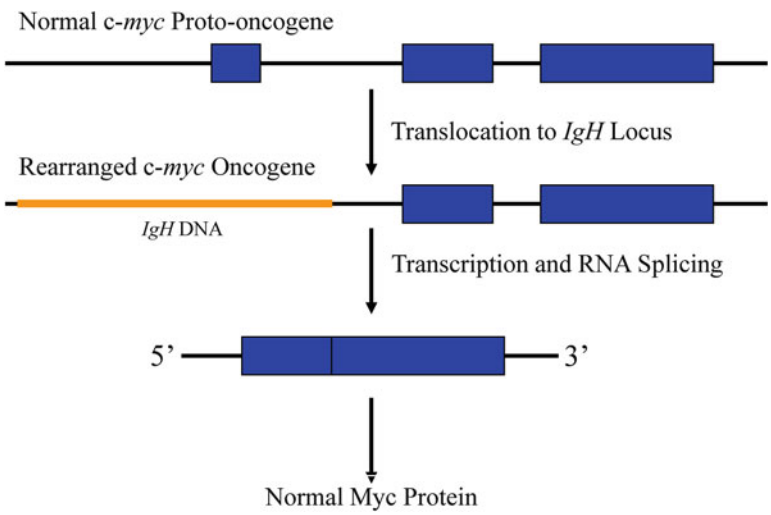

Fig. 7.2 Consequences of the t(8;14) chromosomal translocation. The c-*myc* gene of chromosome 8 is normally not expressed in differentiated B cells. After translocation, it comes under the control of either a cryptic promoter in intron 1 or an enhancer from the immunoglobulin locus of chromosome 14 (*IgH*), leading to constitutive expression of the normal c-myc protein (exon 1 is noncoding). *IgH* gene sequences are depicted in *orange* and c-*myc* gene sequences are depicted in *blue*.

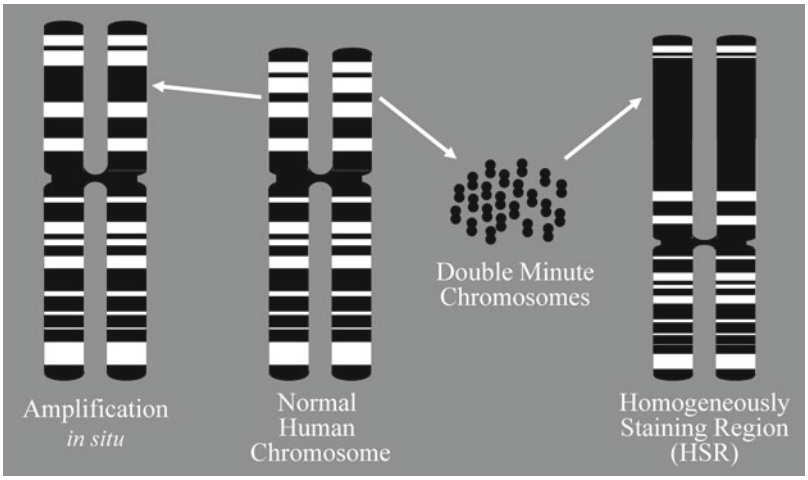

Fig. 7.3 DNA amplification. When DNA is amplified by repeated DNA replication events, the results can sometimes be seen cytogenetically as homogeneously staining regions (HSRs) or double minutes. Double minutes represent the extrachromosomal manifestation of HSRs. Double minutes can insert into any chromosome (the one they are derived form or a different chromosome).

one mechanism leading to drug resistance in mammalian cells [29]. Through direct probing for c-*myc* it was discovered that double minutes and homogeneously staining regions contained amplified copies of the oncogene in human colon carcinoma cells [30]. The c-*myc* gene has been shown to be amplified and overexpressed in a number of human neoplasms supporting the role of DNA amplification as a major mechanism for cellular proto-oncogene activation in neoplastic transformation.

Although the precise mechanism for gene amplification has not been entirely determined, the role amplification plays in cellular transformation in human malignancies is clear, particularly from studies of neuroblastomas and studies involving neoplastic transformation of cells in vitro [31]. Neuroblastoma is one of the most common childhood extracranial solid tumors accounting for approximately 15 % of all childhood cancer deaths [32]. Neuroblastomas exhibit DMs and HSRs that hybridize with probes to the c-*myc* gene. The hybridizing sequences were determined to be related to but distinct from c-*myc* and was designated N-*myc* [33]. The N-*myc* gene is transcribed at higher levels in neuroblastomas that demonstrate gene amplification. N-*myc* amplification is now a major prognostic determinant in neuroblastomas, with high levels of transcription from either a single copy or more commonly from increased gene copy number in the form of DMs or HSRs correlating with poor patient survival [34]. The demonstration of the link between high N-*myc* expression and poor clinical prognosis, and its demonstrated ability to cause neoplastic transformation in cell culture provides strong evidence for the importance of gene amplification in the activation of cellular proto-oncogenes. Table 7.3 lists other cellular proto-oncogenes that have been shown to be amplified in human neoplasms.

Table 7.3 Oncogene amplification in human tumors

Oncogene	Neoplasm
c-*myc* family	
c-*myc*	Leukemias, breast, stomach, lung, and colon carcinomas, neuroblastomas and glioblastomas
N-*myc*	Neuroblastomas, rhabomyosarcomas, retinoblastomas, lung carcinomas
L-*myc*	Lung carcinomas
c-*erb*B family	
c-*erb*B1	Glioblastomas, medulloblastomas, renal cell, squamous cell, breast, gastric and esophageal carcinomas
c-*erb*B2	Breast, salivary gland, gastric esophageal, lung, colon, and ovarian carcinomas
c-*ras* Family	
c-H-*ras*	Bladder carcinoma
c-K-*ras*	Lung, ovarian, breast, ovarian, and bladder carcinomas
c-N-*ras*	Breast, lung, and head and neck carcinomas
Other proto-oncogenes	
*int*2	Breast and squamous cell carcinomas
hst	Breast and squamous cell carcinomas
PRAD-1	Breast and squamous cell carcinomas
c-*abl*	K562 chronic myelogenous leukemia cell line
c-*myb*	Colon and breast carcinomas, leukemias
*ets*1	Lymphoma, breast cancers
gli	Glioblastomas
K-*sam*	Stomach carcinomas
*mdm*2	Sarcomas
11q13 locus	Breast, gastric, esophageal, squamous, ovarian, bladder carcinomas, and melanoma

7.2.3.3 Proto-oncogene Activation Through Point Mutation

Several cellular proto-oncogenes have been shown to be activated through point mutation. However, the c-*ras* family of proto-oncogenes represent the most important subset of proto-oncogenes that are activated through this mechanism. The c-*ras* genes were the first human proto-oncogenes identified using gene transfer assays [9, 10]. This family includes the cellular homologs of the Harvey-*ras* (H-*ras*) and Kirsten-*ras* (K-*ras*) retroviral oncogenes, both of which had previously been shown to induce sarcomas in rats [11]. DNA extracted from various human tumor cell lines have been

shown to induce transformation of mouse fibroblast cell lines in vitro, and the most commonly isolated sequences responsible for neoplastic transformation are members of the c-*ras* family of proto-oncogenes [35, 36]. The activated form of c-*ras* (oncogenic) exhibits markedly different transforming properties from that of the normal c-*ras* proto-oncogene. The activated form consistently and efficiently induces neoplastic transformation in cultured cells, whereas the normal proto-oncogene does not. The critical molecular difference between the two forms of c-*ras* was found in the nucleic acid sequence: the activated form of c-*ras* harbors a point mutation in codon 12 of exon 1, which results in a glycine to valine amino acid substitution [37–39]. Up to 30 % of all human neoplasms are now known to harbor c-*ras* mutations, and mutations in c-H-*ras*, c-K-*ras*, and N-*ras* reflect specific alterations affecting only codon 12 (most mutations), codon 13, or codon 61. An additional mutation in an intron of c-H-*ras* has been shown to upregulate production of the structurally normal gene product, resulting in increased transforming activity [40]. A common theme of c-*ras* mutations is that a single point mutation is capable of drastically altering the biological activity of a normal protein product into one with efficient transforming properties. Mutations of c-*ras* are found in a large number of human tumor types, including thyroid [41–43], gastrointestinal tract [44–48], uterus [49–53], lung [54–58], myelodysplastic syndromes [59], and leukemias [60–63]. The incidence of c-K-*ras* gene mutations is highest for exocrine pancreas and bile duct carcinomas, which has led to the development of ancillary diagnostic techniques for the detection of pancreatic and bile duct carcinomas [64–66].

7.2.4 Protein Products of Oncogenes

Proto-oncogene protein products regulate cell proliferation and differentiation. Oncogene protein products often closely resemble their proto-oncogene protein products, but differ in that they act independently of normal regulatory elements. Events that occur as a part of normal cell growth and differentiation can often be simplified into a series of four steps, all of which involve proto-oncogenes normally, and each of which is subject to disruption during neoplastic transformation: (1) an extracellular growth factor binds to a specific receptor on the plasma membrane, (2) the growth factor receptor is transiently activated, leading to a cascade of signaling cellular events, many of which involve signal-transducing proteins on the plasma membrane, (3) the signal/message is transmitted from the plasma membrane to the nucleus via secondary messenger molecules, and (4) the nuclear regulatory machinery is induced/activated to initiate cell replication and transcription. Within these pathways, there are three major biochemical mechanisms through which these oncoproteins function [67, 68]. The first of these mechanisms involves the phosphorylation of target proteins at serine, tyrosine, and threonine amino acid residues. The second mechanism involves intracellular signal transmission through proteins with GTPase activity. The last of these involves the transcriptional regulation of structural genes in the nucleus.

7.2.4.1 The Fibroblast Growth Factor Family

The discovery of growth factors in the early 1960s led to the isolation of a diverse group of factors affecting all cells. Growth factors are grouped into families that share significant sequence homology and cell surface receptors. One example is the epidermal growth factor (EGF) family which includes among others, EGF and the transforming growth factor TGFα [69, 70]. EGF, one of the earlier growth factors discovered, was shown to be a polypeptide of 53 amino acids that stimulated proliferation of a variety of different cell types. Growth factors were not only capable of promoting growth, but some also concurrently promoted differentiation [71]. In normal cells, growth factors induce cells to exit the resting or G_0 phase and enter the cell cycle, or they may stimulate cells already cycling. It follows that the biochemical and physiologic effect of aberrant expression of growth factors leads to constitutive stimulation of cell growth, potentiating the process of cell transformation.

Platelet derived growth factor (PDGF) is another growth factor shown to have transforming potential, and has an important history in that it provided the first link between two originally disparate tracks of research: (1) biochemical studies of the regulation of cell proliferation (growth factors), and (2) molecular analysis of neoplastic transformation (oncogenes) [72]. In the early 1980s, two groups working independently reported PDGF was the protein product of an oncogene [4, 73, 74]. Each group determined a partial amino acid sequence of PDGF, and with a computer search of a protein sequence database, amino acid sequence homology was demonstrated with the predicted sequence of v-*sis*, the simian sarcoma virus. From this initial work, oncogenes activated by mechanisms described above frequently have been shown to encode growth factors that participate in mitogenic signaling and cell transformation [69].

Further understanding the mechanism of action of transforming growth factors came from a hypothesis forwarded in 1977 by George Todaro [75]. He suggested that because transformed cells are capable of producing growth factors, autocrine stimulation of cell growth could be, at least in part, responsible for transformation [75]. An individual cell abnormally overexpressing a growth factor to which it responds would result in continuous cell proliferation. This hypothesis gained credence with the discovery of the homology between PDGF and the protein product of v-*sis* as well as from work on the EGF family of growth factors (EGF and TGFα, among others). Several human tumors are known to

overexpress TGFα as well as its receptor the epidermal growth factor receptor (EGFR) which substantiated the autocrine mechanism [76, 77]. The transfection of TGFα genes into cultured cells could induce transformation [78, 79]. Finally, the link between mitogenic signaling and cell transformation properties of growth factors and oncogenes was strengthened by data showing TGFα overexpression in transgenic mice results in the development of tumors [4, 80, 81]. Additional growth factors with oncogenic potential are listed in Table 7.4.

7.2.4.2 The EGFR Family of Growth Factor Receptors

The EGFR family is one of many growth factor families capable of promoting neoplastic transformation. The EGFR family includes c-*erb*B1, c-*erb*B2 (also known as *neu*), and c-*erb*B3, and are structurally related to other transmembrane tyrosine kinase proteins with an external ligand binding domain, a transmembrane domain, and an internal tyrosine kinase domain (Fig. 7.4) [82]. These receptors are activated by binding of a ligand which is followed by transduction of a signal into the cell through the kinase activity of the intracellular domain of the receptor protein. Kinase enzymes regulate protein function by phosphorylation of tyrosine, serine, or threonine amino acid residues. Examples of these receptor tyrosine kinase proteins are listed in Table 7.5. The EGFR was implicated as playing a central role as a regulator of normal cellular growth and differentiation primarily because the kinase activity of the EGFR is stimulated by EGF or TGFα binding [83, 84]. Also, the human *EGFR* gene was linked via significant sequence homology to a known avian erythroblastosis virus oncogene, v-*erb*B. Eventually it was determined that the v-*erb*B gene product was a truncated protein derived from the *EGFR* gene. The v-*erb*B gene product lacks the extracellular ligand binding domain (the amino-terminal half

Table 7.4 Growth factors with oncogenic potential

PDGF family	A chain
	B chain (c-*sis*)
FGF family	Acidic FGF (aFGF)
	Basic FGF (bFGF)
	Int-2
	hst (KS3)
	Fgf-5
EGF family	EGF
	TGFα
Wnt family	Wnt-1
	Wnt-3
Neurotrophins	NGF
	BDNF
	NT-3
Hematopoietic growth factors	Interleukin-2
	Interleukin-3
	M-CSF
	GM-CSF

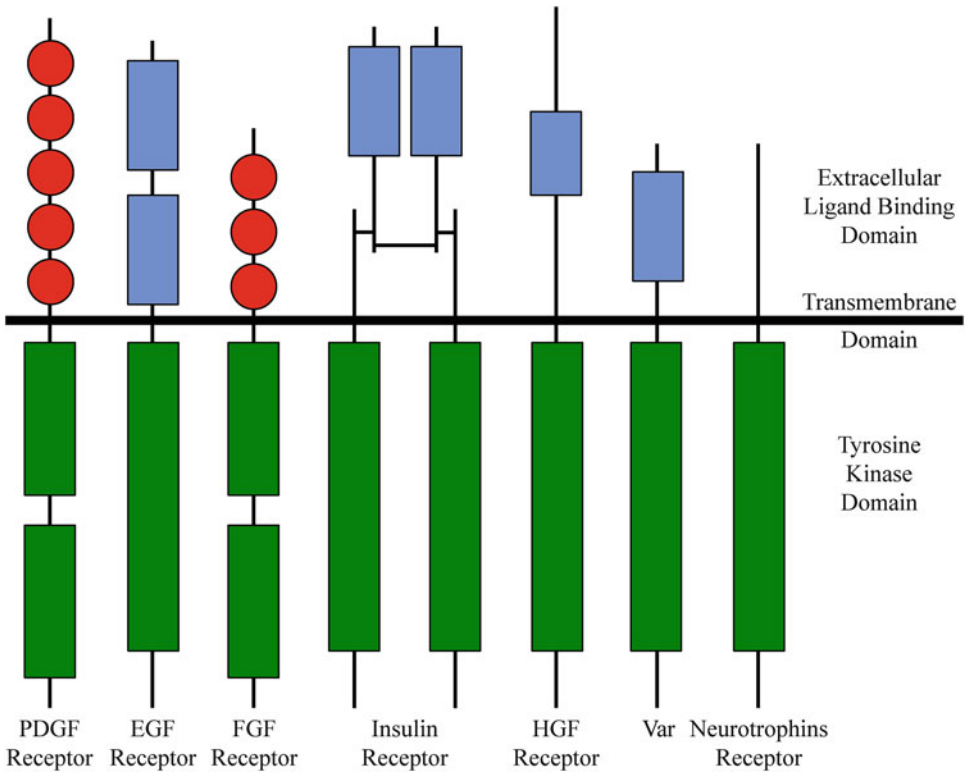

Fig. 7.4 Transmembrane tyrosine kinases. Growth factor receptors with tyrosine kinase activity share similar overall structure with extracellular binding domains, transmembrane domains, and cytoplasmic tyrosine kinase domains. Each receptor is designated according to its prototype ligand. *Red circles* illustrate immunoglobulin-like repeats. *Blue boxes* denote cytosine-rich domains. *Green boxes* represent conserved tyrosine kinase domains. The receptor labeled "Var" depicts the structure for receptors bound by ret, ros, axl, alk, eph, and eck (possibly others).

Table 7.5 Receptor protein-tyrosine kinases

EGF	erbB1 (c-*erb*B)
	erbB2 (*neu*)
	erbB3
	erbB4
FGF	FGF receptor-1 (*fig*)
	FGF receptor-2 (K-*sam*)
	FGF receptor-3
	FGF receptor-4
PDGF	PDGF α-receptor
	PDGF β-receptor
	CSF-1 receptor (c-*fms*)
	SLF receptor (c-*kit*)
Insulin	Insulin receptor (α, β)
	IGF-1 receptor (c-*ros*)
Hepatocyte growth factor	HGF receptor (*met*) (α, β)
	c-*sea* (ligand unknown) (α, β)
Neurotrophin	NGF receptor (*trk*)
	BDNF and NT4 receptor (*trk*-B)
	NT3 receptor *(trk*-C)
Ligands unknown	*eph/elk*
	VEGF Receptor
	eck
	c-*ret*
	axl

Table 7.6 Proto-oncogenes which encode for cytoplasmic serine/threonine kinases and non-receptor tyrosine kinases with oncogenic potential

Serine–threonine kinases
c-*raf* Family
raf-1
A-*raf*
B-*raf*
Protein kinase C family
PKC-β1
PKC-γ
PKC-ε
PKC-ζ
Other serine–threonine kinases
mos
pim-1
akt
cot
tpl-2
Non-receptor tyrosine kinases
yes
fgr
fyn
lck
abl
fps/fes

of the normal protein) that is present in the normal EGFR protein. This structural aberration results in a constitutively activated protein with tyrosine kinase activity. The constitutive cell signaling activity of the truncated receptor drives signal transduction and cell proliferation in the absence of growth factor stimulation. Thus, an oncogene was shown to correspond to a known growth factor receptor, which established a direct link between the two [4, 85–89].

Although structural aberrations play an important role in EGFR-mediated neoplastic transformation, a more common mechanism is overexpression of the normal proto-oncogene product, as is seen in breast cancers. Overexpression occurs not only as a result of gene amplification, but also in the absence of gene amplification, suggesting another as yet undetermined mechanism. Overexpression of EGFR has been found to have prognostic significance in several human tumors [77, 90].

7.2.4.3 Proteins Involved in Signal Transduction

c-abl. Once a cell receives a signal via plasma membrane bound receptors, it is transmitted to the cell nucleus by a cascade of messenger molecules. Because abnormal growth factor receptors can function as oncoproteins by stimulating cell proliferation, it follows that protein messengers coupled to receptors, or even proteins involved in signal transduction that are not associated with receptors, can act as equally potent oncoproteins. In fact, many such oncoproteins have been identified, and they have been shown to mimic the normal function of signal transducing proteins. The signal transducing proteins can be widely grouped into two categories (Table 7.6): (1) protein kinases (non-receptor associated tyrosine kinases, such as the c-*abl* protein product, and cytoplasmic serine/threonine kinases) and (2) receptor-associated GTP-binding proteins (which include the c-*ras* proteins). The c-*abl* protein product is present on the inner surface of the plasma membrane. However, its tyrosine kinase activity is not dependent on coupling with a plasma membrane bound receptor. Rather, negative regulatory domains are lost when c-*abl* of chromosome 9 is translocated to the breakpoint cluster region of chromosome 22. The hybrid protein product has increased enzymatic activity responsible for phosphorylating downstream substrates. This constitutive activity drives cells to proliferate, contributing to neoplastic growth. This form of molecular aberration characterizes chronic myelogenous leukemia (CML) and some forms of acute lymphoblastic leukemia (ALL).

c-ras. The larger category of signal transducing proteins is associated with membrane bound receptors, such as the GTP-binding proteins (G proteins), which include the c-*ras* family of proteins. There are many similarities between the latter two sets of proteins. G proteins are located on the inner face of the cell membrane, where they couple signals received

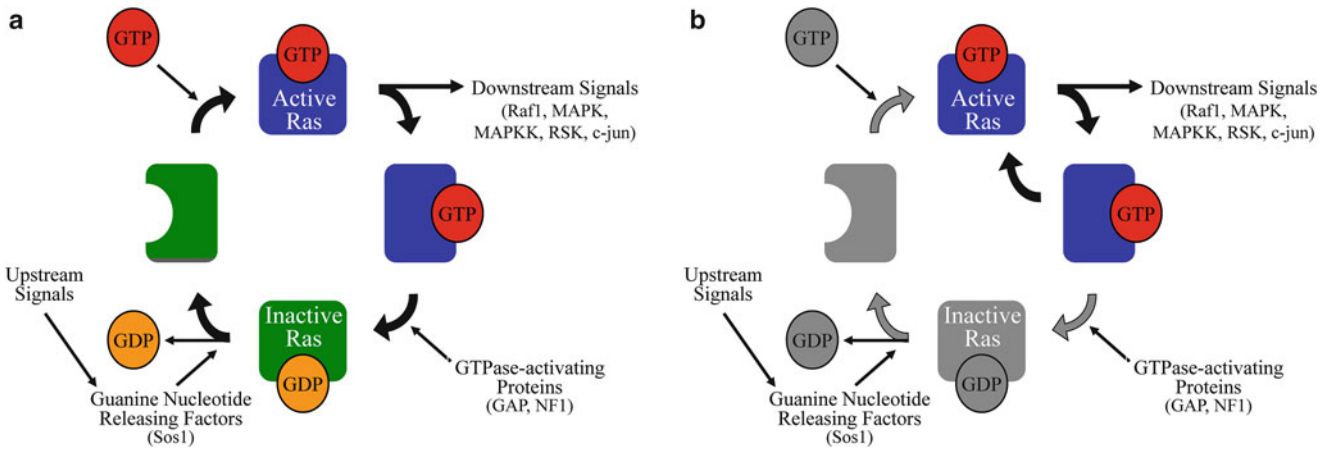

Fig. 7.5 c-ras mechanism of action. The c-ras protein is active when complexed with GTP, and this interaction is facilitated by GEFs in response to growth factor stimulation. Although c-ras has its own GTPase activity, its inactivation by GTP hydrolysis is stimulated by GAPs, such as neurofibromin. Mutated c-ras protein has a decreased ability to hydrolyze GTP, or an increased rate of exchange of bound GDP for free GTP. By either mechanism, the result is increased activated c-ras. (**a**) Depicts normal c-ras regulation (activation and inactivation). (**b**) Depicts aberrant c-ras constitutive activation when mutated.

from activated plasma membrane receptors to other, mainly cytoplasmic, second messengers in a cascade ultimately culminating in the cell nucleus. Cell signaling through G proteins requires GTP binding. Hydrolysis of the bound GTP terminates signaling through a specific G protein molecule.

The c-*ras* proto-oncogenes are activated by point mutations. As would be predicted, the point mutations alter the function of the GTPase activity of its protein product. The mutated protein has a decreased ability to hydrolyze GTP, or has an increased rate of exchange of bound GDP for free GTP (Fig. 7.5). The decreased ability to hydrolyze GTP results from interactions between c-*ras* and GTPase activating proteins (GAPs), an example of which is neurofibromin, the gene product of the tumor suppressor gene neurofibromatosis 1 (NF1). GAPs inactivate normal c-*ras* protein by augmenting the conversion of GTP-*ras* (active form) to GDP-*ras* (inactive form). The activated c-ras oncoproteins bind GAP, but their GTPase activity is stunted, leading to upregulation of active GTP-*ras*. Given the central role of GAPs in c-*ras* regulation, it is not surprising that the loss of activity of the GAP neurofibromin also results in upregulation of *ras*-GTP in affected cells. In response to growth factor stimulation c-*ras* is activated by guanine nucleotide releasing or exchange factors (GEFs). They are responsible for the exchange of GDP for GTP (converting inactive ras-GDP to active ras-GTP). The increased exchange of GDP for GTP produces the same result: constitutively active c-ras protein bound to GTP. Interestingly, a GEF domain is present in the *bcr* protein product as well as in the *bcr-abl* fusion protein product created in the t(9;22) translocation, suggesting a possible role in c-*ras* regulation [4, 35, 91–95].

7.2.4.4 The c-myc Family of Nuclear Regulatory Proteins

The final steps in the mitogenic signaling pathways involve signal entry into and the subsequent events which occur in the nucleus. The protein products of many proto-oncogenes (and tumor suppressor genes) are localized to the nucleus, and function to control the transcription of growth-related genes through interaction with specific regulatory DNA sequences. Regulation of transcription is a key mechanism through which proto-oncogenes (and tumor suppressor genes) exert control over cell proliferation. Nuclear proteins involved in these processes generally bind upstream to a specific gene and function as a transcription factor. Once bound to a specific DNA sequence, they act to increase expression of the target gene by interacting with other proteins involved in transcription. In order to be able to span the interaction between DNA and other proteins, most transcription factors have two functional domains: (1) a DNA binding domain, and (2) a protein binding domain. The DNA binding domain is often either a cysteine rich region, the secondary structure of which binds zinc and forms looped structures (called zinc fingers), or a stretch of basic amino acids proximal to leucine zipper motifs (ZIP) or basic helix-loop-helix (bHLH) domains. The leucine zipper is composed of a stretch of hydrophobic leucine residues and functions in protein dimerization. The bHLH motif is composed of two amphipathic α-helical regions separated by a loop and also functions in protein dimerization [5].

Because the c-*myc* oncogene is commonly involved in human tumorigenesis, there has been tremendous research activity into its mechanism of action. The c-*myc* proto-oncogene is expressed in nearly all eukaryotic cells and its

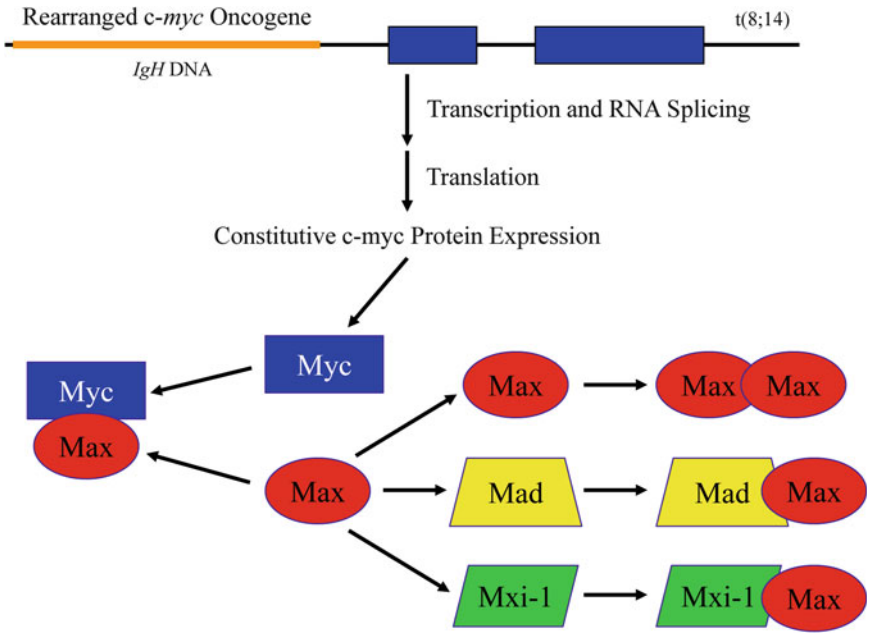

Fig. 7.6 The myc/max/mad/mxi-1 transcription network in Burkitt's lymphoma. The c-myc protein is expressed constitutively as a result of a chromosomal translocation: t(8;14), t(8;22), or t(2;8). The t(8;14) translocation is depicted in the schematic. The Myc protein dimerizes with Max to activate transcription. The Max protein can homodimerize, or form complexes with Mad or Mxi-1. Each of these complexes inhibits transcription. Constitutive expression of c-myc leads to a shift in the equilibrium in favor of transcriptional activation.

mRNA synthesis is rapidly induced when quiescent cells receive a signal to divide. The C-terminal region of c-myc proteins has one basic DNA binding domain followed by bHLH and ZIP domains. The c-myc protein was suggested to function as a transcription factor based upon the pattern of transient expression following cell stimulation by growth factors and sequence similarities with other DNA binding transcription factors. However, it was found that the c-myc protein by itself does not bind DNA well. Rather, c-*myc* binds to DNA with greater affinity when dimerized with another protein possessing c-*myc*-like bHLH and ZIP domains, called max (Fig. 7.6). Max is a small protein that forms homodimers, interacts with all members of the c-myc family, and forms heterodimers with other proteins called mad and mxi1. Although c-*myc*, max, and mad share sequence domains, they differ in that c-*myc* has a transcriptional activation domain at its amino terminus. Thus, the *myc*-max heterodimer represents the functional form of c-*myc*, and upon binding to specific CACGTG DNA sequences, stimulates expression of genes involved in cell proliferation.

Max-max homodimers and heterodimers composed of max-mad and max-mxi1 also binds DNA efficiently. These other max-containing complexes compete with *myc*-max heterodimers for DNA binding. However, the proteins that compose these complexes lack a transcriptional activation domain. Therefore, DNA binding by any of these other complexes results in repression of transcription. The control over cell proliferation is influenced by the balance between transcriptional activation by *myc*-max heterodimers and transcriptional repression by max-max, max-mad, and max-mxi1 complexes. A common theme of c-*myc* activation by chromosome translocation, insertional mutagenesis, or amplification, is overexpression of the c-myc protein. Overexpression of the c-myc protein leads to a shift in equilibrium toward *myc*-max dimers, activating transcription, promoting cell proliferation, and thereby contributing to neoplastic transformation [4, 22, 96–103].

7.3 Tumor Suppressor Genes

While proto-oncogenes are normal cellular genes that act in a positive fashion to promote physiologic cell growth and differentiation, tumor suppressor genes act as the cellular braking mechanism, regulating cell growth in a negative fashion. Normal tumor suppressor proteins exhibit diverse functions and are found in all subcellular compartments. As alterations of tumor suppressor protein function contribute to the development of cancer, their principle normal function is likely the control of cellular proliferation and differentiation. The specific types of mutations in these genes invariably lead to the inability of the encoded protein to perform its normal function. In general, neoplastic transformation requires loss of tumor suppressor protein function, and this requires mutational inactivation or loss (deletion) of both alleles of the tumor suppressor gene. Thus, tumor suppressor genes are termed recessive and alterations of these genes are typically considered loss of function mutations. A list of known or putative tumor suppressor genes is given in Table 7.7.

In the mid and late 1960s theories about the genesis of malignant tumors were enormously influenced by studies of virus and gene transfer experiments. Introduction of a virus

Table 7.7 Putative and cloned tumor suppressor genes

Gene	Chromosomal location	Inherited cancer	Sporadic cancer
Rb1	13q14	Retinoblastoma	Retinoblastoma, sarcomas, bladder, breast, esophageal, and lung carcinomas
p53	17p13	Li–Fraumeni cancer family syndrome	Bladder, breast, colorectal, esophageal, liver, lung, and ovarian carcinomas, brain tumors, sarcomas, lymphomas, and leukemias
DCC	18q21	–	Colorecetal carcinomas
MCC	5q21	–	Colorectal carcinomas
APC	5q21	Familial adenomatous polyposis	Colorectal, stomach, and pancreatic carcinomas
WT1	11p13	Wilms tumor	Wilms tumor
WT2	11p15	Weidemann–Beckwith syndrome	Renal rhabdoid tumors, embryonal rhabdomyosarcoma
WT3	16q	Wilms tumor	–
NF1	17q11	Neurofibromatosis type 1	Colon carcinoma and astrocytoma
NF2	22q12	Neurofibromatosis type 2	Schwannoma and meningioma
VHL	3p25	von Hippel–Lindau syndrome	Renal cell carcinomas
MEN1	11q23	Multiple endocrine neoplasia type 1 (MEN1)	Endocrine tumors such as pancreatic adenomas
nm23	17q21	–	Melanoma, breast, colorectal, prostate, meningioma, others
MTS1	9p21	Melanoma	Melanoma, brain tumors, leukemias, sarcomas, bladder, breast, kidney, lung, and ovarian carcinomas

into cultured cells added new genetic information that led to transformation. Most investigators believed at the time that neoplastic transformation resulted from a simple gain of genetic information, rather than from a loss of some cellular gene. In Mendelian terms, malignancy was thought to be a dominant characteristic. Henry Harris of the University of Oxford in collaboration with George Klein of Stockholm forwarded another approach [104]. Cells from mouse tumor cell lines were fused with non-malignant cells, and the resulting hybrids were evaluated for their tumorigenic potential in appropriate hybrid animals. The hybrid cells produced very few tumors compared with the malignant parent cells. These results were interpreted to mean that normal cells contain one or more genes that act as negative regulators of the neoplastic phenotype. They postulated that malignancy was determined by a loss and not a gain of genetic information. At the time, these results were vigorously challenged [105].

Additional cell hybrid studies strengthened the concept that normal cellular genes can function to suppress the tumorigenic potential of neoplastically transformed cells. A cytogenetic analysis of cell hybrids that reexpressed tumorigenic potential established the chromosomal location of one of the normal genes in mouse that suppressed the malignant phenotype [106, 107]. Similar studies in human hybrid cells derived from the fusion of normal fibroblasts and HeLa cells (cervical carcinoma cell line) showed that reversion to the tumorigenic phenotype followed the loss of chromosome 11. Introduction of the wild-type allele by fusion with a normal cell once again suppressed malignancy, suggesting the presence of a tumor suppressor gene on this chromosome [4, 108, 109].

Some generalizations about tumor suppressor genes can be made (Table 7.8), and are further illustrated with specific examples below. Tumor suppressor gene mutations or deletions are often found as germ-line mutations associated with hereditary syndromes that predispose to the development of specific tumors. Mutations or deletions in the same genes involved in cancers arising in the setting of these hereditary syndromes are also found in sporadic tumors (tumors that arise in individuals known not to have germ-line mutations). Commonly, these somatic mutations can be found in tumors not related to those associated with hereditary syndromes. The latter findings in sporadic tumors suggest a broader role for these genes in tumorigenesis. In many but not all cases, tumor suppressor activity can be demonstrated in gene transfer assays. Tumor suppressor gene products are integral components of cell signaling pathways, in addition to having

Table 7.8 Characteristics of oncogenes and tumor suppressor genes

Characteristic	Oncogenes	Tumor suppressor gene
Number of mutational events required to contribute to cancer development	One	Two
Function of the mutant allele	Dominant (gain of function)	Recessive (loss of function)
Activity demonstrated in gene transfer assays	Yes	Yes
Associated with hereditary syndromes (inheritance of germ line mutations)	Seldom (c-*ret* proto-oncogene)	Often
Somatic mutations contribute to cancer development	Yes	Yes
Tissue specificity of mutational event	Some	In inherited cases, there is often a tissue preference

roles in cell–cell and cell–matrix interactions. Their role in the development of cancers has been demonstrated to be as significant as the role played by proto-oncogenes.

7.3.1 Mechanism of Tumor Suppressor Gene Action

The mechanism of action for the products of tumor suppressor genes is diverse and not fully understood. Conceptually, the products of tumor suppressor genes can be thought of as functioning to receive and process growth inhibitory signals from their surroundings. When a cell loses components of this signaling network, or loses responsiveness to extracellular growth inhibitory signals, the cellular consequences are the same as for unchecked stimulation of cell growth, neoplasia. The tumor suppressor gene products function in parallel with the protein products of proto-oncogenes, but work instead to suppress cell proliferation through the regulation of signal transduction and nuclear transcription. Not all tumor suppressor gene products conform to the growth inhibitory concept. Cell surface and cell matrix molecules are responsible for normal cell morphology, cell–cell interactions, and cell–extracellular matrix interactions. Tumor suppressor genes encode for such proteins, and mutations in these genes lead to altered cellular morphology, loss of normal intracellular signaling pathways, and loss of normal intercellular interactions, all of which are features of neoplastic cells.

7.3.1.1 Tumor Suppressor Gene Products That Regulate Signal Transduction

An example of a tumor suppressor gene whose product regulates signal transduction is the gene product of neurofibromatosis 1 (NF1), which is responsible for the clinical syndrome neurofibromatosis or von Recklinghausen's disease. Neurofibromatosis is one of the more common autosomal dominant disorders in humans and is clinically associated with café-au-lait spots (brown skin macules), benign neurofibromas, and other abnormalities. Patients with neurofibromatosis have a higher incidence of malignant tumors, including neurofibrosarcomas, pheochromocytomas, optic nerve gliomas, and malignant myeloid diseases [110].

The product of the *NF1* gene (neurofibromin) encodes for a GTPase activating protein (GAP). GAP proteins interact with c-ras proteins, which in normal cells are transiently activated upon exchange of bound GDP for bound GTP (Fig. 7.5). The c-ras proteins have intrinsic GTPase activity which is significantly increased upon binding with GAP proteins. Mutations in c-*ras* alter the GTPase activity of its protein product. The ras-GTP oncoprotein is significantly less responsive to GAP augmented hydrolysis [4, 35, 91–95]. It follows that mutations in genes that encode for GAP proteins, such as *NF1*, should be similarly deleterious to affected cells. In fact, *NF1* mutations are associated with and may contribute to the development of not only those tumors found in neurofibromatosis 1, but also adenocarcinomas of the colon, anaplastic astrocytomas, and myeloid malignancies, among others [110].

7.3.1.2 Tumor Suppressor Gene Products That Regulate Transcription

Examples of tumor suppressor genes whose products regulate transcription include *Rb1* and *p53*, the latter representing the gene most frequently involved in human cancers [111–113].

Rb1 gene. The discovery of the retinoblastoma gene (*Rb1*) gene resulted in an intense research effort to understand the mechanism of action of its gene product. In studies of cell cycle regulation, the *Rb1* tumor suppressor gene product was found to be active in a hypophosphorylated state, and inactive in a hyperphosphorylated state. Further, the active hypophosphorylated gene product was present in abundance in the G_0/G_1 stage of the cell cycle as compared to the finding of abundant inactive hyperphosphorylated pRb protein in late G_1, S, G_2, and M, suggesting a role for pRb as a suppressor of cell proliferation between the G_0/G_1 and S phase of the cell cycle. This was found to be true. The pRb protein binds transcription factors, and in particular, the E2F family of transcription factors as well as the product of the c-*myc* oncogene [114, 115]. Cyclin-dependent kinases are responsible for phosphorylating pRb (resulting in inactivation) when cells are stimulated to divide by exogenous growth factors or other mitogenic signals. The pRb protein dissociates from sequestered/bound transcription factors, allowing the cell to progress from G_0/G_1 to the S phase. After mitosis, a phosphatase returns pRb to its active, hypophosphorylated form. Unlike other regulators of transcription, pRb does not directly interact with DNA [116–120].

The inability of pRb to bind and negatively regulate the function of certain transcription factors leads to unregulated cell proliferation, and this can result from deletion or mutation of the *Rb1* gene, or functional inactivation of the pRb protein. In general, mutation of the *Rb1* gene results in truncated and unstable proteins [111]. As might be expected, the significant mutations or deletions in the *Rb1* gene occur in the transcription factor binding domain, also known as the pRb pocket. Functional inactivation of pRb was first recognized in studies of viral oncogenes. DNA viral oncogene products from animals (SV40 large T antigen) and humans (human papilloma virus E7, adenovirus E1A) inhibit pRb function by binding and occupying the pRb pocket. Gene mutations or occupation of the pRb pocket/transcription binding domain have, as a common result, the liberation of activating transcription factors with subsequent uncontrolled cell proliferation. The interplay between the products of these viral oncoproteins and pRb is an illustration of their mutual cooperation, which serves as a paradigm of the multistep

nature of oncogenesis. Inhibition of tumor suppressor genes represents an important way in which oncogenes exert their neoplastic potential [111, 121].

Mechanism of Rb1 Inactivation in Retinoblastoma. Although by the mid-1970s cell hybrid studies clearly established that malignancy is at least in part due to loss of function of critical regulatory genes in malignant cells, the identification of tumor suppressor genes at the molecular level did not occur until more than a decade later. As the prototypic tumor suppressor gene, the mechanism of inactivation and loss of function associated with the *Rb1* are illustrative of the whole class of tumor suppressor genes. The inactivation of both alleles of the *Rb1* gene is required for development of retinoblastoma, an eye malignancy that usually occurs at a very young age. Until the end of the nineteenth century, this tumor was uniformly fatal. During the twentieth century, more of these tumors were recognized and diagnosed at an earlier stage, permitting a surgical cure. It was noted that the offspring of retinoblastoma patients cured by surgery developed the disease at a very high frequency. Pedigree analysis of these families suggested a dominant pattern of Mendelian inheritance [122, 123].

The suggestion that a specific gene was responsible for the disease stemmed not from molecular or cell hybrid analyses, but rather from epidemiological data first reported by Alfred Knudson [123]. He noted that 40 % of the cases of retinoblastoma were bilateral and occurred in young infants (mean age 14 months), who if cured went on to develop secondary tumors (often osteosarcomas). In these patients, there was often a relevant family history of retinoblastoma. In contrast, the remaining 60 % of cases were unilateral and occurred in older children (mean age 30 months), who if cured did not develop secondary malignancies. These patients generally lacked a relevant family history. Knudson proposed that the first group inherited a mutant allele (germline mutation) which conferred a dominant predisposition to cancer to this group of patients. In these patients, a second somatic mutation in retinal cells resulted in retinoblastoma. The second nonfamilial, later onset, form was very rare (occurring in 1 in 30,000 people). Knudson suggested that these patients did not inherit a mutant gene, but rather two independent somatic mutations in retinal cells occurred to give rise to retinoblastoma.

The two-hit model proposed by Knudson did not address the mechanism of action of the gene(s) involved. There were at least two possible explanations. The first is that a dominant mutation of a single proto-oncogene allele is insufficient for the development of neoplasia, or that a second mutation, perhaps in a second proto-oncogene, is required. The second possibility, which proved to be correct, is that two mutations are required for development of retinoblastoma and that these mutations are inactivating mutations. Thus, the loss of both functional copies of the *Rb1* gene is necessary for neoplastic transformation and tumor formation. This conclusion is based upon numerous studies involving cytogenetic, linkage, and restriction fragment length polymorphism (RFLP) analyses of constitutional and tumor DNAs from affected individuals.

Cytogenetic studies demonstrated a loss of the long arm (13q14 region) of chromosome 13 in retinoblastoma tumors, and in the germ line of patients with a hereditary predisposition to retinoblastoma development [124]. Esterase D, a gene present on chromosome 13q14, was used in linkage analysis studies. Assuming that a mutant *Rb1* is closely linked to one of the two esterase D alleles which can be traced back to an affected parent, one can detect offspring who have inherited the mutant *Rb1* allele. Tumors arising in these individuals were shown to be homozygous for one form of esterase D, and by extension, homozygous for the mutant *Rb1* gene [125]. Similar conclusions were drawn from RFLP analysis of retinoblastomas arising in patients with familial predisposition, by comparing tumor DNA with germ-line DNA. Paralleling the findings using esterase D, homozygosity was found in these tumors as well [125–127]. Shortly thereafter, probes from the 13q14 region were used to screen retinoblastomas, and several demonstrated homozygous deletions for at least 25 kb of DNA.

Molecular cloning of the gene was accomplished using probes to human 13q14 to isolate genomic DNA clones corresponding to flanking DNA. The genomic DNA clones from the tumor suppressor gene region were then used as probes against RNA to compare the pattern of mRNA expression between retinoblastoma and normal retinal cells. The retinoblastoma gene was found to have 27 exons extending over approximately 200 kb of DNA and encoding a 928 amino acid protein [127–129]. Once the *Rb1* gene was isolated, mechanisms of inactivation were found and included deletions and inactivating point mutations [130, 131]. Importantly, tumor cell lines into which a cloned normal *Rb1* gene was introduced lost their malignant phenotype, confirming the tumor suppressing action of *Rb1* [132]. These studies definitively established the role of deletion and/or loss of function as a major genetic mechanism involved with neoplastic transformation, and solidified the existence of a new class of genes, the tumor suppressor genes.

The *Rb1* gene has since been found to be associated with many human neoplasms, usually through a genetic mechanism involving mutations or deletions. Most other tumor suppressor genes have similar mechanisms of inactivation as described for *Rb1*. Specifically, any alteration in DNA or functional inactivation of the gene protein product that results in a loss of function of both copies or alleles of the gene is required for the development of tumors. These alterations include inactivating point mutations, deletions, or

insertions. In general, all of these mechanisms have been described for many of the tumor suppressor genes, and it is possible to find in one inactivated gene of a clonal cell population a combination of two mechanisms of inactivation, a different one for each allele.

p53 gene. The *p53* gene is briefly mentioned because of its important and frequent involvement in human neoplasia [82, 112]. Like the *Rb1* gene product, p53 is a nuclear phosphoprotein and functions primarily as a regulator of transcription [113]. Specifically, when the genome sustains mutagenic damage (from radiation or a chemical insult) wild-type p53 protein accumulates in the nucleus where it binds DNA and causes cells to halt in the G_1 phase of the cell cycle, where the genetic damage is repaired. If the damage is not repaired, p53 induces apoptosis [133]. When first discovered, *p53* was thought to be a proto-oncogene because overexpression of cloned *p53* genes was shown to be related to transformation in gene transfer assays. It was later shown that the clones used were actually mutant forms of the *p53* gene. When the wild-type gene was cloned and used in similar transfection studies it did not demonstrate the ability to participate in the neoplastic transformation of normal cells. Rather, overexpression led to inhibition of neoplastic transformation, suggesting it to be a tumor suppressor gene.

Mechanism of p53 inactivation in cancer. The tumor suppressor function of p53 can be inactivated by either mutational events or through negative protein-protein interactions. The *p53* gene is composed of 11 exons spanning 20 kb of genomic DNA, encoding a protein of 393 amino acids. Mutations have been described for each exon, but appear to be most common in exons 5–9. A similar distribution of mutations is found in the Li Fraumeni syndrome, in which patients inherit a germ-line mutation in the *p53* gene. This syndrome is characterized by a familial predisposition to many tumor types, including breast (among other epithelial) carcinomas, soft tissue sarcomas, brain neoplasms, and leukemias [133]. Mutant p53 protein products lose their ability to suppress transformation, and gain the ability to inactivate wild-type p53. A cell with one mutant and one wild type copy of p53 behaves as if it has no wild-type p53 at all. This type of mutation is referred to as dominant negative because the mutant allele acts in a dominant fashion to alter the functioning of the normal allele [134–136]. The half-life of mutant species of the p53 protein tend to be increased compared to the very short half-life of the wild-type protein. This increase in protein half-life enhances the dominant negative effects of the mutant protein. Furthermore, when complexed with oncoprotein products such as from DNA tumor viruses (SV40 large T antigen, adenovirus E1B protein, and the E6 protein of human papilloma virus), the p53 protein is functionally inactivated in a manner analogous to the oncoprotein inactivation of the pRb [136].

7.3.1.3 Tumor Suppressor Gene Products That Function as Cell Surface/Cell Matrix Molecules

Properties of malignant cells include not only uncontrolled proliferation capabilities, but also changes in cell morphology, loss of contact inhibition or cell–cell interactions, and loss of cell–extracellular matrix interactions. This results in an altered phenotype, including morphologic changes which allow recognition by microscopy as malignant, inability to process inhibitory or other signals from adjacent cells, and loss of adhesion properties resulting in metastatic potential. Cell surface and cell matrix molecules thought to play a role in these processes include products from the neurofibromatosis 2 (*NF2*) gene, the adenomatous polyposis coli (*APC*) gene, and the deleted in colon cancer (*DCC*) gene, among others.

APC gene. The *APC* gene was isolated in 1991 and is responsible for the familial adenomatous polyposis (FAP) syndrome, an uncommon autosomal dominant disease affecting approximately 1 in 8000 individuals [137–140]. Patients with FAP typically develop 500–2500 adenomas of the colonic mucosa. The frequency of progression to colon adenocarcinoma approaches 100 % necessitating prophylactic colectomy by the second or third decade of life, and early surveillance of siblings and first degree relatives [140]. The *APC* gene was found to be located on the long arm of chromosome 5 (5q21), a locus known to be deleted frequently in colonic adenocarcinomas. Patients with FAP carry germ-line *APC* mutations leading to the production of truncated APC proteins, the detection of which has provided a diagnostic assay [141]. In addition to its role in the development of hereditary FAP, *APC* mutations have been found in sporadic adenomas, the majority of sporadic colorectal cancers [141, 142], and other human malignancies of the pancreas, esophagus, stomach, and lung [142–146]. Sporadic mutations also lead to the production of truncated proteins. Tumor suppressor activity has not been demonstrated in gene transfer assays using the *APC* gene.

The *APC* gene is large, extending over 8500 nucleotides, and encoding a protein of approximately 2840 amino acids. The protein interacts with catenins, which are cytoplasmic proteins thought to play a role in signal transduction because of their interactions with cadherins, a family of cell surface molecules. Cadherins have been shown to regulate cell–cell interactions and morphogenesis. Based on these observations, it has been postulated that the APC-catenin complex plays a role in cell adherence, and possibly signal transduction such as contact inhibition of cell growth [146]. In FAP, mutations in the 5′-portion of the gene have been shown to correlate with an attenuated form of the disease [147], while mutations at codon 1309 are associated with an early onset of colon cancer in FAP families [148].

DCC gene. The *DCC* gene was discovered using polymorphic DNA markers that showed a loss of heterozygosity (LOH) of the long arm of chromosome 18 in colorectal tumors [46]. The gene was subsequently isolated and shown to be composed of more than 29 exons spanning more than 1×10^6 nucleotides [149, 150]. The *DCC* gene encodes for a transmembrane molecule with unknown function which has homology to cell adhesion molecules (CAMs) involved in cell–cell or cell–extracellular matrix interactions [151]. It may play a role in transmitting negative signals, and inactivation of *DCC* function through deletion or mutation (a rare event) may lead to loss of contact inhibition and subsequent uncontrolled cellular proliferation [151]. LOH at the 18q locus was initially described in colorectal carcinomas as one of several steps involved in the sequence of events from premalignant adenomas to invasive carcinomas. Although no hereditary conditions involving germ-line alterations of the gene have been described (in contrast to most other tumor suppressor genes), *DCC* abnormalities are associated with several tumor types other than colon cancer, including stomach cancer, pancreatic cancer, and leukemias [152–155]. The *DCC* gene has not been formally classified as a tumor suppressor gene because the predicted tumor suppressor activity has not been demonstrated in gene transfer assays [4, 156, 157].

NF2 gene. NF2 is genetically and clinically distinct from NF1 discussed above. It is a rare autosomal dominant disorder in which patients develop bilateral schwannomas affecting the vestibular branch of the eighth nerve (acoustic neuromas), and other tumors of the central nervous system such as meningiomas and ependymomas (hence its designation as central neurofibromatosis). The gene is located on the long arm of chromosome 22 (22q22) and was isolated in 1993 [158, 159]. Mutations of the *NF2* gene have been found in tumors (breast carcinomas and melanomas) other than those associated with the NF2 syndrome [160], as well as in sporadic meningiomas and ependymomas [161, 162], suggesting a role in tumorigenesis extending beyond that played in NF2. Its protein product (schwannomin or merlin) shows sequence homologies to proteins which act as linkers between cytoskeletal scaffolding components and proteins in the cell membrane. On this basis schwannomin is thought to play a role in cell shape, cell–cell interactions, and cell–matrix interactions [159, 160]. Inactivation of the *NF2* gene may lead to changes in cell shape and loss of contact inhibition [110].

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Oncogenes and the Initiation and Maintenance of Tumorigenesis

8

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8.1 Introduction

Cancer is a complex and multistep process whereby an individual cell acquires a series of mutant gene products culminating in a spectrum of pathophysiologic features including relentless proliferation, growth, blocked differentiation, the inappropriate induction of angiogenesis, tissue invasion, and loss of genomic stability. Given the genetic and biologic complexity of tumorigenesis, it is perhaps surprising that there are circumstances when cancer can be reversed through the repair or inactivation of individual mutant genes. However, recent experiments in transgenic mouse models and clinical results using new pharmacological agents demonstrate that cancer can be treated through the targeted repair and/or inactivation of specific oncogenes. Hence, cancers appear to be dependent upon particular oncogenes to maintain their neoplastic properties, thus exhibiting the phenomena *tumor maintenance* or *oncogene addiction*.

We will describe how proto-oncogenes physiologically play a fundamental role in specific critical signaling processes. Hence, when inappropriately activated, as oncogenes, they can initiate tumorigenesis. In addition to summarizing the major signaling pathways that may be most effectively targeted for the treatment of cancer, we will describe how conditional transgenic model systems have been exploited as innovative avenues for discovery and validation of oncogenes as therapeutic targets. Then, we will highlight recent successes and current challenges in the use of targeted therapeutics for the treatment of cancer. Finally, we will discuss current thoughts on why the targeted inactivation of specific oncogenes results in cancer regression.

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8.2 Oncogene Signaling Pathways

Oncogenes were first identified as the etiologic agents of retrovirus mediated malignant transformation. Only subsequently was it appreciated that these gene products were mutant versions of normal cellular counterparts [1]. With this epiphany, it rapidly became apparent that cancer was a disease caused by genetic events that resulted in the perturbation of gene products that were intimately involved in the regulation of cellular processes that governed when and how a cell will proliferate and grow. Now, hundreds of oncogenes have been identified. In many cases specific oncogenes have been associated with specific human diseases. In this chapter, the goal is to choose particular examples of oncogenes that are illustrative, rather than attempt the impossible task of cataloging all the different oncogenes that have been implicated in neoplasia.

At least four different classes of oncogene signaling molecules are commonly involved in the pathogenesis of cancer. These include gene products that have several distinct functions: (1) receptors such as ErbB, (2) small GTPases such as Ras, (3) kinases such as BCR-ABL, and (4) transcription factors such as MYC. Correspondingly, the oncoproteins in these interacting signaling pathways have been the most intensely studied as potential targets for the treatment of cancer (Fig. 8.1).

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_8

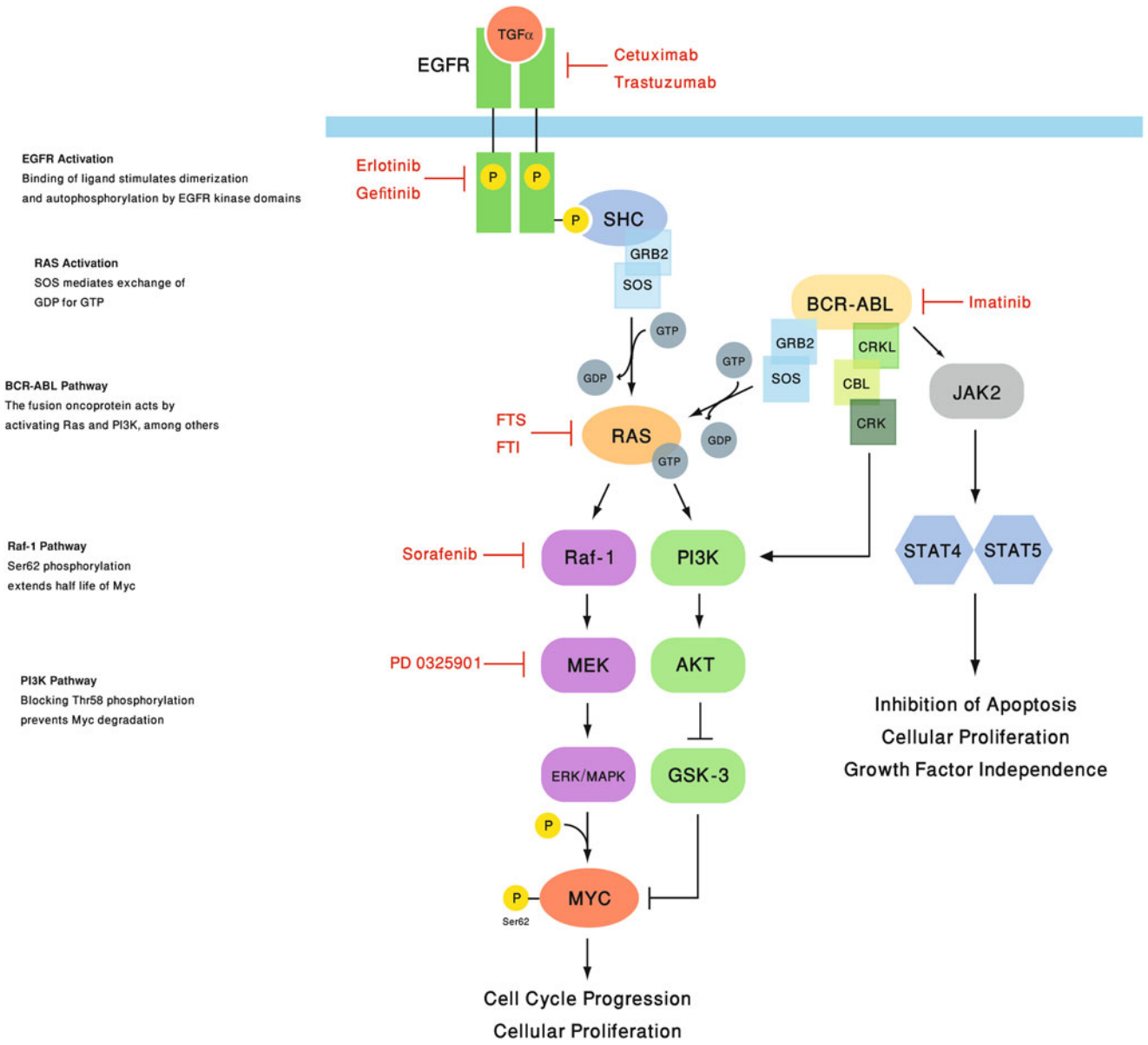

Fig. 8.1 Critical signaling pathways as drug targets for cancer therapy. (**a**) Surface receptors dimerize and activate downstream effectors such as RAS (a GTPase). Many surface receptors are targeted by antibodies, and EGFR has been targeted through cetuximab and trastuzumab. (**b**) RAS activation is caused by SOS-mediated exchange of GDP for GTP on RAS. G protein signaling molecules, such as RAS, have been targeted by farnesyltransferase inhibitors (FTI) and *S*-farnesylthiosalicylic acid (FTS). (**c**) The BCR-ABL fusion protein activates RAS, PI3K, and other oncogenic signaling molecules. Tyrosine kinases are often targeted with small molecules. BCR-ABL has been targeted by Imanitib. (**d**) Transcription factors are often the terminal effectors of a pathway. To date, transcription factors are yet to be successfully targeted. All drugs are in red at their sites of action.

8.2.1 Receptor Signaling

Cell surface receptors are the vehicles of communication amongst cells. They are responsible for initiating the response of cells to their tissue microenvironment and mediate many signaling cascades. Growth factor receptors were some of the first proto-oncogenes discovered [2]. A multitude of cell surface receptors have been implicated in tumorigenesis including the epithelial growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and insulin-like growth factor receptor (IGFR) [3]. Each of these receptors has been implicated in the pathogenesis of specific types of human neoplasia.

In many regards the ErbB family of transmembrane tyrosine kinase receptors is prototypical of cell surface receptors. This gene family includes four members: (1) EGFR (ErbB1), (2) HER2/NEU (ErbB2 hereafter referred to as HER2), (3) ErbB3, and (4) ErbB4. Ligands have been identified for each

of the ErbB family members with the notable exception of HER2 that likely heterodimerizes with EGFR or HER3. Downstream effectors of these molecules include many other proto-oncogenes including the MAPK/RAF/RAS, PI3K/AKT, and STAT signaling. Activation of these receptor pathways induces cellular proliferation, enhanced survival, and increased cellular motility [4].

Many of these gene products have multiple alleles that appear to exhibit tissue-specific expression. For example, each of the ErbB receptors exhibits unique expression patterns. Correspondingly, mutated receptors have been implicated in particular types of human cancer [5]. For example, EGFR is commonly overexpressed in lung carcinoma [6], while Her2 overexpression is more commonly associated with breast cancer [7]. Thus, the biology and pathophysiology associated with these gene products is associated with specific cellular lineages.

Cell surface receptors are particularly attractive candidates as drug targets. First, receptors are specific to particular tissue types thus allowing for target specificity. Second, pharmacologically blocking a ligand-binding site is an obvious and frequently successful strategy to inactivate a receptor. Finally, drugs that target receptor molecules do not need to be able to transit through cellular membranes.

8.2.2 GTPases

GTPases are a large family of gene products that mediate receptor signaling [8]. A multitude of GTPases have been implicated as oncogenes. A characteristic feature of the biology of GTPases is that they only become activated after post-translational modification that permits their localization to the plasma membrane [9]. Moreover, the enzymes that mediate these modifications are possible targets for the treatment of cancer.

The RAS family represents a prototypical example of GTPases. There are three members: (1) H-RAS, (2) K-RAS, and (3) N-RAS [8]. A mutant RAS was one of the first oncogenes identified to be mutated in a human cancer. Over 25 % of human cancers exhibit activating point mutations of a RAS gene [10]. RAS proteins are known to play a critical role in the regulation of a multitude of cellular programs including cellular growth, proliferation, apoptosis, and angiogenesis [9, 11]. The RAS/RAF/MAPK pathway is a frequent target for pharmacologic treatment of human cancers.

8.2.3 Protein Kinases

Protein Kinases are among the most abundant signaling molecules with approximately 500 members. The phosphorylation of proteins at serine, threonine, or tyrosine represents one of the most common and fundamental mechanisms of cellular signaling. Phosphorylation has been implicated in the regulation of the activity of cellular receptors, signaling molecules, transcription factors, DNA repair molecules, and chromatin structure proteins amongst many different functions. Many of these gene products when mutated exhibit oncogenic function through constitutive activation [12]. The recent ability to pharmacologically target the ATP binding domains of kinases has made these gene products one of the most frequently pharmacologically targeted molecules [13, 14].

An important protein kinase associated with neoplasia is the BCR-ABL fusion protein. BCR-ABL results from a chromosomal translocation between the ABL proto-oncogene and the BCR locus. BCR-ABL overexpression has been implicated in the pathogenesis of both chronic myelogenous leukemia and acute lymphocytic leukemia. Targeted inactivation of BCR-ABL through the drug imatanib mesylate, is the most regarded example of a successful targeted therapeutic (Gleevec, STI-571) [15–18].

8.2.4 Transcription Factors

Transcription factors are some of the most potent oncoproteins in the induction of cancer. By regulating the expression of numerous gene products they can exert broad, concerted, and sustained effects on cellular programming. They include a multitude of gene products including steroid receptors and nuclear transcription factors such as MYC, MYB, and FOS/JUN.

The family of MYC proto-oncogenes (c-MYC, n-MYC, l-MYC) have been implicated in the regulation of many physiologic programs cellular growth, proliferation, differentiation, immortalization/senescence, apoptosis, angiogenesis, adhesion, motility, and DNA repair. MYC is overexpressed in over half of all human cancers suggesting that it plays a role in the pathogenesis of most human cancers [19]. MYC has been shown to regulate the transcription of up to thousands of target genes [20–22], suggesting that these gene products function as grand coordinators of gene expression programs. However, MYC has also been shown to play a role in the regulation of ribosomal biogenesis, RNA processing, DNA replication, and DNA repair.

c-MYC was one of the first proto-oncogenes identified [23]. c-MYC is activated in human cancer through at least three different mechanisms. The chromosomal translocation of the MYC locus to the immunoglobulin or T-cell receptor loci is associated with Burkitt's lymphoma. n-MYC is commonly expressed in neuronal cells and is often overexpressed as a result of genomic amplification in neuroblastoma. l-MYC was first identified in lung tissue and is associated with small cell lung carcinoma [19]. MYC has also shown to be overexpressed as a consequence of the disruption of the

regulation of its transcription regulations, for example, through the disruption of the beta-catenin pathway through mutation of the APC gene. Although MYC and other transcription factors play such a fundamental role in the pathogenesis of cancer, they are yet to be successfully targeted therapeutically.

8.3 Interrogating Oncogenes via Mouse Models

Our understanding of the physiologic function of proto-oncogenes and the contribution of oncogenes to the initiation and maintenance of tumorigenesis has been markedly enhanced by the ability to generate mouse models. Most notably, the development of transgenic mouse models has provided the unprecedented opportunity to model the contribution of specific gene products to the pathogenesis of neoplasia in vivo. The use of conditional transgenic models has made it possible to directly interrogate when and how the inactivation of oncogenes can result in cancer regression.

Several recent reviews have extensively described the application of conventional transgenic mouse models for the development of therapeutics for cancer [24–26]. Here we will focus on the use of conditional transgenic models can be used to define when and how oncoproteins can be used as targets for the treatment of cancer.

8.3.1 Experimental Approaches

Three different strategies have been most commonly utilized to conditionally regulate gene expression in transgenic mouse models: (1) the Tet System, (2) the Tamoxifen System, and (3) the TVA system [24, 27, 28].

8.3.1.1 The Tet System

The Tetracycline Regulatory System (Tet System) was developed as a strategy to regulate the transcription of genes in eukaryotic cells utilizing prokaryotic transcriptional regulatory proteins [29, 30]. There are two variations of this system—one activates transgene expression in the presence of a tetracycline such as doxycycline (Tet-on), while the other system shuts off transgene expression upon doxycycline addition (Tet-off). In both variations, two different transgenes are generated. The first transgene uses a tissue-specific promoter to drive the expression of a tetracycline transactivator (tTA or rtTA). The second transgene contains a tetracycline response element (Tet-O) adjacent to a target gene of interest. The tTA or rtTA protein binds to the Tet-O promoter regulating gene transcription. The presence of doxycycline, prevents binding of the tTA protein to the Tet-O element turning off gene expression (Tet-off) or promotes the binding of the rtTA protein to the Tet-O element turning gene expression on (Tet-on). The Tet system facilitates monitoring of transgene expression at the transcription level in specific tissues within the mouse.

8.3.1.2 The Tamoxifen System

The Tamoxifen System also has been employed to conditionally regulate gene activation posttranscriptionally. MYC fused with the estradiol receptor exhibited conditional oncogene activation [31]. A mutant version of the estradiol receptor, which binds tamoxifen, is utilized to prevent endogenous estradiol from activating gene function [32]. Upon addition of tamoxifen, MYC is active, and withdrawal leads to an inactive product. Subsequently, this strategy was shown to be useful in the generation of conditional transgenic mice [33, 34].

8.3.1.3 The RCAS-TVA-Tet System

The Tet-system can be combined with the RCAS-TVA system [35, 36]. In this approach a tissue-specific promoter is used to drive the expression of the avian retroviral receptor (TVA) in transgenic mice. The cells of these mice also contain a Tet-O regulated transgene, but lack the rtTA protein. Avian retroviral vectors (RCAS) are used to deliver the rtTA transactivator to cells that express the TVA. The successfully infected cells now contain a transgene whose expression can be regulated by doxycycline.

8.3.2 Defining When Cancer Is Reversible

Conditional transgenic models have been used to evaluate the consequences of oncogene inactivation in vivo. From these studies, several general themes emerge regarding the role of oncogenes in the initiation and maintenance of tumorigenesis, as we have described [27, 37–42]. Oncogene inactivation can reverse tumorigenesis by inducing sustained cancer regression through differentiation, proliferative arrest, and/or apoptosis (Table 8.1).

The specific consequences of the inactivation of an oncogene depend upon the type of cancer. In some cases, even briefly inactivating an oncogene may be sufficient to induce sustained cancer regression [43, 44], but in other cases, this has not been observed [45]. Oncogene inactivation may uncover the stem cell properties of cancer cells and induce a state of cancer dormancy [45–49]. Finally, the genetic context can affect whether inactivation of an oncogene will induce sustained regression, or if the cancers can relapse acquiring additional genetic events [45, 50–52]. Conditional transgenic models have been generated for many types of signaling molecules implicated in the pathogenesis of neoplasia.

Table 8.1 Consequences of oncogene inactivation in transgenic mouse models

Oncogene	Model	System	Tumor type	Response to inactivation	Mechanism of tumor regression	References
BCL2	MMTV-tTA Tet-O-BCL-2 Eμ-MYC	Tet-off	Lymphoblastic leukemia	Regression	Apoptosis	[142]
BCR-ABL	MMTV-tTA Tet-O-BCR-ABL	Tet-Off	B-cell leukemia	Regression[a]	Apoptosis	[59]
	SCL-tTA Tet-O-BCR-ABL	Tet-Off	CML	Regression	ND	[60]
FGF-10	CCSP-rtTA or SPC-rtTA Tet-O-CMV-FGF10	Tet-on	Pulmonary adenomas	Regression	ND	[143]
HER2/NEU	MMTV-rtTA Tet-O-NeuNT	Tet-On	Mammary Carcinomas	Regression[a]	Decreased proliferation and apoptosis	[53]
MET	LAP-tTA Tet-O-MET	Tet-On	Hepatocellular carcinoma	Regression	Decreased proliferation and Apoptosis	[144]
c-MYC	EμSRα-tTA Tet-O-MYC	Tet-Off	T- and B-cell lymphoma, acute myeloid leukemia	Regression[a]	Cell cycle arrest, differentiation and apoptosis	[61–63]
	EμSRα-tTA Tet-O-MYC	Tet-Off	Osteosarcoma	Regression	Differentiation	[44]
	MMTV-rtTA Tet-O-MYC	Tet-On	Breast adenoma	Partial regression	ND	[45, 50]
	LAP-tTA Tet-O-MYC	Tet-Off	Hepatocellular carcinoma	Regression	Apoptosis and differentiation	[46, 64]
	Plns- MycER^{Tam}	Tamoxifen	Pancreatic Islet cell	Regression	Growth arrest, differentiation, cellular adhesion, vascular collapse	[33]
	Involucrin-MycER ^{Tam}	Tamoxifen	Papillomas	Regression	Growth arrest and differentiation	[34, 43]
RAS	Tyr-rtTA H-Ras$^{(V12G)}$ Ink4a$^{-/-}$	Tet-On	Melanoma	Regression[a]	Apoptosis, EGFR expression required	[54, 55]
	SP-r-rtTTA RtTA-KiRas$^{(G12C)}$	Tet-On	Lung adenoma	Regression	ND	[57]
	CCSP-rtTA Tet-O-KiRas$^{(G12C)}$	Tet-On	Lung adenoma	Regression	ND	[57]
	CCSP-rtTA Tet-op-K-Ras4B$^{(G12D)}$	Tet-On	Lung adenoma	Regression	Apoptosis	[58]
	Nestin-TVA RCAS-tTA RCAS-Akt RCAS-Tet-O-KRas	RCAS	Glioblastoma	Regression	Apoptosis	[145]
WNT	MMTV-rtTA Tet-O-WNT1 P53$^{-/-}$	Tet	Mammary adenoma	Regression[a]	ND	[146]

ND not determined

[a]While most of the tumors regressed upon oncogene inactivation, some of the mice relapsed while the oncogene was inactivated

8.3.2.1 Conditional Models of Receptor Induced Tumorigenesis

The Tet system has been used to conditionally overexpress receptors including an oncogenic form of HER2 containing an activating point mutation in its transmembrane domain [52, 53]. Expression was directed to the breast by utilizing the mouse mammary tumor virus (MMTV) promoter to drive the expression of the rtTA protein. Within 4 days of HER2 activation by doxycycline administration, the mice developed hyperplastic abnormalities. Six weeks following oncogene activation, all of the mice developed multiple invasive mammary carcinomas. The tumors were solid invasive carcinomas that often metastasized to the lung. Following 48 h of HER2 inactivation through doxycycline withdrawal, the cancer cells exhibited proliferation arrest and increased apoptosis. The primary carcinomas rapidly and completely regressed in over 90 % of the mice with a mean regression time of 17 days. Within 30 days, the pulmonary metastases

had also completely and rapidly regressed. Thus, tumorigenesis appears to be reversible.

However, a majority of the cancers that had a complete regression upon HER2 repression eventually reoccurred. When the primary cancers and metastases were transplanted into syngeneic hosts, they completely regressed only 55–70 % of the time. The relapse tumors all uniformly lacked both endogeneous and transgene protein expression indicating that the cancers had all become HER2 independent [53]. Subsequently, Snail, a transcriptional repressor was found to be activated in relapsed cancers [52]. Therefore, although oncogene inactivation can cause cancer regression, some transgenic tumors are capable of becoming independent of their initiating oncogenic event.

8.3.2.2 GTPases and Cancer Regression

The Tet-On system has been used to generate a conditional model of mutant H-RAS induced melanomas [54, 55]. The tyrosinase gene promoter (Tyr) was used to conditionally overexpress an H-RAS bearing an activating point mutation (V12G) in an Ink4a-deficient background. Approximately 25 % of the mice developed melanomas within 60 days of H-RAS activation. The melanomas were invasive, highly vascular, and notably amelanotic. The tumors exhibited expression of tyrosinase-related-protein-1 (TRP-1), an early melanocyte-specific maker. Within 48 h of H-RAS inactivation through doxycycline withdrawal, the cancers decreased their proliferation and exhibited robust apoptosis. Within 14 days of H-RAS inactivation, the cancers had completely regressed with only microscopically detectable scattered cancer foci. Notably, melanomas transplanted into SCID hosts also regressed upon inactivation of mutant H-RAS. Approximately 30 % of the melanomas resumed growth, even in the absence of H-RAS, but relapsed tumors failed to express TRP-1, suggesting that these tumors were phenotypically different from the primary cancers [55]. Additionally studies have shown that EGFR signaling is required for maintenance of a tumorigenic phenotype in H-RAS induced melanomas. A dominant-negative EGFR reduced the tumorigencity of melanomas, and sustained expression of EGFR can delay cancer regression [56].

The Tet-System has also been used to generate conditional models of mutant K-RAS induced lung adenocarcinoma [57, 58]. The Clara cell secretory protein (CCSP) promoter was used to regulate gene expression in alveolar epithelial cells. Within 7–14 days post induction of K-RAS overexpression, type II pneumocytes exhibited focal hyperplasia, and after 2 months, multiple solid adenomas or adenocarcinomas were present in the lung. The solid adenomas contained a population of macrophages, but lacked invasive growth and stromal elements. The adenocarcinomas had fewer macrophages and cytoplasmic inclusions, but had local invasion of the pleura. Within 3 days of K-RAS inactivation through doxycycline withdrawal, cancers exhibited decreased cellular density and an increased rate of apoptosis. Within 7 days of K-RAS inactivation, only a few patches of hyperplasia were found, and within a month no residual cancer tissue was found in 5/5 mice. The same mice were generated in either a p53 or an Ink4A/Arf deficient background. Cancers grew rapidly in these mice after K-RAS induction, but regressed with the same kinetics. TUNEL assays revealed that regardless of the genetic context cancers were regressing associated with apoptosis [58]. Similarly, CCSP regulated K-RAS (G12C) induced lung adenomas regressed upon oncogene inactivation [57]. Hence, even aggressive lung cancers in a tumor suppressor-deficient background regress upon the inactivation of a single oncogene.

8.3.2.3 Protein Kinases and Cancer Regression

A conditional transgenic model for BCR-ABL leukemias was generated using either the MMTV or SCL (stem cell leukemia) promoter to drive the expression of tTA [59, 60]. Upon induction of BCR-ABL the mice developed B-cell leukemia associated with lymphadenopathy, splenomegaly, and bone marrow infiltration. A third of mice with BCR-ABL under the control of the SCL promoter developed B-cell lymphoblastic disease resembling blast crisis, closely mimicking what is observed in patients with chronic myelogenous leukemia (CML). Inactivation of BCR-ABL induced rapid cancer regression in all mice. BCR-ABL inactivation was associated with the apoptosis of 80 % of the cancer cells within 20 h and complete tumor regression within 5 days. Sustained regression of cancer was observed in tumors arising from three of the four founder lines, as long as the mice had BCR-ABL continuously inactivated. Upon reactivation of BCR-ABL the cancers rapidly recurred. Interestingly, all the mice derived from the fourth founder relapsed within 4 weeks after complete regression. Relapsed cancers lacked continued expression of BCR-ABL protein and mRNA, suggesting that they had become independent of BCR-ABL expression.

8.3.2.4 Nuclear Transcription Factors

The Tet and Tamoxifen systems have been used to demonstrate that MYC inactivation can induce cancer regression in a multitude of different types of cancer (Table 8.1). The Tet-Off System was used to regulate human c-MYC in lymphoid cells under the regulation of the EμSRα promoter [61–63]. When MYC is constitutively activated, 100 % of the mice developed hematopoietic tumors within 5 months. Upon gross examination, the mice exhibited enlargement of the thymus, liver, spleen, and gastrointestinal lymph nodes. Histological examination revealed that cancer cells had invaded all hematopoietic organs as well as liver, kidney, blood, and the lamina propia of the intestines. In one study, tumors were generally immature CD+4/CD+8 T-cell lymphomas and were rarely acute myeloid leukemias [61]. In another

Table 8.2 Oncogene inactivation in the therapeutic setting

Target	Target type	Drug	Cancer	Clinical efficacy	References
EGFR	Receptor tyrosine kinase	Cetuximab	Colorectal cancer	Synergism with irinotecan in irinotecan-refractory colorectal cancer	[75]
		Erlotinib (Tarceva)	NSCLC	Approved for refractory NSCLC; disappointing results of addition to chemotherapy in initial treatment of NSCLC	[68]
		Gefitinib (Iressa)	NSCLC	Approved for refractory NSCLC; disappointing results of addition to chemotherapy in initial treatment of NSCLC	[66, 69, 70]
ERBB2 (Her2/Neu)	Receptor tyrosine kinase	Trastuzumab (Herceptin)	Breast cancer	Increases response rates and improves survival when added to chemotherapy for metastatic HER2 overexpressing breast cancer	[149]
VEGF	Receptor tyrosine kinase ligand	Bevacizumab (Avastin)	Metastatic colorectal cancer	Significant prolongation of survival in combination therapy	[152]
RAS	GTPase	Zanestra	Colorectal and pancreatic cancer	No effect	End et al. (2001)
		ISIS 2503	Pancreatic adenocarcinoma	Unclear benefit in combination therapy	[85]
BCR-ABL	Tyrosine kinase	Imatinib mesylate (Gleevec/STI-571)	CML; GIST	Complete hematologic and cytogenetic remissions in most CML patients; partial response in more than half of GIST patients	[148] and [147]
RAF-1	Tyrosine kinase	Sorafenib	Metastatic renal cell carcinoma; advanced melanoma	Improves time to progression in metastatic renal cell carcinoma and produces partial responses in combination therapy against advanced melanoma	[151] and [150]

study, tumors were either B-cell or T-cell lymphomas [63]. In both studies, the resulting hematopoietic tumors exhibited a high degree of genomic instability reflected by chromosomal gains, losses, or translocations [61, 63]. Despite this genomic complexity, the inactivation of MYC resulted in rapid and sustained cancer regression. Upon MYC inactivation, cancer cells arrested, differentiated, and underwent apoptosis. Over 50 % of tumors exhibit sustained regression for over 30 weeks. Thus, MYC inactivation can induce sustained regression of hematopoietic tumors.

Conditional transgenic mice expressing c-MYC under the control of the Eμ-promoter occasionally developed highly metastatic osteosarcomas [44]. Histological examination of the primary cancer reveals the presence of disorganized bone matrix. MYC inactivation induced rapid cancer regression associated with the differentiation of cancer into mature bone. Continuous video time-lapsed microscopy (CVTL) revealed that upon MYC inactivation, cancer cells ceased to proliferate and differentiated, as confirmed by immunocytochemical analysis. Identically, MYC inactivation in cancers in vivo was associated with the differentiation of malignant cells into mature osteoid. Upon MYC reactivation less than 1 % of the cells were able to regain a proliferative phenotype. Surprisingly, MYC reactivation was also associated with the apoptosis of the now differentiated cancer cells. Moreover, even the transient inactivation of MYC was found to increase the survival of mice with these cancers. Hence, at least in some circumstances even brief oncogene inactivation can induce sustained loss of a neoplastic state.

The tamoxifen system also has been used to evaluate the consequences of MYC inactivation in different types of tumors using MycER^{TAM} (Table 8.2). MycER^{TAM} has been expressed in the skin through the involucrin promoter [34, 43]. MYC activation resulted in increased proliferation and blocked differentiation of the suprabasal epidermis. Sustained MYC activation resulted in hyperplasia, dysplasia, angiogenesis, and papillomatosis. MYC inactivation resulted in regression of blood vessels, restoration of cellular differentiation, and the regression of papillomas. A brief inactivation of MYC in keratinocytes caused the cells to differentiate and become unresponsive to MYC reactivation. MYC reactivation could not restore a proliferative phenotype to the differentiated keratinocytes and eventually the cells were sloughed off the skin [43]. Hence, brief inactivation of MYC can induce the sustained loss of neoplastic features in some skin tumors.

MycER^{TAM} was also expressed under the control of the insulin (plns) promoter to induce pancreatic islet cell carcinomas [33]. Within 24 h of MYC activation, virtually all β-islet cells were rapidly proliferating. By 72 h of MYC acti-

vation 4–7 % of β-cells were undergoing apoptosis, and within 6–10 days there were almost no β-cells detectable. MycER^{TAM} was expressed in the presence of BCL-x_L to address the consequences of MYC activation when apoptosis is repressed. Within 7 days of MYC activation β-cells became hyperplastic, ceased insulin production, and decreased expression of the intercellular adhesion molecule E-cadherin. Within 6 weeks pancreatic islet cell carcinomas had formed highly vascularized cancers. Upon MYC inactivation, these cancers regressed completely. The cancers decreased proliferation, differentiated, increased expression of E-cadherin, and exhibited vascular collapse. While these cancers initially regressed upon MYC inactivation, transient MYC inactivation did not result in sustained cancer regression.

The Tet-System has been used to explore the role of MYC in the initiation and maintenance of liver cancer by utilizing the liver activator protein (LAP) promoter to express tTA [46, 64]. The latency of tumorigenesis was inversely correlated with the age at which MYC was activated [64]. When MYC was activated during embryonic development, mice would succumb to neoplasia within 10 days of birth. In contrast, if MYC was activated in adult mice, the mean latency of cancer onset was 35 weeks. The cancers generated in adult mice histologically resembled either hepatocellular carcinomas (HCC) and/or hepatoblastomas. MYC was found to be able to induce proliferation in embryonic or neonatal liver cells, but induce cellular hypertrophy without cellular proliferation in adult liver cells. In part, this was explained by the observation that MYC induced a p53-dependent arrest in cellular division in adult hepatocytes. Thus, it appears that the ability of MYC to induce tumorigenesis depends upon epigenetic parameters dictated by developmental state.

The same Tet system model was used to examine the consequences of MYC inactivation in liver cancer [46]. The liver cancers were locally invasive, occasionally metastasized to the lung, and were readily transplantable into SCID mice. Within 4 days of MYC inactivation, cancer cells stopped proliferating, differentiated into normal liver cells, and subsequently underwent apoptosis [46]. Even after 5 months of continuous MYC inactivation, a residual population of tumor-derived cells remained detectable. However, MYC reactivation immediately resulted in resumption of a tumorigenic phenotype. Thus, these results were in marked contrast to earlier reports that brief inactivation of MYC can result in a permanent loss of a neoplastic phenotype. One possible explanation for these results is that MYC inactivation uncovers the latent stem cell properties of cancer cells that now can differentiate into normal liver, but some of these cancer stem cells retain the capacity to regain their neoplastic features. In support of this hypothesis, upon MYC inactivation, some of the residual cells expressed the liver stem cell marker cytokeratin 19 (CK-19) [46]

MYC also has been conditionally expressed in mammary epithelium using a Tet-on system with the MMTV promoter [45, 50]. MYC activation resulted in mammary adenocarcinomas with a mean latency of 22 weeks in approximately 86 % of mice. Histologically, the cancers exhibited focal hyperplasia, increased proliferation, and dysplasia. Upon MYC inactivation, less than half of the adenocarcinomas completely regressed, and a majority of the cancers that completely regressed spontaneously relapsed. Some relapsed cancers had either sustained expression of transgenic MYC in the absence of doxycycline or had reactivated MYC target genes in the absence of transgenic MYC. The majority of cancers relapsed by becoming independent of MYC. Half of the tumors exhibited an activating mutation in either K-RAS2 (17/23) or H-RAS2 (6/23). Thus, MYC inactivation can induce sustained regression of breast cancers unless they have acquired mutations in K-RAS or H-RAS. These important results underscore the importance of genetic context on the consequences of oncogene inactivation in a cancer.

8.4 Targeted Oncogenes to Treat Cancer

The pharmacologic inactivation of oncogenes has been effective in the treatment of some cancers including acute promyelocytic leukemia, breast cancer, colorectal cancer, and lung adenocarcinoma (Table 8.2). Two general concepts have emerged: (1) small molecules can be used to target mutant signaling proteins, and (2) receptors can be targeted with monoclonal antibodies. However, many gene products are yet to be successfully targeted.

8.4.1 Erlotinib, Gefitinib, and EGFR

EGFR has been successfully targeted by many agents. Gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva) were approved by the FDA in 2004 as single agents for the treatment of non-small-cell lung carcinoma (NSCLC) that had proven refractory to standard chemotherapy. Both drugs are members of the anilinoquinazoline class of small molecules and act by competitively inhibiting ATP binding to the intracellular tyrosine kinase domain of EGFR, thereby preventing the autophosphorylation that is necessary for receptor tyrosine kinases to initiate their signaling cascades (Fig. 8.1). Gefitinib was approved based upon results from two studies, the IDEAL-1 and IDEAL-2 trials, that describe an 11–19 % response rate for the drug as a single agent in patients with refractory disease [65–67].

Erlotinib yielded partial responses in 9 % of patients when administered as a single agent to those who had failed one or two regimens of standard chemotherapy [68]. Advanced or metastatic NSCLC entails very poor survival, so gefitinib and erlotinib were at first considered to provide real benefits over existing therapy despite the low response rates associated with their use. Additional studies (the INTACT trials)

illustrated that adding these two drugs to regimens consisting of standard chemotherapy agents yielded no additional benefit [69, 70]. Moreover, almost all patients who showed an initial response eventually relapsed with disease that was resistant to EGFR inhibitors [71]. The prospect of successfully inhibiting EGFR with small molecules was thus cast in doubt, and a concerted effort was made to explain the contradictory findings of the IDEAL and INTACT studies.

One possibility is that a given tumor's response to gefitinib will be dependent on activating mutations in exons 19 and 21 of EGFR [72]. Analysis of the crystal structure of EGFR revealed that these mutations occurred in a crucial ATP binding pocket where the anilinoquinazolines act as competitive inhibitors [73]. Hence, although gefitinib therapy may not be broadly applicable to all lung cancers, genetic subpopulations may be identified that exhibit an increased susceptibility to EGFR inhibitors. Another clue to the possible mechanism of action of these agents is that cells transfected with mutant EGFR uniquely activate anti-apoptotic pathways involving Akt and STAT signaling [74]. Therefore, cells carrying the mutant receptor although resistant to the apoptotic effects of standard chemotherapeutic agents, in combination with EGFR inhibitors may synergize with these traditional chemotherapies for a clinical response.

An important example of a possible synergistic effect between a targeted therapy and conventional chemotherapy is cetuximab (a monoclonal antibody to EGFR) combined with irinotecan (a topoisomerase I inhibitor). Patients with irinotecan-refractory colorectal cancer had higher response rates and modestly prolonged survival when treated with cetuximab in combination with irinotecan than patients treated with cetuximab alone [75] suggesting that cetuximab may reverse resistance to irinotecan. Both preclinical and clinical studies have shown synergy between platinum-based chemotherapy and trastuzumab, a monoclonal antibody against the ErbB family member HER2 [76, 77].

Other possible synergistic effects amongst therapeutics may be anticipated through insight into the mechanisms of action. EGFR acts upstream of the RAS signaling pathway (Fig. 8.1). RAS and EGFR mutations seem to be mutually exclusive, implying that they are interchangeable in tumorigenesis [71]. RAS mutations may allow cancers to circumvent EGFR inhibition [78]. Therefore, it seems logical that targeting the RAS pathway may also be required for inhibitors of EGFR to prevent cancer growth. Whether this proves to be the case in patients with cancer remains to be confirmed.

8.4.2 Targeting RAS

The RAS signaling pathways have been targeted using several strategies. RAS must be localized to the plasma membrane to become activated, so pharmacological strategies that prevent localization have been developed. Farnesyltransferase inhibitors (FTIs) were developed to interfere with the post-translational farnesylation process and thereby abrogate native RAS activity [79]. Initially, these compounds showed promise against tumors driven by activated H-RAS [80]. However, the activity of these drugs was not reproduced in cancer cells with K-RAS and N-RAS, the two isotypes that are much more common in human malignancies.

N-RAS and K-RAS are resistant to FTIs because they can alternatively be geranylgeranylated when farnesylation is not possible and can subsequently carry out their tumorigenic functions [81]. Unfortunately, inhibitors of geranylgeranyltransferase (GGTIs) were found to be exceedingly toxic in mice when used in combination with FTIs [82], and therefore are not a possible treatment option. More recently, *S*-farnesylthiosalicylic acid (FTS) has been identified as a drug that dislodges activated RAS from the plasma membrane. FTS has been shown to inhibit growth of human cancers xenografted into nude mice [83, 84]. Clinical studies of FTS in human trials are pending.

As a final strategy worthy of mentioning, RAS has also been targeted at the transcriptional level through the use of antisense RNA. One antisense oligonucleotide, ISIS 2503, has been administered to patients with very little toxicity. In a Phase II trial ISIS 2503 exhibited some clinical activity in patients with pancreatic cancer [85]. However, more recently interest in this clinical approach has waned, because it is now less clear that these agents can ever be efficacious in vivo.

8.4.3 Imatinib Mesylate and BCR-ABL

One of the most exciting discoveries has been the identification of imatinib mesylate for the inhibition tyrosine kinases for the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors [15–18]. Importantly, the possible importance of such a drug would perhaps never have been appreciated if it were not well known that BCR-ABL exhibits tyrosine kinase activity that is essential for its ability to mediate malignant transformation [86, 87].

Imatinib mesylate is derived from a 2-phenylaminopyrimidine backbone that exhibits inhibition of multiple kinases including ABL tyrosine kinases, PDGFR, and the c-kit tyrosine kinase [15, 88, 89]. Imatinib mesylate restricts enzyme activity by competitively inhibiting ATP access to the binding pocket of the kinase thereby preventing substrate phosphorylation. Preclinical studies confirmed that imatinib mesylate inhibits BCR-ABL activity [90].

Early phase I trials of imatinib mesylate revealed that the drug was well tolerated, and almost all patients exhibited a positive clinical response [91]. Subsequently, a phase II trial in patients with late chronic-phase CML that failed to respond to interferon-based therapy reported that 41 % experienced a complete cytogenetic response, 60 % showed a major cytogenetic response, and 89.2 % benefited from

progression-free survival at 18 months [92]. Based upon these results, the FDA approved imatinib mesylate for the treatment of CML in advanced phase disease and after failure of interferon therapy. The results of a phase III study (the IRIS trial) proved imatinib mesylate was significantly superior to interferon alpha plus cytarabine in achieving complete hematologic response, major cytogenetic response, complete cytogenetic response, and progression-free survival [93]. Imatinib mesylate was then approved as a first-line treatment for CML in the USA and Europe.

Unfortunately, cancers can become resistant to imatinib mesylate through multiple mechanisms. First, mutations occur in the kinase domain of the BCR-ABL oncoprotein that confer varying degrees of drug insensitivity [15]. Sometimes tumors with mutant BCR-ABL will respond to increased doses of imatinib mesylate [94]. Resistant tumors also appear to respond to new drugs that are not affected by these mutations [95, 96]. Second, tumors can become resistant to imatinib mesylate through BCR-ABL gene amplification and mRNA overexpression [97, 98]. Importantly, resistance to imatinib mesylate is almost always associated with reactivation of BCR-ABL signaling, suggesting that this oncogene is required to sustain tumorigenesis in CML [99].

8.4.4 Future Directions

Ideally, we would understand which oncogenes are best to target and every oncogene could be targeted for the treatment of cancer. However, we do not know for most cancers what to target. Even when there are encouraging experimental and clinical evidence that the targeted inactivation of specific gene products may be useful in cancer treatment, many gene products cannot be targeted using existing pharmacological approaches.

Notably, there are no drugs that clinically target transcription factors. We discuss some of the possible approaches to target MYC that have been employed in experimental models such as: antisense oligonucleotides (ASOs), RNAi, and cationic porphyrin TMPyP4 have been used to target MYC at the transcriptional level; peptides and small molecules have been used to disrupt MYC binding to MAX; and triple helix forming oligonucleotides (TFOs) have been used to target MYC at the posttranslational level [100–102]. Additionally, MYC may be targeted at the posttranslational level by targeting its phosphorylation sites.

ASOs and RNAi both work by hybridizing to MYC mRNA and targeting it for degradation. ASO and RNAi treatments have led to decreased MYC protein levels both in vivo and in vitro. [103–105]. The decrease in MYC results in cellular differentiation, reduced proliferation, and inhibition of G1/S progression in leukemia and lymphoma cells [106–111]. In transgenic mouse models, ASOs have prevented or delayed the onset of Burkitt's lymphoma [112–114]. An ASO type molecule with a morpholino backbone (AVI-4126) has been administered to patients in a Phase I clinical trial and was found to concentrate in the tumor tissues of breast and prostate cancer patients, with little toxicity, thus making it a viable treatment option [115, 116]. Cationic porphyrin TMPyP4 has also been used to target MYC at the transcriptional level. TMPyP4 blocks DNA structures formed during transcription in G-rich regions of DNA (G-quadruplexes) where MYC binds [117, 118]. Additionally, TMPyP4 was able to inhibit in vitro transcription of MYC and decreased growth of Burkitt's lymphoma cells [119].

Peptides and small molecules have been used to disrupt MYC binding to its dimerization partner MAX. Small peptides that mimic the helix–loop–helix (HLH) domain of MYC were found to sequester MYC and prevent MYC–MAX dimerization [120–122]. Interference with this protein–protein interaction leads to decreased proliferation of breast cancer and colon cancer cell lines [123, 124]. Peptide inhibitors are also capable of achieving high concentrations in mouse organs and may therefore be used to treat cancers [123]. Unfortunately, peptides tend to be unstable and must be administered through intravenous injection, leaving a need for the development of small chemical molecules that can effectively prevent MYC–MAX dimerization. Peptidomimetics are an attempt to increase the stability and activity of peptides. Several peptidomimetics have been made that increase stability and activity of proteins that inhibit MYC–MAX dimerization but have not been used in human trails [122, 123]. Additionally, there has been an effort to target MYC–MAX interaction using small chemical molecules [125–127]. One of these approaches uses molecular credit cards, which are planar chemicals designed to physically prevent protein–protein interaction by sliding between the two protein surfaces. In one study, potent inhibitors of MYC–MAX interaction were identified that partially prevented oncogenic transformation of chicken embryonic fibroblasts [126].

MYC has also been targeted at the DNA–protein interaction level. Triple helix forming oligonucleotides (TFOs) can disrupt transcription factor binding to DNA by attaching to purine-rich regions of DNA often found in promoter regions [128, 129]. TFOs against the promoter for MYC have been able to induce apoptosis and cell cycle arrest in several cancer cell lines [130–132]. TFOs may also be conjugated to DNA damaging agents such as daunomycin. This combination agent leads to MYC downregulation in prostate and breast cancer cell lines [133].

MYC may be marked for degradation at the posttranslational level by targeting its phosphorylation sites. MYC has two phosphorylation sites: Thr58 and Ser62. Phosphorylation of Ser62 increases MYC stability and occurs in a RAS-dependent manner. The phosphorylation of Ser62 is followed

by phosphorylation of Thr58 and leads to the degradation of MYC [134, 135]. Thus, it may possible to screen for small molecules that inhibit MYC stabilization, or facilitate MYC degradation.

8.5 The Phenomena of Oncogene Addiction

Observations in experimental transgenic mouse models and in the clinic have a central theme—although cancers are genetically complex and genomically unstable, they appear to be dependent upon the continued activation of specific oncogenes to maintain their neoplastic properties, exhibiting the phenomenon *oncogene addiction*. Thus, specific gene products that mediate critical signaling processes appear to be the most likely best targets for the treatment of cancer. Conditional transgenic mouse models have been particularly useful for validating which signaling molecules are the most useful targets, for gaining insight into the mechanism of cancer regression, and for anticipating mechanisms by which cancers can escape dependence on signaling processes. In several cases, drugs have been identified that target receptors, GTPases, and/or kinases to successfully treat humans with cancer. However, these drugs, although effective in prolonging the survival of some patients, do not cure cancer. The hope is in the future, the correct combination of drugs we will be able to cure cancer. However there are several potential difficulties.

One major difficulty is that many gene products, in particular transcription factors, are yet to be successfully targeted. Thus, we may not be able yet to target the most critical gene products. Many new approaches suggest that even these gene products can be inhibited through small molecules. It may be possible to develop effective pharmacological strategies to target critical signaling molecules for the treatment of cancer. Another problem is that we as of yet do not understand why the repair or inactivation of specific gene products induces cancer regression. Several possible cell-autonomous and cell-dependent mechanisms could account for oncogene addiction. Bernard Weinstein, who was one of the first to articulate the notion of oncogene addiction, proposed that because oncogenes in cancer cells upregulate cellular programs that drive proliferation and block apoptosis, compensatory programs such as proliferative arrest and pro-apoptotic programs are also upregulated. Thus, when a mutant oncogene is inactivated, the balance in the cancer cell shifts to proliferative arrest and/or apoptosis [136].

A final issue to consider is that cancer is not just caused by genetic events and so fixing these events may not be sufficient to reverse the process of tumorigenesis. Thus, it has recently been suggested that cancers consist of cancer stem cells, of generally low abundance, and progenitor cells, that are the majority of the tumor. The cancer stem cells have the capacity for self-renewal, whereas the progenitor cells have a limited capacity of self-renewal. Such a model, provides just one example of a circumstance where the properties that are essential to the neoplastic phenotype are not necessarily dictated by genetic events involving signaling molecules.

Observations in transgenic mouse models have led investigators to reflect on several nonmutually exclusive mechanisms for oncogene addiction. Thus, it has been proposed that oncogenes initiate tumorigenesis by hijacking stem cell features in cells such as proliferation and self-renewal [46, 64], thus driving cell cycle transit despite ongoing genomic instability [137]. Hence, upon oncogene inactivation, cancer cells recover their differentiated physiologic program, undergo proliferative arrest, become aware of their genomic damage, and/or undergo apoptosis.

Many have illustrated that upon oncogene inactivation cell nonautonomous programs such as angiogenesis are shut down, which may also contribute to cancer regression [55, 138, 139]. Hence, cell-autonomous and cell-dependent mechanisms may play a role in oncogene addiction.

One possible unifying mechanism is that oncogene inactivation restores physiologic programs that allow cancer cells to recognize that they are genomically disrupted, hence triggering programs of proliferative arrest, differentiation, cellular senescence, and apoptosis. Indeed, we have recently described that upon MYC inactivation several different cancers variously undergo cellular senescence [140]. However, other mechanisms must also be involved. Thus, oncogene inactivation also shuts down angiogenesis, suggesting that it is not only internal physiological cues that are being restored but also how the cancer interacts with the tissue microenvironment [141]. Other mechanisms are also likely to be involved that are yet to be investigated, such as the potential role of immune or inflammatory mechanisms or the possible that cellular homeostatic regulatory mechanisms are important. Ultimately, insights into the mechanism of oncogene addiction will be essential towards the development of true targeted therapeutics for the treatment and cure of cancer.

Acknowledgements We thank the members of the Felsher laboratory for their many helpful discussions. This work was supported from grants from the NCI and related programs (ICBP, ICMIC, CCNE), Emerald Foundation, Leukemia and Lymphoma Society, Damon Runyon Foundation, Burroughs Wellcome Fund, and Stanford Digestive Disease Center (D.W.F.), as well as the Developmental and Neonatal Biology NIH Training Grant (C.A.).

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Positive Mediators of Cell Proliferation in Neoplasia: Growth Factors and Receptors

9

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9.1 Introduction

Complex signaling networks underlie the regulation of fundamental processes such as embryonic development, tissue differentiation, and systemic responses to wounds and infections. Among the major mediators of these events, growth factors are in large part responsible for the control of cell proliferation, differentiation, and survival. The specific interaction of a growth factor with its receptor initiates a cascade of intracellular biochemical reactions that ultimately mediate the biological response in the target cells. The cytoplasmic molecules that participate in these pathways have been termed second messengers—their activation allows the transmission of the signals to the nucleus and eventually the regulation of the expression of genes involved in mitogenic and differentiation responses.

Because of their important role in cell proliferation and survival, the genes involved in growth factor signaling pathways are frequently targeted in tumorigenesis. The pathogenic expression of growth factors can contribute to altered cell growth associated with malignancy, such as in the case of the *v-sis* oncogene of simian sarcoma virus (SSV), which encodes a protein homologous to the B chain of human platelet-derived growth factor (PDGF-B) [1, 2]. Other oncogenes have been shown to encode membrane-spanning growth factor receptors [3, 4] or genes involved in growth factor signal transduction [5, 6]. Present knowledge indicates that the constitutive activation of growth factor signaling pathways through genetic alterations affecting these genes contributes to the development and progression of most, if not all human cancers.

The focus of this chapter is on the normal aspects of growth factor signaling and the alterations that have been implicated in the etiology of human malignancies. Because of the limits of space, the discussion will mainly cover growth factor signaling mediated by receptors with intrinsic protein tyrosine kinase activity. Finally, we will discuss how a better understanding of growth factor signaling may be useful in efforts to design new approaches toward therapeutic intervention with the malignant process.

9.2 Background

The first growth factor was discovered in the 1950s by neurobiologist Rita Levi-Montalcini, who observed that a soluble factor released by mouse sarcoma cells dramatically stimulated the growth of sensory and sympathetic neurons in chicken embryos. This nerve growth factor (NGF), which consists of two chains of 118 amino acids, was subsequently purified by Stanley Cohen from snake venom and mouse salivary glands [7]. During these studies, Cohen discovered another factor present in mouse salivary glands and capable of promoting precocious eyelid opening and tooth eruption in newborn mice. Because of recognition of its effects on epithelial cells, this factor was designated epidermal growth factor (EGF) [8]. The isolation and characterization of NGF and EGF opened the way to the identification, in a number of laboratories, of a series of growth factors, which were often given names based on the tissue or cell of origin, or the target cell initially found to be stimulated.

An important discovery concerning growth factors came from the demonstration of a unique enzymologic activity associated with binding of EGF to its receptor (EGFR) [8]. Studies of the product of the viral oncogene, *v-src*, had led to the demonstration of its ability to act as a protein kinase

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_9

[9, 10]. It was well established that phosphorylations and dephosphorylations affected the activities of a variety of proteins, and many protein kinases had been previously identified. However, unlike theses kinases, which have the capacity to phosphorylate serine and/or threonine residues, the *src* product was shown to phosphorylate tyrosine residues [11]. Cohen then demonstrated that addition of EGF led to phosphorylation of its purified receptor on tyrosine residues [8]. Subsequent studies have established that tyrosine kinase activity is central to the functions of a large number of mitogenic signaling molecules.

Several major modes of action for growth factors have been described. Sporn and Todaro [12] defined autocrine and paracrine as major modes of action for growth factors in addition to the classical means by which hormones travel great distances from their sites of production. The autocrine mode refers to the ability of growth factor to act on the same cell releasing it. In the paracrine mode, the released growth factor from one cell acts on a nearby or adjacent cell. Certain growth factors also exist as membrane-anchored forms, which can bind and activate membrane receptors only on adjacent cells. This process, considered a variant of the paracrine mode, has been termed juxtacrine [13] and is capable of delivering spatially localized intercellular stimuli. A number of researchers have observed that factors that are produced in cells, but are not detectably secreted, nevertheless can induce observable phenotypic changes in those cells. The suggestion has been made that this represents an intracrine mode of action, whereby the factor interacts with its receptor directly inside the cell [14]. An example of this mechanism has been shown in acute myeloid leukemia, where the vascular endothelial growth factor (VEGF) internally activates its cognate receptor VEGFR-2/KDR/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1) to promote cell survival [15]. A sixth mode of action, in which the growth factor is bound to and stored within the extracellular matrix before presentation to the receptor on the cell surface, has also been demonstrated [16].

9.3 Growth Factor Receptors with Tyrosine Kinase Activity

9.3.1 RTK Structure

Many growth factors mediate their biological function through the binding to a family of cell surface receptors with intrinsic tyrosine kinase activity (RTKs). These receptors present a general common structure: a large, glycosylated, extracellular ligand-binding domain, a single transmembrane region, and a cytoplasmic portion with a conserved protein tyrosine kinase domain, which catalyzes the transfer of the γ-phosphate of adenosine triphosphate (ATP) to tyrosine residues within the receptor and to other substrates (Fig. 9.1). In addition to the catalytic domain, a juxtamembrane region and a carboxyl-terminal tail can be identified in the cytoplasmic portion [17]. Because of their structure, RTKs can be visualized as membrane-associated allosteric enzymes with the ligand binding and protein tyrosine kinase domains separated by the plasma membrane [18].

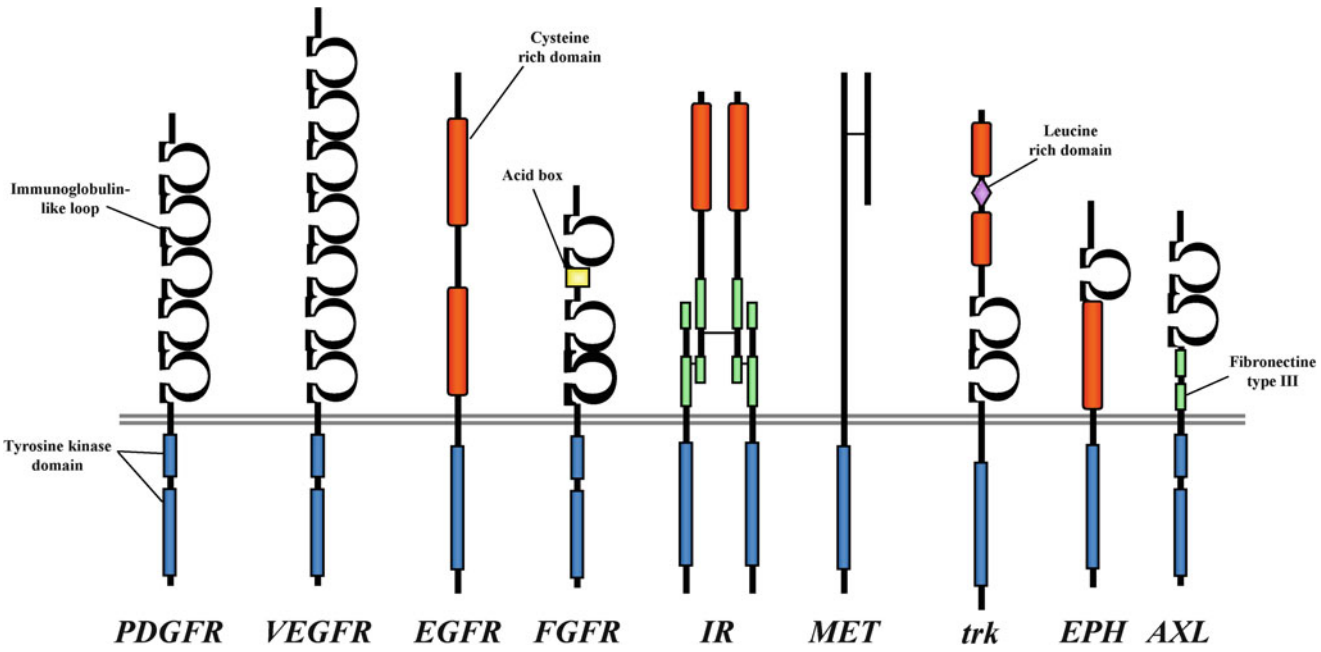

Fig. 9.1 Receptor tyrosine kinase families. Simplified scheme depicts the structural features of the major families of tyrosine kinase receptors.

The extracellular domain of RTKs is poorly conserved among the different subfamilies and displays characteristic structural features that help to determine specificity for ligand binding. These include cysteine-rich motifs, immunoglobulin-like repeats (Ig-like), fibronectin (FN) type III repeats (FNIII), and EGF motifs that can be present singly or in different combinations.

The main function of the transmembrane domain is to anchor the receptor in the plane of the plasma membrane, thereby connecting the extracellular environment with internal compartments of the cell. It was initially thought that this domain represented a passive anchor of the receptor to the membrane [19]. However, point mutations in the transmembrane domain of *neu*/erbB-2 enhance receptor dimerization and consequently its activity and transforming properties [20–22], implying that the transmembrane domain can play an important role to determine the correct conformation of the receptor.

The juxtamembrane sequence that separates the transmembrane and cytoplasmic domains is highly similar within the same receptor family but it is not conserved among different RTK groups. Studies indicate that this domain plays a role in modulation of receptor functions by heterologous stimuli, a process termed receptor transmodulation [19]. For example, addition of PDGF to many types of cells causes a rapid decrease in high affinity binding of EGF to its receptor. This has been shown to be a downstream effect of PDGF receptor (PDGFR) activation in which protein kinase C, itself a serine protein kinase, is activated and in turn phosphorylates a site in the juxtamembrane domain of the EGFR [23]. This region may also play a role in signaling, through the binding to specific substrates in a ligand-dependent manner. For example, it has been shown that *eps8* directly binds to the juxtamembrane domain of EGFR in a phosphotyrosine-independent and SH2-independent manner [24].

The high degree of conservation between the tyrosine domains of receptors belonging to different RTKs families has allowed the identification of several new members by molecular cloning. This domain is absolutely required for receptor signaling: mutation of a single lysine in the ATP binding site, which blocks the ability of the receptor to phosphorylate tyrosine residues, completely inactivates receptor biologic function. Conversely, activating mutation in the tyrosine kinase domain of some RTKs, such as EGFR, have been observed in cancer [25]. These EGFR mutations have a great impact on the choice of the best therapeutic strategy. The kinase domain of some receptor tyrosine kinases, e.g., PDGFR and fibroblast growth factor receptor (FGFR), is split by insertions of up to 100, mostly hydrophilic, amino acid residues. Kinase insertion sequences are highly conserved between species, suggesting an important role of this domain in receptor function. In fact, this region contains important autophosphorylation sites that mediate the coupling with signal transduction [19, 26].

The carboxyl-terminal domains are among the most divergent between known RTKs [23] and they are involved in the regulation of the kinase activity. The tyrosine residues typically present in this region are phosphorylated following ligand stimulation and play an important role in the specific activation of second messengers by RTKs.

9.3.2 Mechanisms of RTK Activation

There is substantial evidence that ligand-induced activation of the kinase domain and its signaling potential are mediated by receptor oligomerization [19]. This event stabilizes interactions between adjacent cytoplasmic domains and controls the activation of kinase activity. Dimerization can take place between two identical receptors (homodimerization), between different members of the same receptor family, or in some cases between a receptor and an accessory protein (heterodimerization) [26]. Heterodimerization of RTKs has been shown to increase the repertoire of ligands that can be recognized by each receptor alone and to expand the diversity of signaling pathways that can be recruited by a given receptor.

How ligands bind to the receptors and induce oligomerization seems specific for each class of RTKs [27]. PDGF, for example, induces receptor dimerization by virtue of its dimeric nature [28]. EGF, instead, binds its receptor as a monomer and dimerization occurs by subsequent association of two EGF-EGFR complexes [29]. FGF is a monomeric ligand like EGF, but it needs the association of heparan sulfate proteoglycans to induce receptor dimerization [30]. Interestingly, the insulin receptor (IR) family exists as disulfide-bonded homodimers or heterodimers of receptor subunits. Thus, ligand binding does not induce receptor dimerization, but presumably causes a conformational change in the preformed dimeric receptor, which leads to receptor activation (Fig. 9.2).

In the unphosphorylated state, RTKs possess a low catalytic activity due to the particular conformation of a specific domain in the kinase region, which interferes with the phosphotransfer event. Although this kinase activity is at a low basal level in the monomeric state, it is sufficient to induce *trans*-autophosphorylation in the cytoplasmic portion of the RTK once the dimer forms. This event stabilizes the tyrosine kinase domain in an active conformation, leading to further phosphorylation in *cis* of other tyrosine residues within the catalytic domain and the carboxyl-terminal tail, creating docking sites for downstream signal transduction molecules.

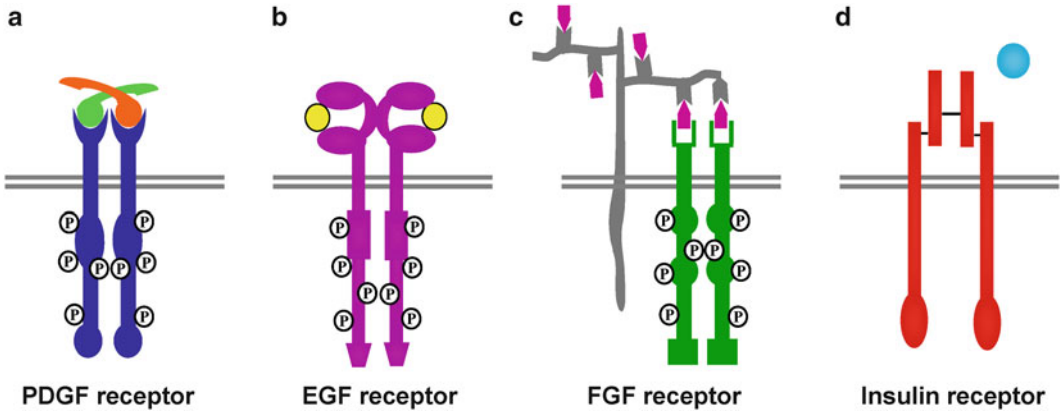

Fig. 9.2 Dimerization and activation of RTKs. Diagram illustrating different mechanisms for dimerization and activation of RTK. (**a**) PDGF ligands are dimers that induce dimerization by their cognate receptors. (**b**) EGF induces a conformational change within its receptor that allows receptor dimerization. (**c**) FGF ligands associate with heparan sulfate proteoglycans that contribute to the dimerization of their cognate receptors. (**d**) The insulin receptors exist as inactive dimmers, and the ligands cause a conformational change that induces receptor activation.

9.4 The Role of Growth Factors and Their Receptors in Malignancy

9.4.1 Platelet-Derived Growth Factor Family

PDGF is the major protein growth factor in human serum and is a markedly heat-stable, cationic protein that consists of four related but nonidentical polypeptide chains. While PDGF-A and PDGF-B have been extensively studied since the 1970s, PDGF-C, and PDGF-D have been only identified in 2000 [31–33] and 2001 [34–36], respectively, using bioinformatics approaches. The PDGF ligands contain a characteristic motif named cystine knot and formed of regularly spaced cysteine residues that allow intramolecular and intermolecular bonds [37]. All PDGF molecules form disulfide-linked homodimers, but PDGF-A and PDGF-B can also exist as heterodimers. Unlike PDGF-A and PDGF-B, which are secreted as active ligands, PDGF-C and PDGF-D become active following cleavage of their N-terminal domain [38]. The two PDGF receptors, PDGFRα and PDGFRβ, exhibit distinctive features, including the five immunoglobulin-like domains in the extracellular portion [39] and the presence of a large kinase insert within the tyrosine kinase domain. There are reports that the kinase insert is required for interaction with certain substrates [40], and deletions in this domain impair receptor mitogenic signaling [41]. As the four PDFG ligands, PDGFRs are encoded by distinct genes [42] and form homo-dimers or hetero-dimers [43]. Ligands have different affinity for the different receptor combinations: AA binds αα, CC and AB bind αα and αβ, BB binds all the three receptor dimers, and DD binds αβ and ββ [38]. Receptor dimerization caused by binding to the ligand induces cross phosphorylation in the cytoplasmic domain on several tyrosine residues, which act as docking sites for protein complexes mediating the activation of second messengers. This is an example of the fine degree of regulation that can evolve in the interactions of ligands with their receptors. Presumably, in the case of PDGF, this relates to quantitative regulation of responses based upon differential availability in tissues of ligands and receptors, since there is evidence that the two PDGF receptors themselves are each capable of mediating the major known PDGF responses including mitogenic signaling and chemotaxis [44].

Gene inactivation of the PDGF receptors and ligands has demonstrated that this signaling is essential during embryonic development. In particular, these studies have shown that PDGF-B and PDGFRβ are crucially involved in vessel formation, while PDGF-A/C and PDGFRα play a broader role in different processes, such as central nervous system, neural crest, and organ development [37, 45]. More specific strategies, including conditional knockout and knockin approaches, have been used to address the primary functions of PDGF signaling during development [45, 46].

The gene for the PDGF-B chain is the human homologue of the *v-sis* oncogene of SSV [1, 2]. The transforming protein expressed by SSV shares close structural similarities with PDGF-B chain homodimers [47]. PDGF-B has been detected in human cancer cells [48, 49] that also possess PDGF receptors. These findings, taken together with the demonstration that the normal PDGF-B gene can act as an oncogene when expressed at high levels [50], suggest that PDGF-B plays a role in the development of certain human cancers (Table 9.1). The PDGF-A chain is frequently expressed by human cancer cells, and AA homodimers are produced by osteosarcoma [51], melanoma [52], and glioblastoma cells [53], which can display autocrine activation of PDGF signaling [54]. Of note, a translocation between chromosomes 17 and 22 in dermatofibrosarcoma protuberans has been shown to bring the PDGF-B gene

Table 9.1 Role of RTKs and their ligands in human cancer

Family	Gene	Mechanism	Tumor
PDGF	*PDGF-A*	Autocrine loop	Glioblastoma, melanoma, osteosarcoma
	PDGF-B	Translocation-autocrine loop	Dermatofibrosarcoma protuberans
	PDGF-C, PDGF-D	Autocrine loop	Glioblastoma, Ewing sarcoma, medulloblastoma, and prostate cancer
	PDGFRα	Activating mutation	GIST
	PDGFRα, PDGFRβ	Translocation	Hematological malignancies
	CSF-1	Paracrine	Breast carcinoma
	c-kit	Activating mutation	GIST
	SCF	Autocrine, paracrine	SCLC, testicular germ cell tumor
	Flt3	Activating mutation	AML
VEGF	*VEGFs*	Paracrine (angiogenesis and lymphangiogenesis)	Several tumors
	PlGF	Autocrine, paracrine	ALL, Ewing's sarcoma
EGF	*TGF-α*	Overexpression	Lung, ovary, colon, prostate, pancreas tumors
	NRG1	Overexpression	Mammary adenocarcinoma
	EGFR	Overexpression	Head and neck tumors
	EGFR	Activating mutations	NSCLC, brain tumors
	ErbB-2	Gene amplification	Breast and ovarian carcinoma
FGF	*FGF-2*	Autocrine	Pituitary adenoma, prostatic and pancreatic cancer
	FGF-3	Autocrine	Mammary and prostate hyperplasia, NSCLC
	FGFR1	Translocation	Hematological malignancies
	FGFR2, FGFR3	Mutations	Gastric cancer, bladder and cervical carcinomas, myelomas, and skin cancer
Insulin	*IGF-I, IGF-II*	Overexpression	Glioblastoma, medulloblastoma, breast, colorectal and pancreatic carcinomas, ovarian cancer
	IGF-IR	Overexpression	Breast and prostate tumors
	IGF-IR	Gene amplification	Breast and pancreatic cancer, melanoma
HGF	*MET*	Mutation (familial)	Hereditary papillary renal carcinoma
	MET	Mutation (sporadic)	Sporadic papillary renal carcinoma, hepatocellular carcinoma, gastric cancer
	MET	Overexpression	Hepatocarcinoma, colorectal, pancreas, stomach, prostate, ovary and breast carcinomas
	RON	Mutations	Gastrointestinal tumors
NGF	*trkA*	Translocation	Papillary thyroid carcinoma
	trkB	Overexpression	Pancreatic and prostatic carcinoma
	trkB, trkC	Mutations	Colon cancer
	trkC	Translocation	Congenital fibrosarcoma, secretory breast carcinoma
Axl	*Axl*	Overexpression	Lung cancer, breast carcinoma, glioma
Ephrin	*EPHB*	Loss of expression	Colorectal tumor
GDNF	*RET*	Mutation (familial)	MEN 2 (medullary thyroid cancer, pheochromocytoma)
	RET	Mutation (sporadic)	Medullary thyroid cancer
		Chromosomal rearrangements	Papillary thyroid cancers
DDR	*DDRs*	Overexpression	Breast cancer
Orphan receptors	*c-ros*	Overexpression, translocation	Brain tumors

under the control of the widely expressed collagen type 1 alpha 1 promoter, leading to an autocrine loop [55]. The more recently discovered PDGF-C and PDGF-D ligands have been reported to possess transforming capacity in some glioblastomas [56] and may be involved in different types of cancers, including Ewing sarcoma, medulloblastoma, and prostate cancer [37]. Aberrant stimulation of PDGF signaling in cancer can also be caused by ligand-independent activation of PDGFRs. For example, mutations in the catalytic and juxtamembrane domains of PDGFRA have been identified in gastrointestinal stromal tumor (GIST), a rare malignancy of mesenchymal origin [57]. Of note, PDGFRA and c-kit mutations in GIST are mutually exclusive, indicating that these RTKs activate common oncogenic downstream pathways in this type of cancer [57, 58]. Another mechanism of ligand-independent activation of PDGFRs has been described in hematological malignancies, where gene translocations can provoke the fusion of PDGFRα or PDGFRβ catalytic domain with different partner proteins [57].

Colony stimulating factor 1 (CSF-1) or macrophage-colony stimulating factor (M-CSF) also belongs to the PDGF family of RTKs [59]. This molecule promotes the growth and maturation of monocytes and macrophage precursors. It also enhances the phagocytic and tumoricidal activity of human macrophage/monocytes and induces them to secrete a variety of different cytokines [60]. Two active forms, one of which is secreted and the other cell associated, arise from differential splicing. The *c-fms* proto-oncogene encodes the CSF-1 receptor [4], another member of this RTK family, which is expressed in cells of the monocyte/macrophage lineage, as well as in the central nervous system (CNS) and placental trophoblasts [61]. Several studies have reported elevated CSF-1 serum concentration in patients affected with different types of cancer [62], and it has been proposed that CSF-1 can stimulate tumor growth and progression to metastasis [63]. For example, one study showed that the invasiveness of breast carcinoma is promoted through a paracrine positive loop between cancer cells secreting CSF-1 and macrophages producing EGF [64].

Stem cell factor (SCF), also designated kit ligand, mast cell growth factor, or steel factor, is a hematopoietic and tissue growth factor consisting of a glycosylated 165 amino acid polypeptide that forms dimers [65]. SCF exists in a secreted or a membrane bound form, resulting from alternative splicing. The SCF/kit ligand is present at relatively high levels in human plasma and it has been shown to augment the in vitro proliferation of both myeloid and lymphoid hematopoietic progenitor cells in the presence of other cytokines [66, 67]. SCF binds to a RTK encoded by the *c-kit* proto-oncogene. The extracellular portion of c-kit contains five immunoglobulin-like domains, which determine the binding specificity and facilitate dimerization. An intracellular juxtamembrane domain separates the transmembrane region and the kinase domain. It has been shown that *c-kit* is frequently mutated in GIST. Activating mutations in *c-kit* gene have been reported on exon 9, 11, and 13, encoding the extracellular dimerization domain, the juxtamembrane domain and the kinase domain, respectively [68]. Other malignancies showing activation of SCF-c-kit signaling through an autocrine or paracrine mechanism include small cell lung cancer (SCLC) and testicular germ cell tumor [68].

The Flt3 ligand is a transmembrane protein that undergoes proteolytic cleavage to generate a soluble factor with a certain similarity in the conserved cysteine residues with the kit ligand [65]. The Flt3 receptor was cloned by low stringency hybridization with a c-fms probe [69] and contains, as the other members of this family of RTKs, an extracellular domain with five immunoglobulin-like domains, a transmembrane region, a cytoplasmic juxtamembrane domain, and two kinase domains liked by a kinase-insert domain [70]. Flt3 is expressed at high levels in CD34+ short-term hematopoietic stem cells, and the Flt3 ligand exerts a proliferative effect on both the myeloid and lymphoid lineages, usually in combination with other cytokines [70]. Activating mutations of Flt3 are the most frequent genetic abnormality in acute myeloid leukemia (AML) and include in-frame tandem duplications in the juxtamembrane domain or missense point mutations in the kinase domain [70].

9.4.2 Vascular Endothelial Growth Factor Family

Efforts to identify factors that control angiogenesis led to the identification of a potent mitogen for vascular endothelial cells of small and large vessels [71, 72]. VEGF was initially isolated from conditioned medium of folliculostellate and bovine pituitary follicular cells [73]. Similar to PDGF, VEGF contains a cystine-knot domain, and the two growth factors are thought to derive from a common ancestor polypeptide [74]. The most abundant and active member of the family, VEGF-A, is a glycosylated, dimeric heparin binding protein capable to stimulate angiogenesis and to increase the permeability of capillary vessels to different macromolecules. Alternative splicing originates several isoforms of VEGF-A. The predominant human isoforms, VEGF-A-121 and VEGF-A-165, are secreted from producing cells, whereas VEGF-A-189 and VEGF-A-206 are not efficiently secreted and seem to bind tightly to cell surface heparin-like molecules [75]. Substantial evidence indicates that the binding of the best-studied form VEGF-A-165 to its receptor is dependent on cell surface associated heparin-like molecules. Four additional endothelial growth factors, which are structurally related to VEGF, have been reported [76–78]. VEGF-B and its alternatively spliced isoform, described as

VEGF-related factor (VRF) [79], are predominantly expressed in embryonal and adult myocardial and skeletal muscle tissues. VEGF-B isoforms can bind VEGFR-1 but not VEGFR-2 or VEGFR-3 [77]. Inactivation of the gene encoding VEGF-B suggested an involvement of this growth factor in heart development and inflammatory angiogenesis [77]. VEGF-C is a ligand for VEGFR-2 and VEGFR-3 receptors, and it is expressed in different organs including the heart, small intestine, placenta, ovary, and the thyroid gland [77]. Mice lacking both VEGF-C alleles die of edema due to incomplete development of lymphatic vessels, while the loss of one VEGF-C allele provokes less severe defects in lymphatic vasculature [78]. VEGF-D binds VEGFR-2 and VEGFR-3 and has both angiogenic and lymphangiogenic properties. VEGF-D is present in most human tissues, with highest expression levels in the lung and skin during development [78]. It has been shown that both VEGF-C and VEGF-D play important roles in promoting tumor-induced lymphangiogenesis [80, 81]. The last member of the VEGF family of growth factors, VEGF-E, was discovered in the genome of the parapoxvirus, which provokes skin lesions with extensive capillary proliferation [82]. VEGF-E is a potent angiogenic factor that binds with high affinity to the VEGFR-2 receptor [78]. More recently, a new VEGF was identified in snake venom and called VEGF-F [78].

Three high-affinity receptors for VEGF have been identified [75, 78]. These receptors, termed VEGFR-1/Flt1 [83], VEGFR-2/KDR/Flk-1 [84] and VEGFR-3/Flt4 [85] are characterized by an extracellular region containing seven (VEGFR-1 and VEGFR-2) or six (VEGFR-3) Ig-like domains and a tyrosine-kinase interrupted by a large kinase insert. VEGFRs are expressed by vascular endothelial cells although expression has also been detected in certain hematopoietic cells, such as monocytes, and in melanoma cell lines. VEGFR-1 was initially considered a decoy receptor reducing the levels of circulating VEGF, but more recent studies have shown that VEGFR-1 can mediate mitotic signals and be involved in the recruitment of endothelial progenitor cells [86]. VEGFR-2 is reported as a major regulator of vasculogenesis and angiogenesis. Its biologic role has been clarified by disruption of this gene in mouse embryos, which failed to develop both endothelial and hematopoietic cells [87]. Heparan sulfate proteoglycan and neurophilin can act as co-receptors by enhancing binding of several VEGF isoforms to VEGFR-2 [75]. VEGFR-3 expression pattern and knockout studies suggest that this receptor is essential during embryogenesis for the development of blood vessels, but it becomes redundant in mature vessels [77]. It has been shown that VEGF-A can promote cell migration and proliferation through direct activation of PDGFR-α and PDGFR-β in human mesenchymal stem cells, providing evidence of cross talk between VEGF and PDGF signaling pathways [88].

Placenta-derived growth factor (PlGF) is another member of this family originally discovered in the placenta [89] and also expressed in the heart and lungs. PlGF forms homodimers or heterodimers with VEGF and binds to VEGFR-1 [78]. PlGF inactivation revealed that this growth factor is not involved in embryonic angiogenesis but it is required in angiogenesis, plasma extravasation, and collateral growth in response to ischemia and inflammation or during wound healing [90]. PlGF has been implicated in colorectal cancer, acute lymphoblastic leukemia (ALL), and Ewing's sarcoma [78].

9.4.3 Epidermal Growth Factor Family

EGF consists of 53 amino acids constrained by three internal disulfide bonds and is generated from a 1200 residue precursor containing 8 units similar to EGF. This precursor is a glycosylated transmembrane protein that undergoes proteolytic cleavage by cell-surface proteases to release the mature form of the ligand [91–93]. Other members of this widely expressed EGF family include transforming growth factor-α (TGF-α) [94], amphiregulin (AR) or schwannoma-derived growth factor (rat homologue of AR), heparin-binding EGF (HB-EGF), betacellulin, the poxvirus mitogens (vaccinia, Shope, and myxoma growth factors), epiregulin, and the neuregulin family. All of these molecules share sequence similarity, at least 28 % sequence identity, and 100 % conservation of the six cysteine residues within the mature sequence of EGF. This EGF-like motif (XnCX7CX2-3GXCX10-13CXCX3YXGXRCX4LXn) is also present in diverse proteins found associated with the cell surface or extracellularly but that are not ligands for the EGF receptor [95]. With the exception of the neuregulins, all of these proteins are able to bind to the EGF receptor and show mitogenic effects on EGF-responsive cells [95, 96]. The membrane-bound form of some of these ligands, including EGF and TGF-α, may interact with receptors on the surface of adjacent cells, thereby potentially contributing to cell–cell adhesion and to cell–cell stimulation [94].

Different ligands of the EGF family bind and activate the same receptor, implying a substantial functional redundancy within the family. Nonetheless, quantitative differences in their biologic activities have been demonstrated. TGF-α was initially identified in culture fluids from various oncogenically transformed cells [97], which gave rise to its designation as a transforming growth factor. TGF-α and EGF display similar ability to bind, activate, and down-modulate the EGF receptor in mammalian cells [97]. EGF is normally expressed in kidney and submaxillary glands and is produced in response to gastrointestinal tract injury [8], while TGF-α appears to be normally expressed by a variety of epithelial cells [94].

AR was initially purified from conditioned medium of a human breast adenocarcinoma, MCF-7, treated with phorbol 12-myristate 13-acetate [98]. Relative to EGF, AR contains a very basic 40 amino acid stretch at its N-terminus, which is also rich in potential N-linked and O-linked glycosylation sites. AR is a heparin-binding growth factor, whose bioactivity can be inhibited by heparin sulfate [99]. It has also been shown that extracellular heparan sulfate proteoglycans are essential for mediation of its mitogenic signal by EGFR [100]. Studies have demonstrated the important role of AR in kidney and postnatal mammary development [101, 102], as well as liver regeneration [103].

HB-EGF was initially purified from conditioned medium of macrophage-like U937 cells, and it is a more potent mitogen for smooth-muscle cells than either EGF or TGF-α [104]. It is also active on fibroblasts, but not endothelial cells. Like TGF-α and AR, HB-EGF is secreted by means of proteolytic cleavage of a transmembrane precursor. In some instances, this processing does not occur with a high degree of fidelity and at least five different forms with amino-terminal heterogeneity have been identified [105]. It has also been demonstrated that the membrane-anchored form of HB-EGF acts as the diphtheria toxin receptor [106]. Betacellulin, initially isolated from an insulinoma-derived cell line, is a potent mitogen for retinal pigment epithelial cells and vascular smooth-muscle cells [107]. Epiregulin was identified as a novel EGFR ligand, isolated from the conditioned medium of a tumorigenic clone of NIH3T3 fibroblasts. It was shown to be capable of inhibiting the growth of several epithelial tumor cells and stimulating the proliferation of fibroblasts, hepatocytes, and smooth-muscle cells [108].

Purification of rat and human stimulatory proteins for the second member of the EGF receptor family led to the isolation of cDNAs encoding novel EGF-related proteins. The 44 kDa rat factor, termed Neu differentiation factor (NDF), stimulates p185neu tyrosine phosphorylation and induces the production of milk components in certain breast carcinoma cell lines [109]. The homologous human factors, termed heregulins (HRGs) or neuregulins (NRGs), were found to be mitogenic for certain mammary tumor cells [110]. These factors are encoded by the same gene, NGR-1, through alternative splicing of at least six recognizable domains: the N-terminal region, an immunoglobulin (Ig) motif, a glycosylation-rich spacer motif, an EGF-like domain, a hydrophobic transmembrane domain, and a cytoplasmic tail [111]. At least 26 different NRG-1 isoforms have been described in different species, including the acetylcholine receptor inducing activity, and glial growth factors (GGF). Based on the N-terminal domain, these isoforms are divided into three types: HRG (type I), GGF (type II), and sensory and motor neuron derived factor (SMDF, type III) [112]. Another characteristic used to classify NRG-1 isoforms is the presence of exon α or β, which encode the last cysteine of the EGF motif. It has been shown that β isoforms have higher receptor affinity and are generally more potent than α isoforms [112]. Like TGF-α, NRGs display a wide distribution in many tissues and organs. Moreover, the expression patterns of some isoforms are tissue specific. For example, the α2 isoform is the predominant form in mesenchymal tissues, whereas the β1 isoform is enriched in brain tissue and spinal cord [111, 112]. Three other genes with a high degree of homology to NRG-1 and displaying different affinities for EGFRs have been identified. NRG-2 and NRG-3 are expressed predominantly in neural tissues both in embryos and adults. NRG-4 was detected in adult pancreas and weakly in muscle [96, 112].

The EGFR was identified and isolated by biochemical techniques and shown to be the cellular homologue of the *v-erbB*, a retroviral oncogene [113]. The other members of the EGF/ErbB receptor family were isolated from genomic DNAs and cDNA libraries by low-stringency hybridization techniques using conserved tyrosine kinase domain probes and include ErbB-2, (also known as HER-2, for homologue of the human EGF receptor, or c-neu for homologue of the rat proto-oncogene neu), ErbB-3, and ErbB-4 [96, 114]. The extracellular domains of each of these molecules contain cysteine-rich motifs in two distinct regions and an uninterrupted tyrosine kinase domain. Whereas the EGFR and ErbB-2 are expressed in a wide variety of cell types, the expression of ErbB-3 is restricted to cells of epithelial or neuroectodermal origin. The four members of this family are normally co-expressed in various combinations in many tissues other than the hematopoietic system [96, 115]. ErbB-2 and ErbB-3 are nonautonomous receptors: ErbB-2 lacks the capacity to interact with a growth factor, whereas ErbB-3 lacks functional kinase domain. However, these two receptors are capable of generating a potent signal through heterodimerization with EGFR, ErbB-4, or each other [93]. EGF family ligands have been classified based on their affinity for EGF/ErbB receptors. The first group of ligands binds to the EGFR and includes EGF, TGF-α, AR, HB-EGF, betacellulin, and epiregulin. The second group, represented by betacellulin, HB-EGF, and epiregulin, binds the EGFR and ErbB4, while all NRGs bind to ErbB3 and ErbB4. ErbB receptor heterodimerization allows the receptors to work synergistically by expanding the array of signaling events that can be activated by a single molecule [116]. Gene inactivation of different components of the EGF signaling showed its crucial role in development, in particular in skin, gastrointestinal tract, kidney, mammary gland, and heart [93, 117]. An aberrant activation of the EGF signaling is a common event in many types of cancer and it can be due to different mechanisms. For example, autocrine production of EGF, TGFα, or NRGs is associated with reduced patient survival in different cancers [93, 118]. Studies have shown that over-expression of EGFR occurs in 80 % of head and neck

cancers, whereas ErbB-2 amplification is frequent in breast and ovarian carcinomas [93, 96]. Large deletions in the dimerization and ligand-binding domain of EGFR are relatively common in brain tumors, while activating mutation in its catalytic domain are present in 10–35 % of non-small-cell lung cancers (NSCLC), according to the population studied [25, 93]. Of note, rare mutations have also been reported for the kinase domain of ErbB-2 in NSCLC with wild-type EGFR and KRAS [119].

9.4.4 Fibroblast Growth Factor Family

FGFs comprise a family of growth factors present throughout evolution from nematodes to human that exert mitogenic activities toward a wide variety of cells of mesenchymal, neuronal, and epithelial origin. While *Caenorhabditis elegans* and *Drosophila* possess only two and three FGF genes, respectively, vertebrate FGF families are larger, ranging from ten genes in Zebrafish to 22 in mouse and human, including acidic FGF (aFGF, FGF-1), basic FGF (bFGF, FGF-2), int-2 (FGF-3), hst/KS3 (FGF-4), FGF-5, FGF-6, keratinocyte growth factor (KGF or FGF-7), androgen-induced growth factor (AIGF or FGF-8), and glia activating factor (GAF or FGF-9) [120–122].

The first to be isolated, bFGF, was recognized in certain hormone preparations by its mitogenicity for fibroblasts and chondrocytes and was later purified as a heparin-binding polypeptide from bovine pituitary. aFGF was purified independently from acidic extracts of bovine brain [123]. Both acidic and basic FGF are pleiotropic factors, capable to induce a strong angiogenic response in vivo, but are also involved in other processes, including neuronal regeneration and bone development [124]. Because of their proliferative effects, aFGF and bFGF have been implicated in different types of cancer [124, 125]. These two growth factors are single-chain polypeptides of about 17 kDa and share 55 % amino acid sequence identity. A striking feature of their structures, in contrast to those of most other family members, is the lack of a consensus secretory signal peptide. This has generated a great deal of speculation regarding their mode of release from cells. It has been shown that under stress conditions, such as hypoxia and low serum, cells secrete aFGF through the formation of a Cu^{2+}-dependent multiprotein complex that includes the S100A13 protein [126]. Conversely, the release of bFGF is constitutive, and it depends on Na^+/K^+ ATPases [124, 126]. Of note, higher molecular weight isoforms of bFGF have been identified that are expressed not only in the cytosol and the extracellular environment, but also in the nucleus, suggesting that products of the FGF-2 gene may act through alternative mechanisms in addition to signaling through their cell surface receptor [124].

Analysis of DNA of mammary tumors induced by mouse mammary tumor virus (MMTV) revealed that the viral genome frequently integrates within a genetic locus termed *int-2* and thereby activates expression of this gene by insertional mutagenesis. The protein encoded by *int-2*, renamed FGF3, is predicted to be 245 amino acids long and highly similar to aFGF and bFGF. The normal expression of FGF3 is apparently limited to embryonic tissues, and there is evidence from in vitro translation studies that it is a weak mitogen for mammary epithelial cells. A recent study revealed that individuals affected with a particular form of deafness presented a missense mutation in the *FGF3* gene, indicating an important role of this growth factor in the development of the inner ear [127]. Transgenic mouse experiments have shown that FGF3 expression leads to mammary gland hyperplasia in female mice and benign epithelial hyperplasia in the prostate of males [128]. A potential role of FGF3 has also been proposed for NSCLC, where it is frequently overexpressed together with EGFR [129].

FGF-4 and FGF-5 were uncovered during searches for oncogenes in human tumor cells [123, 130]. FGF-4 was isolated independently from a human stomach tumor (hst) and a Kaposi's sarcoma (KS3). It is mitogenic for vascular endothelial cells, human melanocytes, and mouse NIH/3T3 fibroblasts [123]. The FGF-5 gene was also isolated by DNA transfection, but by use of a selection system in which cell proliferation was dependent on abrogation of growth factor requirements. FGF-5 was activated by a DNA rearrangement that juxtaposed a retrovirus transcriptional enhancer upstream of its natural promoter. FGF-5 was found to be mitogenic for mouse fibroblasts and bovine heart endothelial cells [123].

An elegant demonstration of the critical importance of different FGF family members at specific phases of normal development derives from gene knockout experiments in mice. Such studies have shown that FGF-4 is required at a very early stage of development involving implantation of the embryo [131]. The absence of FGF-5 is associated with a very different phenotype, in which affected mice develop apparently normally and show only increased hair length following birth [132].

Isolation of additional members of gene families is sometimes possible by low-stringency molecular hybridization employing probes derived from the most highly conserved sequences. FGF-6 was isolated by this approach from a cosmid library prepared from a human lymphoblastoid cell line and was shown to act as a transforming gene for NIH/3T3 cells by transfection analysis [121]. KGF was isolated from medium conditioned by human embryonic lung fibroblasts and was found to be a potent mitogen for epithelial cells but to lack activity on fibroblasts or endothelial cells [133]. Thus, KGF was distinct in its target cell specificity not only from other members of the FGF family, but from other

known polypeptide growth factors as well. Molecular cloning and sequence analysis established KGF as a member of the FGF family, whose predicted amino acid sequence is about 38 % identical to those of aFGF and bFGF. KGF is expressed by stromal, but not epithelial, cells of most major epithelial tissues [134]. There is evidence that KGF plays a role in epithelial renewal during wound repair [135] and as a stromal mediator of epithelial cell proliferation/differentiation in sex hormone-responsive tissues [136], supporting the concept that this factor is important in the normal mesenchymal stimulation of epithelial cell growth. More recently, it has been shown that KGF and the related FGF-10 and FGF-22 share a role in the presynaptic differentiation of the mossy fibers in the cerebellum [137].

AIGF [138] was isolated from media conditioned by a cell line derived from a testosterone-dependent mouse mammary tumor cell line, and it is approximately equidistantly related to the other FGFs. Target cells include epithelial and fibroblast cells, and it appears to act in an autocrine fashion to stimulate the proliferation of mammary carcinoma cells, from which it was isolated. It appears to be restricted to expression in the testes in the adult, and its expression during development is maximal during the period of reproductive tract development [121].

GAF/FGF-9 was purified from supernatants of a cultured human glioma cell line [139]. It lacks a canonical signal sequence but was found to be efficiently secreted from COS cells transfected with the cDNA. Several other FGF family members have been identified by degenerate PCR or by searching genome databases for homology with known FGFs. The role of many of this factors, some of which lack signal peptides and contain nuclear localization sites, are still under investigation.

The prototype FGF receptor closely resembles the PDGF receptor family, but instead contains extracellular domain variants with two or three immunoglobulin-like motifs instead of five. Between the first and the second Ig domain, FGFRs contain a short domain referred to as the acid box domain. In FGFR1, this domain has a core sequence of eight consecutive acidic residues. In the cytoplasmic region, FGFRs contain a shorter (14 amino acids) insert within the tyrosine-kinase domain as compared to the PDGF receptor. Following the TK domain is a carboxyl terminal domain of approximately 55–65 amino acids containing multiple sites of autophosphorylation. The FGF receptor family includes four members (FGFR1, FGFR2, FGFR3, and FGFR4) displaying different affinity for the FGF ligands [121, 140]. Adding to this complexity, alternative splicing generates various isoforms of FGFR1, FGFR2, and FGFR3, including both cell-bound and secreted forms [141]. Of note, it has been shown that alternative splicing of the region encoding the third Ig domain has a profound impact on ligand–receptor binding specificity [122]. Another level of regulation in the signaling is the different spatial and temporal pattern of FGFR expression: FGFR1 is preferentially expressed in developing mesenchyme, FGFR2 predominantly in glial and epithelial cells, FGFR3 mainly in cartilage and blood tissues, and FGFR4 mostly in endoderm-derived tissues, such as the gastrointestinal tract and muscle [141]. Different types of malignancies, including gastric cancer, bladder and cervical carcinomas, myelomas, and skin cancer, contain sporadic mutations in FGFR2 and FGFR3 that cause ligand-independent dimerization or constitutive activation of the tyrosine kinase domain [142–144]. Conversely, fusion between FGFR1 and other genes, including the transcription factor ZNF198 and BCR, have been reported in some hematologic malignancies [144].

9.4.5 The Insulin Family

The diversity of metabolic effects of insulin has been studied intensively for decades [145]. Its primary in vivo functions involve the regulation of rapid anabolic responses, such as glucose uptake, lipogenesis, as well as amino acid and ion transport. In addition to its effects on metabolism, insulin stimulates DNA synthesis and cell growth. The insulin-like growth factors, IGF-I and IGF-II, were first recognized as serum factors, antigenically distinct from insulin. These molecules are induced by growth hormone and serve as its effectors in stimulating growth of skeletal tissues. IGF-I and insulin share 48 % of their amino acid sequences, and their similarity to IGF-II is 50 % [146]. Insulin is synthesized as a 109 amino acid precursor (preproinsulin), which is cleaved to produce an 86 amino acid single chain proinsulin. Proinsulin is further processed in the Golgi complex to insulin, which consists of two chains of 21 and 30 amino acids (A and B), linked by two disulfide bonds. IGF-I and IGF-II are single chain polypeptides with a structure analogous to proinsulin. The IGFs contribute to the insulin-like effects of serum on muscle and adipose tissue, but there are major differences between insulin and the IGFs. For example, whereas insulin levels fluctuate widely according to carbohydrate level, the IGFs are bound to carrier proteins and are maintained at steady concentrations in the blood stream. The carrier proteins belong to a class of proteins that have high affinity and specificity for the IGFs and are designated as insulin-like growth factor binding proteins (IGFBP). Six different IGFBPs have been identified in humans, and they are well conserved among mammals. The IGFBPs are involved in modulation of the proliferative and mitogenic effects of the IGFs at endocrine, paracrine, and autocrine levels [147, 148].

The insulin receptor, IR, is the prototype for a family of RTKs, whose distinctive structural feature is to function as a heterotetrameric aggregation of two α and two β subunits.

The extracellular ligand-binding subunit, containing a single cysteine-rich cluster, is disulfide linked to the transmembrane β subunit, which includes the cytoplasmic tyrosine kinase domain [149]. The IR binds insulin with approximately 100-fold greater affinity than it does IGF-I or IGF-II (100 pM versus 10 nM). The IGF-I receptor is closely related to the IR in sequence and structure, but binds IGF-I with highest affinity (100 pM), followed by IGF-II and insulin [150]. IGF-II is also bound by another receptor, which has been shown to be identical to the cation-independent mannose-6-phosphate receptor. The IGF-II receptor binds IGF-II and with a lower affinity IGF-I, but does not bind insulin, and it is thought to act as a scavenger receptor [151]. A gene encoding another family member, insulin receptor-related receptor (IRR), was identified by low-stringency hybridization of Southern blots of human and guinea pig genomic DNA probed with fragments of IR cDNA. Like the other family members, the IRR is synthesized as a single polypeptide precursor that is proteolytically cleaved into α and β subunits. It has been shown that the IRR does not bind insulin or the other related molecules [152]. However, the intrinsic kinase activity appears to phosphorylate endogenous proteins with a specificity very similar to that of the other two receptors of this family [153]. No ligands for IRR have been identified so far, and it is possible that this receptor forms heterodimers with the IR or IGF-R, in a way analogous to the ligandless ErbB2 in the EGF signaling.

Increased expression of IGF-I, IGF-II, IGF-IR, or combinations thereof have been observed in different types of cancer, including glioblastoma, medulloblastoma, breast, colorectal and pancreas carcinomas, and ovarian cancer [151]. These data, together with studies using cellular and animal models, suggest a role of this signaling in cancer growth and metastasis, and support the idea of the IGF system as a potential target for cancer therapy [151, 154].

9.4.6 Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) was isolated from plasma or platelets as a mitogen for hepatocytes in culture [155–157]. Independent studies led to purification of a fibroblast-derived factor that promotes dissociation and motility of epithelial cells, termed scatter factor (SF) [158], which is identical to HGF. The biochemical and biologic properties of HGF/SF were found to differ from those of other known growth factors. The molecular weight of native HGF is around 90 kDa and consists of two polypeptide chains of about 70 and 34 kDa linked by disulfide bonds. Cloning of HGF/SF cDNA showed that the growth factor is encoded as a single transcript whose 728 amino acid product is processed by proteolytic cleavage into heavy and light chains [157]. The predicted amino acid sequence of HGF/SF was found to be related to plasminogen. In addition to their 38 % sequence identity, both molecules contain serine-protease domains and disulfide bond-linked intrachain structures known as kringles. Neither plasminogen nor plasmin have HGF-like activity, and HGF is not likely to be a protease since the histidine and serine residues in the region corresponding to the catalytic site are replaced by other amino acids [157].

HGF/SCF has been shown to have an expanding array of biologic activities. It is mitogenic for a variety of epithelial cells, as well as endothelial cells and melanocytes. HGF/SCF is also capable of inducing certain cell types to undergo morphogenesis when suspended in a semisolid matrix. For example, it induces tubule formation in canine epithelial cells, which undergo scattering under standard culture conditions. Thus, HGF/scatter factor is a mitogen, a motogen, and a morphogen [159, 160]. The HGF gene knockout is an embryonic lethal, leading to obvious abnormalities in liver and placenta development, as well as in the migration of myoblasts from the somites to the limbs and in motoneuron axon guidance [61, 161]. The increase of HGF/SCF levels following liver, kidney, or heart injury, and the expression of HGF/SCF and its receptor in mesenchymal stem cells strongly suggest the involvement of this growth factor in wound healing and tissue repair [162, 163].

A ligand related to HGF, termed HGF-like or macrophage-stimulating protein (MSP) [164], is a heterodimer of a heavy chain of 53 kDa (α) and a light chain of 25 kDa (β). MSP, which is also referred to as scatter factor 2 (SF-2), shares with HGF the overall four kringle/protease domain-like structure. Liver appears to be the main source of MSP, and its major activity to date is stimulating macrophage migration [165].

c-met was initially identified as a rearranged oncogene in a human osteogenic sarcoma cell line transformed in vitro with a chemical carcinogen [166]. This proto-oncogene encodes a 190 kDa glycoprotein that is processed to form a heterodimer comprised of a 50 kDa α-chain and a 145 kDa β-chain. The extracellular, membrane spanning, and tyrosine kinase domains are located on the β-chain. The juxtamembrane domain contains serine and tyrosine residues that, upon phosphorylation, are involved in the degradation of the receptor, while the C-terminal tail is a unique docking site for the recruitment of several downstream signaling molecules [167]. MET is expressed in a variety of tissues and cell types, but the highest levels are found in epithelial cells. The expression of HGF/SF was first detected in mesenchymal cells of various organs. The MET receptor initiates all of the known responses to HGF/SF mitogenesis, including motility and morphogenesis [159, 160]. Although MET mutations are present in hereditary papillary renal carcinomas and in some sporadic cancers, the most frequent mechanism of HGF/MET activation in human tumors is overexpression of

the receptor, which is associated with increased invasiveness and a poor prognosis [161].

Ron/Stk was identified and isolated as a *c-met*-related gene from cDNA libraries of human keratinocytes and a gastric carcinoma by means of degenerate oligonucleotides [168]. Ron cDNA encodes a glycosylated protein, which shares overall similar topology with the HGF receptor, displays 63 % sequence identity in its catalytic domain and has a similar tissue distribution. The p185 RON product, like MET, is synthesized as a single-chain precursor, which is converted into the mature form by proteolytic cleavage. The ligand for RON is MSP [169]. Truncated variants of RON have been identified in tumors of the gastrointestinal tract, which display constitutive activation and transforming activity [165].

9.4.7 Neurotrophin Family

In addition to NGF [7], the neurotrophin family includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). These factors are crucially involved in central and peripheral nervous system (PNS) development and function, mediating processes such as survival, differentiation, and synaptogenesis [170]. Neurotrophins are 12–13 kDa proteins derived from 30–35 kDa precursors, proneurotrophins, by proteolytic cleavage. Both neurotrophins and proneurotrophins are secreted and can have opposite effects on target cells, such as survival versus apoptosis or synaptic long-term potentiation versus depression [171]. NGF is a basic 118 amino acid protein that acts in sensory and sympathetic neurons in the PNS [7]. NGF is also present in the brain, where it serves a trophic function in the development and maintenance of cholinergic neurons of the basal forebrain [170]. BDNF is necessary for the formation of the vestibular ganglia and have a key role in synaptic plasticity [170, 171]. NT-3 shows a strong sequence similarity to both NGF and BDNF and displays a high degree of regional specificity. Gene inactivation studies have shown that NT-3 participates in the development of the CNS and PNS, in particular for sensory neurons and the enteric nervous system [170, 172]. NT-4 was isolated from *Xenopus* as a molecule with the capacity to stimulate sensory neurons in culture. Another growth factor was almost simultaneously isolated from human and rat and termed NT-5, but was later shown to reflect the same gene. NT-4/5 is the less conserved factor among the family, and the inactivation of its gene provoked a mild phenotype, with reduced visceral afferent fibers in the small intestine and defects in nutrient feedback from the gastrointestinal tract [173, 174].

The neurotrophins interact with two distinct classes of receptors [175, 176]. One is represented by the p75 low-affinity neurotrophin receptor (p75NTR) protein [177]. This receptor is a highly conserved glycoprotein which is broadly expressed in neuronal and non-neuronal tissues. The extracellular region of the p75NTR contains a cysteine-rich domain, also found in the tumor necrosis receptor (TNFR) superfamily [178]. The intracellular domain is not related to any known protein and has no known enzymatic function. p75NTR acts as a co-receptor that allows the other type of receptor, Trks, to respond to low concentration of neurotrophins [178, 179]. p75NTR was recently found to function as a co-receptor for other ligands. Using a furin-resistant form of proNGF, Hempstead and colleagues showed that the NGF precursor binds with high affinity p75NTR and its co-receptor sortilin, but not Trks, and induces apoptosis in neuronal and glial cells [180, 181]. Finally, p75NTR can form a complex with the co-receptors NogoR and LINGO-1 to mediate the effects of the myelin-based growth inhibitors, Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein [178]. The second class of neurotrophin receptors is encoded by the *trk* genes. *Trk* or *trkA* was discovered in efforts to isolate oncogenes from human tumor cells [182]. The other two members of the family, *trkB* [183] and *trkC* [184], were isolated by screening mammalian cDNA libraries with the *trkA* proto-oncogene as probe. In contrast to the p75NTR, the cytoplasmic regions of trks contain tyrosine kinase catalytic domains. Their extracellular regions contain Ig-like and FNIII domains, in addition to cysteine clusters alternated with leucine motif repeats. Structural studies have shown that NGF binds the membrane-proximal Ig-C2-like domain of trkA, which induces receptor dimerization, phosphorylation of cytoplasmic tyrosine and recruitment of adaptor proteins that mediate the signaling cascades [179, 185]

Each member of the trk family can bind at least one member of the neurotrophin family [176, 186]. Although there are obvious preferences for binding of a particular neurotrophin to one of the trk family members, there is some promiscuity. In particular, the presence or absence of short sequences of amino acids in the juxtamembrane region of each trk, determined by alternative splicing, have profound effects on the specificity of the receptors for the different neurotrophins. In summary, trkA binds and becomes activated by NGF and NT-3 in the presence of the insert region. TrkB binds and is activated by BDNF, while the splice variant containing the insert in the juxtamembrane region also binds NT-3 and NT-4. The *trkC* product is activated by NT-3 but not NGF or BDNF [185]. Thus, at least three high-affinity receptors confer different but not absolute specificities for these related ligands.

The expression of neurotrophins and their receptors have been shown to affect the progression of different types of cancers. For example, activation of NT-3/trkC and NGF/trkA

can induce apoptosis in medulloblastoma and neuroblastoma cells, respectively [187]. Conversely, trkB, which is frequently overexpressed in cancer, such as pancreatic and prostatic carcinomas, is a potent inhibitor of anoikis, an apoptotic process resulting from loss of cell–matrix interactions and which is thought to act as a barrier to metastasis [188]. Of note, the kinase activity of trkB was shown to be required and sufficient to suppress anoikis [189], and mutations in the catalytic domain of trkB and trkC have been identified in colon cancer [190]. Another example of the implication of neurotrophin signaling in cancer is the translocation that generates the *ETV6-trkC* gene fusion. This constitutively active chimera, containing the dimerization domain of the ETV6 transcription factor and the catalytic domain of trkC, displays a strong transforming activity and it has been identified in congenital fibrosarcoma and secretory breast carcinoma [191–193]. *TrkA* is also subject to oncogenic rearrangements, and fusions of trkA catalytic domain with other genes, including *tropomyosin 3*, *translocated promoter region gene*, or *trk fused gene*, have been identified in papillary thyroid carcinomas [194].

9.4.8 Axl Family

Protein S, a protease regulator that functions as a potent anticoagulant [195], and Gas6 [196] have been shown to be the ligands for the Axl family of RTKs [197–199]. The critical role of protein S in the coagulation process is illustrated by the massive thrombotic complications suffered by infants homozygous for protein S deficiency. This 70 kDa protein contains several modules, including an N-terminal region containing 11 γ-carboxyglutamic acid residues (Gla), a thrombin-sensitive module, a series of EGF-like repeats that undergo hydroxylation modification, and a module with homology to steroid-binding globulin. The Gla region is posttranslationally modified in the endoplasmic reticulum by γ-glutamyl carboxylase, an enzyme that requires vitamin K as a cofactor [198]. Gas6, first cloned as a growth arrest-specific gene [200], was shown to activate Axl receptor and shares all but the thrombin-sensitive module with protein S [198]. While the majority of the studies on protein S focus on its anticoagulant properties, Gas6 is a more typical growth factor, promoting survival and proliferation. Inactivation of this gene by two independent groups showed that Gas6 promotes glomerular cell proliferation in a model of nephritis [201] and can act as an amplifier of the platelet response during thrombosis [202]. It has been previously shown that other coagulation factors such as thrombin are able to bind and activate intracellular signaling *via* G protein-coupled cell surface receptors. It is possible that proteases and protease regulators, which activate specific cell surface receptors, may serve to integrate coagulation with associated cellular responses required for tissue repair and growth [203].

Axl (from the Greek word *anexelekto* or uncontrolled), was identified and isolated independently as a transforming gene from patients with chronic myelogenous leukemia and myeloproliferative disorders, respectively, by DNA transfection-tumorigenicity analysis [204]. The murine homologue, adhesion-related kinase (*Ark*), was cloned on the basis of relatedness to the tyrosine kinase domain of one of the FGF receptors. The encoded Axl/Ark proteins define a family of RTKs, which feature a new sequence in their cytoplasmic tyrosine kinase domains and an extracellular domain that juxtaposes two Ig-like domains and two FN type III repeats. A similar external domain topology has been observed among several neural cell adhesion molecules and a receptor tyrosine phosphatase [205]. Axl expression has been detected in the majority of cell types examined, with the exception of lymphocytes and granulocytes. Another member of the Axl family was designated Tyro3 (Sky) [206]. The encoded protein showed around 64 % amino acid identity with Axl. Northern blot analysis further revealed that its mRNA is expressed predominantly in the brain, kidney, testis, and ovary [198]. A third family member, *c-mer*, was isolated from a human B-lymphoblastoid cDNA expression library [207]. Sequence comparisons showed that this gene is the human homologue of the chicken retroviral oncogene, *v-nyk* (renamed *v-eyk*), a truncated tyrosine kinase whose expression by retroviral infection produced sarcomas in chickens. The designation *c-mer* was based on its expression pattern in monocytes and tissues of epithelial and reproductive origin. Gas6 is considered the main ligand for this family of RTKs, while protein S can activate Sky and Mer, but only at high, and possibly not physiological, concentrations [198, 208]. The crystal structure of a minimal Gas6-Axl complex has been recently published [208]. The two Ig-like domains of an Axl monomer are linked to the steroid binding globulin domain of a Gas6 molecule. Lateral diffusion of these 1:1 complexes induces the formation of a circular 2:2 assembly [198, 208]. Of note, it has been shown that Axl constitutively interacts with interleukin (IL) 15 receptor α, and that both receptors can be triggered by IL-15 in mouse fibroblasts [209].

Axl signaling has been implicated in the invasiveness of different types of cancer, including lung cancer [210, 211], prostatic carcinoma [212], and gastric cancer [213]. Of note, Axl downregulation by RNAi in a xenograft model of breast carcinoma revealed a role of this RTK in neovascularization [214], while overexpression of a dominant-negative form of Axl inhibited glioma cell proliferation, migration and invasion [215].

9.4.9 The Ephrin Family

The EPH receptors are the largest subfamily of RTKs [216], with at least 16 different members described to date. These genes encode proteins of approximately 130–135 kDa, characterized by an extracellular domain containing two regions with weak similarity to an Ig-like loop and a cysteine-rich region, followed by two FN type-III domains [216]. The TK domains of the EPH receptors do not contain kinase insert sequences and are followed by a carboxy-terminal domain of approximately 90–100 amino acids. Of note, in the TK domains of two of these receptors, EphA10 and EphB6, residues normally essential for the catalytic function are not conserved, suggesting that these receptors might signal through a different mechanism [217]. EPH receptors are classified into two groups (A and B), based on relatedness of their extracellular domain sequences and their affinity for the two groups of ligands. This family of RTKs binds to membrane-bound molecules named ephrins [218]. The ephrins are divided into two groups: ephrin-A subclass, which is anchored to the membrane by a GPI linkage, and ephrin-B, which possesses a transmembrane domain [216]. With few exceptions, ephrin-A binds to EPHA receptors, whereas ephrin-B binds to EPHB receptors [217]. Ephrins are able to function when presented in a membrane-bound form, indicating that they require direct cell to cell contact to activate their receptors. Ephrins bind EPH on juxtaposed cell surfaces with nanomolar affinity, which then leads to the formation of a tetramer comprising two receptors and two ligands. These complexes can aggregate in larger clusters according to the density of Ephrin and EPH on the cell surfaces [217]. Activation by Ephrin induces EPH phosphorylation by the receptors themselves (transphosphorylation) or by other kinases such as those belonging to the SRC-family [217]. It has been shown that the transmembrane ephrin-B molecules can signal bidirectionally following binding and activation of receptors in a neighboring cell [219].

The best characterized role of the ephrin-EPH signaling is the establishment of organized neuronal connections and circuits in several regions of the brain [217]. This RTK family is also involved in the regulation of cell shape in other tissues, such as the vasculature, as well as in cell migration, proliferation and differentiation. In particular, it has been shown that ephrins can modulate the balance between self-renewal and differentiation in different types of precursor and stem cells [217]. Different EPHs have been shown to act as tumor suppressors in cancer, including prostate and colorectal [217, 220]. For example, recent studies indicated that loss of EPHB expression is important for the adenoma–carcinoma transition in colorectal tumors initiated by activation of Wnt signaling, suggesting that the specification of cell positioning can play an important role in inhibiting tumor progression [220, 221].

9.4.10 Agrin Family

Agrin, named from the Greek word *agrein* (to aggregate), was identified as a protein capable of inducing aggregation of acetylcholine receptors (AchRs) in myotubes at the postsynaptic membrane [222]. Agrin is a large heparan sulfate proteoglycan in the extracellular matrix that is capable of inducing aggregation not only of AchRs but also of other synaptic proteins including laminin, heparan sulfate proteoglycan perlecan, rapsyn, vinculin, and dystroglycan [223]. Agrin is synthesized by a large number of tissues and, based on its pattern of expression during fetal development, it has been suggested to play a role in neurogenesis, skeletal muscle development, and mesenchymal–epithelial interaction during organ formation [224]. The effects of agrin on myotubes were abolished by inhibitors of tyrosine phosphorylation. These investigations led to the identification of MuSK (muscle-specific kinase), a novel RTK characterized by an extracellular portion containing four immunoglobulin-like domains [225]. MuSK is expressed at the postsynaptic muscle-fiber membrane, and it is rapidly phosphorylated in the presence of agrin, leading to tyrosine phosphorylation of the AChR β subunit, which probably facilitates AChR clustering [226]. The similar phenotypes of Agrin and MuSK knockout mice confirmed that MuSK is the receptor for agrin at the neuromuscular junctions [227, 228]. It has been shown that the functions of agrin in the central nervous system are probably mediated by another receptor, the α3 subunit of the Na^+/K^+-ATPase [228].

9.4.11 GDNF Family

Glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ART), and persephin (PSPN) are members of the GDNF family of neurotrophic factors that, based on their cystine knot structure, represent a subgroup of the TGF-β protein superfamily [229]. GDNF was initially identified as a trophic factor for midbrain dopaminergic neurons [230], but it was shown to promote survival of many neurons belonging to the PNS and CNS. GDNF is the most potent survival factor for motor neurons yet identified. It has received attention as a potential therapeutic agent for the treatment of neurodegenerative diseases, in particular for Parkinson's disease, caused by the loss of dopaminergic neurons in the *substantia nigra*, and amyotrophic lateral sclerosis, provoked by the degeneration of motor neurons [231, 232]. NRTN and PSPN promote the survival of basal forebrain neurons [233], while ART supports the survival of sympathetic and sensory neurons [232]. The effects of all four GDNF family members are mediated by the RTK rearranged during transfection (RET). RET contains a typical intracellular tyrosine kinase domain,

but it is characterized by an unusual extracellular domain, comprising four cadherin-like domains and a cysteine rich domain. Unlike other RTKs, RET cannot bind GDNF directly, but it requires co-receptors called GDNF family receptor α (GRFα). The four GRFα receptors provide specificity for the ligand, with GDNF preferentially binding GRFα1, NRTN to GRFα2, ARTN to GRFα3, and PSPN to GRFα4. A dimeric GDNF family member first binds to GFRα and this complex then interacts with RET to induce its dimerization and activation [232, 234]. Germ line activating mutations of RET are found in multiple endocrine neoplasia type 2, causing medullary thyroid cancers (MTC) and pheochromocytomas. Somatic mutations of RET are also present in 50 % of sporadic MTC, and chromosomal rearrangements resulting in constitutively activated RET are frequent in sporadic papillary thyroid cancers [235].

9.4.12 Angiopoietins

The Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) family of RTKs includes two receptors that are almost exclusively expressed in endothelial cells and hematopoietic stem cells [236, 237]. Tie1 and Tie2 are characterized in their extracellular domains by two Ig-like loops separated by three EGF-like domains, followed by three FNIII repeats. The angiopoietins were originally identified as ligands for Tie2: Ang1 and Ang2 are the best characterized. Ang4 and its mouse ortholog Ang3 were identified later, and were found to act on human endothelial cells as Tie2 agonists and antagonists, respectively. Later studies revealed that Ang3 is instead an agonist in murine endothelial cells, indicating a species-specific activity [238]. No specific ligand has been identified for Tie1, but it has been shown that at high concentrations Ang1 binds to Tie1 through integrins [237]. While Ang1 and Ang2 bind to the same region in the extracellular portion of Tie2, only Ang1 is capable of promoting Tie2 autophosphorylation. Tie2 activation by Ang1 is required to maintain the resting state of the endothelium, whereas Ang2 destabilizes the quiescent endothelium and primes the response to exogenous stimuli [238]. Ang1 or Tie2 knockout mice are embryonic lethal and show a similar phenotype, with severe vascular remodeling defects. Interestingly, Ang2 transgenics phenocopy Ang1-deficient or Tie2-deficient mice, while inactivation of the Ang2 gene only induces a very mild phenotype [237]. There are conflicting findings concerning the role of angiopoietins in cancer, but a better understanding of the molecular mechanisms involved in their signaling could have important therapeutic outcomes, particularly for certain aspects of cancer biology, such as tumor angiogenesis, inflammation and vascular extravasation [239].

9.4.13 DDR Receptors

The discoidin domain receptor (DDR) family of RTKs includes two members that are activated by different types of collagen [240]. The discoidin domain was first identified in a protein isolated from the amoeba *Dictyostelium discoideum* and it is present in several extracellular and membrane proteins, including blood coagulation factors, enzymes and receptors [241]. The discoidin domain on the N-terminus of DDRs is necessary for the binding to collagen [242], but it has been proposed that DDRs may bind other factors, such as components of myelin in the CNS [241]. Another characteristic of this family of RTKs is the long juxtamembrane region, which includes 176 and 147 amino acids for *DDR1* and *DDR2*, respectively [240]. In certain conditions, DDR1 is processed into a membrane-anchored and a soluble subunit by proteases belonging to the ADAM family [240]. While inactivation of DDR1 and DDR2 genes in mice provokes dwarfism, the phenotypes are different. In particular, DDR1 appears to be involved in the morphogenesis and proliferation of the mammary gland, the vasculature, and the kidney, while DDR2 plays an important role in wound healing [240]. DDRs are often overexpressed in cancers of different origin, and it has been suggested that these RTKs may participate in cancer invasion and metastasis through their interaction with the collagenous extracellular matrix [240, 241]. Of note, DDR1 is a direct transcriptional target of the tumor suppressor p53, and it seems to alleviate the adverse effects of stress mediated by p53 [243].

9.4.14 Orphan Receptors

Several RTKs await the assignment of ligands. *Ros* is the cellular counterpart of the retroviral oncogene, *v-ros*, originally identified in the avian sarcoma virus UR2 [244]. The gene encodes a receptor-like PTK with an unusually large extracellular domain of nearly 2000 amino acids. It is closely related to the product of the *sevenless* gene from *Drosophila*, which determines cellular fate during development of the compact eye. However, *c-ros* does not appear to be expressed in avian or mammalian eyes [245]. In fact, in situ hybridization, studies have indicated that *c-ros* is mainly expressed in epithelial cells of the renal collecting ducts and intestinal villi and crypts [245]. A likely developmental role for *c-ros* has been inferred since expression of the proto-oncogene is detected only transiently during embryogenesis. Aberrant expression of c-ros is common in human brain tumors and a fusion of c-ros carboxy terminal domain with the protein Fused in Glioblastoma (FIG) was detected in U118MG human glioblastoma cell line, resulting in the constitutive activation of c-ros kinase domain. The c-ros-FIG fusion

protein cooperates with the loss of the tumor suppressor *Ink4a;Arf* to induce glioblastoma in mice, strongly suggesting that this orphan RTK can play a role in the onset of human brain tumors [246, 247]. The identification of a specific ligand should make it possible to better understand the functions of this protein.

Leukocyte tyrosine kinase (LTK) is expressed in B lymphocyte precursors, forebrain neurons in the mouse, as well as in placenta and hematopoietic cells in human [248]. Another member was identified as rearranged with the NPM nucleolar phosphoprotein gene in most anaplastic large-cell non-Hodgkin's lymphomas [249]. This gene, termed *anaplastic lymphoma kinase* (ALK), is normally expressed in the small intestine, testis, and brain, but not in normal lymphoid cells [250]. It has been suggested that ALK is the receptor for pleiotrophin family members [251], but this idea is still controversial.

ROR1 and ROR2 [252] define a family of receptors whose extracellular domains contain Ig-like, cysteine-rich, and kringle domains. Their tyrosine kinase domains are followed by serine/threonine-rich and proline-rich motifs. These receptors were originally identified on the basis of similarity of their TK domains to the Trk family of neurotrophin receptors. Both are widely expressed and at high levels during early rat embryonic development. Dror is the corresponding gene in *Drosophila* [253], where it is expressed specifically in the developing nervous system. The ROR receptors and NSK2 extracellular domains also show sequence similarity with the cysteine-rich domain of the frizzled proteins, which are non-tyrosine kinase receptors. Wnt ligands appear to bind this domain of frizzled. Indeed, ROR2 has been shown to bind to Wnt5a and possibly act in β-catenin-independent Wnt signaling [254, 255].

KLG and RYK represent two other distinct tyrosine kinase receptors. KLG (for kinase like gene) was isolated from a cDNA library prepared from embryonic chicken tissues using as a probe the *v-sea* oncogene [256]. KLG is a member of the immunoglobulin gene superfamily, with seven Ig-like loops in its extracellular domain. Its human homologue CCK-4, also called PTK7, was isolated from a colon carcinoma [257]. RYK (for related to tyrosine kinase) [258], a ubiquitously expressed gene, encodes a protein containing two putative transmembrane segments and two leucine-rich motifs in the extracellular domain [259]. The functional significance of these two genes is not known because efforts to demonstrate their tyrosine kinase activity have not as yet been successful. Both proteins contain several sequence substitutions in the conserved TK catalytic domain, similar to those present in the tyrosine kinase defective ErbB3. Ryk, which contains a Wnt-inhibitory factor-1 like domain in its extracellular portion, has recently been implicated in Wnt signaling [260, 261].

9.4.15 Signaling Pathways of Tyrosine Kinase Receptors

The activation of a RTK by its ligand or by other oncogenic mechanisms, such as receptor overexpression, mutation, or gene fusion, initiates a series of events that mediates the biological response in normal or cancer cells. An important role in these processes is played by a group of adaptor proteins that serve as a link between molecules of a signaling cascade and RTKs. It is generally considered that stimulation of effector proteins by RTKs can occur with three different mechanisms. In the first case, the activated receptor can induce membrane translocation of factors involved in signaling. For example, PDGF-induced stimulation of phosphatidylinositol 3 kinase (PI3K) generates the second messengers PtdIns(3,4)P2 and PtdIns(3,4,5)P3 that recruit to the membrane and activate Akt and PDK1 [PtdIns(3,4,5)P3-dependent kinase]. Another mechanism involves a conformational change, which results in the stimulation of an enzymatic activity. This is the case, for example, of Src, whose protein kinase activity is induced when its SH2 domain binds to tyrosine autophosphorylation sites of PDGFR. Finally, the signaling cascade can be triggered by RTK direct phosphorylation of the effector proteins, such as phospholipase Cγ, which can be tyrosine phosphorylated by EGF, PDGF, and FGF receptors [262] (Fig. 9.3). The signaling pathways activated by RTKs are extremely complex and interconnected. These pathways regulate fundamental processes, such as cell proliferation, survival, and differentiation, and they are frequently targeted by oncogenic events, which bypass the growth factor receptor–effector normal cascade, resulting in the constitutive activation of the signaling. For example, the oncogene Ras and the tumor suppressor PTEN are important players in two of the major signaling cascades activated by RTKs, the Ras-MAP kinase and the PI3K pathway, respectively. The limitation of space precludes a detailed discussion of the events downstream RTK activation. The last part of this chapter will focus on the role of growth factor signaling as a target for therapeutic strategies against cancer.

9.5 Implications for Cancer Therapy

We have seen in this chapter that growth factor signaling is often activated in cancer. In some cases, the activity of a particular pathway is essential for the tumor cell, a condition called oncogene addiction. In other tumors, the growth factor signaling contributes to the progression to a more aggressive disease. In both cases, the aberrantly activated signaling pathway represents a target of choice for therapeutic strategies. This type of therapy has great potential as it relies on blocking specific molecules rather than the traditional chemotherapy or

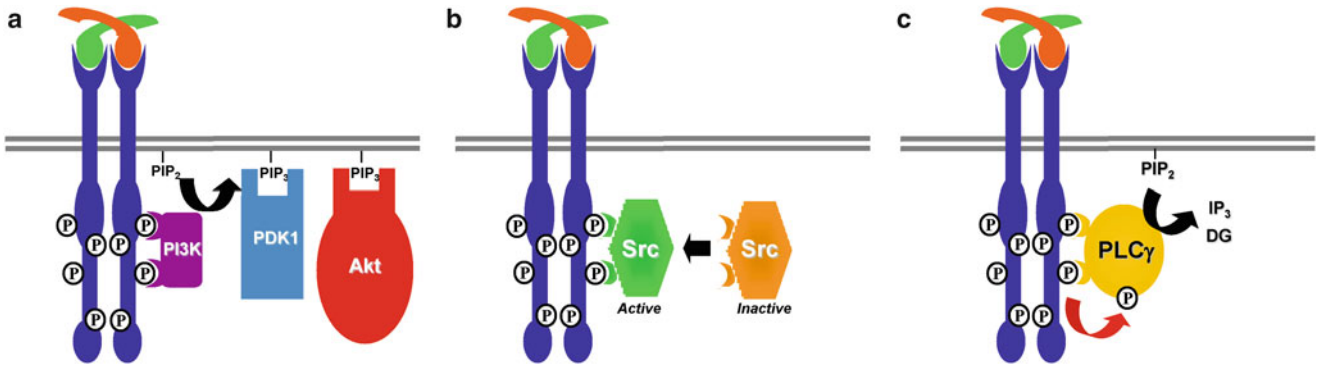

Fig. 9.3 Mechanisms for PDGFR-mediated activation of effector proteins. Simplified diagram illustrates different mechanisms of signal transduction following PDGFR activation. (**a**) PDGFR-induced stimulation of PI3K generates the second messenger PIP_3 that in turn recruits to the membrane and activates Akt and PDK1. (**b**) Src is activated by a conformational change following its binding to the PDGFR. (**c**) PDGFR can directly phosphorylate and activate PLCγ, which then generates the second messengers IP3 and diacylglycerol.

Table 9.2 Examples of Food and Drug Administration approved cancer drugs targeting RTKs

Drug	Target(s)	Disease
Monoclonal antibodies		
Trastuzumab (Herceptin)	ErbB-2	Breast cancer
Cetuximab (Erbitux)	EGFR	Colorectal cancer, head and neck cancer
Panitumumab (Vecitibix, ABX-EGF)	EGFR	Colorectal cancer
Bevacizumab (Avastin)	VEGF	Colorectal cancer, NSCLC
Small molecules		
Imatinib (Gleevec)	PDGFR, c-Kit, (ABL)	GIST
Gefitinib (Iressa)	EGFR	NSCLC
Erlotinib (Tarceva)	EGFR	NSCLC, pancreatic cancer
Lapatinib (Tykerb)	EGFR, ErbB-2	Breast cancer
Sorafenib (Nexavar)	VEGFR2, PDGFRβ, c-Kit, (Raf)	RCC, HCC
Sunitinib (Sutent)	VEGFR1-3, PDGFRα, RET, c-Kit	GIST, RCC

Some of these drugs can target non-RTK proteins. *NSCLC* non-small-cell carcinoma, *CML* chronic myeloid leukemia, *GIST* gastrointestinal stromal tumors, *RCC* renal cell carcinoma, *HCC* hepatocellular carcinoma

radiation therapy. Tremendous strides have been made in developing therapies that target oncogene products expressed at the cell surface, such as growth factor receptors using humanized monoclonal antibodies (Mabs) that either inhibit ligand/receptor interactions or cause receptor downregulation and host mediated immune responses [263–267]. One of these Mabs, Herceptin, which recognizes ErbB2, showed for the first time that a targeted therapy might be effective as a single agent and became the first clinically approved drug against an oncogene product (Table 9.2). These Mabs have been particularly effective when used in combination with traditional agents, such as in the case of Erbitux, a Mab targeting EGFR that has been approved for the treatment of colorectal and head and neck cancers in combination with chemotherapy and radiotherapy, respectively [117, 265, 268]. In addition to targeting an overexpressed receptor on which tumor proliferation is dependent, this approach could provide a means of targeting chemotherapeutic agents internalized by the receptor to sites within the tumor cell. As with other receptor targeting strategies, the efficacy of this approach would be expected to depend upon the differential magnitude of receptor expression by the tumor, as opposed to normal cells, as well as accessibility of tumor cells to the systematic administration of such agents [269].

Increased understanding of the important role of growth factor signal transduction in cancer has led to intensive efforts focused on the development of small molecule inhibitors of specific target molecules including RTKs and their

downstream effectors. The most exciting progress to date has come from investigations of Gleevec, a low molecular weight inhibitor of the tyrosine kinase activity of the PDGFR, c-kit, and the non-receptor tyrosine kinase Abl, which is translocated in chronic myelogenous leukemia (CML) as part of the Philadelphia chromosome to create the *Bcr-Abl* oncogene [270]. This inhibitor blocks ATP binding to the tyrosine kinase domain, preventing phosphorylation reactions involved in signaling [271]. Gleevec is used in the treatment of CML and it has been approved for GISTs, which contain c-kit or PDGFRA mutations [272, 273]. Other tumors with *c-kit* and *PDGFR* activating mutations are currently being evaluated for treatment efficacy by Gleevec [271, 274].

Iressa and Tarceva are examples of small molecules targeting growth factor receptors in cancer therapy. These compounds are reversible inhibitors of EGFR that have been approved for patients with advanced NSCLC who failed to respond to conventional chemotherapy. The first clinical trials showed that only a relatively small fraction of NSCLC patients responded to these drugs. Later studies revealed that sensitivity to these inhibitors correlates with activating mutations in the EGFR kinase domain, present in ~10 % of NSCLC patients, usually nonsmokers, more frequent in women and in individuals of Asian descendent [25]. Despite the initial remission, NSCLCs responsive to Iressa or Tarceva invariably develop resistance within 6–12 months of treatment, in half of the cases because of an additional mutation in EGFR (T790M) that weakens the interaction of the inhibitors with the kinase [25]. Of note, it has been shown that *MET* amplification and the resulting activation of ErbB3 is another mechanism responsible for resistance to these drugs [275], underlining the need of a therapeutic strategy that simultaneously targets different signaling pathways. Small molecules have already been generated or are under development to inhibit other growth factor receptors, such as MET [275] and IGF-R [276], as well downstream effectors, which should provide complementary therapies for a given type of cancer.

Another treatment modality that derives from increased understanding of growth factor signaling that occurs in the microenvironment of a tumor is the application of a Mab directed against the angiogenic growth factor, VEGF, which has been approved as a novel therapy in combination with other modalities. Whether this Mab acts by inhibiting tumor angiogenesis or by normalizing such vessels and actually improving access of traditional agents to the tumor is being evaluated, but there is little question that this approach can have therapeutic effects [266]. Other approaches to target the tumor microenvironment, including small molecules such as the VEGFR and PDGFR inhibitor Sunitinib, are also being used [266, 271, 277–279].

In the future, cancer treatment will most likely combine different target specific agents tailored to particular tumors. Novel therapeutics based on the knowledge of growth factor signaling pathways are already in the clinic, and there is no doubt that the number of rationally based strategies targeting these molecules will continue to grow.

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Senescence, Apoptosis, and Cancer

10

Manjari Dimri and Goberdhan P. Dimri

10.1 Senescence and Cancer

In 1961, in a landmark paper, Hayflick and Moorehead suggested that normal human fibroblasts do not proliferate indefinitely in culture [1]. Their observation led to discovery of cellular senescence, which now appears to be a basic fundamental property of all normal human somatic cells. The process of cellular senescence is a function of number of population doublings (PDs) versus proliferative capacity (Fig. 10.1). At increasing PDs, the proliferative capacity of cells decreases in culture. As a result, the increase in cell number reaches a plateau after a fixed number of PDs, and cells cease to proliferate. However, in case of tumor-derived cells, the increase in cell number does not reach a plateau and cells keep proliferating indefinitely. Such cells are immortal. Although it is mostly studied in culture, cellular senescence is now known to occur in vivo and play an important role in cancer and aging [2]. The vast majority of literature suggests that cellular senescence is involved in every aspect of cancer from its development, progression to treatment [3].

10.1.1 Cellular and Molecular Features of Cellular Senescence

Cellular senescence traditionally has been confused with cell death. Contrary to cell death, senescent cells are metabolically active and resistant to apoptosis [3, 4]. Initial characterization of senescent phenotype in culture from various laboratories suggested that senescent cells stop synthesizing DNA as determined by tritium-labeled (^{3}H) Thymidine or 5-bromo-2′-deoxyuridine (BrdU) incorporation, and are irreversibly arrested with a G1 DNA content [5]. The morphological features of senescent cells include big flat and vacuolated appearance [5]. Senescent cells express a senescence-associated β-galactosidase (SA-β-gal) marker, which is biochemically detected using X-gal as a substrate at pH 6.0 in human cells [6]. Increase in this enzyme activity probably reflects an increase in lysosomal compartment of senescent cells [7, 8]. The marker offers simple yet powerful tool to identify senescent cells in culture and in vivo, and has been widely used in several cancer-related studies.

The molecular features of senescent cells include altered expression of several genes [5, 9]. For example, several proliferation-associated genes such as gene encoding c-Fos, c-Myc, cyclin A, cyclin B, polo kinase PLK, centromere-associated protein CENP-A and CENP-F, and c-Myb and c-Myc are expressed, but downregulated in senescent fibroblasts [5, 9]. On the other hand, genes which encode proteins associated with maintenance and remodeling of ECM (extracellular matrix), such as stromelysin 1, stromelysin 2, collagenase, gelatinase, human macrophage metalloproteinase (HME), tPA, uPA, PAI 1, PAI 2, cystatin M, thrombospondin, dermatopontin, fibromodulin, collagens VI and XV, elafin, cathepsin D, cathepsin L, serpin b2, and other ECM-associated proteins are upregulated in senescent fibroblasts [2, 9].

10.1.2 Types of Cellular Senescence

The senescence phenotype which Hayflick and Moorehead first described is now known as replicative senescence. Replicative senescence is caused by repeated cell division or mitoses (Fig. 10.2). The prime cause of replicative senescence is telomere shortening or dysfunction [10]. However, it is very well established that a similar phenotype also results due to non-telomeric signals such as stress signals (Fig. 10.2) [10]. For example, cellular senescence in cultured murine cells is thought to be due to stress induced by culture conditions [11]. In addition, oncogenic and mitogenic signals, such as strong oncogenic signal provided by

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_10

activated H-RAS can induce senescence in primary cells (Fig. 10.2). Thus, senescence inducing signals can be telomeric or non-telomeric (Fig. 10.2). The non-telomeric signals include oncogenic, mitogenic, DNA damage, and undefined stress signals (Fig. 10.2). Senescence induced by non-telomeric signals is termed accelerated senescence, premature senescence, *st*ress or *a*berrant *s*ignaling *i*nduced *s*enescence (STASIS), and extrinsic senescence [12, 13].

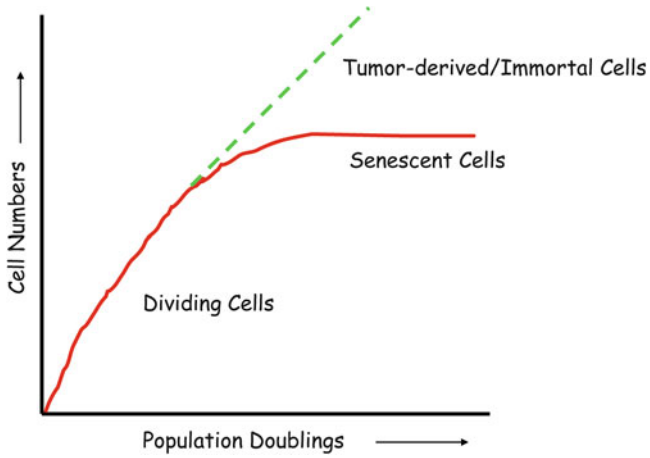

Fig. 10.1 In cell culture systems, cellular senescence is described as a decrease in proliferative capacity and eventual cessation of proliferation with increasing number of population doublings (PDs). This property of normal cells is also described as finite life span or Hayflick limit in the literature. Tumor-derived and stem cells contain telomerase activity and/or maintain telomere length. Such cells are immortal and do not exhibit cellular senescence in culture.

10.1.3 Mechanisms of Cellular Senescence

A large number of telomere binding proteins cap the end of chromosome known as telomeres, which consist of long stretch of repetitive sequences, such as (TTAGGG)*n* in humans [14]. In human somatic cells, progressive telomere shortening or telomere dysfunction appears to be the primary cause of cellular senescence [14, 15]. In most cases, the enzyme telomerase and telomere associated proteins maintain telomere homeostasis and telomere length. The catalytic subunit of telomerase (TERT) together with its RNA component (*TERC*), builds telomere repeats at the chromosome ends, which otherwise, owing to asymmetric DNA replication, are progressively lost [14].

Most human somatic cells do not contain sufficient telomerase to maintain telomere length [16], resulting in telomere shortening after each round of cell division. Consistent with this finding, exogenous expression of telomerase can either increase or stabilize telomere length in normal human cells, and in some cases results in cell immortalization [17]. It is thought that telomere dysfunction or uncapping triggers a DNA damage response, which activates p53 pathway resulting in proliferation arrest [18–20]. Consistent with this model, senescent cells are enriched in the nuclear foci of phosphorylated histone H2AX (γ-H2AX), p53 binding protein 53BP1, NBS1, the phosphoS966 form of SMC1 and MDC1, and inactivation of CHK1 and CHK2 (checkpoint kinases) can restore S phase progression in senescent cells [18–20]. Oncogene-induced senescence (OIS) has also been shown to be a DNA-damage checkpoint-response mediated by ATM and CHK2 [21, 22].

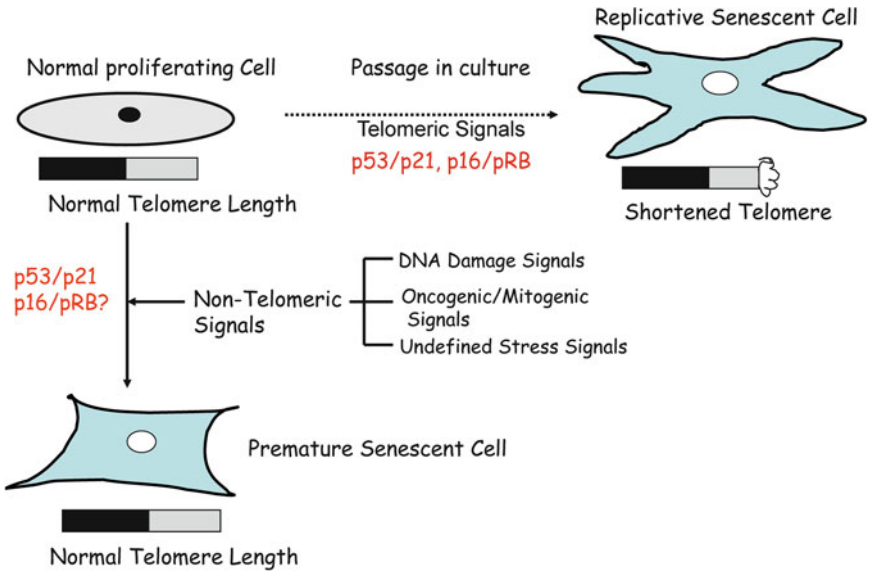

Fig. 10.2 Normal human cells undergo two types of senescence-replicative and premature senescence. Replicative senescence is caused by cell divisions (mitoses); during each cell division, telomeres shortens about 50–100 bp (base pairs). When telomeres length gets critically short to 1–2 kb (kilo base) from 8–12 kb, cells stop dividing and acquire a distinctive morphology. On the other hand, premature or accelerated senescence is caused by non-telomeric signals such as DNA damaging agents, constitutive oncogenic/mitogenic signaling, and undefined stress signals. During premature senescence, telomere length remains unchanged; however, the distinctive senescent morphology is still acquired by the cells.

The constitutive activation of p53 via ATM pathway results in induction of p53 target genes most notably p21 [23], which inhibits the activity of CDK2/cyclin E kinase. Inhibition of CDK2/cyclin E kinase activity results in hypophosphorylation of pRB (retinoblastoma tumor suppressor), which very likely mediates cell cycle arrest during senescence (Fig. 10.3). In addition to p21, other growth inhibitory p53 target genes may also play a role in cellular senescence [10]. Due to p53 and p21 activation and resulting G1 arrest, many proliferation-associated genes such as *CDK1*, *CDK2*, *cyclin A*, *cyclin B*, and *E2F1* remain repressed in senescent cells. In a parallel pathway, $p16^{INK4a}$ (CDKN2A) is upregulated during senescence [10]. $p16^{INK4a}$ inhibits the activity of other CDK-cyclin kinases such as CDK4-cyclin D1 and CDK6-cyclin D1. CDK4, CDK6, and cyclin D are expressed in senescent cells, but the activity of CDK-cyclin D complexes is lost during senescence. Thus, the repression of CDK kinases via multiple mechanisms results in hypophosphorylation of pRB. The hypophosphorylated or unphosphorylated pRB maintains permanent growth arrest phenotype of senescent cells (Fig. 10.3) [10]. Expression of $p16^{INK4a}$ is negatively regulated by BMI-1 oncoprotein [13], while p53 is negatively regulated by MDM2, PIRH2, COP1, and ARF-BP1 [24]. These regulators of $p16^{INK4a}$ and p53 are likely to impact cellular senescence and replicative life span of cells in vitro and in vivo (Fig. 10.3).

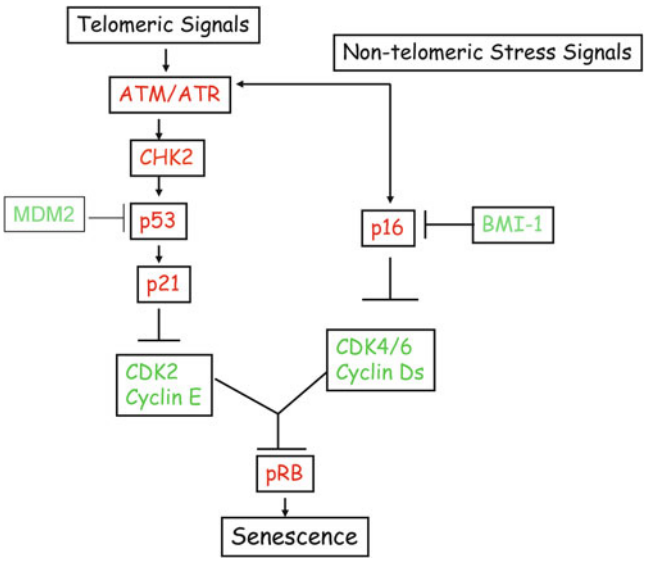

Fig. 10.3 Telomeric and non-telomeric stress signals induce senescence via tumor suppressor pathways. These signals activate a DNA damage response pathway mediated by ATM and CHK2. These DNA damage inducible kinases activate p53, which via p21 suppresses the CDK2/cyclin E activity to maintain pRB in unphosphorylated or hypophosphorylated (growth inhibitory) state. Stress signals in culture also activate $p16^{INK4a}$ (independent of p53). Very little is known about the upstream signaling molecules that mediate $p16^{INK4a}$ induction. p16 primarily inhibits CDK4/6 cyclin D activity. $p16^{INk4a}$ together with p21 and possibly other CDK inhibitors maintain pRB in a permanent growth inhibitory state, which ensures stability and irreversibility of senescent phenotype. $p16^{INK4a}$ is negatively regulated at the transcription level by polycomb proteins, in particular BMI-1. p53 is negatively regulated at the protein level by MDM2 and other ubiquitin ligases.

10.1.4 Telomere-Dependent Senescence and Cancer

Telomeres and Telomerase expression are linked to cancer in many ways. First, more than 90 % of tumors express telomerase [25] and tumor-derived cells usually maintain telomere lengths of their chromosomes. Second, several in vitro studies have shown that telomere attrition can induce senescence or apoptosis in tumor cells [26]. Third, the ectopic expression of telomerase in somatic cells prevents telomere shortening and in some cases results in immortalization of cells [17, 26], which may increase the possibility of accumulation of additional genetic abnormalities resulting in cancer initiation and progression. Indeed several studies have shown that hTERT overexpression is one of the four pivotal steps required to convert a normal human somatic cell into a cancer cell [27, 28]. These studies also showed that bypass of senescence by inactivating pRB and p53 is one of the other four requirements for in vitro transformation of human cells [10, 28, 29]. Based on these studies, one can hypothesize that telomere shortening should limit cancer progression in vivo. Until now, compelling evidence for such a hypothesis has been lacking because the laboratory mouse which is the most studied in vivo cancer model harbors very long telomeres.

Taking advantage of a mouse knockout model of telomerase ($mTR^{-/-}$), which lacks the essential RNA subunit of the enzyme telomerase, Felder and Greider recently showed that indeed short telomeres limit tumor growth in vivo by inducing cellular senescence [30]. Felder and Greider crossed $mTR^{-/-}$ mice with Eμ-Myc transgenic mice, which develop very aggressive Myc-driven Burkitt's lymphomas. It was found that at generation six (G-6), when the telomeres in $mTR^{-/-}$ mice get short enough, the progression of Eμ-Myc-induced Burkitt's lymphomas was completely blocked by apoptosis. To negate apoptosis in these animals, the investigators used Bcl-2 overexpression, and showed that short telomeres still inhibited lymphoma formation in these animals despite inhibition of apoptosis. The staining for SA-β-gal marker, $p16^{INK4a}$, and $p15^{INK4b}$ indicated that precancer cells in this setting were undergoing cellular senescence, implying that short telomere-driven senescence acts as a strong barrier to neoplasia in vivo [30]. Importantly, loss of p53 abrogated short telomere-induced senescence and promoted cancer progression [30]. Cosme-Blanco et al. also demonstrated that dysfunctional telomeres induce p53-dependent cellular senescence, which abrogated spontaneous tumorigenesis in $mTR^{-/-}$p53 (R172P) (telomerase knockout with mutant p53 knockin) mouse model [31].

Thus, telomere-dependent senescence also acts as a strong barrier to tumor-progression in vivo.

If the telomerase expression is required for oncogenesis, then inhibitors of telomerase should be considered attractive cancer therapy reagents. Indeed, inhibitors of telomerase are known to induce senescence and/or apoptosis in cancer cells. For example, Damm et al. reported that a highly selective small molecule inhibitor of telomerase caused progressive telomere shortening and induced senescence in p53-positive as well as p53-negative cancer cell lines [32]. Similarly, synthetic telomerase inhibitors MST-312, MST-295, and MST-199 were reported to induce senescence in cancer cells [33]. Riou et al. also demonstrated that a series of G-quadruplex DNA ligands can inhibit telomerase activity, cause telomere shortening, and induce cellular senescence [34]. Several other inhibitors of telomerase such as BIBR1532 and GRN163L exhibit growth suppressive and anticancer activity [35]. These studies suggest that senescence induced by telomere shortening is a tumor suppressive mechanism, and compounds that inhibit telomerase activity and induce senescence can be used as cancer therapy reagents.

10.1.5 Oncogene-Induced Senescence and Cancer

In 1997, Serrano et al. first reported that oncogenic RAS did not transform primary human cells but instead invoked a phenotype similar to cellular senescence, suggesting that senescence can be prematurely activated in response to an oncogenic stimulus [36]. This phenomenon of premature senescence by oncogenes is now popularly known as OIS [37]. Apart from oncogenic RAS, OIS is known to be induced by RAF, MYC, E2F1, and constitutive MAPK signaling in normal cells [10]. Recently, *MOS*, *cyclin E*, and *CDC6* oncogenes were also shown to cause OIS in human fibroblasts [21]. Since the discovery of Serrano et al. and others, OIS is now recognized as an important body defense mechanism against oncogenic insults. The in vivo evidence for such a role for OIS was reported in a series of papers published in 2005 [38–41]. It was found that the expression of B-RAF, K-RAS, and Eμ-N-RAS oncoproteins, or inactivation of the tumor suppressor PTEN induced a senescence response in vivo, and as a result, cancer progression was blocked at a very early stage [38–41]. Importantly, in these studies it was shown that cancer progression was only facilitated by additional mutations such as loss of p53, which caused bypass of senescence. Accordingly, malignant tumors that eventually arose in these models were largely devoid of biomarker of senescence including SA-β-gal.

Michaloglou et al. reported that benign nevi in human patients, which express oncogenic form of B-RAF, by in large are composed of senescent melanocytes as these cells express markers of senescence such as SA-β-gal and $p16^{INK4a}$, while the malignant melanomas were devoid of these senescence markers [41]. In a transgenic model of lung cancer progression by K-RAS, it was shown that the premalignant tumors but not the malignant tumors contained senescent cells [40]. In the Braig et al. study, OIS was shown as an initial barrier in lymphoma development in Eμ-N-RAS transgenic mouse model [38]. It was shown that the deficiency of Suv39h1, a methyltransferase, which silences proliferative genes to induce senescence, is required for lymphomagenesis induced by Eμ-N-RAS transgene. Finally, Chen et al. showed that PTEN inactivation per se leads to induction of p53-dependent premature senescence in vitro and in vivo [39]. It was also shown that early stage benign human prostate cancer samples but not the late stage prostate cancer samples contained senescent cells [39]. Collectively, these studies strongly suggest that OIS acts as an initial barrier to cancer progression in vivo [42].

10.1.6 Senescence and Cancer Therapy

Both OIS and telomere-based senescence are important for cancer initiation and progression. Apart from cancer initiation and progression, senescence is also important for cancer treatment. Several recent studies have shown that chemotherapeutic drugs and radiation can induce senescence in cancer cells [43]. Induction of senescent phenotype as measured by the expression of SA-β-gal marker has been reported in a number of cancer cell lines by doxorubicin [44–46], cisplatin [47–49], hydroxyurea [50–52], camptothecin [53–55], etoposide [55], and other chemotherapeutic agents.

It has been suggested that DNA-interactive chemotherapeutic drugs such as doxorubicin, aphidicolin, cisplatin, and cytrabine induce a robust senescence-like growth arrest while other cytotoxic drugs, such as taxol and vincristine, induce it only minimally [56]. Apart from in vitro studies, recent studies provide compelling evidence that cellular senescence can be induced in vivo by chemotherapeutic drugs, and that senescence induction by chemotherapy drugs might be an important mechanism of cancer growth inhibition by these drugs [55, 57, 58]. te Poele et al. reported that 41 % of breast cancers from patients who had undergone a chemotherapy regimen stained positive for SA-β-gal marker and $p16^{INK4a}$ protein, while normal tissues adjacent to tumors were devoid of senescence markers [55]. The investigators concluded that like apoptosis, senescence is a p53-induced cellular response to chemotherapeutic drugs in vivo and that it is an important factor in determining treatment outcome [55]. The induction of premature senescence by chemotherapy treatment of human lung cancer was also recently demonstrated [57].

A study by Schmitt et al. provides clear evidence for the induction of senescence by cancer chemotherapy reagents in

a mouse model [58]. The investigators generated Eμ-Myc-derived lymphomas in control-MSCV (vector), control-Bcl2, *p53* null-MSCV (vector), and *p53* null-Bcl2 mice. Mice bearing these four sets of lymphomas were treated with a chemotherapy drug CTX (cyclophosphamide). Using staining for SA-β-gal marker, $p16^{INK4a}$, and p53, the investigators showed that CTX is able to engage a senescence program when apoptosis is inhibited by Bcl2 overexpression (control-Bcl2 set) [58]. Furthermore, as a result of senescence induction, control-Bcl2 lymphomas did not progress and these animals had a better prognosis [58]. This is in contrast to *p53* null-MSCV and *p53* null-Bcl2 lymphomas, which progressed rapidly [58]. Induction of a senescence-like phenotype has also been reported in rat mammary tumors undergoing treatment with chemopreventive agents [59]. Thus, chemotherapeutic drugs can induce a senescence-like stage in vivo and in vitro by upregulating p53 and/or $p16^{INK4a}$, which may be an important mechanism of cancer growth inhibition by these drugs [10].

10.1.7 p53-Mediated Senescence in Cancer Treatment

p53 is mutated in a large number of human malignancies [60, 61]. p53 is also a major regulator of senescence and enforced expression of p53 or p21 in many cell types can induce a senescence-like phenotype [10]. Thus, it is natural to ask—does restoration of p53 in p53-deficient tumors induces senescence and stop cancer growth? Two elegant studies suggest that p53 restoration in a mouse model can cure cancer via induction of senescence and tumor clearance [62, 63]. In the first study, Xue et al. used RNA interference (RNAi) approach to conditionally regulate p53 expression in liver carcinoma in a mouse model [63]. The investigators showed that even a brief reactivation of endogenous p53 caused complete regression of the liver tumor. Importantly, the primary cause of cancer regression was not apoptosis, as one would have thought. Instead, cancer regression in this model involved induction of the cellular senescence program accompanied by the production of inflammatory cytokines [63]. The inflammatory cytokines triggered an innate immune response which enhanced tumor clearance. Thus, it was shown that p53-mediated induction of cellular senescence together with innate immune response can cure murine liver carcinomas [63].

Ventura et al. used a *Cre-loxP*-based conditional strategy to activate or inactivate endogenous p53 in a mouse model [62]. The investigators generated mice with a reactivatable p53 knockout allele by inserting a *loxP* flanked STOP cassette in the first intron of p53. This STOP cassette created a p53 null phenotype, which could be reversed to the p53-positive phenotype by removal of *loxP* sites (and STOP cassette) using a tamoxifen inducible Cre-recombinase. The p53-null mice in this setting developed lymphomas and sarcomas. After the tumors grew to a detectable size as measured by MRI imaging, the investigators restored p53 expression by administration of tamoxifen, which removed the STOP cassette and *loxP* sites. Within 24–48 h of activation of p53, it was shown that lymphomas regressed by apoptosis as determined by markers of cell death [62]. On the other hand, regression of sarcomas was only modest by 48 h and took several days for complete regression. The regressing sarcomas showed no apoptosis but rather were positive for markers of senescence such as SA-β-gal, $p16^{INK4a}$, and $p15^{INK4b}$. Thus, the investigators concluded that p53 restoration caused tumor regression in both lymphoma and sarcomas, although the outcome in the first case was based on apoptosis, and in the second case was based primarily on senescence induction [62]. The reasons for cancer clearance after senescence induction are not clear, but could involve immune response by the host.

10.2 Apoptosis and Cancer

Long before the role of senescence in pathogenesis of cancer was recognized, it was firmly believed that inhibition of apoptosis is an integral part of cancer development [64–66]. Current literature only reinforces this notion that apoptosis is an important determinant in the pathogenesis of cancer. Selective elimination of cancer cells by apoptosis is still the long sought promise of many cancer therapy drugs [67]. The process of apoptosis is irreversible and characterized by chromatin condensation, nuclear fragmentation, membrane blebbing and cell shrinkage, and formation of apoptotic bodies [68]. These properties of apoptotic cells are distinct and usually not associated with other forms of cell death such as necrosis, autophagy, and mitotic catastrophe [69]. In view of its highly regulated nature, apoptosis is also termed programmed cell death (PCD). Apoptosis is involved in many physiological processes such as embryonic development, morphogenesis and tissue homeostasis [70, 71]. It is dysregulated in many human diseases such as autoimmune diseases, AIDS, neurodegenerative diseases, and cancer [72]. Here, we briefly review the mechanisms of apoptosis, the role of apoptosis in cancer progression and induction of apoptosis by cancer therapeutics.

10.2.1 Mechanisms of Apoptosis

In mammalian cells, apoptosis is activated and executed through two major signaling pathways, the intrinsic and the extrinsic pathways (Fig. 10.4) [73, 74]. The intrinsic pathway is mitochondria-mediated and is triggered by intracellular

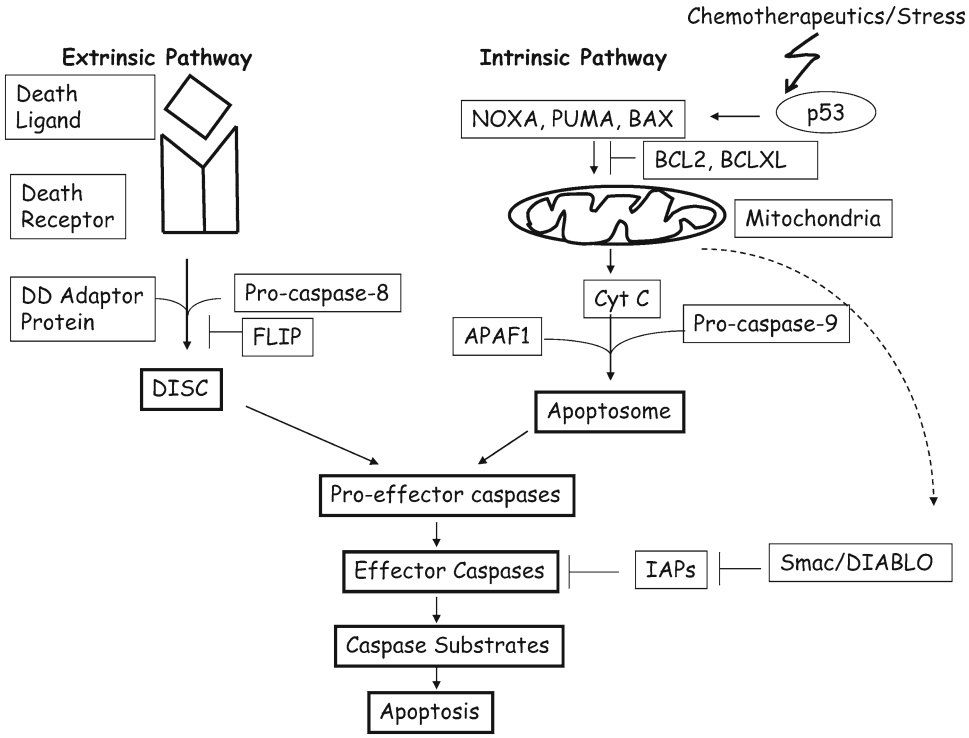

Fig. 10.4 A simplified cartoon of apoptotic pathways in mammalian cells. The extrinsic pathway of apoptosis is activated when a death ligand such as FASL, binds to pro-apoptotic receptor such as FAS. The downstream signaling is mediated by interaction of death ligand–death receptor complex with a death domain (DD) protein such as FADD. The association of these proteins with pro-caspase-8 results in DISC assembly, which activate effector caspases by proteolytic cleavage. Effector caspases cleave various caspase substrates to complete the process of apoptosis. FLIP inhibits DISC assembly by binding to caspase-8 and DD adaptor proteins. The intrinsic pathway of apoptosis is primarily mediated by mitochondrial release of Cyt-c. In response to DNA damage or other stresses, p53 is activated, which transcriptionally induce pro-apoptotic proteins such as BAX, NOXA, and PUMA. These proteins help release of Cyt-c from mitochondria. Release of Cyt-c is inhibited by BCL2 and BCLXL. Cyt-c release facilitates APAF1 and pro-caspase-9 assembly known as apoptosome, which activate casapase-9. Caspase-9 binds pro-effector caspases and activates them. IAPs negatively regulate effector caspases such as caspase-3, caspase-6, and caspase-7. Certain IAPs such as XIAP also binds and inhibits caspase-9. In response to apoptotic signals, mitochondria also releases an inhibitor of IAPs known as Smac/DIABLO. The role of these pro- and anti-apoptotic proteins is described in the main text.

cues such as DNA damage [73, 74]. The intracellular signals activated by DNA damage or other stresses result in the release of electron-transport chain intermediate Cytochrome-c (Cyt-c) to the cytoplasm from mitochondrial intermembrane space (IMS). Once released into the cytoplasm, Cyt-c facilitates the binding of procaspase-9 to APAF-1, which results in its activation and formation of the apoptosome containing activated caspase-9 [73, 74]. Release of Cyt-c from IMS is regulated by a balance between pro-apoptotic and anti-apoptotic members of the BCL2 (Bcl-2) family of proteins [74].

The detailed mechanism of action of these pro-apoptotic and anti-apoptotic proteins is still not clear. It is thought that pro-apoptotic BH3 (BCL2 homology 3)-only proteins such as BIK, BAD, BIM, NOXA, PUMA, and multi-domain BH proteins such as BAX, BAK, BCLB, BLCG, and others increase the permeability of mitochondrial membrane by forming pores. On the other hand, anti-apoptotic BCL2 family members such as BCL2, $BCLX_L$, BCLB, BCLW, and MCL1 prevent permeabilization of mitochondrial outer membrane by inhibiting the function of pro-apoptotic members of BCL2 family of proteins [74–76]. Several of these pro-apoptotic proteins such as NOXA, PUMA, and BAX are direct transcriptional targets of p53 [77–79], which is activated by DNA damage and other stress signals. p53 can also directly cause Cyt-c release from mitochondria and thus cause apoptosis without transcriptional activation of its target genes [80, 81]. Other molecules which regulate intrinsic pathway of apoptosis are IAPs (inhibitor of apoptosis) such as XIAP, which inhibits caspase-3, caspase-7, and caspase-9 [82]. XIAP in turn is inhibited by a pro-apoptotic protein Smac/DIABLO, which is released by mitochondria during apoptosis (Fig. 10.4) [83, 84].

The extrinsic pathway is death receptor-mediated and is activated at the cell surface by extracellular cues, whereby pro-apoptotic (death) ligand binds such as FASL, TNF, and TRAIL, bind and activate pro-apoptotic (death) receptors CD95/FAS, TNFR, and DR4/5 respectively [73, 74]. Death receptors contain related intracellular motifs known as death domains (DD), which interact with similar domains in corresponding adaptor molecules such as FADD and

TRADD. Through the interaction of DD of death receptors and corresponding adaptors, DISC (death-inducing signaling complex) is assembled at the plasma membrane. Adaptor molecules also contain death effector domains (DED), which help recruit procaspase-8 via its own DED domain to DISC [73, 74]. Once at DISC, high local concentration of procaspase-8 leads to its activation via autoproteolytic cleavage. Subsequently, caspase-3, caspase-6, and caspase-7 are activated, which ultimately lead to apoptosis by cleaving their substrates (Fig. 10.4) [73, 74].

Caspases belong to a family of cysteine-dependent aspartate-directed proteases [85]. There are two types of caspases, upstream or initiator caspases (caspase-8, caspase-9, and caspase-10), and effector or executioner caspases (caspase-3, caspase-6, and caspase-7) [85–87]. The upstream caspases activate effector or executioner caspases. The executioner caspases then cleave selected set of target proteins after an aspartate residue in their amino acids sequence [85–87]. The target of caspases include caspase-activated DNAse (CAD), nuclear laminins, cytoskeletal proteins, apoptosis inhibitory proteins such as BCL2 and BCL-X_L, poly(ADP-ribose) polymerase (PARP), and other proteins [85–87]. Cleavage of these various substrates ultimately results in nuclear fragmentation, membrane blebbing, and other morphological changes seen in apoptotic cells. Apart from caspases, non-caspase proteases such as cathepsins, calpines, and granzymes are also implicated in apoptosis [86, 88].

10.2.2 Apoptosis and Cancer Progression

The role of inhibition of apoptosis and cell death comes from epidemiological studies about cancer mutations, and in vivo mouse models where apoptosis related genes have been knocked out or overexpressed in transgenic models.

10.2.2.1 Apoptosis Genes and Cancer

Given the prime role of apoptosis in inhibition of proliferation and growth, and the clearance of deleterious cells, it is not surprising that many apoptosis-related genes are altered in cancer [89]. Many tumor suppressors that directly or indirectly inhibit apoptosis are mutated, deleted, or underexpressed in a variety of cancer cells. Here, we summarize genetic aberrations in few important apoptosis-related genes that are found in human cancer.

p53

The most prominent tumor suppressor in this category is p53 encoded by gene *Tp53*, which is mutated in more than 50 % of human cancers [60, 61] (also see http://p53.free.fr and www-p53.iarc.fr). Most mutations in p53 are missense and occur in its DNA binding domain (up to 80 %) [60, 61]. p53 primarily modulates intrinsic pathway of apoptosis by transcriptional control of pro-apoptotic genes such as BAX, NOXA, PUMA, and APAF-1 [79]. p53 is also involved in extrinsic pathway of apoptosis, where it transcriptionally regulates FAS and DR5 [90, 91]. Recently it was shown that p53 can also transcriptionally upregulate caspase-3 [92], which is an executioner caspase involved in both extrinsic and intrinsic pathways of apoptosis.

BCL2 and BCL2 Family Members

BCL2 (B-cell CLL/lymphoma 2) gene, which is located at chromosome 18q21.3 in human is frequently overexpressed in acute and chronic leukemia and non-Hodgkin lymphoma. It is also overexpressed in several solid cancers such as prostate, breast, lung, gastric, and renal cancers [93, 94]. It was identified as a gene located at the break point of the t(14;18) translocation found in follicular lymphoma, and the protein encoded by it was found to be a 26-kDa protein, which is homologous to a predicted Epstein-Barr virus protein [95]. BCL2 is a very potent inhibitor of apoptosis, although mechanism of inhibition of apoptosis by BCL2 is not clear. BCL2 is localized to outer mitochondrial membrane. It is thought that BCL2 preserves mitochondrial integrity by preventing oligomerization of BAX/BAK and by inhibiting the release of Cyt-c and other apoptogenic molecules from mitochondria [93, 94]. Apart from regulating the release of Cyt-c, BCL2 can also regulate the activation of initiator caspases independent of Cyt-c [96].

$BCLX_L$, a splice variant from gene *BCLX*, exhibits 44 % homology to BCL2 and protects cells from apoptosis. $BCLX_S$ another splice variant of BCLX, acts as a dominant negative inhibitor of BCL2 and $BCLX_L$, and is a pro-apoptotic protein. Increased expression of $BCLX_L$ is detected in several human cancers such as hepatocellular and renal carcinoma, and pancreatic cancer [93]. BCLW, another BCL2 like gene was found to be overexpressed in colorectal and gastric carcinomas [93]. Likewise two other anti-apoptotic protein A1/BFL1 and MCL1, with significant homology to BCL2, are overexpressed in gastric cancer and the myeloid leukemias respectively [93]. Consistent with their tumor suppressor function, the pro-apoptotic members of BCL2 family, such as BAX and BAK, are underexpressed in human cancers. For example, downregulation of BAX has been reported in breast cancer and hepatocellular carcinoma [93, 97]. In addition, loss of function and frame shift mutations in BAX have been reported in colon cancer and hematological malignancies [93]. Similarly, BAK mutations and its reduced expression have been reported in gastric and colorectal cancers [98].

APAF1

APAF1 (Apaf-1) is essential for activation of caspase-9 following Cyt-c release [99]. It is mutated, transcriptionally silenced, or downregulated in various cancers such as melanomas [100], colorectal cancer [101], bladder and kidney

cancers [102], leukemia [103], and multiple myelomas [104]. APAF1 is p53 transcriptional target and is essential for DNA damage induced apoptosis in several cell types [105].

IAPs

IAPs family of proteins acts as inhibitors of apoptosis by negatively regulating caspases. Members of IAPs family of proteins are dysregulated in numerous cancers, and are involved in tumor resistance to chemotherapeutics [106]. cIAP-2, survivin, livin, and XIAP were reported to be overexpressed in pancreatic cancer [107]. Survivin, cIAP1, cIAP2, NAIP, and XIAP are also overexpressed in esophageal cancer than in normal mucosa [108]. Livin/ML-IAP is overexpressed in lung cancer and melanomas [109]. Survivin overexpression is correlated with bladder cancer progression [110]. It is also overexpressed in breast cancer and is an independent prognostic marker of poor survival [111, 112]. Survivin is reportedly overexpressed in several other cancers such as pancreatic cancer [113], pediatric cancers [114], esophageal and squamous cell carcinomas [115], thyroid cancers [116], non-small-cell lung cancer [117], ovarian cancer [118], and gastric cancers [119]. Because of its overexpression in a variety of cancers and its role in chemoresistance and radioresistance, survivin is considered an attractive target for cancer therapy [120].

Other Apoptosis Related-Genes in Cancer

Several other genes involved in extrinsic pathway of apoptosis such as death receptors, death ligands, and adaptor proteins, and caspases are dysregulated in cancer. FAS (CD-95), which initiates the extrinsic pathway of apoptosis is mutated or downregulated in human malignancies such as gastric cancers [121], non-small-cell lung cancers [122], ovarian cancers [123], colon cancer [124], esophageal cancer [125], and lymphomas [126, 127]. Similarly, TRAIL receptors are mutated in breast and other cancers [128]. Caspases are also found to be downregulated and contain inactivating mutations in breast cancer and breast cancer cell lines [129]. Several other genes related to apoptosis and cell survival are altered in cancers; tumor suppressors such as PTEN, p14ARF, pRB, ATM, and CHK2 are often mutated, deleted, or underexpressed, while oncogenes such as *AKT*, *NF-kB*, *PI3K* subunits, and *RAS* genes are activated and/or overexpressed in various human cancers [89].

10.2.2.2 Mouse Models of Apoptosis and Cancer

A direct role for cell death or apoptosis-related genes in cancer comes from knockout and transgenic studies. It is very well established that deficiency of p53, which is a prime regulator of apoptosis, results in a wide spectrum of cancers, most notably soft-tissue sarcomas, and lymphomas in knockout mouse models [130] (also see http://p53.free.fr/p53_info/Mouse_model/p53_mouse_models.html). *Tp53*−/− mice develop normally but succumb to cancer in less than a year, suggesting that the apoptotic role of p53 is not essential for normal development, but is important for tumor suppression [130]. However, the cause of high rate of spontaneous tumorigenesis in *Tp53*−/− knockout mice is poorly understood. Recent in vivo knockin studies using wild-type or mutant p53, expression of inducible p53, and inducible/regulated knockout mice models has produced insight into the particular functions of p53 that relate to its regulation of apoptosis and senescence.

A recent surprising finding suggest that expression of human p53 in *Tp53*−/− knockout mice (SWAP mice) did not complement p53-mediated transactivation, apoptosis, and G1-S checkpoint, although the mice displayed normal p53-mediated centrosomal checkpoint and p53-mediated transrepression [131]. The SWAP mice apparently developed tumors similar to p53 deficient mice suggesting that the role p53 in spontaneous tumorigenesis does not fully rely on apoptosis [131]. However, a different study where a chimeric human-mouse p53 was used for knockin studies suggests that the chimeric p53 can fully rescue mouse p53 deficiency, restores apoptosis response to DNA damage, and blocks accelerated development of spontaneous cancers [132, 133]. Christophorou et al. investigated the tumor suppressor functions of p53 using a 4-hydroxytamoxifen (4-OHT) inducible knockin gene replacement ($p53ER^{TAM}$) mouse model [134]. It was shown that depending on the presence or absence of 4-OHT, such mice can be rapidly toggled between wild-type and p53 knockout states. Using this rapid perturbation model, authors showed that DNA damage induced apoptosis requires p53 in vivo [134]. To dissect out the function of p53 required for tumor-suppression in this model, $p53ER^{TAM}$ mice were crossed with lymphoma prone Eμ-Myc mice [135]. The composite transgenic mice could be rapidly converted into p53-deficient and p53-sufficient mice with 4-OHT. In the absence of 4-OHT, these mice rapidly developed lymphoma, while the restoration of p53 using 4-OHT significantly delayed tumorigenesis [135]. Furthermore it was shown that in this setting rapid induction of p53 induced tumor apoptosis as determined by annexin V/PI and TUNEL staining [135].

Restoration of p53 function using another 4-OHT inducible *Cre-loxP* system in mouse was shown to cause rapid regression of lymphomas by inducing apoptosis and not cellular senescence as determined by markers of apoptosis and senescence [62]. Several other p53 knockin mouse studies have clearly demonstrated that apoptosis-related function of p53 is required for its tumor suppressive function [136]. For example, Liu et al. showed that in R172P (mutant p53) knockin mice, the mutant p53 retained cell-cycle arrest function but lost its apoptotic function, and that these mice showed delayed cancer onset and stable chromosome number when

compared to Tp53−/− mice [137]. Short-telomeres in telomerase knockout mice (mTR−/−) at later generations (G-5/6) has been shown to limit tumor formation via induction of p53-dependent apoptosis [138, 139]. Furthermore, in this model, it was shown that p53 deficiency abrogates the short telomere-induced apoptotic response, and enhances tumor formation [138].

There are several reports of *BCL2* cooperating with other oncogenes such as *RAS* and *MYC* to develop tumors in transgenic mouse models [140]. BCL2 overexpression alone in B-Cells demonstrated extended survival and follicular lymphoproliferation, while the translocated BCL2 (t(14:18)) transgenic mice developed high-grade malignant lymphoma [141, 142]. BCL2 overexpression using prostate-specific Probasin promoter has been shown to facilitate multistep progression of prostate cancer in TRAMP (TRansgenic Adenocarcinoma Mouse Prostate) mice that express the SV40 early genes (T/t antigens) [143]. Similarly, cooperation between H-RAS and BCL2 has been demonstrated in multistep skin carcinogenesis in a mouse model [144]. Using a conditional BCL2 and constitutive MYC in a transgenic mouse model, Letai et al. have demonstrated that the suppression of apoptosis by BCL2 is required to enhance and maintain leukemia [145]. BCL2 also synergizes with MYC during mammary carcinogenesis in a WAP-Bcl-2/MMTV-c-Myc transgenic mouse model [146]. Overexpression of $BCLX_L$, which functions similar to BCL2 in a nonredundant way, has been shown to enhance transformation of pancreatic β cells by SV40 large T in a mouse transgenic model [147]. $BCLX_L$ also promotes chemically induced skin carcinogenesis in the transgenic mouse model [148]. Studies from several other mouse knockout and transgenic models of BCL2 family members strongly implicate apoptosis in tumor progression and tumor resistance to chemotherapy and radiotherapy reagents [93].

10.2.3 Apoptosis and Cancer Therapy

Considering the important role of apoptosis in cancer progression and maintenance of the tumorigenic phenotype, it is not surprising that the major goal of oncology companies is to facilitate apoptosis in cancer. When the survival pathways are blocked, most cells, normal or neoplastic, can be killed via induction of apoptosis, necrosis, mitotic catastrophe, and autophagy. This is what chemotherapy reagents and cytotoxic drugs do to cancer cells. Intriguingly, cancer cells are more susceptible to apoptosis than normal cells, which makes apoptosis-inducing reagents more appealing. Several cancer therapy reagents that are in preclinical or clinical trials directly affect components of apoptotic pathways. Upon administration, these ligands activate extrinsic pathway of apoptosis mediated by their respective receptors resulting in caspase activation and ultimately apoptotic cell death in cancer cells.

Death ligands such as TNFα, FASL (CD95L), and TRAIL are in clinical trials for treatment of various cancers. TNFα went through extensive clinical testing in the past but was found to be very toxic for systemic administration due to its detrimental effect on normal cells in addition to cancer cells. Similar to TNFα, FASL is also toxic when systemically administered. Clinical trials with TRAIL and TRAIL receptor agonists are now under way for the treatment of cancer [149, 150]. These reagents are less toxic to normal cells because TRAIL receptors (DR4 and DR5) are more selective to cancer cells [149, 150]. Although TRAIL therapy is promising, many cancer cells are resistant to TRAIL. Therefore, use of TRAIL in combination therapy is being developed against tumors that are refractory to treatment with either agent alone [151]. Several reagents such as COX-2 inhibitors and Casein kinase inhibitors can also sensitize cells to TRAIL and FASL by enhancing death receptor clustering, and recruitment of FADD and caspase-8, respectively [67]. Other apoptosis-related reagents that are being developed for cancer treatment include BCL2 antisense oligonucleotide, small molecule antagonist (SMA) of BCL2, $BCLX_L$ antisense oligonucleotides, SMA of $BCLX_L$, XIAP antagonists, survivin antisense oligonucleotides, and inhibitors of PI3K/AKT pathway. Apart from these reagents, p53 reactivating small molecules such as CP-31398, PRIMA-1, MIRA-1 are also being used to restore p53 functions and apoptosis in cancer [152]. Similarly, MDM2 antagonists such as Nutlin, RITA, and other compounds can be used to activate and potentiate wild-type p53 function to cause apoptosis and senescence in cancers that overexpresses MDM2 but contain wild-type p53 [153–158].

10.3 Concluding Remarks

Arguably, cancer is the most complex disease of our time. Multiple genetic pathways are deregulated in cancer. Loeb et al. have suggested that a human cancer of 10^8 cells likely contains a billion different mutations [159]. Loeb et al.'s calculations may be artificially high and are based on a postulated mutator phenotype of tumors, which may not be common to all cancers, and is debatable. However, there is no argument that cancers contain multiple mutations (perhaps not a billion) and genetic aberrations. At least four steps, which may involve several genes, are required for the conversion of normal cells into malignant tumor cells [65]. Recently, Sjoblom et al. analyzed 13,023 individual genes in 11 breast and 11 colorectal cancers to conclude that individual tumors contain an average of approximately 90 mutant genes [160].

Quite often, it is difficult to pinpoint what is the key primary mutation in the development of a specific cancer, and what are the vast majority of passenger or secondary mutations, which accumulate in a genetically unstable tumor

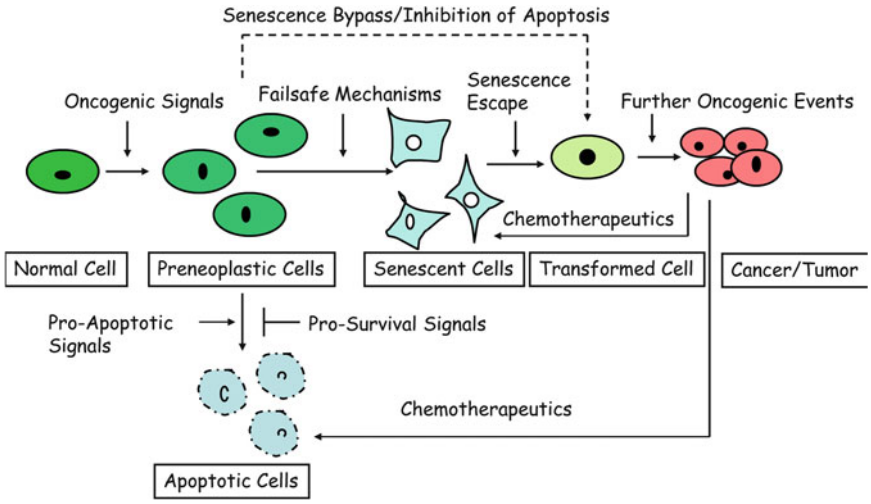

Fig. 10.5 A model of cancer progression suggesting that senescence bypass and inhibition of apoptosis due to mutations in these pathways results in cancer development. Various oncogenic signals prime a cell to a transitional stage known as preneoplastic stage. Preneoplastic cells can undergo senescence or apoptosis by fail-safe or genome surveillance mechanisms. Some cells can escape senescence and become immortal and transformed. Cells that have bypassed senescence or cannot undergo apoptosis in response to oncogenic signals become transformed. Accumulation of further oncogenic events leads to full-blown tumor capable of angiogenesis and metastasis to other organs. Treatment of tumors with chemotherapy drugs can induce either senescence or apoptosis, and ultimately tumor clearance by immunosurveillance mechanisms occur (not shown).

cell. Despite these undesirable traits of cancer cells, a few key steps are vital for cancer development, progression, and maintenance. Two of these key steps are senescence bypass and inhibition of apoptotic pathways (Fig. 10.5). Senescence bypass is essential for cancer initiation and development, while inhibition of apoptosis and induction of survival pathways is essential for cancer progression and maintenance (Fig. 10.5). Cancers achieve these goals by mutation in multiple pathways involved in these key processes. Therefore, the primary goal of cancer treatment strategies remains with the induction of apoptosis and/or senescence by interfering with the mutations that were responsible for senescence bypass and inhibition of apoptosis. Apart from these generalized treatment strategies, we now know that targeted therapies can also be used to induce senescence and/or apoptosis in tumors.

Very recent reports from laboratories of Drs. Lowe, Jacks, and Evans clearly show the promise of turning on apoptosis and senescence by a single gene *TP53* in a mouse model of cancer. Turning on *TP53* in hepatocellular carcinomas and sarcomas resulted in cellular senescence, which eventually caused regression of these tumors, and completely cured mice of these cancers [62, 63]. On the other hand, p53 induction caused apoptosis and a rapid regression of lymphoma in mice to restore normal life span [62, 135]. These reports are of extraordinary significance. We have learned a lot about cancer from animals models. We can add one more chapter of learning from these mice models that perhaps treating human cancer is not impossible; we just need to find ways to restore apoptosis and/or senescence in tumors.

Acknowledgements Grant support from National Cancer Institute (R01 CA094150) and the Department of Defense (DAMD17-02-1-0509) to GPD, and contribution of our past and present collaborators is gratefully acknowledged.

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Novel Cancer Therapies Targeting Angiogenesis

11

Monte S. Willis and Nancy Klauber-DeMore

11.1 Tumors Overcome Growth Limitations by Directing the Formation of New Blood Supply

There are four general strategies by which cancers can enhance their blood supply. They can (1) stimulate angiogenesis, (2) utilize existing vessels directly, (3) induce vasculogenesis, and/or (4) form vasculogenic networks without vascular cells. The secretion of proangiogenic factors and/or inhibition of antiangiogenic factors induce vascular sprouting from preexisting capillaries and venules constitutes the process of angiogenesis. This is the most common way cancers gain access to the vasculature. Cancers may also utilize existing vessels directly by growing along beside them, as is the case in astrocytomas [1]. The strategy of vasculogenesis involves the formation of blood vessels from bone marrow precursors. Vasculogenesis differs distinctly from angiogenesis in that the source of the cells that make up the vessels are from the bone marrow and not from preexisting vessels. However, many of the soluble mediators that initiate this process, notably VEGF, parallel those found in angiogenesis [2]. Lastly, tumors themselves can form lumens which can be used to transport blood, lacking endothelial cells or other vascular components. The contribution of these types of networks to cancer progression has been debated [3, 4]. Multiple strategies may be in play in a particular tumor, depending on their stage and malignancy. We focus here on the major pathways of angiogenesis as its contribution is most common as applied to therapeutic intervention.

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11.2 Evidence that Angiogenesis is Necessary for Cancer Progression

The initial evidence that angiogenesis is necessary for the growth of cancer came from studies transplanting cancer cells into the avascular corneas of rabbits [5]. In these studies, tumors did not grow in rabbit corneas before sprouting vessels were able to grow to connect to the tumor. Moreover, inhibiting vessel formation would prevent cancer growth beyond 0.4 mm [5]. Other investigators similarly found that tumors placed in chicken embryo chorioallantoic membranes shrank during the first three days after placement [6]. However, new vessel formation could be seen to form from existing vessels after the cancers were placed. When these new vessels connected to the tumor, cancer growth continued. These studies not only identified a significant role of vessel formation in cancer progression but also determined that cancers elicit the growth of vessels from existing vessels. This suggests that cancer release diffusible factors that initiate angiogenesis (vessel formation from existing vasculature) to continue and maintain their growth.

The ability of cancer cells to induce angiogenesis is not a constitutive trait. In mice, the ability to induce angiogenesis appears to be initiated by tumor progression in models of pancreatic cancer [7, 8] and dermal fibrosarcomas [9–11]. The initiation of a program to induce angiogenesis also occurs in the development of human tumors. Evidence for this comes from comparing vasculature in precancerous and cancerous lesions of the breast, where the microvessel density has been reported to be relatively increased [12]. Similarly, increased capillary density has been reported to increase in cervical squamous cell carcinoma as the stage progresses [13, 14], suggesting that as a cancer becomes more aggressive, its ability to stimulate angiogenesis increases. It is believed that the switch that cancer undergo to induce angiogenesis not only come from the malignant tissue itself, but also from the surrounding tissue and infiltrating immune cells [15]. The targets of these soluble factors are endothelial cells as well as other vascular cells in the

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_11

microenvironment of the tumor [15]. The interplay between the soluble proangiogenic and antiangiogenic factors and their cognate receptors on the targets is the basis of tumor-induced angiogenesis necessary for tumors to continue their unrestrained growth. Each step in the angiogenic process presents an opportunity for targeted inhibition of angiogenesis. This review focuses on the main steps of angiogenesis for which targeted therapy is being developed.

11.3 Growth Factors/Receptor Tyrosine Kinases

11.3.1 Vascular Endothelial Growth Factor Family

An essential mediator of angiogenesis is the vascular endothelial growth factor (VEGF) family, which consists of five family members of secreted proteins (VEGFA, VEGFB, VEGFC, VEGFD, VEGFE), and platelet-derived growth factor (PDGF) [16], that bind and activate three receptor tyrosine kinases (VEGFR-1, VEGFR-2, and VEGFR-3). VEGFA stimulates endothelial cell proliferation, migration, tube formation, and vascular permeability. VEGFB was identified as an endothelial cell growth factor expressed in heart and skeletal muscle [17]; however, its function as an angiogenesis factor is not clearly defined. VEGFC and VEGFD play a critical role in lymphangiogenesis and expression has been correlated with the development of lymph node metastases [18]. Placental growth factor (PGF) promotes the survival of endothelial cells and modulates the activity of VEGF signaling [19].

Regulation of VEGF family genes expression is under the control of hypoxia, along with other forms of stress such as acidity and hypoglycemia, which stimulate transcription and increase mRNA stability, resulting in increased protein expression. Under normoxic conditions, prolyl residues in hypoxia-inducible factors (HIF) proteins are hydroxylated by prolyl hydroxylase in a reaction that uses molecular oxygen. Hydroxylation of HIF proteins targets them for ubiquitin-mediated proteolysis. Reduction in the concentration of oxygen decreases the efficiency of this process: HIF proteins are stabilized and therefore become available to bind to hypoxia-response elements in the promoters of target genes, thereby activating transcription [20].

VEGF proteins bind to receptor tyrosine kinases (VEGFR-1, VEGFR-2, and VEGFR-3) [21], which then mediate cell signaling resulting in the biologic effects of VEGF. VEGFR-1 (Flt-1) binds three of the VEGF family ligands, VEGF-A, VEGF-B, and PGF. Activation of VEGFR-1 results in embryonic vessel development, hematopoiesis, macrophage chemotaxis and recruitment of endothelial progenitor cells to tumor blood vessels from the bone marrow [22]. VEGFR-2 (Flk-1/KDR) is the key mediator of VEGF-stimulated tumor angiogenesis and is critical in embryonic vascular development. When VEGF ligands VEGFR-2, the receptor is phosphorylated and activates downstream signaling molecules including phospholipase C, protein kinase C, Raf, MAP kinase, PI3K, and FAK pathways, resulting in endothelial cell proliferation, migration, and tube formation, and anti-apoptosis [23]. VEGFR-3 binds VEGF-C and -D and is implicated in the formation of lymphatics in normal tissue and tumors [24].

In 2004 the humanized version of a monoclonal antibody to VEGFA, bevacizumab (Avastin; Genentech), became the first Food and Drug Administration (FDA)-approved antiangiogenic drug in the United States [18]. It was approved as a first-line treatment agent for metastatic colorectal cancer, in combination with 5-fluorouracil [25] and was subsequently approved for treatment metastatic non-squamous-cell lung cancer, breast cancer, and glioblastoma multiforme [26]. Ranibizumab (Lucentis; Genentech), another monoclonal antibody recognizing VEGFA, and pegaptanib (Macugen; Pfizer, New York, NY), a single-stranded nucleic acid aptamer that binds specifically to the heparin-binding domain of VEGFA165, are FDA-approved antiangiogenesis inhibitors in use for treating the wet type of age-related macular degeneration [27].

Additional FDA-approved drugs that block VEGF signaling are sorafenib and sunitinib, both receptor tyrosine kinase inhibitors, which are both administered orally. Sorafenib, in addition to blocking VEGFR signaling, also blocks FLT3, PDGFRB, and KIT signaling [28]. Similarly, sunitinib blocks signaling from VEGFR1-3, FLT3, RET, PDGFRA, and PDGFRB [28]. Sorafenib has been approved for unresectable hepatocellular carcinoma and advanced renal cell carcinoma, whereas sunitinib has been approved for gastrointestinal stromal tumors and metastatic renal cell carcinoma [18] and neuroendocrine tumors [29].

11.3.1.1 Fibroblast Growth Factor

Fibroblast growth factors (FGFs) are involved in maintaining endothelial cell function. FGF1 and FGF2 promote endothelial cell migration and proliferation and stimulate angiogenesis [30]. FGFs produce their biological effects by binding to transmembrane tyrosine kinase receptors, FGFR1 through FGFR4 [31]. FGFR can activate PLC-γ, thereby stimulating the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). This, in turn, releases intracellular calcium and activates Ca^{2+}-dependent PKCs. The activation of the PI3K-Akt cell survival pathway is one of the important biological responses induced by FGF2 in endothelial cells [32].

There are several inhibitors of FGF signaling in clinical trials, including FP-1039 (FGFR1:Fc), a soluble fusion protein consisting of the extracellular domain of human fibroblast growth factor receptor 1c (FGFR1) linked to the Fc portion of human IgG1. FP-1039 prevents FGFR1 ligands from binding to any of their cognate receptors within the family of seven FGF receptors, and may mediate both direct antitumor and

antiangiogenic effects. Both E-3810 and TKI258 are dual VEGFR and FGFR tyrosine kinase inhibitors [30].

11.3.1.2 Notch

The Notch signaling pathway is important for cell-cell communication, involving gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life. Notch signaling has been directly implicated in tumor angiogenesis and in the process of activating dormant tumors. VEGFA also induces expression of the endothelium-specific Notch ligand Delta-like 4 (DLL4). When DLL4 activates the Notch signaling pathway in adjacent cells, the effect is to inhibit dorsal sprouting. When expressed in tumor cells DLL4 can activate Notch signaling in host stromal cells, thereby improving vascular function [33]. Inhibition of DLL4-mediated Notch signaling promotes a hyperproliferative response in endothelial cells, a process that leads to an increase in angiogenic sprouting and branching. Despite this increase in vascularity, tumors are poorly perfused, hypoxia increases, and cancer growth is inhibited. Neutralizing anti-Dll4 antibodies have been demonstrated to inhibit cancer growth in vivo [34]. These findings point to the Notch pathway as a potential therapeutic target.

11.3.1.3 TGFβ

TGFβ is a paracrine polypeptide with three homologous forms (TGFβ1, TGFβ2, and TGFβ3). TGFβ is produced in latent form as a zymogen and after secretion a latency associated peptide is proteolytically cleaved to release active TGFβ. Active TGFβ signals by binding to constitutively active type 2 receptors (TGFBR2) to activate type 1 receptors (TGFBR1) in a heteromeric complex that controls transcription through the action of a family of SMAD proteins [35]. TGF-β is a strong proangiogenic agent despite the fact that TGFβ causes growth arrest and apoptosis of endothelial cells in vitro. This paradoxical behavior may be explained by the fact that TGFβ activates the secretion of fibroblast growth factor 2, which acts as an autocrine signal to stimulate the expression of VEGF. VEGF, in turn, acts in an autocrine manner through its receptor VEGFR-2 to activate the MAPK pathway (specifically p38MAPK). However, TGFβ will reverse the protective action of VEGF, promoting apoptosis, which occurs in the pruning process, to form the final vascular network [36]. Endothelial cells subsequently become refractory to TGFβ-mediated apoptosis and TGFβ then directly promotes capillary lumen formation. Therapeutic approaches for targeting TGFβ signaling include antagonism of TGFβ ligand binding to the heteromeric receptor complex with isoform-selective antibodies, such as lerdelimumab (TGFβ2) and metelimumab (TGFβ1) or the pan-neutralizing antibody GC-1008, and intracellular inhibition of the type I TGFβ receptor kinase with small-molecule inhibitors, such as LY550410, SB-505124, or SD-208 [37].

11.3.1.4 Angiopoietin/Tie Receptors

Angiopoietins are another family of endothelial cell-specific molecules that play an important role in vessel growth, maintenance, and stabilization by binding to Tie receptors [38]. There are four types of angiopoietins: Ang-1, Ang-2, Ang-3, and Ang-4. The Tie1 receptor is highly expressed in embryonic vascular endothelium, angioblasts, and endocardium, and in adult tissues expressed strongly in lung capillaries tissues [39]. The Tie2 receptor takes part in vessel maturation by mediating survival signals for endothelial cells. Ang-1 is an agonist that promotes vessel stabilization in a paracrine fashion, whereas Ang-2 is an autocrine antagonist that induces vascular destabilization. Ang-2 is increased during vascular remodeling and is implicated in tumor-induced angiogenesis and progression [40]. AMG 386 is an investigational peptide-Fc fusion protein that inhibits angiogenesis by preventing the interaction of Ang-1 and Ang-2 with their receptor, Tie2, and is being studied in clinical trials [41].

11.3.1.5 Epidermal Growth Factor

The epidermal growth factor (EGF) family consists of 11 members which bind to one of four epidermal growth factor receptors (EGFR) [42]. All of the receptors, except HER3, contain an intracellular tyrosine kinase domain [43]. Activation of EGFR contributes to angiogenesis in xenograft models [44], in addition to cellular proliferation, survival, migration, adhesion, differentiation, and cancer metastasis [43]. Activation of the EGFR pathway upregulates the production of proangiogenic factors such as VEGF, and therefore the EGFR pathway is more of an indirect regulator of angiogenesis. There are three FDA-approved EGFR inhibitors: cetuximab and panitumumab, which are monoclonal antibodies, and erlotinib, a tyrosine kinase inhibitor that specifically targets EGFR [18].

11.3.1.6 Insulin-Like Growth Factor Pathway

The insulin-like growth factor pathway plays a major role in cancer cell proliferation, survival and resistance to anticancer therapies in many human malignancies [45]. Insulin-like growth factor-1 (IGF-1) contributes to the promotion of angiogenesis through increasing VEGF expression via HIF-1a [46]. The two main strategies in development for blocking IGF signaling as an anticancer therapeutic are receptor blockade and tyrosine kinase inhibition [47]. Receptor blockade with the use of monoclonal antibody therapies against the IGF-1R (such as Figitumumab) [48] is being investigated. Tyrosine kinase inhibition is another strategy being developed. In general, these therapies will indiscriminately inhibit the kinase domains of all IGF system receptors. The exception to this is the NVP-AEW541 and NVP-ADW742, which has 15- to 30-fold increased potency for IGF-1R kinase inhibition compared to IR kinase inhibition in cellular assays [48].

11.3.2 Cell Adhesion Molecules

Integrins are heterodimer transmembrane receptors for the extracellular matrix composed of an alpha and beta subunits [49]. Integrins bind ligands by recognizing short amino acid stretches on exposed loops, particularly the arginine–glycine–aspartic acid (RGD) sequence. Upon ligation, either alone or in combination with growth factor receptors, integrins mediate signaling events that regulate angiogenesis, cell adhesion, proliferation, survival, and migration. Pathways that are activated include integrin-linked kinase, protein kinase B (PKB/Akt), mitogen-activated protein kinase (MAPK), Rac, or nuclear factor kappa B (NF-kB). In inactive vessels, integrins interact with the basal membrane, thereby maintaining vascular quiescence. During angiogenesis, integrins are essential for endothelial cell migration, proliferation, and survival. In preclinical studies, inhibition of integrin function suppresses angiogenesis and tumor growth. Of the 24 known integrin heterodimers, αVβ3 [50] and αVβ5 [51] were the first vascular integrins targeted to suppress tumor angiogenesis. Three classes of integrin inhibitors are currently in preclinical and clinical development: monoclonal antibodies targeting the extracellular domain of the heterodimer (Vitaxin; MedImmune), synthetic peptides containing an RGD sequence (cilengitide; Merck), and peptidomimetics (S247; Pfizer), which are orally bioavailable nonpeptidic molecules mimicking the RGD sequence [52].

Focal Adhesion Kinase (FAK) is a protein that plays a critical role in intracellular processes of cell spreading, adhesion, motility, survival, and cell cycle progression, and has shown to play a role in cancer angiogenesis [53]. The FAK gene encodes a non-receptor tyrosine kinase that localizes at contact points of cells with extracellular matrix and is activated by integrin (cell surface receptor) signaling. Recently Novartis Inc. developed novel FAK inhibitors downregulation its kinase activity [54]. The novel Novartis FAK inhibitor, TAE-226 was employed in brain cancer and effectively inhibited FAK signaling and caused apoptosis in these cells. Another, ATP-targeting site inhibitor of FAK, Pfizer-PF-573,228 has been described [55].

11.3.3 Calcineurin/NFAT Signaling

One of the important intracellular pathways stimulated by a variety of angiogenic growth factors, including VEGF, FGF, and a novel angiogenesis factor secreted frizzled-related protein 2 (SFRP2) [56] is activation of calcium signaling. Signaling is mediated through transient increases in cytoplasmic free calcium which activate the phosphatase calcineurin. Activated calcineurin dephosphorylates NFAT, which then translocates to from the cytoplasm to the nucleus [57]. There is increasing data supporting a critical role of NFAT in mediating angiogenic responses [58, 59]. Importantly, NFAT activation was identified as a critical component of SFRP2 [56, 60] and VEGF-induced angiogenesis and linked to the induction of cyclooxygenase-2 [61], which is also a critical player in angiogenesis. Activation of the Ca^{2+} pathway induces cell proliferation and inhibits apoptosis in cultured endothelial cells, suggesting a proangiogenic activity in vivo. FK506 is a calcineurin inhibitor that blocks NFAT activation, and has been shown to inhibit angiogenesis in vitro and tumor growth in vivo [56, 60].

11.3.4 Endogenous Angiogenesis Inhibitors

The activities of a variety of endogenous angiogenic inhibitors have been described to regulate cancer endothelial cell growth. These include thrombospondin-1 [62], angiostatin [63], endostatin [64], and 2-methoxyestradiol [65]. One of the most extraordinary developments in the discovery of endogenous inhibitors came again from the Folkman laboratory [63] via a Lewis Lung Carcinoma mouse model in which lung micrometastases seeded from primary subcutaneous cancers failed to develop further when the cancer was intact but grew rapidly after the primary cancer had been surgically removed. It was hypothesized that the primary tumor itself was producing a circulating antiangiogenic agent that inhibited blood vessel growth in the lung micrometastases. After resection of the primary cancer, the source of the endogenous angiogenesis inhibitor was removed, and the lung metastases therefore grew rapidly. O'Reilly and Folkman isolation of a protein they called angiostatin from the urine of mice with intact primary tumors. Angiostatin is a fragment of the protein plasminogen that occurs normally in the circulation, and the cleavage of plasminogen to produce angiostatin occurs in the tum or itself [63]. Purified angiostatin given daily to mice after resection of the primary tumor completely prevented the development of micrometastases. Angiostatin was subsequently shown to be active against primary cancer xenografts established in mice from inoculated human cancer cells, and it also inhibits the proliferation of endothelial cells in culture, but had no effect on cancer cell proliferation. Additional proteolytically activated antiangiogenic proteins have been isolated, including endostatin derived from collagen XVIII [64].

11.3.5 Tumor Endothelial Markers

A recent strategy to discovery novel angiogenesis targets is to explore differences in gene expression profiles between cancer and normal endothelium. This was first performed by St Croix who isolated endothelial cells using magnetic bead selection from a human colon cancer and adjacent normal colon [66].

Among the novel genes identified as being overexpressed by cancer endothelium was TEM8. TEM8 is the anthrax toxin receptor and successful targeting of this receptor in preclinical tumor models make this molecule a particularly attractive candidate for future vascular targeting studies [67]. Subsequent studies have used laser capture microdissection of blood vessels from breast cancer and normal breast tissue [68], or ovarian cancer and normal ovarian tissue [69], to identify novel targets that are presently under investigation.

11.4 Summary

Angiogenesis involves complex biological signaling pathways with a wide variety of antiangiogenic targets. Antiangiogenic therapy has been shown to increase survival in human tumors, but further research is needed to inhibit tumors that are not responsive to, or become resistant to, current antiangiogenic therapy.

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Beyond the Primary Tumor: Progression, Invasion, and Metastasis

12

Danny R. Welch and Douglas R. Hurst

12.1 Introduction

The cure rate for the majority of cancer patients is >90 % if the diagnosed tumor has not spread beyond the tissue of origin. However, when tumor cells have established colonies elsewhere, the cancer is often incurable [1, 2]. Neoplasms have been diagnosed for approximately four millennia and even the earliest medical practitioners recognized that the most lethal attribute of neoplastic cells is their ability to disseminate and colonize secondary sites. Despite these well-recognized facts, the majority of cancer research funding still focuses on primary tumorigenesis. Relatively few funded grants from the NIH include the word metastasis and significantly fewer actually perform metastasis research [3]. In recognition of the need to study metastasis, a major push has been toward the last frontier of cancer research [4]. This chapter provides a brief overview of what is known regarding the cellular and molecular events of a primary tumor mass progressing to form a distinct secondary macroscopic lesion.

The evolution of a normal cell into a life-threatening metastatic cancer cell is referred to as tumor progression (Fig. 12.1). Although a secondary mass is the ultimate consequence of progression of a primary tumor cell, metastatic cells are now recognized to be behaviorally and genetically distinct from the cells remaining at the site of primary origin. These behavioral differences arise at multiple levels including the cellular level (genetic and epigenetic heterogeneity) and from the physical environment (positional heterogeneity). The molecular mechanisms underlying the phenotypic differences that characterize a metastatic cell are currently being elucidated [5, 6]. Understanding the metastatic cell and the surrounding environment may enable the design of therapeutics that target metastasis, the deadliest aspect of tumor progression [1].

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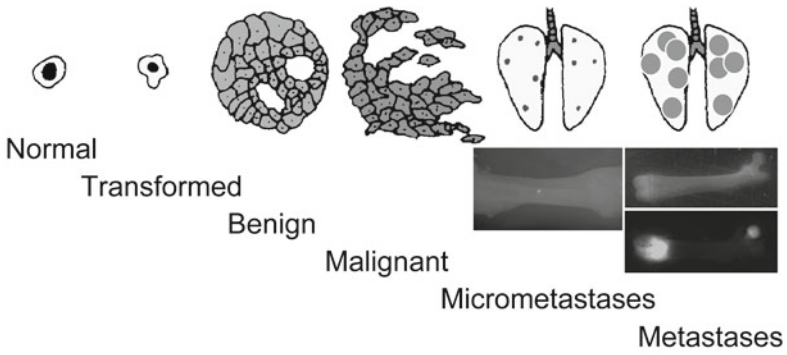
12.2 Tumor Heterogeneity: Generation of a Metastatic Cell

The definition of metastasis is the dissemination of neoplastic cells to discontiguous nearby or distant secondary (or higher order) sites where they proliferate to form a mass [1, 7] (Fig. 12.2). Fortunately, this process is highly inefficient [8]. Of the millions of cells that enter the vascular compartment per gram of primary tumor per day, only small fractions (much less than 0.1 %) actually form a macroscopic mass [9, 10]. Just as most tumors are clonal in origin [5, 11, 12], each metastasis arises from a single cell [6, 13, 14]. By the time the primary mass is apparent to the individual or the diagnosing physician, it is usually composed of 10^{10} or 10^{11} cells based on the fact that a cubic centimeter of tissue contains approximately 10^9 cells. Although not all cells in a neoplasm are capable of completing the required steps for metastasis, histological examination reveals that these cells are pleiomorphic and that single cell clones isolated from a tumor vary dramatically in terms of biological behavior [5, 6, 11].

Heterogeneity describes how cancers are composed of cells which are inherently and/or behaviorally different. The basis of the behavioral differences can be genetic, epigenetic, positional, and temporal. Genetic refers to the inherent properties of tumor cells themselves. Genetic heterogeneity is demonstrated by isolation of single cell clones that stably differ for a given phenotype. Like genetic heterogeneity, epigenetic heterogeneity refers to the chemical modifications of DNA and chromatin that lead to the selective regulation of gene transcription. Epigenetic changes can occur because of position (positional heterogeneity), for example the accessibility of a cell to external stimuli (O_2, pH, growth factors, cytokines, chemokines, etc.). An example is noted with

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_12



Self-sufficient growth	+/-	+	+	+	+
Resist growth inhibitory signals	+/-	+	+	+	+
Evade apoptosis	+/-	+	+	+	+
Limitless replicative potential	+	+	+	+	+
Sustained angiogenesis	-	+	+	+	+
Genomic Instability	+	+	+	+	+
Invasion	-	-	+	+	+
Metastasis	-	-	-	+/-	+

Fig. 12.1 Hallmarks of cancer in the tumor progression continuum. The indicated phenotypes along the tumor progression continuum do (+), do not (−), or sometimes (+/−) involve the indicated properties as described by Hanahan and Weinberg [79]. Note that not all of the hallmarks are present at each stage along the entire progression. The invasion and metastasis hallmarks have been separated according to the discussion in the text. Also, the distinction has been made between the potential to form micrometastasis from development of macroscopic metastases. Examples of mouse femurs with microscopic and macroscopic metastasis are shown. The fluorescent images are from human breast cancer cells labeled with GFP that were injected intra-cardiac into athymic mice.

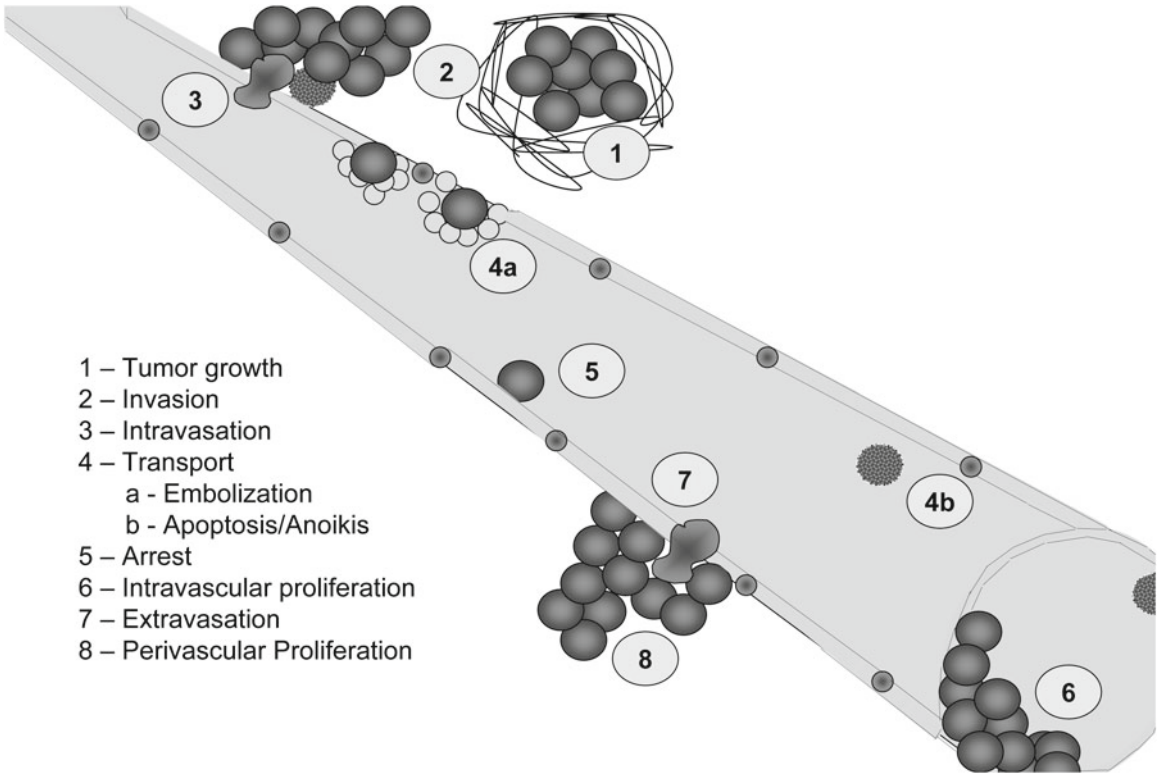


Fig. 12.2 Steps of cancer metastasis. The necessary steps for metastasis are listed in order. In the first step, tumor growth is noted with an increase in desmoplasia (1) host response to the presence of the tumor mass. Tumor cells invade away from the primary tumor (2) and eventually intravasate (3) into a circulatory compartment. Emboli involving multiple tumor cells and platelets/thrombin (4a) often occur. However, most tumor cells succumb to sheer or anoikis (4b) following entry into the vasculature. Tumor cells then specifically adhere to endothelial cells lining vessels (5), depending upon reciprocal recognition of surface molecules or arrest due to size limitations (6) at vessel bifurcations. Some cells can proliferate intravascularly (6); whereas, others extravasate (7) prior to proliferating at the secondary site (8).

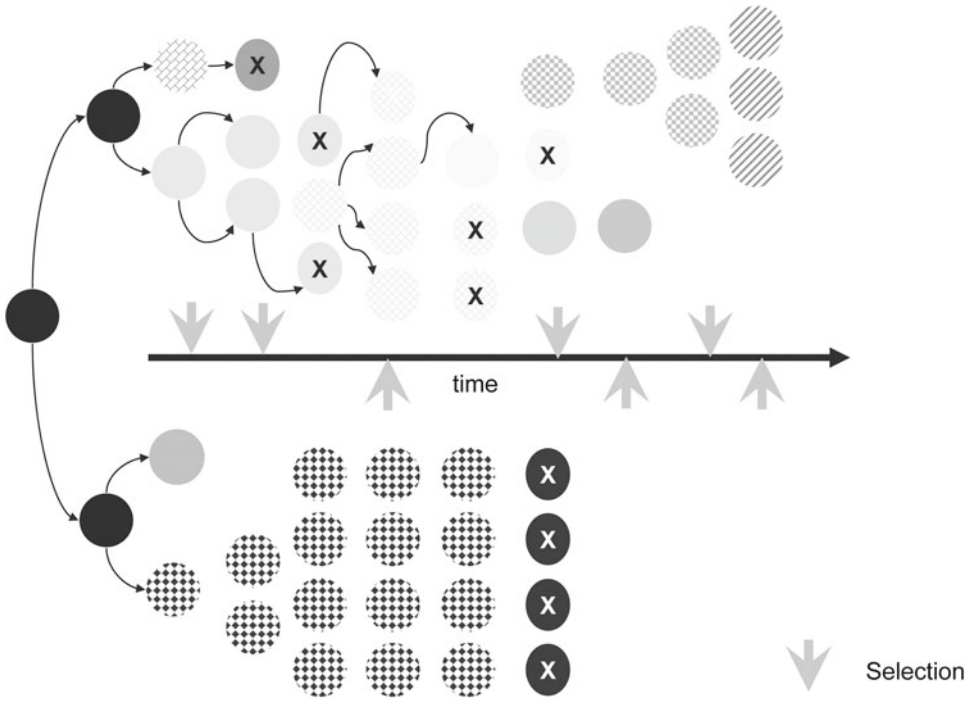


Fig. 12.3 Mutation-selection theory of tumor progression. Although mutation rates can be highly variable according to specific cell types, tumor cells generally have higher mutation rates than normal cells. Tumor cells with higher mutation rates (*upper series*) have increased chances for survival after lethal selection pressures. Variants are selected continually and are either eliminated by selection or are overwhelmed by other cells with more robust growth characteristics. Populations of cells with low mutation rates are more susceptible to lethal selection pressures (*bottom series* of cells).

radiation sensitivity being proportional to oxygenation. Thus, two identical cells would exhibit differences in radioresponse depending upon distance from a capillary since oxygen tension is inversely proportional to distance from blood. Heterogeneity can be observed temporally as well, as cells change due to cyclical signals. One example would be that cells in the G_0/G_1 phase of the cell cycle would be less sensitive than cells in S phase to drugs targeting DNA replication.

At first glance, isolation of multiple cell subpopulations from virtually every tumor mass would appear to support the hypothesis that cancers are of multicellular origin. However, tumors express either maternal or paternal isoenzymes, but rarely both, strongly supporting unicellular origination. In addition, analysis of karyotypes reveals that virtually all cells within a tumor share common abnormal chromosomal changes upon which additional karyotypic abnormalities may be superimposed. Therefore, the majority of tumors are monoclonal in origin and it is the divergence of single transformed cells into multiple phenotypically distinct progeny that generates genetic heterogeneity [5, 11].

As with most aspects of tumor biology, development of heterogeneity is not unique to tumor progression. Pluripotent hematopoietic stem cells generate cells along multiple lineages, and a zygote yields a multicellular organism with organs and tissues. Stem cells have the capacity for self-renewal and progenitor production via asymmetric cell division and are regulated by the microenvironmental niche [15, 16]. Although stem cell theory accommodates diversification, the molecular mechanisms underlying differentiation and diversification of normal cells are still being elucidated [17]. Additionally, the relevance of cancer stem cell theory is still being debated by many researchers to account for the diversification of cancer cells [18–23].

One of the first formalized conceptual frameworks of tumor progression was introduced by Peyton Rous, who described the steps involved in the transformation of skin carcinomas from the study of tar tumors in rabbits [24]. His concepts were expanded by Leslie Foulds, who studied the acquisition of hormone independence by mammary tumors. Foulds defined progression as "…*the acquisition of permanent, irreversible qualitative changes of one or more characteristics in a neoplasm*…" [25]. Both Rous and Foulds provided evidence that tumor progression occurs in a constant, unique and stepwise pattern. The trend is toward increased autonomy; however, individual characteristics within a tumor independently assort [6].

The mutation-selection theory of tumor progression proposes that genetic instability drives the process by random generation of variants (Fig. 12.3). Expanding upon Theodor Boveri's original observations that alterations of chromosomal material were significant in the generation and progression of tumors [26], Peter Nowell proposed that neoplastic cells are more genetically unstable than normal

counterparts [27]. Fluctuation analyses for a variety of genes and phenotypes show that transformed cells are significantly (frequently 10,000- to 100,000-fold, but as high as 10^7-fold higher) more genetically unstable than normal counterparts [11]. Progression is believed to occur as a result of mutation and coupled selection via Darwinian selection principles [6]. Although not formally proven, subpopulations of cells that have acquired the ability to migrate and establish themselves at other sites are thought to have a selective advantage since these tumor cells are not limited by space or location.

Isaiah "Josh" Fidler and Margaret Kripke tested these hypotheses with regard to the metastatic phenotype using combinations of cloning and Luria–Delbrück fluctuation analysis [28]. Single cell clones isolated from a single tumor varied considerably in their metastatic potentials. George Poste and colleagues later showed that highly metastatic cells, when grown in culture continuously and re-cloned, yielded populations that contained non-metastatic or poorly metastatic cells [29, 30]. Likewise, continuous culture of poorly metastatic cells yielded subpopulations that were highly metastatic. In other words, the clonal populations did not remain homogeneous.

At an organismal (i.e., tumor) level, progression typically follows a sequence (Fig. 12.1). Prior to becoming tumorigenic, cells lose the ability to differentiate fully, are no longer contact inhibited or anchorage dependent and have acquired genetic instability. The ability to form a neoplastic mass typically goes through a phase with expansile growth in the absence of invasion. While cells may be pleiomorphic at this stage, they are often encapsulated by a dense fibrous network (i.e., desmoplasia) [31, 32]. Tumors that have failed to invade through a basement membrane are referred to as benign or as carcinoma in situ. With continued generation of variants and selection, subsets of the cells acquire the ability to escape through a basement membrane, the hallmark of malignancy. Acquisition of the ability to detach from the primary tumor and move elsewhere is required for metastasis. It is important to emphasize that tumor progression is typically measured in terms of the tumor mass, rather than the individual cells within it. The stage of a tumor is defined by the most malignant cells found. Even if 99.9 % of cells are indolent, a tumor is defined as malignant if a single cell has penetrated a basement membrane.

At a molecular level, certain chromosomal and genetic changes are more prevalent in early versus late stages of tumor progression, despite unpredictability in specific genetic changes occurring within a cell. Use of this information has allowed prediction of genetic underpinnings controlling tumorigenesis, invasiveness, and metastasis [6]. The complexity of tumor progression leading to a metastatic cell, as described above, shows that a single genetic change is not enough to accurately predict whether a person will have an increased chance of a lesion becoming metastatic and defined subsets of genes have become more predictive as prognostic tools [33, 34].

However, the mutation-selection theory of tumor progression is not without its detractors. Some argue that the acquisition of invasive and metastatic behaviors is more a recapitulation of a process that occurs during embryogenesis—the epithelial–mesenchymal transition (EMT) [35]. Since invasive cells frequently dramatically change their cell shape to a non-polarized, motile, spindle shaped cell resembling a fibroblast, some hypothesize that neoplastic cells dedifferentiate to a more motile mesenchymal cell phenotype. Developmental EMT and cancer EMT are not necessarily equivalent at a molecular level, but share some characteristics. Cancer EMT is characterized molecularly by the loss of epithelial-specific E-cadherin from the adherens junctions, and a switch from the expression of keratins as the major intermediate filament to the mesenchymal intermediate filament vimentin. Ultimately, some believe that more epigenetic mechanisms may be driving tumor progression toward malignancy than mutation and selection [36].

12.3 Invasion

Invasion, the distinguishing feature of malignancy, is the capacity for tumor cells to disrupt the basement membrane and penetrate underlying stroma. Although invasion is required for metastasis, the ability to invade is not sufficient. This point is highlighted by clinical observations. Some tumors are highly aggressive, forming secondary lesions with high frequency (melanoma, pancreatic carcinoma, small cell carcinoma of the lung), whereas others are rarely metastatic despite being locally invasive (basal cell carcinomas of the skin, glioblastoma multiforme). It should be emphasized that if an invasive cell cannot complete any of the subsequent steps in the metastatic cascade, it will not form a metastasis.

The process of invasion requires major changes to the cell (morphology and phenotype) and to the surrounding environment. During invasion, three important processes are dynamically regulated that include adhesion, extracellular matrix (ECM) reorganization, and motility. Normal epithelial cells form polarized sheets that are maintained by tight junctions and desmosomes. They are anchored to the basement membrane by hemidesmosomes and their associated intermediate filaments, and integrin contacts that organize actin. Therefore, to be invasive, cells must alter cell-to-cell and cell-to-matrix adhesion in conjunction with reorganization of the ECM and cellular motility [37, 38]. The structural and functional proteins that regulate cell adhesion and migration are key downstream targets of oncogenes and tumor suppressor-controlled signaling pathways and provide insights into how oncogenic transformation results in progression to an invasive phenotype. Many of the proteins involved in tumor invasion have also been observed to affect other processes that are part of the hallmarks of cancer including cell survival, growth, apop-

tosis and angiogenesis. This highlights the intricate network of interrelated pathways controlling cell behavior [39].

The dramatic changes to a tumor cell morphologically during invasion have been referred to as EMT, which describes the conversion from an epithelial morphology to a non-polarized, motile, spindle shaped cell resembling a fibroblast [35, 40, 41]. EMT is associated with the loss of epithelial-specific E-cadherin from the adherens junctions, and a switch from the expression of keratins as the major intermediate filament to the mesenchymal intermediate filament, vimentin. EMT is influenced by the tumor microenvironment, and has been observed primarily at the edge of the tumor mass that is in contact with the tumor stroma [42]. A key regulator of EMT is transforming growth factor beta (TGF-β) signaling but other mediators include hepatocyte growth factor/scatter factor (HGF/SF), PI3 kinase signaling pathway, MAP kinases, and the transcriptional regulators Twist and Snail. Other signaling pathways implicated in stem cell maintenance that are linked to EMT are Wnt, Notch and Hedgehog. Tumor cells may also reverse the process and undergo a mesenchymal-to-epithelial transition (MET) in the absence of EMT-inducing signals. This transient nature of EMT helps explain why metastatic cells can morphologically resemble cells in the primary tumor despite the fact that they by necessity accomplished all the steps of the metastatic cascade [40, 43].

Epithelial cell–cell interactions are mediated primarily by cadherins, transmembrane glycoproteins that form calcium-dependent homotypic complexes. The loss of E-cadherin that occurs during EMT correlates with increased invasion and metastatic potential in most tumor types and reexpression in experimental models can block invasion. This observation suggests that loss of E-cadherin is causative for invasion. E-cadherin loss occurs at multiple levels including transcriptional repression and proteolytic degradation. The zinc finger transcriptional repressors Snail and Slug in particular have been implicated in regulating EMT by virtue of their ability to repress E-cadherin transcription. Cadherins are regulated by catenins (α, β, γ and p120 catenins), cytoplasmic proteins that functionally link the cadherin complex to the actin cytoskeleton. β-Catenin is both a cell adhesion protein and a transcription factor. In addition to its role in adherens junctions, it participates in canonical Wnt signaling, a signaling pathway important in development and cancer. E-cadherin levels and function are also disrupted by loss of p120 catenin, which occurs in many tumor types and may also contribute to tumor metastasis.

E-cadherin is not the only cell–cell adhesion molecule associated with invasion and metastasis. NCAM, a member of the immunoglobulin cell adhesion molecule Ig-CAM family, is downregulated in several tumor types, and NCAM loss results in an increased ability of tumor cells to disseminate [44, 45]. Other Ig-CAMs, such as DCC, CEACAM1, and Mel-CAM, also demonstrate reduced expression in specific cancer types. It should be noted, however, that not all cell–cell adhesion molecules can be viewed as potential invasion suppressors. Several are overexpressed in advanced cancers and have functions associated with cancer progression including Ig-CAMs such as L1, CEA, and ALCAM. Additionally, N-cadherin promotes cell motility. This complexity may be explained by direct or indirect signaling functions for these molecules that are distinct from their role in cell–cell adhesion. The interrelatedness of tumor growth and tumor invasion, and limitations of experimental model systems, does not always allow a distinction between growth effects that influence the appearance of an invasive phenotype and an effect on actual cellular invasion.

Cells induced to undergo EMT not only exhibit enhanced motility but are resistant to apoptosis, another key requirement for successful metastasis. However, some cancer cells use EMT independent modes of migration including collective and amoeboid [38]. Although EMT and MET are well accepted processes necessary for normal development and can be demonstrated and manipulated in many experimental tumor models, it has been questioned whether they are actual requirements for human cancer progression.

The ECM provides a scaffold for the organization of cells and spatial cues that dictate cell behavior [46]. This matrix is composed of proteins, primarily triple-helical collagens, glycoproteins such as laminins and fibronectin, and proteolgycans. The basement membrane is an organized ECM that forms a barrier separating polarized epithelial, endothelial, and muscle cells from the underlying tissue. Interstitial matrix provides the structural characteristics of connective tissues. The molecular composition of the ECM varies between tissues and organs, and provides important contextual information to cellular constituents. In addition, the ECM interacts with many secreted molecules to serve as a repository for regulatory proteins and growth factors. Thus, the interaction of cells with ECM molecules dictates their ability for survival, growth, differentiation, and migration. Moreover, selective proteolysis of ECM components leads to release of fragments that further regulate protein function and may be involved in cell signaling. These factors are collectively known as matrikines.

Adhesion of cells to their surrounding matrix occurs primarily through a family of transmembrane glycoproteins known as integrins that are assembled as specific combinations of 18 alpha and 8 beta subunits [47, 48]. Each combination of integrin subunits binds to distinct but overlapping subsets of ECM components and may be either tumor promoting or inhibitory. During tumor progression, cancer cells tend to downregulate the integrins that mediate adhesion and maintain a quiescent, differentiated state, and upregulate integrins that promote survival, migration, and proliferation. Although there is a cell-type dependency on integrin function, generally

integrins α2β1 and α3β1 are viewed as suppressors of tumor progression, while αvβ3, αvβ6, and α6β4 promote cellular proliferation and migration. Integrins mediate signals in both directions, so that changes in intracellular signaling pathways can modulate cellular adhesion, and changes in cellular adhesion can alter cellular phenotype. A well-described and important mechanism whereby integrin-ECM interactions modulate cell function is by cooperative signaling with different growth factor receptors. Many of the cellular responses induced by activation of tyrosine kinase growth factor receptors are dependent on the cells being able to adhere to an ECM substrate in an integrin-dependent fashion. Signaling in response to ECM interaction usually activates focal adhesion kinase (FAK) and non-receptor tyrosine kinases of the src-family.

The ECM is remodeled by degradative enzymes that are produced by the tumor cells themselves and additionally by the resident and infiltrating cells as a response to the tumor. These enzymes contribute to matrix degradation and facilitate tumor cell invasion. Proteolytic enzymes of many classes have been implicated in tumor cell invasion, including but not limited to the serine proteinases plasmin, plasminogen activator, seprase, hepsin, and several kallikreins, the cysteine proteinase cathepsin B, the aspartyl proteinase cathepsin D, and metal-dependent proteinases of the matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase (ADAM) families. Other matrix degrading enzymes such as heparanase, an endoglycosidase which cleaves heparin sulfate proteoglycans, and hylauronidase cleavage of its substrate hylauronic acid, have also been causally associated with tumor progression and invasion.

Liotta and colleagues observed that metastatic potential correlates with the degradation of type IV collagen found predominantly in the basement membrane and focused attention on the metal-dependent type IV collagenases or gelatinases that are now recognized as MMP-2 and MMP-9. Subsequently, many of the 23 members of the MMP family of matrix-degrading metalloproteinases have been associated with tumor progression. Elevated MMP levels correlate with invasion and metastasis and poor prognosis in many cancer types, and animal models provide evidence for a causal role for MMP activity in cancer progression [49–51]. Additionally, the plasminogen activator/plasmin system has been causally implicated in cancer invasion, and urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) are validated prognostic and predictive markers for breast cancer.

Regulation of matrix proteolysis occurs at multiple levels and in addition to the expression of proteases by both the tumor cells and adjacent resident and infiltrating cells, they produce specific endogenous inhibitors including the tissue inhibitors of metalloproteinases (TIMPs), serine proteinase inhibitors (SERPINs), and cysteine protease inhibitors (CYSTATINs). Some of these inhibitors are stored in high concentrations within the ECM and paradoxically may be required for activation of the protease. The conversion of pro-MMP-2 to active MMP-2 requires the activity of MT1-MMP (MMP-14), a transmembrane MMP that is activated intracellularly by the propeptidase family member furin, and TIMP-2 (Fig. 12.4). The concentration of each of these molecules is critical for proper function and regulation. Another proteolytic cascade important for regulating protease activity during the degradation of ECM is cathepsin(s) → uPA → plasmin → MMP. Each of the proteases in this cascade is capable of cleaving components of the ECM. Therefore, activity cascades are important regulators of proteolytic function in addition to endogenous inhibitors, protease degradation, and activation.

The original view that proteolytic enzymes function predominantly to remove physical ECM barriers has been expanded with the realization that proteolysis regulates multiple steps of tumor progression. For example, MMP substrates in the matrix or on the cell surface that modulate cellular growth, differentiation, apoptosis, angiogenesis, chemotaxis, and migration have been identified. The abundant evidence for a role for MMPs in tumor progression led to the design and testing of synthetic MMP inhibitors for cancer therapy. These inhibitors proved to be ineffective in clinical trials, results that have been explained by problems with inhibitor or clinical trial design, as well as a lack of understanding of the broad range of MMP activities resulting in both cancer-promoting and cancer inhibitory effects.

In addition to ECM remodeling, locomotion of a cell occurs through the coordination of polymerization and depolymerization of the actin cytoskeleton to extend a pseudopod at the leading edge of the cell, followed by contraction associated with disassembly of cell–matrix adhesive contacts at the trailing edge [52]. Lamellipodial protrusions at the leading edge are nucleated by a branched actin network involving the Arp2/3 complex and its regulators, the WASP family, cortactin, and the GTPase Rac. Actin contractility is regulated by myosin light chain kinase and upstream small GTPases, in particular Rho and its effector ROCK. As alluded to above in the discussion of EMT, single cells migrate either with a spindle-shaped morphology, referred to as mesenchymal migration, or with the less adhesive ellipsoid shape used by leukocytes and *Dictyostelium* termed amoeboid migration. Collective migration can occur when the cells retain cell–cell junctions and clusters of cells move in single file through a tissue.

Tumor cells can secrete factors that stimulate motility in an autocrine fashion. Tumor cell-produced lysophospholipase D (autotaxin) stimulates motility, as does lysophosphatidic acid (LPA), which can be produced by lysophospholipase D activity on lysophosphatidylcholine. Hepatocyte growth factor/scatter factor (HGF/SF) interacts with its receptor, c-met, to induce chemokinetic activity of epithelial cells, resulting in an invasive phenotype. Directional motility is a chemotac-

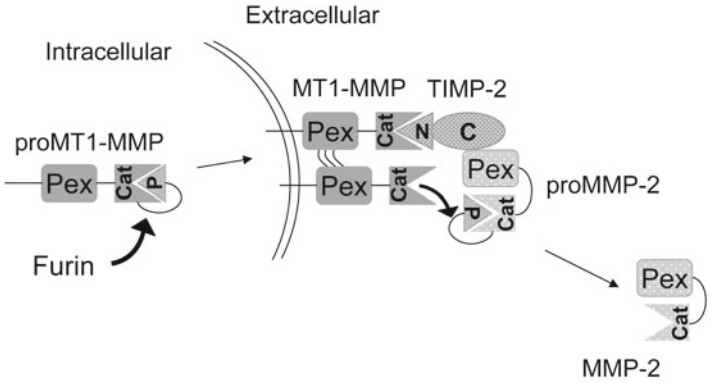


Fig. 12.4 Example of a proteolytic activation cascade. The proteolytic activities are intricately regulated at several levels during the MMP-2 activation cascade. The activation of proMMP-2 occurs by the balance of TIMP-2, MT1-MMP, and itself. MT1-MMP is activated intracellularly by furin and localized to the plasma membrane. The TIMP-2N-terminal (N) domain binds to and inhibits MT1-MMP activity and recruits proMMP-2 by interaction of TIMP-2 C-terminal (C) domain to proMMP-2 hemopexin domain (Pex). An adjacent MT1-MMP activates proMMP-2 to MMP-2 through cleavage of the pro-domain (P).

tic (following a soluble concentration gradient) or haptotactic (following an insoluble concentration gradient) effect in response to a gradient of soluble or localized factors, respectively. Chemotaxis is often the result of growth factors such as insulin-like growth factor (IGF), and chemokines of the CCR and CXC family. Haptotaxis is characterized as a response to gradients of ECM components such as laminin-5 and fibronectin, and can be modulated positively or negatively by proteolysis [53].

The coordination of cell–cell and cell–matrix adhesion, matrix degradation, and cytoskeletal activity is required for cellular invasion. The type of cell migration (collective, mesenchymal, or amoeboid) is influenced by the relative levels of adhesion mediated by cadherins and integrins, proteolytic activity, and actin contractility. Modulation of any of these factors is thought to convert one type of motility into another. Structures identified in invading cells in three dimensions that represent the physical convergence of the adhesive, proteolytic, and motility component of invasion are known as invadopodia [54]. Invadopodia are actin-rich organelles that protrude from the plasma membrane and contact and locally degrade the ECM [55–58]. Invadopodia contain adhesion molecules, including several β1 integrins and CD44, and the proteinases seprase, dipeptidyl dipeptidase IV, and several MMP and ADAM metalloproteinases. Inside the plasma membrane, invadopodia contain actin and actin assembly molecules, as well as multiple signaling molecules including FAK, src associated proteins such as p130Cas and Tks5/FISH, and the small GTPases cdc42, Arf1, and Arf6. Thus, invadopodia are implicated as key cellular structures that are used to coordinate and regulate the various components of the process of cancer invasion.

12.4 Further Steps Required for Metastasis

Invasion and metastasis are not equivalent phenotypes. Although invasion is prerequisite for metastasis, the ability to invade is insufficient to develop a metastasis. Once cells have invaded away from the primary tumor, they disseminate. Most commonly, metastasis is described in terms of hematogenous (blood-borne) dissemination. However, secondary tumors can arise because tumor cells have migrated via lymphatics (i.e., lymph nodes are common sites of metastasis for most carcinomas) or across body cavities (e.g., ovarian carcinoma cells most frequently establish secondary tumors by dissemination in the peritoneum while rarely forming metastases via hematogenous spread) [59]. Additional examples are dissemination of melanoma cells along the space between endothelium and basement membrane or perineural spread in pancreatic and prostatic carcinomas. Some question whether non-hematogenous spread is actually metastasis. We submit that the route of dissemination is irrelevant to the definition of metastasis, as long as the cells are discontinuous from the primary tumor mass. Nonetheless, for the remainder of this chapter, we will focus on blood-borne metastasis since this route is responsible for the majority of visceral metastases.

12.4.1 Intravasation

Following local invasion, the next step in dissemination is the entrance of tumor cells into the vasculature, a process termed intravasation. The mechanisms of tumor cell entry into the blood stream are not clearly understood. Both active and passive processes are involved. The growth of a tumor exerts a

hydrostatic pressure, and studies imply that tumor cell invasive cords follow lines of least resistance. Angiogenesis is likely to be a prerequisite for metastasis, but this has not been formally proven. Tumor cell entry into intact blood vessels is an active process that requires serine- and metallo-proteinase activity in an experimental model of intravasation. Tumor blood vessels, however, are highly abnormal with fewer pericytes and increased permeability compared to normal vessels, and presumably provide an easier route of entry for tumor cells. Tumor-associated lymphatic vessels are also abnormal, but their role in intravasation is unclear [60]. Irrespective of the route, tumor cells enter the circulation in great numbers: measurements of 3–4 million cells/day/g of tumor have been reported [9]. The number of tumor cells in the peripheral blood, however, does not necessarily predict if the patient will develop metastases [61–63].

12.4.2 Transport

Once tumor cells enter the blood stream, they can move actively by motility mechanisms or passively, carried or pushed along with fluid flow. Intravital microscopy has shown that most tumor cells do not float along, but rather roll like leukocytes. Despite having weak adhesion while rolling in a vessel, tumor cells are subject to anoikis, a specialized type of apoptosis in which cells that are anchorage-dependent are induced to die. In general, metastatic cells are more resistant to anoikis than non-metastatic cells.

Experiments designed to assess tumor cell fate following vessel entry often involve bioassays to quantify cells following intravenous injection. By the time it takes to remove various tissues for assay (2–3 min), the majority of cells have died. What is the basis for the cell loss? Tumor cells are killed by immune recognition, typically natural killer (NK) or monocytes. However, the majority are thought to be killed by exposure to hemostatic sheer forces.

The average tumor cell has a diameter of 20–30 μm but must navigate through vessels significantly smaller (capillaries are 6–7 μm). Therefore, tumor cells must deform and squeeze through the vascular passages. Biophysical parameters such as membrane fluidity, cellular elasticity and cytoskeletal organization influence whether the cells will remain intact or be broken by sheer. Deformability of the cell is also impacted by the hemodynamic pressures found within various tissues. In contrast to the sheer forces usually encountered in the vasculature, blood flow in bone sinusoids is sluggish (~30-fold lower than capillaries and post-capillary venules), and diameter is not limiting.

During transport, tumor cell behavior is often determined by their presence as either single cells or as emboli. Embolization can either be homotypic (tumor cell-tumor cell) or heterotypic (tumor cell-leukocyte, tumor cell-platelet, tumor cell-fibrin). The association of tumor cells with blood cells can be the result of altered cell surface glycosylation and expression of sialyl Lewis X and sialyl Lewis A on the tumor cell that permits interaction with a class of vascular adhesion molecules found on normal leukocytes and endothelium, the selectins. Alterations in the adherence of tumor cells to endothelium via E-selectin, platelets via P-selectin, and to leukocytes via L-selectin alters metastatic potential in animal models. However, the cellular specificity of adhesion to the selectins may not be so clear cut, as recent evidence indicates that bone endothelium expresses P-selectins and L-selectins. Embolus size can also contribute to the protection of the tumor cells from biophysical forces or immune attack as well. In essence, putting tumor cells into a cellular or molecular cage helps to protect them. As a result of the consequence of embolus formation, heparin, and inhibitors of selectin/glycan interactions, have been considered for anti-metastatic therapy.

12.4.3 Arrest

After tumor cells have spread to distant sites, they arrest either by physical trapping or selective adhesion to the walls of the microvasculature. Both processes have been observed, and the relative importance of these mechanisms in specific organs is debated.

In higher vertebrates, three types of endothelial structures are found—continuous, discontinuous and fenestrated. The majority of endothelial cells form tight junctions with their neighbors and have a continuous, unbroken basement membrane beneath them. However in certain organs, such as liver and spleen, the endothelial cells and the basement membrane have gaps, or discontinuities, in their structure. In the kidney, a fenestrated endothelium, there are gaps between endothelial cells but a membrane-like structure connects them and the entire structure overlaps in a continuous basement membrane. The structure of these endothelial/basement membrane barriers contribute to the normal function of the tissues and form different barriers through which tumor cells must pass. The structures also provide different ligands to which the tumor cells might adhere.

Adhesion of circulating cells to organ microvascular endothelial cells is key to metastasis, especially organ-specific metastasis. In general, higher rates of tumor cell–endothelial adhesion correlate well with metastatic potential. The initial attachment of cancer cells occurs preferentially at endothelial cell junctions and at sites of active inflammation. The latter is most likely related to endothelial cell surface alterations concomitant with inflammatory processes. In general, tumor cells utilize the same molecules and mechanisms to adhere to and traverse endothelium as inflammatory cells.

After tumor cells bind endothelium, they induce endothelial cell retraction. Eventually, the endothelial cells overlap the tumor cell. Tumor cells are then directly in contact with the basement membrane and can initiate the next step of the metastatic cascade.

12.4.4 Extravasation

Extravasation is the process of tumor cells exiting a vessel into the organ parenchyma. Extravasation was viewed as a key rate limiting step for metastasis formation, but intravital microscopy studies have indicated that extravasation can be a remarkably efficient process, at least in some situations. For example, Ann Chambers and colleagues showed that nearly 90 % of B16F1 murine melanoma cells that were injected into the mesenteric vein arrested in the liver 90 min after injection. Of the injected cells, 83 % were in the liver parenchyma by 3 days, meaning that >95 % of the arrested cells extravasated [64]. The molecular mechanisms underlying extravasation are thought to be similar to those involved in invasion, and in vitro assays for extravasation reveal a contribution of cellular adhesion molecules, proteinases, and motility factors.

There is controversy as to whether extravasation is required for metastasis. In the case of some pulmonary metastases, there is evidence that tumor cells can attach to the lung endothelium, survive and grow intravascularly [65]. Extravasation occurs in this model only when the intravascular foci outgrow the vessel.

12.4.5 Colonization

Colonization, the formation of clusters of tumor cells at ectopic sites, represents one of the most highly inefficient steps in the metastatic cascade. A tumor cell must first survive and then grow in a foreign environment. In some tumor types, it is not uncommon for metastases to arise decades after the primary tumor had apparently been treated. Those clinical observations demonstrate that tumor cells can survive in a dormant state for long times. It is unclear whether cells at these sites persist as solitary cells, or whether their overall growth rate is balanced by the rate of apoptosis or differentiation. Conversion to a clinically detectable, macroscopic metastatic lesion requires the subsequent initiation of angiogenesis.

The ability of a tumor cell to establish a metastatic lesion is very much dependent on the microenvironment. The growth of the cells is dependent on several factors, primarily soluble growth factors, present at the site of colonization. Although it is natural to focus upon molecules that promote tumor cell growth, there is ample experimental evidence showing that some tissues are hostile to tumor cells. So, resistance to growth inhibitory factors is required as well.

12.4.5.1 Organ Selectivity

There is a clear tendency for primary tumors to form metastatic lesions in specific organ sites (Table 12.1). Common regional sites of metastatic involvement can often be explained by anatomical or mechanical considerations (efferent venous circulation or lymphatic drainage). Tumor cells often arrest in the first capillary bed or lymph node encountered. Since the vast majority of tumor cells enter the vasculature in small veins or capillaries, the most common sites of metastasis are lung and liver. However, distant metastases are typically more site specific.

In 1889, Stephen Paget analyzed postmortem data of women with breast cancer and noticed a higher frequency of skeletal metastasis than would be expected based solely upon vascular perfusion. He concluded that the pattern was not simply by chance, and suggested that metastases develop only when the seed (tumor cells with metastatic potential) and the soil (organs or tissues providing growth advantages to seeds) are compatible.

Experimental data supporting Paget's "…*seed and soil*…" hypothesis is abundant, including preferential invasion and growth of tumor cells in specific organs and tissues as well as selection of tumor subpopulations with preferential sites of metastasis [66]. Furthermore, David Tarin and colleagues observed in women with advanced ovarian cancer that palliative treatment to reduce large ascites burden by peritovenous shunts did not result in disease outside the peritoneal cavity [67]. By introducing a Levine shunt, a tube that drains the peritoneal ascites into the vena cava, billions of viable tumor cells were introduced into the circulation daily. Despite this, metastases to the lung (the first capillary bed encountered) were rare. This single clinical observation highlights the inefficiency of the metastatic process and, more importantly, demonstrates that merely seeding cells in different tissues is not adequate to develop metastases.

What, then, are the molecular mechanisms underlying organotropism of tumor cells? Tumor cells adhere more selectively to organ-derived microvascular endothelial cells than large vessel endothelial cells, and variants of the B16 melanoma previously selected for metastases to brain, lung, ovary or liver adhere at a more rapid rate to brain, lung, ovary or liver endothelial cells, respectively [66]. Using phage display technology, endothelial cells in different tissues were shown to express unique markers, and tumor cells recognize the molecular addresses in order to adhere in a selective manner [68]. Tumor cells can also recognize subendothelial basement membrane differences. In vitro studies demonstrate the selective growth of tumor cells in organ-derived soluble growth factors or cells. Some tumor cells even respond to tissue derived chemotactic factors. For example, breast tumor cells that express the chemokine receptor CXCR4 preferentially metastasized to tissues that expressed the ligand, SDF1/CXCL12 [69].

While the chemoattractant data support the notion that there are soluble factors produced in different tissues to which tumor cells can respond, the process of homing has not been observed. Strictly speaking, homing would require

Table 12.1 Predilection of metastasis for certain tissues

Primary tumor	Common site(s) of metastasis
Breast	Bone, lung (pleura), liver, brain, adrenal, axillary lymph nodes, contralateral breast, ovary
Colon	Liver, lymph node, lung, direct extension into urinary bladder or stomach
Kidney	Lung, liver, bone
Lung	Bone, brain, lymph nodes, pleura, diaphragm (by direct extension), liver, kidney, adrenal, thyroid, spleen
Ovary	Diaphragm, peritoneal surfaces, lymph nodes
Pancreas	Liver, stomach (by direct extension), colon, peritoneum
Prostate	Bone (particularly vertebrae and pelvis), lymph nodes
Stomach	Liver, lymph nodes, lung, bone
Testes	Lymph nodes, lung, liver
Urinary bladder	Lung, rectum (by direct extension), colon, prostate, ureter, vagina, bone, lymph nodes, peritoneum, pleura, liver, brain
Uterine endometrium	Lung, lymph nodes, liver, ovary

Adapted from [52]

directed movement throughout the transit of tumor cells as they leave the primary tumor. Rather, tumor cells distribute according to circulatory patterns initially but may home once they are more proximate. More likely, they may be retained at sites more readily.

Some of the strongest new evidence supporting organ selectivity of cancer cells comes from data showing the selection of variants that colonize different tissues. The first selections were done by repetitive isolation of lung metastases from the B16 melanoma followed by reinjection and recolonization. Similar approaches were used to select B16 melanoma cells with brain, ovary, and liver predilection. More recently, MDA-MB-231 human breast carcinoma cells were used to select variants that colonize bone, lung and adrenal gland. Using these breast carcinoma cell lines coupled with comparison by cDNA microarray, Joan Massagué and colleagues demonstrated the requirement for coordinated expression of multiple genes for metastasis [34, 70, 71]. Transcriptomes were compared between parental and bone-selective variants and overexpressed and underexpressed genes were identified. Transfection of individual cDNAs only modestly increased site-specific metastatic efficiency, whereas co-transfection of gene combinations into the parental cells resulted in populations as efficient as the bone or lung colonizing selected variants. These data highlight that there is probably a hierarchy in the genetics of metastasis. Superimposed upon the metastasis competency transcriptome are gene expressions which determine metastasis localization.

Yet, not all organ selectivity may be determined by the seed and the soil alone. There is a recent concept that tumor cells colonize in pre-metastatic niches initiated in target organs by tumor cell-generated soluble factors that induce the migration of hematopoietic stem cells to sites where they induce expression of fibronectin by resident fibroblast-like cells [72, 73]. Bone marrow-derived cells that express the vascular endothelial cell growth factor receptor 1 and the integrin α4β1 selectively adhere to these regions, produce the proteinase MMP-9 and the chemokine SDF1/CXCL12, and provide a permissive niche for the colonization of tumor cells. In a sense, the tumor cells ensure fertilization of the soil using this mechanism.

12.5 Genetic Determinants of Metastasis

Primary tumor formation and metastasis are distinct processes—locally growing tumors can grow and progress without the development of metastases. This observation prompted the hypothesis that the molecular processes regulating tumorigenicity and metastasis are distinguishable. The existence of metastasis-controlling genes is supported by data from several laboratories showing that specific cDNAs block metastasis but not tumorigenicity [74, 75]. Such genes are termed metastasis suppressors and, by definition, are distinct from tumor suppressors (Table 12.2). Tumor suppressors block both tumor formation and metastasis since the former is prerequisite to the latter.

The identification of nm23 (non-metastatic 23), the first metastasis suppressor, provided functional evidence for the existence of molecules that specifically regulate metastasis. Subsequently, several laboratories have used various unbiased approaches to identify more than 20 metastasis suppressors. Importantly, the use of in vivo assays was required because in vitro assays were often of inadequate complexity to sufficiently model the entire process of metastasis. Several of the known metastasis suppressors block the last step of the cascade, colonization. And since there is not a reliable in vitro model of the last step of metastasis, determining the molecular underpinnings of the suppression has moved more slowly than desired.

Table 12.2 Examples of metastasis suppressors and known functions

BRMS1	Transcriptional co-repressor Component of mSin3A histone deacetylase complexes Regulates NFkB and PI3 kinase signaling Restores homotypic gap junctional intercellular communication
Cadherin-11	Cellular adhesion, cytoskeletal structure
Caspase 8	Pro-apoptotic enzyme
CD44 (family)	Cellular adhesion (hyaluronate) Potentiates HER signaling
Claudins 1 and 14	Cell–cell tight junctions
CRSP3 (cofactor required for SP1)	Transcriptional co-activator/co-repressor
CTGF (connective tissue growth factor)	Cellular adhesion (integrin binding)
DCC	Regulates cytoskeletal organization
DLC1	RhoGTPase activating protein
Drg-1	Unknown
E- and N-cadherin	Cell–cell adhesion (calcium-dependent)
GAS1	Inhibit cell cycle
Gelsolin	Actin polymerization
HUNK	Protein kinase
JNKK1/MKK4	Activates p38 MAP kinase and/or JNK kinase
KAI1/CD82	Binds DARC on endothelial cells Integrin interactions EGFR desensitization
KISS1	Ligand for G-protein coupled receptor
KISS1R	G-protein coupled receptor
KLF17	Transcription
LSD1	Chromatin remodeling
MKK6	Activates p38 MAP kinase
MKK7	Activates JNK kinase
Nm23-H1	Histidine kinase Phosphorylated kinase suppressor of Ras (KSR) Nucleotide diphosphate kinase
OGR1	GPCR signaling
p38	Stress-activated MAPK signaling
RECK	Regulates matrix metalloproteinases
RhoGDI2	Cellular motility: regulates Rho and Rac functions
RKIP	Inhibits RAF-mediated MAP/ERK kinase phosphorylation
RRMI	Increases PTEN expression; decreases FAK phosphorylation
Src-suppressed C kinase substrate	Cellular motility: regulates Rho signaling
TIMPs	Inhibit metalloproteinases; signaling
TXNIP (thioredoxin interacting protein)	Inhibitor of thioredoxin; transcriptional regulator

Adapted from [4, 48, 53, 54]

Table 12.2 lists the proteins that have bona fide metastasis suppressor activity in vivo (suppression of metastasis following ectopic expression into metastatic cell lines). Metastasis suppressors vary widely in their cellular locations and biochemical functions, and many would not have been predicted a priori based upon their known cellular functions. Many metastasis suppressors are involved in cellular responses to exogenous signals, highlighting the importance of tumor-stromal interactions. Cells respond to external stimuli in a spatiotemporal manner by utilizing a relatively limited number of signaling pathways. In this light, metastasis formation can be viewed as the result of a tumor cell's ability to respond to multiple growth milieus (i.e., orthotopic and ectopic) as opposed to being restricted to growth at orthotopic sites.

In addition to the genetic changes associated with the metastatic cell, the importance of the host genome in controlling metastasis has been elegantly demonstrated by Kent Hunter and colleagues [76, 77]. Transgenic mice expressing the polyoma middle T oncogene (PyMT) under control of the mouse mammary tumor virus (MMTV) promoter develop metastatic mammary tumors. When bred to non-syngeneic mice, metastatic potential was enhanced or inhibited, depending upon the mouse strain. Since all tumors were initiated by the same oncogene, differences in metastasis can be

explained by genetic background differences. Specific loci contributing to metastatic efficiency have been identified.

12.6 Microenvironmental Factors Influencing Metastasis

The Hunter data highlights contributions of the host to the development of metastases. As described above, metastasis is regulated by tumor and stromal interactions at every step. Tumor cells can co-opt already existing endothelial cells to create an unimpeded vascular supply. Many of the proteases involved in tumor cell invasion are produced by stromal cells rather than the tumor cells themselves. Carcinoma-associated fibroblasts can stimulate tumor cell growth and/or invasion while normal fibroblasts are growth-neutral or growth-suppressing. Adhesion to endothelium, and growth in response to organ-specific factors are all dictated by microenvironmental cues.

In experimental models, metastatic capacity is very much dependent on the site of injection. For example, human colon cancer cells injected subcutaneously do not metastasize, although the same cells colonize the liver following orthotopic injection into the GI tract. Similar findings have been observed with renal cell carcinomas, mammary carcinomas and melanomas. Although mechanical factors certainly contribute to this effect, molecular differences in the tumor microenvironment are also contributing factors.

Infiltrating immune and inflammatory cells can have dual effects on tumor metastasis. On the one hand, recognition by the immune system that a tumor cell is foreign often leads to the destruction of the tumor cell in the elimination of the cancer. However, tumor cells have been known to subvert the immune system and often take advantage of properties inherent to the inflammatory cells, such as invasion. Tumors induce macrophage and neutrophil infiltrates which have been associated with increased invasion across ECM in vitro. Recent data even indicate that tumor cells recruit hematopoietic stem cells to the primary tumor where the immune cells are activated to assist with invasion. Co-injection of tumor elicited macrophages or neutrophils increased metastasis in animal models as well. The inflammatory cells invaded through basement membranes followed by tumor cells. In addition, the inflammatory system in various tissues produces factors that are either stimulatory, or inhibitory, for tumor growth.

12.7 Therapeutic Challenges and Opportunities

The most devastating aspect of cancer cell behavior is the ability to metastasize. When tumor cells are localized, surgery is curative. However, when cells colonize secondary sites, cure rates drop precipitously. If no (neo)adjuvant therapy is given, metastases invariably kill the patient. Since the introduction of adjuvant therapies, the long-term survival rates have increased significantly (to approximately 25–30 % for breast and prostate cancers, for example). Such statistics are encouraging beginnings, but are certainly not adequate.

How, then, can understanding the molecular basis of metastasis lead to improved cancer therapies? First, by identifying what is unique about metastatic cells, one can theoretically target the therapy to the most dangerous populations of cancer cells. Unfortunately, the situation is not so simple. Cellular behavior in metastatic cells is governed by the same mechanisms that are present in normal cells and under normal physiology. The ability to invade is not unique to cancer cells. Leukocytes and neurons invade as part of inflammation and normal development, respectively. Similarly, leukocytes and stem cells exhibit intermittent adhesion as part of their normal function. And while moving around the body, they are certainly resistant to anoikis. These cells exert an influence at the secondary site. During inflammation, for example, leukocytes and fibroblasts degrade and reconstitute extracellular matrix. Proliferation of cells at two different locations would seemingly distinguish metastatic cells from normal counterparts; however, macrophages and stem cells (e.g., angioblasts, recruited hematopoietic stem cells) can proliferate at secondary sites, and in the case of stem cells they can proliferate long after initial seeding. Together, the distinctions between metastatic tumor cells and normal cells have been incredibly difficult to identify. Making matters even more complicated, metastatic cells use essentially the same molecular mechanisms for accomplishing each of the steps as do their normal counterparts.

Nonetheless, there is hope. There are some essential differences between normal cells and metastatic cells. Importantly, all of the properties necessary for metastasis must coexist within a single cell (including the properties necessary to recruit another cell to complement a specific defect) since metastases are clonally derived. Conceptually, this offers opportunities. Molecules that block adhesion to tissue-specific endothelium and/or the underlying basement membrane components offer some specificity in response. Invadopodia appear to be specialized structures found in only a limited number of normal cells, and presents an opportunity for selective targeting. In addition, unlike stem cells that can enter a secondary site, proliferate and differentiate, metastatic cells do not differentiate fully at a secondary site. Hence, another hallmark of metastatic cells is their ability to persistently proliferate without fully differentiating.

A therapeutic opportunity for controlling metastasis may not lie so much in understanding the unique characteristics of the tumor cell, as in understanding the controls exerted by the tumor microenvironment [1, 78]. Targeting normal cells, as opposed to genetically unstable tumor cells, lessens the chance of drug resistance (since normal cells are not as genet-

ically unstable). The colonization stage of metastasis offers exceptional therapeutic opportunities, since the cells can remain alive but dormant or pre-angiogenic for long periods of time. As long as the resident tumor cells do not impinge upon organ function, cancer could be rendered a chronic, controllable disease rather than an acute, deadly one.

Acknowledgements The authors would like to thank members of the Welch and Hurst labs for discussions and critical reading of this chapter. The authors are extremely grateful for the financial support supporting our research, some of which is cited in this chapter: U.S. National Cancer Institute R01-CA62168 (DRW) CA87728 (DRW), CA134981 (DRW), P30-CA168524 (DRW), Susan G. Komen for the Cure SAC11037 (DRW), National Foundation for Cancer Research-Center for Metastasis Research (DRW) and the Kansas Bioscience Authority (DRW), and American Cancer Society (RSG-11259-01-CSM), METAvivor Research and Support, Inc. (DRW and DRH), and National Institutes of Health (F32CA113037) (DRH). DRW is the Hall Family Professor of Molecular Medicine and is a Kansas Bioscience Authority Eminent Scholar.

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Simple Sequence Mutations

13

Christophe Béroud

13.1 Introduction

Mutations are defined as modifications of the DNA that can be transmitted from one cell to its offspring. Thus, mutations occurring in the germ line can impact the next generation, while mutations happening in other tissues affect the host organism. We can therefore distinguish hereditary diseases resulting from germ line mutations and acquired genetic diseases resulting from somatic mutations. Indeed, hereditary forms of cancers account for a limited fraction of cancers. Moreover, cancers are complex genetic diseases characterized by multiple somatic alterations in the pathways that control cell growth, proliferation, and differentiation. Almost 25 years ago a first attempt was made to decipher the various steps of colorectal tumor development. It led to the creation of a model of colorectal tumorigenesis in which the steps required for the development of cancer involve the mutational activation of an oncogene coupled with the loss of several tumor suppressor genes [1]. This model relied on the mutational and gene expression analysis of known oncogenes and tumor suppressor genes. It was further completed with data from more recent techniques such as the Comparative Genomic Hybridization (CGH) [2] and its latest developments the cDNA microarrays [3, 4] and the oligonucleotide microarray analysis (ROMA) [5]; cDNA microarrays and oligonucleotide microarrays used to analyze gene expression [6, 7] as well as the Serial Analysis of Gene Expression (SAGE) [8], and the study of epigenetic changes that result in loss of gene expression that can be performed by various methods such as the Restriction Landmark Genomic Scanning (RLGS) [9] or DNA bisulfite treatment [10]. Recent progression models of breast, prostate, lung, and colorectal cancer have therefore been proposed [11].

Cancers are unique genetic diseases as they involve numerous somatic alterations. These alterations are subject to a selection pressure in the specific context of the human body. The study of these variations can therefore be approached both by a functional and by an evolutionary point of view. The purpose of this chapter is not to analyze all genetic variations found in cancers but instead to focus on simple mutations. These simple mutations can be defined as substitutions that can result in synonymous or nonsynonymous changes, nonsense or aberrant splicing, as well as small deletions and insertions of a few nucleotides.

The development of new sequencing technologies has led various teams to develop global approaches in order to search for new genes involved in cancer initiation and/or progression. Thus by the end of 2005, the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) launched a new project called the Cancer Genome Atlas (TCGA) that is a comprehensive and coordinated effort to accelerate our understanding of the molecular basis of cancer through the application of genome analysis technologies, including large-scale genome sequencing. The first results from the pilot study on lung, brain, and ovarian cancers have been released [12]. Before this initiative, the Wellcome Trust Sanger Institute had produced a large-scale genome sequencing of 518 protein kinases in 26 primary lung neoplasms and seven lung cancer cell lines [13]. In these various sequencing projects, as expected, many sequence variations have been identified in each cancer. One major challenge is to distinguish mutations involved in tumorigenic phenotype from other randomly co-selected mutations, especially for missense mutations.

This brings us back to a fundamental concept of evolution. In 1974, the hitchhiking concept of sequence variations was proposed by Smith and Haigh [14]. It supposes that a favorable allele can co-select adjacent variations. Therefore a neutral or even deleterious allele can spread in a population only because it is in strong linkage disequilibrium

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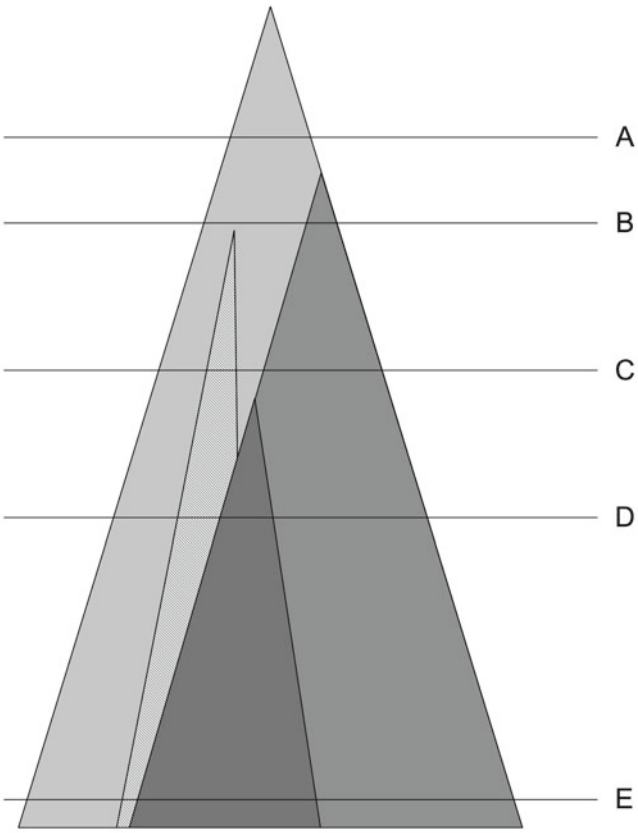
W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_13

with a favorable allele. Because this phenomenon is also observed in cancer, there is selection pressure between cancer cells. Nevertheless a major difference has to be underlined: in the cancer development model, cells accumulate mutations that can result in a favorable phenotype and therefore be selected but there is no mating and recombination between different partners. Therefore hitchhiking mutations (also called passenger mutations) can be co-selected with driver (or causal) mutations without any localization constraint (linkage disequilibrium). Thus, the observation of genetic changes should mainly be driven by the mutation rate of the human genome.

Recent studies have shown that point mutation rates vary across mammalian genomes not only at the gene level [15, 16] but also when noncoding sequences are considered [17]. Analyzing a large set of genes and assuming that the rates at which neutral substitutions accumulate in protein-coding genes allows the indirect estimation of the mutation rate. Kumar et al. [18] have shown that it is approximately constant per year and largely similar among genes. They suggest that the average mammalian genome mutation rate is 2.2×10^{-9} per base pair per year. These data should be compared to results obtained from the analysis of the hemophilia B locus for which mutation rates have been estimated as follow: transitions at CpG sites 1.3×10^{-7}, other transitions 9.9×10^{-9}, transversions at CpG sites 7.3×10^{-9}, other transversions 9.4×10^{-9}, and small deletions/insertions causing frameshifts 6.5×10^{-10} with an overall mutation rate of 2.14×10^{-8} per base per generation, or 128 mutations per human zygote [19]. Data produced by these studies rely on comparison of mutations among individuals and species. Thus, these calculations take into account the final result of evolution that involves genetic drift, natural selection, mutation, and migration. The specific contribution of each basic mechanism is almost impossible to establish. The cancer situation should be viewed as a simpler model as it corresponds to the evolution of cells without resort to mating. The genetic drift is therefore reduced and we can consider that no migration influence is involved in comparison to population's migrations from the evolution theory. Thus only two mechanisms remain: (1) occurrence of mutations, and (2) selection pressure. The non-clonal compound of cancer nevertheless complicates this apparent simplicity. In fact in a cancer, the cell population constantly evolves and the genetic sequence is highly polymorphic at the individual cell level. As illustrated in Fig. 13.1, the global study of a cancer sample can give various results depending on which period the analysis is performed. In addition, these results do not give information about the genetic content of each group of cells that can be found in the cancer and thus complicate the interpretation of variations.



	A	B	C	D	E
w	100	100	100	100	100
x	-	-	14	14	4
y	-	23	53	65	78
z	-	-	-	17	37

Fig. 13.1 Genetic heterogeneity of cancer cells. All cancer cells are derived from a common ancestor cell (*black dot*) that was subject to the pathogenic mutation (w). After an initial homogenous division period, other mutations (x, y, z) appear in various cells. They are subject to selection pressure and the ratio of cells harboring a specific mutation will vary over time. The analysis of the genetic content of the tumor will reveal different results depending on the stage where is performed the analysis (A, B, C, D, or E). The interpretation of the results can thus be hazardous. In fact, x and z mutations are present at the same level at stage D (respectively present in 14% and 17% of cells) but only the z mutation will subsequently be selected while the x mutation will progressively disappear from the tumor (stage E). The x mutation could therefore be considered as a passenger mutation while the z mutation could be considered as a disease causing mutation that will contribute to the cancer phenotype.

13.2 Mutations in Human DNA

The number of somatic mutations in cancer is the sum of the mutations acquired at each round of DNA replication in the normal and neoplastic cellular lineage leading from the fertilized egg to the progenitor cell of the neoplastic clone. Variation in mutation number may therefore be determined

by variation in the number of mitoses and/or by the factors influencing the mutation rate, including mutagenic exposures and DNA repair defects.

Most analyses have been performed on germ line mutations. They have shown that the most common substitution for A (adenine) is G (guanine), for C (cytosine) it is T (thymine), for G it is A, and for T it is C. In addition, it has been shown that CpG dinucleotides mutate to TpG at a frequency five times higher than mutations in all other dinucleotides [20–22]. This event can occur on both DNA strands and can result either in C>T or G>A on the coding strand. It is therefore frequently referred to as CG>TA transitions.

Spontaneous transitions occur during DNA replication through inappropriate pairing of nucleotides, due to a shift of one of the nucleotides to a rare tautomeric form. For example adenine and cytosine can change to an imino form instead of the regular amino form. Another mechanism of transition is the deamination of a methylated cytosine residue. This is the most frequent source of transitions in eukaryotes as a significant proportion of cytosines are methylated (Fig. 13.2).

Spontaneous transversions arise through a combination of two events: tautomerization and base rotation. This rotation causes the so-called syn-conformation of DNA or Z-DNA. As only 10 % of the DNA is present in the syn-conformation at any given time, and as the transversion mechanism involves an additional step in comparison to transitions, the frequency of spontaneous transitions is reduced. Nevertheless, transitions and transversions can also result from the exposure of the DNA molecule to various carcinogens. Therefore, the mutational event spectrum of a specific gene should be viewed as a combination of both spontaneous and induced mutations.

Deletions or insertions of a few nucleotides are also common in the human genome. Data from the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk), which contained by February 2007 an excess of 67,030 different lesions detected in 2478 different genes [23] show that small deletions and insertions account for 15 % and 6.6 % of germ line mutations, respectively. Germ line deletions of 1–3 base pairs (bp) account for 70 % of small deletions and frequently result in an alteration of the reading frame (78 %). The mechanisms by which these micro-rearrangements occur remains unknown, but it has been shown that (1) deletion of one or a few nucleotides frequently occurs in runs of the same nucleotide [24], and (2) larger deletions involve inverted repeats and symmetric elements [25, 26]. Micro-insertions are three times less frequent than micro-deletions, and nearly half of these involve the insertion of only one nucleotide.

Somatic mutations are acquired in somatic tissue during the subject's lifetime and predominantly result in neoplastic disease. These mutations can either be spontaneous or induced by exogenous compounds such as UV, carcinogens, or radioactivity.

The analysis of somatic mutations extracted from the UMD-TP53, UMD-APC, and UMD-VHL databases reveal that micro-deletions represent respectively 8 %, 51.4 %, and 28.6 % of somatic mutations, while micro-insertions account for 2.6 %, 12.2 %, and 7.5 % [27–29]. As for germ line mutations, an excess of deletions is observed with an average ratio

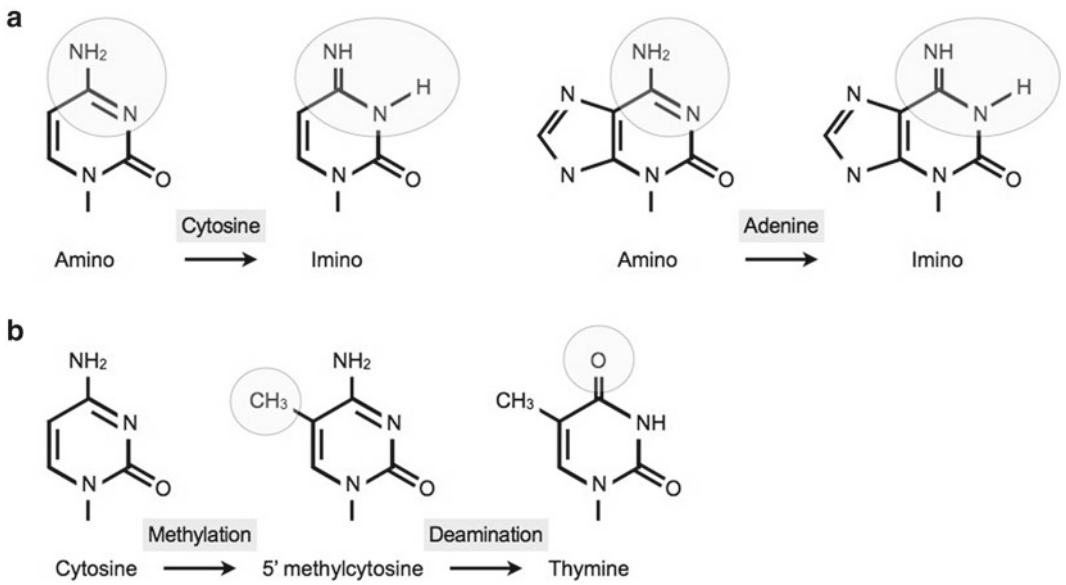


Fig. 13.2 The most frequent events that lead to a transition. (**a**) Shift to a tautomeric form cytosine and adenine shift to an imino form. (**b**) Schematic presentation of the transversion from a cytosine to a thymine after methylation and oxidative deamination.

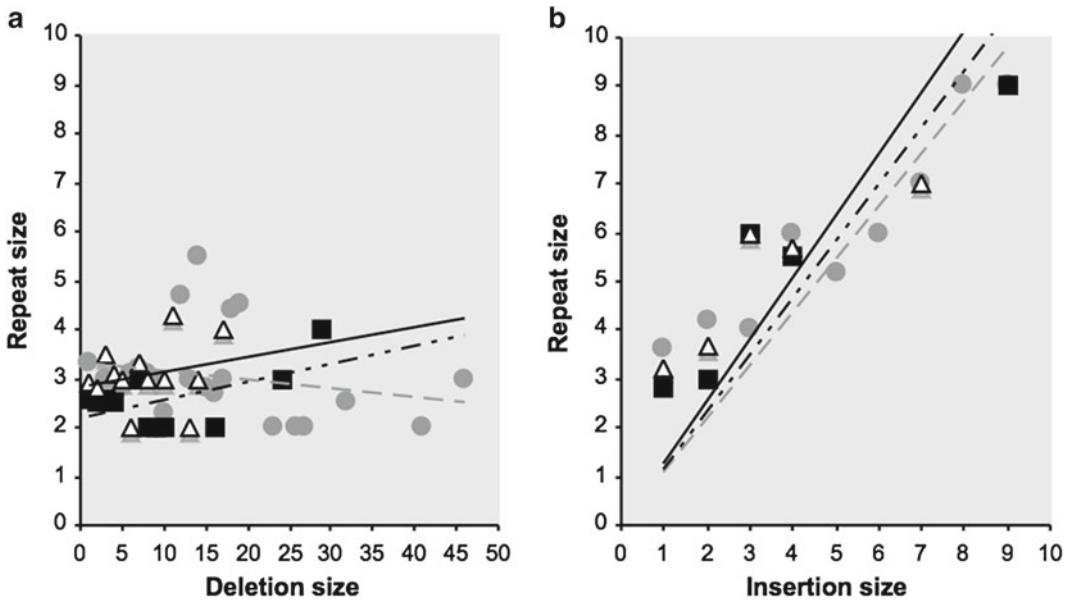


Fig. 13.3 Distribution of repeated elements surrounding deletions (**a**) and insertions (**b**). *Dark squares* = data from the VHL gene; *grey circles* = data from the TP53 gene and *white triangles* = data from the APC gene. *Dotted line* = fitting curves for VHL, *grey lines* = fitting curves for TP53, and *black lines* = fitting curves for APC.

of 3.4 deletions per insertion (this ratio is close to 3 for germ line mutations reported in HGMD). The study of repeated sequences surrounding the deletion reveals that the size of the deletion is not related to the size of the repeated sequences (Fig. 13.3a). In contrast, the study of micro-insertions show that the insertion is sequence dependent and will result in the creation of a repetition of whom the size is directly proportional to the insertion size (Fig. 13.3b).

13.3 Consequences of Mutagen Exposure

Mutagens are usually defined as chemical agents that increase the rate of genetic mutation by interfering with the function of nucleic acids. A clastogen is a specific mutagen that causes breaks in chromosomes. Mutagens that specifically result in nucleotides substitutions usually have a two step mechanism. The first event is the production of a DNA adduct, the second one being the inability of the cell to correctly repair this abnormal complex. In fact DNA adducts are chemical complexes that result from various chemical reactions between DNA and small molecules able to induce these reactions. These DNA adducts mostly involve one or more nucleotides, such as pyrimidine dimers, from a single DNA strand resulting in a mismatch between the two DNA strands. To preserve the genomic integrity, eukaryotic cells employ complex surveillance mechanisms called checkpoints to counteract DNA damage. The complex DNA damage checkpoint network is composed of DNA damage sensors, signal transducers, and various effector-pathways, and its major components are the phosphoinositide 3-kinase related kinases (PIKKs), ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), and DNA-PK (DNA-dependent protein kinase) [30–34]. ATM with its regulator MRN (Mre11-Rad50-NBS1) complex to sense double-strand breaks (DSBs) [35] whereas ATR with its regulator ATRIP (ATR-interacting protein) sense single-strand DNA (ssDNA) generated by processing of DSBs, as well as ssDNA present at stalled replication forks. Both kinases then initiate a signaling cascade that includes mediators, transducers, and effectors. About 25 ATM and ATR substrates have been identified [36]. Depending on DNA lesions, activated checkpoints can then mediate cell cycle arrest in G1, S, or G2 phases, DNA repair or even cell death by apoptosis. Matsuoka and coworkers have performed a large-scale analysis to identify proteins phosphorylated in response to DNA damage [37]. They have identified more than 700 candidate proteins that are involved in various biological processes (Fig. 13.4). Progress made in the decoding of these extraordinarily complex pathways reveal that the DNA-damage response network profoundly alters the cell.

In addition to this first level of response to DNA damage, a second barrier against cell alterations has been identified, oncogene-induced senescence [38–41]. Oncogene-induced senescence belongs to a heterogeneous group of cellular responses that include replicative senescence, which is induced by telomere attrition and depends of the activation of the DSB checkpoint [42]. In contrast, oncogene-induced senescence is a telomere-independent form of senescence associated with precancerous lesions [43]. Indeed, it is

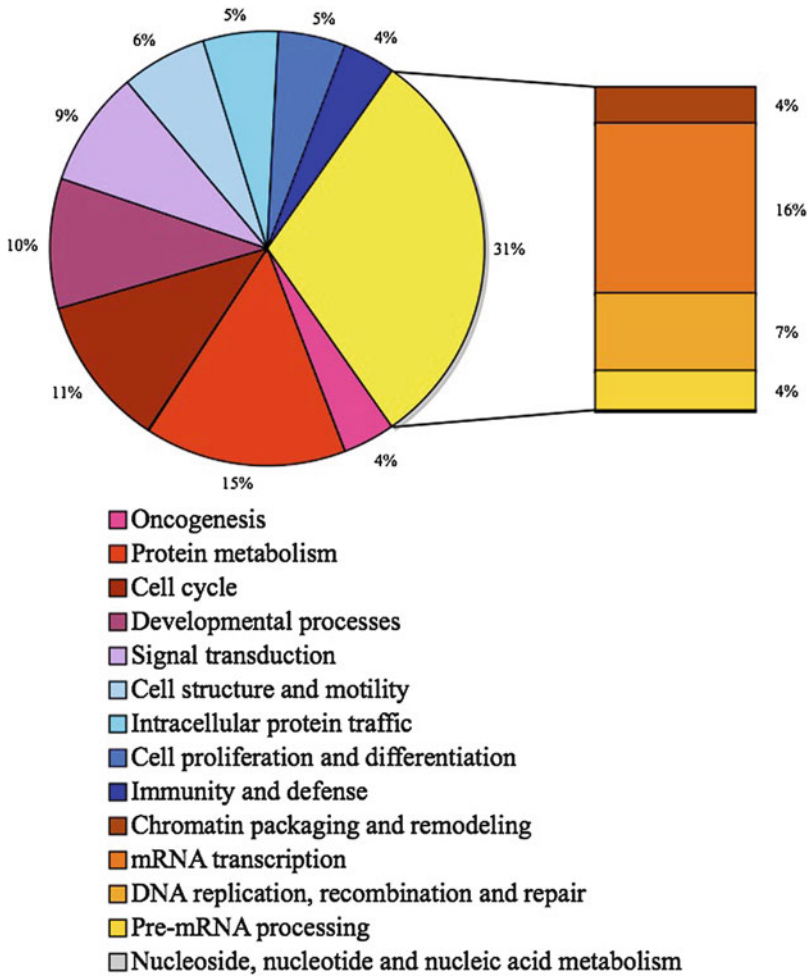


Fig. 13.4 Biological process of the more than 700 candidate ATM and ATR substrate proteins. Data adapted from Matsuoka et al. [37].

believed that this senescence is recruited to terminate a pre-malignant condition before a fully transformed stage can develop. Various experiments have clearly demonstrated that senescence can act as a key barrier to oncogene-mediated transformation in vitro but only few data have been collected in vivo. Melanie Braig and coworkers have provided a nice example about lymphoma development. They have shown that Ras-induced lymphomagenesis can be efficiently regulated by the histone methyltransferase Suv39h1 that methylates H3K9 into H3K9me creating binding sites for HP1 proteins to form constitutive heterochromatin locally. Thus, mice lacking at least one Suv39h1 allele developed lymphomas, whereas most transgenic control animals remain free of lymphoma. They also showed that this phenomenon is dependent on ARF and p53. Thus, an alteration of one of these two senescence regulators leads to the development of lymphomas [44].

Eukaryotic cells harbor complex and efficient networks to avoid mutations. These networks integrate a recognition step that will target DNA damage/intermediates such as uracil, 7,8-dihydro-8-oxoguanine (8-oxoG), 3-methyladenine, apurinic/apyrimidinic (AP) sites, and SSBs followed by: the excision of the inappropriate base moiety (e.g., 8-oxoG), the incision at the resulting abasic site, the replacement of the excised nucleotide, the cleanup of the terminal end(s), and the sealing of the final nick [45]. One of the most studied pathways is the repair step that involves the uracil-DNA glycosylase (UNG), which is responsible for the removal of uracil from DNA. This uracil is usually the result of an erroneous incorporation of dUMP opposite to adenine during the DNA synthesis or the result of a deamination of a methylcytosine (Fig. 13.5) that results in a mispairing between U and G and ultimately to a C to T transition if a DNA polymerase replicates across the mismatch [46]. UNG is a highly conserved enzyme found in many species from *E. coli* to human. All UNG appear to have very similar properties: they are able to cleave uracil from both single- and double-stranded DNA, whether it is in a U/A base pair or any type of base mismatch [47]. This enzyme hydrolyzes the N-glycosidic bond linking the uracil to the sugar and initiates the base excision repair (BER) process [48]. Other base alterations are frequently found in DNA. Among them, the 7,8-dihydro-8-oxoguanine (8-oxoG), also called 8-hydroxyguanine, is a by-product of normal aerobic metabolism. It is strongly mutagenic and

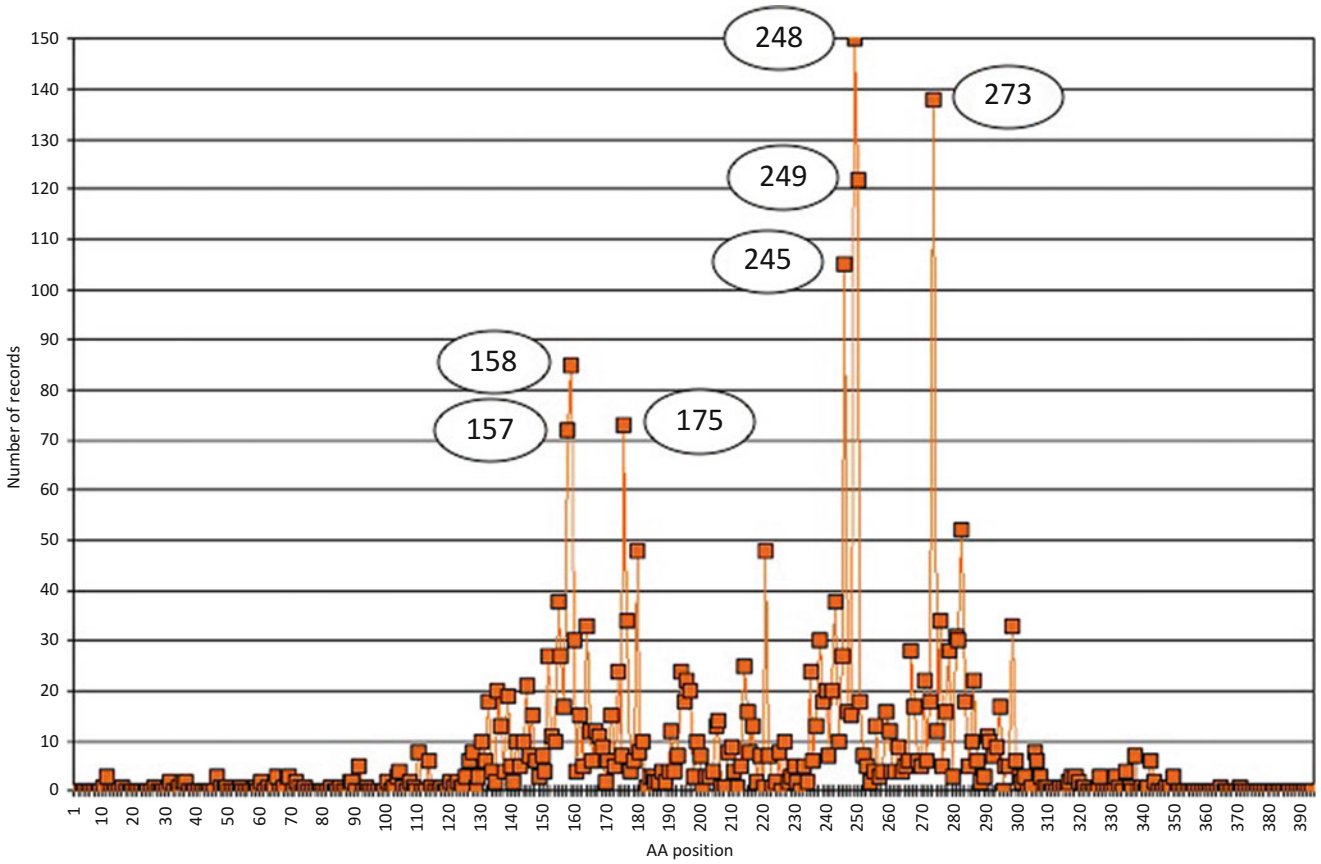


Fig. 13.5 Distribution of TP53 gene mutations in lung cancers. Data were extracted from the UMD-TP53 (http://www.umd.be) that contains 2784 mutations from lung cancers (July 2007 release). *X*-axis = amino acid residues, *Y*-axis = number of records. *Circles* indicate hotspot positions.

able to base pair with adenine and cause G:C → T:A transversion mutations. The OGG1 DNA glycosylase is the major activity excising 8-oxoG from DNA [49].

13.4 Gene Targets

Because of the high efficiency of the BER, the DNA damage checkpoint network, and the oncogene-induced senescence mechanisms, the probability for a mutation to arise is very low. Therefore, mutation of key genes from one of these pathways, as well as oncogenes or tumor suppressor genes, will more efficiently be selected and they are therefore good candidates for cancer-associated genes.

Historically, the identification of cancer genes relied on virus and linkage analysis using large kindreds with a high incidence of cancers. These two approaches led to the identification of oncogenes and later on to tumor suppressor genes associated with a predisposition to develop cancers. The first human retrovirus HTLV-1 was discovered in the late 1970s) [50], and the first tumor suppressor gene identified was the RB1 gene involved in retinoblastoma [51]. More recently, mismatch repair genes (hMLH1, hMSH2, and others) involved in microsatellites instability and colorectal tumors were discovered [52, 53]. These early discoveries paved the way to the identification of a large set of genes associated with cancers. The list being now too long to be printed here, additional information can be found at the Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/Genes) where more than 220 oncogenes and 260 tumor suppressor genes are recorded.

Concomitantly to these efficient approaches to identify genes involved in the early steps of cancer development (i.e., genes involved in the cancer initiation), three other approaches have been developed to identify cancer genes.

13.4.1 DNA Adducts

A DNA adduct is the first step to DNA damage that will eventually result in a mutation. Various examples have shown that levels of DNA adducts vary with a number of lifestyle, environmental, and chemical exposure factors. It has also been shown that increased dietary intake of antioxidants and essential metals, especially zinc, is protective. The detection of DNA adducts as pro-mutagenic markers could enable an understanding of cancer risk. In fact, cancers with

poorly defined etiology may be explained once DNA adducts have been identified. Various assays and in vivo protocols are available to evaluate the mutagenicity of a single agent but, as for drugs interactions, the evaluation of complex mixtures for mutagenicity is difficult. In addition, it is often challenging to predict if the mutagenic compound identified in vitro will reach the target organ at a sufficient concentration. The short-lived positron-emitting radionucleides molecular imaging is an approach as well as the use of IR microspectroscopy that can be used as a high-throughput technology [54], but the need for an inexpensive in vitro system remains [55]. Despite these limitations, numerous studies have been able to demonstrate a relation between DNA adducts and mutations at the gene level. An interesting illustration is given by the study of chromium in the context of lung cancers. Chromium (Cr) is a ubiquitous environmental contaminant that is also used in various occupations (artistic painting) or industries (chemical industry, anticorrosion paints, and others). In addition, Cr is also present in cigarette smoke. Hence, it has been suggested that chromium exposure and cigarette smoke may have a synergistic or additive effect in inducing lung carcinogenesis [56]. In addition, while the direct exposure to Cr (III) is not harmful as this compound is not able to penetrate human cells, the exposure to other compounds such as Cr (VI) will ultimately lead to the production of Cr (III) after various intracellular reducing reactions. This intracellular Cr (III) is then able to form covalent binary and ternary DNA adducts that have mutagenic potencies [57]. It has been shown that these DNA adducts preferentially form in NGG sequences, which include codons 245 (GGC), 248 (CGG), and 249 (AGG) of the p53 gene, the mutational hotspots in cigarette smoke-related lung cancer. While polycyclic aromatic hydrocarbons (PAHs), the major carcinogen found in cigarette smoke, bind to the p53 mutational hotspots for lung cancers including codon 157 (GTC), 158 (CGC), 245 (GGC), 248 (CGG), and 273 (CGT), while codon 249 (AGG) is not a preferential site for PAH binding [58, 59].

The finding that Cr (III) strongly binds at codon 249 suggests that the etiological agent for lung cancer with codon 249 mutations is Cr (III). Mutations involving this codon account for 4.4 % of mutations from lung cancers. Mutational events involving this residue are presented in Fig. 13.6.

Mutations involving the second base of codon 249 are more frequent for smokers (21/41–51.2 %) than for nonsmokers (17/67–25.4 %). However, results show that Cr (III) binds at the second base two times more frequently than at the third base of this codon. It has thus been suggested that the Cr (III)–DNA adducts formed at the second and third bases of this codon are repaired with different efficiencies and/or that they affect the fidelity of DNA replication differently [60]. The DNA adducts approach is usually restricted to the study of a particular component and its relationship with mutations from a previously identified gene. It is thus not frequently used to identify new cancer genes.

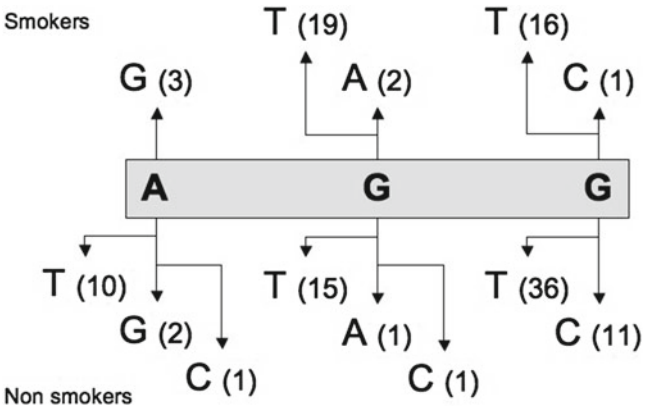


Fig. 13.6 Simple mutational events involving codon 249 of the TP53 gene in lung cancers. *Top* = mutational events reported in smokers; *Bottom* = all mutational events. Data were extracted from the UMD-TP53 (http://www.umd.be) that contains 2784 mutations from lung cancers (July 2007 release).

13.4.2 Cancer Transcriptome

Another approach is the analysis of the cancer transcriptome. With the completion of the Human Genome project [61], life scientists were challenged with the task of analyzing the expression levels on a global scale. In 1995, microarrays were developed as a high-capacity system to monitor the expression of numerous genes in parallel. These microarrays were prepared by a high-speed robotic printing of cDNAs on a glass support. This matrix was further used for quantitative expression measurements of the corresponding genes [62]. In parallel to these microarrays produced by a deposition of cDNA fragments or oligonucleotides on slides, a new in situ synthesis technology that combines photolithography and chemical DNA synthesis was developed [63–65]. This technology enabled a further miniaturization of the assay and the manufacturing of high density oligonucleotide microarrays. The GeneChip® Human Gene 1.0 ST Array is a product in the family of Affymetrix expression arrays offering whole-transcript coverage. It includes 764,885 25-mer probes to address each of the 28,869 genes with an average of 26 probes per gene (http://www.affymetrix.com).

These technical developments allowed testing of the hypothesis that identification of cancer subtypes could be accomplished through detection of all genetic modifications found in cancer cells. This was driven by the fact that cancer cells accumulate genetic abnormalities and that, while they share common genetic defects, specific alterations will be found only in a homogeneous subtype of tumors. The implications of this hypothesis would range from diagnosis (development of classifications on the basis of gene expression patterns) to therapeutic (patient prognosis and the response to a particular treatment). The hundreds of analyses performed have shown that known types and subtypes of cancer can be distinguished by their gene-expression profile. In addition,

new molecular subtypes have been discovered that are associated with various properties such as the propensity to metastasize. While microarrays can be used to search for cancer genes among a list of candidate genes, the two main foci of microarray investigations are to improve our understanding of the pathophysiology and/or the molecular etiology of a specific cancer and the detection of genetic markers that could improve the differential diagnosis. Early results were very promising [66]. With the accumulation of data from various teams, limitations of the technology were soon recognized. In fact, the power of microarray studies depends not only on the quality of the array design and production, but also on the statistic and bioinformatics approaches used to analyze the data. Thus, because this technology offered the possibility to investigate a multitude of genes in a small number of samples, it also introduced the most challenging problem of microarray analysis, which is the problem of multiple comparisons. Therefore the Westfall-Young step-down permutation correction should be mandatory. In addition, hierarchical cluster analysis has become the most popular and most frequently used multivariate technique to analyze microarray data. This method defines a distance between two tumors based on the difference in gene expression. It produces a complete tree with leaves as individual patterns and the root as the convergence point of all branches. However, given enough genes, the genes will always cluster. Therefore, there is only minor scientific value in the fact that there are genes that behave in a similar way. It has been shown that these approaches have numerous limits and that clustering trees can even be produced when starting with very poor quality signals questioning about the significance of reported data [67]. Similarly, it has been recognized that the clustering is today overused to interpret microarray data [68].

In 2005, Rhodes reported the clinical utility of array-based gene profiles in breast cancer based on data from van de Vijver et al. [69], 2 years later a review by Michiels et al. [70] gave quite different conclusions. They underlined that most prediction rules using gene expression have not provided a substantially and significantly improved prognostic classification when compared to conventional prognostic factors [71, 72]. Thus they concluded that these results could be interpreted as disproving the initial assumption and stated that if published results are correct to the extent that published combinations of genes have some prognostic value, many other gene combinations would be as good. Besides, none have been shown to add much to the clinical information that is routinely available. The example of breast cancer illustrates a problem that is central to the interpretation of microarray data. Studies with a solid experimental design and large sample sizes are required before gene expression profiling can be used in the clinic to predict outcome [70].

Eszlinger et al. arrived at similar conclusions in the context of thyroid malignancies [73]. Reviewing microarray data they reported that the use of different platforms and experimental designs (intra-individual or inter-individual comparisons) as well as the use of various control tissues (non-nodular healthy tissue or benign lesions such as goiter or follicular adenoma) complicate cross-analysis. In addition, the studies are characterized by strong differences in data analysis methods, which vary from simple empiric filters to sophisticated statistic algorithms.

Microarray technology is still in its early days of development. In order to standardize its usage strong improvements have to be made to standardize experimental designs and analysis. Quality controls [74] need to be defined and the various platforms should include common probes to allow meta-analysis. Despite these limitations, this technology is promising and should in the next years give valuable information to improve understanding of the pathophysiology and/or the molecular etiology of cancers and the detection of genetic markers that will improve the differential diagnosis and the prognosis of drug response. This technique is not the method of choice to search for cancer genes mutated in tumors as most of the down-regulated or up-regulated transcripts are the result of loss of cellular differentiation in cancer.

13.4.3 Large-Scale Sequencing of Human Cancers

A third approach to identify cancer genes is to perform a systematic resequencing of cancer samples. Ideally, a normal tissue from the same patient is also sequenced in order to identify somatic mutations. A proof-of-principle for this approach was given by work from Stephens et al. who sequenced the gene encoding the transmembrane protein kinase ERBB2 in a series of 120 primary lung cancers [75]. They identified mutations in 4 % of samples and 10 % in the adenocarcinoma subtype. This previous study was followed by a larger analysis of the whole protein kinase gene family or kinome, which is of primary interest as targets for inhibition in cancer treatment. Thus, in 2005 were published results from the sequencing of 518 protein kinases representing about 1.3 Mb of DNA per sample [76]. As underlined by Collier et al. many of the samples used in this work were cell lines and the interpretation of data should thus be done with caution [77]. In fact, 89 % of breast cancer cell lines displayed at least one kinome mutation while only 25 % of primary breast cancers possessed one. These results could be linked to the fact that cells were forced to grow in culture and thus accumulate somatic mutations. In addition, the physiological significance of most mutations remains unclear, and only a functional validation of these mutant alleles could reveal their pathogenic effect. Nevertheless, these early high throughput-sequencing projects demonstrated the ability to

identify somatic mutations in cancers. The same group from the Wellcome Trust Sanger Institute released their data from the sequencing of the 518 protein kinases in 210 diverse cancers. For this analysis they used 169 fresh cancer specimens with matched normal DNA in order to avoid artifacts mentioned by Collier et al. [77] regarding the use of cell lines. Cancers types were: acute lymphoblastic leukemia, bladder, breast, colorectal, gastric, glioma, head and neck, lung, melanoma, ovarian, renal, and testis. During this analysis a total of 274 Mb of cancer genome was sequenced and 1007 somatic variations identified. As expected the number of somatic events varied between cancers with a higher prevalence for cancers with defective mismatch repair or from patients previously treated with anticancer drugs known to induce mutations. About 30 % of other cancers showed no somatic mutations while the mutation rate varied between 4.2 per Mb (lung carcinomas) to 0.2 per Mb (breast cancers) [78]. In order to evaluate the number of driver or passenger mutations the investigators examined the observed ratio of nonsynonymous and synonymous mutations compared with that expected by chance alone [79, 80]. This approach is different from the one reported by Sjoblom et al. who did not take into account the synonymous mutations [12]. The overall approach of these 2 large-scale sequencing projects is shown in Fig. 13.7.

Large-scale sequencing projects mostly rely on bioinformatics tools to extract driver mutations from among all variations detected. Thus, in the project from Sjoblom et al., during the first round they identified 816,986 putative mutations, and after various selection steps they discarded 99.84 % of these mutations (Fig. 13.7). Almost all large-scale sequencing projects rely on the same selection procedure. The first model is known as a codon substitution model and account for potential factors in the mutation process. It is based on the assumption that the genetic code allows some codons to mutate to missense or stop codons more readily than others. Yang et al. have thus developed a new codon-based method to estimate the relative rate of substitution and of nonsense versus missense mutations in different functional domains [79]. It includes differences in mutation rates between transversions and transitions, as well as between silent and non-silent mutations. This model is based on a continuous-time Markov processes, reflecting the hypothesis that mutations are random events that occur independently of one another.

Greenman et al. [80] recently described a second model where the selection is explicitly separated from the mutation. They used an approach related to phylogenetic methods. The basic principle is that silent (synonymous) somatic mutations are passenger mutations. The set of silent mutations can thus be used as a control group to estimate the number of non-silent mutations that would be expected to occur by chance, under the null hypothesis of no association between mutations and cancer development.

The limit of these statistical approaches is that they were validated with small data sets (somatic mutations of the *TP53* gene for example). As proteins have usually specific

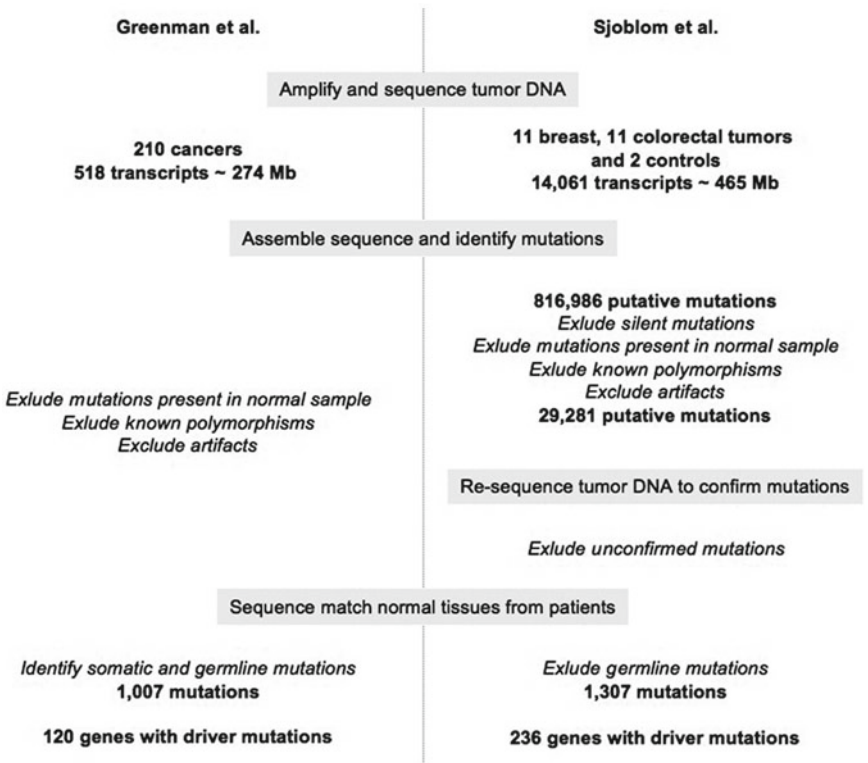


Fig. 13.7 Schematic of mutations detection for the two main large-scale sequencing projects. *Left* = steps for the kinome study by Greenman et al. [78]. *Right* = steps from the study of a major fraction of human genes in two common tumor types, breast and colorectal cancers by Sjoblom et al. [12].

features this approach can result in the misinterpretation of many putative mutations. In addition, as underlined by Greenman, they consider that synonymous mutations are passenger mutations. Recent progress in our knowledge of germ line mutations have shown that these synonymous, but also at least 15 % of missense mutations in fact do affect splicing either by the creation of a new donor or acceptor splice site but also by the inactivation of an exonic splicing enhancer or the creation of an exonic splicing silencer [81].

Overall, these large-scale sequencing projects appear to be the most efficient approach to identify cancer genes in the future. The preliminary results show that the patterns of somatic mutations in human cancers are highly variables. Thus, it is necessary to study large sets of homogenous subtypes of cancers to identify specific signatures and decipher the mutagenic process of cancer progression. In addition, as most somatic mutations are passenger mutations, new tools need to be developed to efficiently recognize driver from passenger mutations. The development of Locus Specific Database (LSDB) [82] will facilitate the rapid interpretation of mutations from common cancer genes and specific tools to predict the pathogenic impact of missense mutations integrated to LSDB software such as the UMD tool will be of valuable help in the selection process [83, 84].

13.5 How These Simple Sequence Mutations Contribute to Cancer Development?

The last 30 years of cancer research has generated enough information about cancer development to build a global model. Thus, it is today recognized that cancer cells have defects in regulatory process that govern cell proliferation and homeostasis. Despite the highly variable set of genes involved in a specific cancer, most abnormalities can be classified in six major alterations in cell physiology as suggested by Hanahan et al. [85] (Fig. 13.8).

The mechanisms by which a cell can detect and correct a DNA damage are numerous and efficient. It is therefore not surprising to observe a multistep progression of cancer cells that will progressively acquire new capacities in order to avoid defense mechanisms. Depending on the cancer progression stage (Fig. 13.1) only few of these mechanisms could be involved but ultimately almost all cancers will acquire these new capacities. The number of genetic changes necessary to acquire these new capacities will vary among cancers. Thus, in some situations the alteration of a single gene will confer several capabilities simultaneously, while for other cancers the alteration of multiple genes will be necessary to acquire a single capacity. In addition a single capacity may be acquired by different ways.

Because the mechanisms and pathways involved in cancers are too numerous to be included in this chapter, we will focus on the example of the discovery of the Insulin-like growth factor binding protein 2 (*IGFBP2*), which plays a major role in the development of astrocytomas.

IGFBP2 was initially reported to be a gene that is overexpressed in high-grade gliomas, and overexpression was associated with poor patient survival [86, 87]. While in vitro studies showed that IGFBP2 promotes glioma cell migration and invasion, it has not been determined whether IGFBP2 plays a causative role in glioma development in vivo. We were thus facing the central question about cancer genes: Because this gene is associated with cancer does that mean that it is actually a cancer gene? To answer this question, Zhang et al. used a viral gene transfer delivery agent known as RCAS that infect only glial cells. They found that the delivery of IGFBP2 alone did not lead to glioma development, but that the co-delivery of IGFBP2 with the activated K-Ras oncogene led to development of astrocytomas [88]. These data can be compared to previous reports that neither K-Ras nor Akt alone are enabled to cause brain cancers while the association of K-Ras and Akt is [89]. Therefore, they tried the combination of Akt and IGFBP2 and no tumor formed, suggesting that these two genes belong to the same molecular pathway. In addition, they showed that the combination of IGFBP2 and the platelet-derived growth factor beta (PDGFB) results in a higher-grade form of the cancer than that caused by PDGFB alone. As PDGFB has been previously shown to initiate oligodendroglioma formation independently from the Akt pathway [90], they thus postulated and demonstrated that IGFBP2 activates the Akt pathway. This was further confirmed by the use of an Akt inhibitor, which killed more IGFBP2-PDGFB infected cells than those infected only with PDGFB.

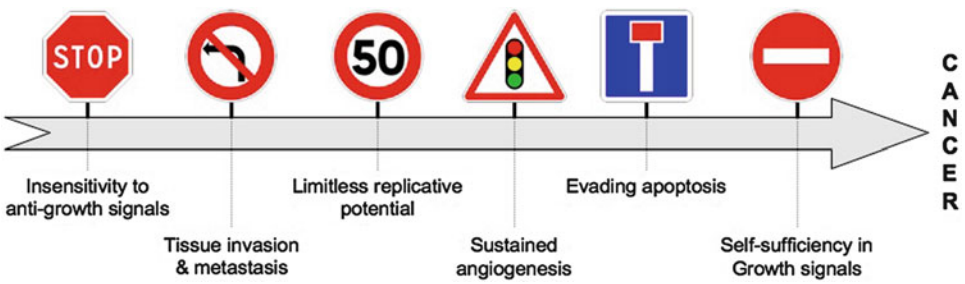


Fig. 13.8 Major acquired functional capacities for cancer cells. Most cancer cells during their development acquire functional capacities that allowed them to ignore regulation signals.

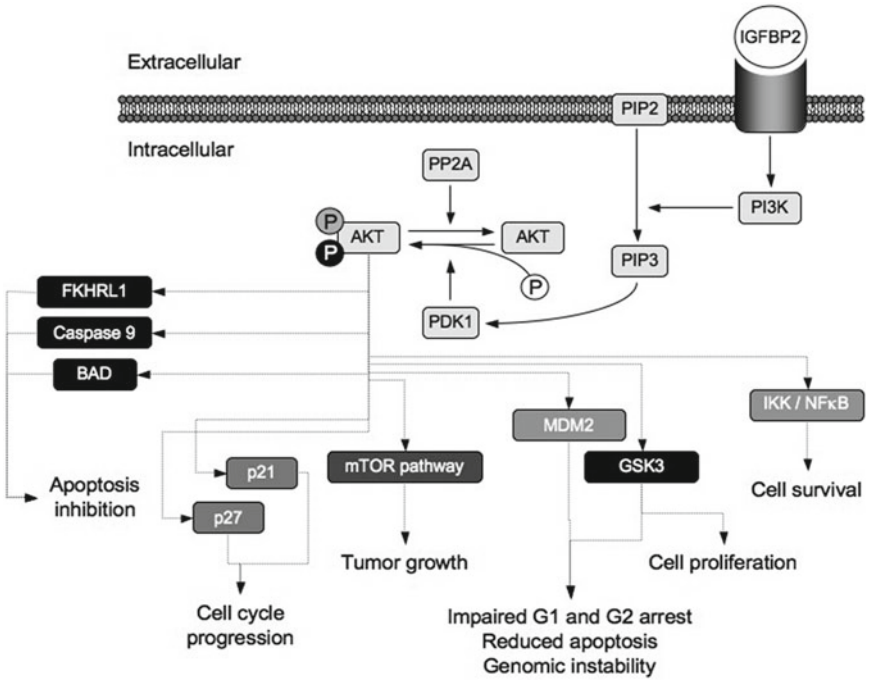


Fig. 13.9 IGFBP2 and PI3K/AKT pathway and its potential roles in cancer. AKT = v-akt murine thymoma viral oncogene homolog 1; BAD = BCL2-antagonist of cell death; FKHRL1 or FOXO3A = forkhead box O3A; GSK3 = glycogen synthase kinase 3 beta; IKK = Ikappa-B kinase; mTOR = mammalian target of rapamycin; NFkB = nuclear factor of kappa light polypeptide; p21 or CDKN1A = cyclin-dependent kinase inhibitor 1A; p27 or CDKN1B = cyclin-dependent kinase inhibitor 1B; mdm2 = transformed 3T3 cell double minute 2, p53 binding protein; PIK3 = phosphoinositide-3-kinase; PIP3 = phosphatidylinositol 3,4,5-trisphosphate; PDK1 = 3-phosphoinositide dependent protein kinase-1; PP2A = protein phosphatase 2. The IGFBP2 circulating protein binds to cell-surface receptors that will induce the production of second messengers via the activation of PI3Ks. PIP3 signals then activates the kinase PDK1, which in turn activates the kinase AKT, also known as protein kinase B. Proteins phosphorylated by activated AKT will play key roles in cancer development: (1) AKT promotes cell survival and opposes apoptosis by a variety of routes: BAD is a protein in the Bcl-2 gene family that opposes Bcl-2 to induce apoptosis. Similarly, phosphorylation of the protease caspase 9 or forkhead transcription factors by AKT block the induction of apoptosis by these factors; (2) The Akt-dependent phosphorylation of p21 prevents the complex formation of p21 with PCNA and decreases the binding of the cyclin-dependent kinases Cdk2 and Cdk4 to p21. Similarly, the phosphorylation and the cytosolic retention of p27 contribute to cell cycle progression; (3) The phosphorylation of the ribosomal protein S6 kinase (p70S6K) and the eukaryotic translation initiation factor 4E binding protein (4EBP) by mTOR and the downstream phosphorylation of the ribosomal protein S6 (RPS6) and the eukaryotic translation initiation factor 4B (EIF-4B) stimulate translational initiation and contribute to cell growth; (4) AKT plays a critical role in controlling of Mdm2-p53 signaling pathway by regulating Mdm2 stability; (5) AKT phosphorylates and deactivates Gsk3-beta, thus subsequently resulting in cell proliferation; (6) The phosphorylation of IKKs lead to activation of the transcription factor NF-kB to oppose apoptosis. Data were extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) website (http://www.genome.jp/kegg/kegg2.html) and the Biocarta website (www.biocarta.com).

Concomitantly to this discovery, Carpten et al. announced the identification of a recurring mutation of the *AKT1* (v-akt murine thymoma viral oncogene homologue 1) gene in breast, colorectal, and ovarian cancers [91]. To identify the AKT1 mutation, the researchers analyzed 162 tumor samples from patients with either breast [62], colorectal [51] or ovarian cancer [50]. They showed that 8 % of breast, 6 % of colorectal and 2 % of ovarian tumors had the c.49G>A (p.Glu17Lys) AKT1 mutation. They also demonstrated that this missense mutation can cause tumor proliferation and may play a role in making cells resistant to certain types of therapies.

It has been known for many years that hormone and growth factor stimulations of phosphoinositide 3-kinases (PI3-Kinases) result in the production of lipid second messengers at the plasma membrane, which initiate a complex network of signaling pathways that have been implicated in cell growth, cell survival, cell proliferation, and cell migration [92]. Class IA PI3-Kinases are activated by a series of cell surface tyrosine kinase receptors such as insulin growth factor receptor and platelet-derived growth factor receptor. Upon growth factor receptor activation, PI3-Kinases promote the phosphorylation of Akt at Serine 473 and/or Threonine 308. Akt then activates a series of cancer-related functions such as cell proliferation, cell migration, invasion, and decrease of cell death in human tumors [93].

Although AKT1 is central to pathway activation, for years its role in cancer has been that of an intermediary between mutated upstream regulatory proteins and downstream survival signaling proteins. The demonstration of an existing cancer causing mutation is the first evidence of a direct role of

AKT1 in human cancer. This discovery was made possible by the sequencing of clinical samples from cancer patients, while a mutation from this gene has never been detected in cancer cell lines. These data together with the ones from IGFBP2 allow drawing a new pathway linking these two proteins (Fig. 13.9).

The identification of simple sequence mutations has been driven for years by oncogenes identification using viral vectors and by human genetics to isolate oncogenes and tumor suppressor genes by linkage and positional cloning. If these approaches have been fruitful and our knowledge of human cancers has made tremendous progresses, a new area is now opened to search for cancer genes. In the next few years, the large-scale sequencing projects will be able to identify thousands of mutations in each tumor samples. The development of efficient bioinformatics and biological tools to extract driver mutations from passengers ones will allow the establishment of databases of somatic events. The collection of these mutations from thousands of samples will then allow the characterization of molecular signatures of types and subtypes of cancers. These signatures will then be useful both for diagnosis and for the development of new therapeutics. Other strategies such as the analysis of the cancer transcriptome and the DNA adducts studies will be favorably combined to draw the various pathways that can be activated or inactivated in cancer cells and their relationship to exposure to various environmental factors.

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Single Nucleotide Polymorphisms and Cancer Susceptibility

14

Richard A. Hubner and Richard S. Houlston

14.1 Introduction

Cancer susceptibility is determined by exposure to environmental factors and inheritance of genetic factors, which act either alone or in combination to influence likelihood of disease. For most common cancers relatives of affected individuals have an approximately twofold increased risk of developing the disease themselves, which may be a due to shared environment, inherited factors, or a combination of both [1]. However, twin studies have provided evidence that the familial aggregation of cancer results from inherited factors, in particular for prostate, colorectal, and breast cancer [2]. Known highly penetrant rare mutations account for some but not all of this familial risk. For example, mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* account for less than 20 % of the excess risk in relatives of breast cancer patients [3], and similarly less than 5 % of colorectal cancer can be ascribed to high penetrance mutations in *APC*, mismatch repair (MMR) genes, and *MYH*. The nature of the remaining familial risk is unknown, but multiple-case families have failed to provide evidence of linkage to novel loci in recent studies, and it is more plausible that a substantial proportion will be conferred by a number of low-penetrance genetic variants with relatively high population frequency [4]. Individually these polymorphisms will confer only modest increases in risk, but when considered collectively, and in combination with relevant environmental factors, they may confer substantial susceptibility. This concept has been termed the common disease-common variant hypothesis, and single nucleotide polymorphisms (SNPs), which are the most abundant sequence variation in the human genome, are thought to account for the majority of such common low-penetrance cancer susceptibility variants.

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14.2 Distribution of SNPs and Linkage Disequilibrium in the Human Genome

A SNP is a DNA sequence variation that occurs when a single nucleotide in the genome varies between two individuals or between paired chromosomes in the same individual. SNPs are a consequence of point mutations occurring in the reproductive cells of an individual which are passed onto their offspring, and after many generations become established within a population. Each SNP has two alleles, the original sequence and the mutated copy. The less common (minor) allele should have a population frequency of at least 1 % for the genetic variant to be termed a polymorphism, but the frequency of SNPs may vary widely between different geographical populations and ethnic groups [5]. There are estimated to be over 10 million SNPS in the human genome, collectively accounting for over 90 % of sequence variation [6]. They are distributed throughout the genome, occurring approximately every 100–300 base pairs but with marked regional differences, and the vast majority lie in untranslated regions outside of known genes [7].

Adjacent SNPs in the same chromosomal region are not inherited randomly but as a combination of alleles which form haplotype blocks [8]. This phenomenon is termed linkage disequilibrium (LD), and the strength of LD between two adjacent SNPs is dependent on the physical distance between them along the chromosome. Two SNPs that are very close to each other will be tightly linked due to the low probability of a meiotic chromosomal break occurring between them resulting in recombination and loss of linkage. In contrast, widely spaced SNPs will exhibit low LD since recombination during meiosis is more likely. However, the degree of LD between two adjacent SNPs cannot be simply predicted by the chromosomal distance between them since the pattern of LD within the human genome is not uniform [9, 10]. Distinct blocks of high LD occur, and are interspersed with regions in which LD breaks down rapidly [11].

Linkage disequilibrium underlies the principle of gene mapping by association analysis. Linkage disequilibrium

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_14

between a marker allele and a disease susceptibility allele will result in both alleles being inherited together over many generations, thus the same marker allele will be detected in affected individuals in multiple apparently unrelated families. Recombination between the marker and disease susceptibility allele will eventually dissipate the association (as can further mutational events) with the rate of decay being primarily dependent on the distance between the two alleles and the number of generations that has passed [12, 13]. However, the slowness of this decay makes allelic association a useful tool. Additionally, the complexity of analysing a number of different SNPs within a particular gene or locus can be significantly reduced if there is strong LD between them, since the genotype of all the SNPs within the haplotype block can be inferred by the genotyping of only one or a few marker SNPs or tagging SNPs [14]. Thus, linkage disequilibrium can be further exploited in association studies by using tagging SNPs to reduce the number of SNPs that require genotyping, significantly lowering laboratory costs.

14.3 Biological Consequences of SNPs

The likely functional effects of a SNP are dependent on the location and nature of the sequence change. Those occurring in exonic regions of genes are generally thought most likely to confer functional effects. However, SNPs occurring in the 5′ untranslated region (UTR) and 3′ UTR may influence gene transcription or mRNA stability, whilst SNPs in intronic regions of genes or in non-gene regions may also have as yet unknown or poorly defined consequences [5].

Of the exonic SNPs nonsense or frame shift changes that result in protein truncation have the most serious deleterious effects, often resulting in total loss of protein function. Non-synonymous SNPs that result in a change in the amino acid sequence can also have deleterious effects, the functional effects being dependent on the similarity of the original and mutant amino acid, its location within the protein, and consequences to the tertiary structure. Algorithms have been developed to aid prediction of the functional consequences of non-synonymous SNPs. Examples include the Grantham Scale which categorises codon replacements into classes of increasing chemical dissimilarity [6], and the PolyPhen algorithm which predicts the functional impact of amino acid changes by considering evolutionary conservation across different species, physiochemical differences, and proximity of the substitution to predicted functional domains and structural features [15]. Such algorithms can at best only give a very general prediction, placing amino acid changes into a hierarchy of four or five categories of likely functional significance. To provide more detailed data in vitro experiments with different enzyme isoforms are required, and even where such experiments have been performed extrapolation to the in vivo situation may be problematic [16]. The consequences of synonymous SNPs that do not change the amino acid sequence, intronic SNPs, and SNPs outside of gene regions are unknown. Carriage of these variants may still result in cancer susceptibility, either through mechanisms as yet undiscovered or though linkage disequilibrium with an unknown functional variant. For example, a synonymous SNP in the human dopamine receptor D2 gene has been demonstrated to influence mRNA stability resulting in altered expression [17].

14.4 The Association Study Design for Identifying Low Penetrance Susceptibility Alleles

14.4.1 Linkage Versus Association

Highly penetrant cancer susceptibility alleles result in extensive pedigrees and are most readily localised through linkage studies in which genetic markers co-segregate with disease. Low penetrance alleles conferring more modest risks, typically twofold or less, will rarely cause multiple-case families, and will not be identified efficiently through linkage strategies [18]. The search for low penetrance cancer susceptibility alleles has therefore focused on the association study design where the frequencies of candidate alleles are compared in cancer cases and cancer-free controls. A marker allele is said to be associated with a disease if the allele is found more frequently among cases than in the general population, or in a group of unaffected individuals. Association between a marker allele and disease can be a consequence of either a direct biological action of the marker allele, or linkage disequilibrium between the marker allele and a disease-causing allele [19]. The association study design is advantageous since large numbers of case and control samples may be readily obtained, providing adequate power to detect relatively small effects. To detect a gene with a population frequency of 10 % which confers a twofold increased cancer risk, linkage analysis would require about 10,000 affected sibling pairs, whereas an association study would require 500 unselected cases and 500 controls [20].

In most association studies the risk conferred by a specific variant is estimated by using carrier frequencies in case and controls to derive an odds ratio (OR). Usually the frequencies of the three possible genotypes (homozygous wild-type, heterozygous, and homozygous mutant) are compared using homozygous wild-type individuals as the reference group. Where homozygous mutant individuals are rare they may be combined with heterozygotes in the analysis, but this is only efficient if a dominant model can be assumed [20]. Similarly, combining homozygous wild-type and heterozygous individuals is only appropriate if the allele is acting recessively.

14.4.2 Failure to Replicate Positive Associations

The relative ease of collecting DNA samples from unselected cases and controls and the extensive range of genetic variants that could plausibly be associated with cancer susceptibility has made association studies very popular. However, few reported associations have been established beyond reasonable doubt, and in most instances initially significant associations cannot be replicated in subsequent sample sets [21–23]. This is most likely due to type I errors, the spurious association of genetic variants with disease, compounded by publication bias [24]. The levels of statistical significance appropriate in other contexts ($P=0.05$ or $P=0.01$) may not be suitable for association studies, since the number of possible genetic polymorphisms that could be tested is very large and the prior probability that any particular polymorphism will be associated with disease is low, thus most variants achieving a modest level of statistical significance will be false positives [24]. The false-positive rate can be reduced by setting more stringent levels of statistical significance or by improving the selection of candidate SNPs to increase the prior probability of association.

A second possible explanation for the failure to replicate initially positive associations is inadequate statistical power in the replication study, leading to type II errors or false negatives [20]. For example, fewer than 40 % of the colorectal and breast cancer association studies reviewed by Houlston and Tomlinson [23] and Dunning et al. [21] had 80 % or more power to detect a twofold difference in risk at the 0.05 significance level. Very large sample sizes, in the order of thousands or tens of thousands, are required to identify and confirm, or conclusively refute, genetic variants conferring modest cancer susceptibility.

14.4.3 Population Stratification

A further common explanation for spurious association is population stratification, the existence of multiple population subgroups in what was assumed to be a homogeneous population in which allele frequencies vary between the different subgroups [25]. If cases and controls are selected differentially from these subgroups then allelic association will occur in the absence of a true biological association. One way to circumvent this problem is to use family-based approaches, such as the transmission disequilibrium test (TDT), which assess the evidence for preferential transmission of one allele over another in heterozygous parents. Outside the context of childhood cancers this approach is not suitable since it involves genotyping the affected case and both parents who are often not available, and use of other family members severely reduces power [26]. In reality there are few actual examples to support the suggestion that population stratification is a frequent cause of non-replicable associations, indicating that this problem has probably been overemphasised and that other factors such as type I errors and publication bias are more important [5]. Alternatively, failure to replicate associations may occur if there is genuine heterogeneity in risk in different populations. This could occur if there were population differences in LD patterns, allele frequencies of interacting genes, or interacting environmental exposures [24].

14.4.4 Enriching for Genetic Susceptibility

The population frequency of a putative susceptibility allele is an important consideration when designing an association study, since allele frequency markedly influences power to detect an association, and hence the required number of cases and controls (Fig. 14.1). Assuming two controls per case, 800 unselected cases would be required to achieve 90 % power at a significance level of 0.01 to detect a dominant allele with a population frequency of 5 % conferring a twofold increased risk [20]. In contrast, if the population frequency were only 1 % then 3700 cases would be required to achieve the same level of statistical power. Thus the use of unselected cases in association studies is satisfactory for the evaluation of common alleles, but has limited power if the population frequency is less than 5 %. However, power can be increased in association studies by selecting cases that are enriched for genetic susceptibility by virtue of a family history of cancer (Fig. 14.2). The sample size required is typically reduced by more than twofold if cases with an affected first-degree relative are selected, and by more than fourfold if cases with two affected first-degree relatives are used [27, 28]. In the example above, the number of cases required would be reduced from 3700 to 700 if cases with two affected first-degree relatives were selected.

The identification of the rare protein truncating variant 1100delC in the cell-cycle-checkpoint kinase 2 (*CHEK2*) gene in breast cancer patients illustrates the potential of association studies using familial cases to detect rare susceptibility alleles conferring modest relative risks. This variant has a population frequency of less than 1 % and confers a 1.7-fold increase in breast cancer risk [29]. Among unselected breast cancer cases the frequency of *CHEK2* 1100delC was not significantly increased, but in familial cases not carrying *BRCA1* or *BRCA2* mutations the frequency was markedly increased. Subsequently the relative risk in pooled unselected cases has been demonstrated to be 2.3 [30]. Enrichment for genetic factors may also be achieved by selecting cases with an early age of onset, although this has limited effects on power to detect associations in comparison to family history [28].

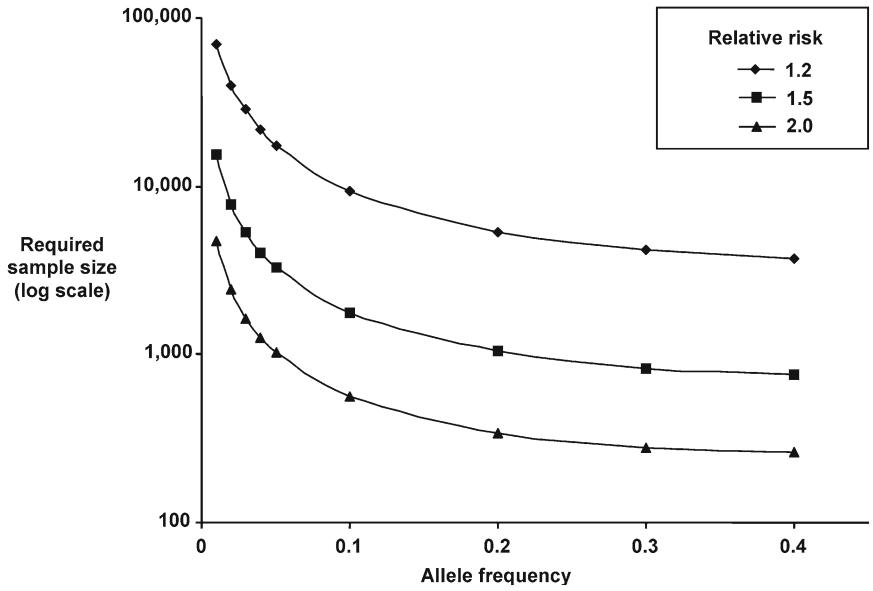

Fig. 14.1 Effect of allele frequency and relative risk on the required sample size to generate 90 % power to show significant associations ($P = 0.01$) for co-dominant susceptibility alleles, assuming one control per case.

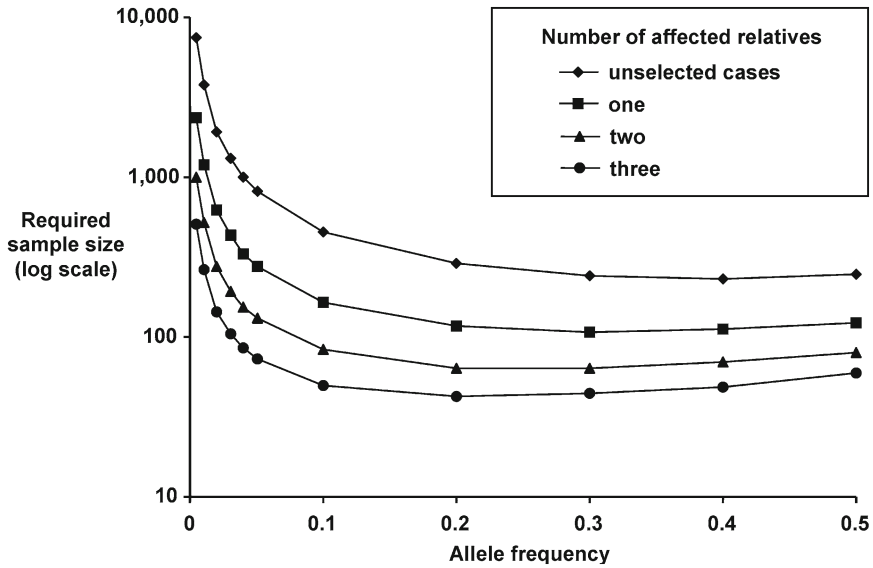

Fig. 14.2 Effect of affected family members on sample sizes required to generate 90 % power to show significant associations ($P = 0.01$) for co-dominant susceptibility alleles conferring a relative risk of 2, assuming one control per case.

14.5 Applications of SNP Genotyping in Cancer Susceptibility Gene Discovery

14.5.1 Direct Association Studies

Most of the known disease alleles in Mendelian cancer syndromes are SNPs within coding regions that result in protein truncation and hence total or very severe loss of function [6]. Ninety-five per cent of germ line mutations in the *APC* gene giving rise to the colorectal cancer susceptibility syndrome familial adenomatous polyposis (FAP), for example, are protein truncating [31]. Low penetrance cancer susceptibility alleles are similarly most likely to be coding variants, and the majority of analyses performed to date have been direct association studies which focus on SNPs that are thought to alter protein function or gene expression [18]. The analysis of functional SNPs has the potential to be a powerful method of cancer gene discovery since the number of common coding SNPs is only a fraction of the total number. It is estimated that there are 50,000–250,000 SNPs which confer a biological effect, most of which are distributed in and around the 30,000 genes [5]. Most cancer association studies have

focused on functional SNPs in candidate genes that encode proteins thought to be involved in carcinogenesis, such as those involved in apoptosis, cell-cycle control, carcinogen metabolism, or DNA repair, or those known to be somatically altered in cancer [24]. Candidate genes may also be identified from linkage analysis, expression array analysis, and comparative genomics. The disadvantages of the direct association approach are that it relies on existing knowledge to select candidate genes, identify potentially functional SNPs within these genes through database searching, and accurately predict their functional effects.

The association between the *MTHFR* C677T polymorphism and colorectal cancer (CRC) risk can be seen as an illustration of the successful application of the direct association paradigm. Folate metabolism impacts on both DNA methylation and DNA synthesis and repair, and aberrations of both these processes are known to be important in colorectal carcinogenesis [32]. Epidemiological studies lend further support for a role of folate metabolism in CRC development with high folate intake individuals generally showing a reduced CRC incidence [33]. The methylenetetrahydrofolate reductase (MTHFR) enzyme occupies a pivotal position in the folate metabolism pathway, directing the flow of one-carbon moieties towards DNA methylation and away from DNA synthesis (Fig. 14.3). Thus, there is strong a priori evidence that genetic variants in folate metabolism genes, and in the *MTHFR* gene in particular, might confer susceptibility to CRC. In vitro studies have demonstrated that the C677T polymorphism in the *MTHFR* gene gives rise to an enzyme with 35 % of normal activity, making this SNP an attractive candidate for direct association studies [34]. A number of such studies in a variety of different populations have been performed, and the pooled estimate from a recent updated meta-analysis indicated that compared to the homozygous wild-type genotype the *MTHFR* 677TT genotype was associated with a 17 % reduction in CRC risk [OR 0.83; 95 % confidence interval (CI): 0.75–0.93] [35]. Although an OR of 0.83 is modest, because of the high frequency of the wild-type allele conferring an increased risk (0.68 in Caucasian populations) this translates into a relatively high population attributable risk, and it was estimated that *MTHFR* C677T genotype contributed to approximately 15 % (95 % CI: 9–22 %) of the total incidence of CRC. It is noteworthy that only a few of the studies included in this meta-analysis individually showed a significant association with CRC risk, and pooling of data from over 12,000 individuals with CRC was required to demonstrate a significant association. This illustrates the order of magnitude of the sample sizes required to generate adequate statistical power to reliably identify low penetrance susceptibility alleles.

The assembly of adequately sized sample sets has been a frequent obstacle to identifying low penetrance susceptibility alleles. Pooling of data from a number of different studies that have analysed the same SNP in independent sample sets has therefore been a popular method of generating adequate statistical power [21, 23, 36]. Examples of genetic variants with evidence from pooled analyses for association with colorectal and breast cancer risk are listed in Table 14.1.

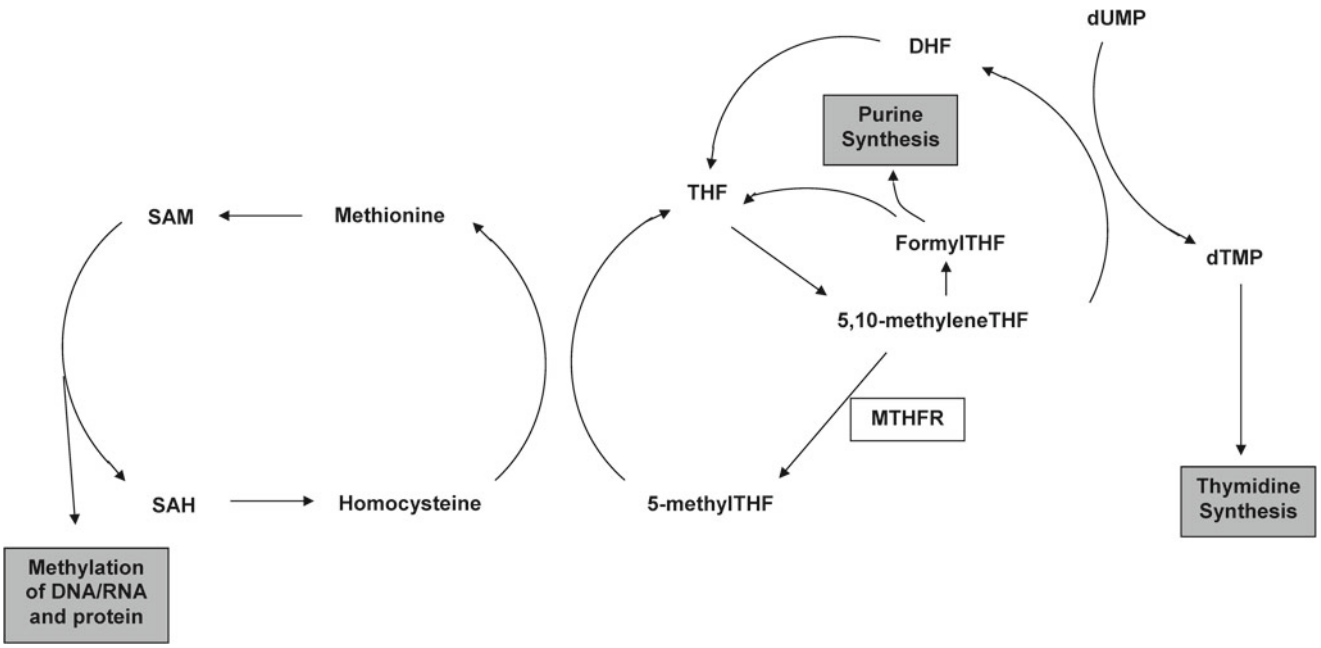

Fig. 14.3 Schematic representation of folate metabolism. *MTHFR* methylenetetrahydrofolate reductase, *THF* tetrahydrofolate, *DHF* dihydrofolate, *dUMP* deoxyuridine monophosphate, *dTMP* deoxythymidine monophosphate, *SAM* S-adenosylmethionine, *SAH* S-adenosylhomocysteine.

Table 14.1 Summary of significant associations in pooled analyses between specific polymorphisms and risk of colorectal and breast cancer

Cancer	Polymorphism	Number of studies (total number of cases)	Risk group	Pooled OR (95 % CI)	Reference
Colorectal	*APC* I1307K	3 (670)	K carriers	1.58 (1.21–2.07)	[23]
	GSTT1 deletion	11 (1490)	Homozygous deleted	1.37 (1.17–1.60)	[36]
	HRAS-1 VNTR	5 (394)	Rare alleles	2.50 (1.54–4.05)	[23]
	MTHFR C677T	25 (12,243)	TT genotype	0.83 (0.75–0.93)	[35]
	NAT2	4 (201)	Fast acetylators	1.67 (1.11–2.46)	[36]
Breast	*CASP8* D302H	14 (16,423)	Per allele[b]	0.88 (0.84–0.92)	[37]
	CYP1b1 V432L	9 (3391)	L carriers	1.50 (1.10–2.1)	[38]
	CYP19 (TTTA)*n*	3 (1404)	$(TTTA)_{12}$ carrier	2.33 (1.36–4.17)	[21]
	IGFBP3 –202C>A	3 (5673)	AA genotype	0.88 (0.80–0.98)	[65][a]
	PGR V660L	5 (7593)	Per allele[b]	1.08 (1.01–1.14)	[65][a]
	TGFB1 L10P	11 (12,946)	Per allele[b]	1.08 (1.04–1.11)	[37]
	*TGFBR1**6A	3 (555)	*6A allele carriers	1.48 (1.11–1.96)	[39]
	TP53 A72P	3 (412)	P carriers	1.27 (1.02–1.59)	[21]

[a]Breast Cancer Association Consortium
[b]Relative risk for each copy of rare allele carried compared to common homozygote

When considering such pooled analyses it is important to identify methodological issues which may affect their reliability. Firstly, an extensive search of all studies potentially suitable for inclusion in the pooled analysis should be performed, and few, if any, exclusion criteria applied in order to avoid ascertainment bias. Authors should be contacted where necessary, if the required data has not been presented in publications. Secondly, evidence of significant heterogeneity between the individual studies included in the analysis makes interpretation of the pooled estimate problematic [40]. Where there is evidence of such between-study heterogeneity, attempts should be made to identify potential sources, such as differences in study design, in particular the use of hospital-based rather than population-based control subjects, differences in ethnicity or geographic location of study subjects, and the methods of genotyping employed [41]. Thirdly, an assessment of publication bias should made, since significant publication bias means the pooled estimate is unlikely to reflect the true influence of the genetic variant under study [42]. Despite these potential methodological problems, carefully designed pooled analyses remain a useful tool, and most of the low penetrance cancer susceptibility alleles identified to date have been confirmed by data-pooling.

14.5.2 Indirect Association Studies

The identification of large numbers of SNPs across the human genome has allowed association studies to progress from the analysis of a small number of specific candidate SNPs, to assessing a much greater proportion of the genetic variation within a particular gene or gene region to detect any allelic association. Such indirect association studies rely on LD between multiple SNPs across a small region, allowing analysis of all SNPs within the LD block through the genotyping of one or a few tagging SNPs. With indirect association studies it is assumed that any cancer causing SNP within the region is unlikely to be analysed directly, rather SNPs in the same LD block will be genotyped, and hence show association with disease. The recent rapid advances in our knowledge of polymorphic variation, and the availability of this information in public databases, has allowed the development of methods and software to select SNPs spanning gene regions such that at least one SNP per LD block is chosen for analysis [43, 44]. This set of tagging SNPs can then be genotyped in a series of cases and controls to test for association. If an association is found, the component SNPs within the LD block should be examined to determine the causal variant, a process that may involve genotyping of additional SNPs to better define the haplotype structure.

Recent indirect association studies have found evidence that common variation in *BRCA2* confers significant susceptibility to breast cancer [45], whilst common variation in *BRCA1* does not [46]. Other significant associations include base excision repair genes and squamous oesophageal cancer [47], *MIC-1* and prostate cancer [48], and *TP53BP2* and gastric cancer [49].

14.5.3 Genome-Wide Association Studies

Until very recently extension of the indirect association approach to evaluation of the whole genome was prohibitively expensive, due to the need to analyse several hundred

thousand SNPs to achieve adequate coverage of the entire human genome. However, the development of novel SNP genotyping technologies means that 500,000 SNPs can now be genotyped simultaneously on a single array at a cost of only a few hundred dollars per sample, making genome-wide studies economically feasible. Evaluation of such large numbers of SNPs presents new problems in terms of interpretation, in particular in relation to thresholds for statistical significance. If 500,000 SNPs are genotyped, a P value of 0.01 for statistical significance will result in 5000 SNPs showing an association by chance alone. Although setting lower thresholds for significance or compensating for multiple tests can help to address this issue, the employment of a multistage study design may be a more efficient solution to this problem [24]. In the first stage of such studies cases and controls are genotyped for the entire set of SNPs. Polymorphisms that are below a nominal level of significance are then selected for genotyping in a second, larger, series of cases and controls to identify the true-positives and false-positives from the first stage. The SNPs that still remain significantly associated may then be tested in a third sample set where available. Genetic variants that are significantly associated with cancer risk in multiple, large, independent sample sets are likely to be genuine low penetrance susceptibility alleles even where there is no a priori evidence for association, probably through LD with unidentified functional variants.

Initial small-scale genome-wide studies provided encouraging results. For example, in a study of squamous oesophageal cancer analysing 11,555 SNPs in samples from 50 cases and 50 controls, significant association with SNPs in 33 different genes was demonstrated [50]. Very recently the results of the first large-scale genome-wide cancer association studies have been published, all of which investigated prostate cancer susceptibility [51–54]. Initially, a study genotyping 1068 microsatellite markers in 871 Icelandic men with prostate cancer reported association with a variant at chromosome 8q24, which was replicated in two independent sample sets, one of prostate cancer cases from Sweden and the second in cases of European American descent [51]. This association was further replicated in a separate study in prostate cancer cases of African American descent [52]. Subsequently two independent studies genotyping 550,000 and over 315,000 SNPs across 1172 and 1453 cases respectively identified a second prostate cancer susceptibility variant also located at 8q24, which showed association independently of the first variant [53, 54]. Interestingly, this association was strengthened when calculations were restricted to cases with an early age of diagnosis, a group in which an increased genetic effect would be expected. Combining the results of these studies indicates extraordinarily strong associations with prostate cancer (adjusted P values $= 4 \times 10^{-29}$ and 1×10^{-19} respectively for the two independent variants), and although the associations are individually relatively modest both risk alleles are relatively frequent, especially among African Americans, thus the estimated population attributable risks are high, ranging from 6–68 % depending on the variant and population considered [55]. The success of these studies highlights the value of genome-wide approaches and the importance of performing multiple complimentary studies in independent populations. The results of similar studies in other cancers are eagerly awaited.

14.5.4 Copy Number Variation

Alterations in gene copy number can lead to loss of tumour suppressor gene function or amplification of oncogenes, thus identification of genomic regions showing such copy number variation can be very informative for identifying novel cancer-related genes. Such allelic imbalances can be identified by analysis of microsatellite markers in paired tumour and normal DNA samples, however genotyping of SNPs may be a more effective method [56]. The abundance of SNPs and the ease and efficiency of high throughput SNP genotyping technologies means that a much denser analysis of a gene region of interest can be performed. Sequential SNPs all yielding homozygous genotypes may indicate loss of heterozygosity, whilst microdeletions may result in stretches of SNPs where no signal is observed. The availability of genome-wide SNP genotyping technologies has allowed extension of this approach to the entire genome. Similar to genome-wide association studies the major advantage of genome-wide copy number variation studies is that a priori knowledge of cancer related genes or chromosomal abnormalities is not required. Studies of this nature have identified novel areas of allelic imbalance in breast cancer [57], prostate cancer [58], ovarian cancer [59], and small cell and non-small cell lung cancers [57, 60].

14.6 Gene–Environment Interactions

Functional SNPs in genes coding for enzymes involved in drug or carcinogen metabolism may not directly influence cancer risk, but may modify the effect of environmental factors, and in this manner alter cancer susceptibility in exposed individuals. Such effects have been termed gene–environment interactions, and are interesting since they may not only confer cancer susceptibility but may also modify an individual's response to anticancer therapies [61]. Investigation of gene–environment interactions can be included in association studies by appropriate measurement of the environmental factor of interest, and analysis of the effect of genotype on disease risk following stratification by exposure.

Examples of gene–environment interactions include the *MTHFR* C677T polymorphism and folate status, and variant uridine diphosphate glucuronosyltransferase 1A6 (*UGT1A6*) genotypes and aspirin use [62, 63]. In both examples the genetic variant has been demonstrated to interact with the environmental exposure to determine colorectal cancer risk. Individuals with the less common *MTHFR* 677TT genotype are at reduced risk of CRC if they have adequate folate status, but at paradoxically increased CRC risk if they have inadequate folate intake [62, 64]. The UGT1A6 enzyme participates in the metabolism of aspirin and other non-steroidal anti-inflammatory drugs, and functional polymorphisms of *UGT1A6* which result in reduced enzyme function, have been reported to modify the protective effect of aspirin on colorectal adenoma (CRA) risk [63]. Individuals with wild-type *UGT1A6* genotypes did not gain benefit from taking aspirin, whilst those with the reduced function genotypes had a lower risk of developing CRA but only if they were exposed to long-term aspirin.

Identification of further gene–environment and gene–gene interactions, where the effect of one genetic variant is modified by the presence or absence of a further variant, will be assisted by the simultaneous analysis of multiple genetic variants within the same metabolic pathway and accurate measurement of appropriate environmental exposures. The only caveat being that adequate assessment of interactions requires sample sizes a further order of magnitude greater than those required for investigation of the main effects of either genotype of environmental exposure separately [61].

14.7 Conclusions

A significant proportion of inherited susceptibility to cancer remains unaccounted for and it is likely that SNPs will be responsible for an as yet unknown fraction of this risk. Alternatively, multiple rare variants could account for the remaining susceptibility, identification of which will require large-scale high-throughput resequencing. The lessons learned from early association studies will aid the design of future studies, and in particular the assembly of multiple, large sample sets will generate the required statistical power to reliably identify low penetrance susceptibility polymorphisms. The advances in SNP genotyping technology have made genome-wide association studies a reality, and it remains to be seen whether such studies will fulfil their promise and identify further low penetrance polymorphisms, perhaps in genes that have not so far been considered as cancer susceptibility candidates.

Acknowledgements RAH is in receipt of a clinical research training fellowship from Cancer Research UK.

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Chromosomes and Chromosomal Instability in Human Cancer

15

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15.1 Introduction

Human cancers show chromosome abnormalities, some of which are frequent and specific cytogenetic changes associated with certain forms of cancer. The most striking findings are nonrandom chromosomal changes in human hematologic neoplasia [1–3]. Specific cytogenetic changes are associated with the diagnosis of leukemia, clinical implications, and prognosis. Since some chromosomal changes in human hematologic neoplasms are simple and linked to molecular changes that create fusion products, such anomalies are currently being utilized as therapeutic markers. These findings have emerged gradually over the last 40 years as increasingly better techniques, for example fluorescence in situ hybridization (FISH), have become available for preparation and analysis of human chromosomes. More recently, small molecules that target fusion protein created by chromosomal changes are clinically available. Thus, detection and monitoring of chromosomal alterations in human cancer cells, especially in human hematologic neoplasms, are important in clinical practice. Nevertheless, the biological and clinical significance of nonrandom cytogenetic changes in neoplasms and chromosomal instability are still unresolved.

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15.2 Overview of Chromosomal Abnormalities in Human Cancer

Chromosomal abnormalities in human cancer can involve both the number, and more frequently, the structure of chromosomes [4]. Cytogenetic changes can be categorized as numerical changes and structural changes. Structural changes include reciprocal translocations (no change in dosage of chromosomal materials), and deletion or addition of chromosomal materials. Numerical changes are sometimes associated with structural changes, as additional cytogenetic changes. Thus, primary cytogenetic changes (termed prototype) are suggested to be essential molecular alterations in carcinogenesis.

In general, chromosomal changes fall into three categories [3]. The first category is reciprocal translocation, which is an exchange of chromosomal materials between two chromosomes. This rearrangement can result in the abnormal expression of translocated genes as a consequence of their new location in the genomes, or it can alter the structure of genes at the site of translocation. The second category involves nonreciprocal exchanges of chromosomal material. These exchanges can result in either deletion or addition of chromosomal regions. Although such changes are frequently observed in tumors, the consequences of this type of chromosomal aberrations are known in only a few instances. For example, deletions at 17p, 13q, or 11p are associated with loss of the tumor-suppressor genes p53, pRB, or WT1, respectively. The third category involves an increasing amount of DNA in a specific region of a chromosome. The increased genetic material results in areas on chromosomes referred to as homogeneously staining regions (HSRs) or double minute chromosome. HSRs are associated with extensive gene amplification [5]. Although the form of gene amplification leads to chromosomal abnormality called double minutes chromosomes (DMs). DMs appear as two small dots of dark staining material in a standard metaphase preparation. It is generally believed that DMS represent the first

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_15

stage in gene amplification and that HSRs result from the integration of DMs into the chromosomes.

Chromosomal abnormalities are designated according to the International System for Human Cytogenetic Nomenclature [6]. To describe the karyotype or chromosomal complement of a cell type or tumor, the total chromosomes number is listed first, followed by the sex chromosomes, and then a complete list of any chromosomal abnormalities.

15.3 Chromosomal Abnormalities in Myeloid Leukemias

15.3.1 Myeloproliferative Neoplasm

Damshek proposed that the entity of chronic myeloproliferative disorders (CMPD) encompassed chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and chronic idiopathic myelofibrosis (CIMF). In 2001, the new WHO classification proposed the CMPD entity to be associated with constitutive upregulation of tyrosine kinase [7] and most recently the entity is called as myeloproliferative neoplasm (MPN), thus the category includes classical MPN, chronic neutrophilic leukemia (CNL), hyper-eosinophilic syndrome (HES), and unclassifiable chronic myeloproliferative disease. Based on this concept, some novel pathways involving tyrosine kinase activity have been identified; some of them were initially found as cytogenetic change (Table 15.1) [7].

The first specific chromosome abnormality found to be associated with cancer was the Philadelphia (Ph) chromosome in CML of which the minute chromosome (22q-) identified as Ph chromosome [8] was a reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)], i.e., Ph translocation [9]. This cytogenetic change is present in the leukemic cells of at least 95% of patients with CML, and 30% of adult acute lymphoblastic leukemia and less than 5% of acute myelogenous leukemia [10]. The Ph translocation occurs in a pluripotent stem cell that give rise to cells of both lymphoid and myeloid lineages. The genetic consequence of the Ph translocation is the translocation of the *ABL* proto-oncogene on chromosome 9 to a location on chromosome 22, termed the breakpoint cluster region (or *BCR* locus) [11–13]. The detection of Ph translocation is still important in determining cytogenetic response even in the imatinib era [14–16], and molecular determination of the reduction level of *BCR-ABL* mRNA is currently a powerful marker in managing Ph-positive leukemia patients, including CML, and predicting imatinib resistant CML [16, 17]. Recently, discontinuation study of tyrosine kinase inhibitors (TKIs), including imatinib and second generation TKIs, after long-lasting complete molecular remission (CMR), the so-called treatment-free remission (TMR), is reported: these results open new avenue for treatment strategy for CML patients [18], though the STOP-TKIs studies are yet under control.

In 2003, Cools et al. reported nine cases of HES with interstitial deletion leading to the formation of the *FIP1L1-PDGFRA* fusion gene; this change was originally detected in HES patients with 4q12 abnormality [19]. This fusion gene encodes an active tyrosine kinase that is able to transform hematopoietic cells and is inhibited by imatinib mesylate. The frequency of the *FIP1L1-PDGFRA* fusion gene in HES has been reported to vary from 0 to 100%, and this anomaly is not detectable by using conventional cytogenetic analysis, thus RT-PCR or FISH analysis is required [20]. The detection of this anomaly is therefore important for determining the indications of imatinib therapy.

Another important discovery in MPN is $JAK2^{V617F}$ mutation in almost all patients with PV and approximately 50% of ET and CIMF. The presence of $JAK2^{V617F}$ mutation in MPN has been speculated, since some PV patients have 9q24 (terminal portion of 9p) changes when the JAK2 gene locates [21–25]. Approximately 30% of PV patients have the homozygous $JAK2^{V617F}$ mutation, therefore, this change could result from mitotic recombination of the 9q region [21]. Again, this mutation is not visible by the microscope, thus cytogenetic change, in combination with JAK2 mutation, might be important in managing MPN patients. In addition there is a possibility to identify new tyrosine kinase involvement in MPN based on cytogenetic studies. More recently, the frequent mutations in the calreticulin gene (*CALR*) occur in MPN has been found, and related study shows that somatic mutations in *CALR* exist in the majority of patients with JAK2 wild-type MPN [26–28].

In CNL, a breakpoint cluster region, referred to as micro-*BCR* (or μ-*BCR*), has been identified, and this reciprocal translocation results in a BCR-ABL fusion protein of 230KD

Table 15.1 Cytogenetic and molecular change in chronic myeloproliferative disorders

Diagnosis	Cytogenetic change	Molecular change	Additional changes
Chronic myeloid leukemia	t(9;22)(q34;q11)	*210KD BCR-ABL*	
CML-AP/BP	t(9;22)(q34;q11)	*210KD BCR-ABL*	+8, +Ph, +19, i(17q), t(3;21)(q26;q22)
Polycythemia vera		$JAK2^{V617F}$ (>90%)	+8, +9, del(20q), del(13q), del(1p11)
Essential thrombocythemia		$JAK2^{V617F}$ (50%)	+8, del(13q)
Idiopathic myelofibrosis		$JAK2^{V617F}$ (50%)	+8, del(20q), −7/del(7q), del(qq1), G15del(13q)
Hypereosinophilic leukemia		FIPL1-PDGFa	+8, dic(1;7), 8p11
Chronic neutrophilic leukemia		*230KD BCR-ABL*	+8, +9, del(20q), del(11q14)
CMPD unclassifiable			

[29]. Maxon et al. demonstrated that activating mutations in the gene encoding the receptor for colony-stimulating factor 3 (*CSF3R*) in approximately 60% of CNL or atypical CML. These mutations segregate within two distinct regions of *CSF3R* thus lead to preferential downstream kinase signaling through *SRC family-TNK2* or *JAK* kinases and differential sensitivity to kinase inhibitors [30].

15.3.2 Acute Myeloid Leukemia

Detection of cytogenetic changes in acute myeloid leukemia (AML) is a powerful tool for determining diagnosis and therapeutic strategy, as well as prognosis [1–3]. Currently mutiplex-PCR is widely used in the detection of known translocation-type cytogenetic changes in acute leukemia. However, cytogenetic analysis is also important in detecting minor populations with additional cytogenetic changes, rare chromosomal change, and numerical chromosome changes. These abnormalities are also important in clinical practice. The genes involved in nonrandom chromosomal translocations are listed in Table 15.2.

Most of all patients with acute promyelocytic leukemia (APL: AML-M3 by the FAB) show t(15;17)(q22;q21) resulting in *PML/RARA* (retinoic acid α receptor); the fusion protein interferes with normal myeloid cell differentiation, possibly through dominant negative effects against the transcriptional activity of RARA [31, 32]. The detection of this cytogenetic change is important in the diagnosis of AML-M3, especially in cases of the variant form of the AML-M3, and to establish the likelihood of therapeutic response to retinoic acid. In contrast, rare AML-M3 patients show t(11;17)(q23;q21) resulting in *PLZF/RARA* fusion, and they are resistant to retinoic acid treatment [33].

In AML, patients with t(8;21)(q22;q22) and inv(16)(p13q22) are now called CBF (core-binding factor) leukemia, and they respond well to cytarabine therapy [34, 35]. The t(8;21)(q22;q22) creates *RUNX1-RUX1T1* (previously designated as *ETO /AML1)* [36] and inv(16)(p13q22) does *CBFβ/MYH11* [37]. Although there are some other chromosome changes, t(15;17)(q22;q21), t(8;21)(q22;q22) and inv(16)(p13q22) are known as favorable cytogenetic change in AML [38, 39]. Intermediate risk cytogeneitc category includes numerical and structural abnormalities, while poor cytogenetic category for AML patients basically includes those with complex cytogenetic changes (more than 3 chromosomes abnormalities. Of note is that the deletion of the long arm of chromosome 7 is categorized as intermediate cytogenetic change, while monosomy 7 is classified as poor cytogenetics (Table 15.3). Other recurring translocations, including t(6;9) [40], abnormalities at 3q26 [41, 42], and abnormalities at 11q23 [43, 44], and the genes involved are listed in Table 15.2.

Detection of normal karyotypes in AML patients is important: these patients are currently separately categorized by the presence of FLT3 internal tandem repeat (FLT3-ITD) and mutation of the nucleophosmin (NPM) gene [45, 46]. These data may show that both cytogenetic analysis and molecular examination are important to predict prognosis and molecular target therapy.

15.3.3 Myelodysplastic Syndromes

Cytogenetic changes in myelodysplastic syndrome (MDS) are currently used in determining the prognosis [47]. Based on the IPSS (International Prognostic Scoring System), chromosomes in MDS are categorized into three groups, i.e., good cytogenetics [normal karyotype, del(20q), del(5q), and −Y], intermediate cytogenetics (other than good or poor cytogenetics) and poor cytogenetics (complex abnormalities: ≥3 chromosomal changes or −7/del(7q) [48]. More recently, detailed cytogenetic classification and survival risk stratification is proposed [49] and thereby revised International Prognostic Scoring System (IPSS-R)

Table 15.2 Chromosomal translocation involving transcription factors in myeloid leukemia

Translocation	Genes	Disease phenotypes
t(8;21)(q22;q22)	*RUNX1-RUNX1T1*	M2
t(3;21)(q26;q22)	*RUNX1-MDS1*	AML
inv(3)(q21q26.2) or t(3;3)(q21;q26.2	*RPN1-EVI1*	AML with thrombocytosis
inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	*CBFB-MYH11*	M4Eo
t(15;17)(q24.1;q21.1)	*PML-RARA*	M3
t(11;17)(q23;q21)	*PML-RARA*	M3
t(12;22)(p13;q22)	*TEL-AML1*	AML, MDS
t(1;22)(p13;q13)	*RBM15-MKL1*	AML (megakaryoblastic)
t(6;9)(p23;q34)	*DEK-NUP214*	M2 (M4) with basophilia
t(7;11)(p15;p15)	*NUP98-HOXA9*	M2 (M4)
t(8;1)(p11.2;p13.3)	*KAT6A-CREBBP/CBP*	Therapy-related AML
t(9;11)(p22;q23)	*KMT2A-MLLT3*	AML-M5
t(11;19)(q23;p13.1)	*HRX (MLL)-MEN*	AML
t(6;21)(q11;p22)	*FUS-ERG*	AML

Table 15.3 Prognosis of acute myeloid leukemia and chromosome abnormality

Risk	Chromosome changes	CR rate (%)	5-year survival (%)
Good	t(8;21)(q22;q22)	98	69
t(3;21)(q26;q22)	t(15;17)(q22;q21)	85	63
	inv(16)(p13q22)	88	61
Intermediate	del(9q)	100	60
	+22	91	59
	+8	84	48
	+21	80	47
	t(11q23)	87	45
	Normal	99	42
	del(7q)	75	23
Poor	Complex	67	21
	del(3q)	63	12
	del(5q)	57	11
	−7	54	10
	−5	42	4

Table 15.4 Revised international prognostic scoring system for myelodysplastic syndromes

IPSS-R score[a]							
Variable	0	0.5	1.0	1.5	2.0	3.0	4.0
Cytogenetics[b]	Very good		Good		Intermediate	Poor	Very poor
BM blasts (%)	≤2		>2–<5		5–10	>10	
Hemoglobin (g/dL)	≥10		8–<10	<8	–		
Platelets ($\times 10^9$/L)	≥100	50–<100	<50	–	–		
ANC ($\times 10^9$/L)	≥0.8	<0.8					

Good = normal, del(5q), del(12p), del(20q), double including del(5q); Int. = del(7q), +8, +19, i(17q), other single or double independent clones; poor = −7, inv(3)/t(3q)/del(3q), complex (3 abnormalities); very poor = complex (>3 abnormalities)
[a]IPSS-R risk groups: very low ≤1.5; Low 1.5–3; intermediate 3–4.5; high 4.5–6; very high >6
[b]Very good = −Y, del(11q)

is utilized for exact prognosis for untreated MDS patient (Table 15.4) [50]. Due to accumulation of cytogenetic data, in combination of hematologic parameters (number of cytopenias and percentage of blasts in the marrow), the prognosis of MDS patients could be predictive and therapeutic strategy is formulated based on this prognostic system, thus indicating that determination of cytogenetic change in MDS patients again is a powerful tool in managing patient care.

15.3.4 Secondary Acute Leukemia/ Myelodysplastic Syndromes

In the WHO classification, secondary AML/MDS was categorized as a separate entity from de novo AML or MDS [51]. Secondary AML/MDS patients are usually classified into two categories based on the causative agents, i.e., alkylating agent/radiation-related and topoisomerase II-inhibitor-related AML/MDS. The alkylating agent/radiation related disorder usually occurs 5–6 years following exposure to the mutagenic agents, and often presents initially as an MDS with low percentage blasts (25 % of this type of patients were refractory anemia with excess blasts 1 or 2), and have short survival. They have unbalanced translocations or deletions involving chromosomes 5 and/or 7 consisting of loss of all or part of the long arm of the chromosomes, and usually show multiple chromosomal abnormalities, referred to as complex cytogenetic changes. Importantly, the prognosis and cytogenetic changes of the secondary AML/MDS are quiet different between these two types. Another type, topoisomerase II-inhibitor-related AML/MDS occurs 12–130 months (median of 33–34 months) after exposure. They usually show AML without a preceding MDS, and predominant cytogenetic finding is a balanced translocation involving 11q23 (the *MLL* gene), primary t(9;11), t(11;19) and t(6;11), or t(8;21) or t(3;21) involving band 21q22 (*AML1* gene). They respond to initial therapy in a manner similar to that of de novo AML.

15.4 Chromosomal Abnormalities in Lymphoid Leukemias

15.4.1 Acute Lymphoid Leukemia

Chromosomal translocation in ALL could be separated into two types, i.e., dysregulation type and chimeric fusion type (Table 15.5) [52–57]. The former group represents involvements of immunoglobulin genes or T-cell receptor genes. Approximately 25 % of T-ALL patients show involvement of T-cell receptor (*TCR*) genes, i.e., *TCRδ* (14q11), *TCRβ* (7q32-36), and altered transcriptional level of involving genes, including *c-MYC* (located at 8q24), *LOM1* (11p15), *LOM2* (11q13), *HOX11* (10q24), *TAL1/SCL* (1p32).

The correlation of cytogenetic changes with morphology in AML leads to the identification and characterization of specific disease-associated chromosome abnormalities, while the correlation between morphology and cytogenetic change is not striking in ALL, except for the 8q24 (c-*MYC*) and ALL-L3 by the FAB classification. In ALL-M3 or Burkitt lymphoma, translocations invariably involve chromosome 8q24 and one of the three chromosomes that carry the immunoglobulin light (2p12: λ-light chain gene and 22q11: k-light chain gene) or heavy chain genes (14q32: heavy-chain gene). These translocations rearrange one allele of *c-MYC* proto-oncogene at 8q24, which encodes a transcription factor consisting of a basic region helix–loop–helix zipper (bHLH) motif, into the immunoglobulin locus carried on one of these chromosomes, resulting in dysregulation of *c-MYC* expression through the strong enhancer of immunoglobulin genes [55, 56].

Table 15.5 Chromosome abnormalities in lymphoid leukemia

Dysregulation		
B-cell lineage		
t(8;14)((q24;q32)	*c-MYC/IgH*	B-ALL
t(14;19)((q32;q13)	*BCL3/IgH*	B-CLL
T-cell lineage		
t(8;14)((q24;q11)	*c-MYC/TCRa*	T-ALL
t(11;14)(p15;q11)	*Rhom-1 (TTG1/LOM1)/TCR δ*	T-ALL
t(11;14)(q13;q11)	*Rhom-2 (TTG2/LOM2)/TCR δ*	T-ALL
t(10;14)(q24;q11)	*HOX11 (TCL3)/TCR δ*	T-ALL
t(1;14)(p32;q11)	*SCL (TAL1,TCL5)/TCR δ*	T-ALL
t(7;9)(q23;q34.4)	*TAN/TCR β*	T-ALL
t(7;9)(q23;q32)	*TAN2/TCR β*	T-ALL
t(4;11)(q21;p15)	*NUP98/RAP1GDS1*	T-ALL
Chimeric fusion gene		
t(1;19)(q23;p13)	*E2A-PBX1*	PreB-ALL
t(17;19)(q22;p13)	*E2A-HLF*	PreB-ALL
t(9;22)(q34;q11)	*BCR-ABL*	cALL
t(12;21)(p21;q22)	*TEL-AML1*	PreB-ALL
t(4;11)(q21;q23)	*MLL-AF4(MLLT2)*	MLL
t(11;19)(q23;p13)	*MLL-ENL (MLLT1)*	MLL

T-cell leukemias (T-ALLs) have a number of different recurring translocations. Most of these involve putative transcription factors, and are not normally expressed in T-cells. As example of this is the *HOX11* gene, located on chromosome 10q24, which is activated by translocations t(10;14)(q24;q11) and t(7;10)(q35;q24) in T-ALL. The *HOX11* gene shares homology with other homeobox-containing genes that normally code for sequence-specific DNA-binding proteins [57]. The t(1;14)(p32;q11) chromosome translocation has been observed in 3 % of T-ALL patients. This translocation results in the juxtaposition of the *SCL* gene (also called *TAL1*) from chromosome 1q32 with the TCRα/δ chain locus on chromosome 14q11 [58]. The *SCL* gene encodes DNA-binding protein containing the bHLH motif, which can dimerize with protein E47. The chromosomal translocation probably causes ectopic *SCL* protein expression, activating specific target genes that are transcriptionally silent in normal T cells.

Another chromosomal translocation type in ALL is chimeric protein formation, for example t(1;19) and t(9;22). The t(1;19)(q23;q13) chromosome translocation affects approximately 25 % of pre-B ALLs, and fuses the N-terminal part of the transcription factor E2A, carrying a transcription domain, to the DNA-binding homeodomain of the transcription factor PBX, replacing the bHLH region of E2A [59, 60]. The E2A PBX fusion protein is recognized by the homeodomain of PBX and can strongly activate transcription, whereas PBX cannot. Target genes that bind the PBXC homeodomain in the E2A/PBX fusion protein may be activated and initiate leukemogenesis.

Translocation (12;21)(p13;q22) is found in 20–30 % of childhood ALL, in contrast to 3–4 % in adult ALL, with favorable prognosis. The translocation results in *TEL (ETV6)-AML1 (CBF2)* fusion and consists of the dimerization domain (HLH) of the *TEL* gene and the most part of the *AML1* protein [61], thus suggesting that homodimer of the *TEL-AML1* or heterodimer suppresses the normal *TEL* function due to a dominant negative effect.

Chromosomal aberrations involving 11q23 is found in 60 % of infantile leukemia (the so-called *MLL* acute leukemia), possibly due to trans-placental chemical exposure [62]. This anomaly is also characterized by (1) acute leukemia with both lymphoid and myeloid phenotypes, and (2) frequently found in therapy-related adult acute leukemia related with topoisomerase II-inhibitor [63]. The *MLL* gene is translocated to more than 30 gene loci, and frequent involvements in adult acute leukemia are t(9;11)(p22;q23), following t(6;11)(q27;q23), t(10;11)(p12;q23), t(11;19)(q23;p13) (Table 15.6) [64]. The *MLL* gene is believed to act to maintain transcription, and the breakpoints of *MLL* gene in 11q23-leukemia are clustered within 8.5-kb spanned exon 5 to exon 11. The 11q23 translocation creates chimera between the 5′-side of the *MLL* region including AT-hook and targeted genes, and loss of the zinc finger domain of the *MLL*

Table 15.6 Translocation involving MLL genes

Trasnslocation	Genes	Function
t(1;11)(p32;q23)	*AF1p*	a-helical coils
t(1;11)(q21;q23)	*AF1q*	Unknown
t(4;11)(q21;q23)	*AF4/FEL*	Transcriptional activating domain
ins(5;11)(q31;q13q23)	*AF5q31*	Partially homologue to AF4
t(6;11)(q27;q23)	*AF6*	a-helical coils, GLGF repeat
t(6;11)(q21;q23)	*AF6q21*	Forkhead DNA binding domain
t(9;11)(p22;q23)	*AF 9*	Homology to ENL
t(10;11)(p12;q23)	*AF10*	Leucine zipper
t(10;11)(p11.2;q23)	*ABL-1*	Homology to c-ABL binding protein
t(11;14)(q23;q24)	*GPHN*	Scaffold protein
t(11;16)(q23;p13)	*CBP*	Transcription co-activator
t(11;17)(q23;q21)	*AF17*	Leucine zipper
t(11;17)(q23;q25)	*MSF/AF17q25*	Septin/GTP binding domain
t(11;19)(q23;p13.1)	*ELL/MEN*	RNA polymerase II transcription
t(11;19)(q23;p13.3)	*ENL*	Transcription domain
t(11;19)(q23;p13)	*EEN*	a-helical coils
t(11;22)(q23;q11.2)	*hCDCrel*	Homology to CDC binding protein
t(11;22)(q23;q13)	*p300*	Transcription co-activator
t(X;11)(q13;q23)	*AFX*	Forkhead DNA binding domain
ins(X;11)(q24;q23)	*SEPTN6*	GTP binding domain

gene might be important in the generation of this type of leukemia [65].

The detection of Ph translocation in ALL is diagnostically and therapeutically important. Ph translocation is also detectable in 30 % of adult ALL. This translocation creates the *BCR-ABL* oncoprotein; some of which have 210KD$^{BCR-ABL}$ (fusion between *ABL* and major *BCR*) which is the transcript similar to those of Ph-positive CML and most patients have 185KD$^{BCR-ABL}$ (fusion between *ABL* and minor-*BCR*). Ph-positive ALL patients have poor prognosis. However, current molecular target therapy, including imatinib or dual SRC-kinase inhibitor, dasatinib, followed by allogeneic stem cell transplantation has yielded promising data for the possibility of obtaining complete remission and maintaining prolonged survival [66, 67], therefore detection of Ph translocation at the ALL diagnosis and incorporation of target therapy is essential in treating such ALL patients.

15.4.2 Malignant Lymphoma

B-cell malignant lymphomas have translocation involving chromosome 14q32, which contains the immunoglobulin heavy (IgH) chain gene. Two specific translocations involving this locus have been characterized. The chromosomal rearrangements occur at precise locations on chromosomes 11 and 18 at the *BCLl* and *BCL2* loci, respectively. The *BCL1* gene, which is aberrantly expressed as a consequence of the t(11;14)(q13;q32) encodes cyclinD1 notable in mantle cell lymphoma (MCL). Cyclin D1 forms a complex with a cyclin-dependent kinase (cdk), and function in cell-cycle regulation [68, 69]. The *BCL2* gene is consistently associated with t(14;18) chromosomal translocation observed in a large percentage of B-cell follicular lymphomas. The *BCL2* gene product is suggested to mediate inhibition of cellular apoptosis [70, 71]. Thus, constitutive activation of the *BCL2* gene may contribute to the formation of follicular lymphoma by blocking programmed cell death.

Anaplastic large cell lymphoma (ALCL) is a variant of non-Hodgkin's lymphomas composed of large pleomorphic cells that usually express the CD30 antigen and is characterized by frequent cutaneous and extranodal involvement. This type of lymphoma frequently exhibits the t(2;5)(p23;q35) chromosomal translocation that fuses the NPM gene (nucleophosmin) on the 5q35 region to the *ALK* (anaplastic lymphoma kinase) on the 2p23 chromosomal region [72, 73]. The *ALK/NPM* fusion protein has tyrosine kinase activity and induces tumorigenesis. ALCL patients with the *ALK/NPM* fusion protein have favorable prognosis compared to those without the fusion protein, thus indicating the presence of a prognostic factor in ALCL patients.

15.4.3 Chronic Lymphoid Leukemia

Trisomy 12 in chronic lymphoid leukemia (CLL) is well known. The t(14;19)(q32;q13.1) chromosomal translocation is noted in some B-CLL. This result in divergent orientation (head-to-head) of the immunoglobulin heavy-chain gene (14q32) and *BCL3* (19q13) [74–76]. The *BCL3* gene is a dis-

tinct member of the IkB family, may function as a positive regulator to NF-kB activity. Thus, overexpression of *BCL3* gene by the translocation may alter the transcriptional activity of NF-kB.

15.4.4 Multiple Myeloma

Conventional metaphase cytogenetic studies are successful in only 40 % of multiple myeloma (MM) patients studied, due to a slowly proliferation of mature B cells. Using metaphase cytogenetics, only one-third of patients show an abnormal karyotype and the remaining two-thirds have normal metaphase cytogenetics. However, current studies utilizing FISH demonstrated that the large majority of such patients have an aneuploid DNA content and abnormal cytogenetics and impact on prognosis. Well known abnormalities in MM are (1) hyperdiploid (≥48 chromosomes), (2) chromosome 13 deletion, (3) an involvement of 14q32 (*IgH*) region, i.e., t(11;14)(q13;q32), t(4;14)(p13;q32), and t(14;16)(q32;q23) [77, 78]. Rare translocations t(6;14) and t(14;20) are also notable. Hyperdiploid MM is associated with improved prognosis, and negative prognostic effector in this group is the concomitant presence of high-risk immunoglobulin heavy chain (IgH) translocation. Chromosome 13 deletion is detectable in approximately 50 % of MM patients with abnormal karyotypes (10–20 % of all MM patients), and associated with shorter survival and lower response rate to treatment. The t(11;14) is associated with oligo-secretary or light-chain-only MM, CD20 expression, and amyloidosis (50 %) and IgM MM (>90 %) [77]. The t(11;14)-MM patients show improved or neutral survivals when treated with high-dose chemotherapy and stem cell transplantation. Translocation (4;14) is found in 15 % of MM patients and this translocation results in *FGFR3* to the IgH switch region locus. Recently, t(14;16), del(17p), and t(4;14) are known to be linked to poor prognosis after conventional therapy, and thereby it is recommended to utilize fluorescent in situ hybridization to detect these genetic changes, in combination with standard metaphase cytogenetics [79].

15.5 Chromosomal Abnormalities in Solid Tumors

15.5.1 Ewing Family of Tumors

Sarcomas are soft tissue tumors that often have chromosomal rearrangements encoding tumor-specific fusion oncoproteins (Table 15.7). Ewing tumors, a subset of sarcomas, occur predominantly in the long bones of children and young adults. Extraskeletal Ewing sarcomas typically involve the soft tissues of the chest wall, paravertebral region, retroperitoneum, and lower extremities. The histogenetic origin of these tumors is now suspected to be neural, a discovery linking Ewing's sarcoma to more differentiated tumors, known as peripheral primitive neuroectodermal tumors (PNET) or neuroepitheliomas [80]. Approximately 80 % of Ewing family tumors exhibit a balanced chromosomal translocation between chromosome 11 and 22, t(11;22)(q24;q12). Molecular analysis revealed that the chromosome 22 breakpoint present in Ewing tumors is located within the *EWS* gene, while the chromosome 11 breakpoint is located within the *Fli1* gene [81, 82]. Reciprocal translocation between these two chromosomes creates a new chimeric protein combining portions of the EWS and Fli1 proteins. The *EWS* gene, located on 22q12, plays a recurrent role in several chromosomal translocations found in soft tissue and bone tumors. This gene is involved in all of the alternative translocations identified in Ewing sarcoma/PNET and in distinct translocations found in desmoplastic small round cell tumors, clear cell sarcomas (soft tissue melanomas), cases of extraskeletal myxoid chondrosarcoma, a subset of angiomatoid fibrous histiocytomas, and rarely in myxoid liposarcomas [83, 84]. The *EWS* and related *TLS/FUS/FUS* gene products have features typical of RNA-binding proteins; their C-termini, which are deleted from fusion oncoproteins, contain R-G-G repeats flanking a highly conserved RNA-binding domain of the RRM type [85, 86]. The *FLI1* gene encodes a DNA-binding protein, Fli1 [85], that appears to function as a transcription factor [81]. The human Fli1 protein shares approximately 70 % sequence identity with the protein encoded by the *ETS1* gene. The EWS-Fli1 fusion protein contains the N-terminal domain of EWS and the DNA-binding domain of Fli1. Evidence suggests that DNA-binding activity and subsequent transcriptional activation of various target genes by the EWS-Fli1 fusion are essential for tumorigenesis of Ewing sarcoma [87]. While other chimeric partners of the *EWS* gene, such as *ERG* [88], *ETV1* [90], ETV4 and *E1AF* [91], are involved as fusion partners less commonly than *FLI1*, and all share homology with *ETS1*.

15.5.2 Myxoid and Round Cell Liposarcoma

Myxoid and round cell liposarcomas are almost invariably associated with translocations involving the *CHOP* gene on chromosome 12q13.1 [92]. The vast majority of tumors contain a reciprocal balanced translocation, t(12;16)(q13;p11), that fuses the 5′ half of the *TLS/FUS* gene (also known as *FUS*) to the *CHOP* coding region, giving rise to a *TLS/*FUS-CHOP oncogene [93, 94]. Rarely, myxoid and round cell liposarcomas are associated with a different translocation, t(12;22)(q13;q12), that fuses the 5′ half of *EWS* to *CHOP* [95]. The clinicopathological features of disease associated with this variant translocation do not differ from those

Table 15.7 Chromosome abnormalities in solid tumors

Type	Genes involved	Tumor type
t(11;22)(q24;12)	*EWSR1, FLI1*	Ewing sarcoma/PNET
t(21;22)(q22;q12)	*EWSR1, ERG*	Ewing sarcoma/PNET
t(7;22)(p22;q12)	*EWSR1, ETV1*	Ewing sarcoma/PNET
t(17;22)(q21;q12)	*EWSR1, ETV4*	Ewing sarcoma/PNET
t(16;21)(p11;q22)	*FUS, ERG*	Ewing sarcoma/PNET
t(2;22)(q33;q12)	*EWSR1, FEV*	Ewing sarcoma/PNET
t(17;22)(q12;12)	*EWSR1, E1AF*	Ewing sarcoma/PNET
inv(22)	*EWSR1, ZSG*	Ewing sarcoma/PNET
t(11;22)(p13;q12)	*EWSR1, WT1*	Desmoplastic small round cell tumor
t(9;22)(q22;q12)	*EWSR1, CHN*	Extraskeletal myxoid chondrosarcoma
t(9;17)(q22;q11)	*RBP56, CHN*	Extraskeletal myxoid chondrosarcoma
t(9;15)(q22;21)	*CHN, TCF12*	Extraskeletal myxoid chondrosarcoma
t(12;22)(q13;12)	*EWSR1, ATF1*	Clear cell sarcoma
t(2;22)(q34;q12)	*EWSR1, CREB1*	Clear cell sarcoma
t(2;13)(q35;q14)	*PAX3, FKHR*	Alveolar rhabdomyosarcoma
t(1;13)(q36;q14)	*PAX7, FKHR*	Alveolar rhabdomyosarcoma
t(12;16)(q13;p11)	*CHOP, TLS(FUS)*	Myxoid/round cell liposarcoma
t(12;22)(q13;12)	*EWSR1, CHOP*	Myxoid/round cell liposarcoma
t(x;18)(q11;q11)	*SSX1, SYT*	Synovial sarcoma
	SSX2, SYT	Synovial sarcoma
t(x;17)(q11.2;q25)	*ASPL, TFE3*	Alveolar soft part sarcoma
t(x;17;22)(q22;q13)	*COL1A1, PDGFB*	Dermatofibrosarcoma protuberans (and fibroblastoma)
t(7;16)(q32-34;p11)	*FUS, CREB3L2*	Low-grade fibromyxoid sarcoma
	FUS, CREB3L1	Low-grade fibromyxoid sarcoma
t(12;16)(q13;p11)	*FUS, ATF1*	Angiomatoid fibrous histiocytoma
t(12;22)(q13;p12)	*EWSR1, ATF1*	Angiomatoid fibrous histiocytoma
t(12;15)(p13;q25)	*ETV6, NTRK3*	Infantile fibrosarcoma (and mesoblastic nephroma)
t(1;3)(p36.3;q25)	*WWTR1, CAMTA1*	Epithelioid hemangioendothelioma
t(x;11)(p11.2;q22.1)	*TFE3, YAP1*	Epithelioid hemangioendothelioma
t(7;9)(q22;q13)	*Unknown*	Epithelioid sarcoma-like hemangioendothelioma
t(2;11)(q31;q12)	*Unknown*	Desmoplastic fibroblastoma
Translocation with 8q12	*PLAG1 fusion*	Lipoblastoma
Translocation with 12q14.3	*HMGA2 fusions*	Lipoma
Translocation with 6p21	*HMGA1 fusions*	Lipoma
t(5;8)(p15;q13)	*AHRR, NCOA2*	Angiofibroma
t(11;16)(q13;p13)	*C11orf95, MKL2*	Chondroid lipoma
del(8)(q13.3q21.1)	*HEY1, NCOA2*	Mesenchymal chondrosarcoma
t(7;17)(p15;21)	*JAZF1, JJAZ1*	Endometrial stromal sarcoma
t(17;22)(p13;q13.1)	*MYH9, USP6*	Nodular fasciitis
inv(12)(q13q13)	*NAB2, STAT6*	Solitary fibrous tumor
t(2;3)(q13;p25).	*PAX8, PPARγ*	Thyroid cancer (follicular)
inv10(q11.2q21.2)	*RET, PTC1*	Thyroid cancer (papillary)
t(10;17)(q11.2;q23)	*RET, PTC2*	Thyroid cancer (papillary)
t(6;11)(p21;q12)	*TFEB, ALPHA*	Renal cell carcinoma
t(X;1)(p11.2;q21)	*PRCC, TFE3*	Renal cell carcinoma
Translocations with 2p23	*ALK, multiple fusion*	Inflammatory myofibroblastic tumor

observed with the typical t(12;16). The *CHOP* gene encodes a member of the CCAAT/enhancer-binding family, which contains a leucine zipper-type dimerization motif and a putative DNA-binding domain [96]. Normally, the CHOP gene product may function in a dominant-negative manner by dimerizing with and inhibiting other transcription-factor complexes. The *TLS/FUS* gene is homologous to both *EWS* and *RBP56/hTAFII 68*; the TLS/FUS gene product contains a glutamine-serine-tyrosine-rich segment, three glycine-rich stretches, and an RNA-binding domain [93]. Of note, a large number of sarcomas are associated with fusion oncoproteins that contain either an EWS, TLS/FUS, or RBP56/hTAFII 68-type N-terminal domain. These include Ewing family tumors associated with EWS-Fli1, EWS-ERG [89], EWS-ETV1 [90], and EWS-E1AF [91], clear cell sarcomas associated with EWS-ATF1 [97], desmoplastic small round cell tumors associated with EWS-WT1 [98], myxoid chondrosarcomas associated with EWS-TEC (also known as EWS-CHN) [99, 100] and RBP56/hTAFII 68-TEC [101], and two tumors associated with TLS/FUS fusions, myxoid liposarcoma (TLS/FUS-CHOP) [93, 94] and myeloid leukemia (TLS/FUS-ERG) [102, 103]. The molecular epidemiology and transforming properties of TLS/FUS-CHOP and EWS-Fli1 point to an important role for TLS/FUS/EWS-type N-terminal domains in oncogenesis [104].

15.5.3 Synovial Sarcoma

Synovial sarcoma is an aggressive tumor that accounts for approximately 10 % of soft-tissue tumors [81] that commonly occur adjacent to limb joints or tendons in children and young adults [81]. A t(X;18) is detected cytogenetically in greater than 90 % of synovial sarcomas, regardless of histologic subtype. At the molecular level, the t(X;18)(p11.2;q11.2) produces a fusion of the *SYT* gene on chromosome 18 with the *SSX* gene on chromosome X, resulting in SYT-SSX fusion gene transcripts [105]. The Xp11 region contains a cluster of closely related *SSX1-SSX5* genes. Although the Xp11 breakpoint typically involves either *SSX1* or *SSX2,* which map to Xp11.23 and Xp11.21, respectively, other members of the cluster can be involved [106]. *SYT* encodes a protein of 387 amino acids; all but the C-terminal eight amino acids are fused to SSX as a result of this translocation. The normal SYT protein does not exhibit homology to any known protein sequences [105, 107, 108]. The *SSX1* and *SSX2* genes encode similar 188 amino-acid proteins. As neither SYT nor the SSX proteins possess recognizable DNA-binding domains, they are presumed to function as transcriptional regulators whose actions are mediated primarily via protein–protein interactions. The SSX2 protein has been shown to exhibit transcriptional repression [109]. Therefore, the SYT-SSX chimeric proteins may be involved in the transcriptional deregulation of specific target genes. Microarray analysis recently identified various genes that are downregulated in synovial sarcoma cell lines [110, 111]. The target genes regulated by endogenous intact SYT and SSX proteins through direct or indirect protein–protein interactions with specific transcription factors are currently unknown.

15.5.4 Clear Cell Sarcoma

Clear cell sarcoma (CCS, malignant melanoma of soft tissue) is an uncommon soft tissue sarcoma that occurs in tendons and aponeuroses, typically of the lower extremities and CCS rarely arises from in the gastrointestinal tract. This tumor is believed to be of neural crest origin. CCS in soft tissue typically possesses the t(12;22)(q13;q12) [112], on the other hand, CCS in gastrointestinal tract shows t(2;22)(q34;q12) . Molecular analyses of the t(12;22) breakpoints have demonstrated that the *EWS* gene on chromosome 22 becomes fused to the *ATF-1* gene on chromosome 12, which encodes a protein containing a leucine-zipper domain and a basic DNA-binding domain [97]. On the other hand, t(2;22) breakpoints have demonstrated that the *EWS* gene on chromosome 22 becomes fused to the *CREB1* gene on chromosome 2 [113]. This translocation produces a fusion transcript in which the RNA-binding domain of the EWS protein is replaced by ATF-1 protein leucine-zipper and DNA-binding domain. In CCS, the DNA-binding and dimerization activities of the fusion protein likely lead to the transcriptional activation of ATF-1 targets.

15.5.5 Alveolar Rhabdomyosarcoma

Rhabdomyosarcoma is a malignant skeletal muscle tumor occurring primarily in children and adolescents. The alveolar subtype (ARMS), the most aggressive, has the poorest prognostic outcome [114]. The typical cytogenetic finding in ARMS is the t(2;13)(q35-37;q14), which has been detected in as many as 70 % of cases [115–117]. A variant translocation, t(1;13)(p36;q14), was identified in a smaller subset of ARMS [118, 119]. These translocations fuse the *FKHR* locus on chromosome 13 to either *PAX3* on chromosome 2 [120] or *PAX7* on chromosome 1 [120]. *PAX3* and *PAX7* encode members of the paired box (PAX) transcription factor family, which is characterized by a conserved paired box DNA-binding domain first identified in *Drosophila* segmentation genes [121]. The FKHR gene product contains a fork head domain, a sequence first identified as a 100 amino-acid region of similarity between *Drosophila fork head* and the rat HNF-3 proteins. This domain was subsequently identified in a large family of genes in multiple species, ranging from

yeast to humans [122]. Of the FOX family of proteins, FKHR is the prototype of a subfamily with highly similar fork head domains and additional regions of sequence similarity [123–125]. Recently, a mouse model of ARMS was developed, in which PAX3-FKHR expression is conditionally activated in developing skeletal muscle [126]. Tumor incidence in these mice increases with the introduction of additional genetic defects, such as deletion of the Ink4A/ARF locus and p53 gene mutations [127]. Thus, PAX3-FKHR plays a primary role in muscle cell transformation and may function at multiple stages in ARMS development.

15.5.6 Desmoplastic Small Round Cell Tumor

The desmoplastic small round cell tumor (DSRCT) is a type of sarcoma characterized by an aggressive clinical course with widespread abdominal involvement and a peculiar histological appearance with prominent desmoplasia [128]. Cytogenetics and molecular characterization of DSRCT have identified a unique chromosomal rearrangement, t(11;22)(p13;q12), in these tumors [129–132]. This translocation involves the *EWS* gene at 22p12 and the Wilms tumor suppressor gene (WT1) at 11p13. The resulting chimeric gene generates a fusion cDNA transcript encoding an aberrant transcriptional regulatory factor containing the N-terminal region of *EWS* and the *WT1* C-terminus [133]. This fusion transcript is similar to other tumor-specific fusions of the *EWS* gene described in other human sarcomas, such as the prototype joining of *EWS* with *ETS* gene family members (*FLI-1, ERG,* or *ETV1*) in Ewing family tumors. In all of these rearrangements, resulting transcripts possess the N-terminal effector region of *EWS* with replacement of the RNA-binding C-terminus by the fusion partner [132, 133]. The chimeric product, which is often transforming, likely functions by dysregulating a critical group of target genes [134, 135].

15.5.7 Dermatofibrosarcoma

Dermatofibrosarcoma protuberans (DP) is a soft tissue tumor typically found in the deep dermis [80]. While it can arise at any site, DP preferentially arises in the subcutaneous tissues of the trunk and proximal extremities. DP typically occurs during early and mid-adult life [80]. Although authentic DP may occur in children, the distinct entity of giant cell fibrosarcoma (GCF) is considered to be the juvenile form of DP [80, 126]. Cytogenetically, DP and GCF both exhibit a unique chromosomal rearrangement, t(17;22)(q22;q13) [136–140]. This translocation, involving the *PDGFB* gene at 22q13.1 and the *COL1A1* gene at 17q22 [141], results in the deregulation of *PDGFB* expression. Unregulated expression of the ligand leads to continuous activation of the PDGF receptor (PDGFR) protein tyrosine kinase, which promotes DP cell growth [142].

15.5.8 Thyroid Cancer

The thyroid gland manifests a wide spectrum of malignant neoplasms. Follicular cell-derived thyroid carcinoma consists of several morphological subtypes, including papillary (PTC), follicular (FTC), Hürthle-cell (HCC), and anaplastic (ATC) carcinomas. These subtypes are phenotypically distinct, exhibiting extremes of malignant potential, from the indolent PTC to the highly aggressive ATC. While chromosomal alterations generating fusion oncoproteins are common in leukemias/lymphomas and sarcomas, they are relatively rare in human carcinomas; such fusions are almost exclusively found in well-differentiated thyroid carcinomas [143]. FTC, which accounts for approximately 20 % of all thyroid cancers, was recently found to contain a genomic rearrangement involving a t(2;3)(q13;p25). This translocation fuses the thyroid-specific transcription factor *PAX8* with *PPARγ*, a ubiquitously expressed transcription factor [144]. The resulting paired box-8 (*P*AX8)/peroxisome proliferator-activated receptor (PPAR)-γ fusion protein (PPFP) has been identified in approximately 50 % of FTC and a smaller proportion of follicular adenomas, the putative precursor lesion. PAX8, which is required for normal thyroid development, is involved in the maintenance of differentiated follicular cell function. As is typical for *pax* family genes, its expression is tightly regulated and controlled. Thus, aberrant expression of PPFP is likely to have oncogenic effects simply by downregulating endogenous PAX8 expression, an effect that has been observed for other fusion genes containing *pax* family genes [145]. In PTC, a single mutation in BRAF, the gene encoding the B-type Raf kinase, changing valine to glutamic acid at position 600 (V600E) is responsible for disease in 40–50 % of patients, especially in older people. This mutation is associated with a poorer clinicopathological outcome. Another important cause of PTC is rearrangements in the RET tyrosine kinase receptor (RET/PTC), which typically recombine the C-terminal region of the RET gene with the promoter and N-terminal domain of a partner gene. This fusion gene results in a protein product exhibiting constitutive activation of RET tyrosine kinase. This rearrangement is responsible for disease in 20–30 % patients, with a higher incidence in younger patients and those receiving radiation [146–149].

15.5.9 Renal Cell Carcinoma

Renal cell carcinoma (RCC) constitutes a heterogeneous group of tumors; specific chromosomal aberrations present in these tumors are important for defining RCC subtypes. The

most common RCC subtypes are clear-cell, papillary, and chromophobe carcinomas, which account for 75, 10, and 5% of malignant kidney tumors, respectively [150]. The most frequent genetic change in clear-cell RCC is loss of the short arm of chromosome 3, which can be caused by terminal deletions, intrachromosomal rearrangements, or unbalanced translocations. Papillary RCCs, which constitute as many as 10% of human kidney tumors, are rare in children. Cytogenetic studies usually identify trisomy or tetrasomy 7 or trisomy 17, 12, 16, or 20 [151]. A subset of papillary RCC exhibits abnormalities at Xp11 in the presence of t(6;11)(p21;q12) [152] or other translocations [153]. The t(6;11)(p21;q12) results in fusion of the 5′ portion of the *ALPHA* gene (11q12) to the transcription factor *TFEB* (6p21) [154, 155].

15.5.10 Other Solid Tumors

Congenital fibrosarcoma (CFS) and cellular mesoblastic nephroma (CMN) are two closely related spindle cell malignancies seen in infancy and young childhood. In addition to clinical and histopathological similarities [156, 157], these tumors share several common genetic abnormalities, including trisomy 11 [158, 159] and expression of the t(12;15)(p13;q25)-associated *ETV6-NTRK3* gene fusion [160].

Several additional neoplasms exhibit distinct genetic abnormalities that are critical in tumorigenesis. Almost all meningiomas exhibit hemizygosity for chromosome 22. Loss of these chromosome 22 loci, specifically the inactivation of the *NF2* tumor-suppressor gene at 22q12, is a critical step in meningioma tumorigenesis [161]. Almost all forms of retinoblastoma contain abnormalities of chromosome 13q14; in a subset of cases, a deletion at 13q14 can be visualized karyotypically. The molecular consequence of this deletion is loss of the *Rb1* tumor suppressor gene. Wilms tumor, a kidney tumor of childhood, typically exhibits deletion or mutation of the short arm of chromosome 11, which inactivates the *WT1* tumor-suppressor gene, located at 11p13 [162]. Inactivation of the *APC* tumor-suppressor gene at 5q21-22 is a critical step in colorectal tumorigenesis [163].

In addition to translocations and deletions, tumor cells often exhibit increases in chromosomal material, with identification of double minutes (DMs) or homogeneously staining chromosomal regions (HSRs). Human neuroblastomas were the first tumors demonstrated to harbor DMs and HSRs; in these tumors, gene amplification involves the *N-MYC* gene [164]. Such amplification of *N-MYC* is typically seen in more advanced tumors (stage III and IV). Amplified *L-MYC* genes in DMs and HSRs [165] have also been found in a subset of small cell carcinomas, typically in the late stages of tumor progression. Additional gene amplifications involving *EGFR, c-ERBB2*, or *BCL1* have been described in various solid tumors.

15.6 Overview of DNA-Repair and DNA-Replication Process

Maintaining genetic stability requires not only an extremely accurate mechanism for replicating the DNA before a cell divides, but also mechanisms for repairing the many accidental lesions that occur in DNA. Most spontaneous changes in DNA are temporary because they are immediately corrected by the various cellular DNA repair processes. There are a variety of repair and replication mechanisms, each catalyzed by a different set of enzymes. DNA repair (including replication-coupled repair mechanisms) involves at least four processes: (1) the altered portion of a damaged DNA strand is recognized and removed by enzymes called DNA-repair nucleases; (2) DNA polymerase binds to the 3′-OH end of the cut DNA strand and fills in the gap by making a complementary copy of the information stored in the residual template strand; (3) during DNA replication, proofreading occurs through the $3' \rightarrow 5'$ exonuclease activity of the DNA polymerase enzyme; (4) post-replication or mismatch repair (MMR), remove any replication errors that were missed by the proofreading exonuclease and remain in the newly synthesized DNA. Inactivation of any of these DNA-repair processes can result in a large increase in spontaneous mutability, and in the case of humans, predisposition to cancer development.

15.7 Nucleotide Excision Repair and Cancer

15.7.1 Mechanism of DNA Excision Repair

There are two main mechanisms for excision of DNA damage. These mechanisms are base excision repair (BER) and nucleotide excision repair (NER) [166]. In general terms, BER removes and abnormal base from the DNA strand. Release of the damaged base from the DNA is catalyzed by the class of enzymes called DNA glycosylases. Each glucosylase is specific for a particular type or class of DNA lesion. The resulting apurinic or apyrimidinic site is cleaved by an AP endonuclease, DNA polymerase fills the gap, and DNA ligase seals the remaining nick.

Another model for damaged DNA excision is NER, which involves the removal of the section of DNA containing the lesion, followed by repair synthesis using the remaining intact DNA strand as a template for restoring the gap [166–168]. Once a bulky DNA lesion is found, the phosphodiester backbone of the abnormal strand is cleaved on both sides of the distortion, and the portion of the strand containing the lesion is peeled away from the DNA double helix by a DNA helicase enzyme. The gap produced in the DNA helix is then repaired in the usual manner by DNA polymerase and DNA

ligase. The importance of these repair processes is indicated by the large investment that cells make in DNA-repair enzymes. Individuals with the genetic disease xeroderma pigmentosum (XP), for example, are defective in a NER process that can be shown by genetic analysis to repair at least seven different gene products [167, 168].

15.7.2 Proteins Involved in NER

Most proteins involved in NER have been discovered through analysis of XP [169]. These proteins are summarized in Table 15.8. The damage recognition step involves the DNA-binding proteins XPA and XPE [170, 171]. Mammalian XPA and XPE proteins show a preference for binding damaged over undamaged DNA. Differential recognition by XPA may be an important factor in determining faster repair. The next step of NER involves the introduction of two incisions into the damaged DNA strand, one on each side of the DNA lesion. The size of the repair patch formed during NER is about 25–30 nucleotides long. Two different nucleases are used to create the dual incisions. In mammalian cells, these are the XPG/ERCC5 protein, which makes the 3′-incision [172], and the complex consisting of XPE/ERCC4 and ERCC1, which makes the 5′-incision [173]. The portion of the strand containing the lesion is peeled away from the DNA double helix by a DNA helicase enzyme. These processes combine to remove the damaged segment of DNA, leaving a single-strand gap in the DNA at the site of the lesion. This gap is subsequently filled through the action of DNA polymerase enzymes.

The multiprotein complex TFIIH participates in both basal transcription and in NER. TFIIH is normally found in the initiation complex at promoters transcribed by RNA polymerase II. Known NER proteins are components of TFIIH [174, 175]. Human TFIIH contains the XPB (ERCC3) and XPD (ERCC2) proteins. XPB and XPOD contain seven conserved helicase domains, and they have $3' \rightarrow 5'$ and $5' \rightarrow 3'$ DNA helicase activities, respectively. TFIIH also contains a kinase, composed of cdk7 and cyclin H. TFIIH may regulate transcription initiation by phosphorylating DNA polymerase II. It has been reported that the p53 tumor-suppressor protein interacts both physically and functionally with the TFIIH complex [176]. This interaction might provide an immediate and direct link between p53 and the multiple function of TFIIH in transcription.

15.7.3 Mutations of NER and Cancer Family Syndromes

Defective excision repair in human in association with some autosomal recessive diseases strongly predispose to malignant disease [177]. XP is caused by absence or greatly reduced levels of excision inheritance. The clinical manifestations of XP result from DNA damage from exposure to the defective NER in these patients. Cell-fusion studies have identified 7 XP complementation groups, XPS. A through XPG, suggesting that many distinct gene products are involve in NER [178].

Bloom's syndrome (BS) is a rare disorder with autosomal recessive inheritance, and major clinical manifestations are growth deficiency, unusual facies, and sun-sensitive facial erythema [179]. Increased sister chromatid changes (SCE) and chromosomal instability are typical in vitro findings demonstrable in lymphocyte and fibroblast cultures from BS patients [180]. These patients also have an increase susceptibility to cancer [181]. The tumor spectrum exhibited by BS patients includes various rare tumor types, acute leukemias and lymphomas. The first step in positional cloning effort to isolate the BS gene (BLM) was to identify genetic linkages between the BLM locus and mapped polymorphic markers [182]. BLM protein is a member of a growing helicase subfamily, including *recQ* in *Escherichia coli*, *RECQL* in human, *SGS1* in yeast, and the gene product encoded by the Werner syndrome (WS) gene *WRN*. RecQ genes are suppressors of illegitimate recombination in E. coli and would lead to DNA instability in humans. Gene products of SGS1 and WRN are especially similar to BLM, and contain the 7 conserved helicase motifs [183]. These proteins are likely to play similar roles in DNA metabolism. Because helicase activity is important for the DNA excision-repair process, cancer susceptibility in BS patients may result from abnormality of the DNA-repair system.

Table 15.8 The polypeptides required for excision repairs in humans

XP protein	ERCC protein	Yeast homolog	Activity	Comments
XPA	–	RAD14	DNA binding	Binds damaged DNA
XPB	ERCC3	RAD25	Helicase	3′ to 5′ DNA helicase in TFIIH
XPC	–	RAD4	DNA binding	Binds ssDNA
XPD	ERCC2	RAD3	Helicase	5′ to 3′ DNA helicase in TFIIH
XPE		–		Binds damaged DNA?
XPF	ERCC4	RAD14	Nuclease	Complex with ERCC1, 5′-incision
XPG	ERCC5	RAD25	Nuclease	3′-incision
–	ERCC1	RAD10	Nuclease	Complex with ERCC4, 3′-incision

15.8 Replication Error and Cancer

15.8.1 Proofreading and MMR

An overview of DNA replication is illustrated in Fig. 15.1. Human cells require a high-fidelity DNA-replication and -repair mechanisms to ensure the integrity of the approximately 3×10^9 base pairs of DNA contained in the genome. The fidelity of DNA replication is extremely high, with about 1 error made for every 1×10^9 base pairs of DNA replicated. This level of fidelity is much higher than might be expected, given that the standard complementary base pairing (AT and GC) observed in mammalian DNAs are not the only ones possible. The high fidelity of DNA replication depends on several proofreading and MMR mechanisms that act sequentially to remove errors [184].

One proofreading process depends on specific properties of the DNA polymerase enzyme [185, 186]. Several DNA polymerase enzymes are known in mammalian cells (Table 15.9), DNA polymerase enzymes are able to correct mismatched nucleotides by means of a separate catalytic subunit that removes unpaired residues at the primer terminus. Excision by this $3' \rightarrow 5'$ proofreading exonuclease activity continues until enough nucleotides have been removed from the 3′-end to regenerate a base-paired terminus that can prime DNA synthesis. In this way, DNA polymerase functions as a self-correcting enzyme that removes its own polymerization errors as it moves along the DNA.

Another mechanism that removes replication errors missed by the proofreading exonuclease is the MMR system. In E. coli, two MMR genes are well known, namely *MutS* and *MulL* proteins with the heteroduplex activates a latent endonuclease associated with MutH protein, which cleaves the unmethylated strand at a d(GATC) site. Although little is known about the reaction responsible for unbiased correction of continuous heteroduplexes, nick-directed MMR in humans has been extensively examined. This type of repair depends on the gene products of *MLH1, MSH2, MSH3, MSH6* (or *GTBP*), *PMS1*, and *PMS2*, implicating these human homologous of *MutS* and *MutL* in the reaction [187]. Available evidence suggests that mismatch recognition in human cells is mediated by the MSH2-MSH6 protein het-

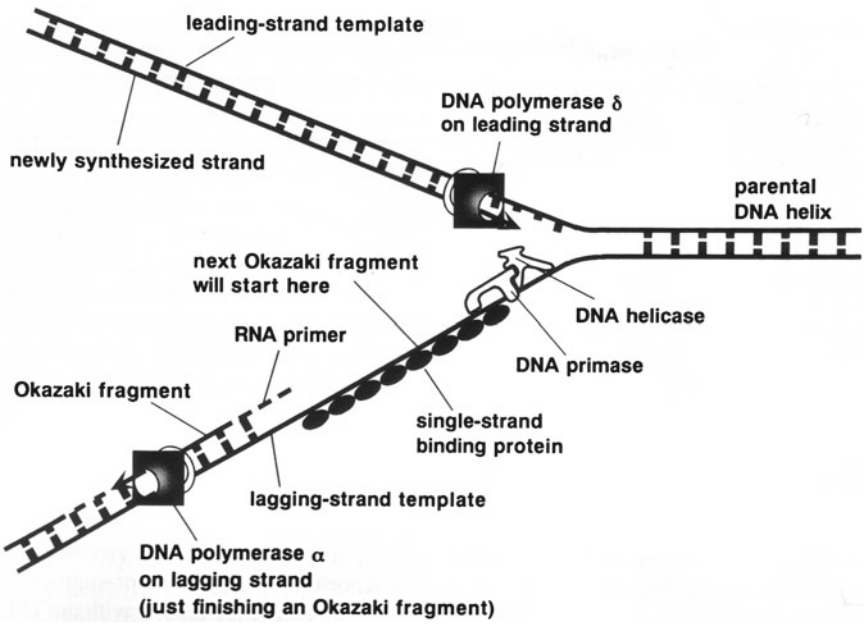

Fig. 15.1 A mammalian replication fork during DNA replication. The major types of proteins that act at a DNA replication fork are illustrated, showing their positions on the DNA. The replication fork makes use of two DNA polymerase enzymes; one for the leading strand and one for lagging strand. The leading-strand polymerase is designed to keep a tight hold on the DNA, whereas that on the lagging-strand must be able to release the template and then rebind each time a new Okazaki fragment is synthesized.

Table 15.9 Properties of DNA polymerase in eukaryotic cells

Exonuclease type	Distributions	3′–5′ Activity	Function
α	Nuclei	–	Synthesis of Okazaki fragment in lagging
β	Nuclei	–	Base excision repair
γ	Mitochondria	+	Replication of mitochondrial DNA repair Proofreading activity
δ	Nuclei	+	Synthesis of reading strand Joining of Okazaki fragment Proofreading activity
ε	Nuclei	+	Replication? Proofreading?

erodimer (designated hMutSa), with the MLH1-PMS2 protein heterodimer (hMutLa) providing MutL function [188].

15.8.2 Microsatellite Instability in Human Cancer

The human genome is punctuated with repetitive nucleotide sequences or microsatellites. These repetitive dinucleotide, trinucleotides, and tetranucleotides are frequently, but not invariably, located between genes and have been classified as junk DNA. Because microsatellites are usually less than 100 bp long and are embedded in DNA with unique sequences, they can be amplified in vitro using the polymerase chain reaction (PCR). Microsatellites are easy to clone and characterize, and they display considerable polymorphism due to variation in the number of units [189, 190]. Microsatellite instability (MSI) may reflect replication error (RER), because RER results in accumulation of changes in the length of microsatellites and other short repeat sequences (Fig. 15.2).

In studies on hereditary non-polyposis colorectal cancer (HNPCC) and sporadic colorectal cancer patients, frequent mutations in the microsatellite-repeat sequences were first described [189–191]. These mutations are typically tumor-specific and indicative of a somatic origin. Since the original description of MSI in HNPCC tumors and sporadic colon cancer, MSI has been described in a significant fraction of sporadic tumors, including colorectal, endometrial, stomach, ovarian cervical, pancreatic, esophageal, and small-cell lung cancer [192–195]. These data suggest that RER followed by MSI could be a common mechanism involved in neoplastic transformation. Moreover, it is demonstrated that a frequent occurrence of MSI in the evolution of CML [196, 197], indicating MSI to be a later genetic event in the evolution of CML to blast crisis. On the other hand, Kaneko et al. reported that MSI contributes to the pathogenesis of myelodysplastic syndromes (MDS) in some patients, especially as an early genetic event [198]. MSI has also been observed in other hematologic malignancies including CLL, Burkitt lymphoma, and HIV-associated lymphomas [199–201].

15.8.3 Mutations of MMR Genes in Human Cancer

The basis of the MSI phenotype has been clarified by genetic analysis of HNPCC families and biochemical assay of tumor cell lines that express MSI. Four genes have been implicated to date in HNPCC: *MSH2, MLH1, PMS1,* and *PMS2* [202, 203]. The majority fraction of HNPCC examined harbor mutations in MSH2 or MLK1, whereas PMS1 and PMS2 mutations appear to be responsible for only a small fraction of HNPCC patients. These data suggest that MSI in tumors arising in patients with HNPCC is due to the presence of mutations in 1 of 4 known (*hMSH2, hMLH1, hPMS1, and hPMS2*) DNA MMR genes. Isolation of *hMutSa* have implicated *MSH6* in mismatch repair, but mutations in the *MSH6* locus have not been identified in HNPCC kindred. However, *MSH6* defects have been identified in several sporadic MSI-positive colorectal cancers [204].

Mutation analysis of these four MMR genes in endometrial carcinomas displaying microsatellite instability revealed that two of nine tumors contained *hMSH2* mutations, suggesting that mutations in these four MMR genes are not responsible for MI in the majority of sporadic endometrial carcinomas [205].

Hangaishi et al. examined alterations of *hMLH1* gene in a total 43 human leukemia cell lines by PCR-SSCP: mutations of the hMLH1 gene were detected in three cell lines from lymphoid leukemias, suggesting that disruption of MMR may play an important role in the development of human lymphoid leukemias [206].

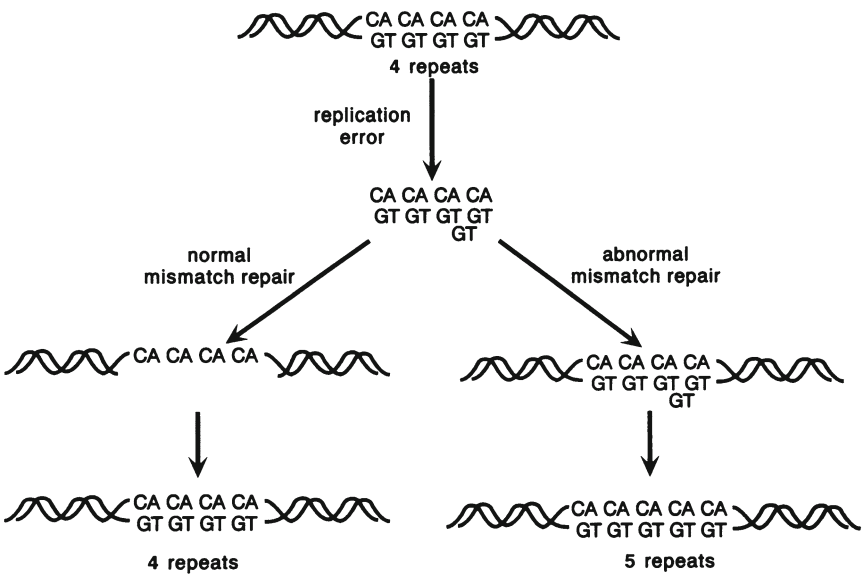

Fig. 15.2 The principle of microsatellite alterations in the disruption of mismatch repair system. During DNA replication, slippage occurs and CA repeats could be altered and not be removed, if the repair system is damaged.

15.8.4 Target Genes of Mismatch Repair Defects

Markowitz et al. demonstrated a strong correlation between defects in the *type II TGF-β receptor* (*TGFβRII*) gene and expression of MSI in tumors cell. The mutations responsible defective TGFβRII function in thee tumor cells were located in microsatellite-repeat units with the coding sequence of the gene, and consisted of expansion (insertions) mutations in an $(A)_{10}$ repeat in several cases and a $(GT)_3$ repeat in one case [207]. Because failure to respond to TGF-β growth inhibition is a characteristic of certain cancers, these findings directly link to RER and a mutational hot spot in a growth control locus. Other genes that are targets for mutations in MSI-positive cells during the course of carcinogenesis have proven elusive. Ouyang et al. examined mutations of the *insulin-like growth factor-2 receptor (IGF2R)* gene in MSI-positive cancers occurring at various primary sites, and found frameshift mutations in some populations of gastric, endometrial, and colorectal cancers expressing MSI [195].

15.8.5 Mutation of DNA Polymerase Enzymes in Human Cancer

In the hereditary form, mutations of MMR genes are usually responsible for instability. However, in many sporadic tumors, mutations of the known MMR genes are apparently absent. Da Costa et al. investigated whether defects in the proofreading DNA-repair function of DNA polymerase δ might contribute to the RER phenotype [208]. They found a variation in exon III of *polymerase* δ was observed in 3 of 8 MMR-positive colorectal cancer cell lines, suggesting that the RER phenotype might be related to mutations in the exonuclease domain of DNA polymerase. DNA polymerase α expression correlates with the extent of malignancy and survival in non-SCLC patients and is therefore considered to be a useful prognostic marker [209]. Moreover, mutations in the gene coding DNA polymerase β were detected in a high percentage of human colorectal cancer [210]. DNA polymerase β gene mutations are also detected in prostate and bladder cancer, although with relative low frequency [211].

15.8.6 Telomere Attrition and Chromosome Instability

Telomeres, the ends of chromosomes, consist of simple tandem repeats. In humans, 10–15 kb of (TTAGGG) repeats are found at the termini of all chromosomes and function to protect chromosomal ends from chromosomal recombination [212–216]. Since telomere overhang does not replicate without the presence of telomerase [217–219], consisting of a RNA component (hTERC) [220], a catalytic subunit (hTERT) [221] and associated proteins [222–225], telomere length progressively shortens on every cell division. Progressive telomere shortening without telomerase activity (or low level telomerase activity) results in chromosomal instability and produces telomere association, for example, dicentric chromosomes or DMS [226, 227]. In normal somatic cells, telomerase activity is only detectable in regenerating tissues, like hematopoietic cells, intestinal cryptic cells, and hair follicle cells. Reduction of telomere length is calculated by the telomere-binding protein (POT-1) and then telomerase activity is controlled via several telomere-binding proteins (TRF-1, TRF-2) [228–230].

In cancer cells, upregulation of telomerase activity was evident and is believed to protect from ultimate telomere shortening that induces cell death [231, 232]. Therefore, telomere loss due to cell division induces chromosomal instability. However, to avoid cell senescence, telomerase activity is upregulated and then tumor cells obtain consistent growth with relatively short but stable telomere length in hematologic neoplasms [233–235]. Although it has been suggested that telomere attrition (and chromosome abnormality) and microsatellite instability cooperate in cancer cells, it remains unresolved whether telomere reduction and telomerase upregulation actually contribute to chromosome abnormalities or instability in cancer cells, especially in solid tumors. Currently, targeted therapy for telomerase or telomere structure has been attempted to reduce telomere length and induce cell death, in combination with chemotherapy [236].

15.9 Cell-Cycle Checkpoints in Cancer

15.9.1 Cell-Cycle Checkpoints and Chromosomal Instability

Cell-cycle checkpoints mechanisms ensure that all process in one phase of the cell cycle are completed before the next phase begins. Such checkpoints are not activated unless an impediment to cell-cycle progression is detected. Therefore, it is natural that DNA damage can trigger cell-cycle checkpoint controls, because DNA damage can interfere significantly with cell-cycle processes and diminish their accuracy.

It has demonstrated been demonstrated that cell-cycle checkpoint abnormalities can lead to the generation of chromosomal aberrations [237, 238].for example, a defect in the S/M checkpoint can result in the improper segregation of chromosomes owing to the incomplete separation of sister chromatids before DNA replication is successfully completed. Defects in the surveillance of the integrity of DNA can cause chromosomal rearrangements, such as deletions, amplifications, and translocations. Defects in spindle surveillance can lead to nondisjunction, generating whole chromosome gain or loss, whereas defects in the surveillance of the spindle poles can lead to change in chromosomal number, i.e., aneuploidy. In such

cells with defective checkpoint machinery, the replicated DNA may not be segregated in time into the daughter cells, which lead to aneuploidy and chromosomal instability However, aurora-kinase inhibitor (target for spindle surveillance) is now ongoing for possible target therapy for various cancers, including Ph-positive leukemia with T315I mutation [239–241].

15.9.2 p53 Gene

Mice deficient for p53 have a predisposition to cancer in multiple organs systems [242]. In human, tumor cells in the Li–Fraumeni syndrome have defects in both copies of p53, whereas the non-tumor cells have a defect in only one. Furthermore, more than 50 % of sporadic cancer patients are found to have p52 mutations [243]. The gene product of the p53 tumor-suppressor gene is a checkpoint factor that mediates G_0/G_1-cell cycle arrest of apoptosis in response to DNA damage. p53 also harbors an intrinsic exonuclease activity and binds to Rad51 recombinase, a factor that facilitates DNA repairs, indicating that p53 is indirectly involved in the DNA repair mechanism [244]. Moreover, p53 seems to be involved in the regulation of chromosome replication and the prevention of DNA replication when a mitotic-spindle inhibitor hampers chromosome replication [245, 246]. Therefore, the loss of p53 function cause enhanced gene amplification, and chromosomal instability [247].

15.9.3 MicroRNA in Cancer

Most currently, it is well known that microRNAs (miRNAs, small noncoding RNAs ranging from 18 to 24 nucleotides in length) regulate gene expression by the RNA-induced silencing complex, resulting in a reduction of the translation and stability of target messenger RNAs [248–250]. Recent studies have shown the presence of miRNA alterations in various human cancers, suggesting that mRNAs play important roles in carcinogenesis and disease progression [251–253]. Moreover, some miRNAs are known to control not only expression of fusion product that creates from chromosomal translocation but also genes link to cell cycle. In the near future, the whole story will be disclosed, in combination with chromosome changes and miRNA profiling, for understanding cancer biology, and thereby contribute to therapeutic approach.

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Epigenetic Mechanisms in Cancer Development

16

José Ignacio Martín-Subero and Manel Esteller

16.1 Introduction

Epigenetics literally means *upon genetics* and comprises various mechanisms that regulate both gene expression and genome stability without modifying the DNA sequence itself [1, 2]. The most widely studied epigenetic changes are DNA methylation of cytosines within CpG dinucleotides and a growing number of chemical modifications at different amino acid residues of histone tails including acetylation, methylation, phosphorylation, and ubiquitination [2–5]. Additionally, other epigenetic factors like nuclear positioning, noncoding RNAs, and microRNAs are also associated with gene regulation and chromatin structure [6–8].

Epigenetics plays a key role in multiple physiological processes like development, establishment of tissue identity, imprinting, X-chromosome inactivation, chromosomal stability, and gene transcription regulation [2]. Therefore, it is not surprising that alterations in the epigenetic code are associated with a wide variety of malignant and nonmalignant diseases [3, 9–13]. Furthermore, multiple environmental factors, e.g., nutrition, exposure to metals, or maternal behavior in early childhood, are able to induce epigenetic changes [9, 14]. These environmentally induced epigenetic changes are in turn related to susceptibility to several diseases in adults, including cancer [15]. Interestingly, there is evidence showing that monozygotic twins acquire epigenetic and phenotypic changes throughout life [16], which supports the concept that lifestyle influences the phenotype through epigenetic modifications.

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CpG dinucleotides are not randomly distributed across the genome but concentrated in the promoter regions of genes (called CpG islands) and in repetitive genomic sequences [3]. In general, it can be said that repetitive sequences are heavily methylated in normal cells whereas most promoter regions associated with CpG islands are unmethylated, which allows gene expression if the appropriate transcriptional activators are present [3]. However, only approximately 70 % of genes contain CpG island-associated promoters. In the remaining 30 % of genes, transcriptional regulation is mostly achieved through histone modifications and not by DNA methylation. But this rule has several exceptions. For instance, expression of some genes lacking the established criteria for CpG islands, e.g., Maspin, is strongly regulated by DNA methylation [10].

As compared to epigenetic patterns in normal cells, cancer cells are characterized by an intense disruption of the epigenomic machinery, which is reflected in multiple aberrations affecting both content and distribution of DNA methylation and histone modifications [3, 10, 11, 17–19]. The aim of this review is to provide an overview on the role of epigenetic modifications in cancer development as well as in cancer management and treatment.

16.2 Gene Expression Regulation by Epigenetic Mechanisms

DNA methylation and histone modifications are tightly regulated by DNA methyltransferases (DNMTs) [20] and a large number of histone modifying enzymes [5], respectively. The best characterized DNMTs are DNMT1, DNMT3A, and DNMT3B. Although DNMTs were originally classified as maintenance or de novo DNMTs (depending on their ability to methylate hemimethylated or unmethylated substrates), several lines of evidence indicate that all three DNMTs not only cooperate, but also may show both de novo and maintenance functions in vivo [21–23]. The knockout cell lines for DNMT1, DNMT3B, and both enzymes demonstrated that in

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_16

single knockouts no effective CpG island demethylation and restoration of gene expression were observed, but the double knockout of DNMT1 and DNMT3B showed complete hypomethylation at the studied CpG islands and corresponding gene activation [22, 24]. The double-knockout cell line has also been shown to be a useful tool for identifying new hypermethylated genes in human cancer [24] thanks to the use of global genomic methylation strategies, such as AIMS and CpG island arrays [3, 25]. Taken together, these results strongly suggest that both enzymes, DNMT1 and DNMT3B, are necessary for effective CpG island methylation.

The information for gene silencing contained by methylated CpG islands is in part read by methyl-CpG-binding proteins (MBDs). MBDs mechanistically link DNA methylation and histone-modifying enzymes that establish a transcriptionally inactive chromatin environment. This family of proteins consists of five well-characterized members (MeCP2, MBD1, MBD2, MBD3, and MBD4) [26]. MBD proteins are associated with hypermethylated CpG island promoters of tumor-suppressor genes and their transcriptional silencing [26], showing remarkable specificity in vitro [27] and in vivo [28–30]. In fact, most hypermethylated promoters are occupied by MBD proteins, whereas unmethylated promoters generally lack MBDs, with the exception of MBD1 [29]. Several promoters are highly specific in recruiting a particular set of MBDs, while other promoters seem to be less exclusive. Thus, it can be speculated that the specific profile of MBD occupancy is gene-type and cancer-type specific [29].

DNA methylation and histone modifications are not independent epigenetic events but function in an orchestrated manner to achieve a chromatin structure associated with gene silencing or gene expression. For instance MeCP2 represses transcription of methylated DNA by recruiting a histone deacetylase (HDAC)-containing complex [31, 32]. Most histone modifications occur in their protruding N-terminal tails. So far, over 60 different residues on histones targeted by modifications like methylation, acetylation, phosphorylation, and ubiquitination have been identified [5]. A specificity in the pattern of modifications under particular conditions led to the proposal of the histone code hypothesis, according to which histone modifications act sequentially or in combination to form a code that may be read by nuclear factors [4, 33]. Several modifications are compatible with gene silencing whereas others are associated with gene activation. In general, histone deacetylation leads to gene silencing whereas histone acetylation leads to gene activation. Other modifications like histone methylation lead to silencing or activation depending on the amino acid targeted, e.g., methylation of lysine 9 of histone 3 leads to repression whereas methylation of lysine 4 of the same histone results in gene activation [5]. Additional connections between DNA methylation and histone modifications have been found: DNMTs are able to recruit HDACs [34, 35], while on the other hand, both DNMTs and MBDs recruit histone methyltransferases (HMTs) that modify lysine 9 of histone H3 [36, 37]. Also, it has been reported that EZH2, a member of the polycomb repressor complex leading to methylation of lysine 27 of histone 3, associates with DNMTs and might be involved in establishing DNA methylation in a subset of target genes [38].

In conclusion, gene expression is highly regulated by various epigenetic mechanisms. Active genes are associated with lack of DNA methylation, acetylation of H3 and H4, and methylation of lysine 4 of H3, whereas inactive genes show deacetylation of histones H3 and H4, methylation of lysine 9 of histone H3, and demethylation of lysine 4 of histone H3 (Fig. 16.1). In cancer, these patterns are profoundly distorted (Fig. 16.1).

16.3 Technical Approaches to Detect Epigenetic Changes

Techniques for the detection of epigenetic changes have dramatically evolved during the last 30 years [39–43]. The initial efforts in the 1970s were focused on the measurement of global DNA methylation content and the study of particular sequences by Southern blot analyses using methylation-sensitive restriction endonucleases. The limitations of the latter method (e.g., large amounts of high-quality DNA, sequence biases, and problems with incomplete digestions) made the study of specific sequences time consuming and not widely applicable. It was not until 1992, with the introduction of the sodium bisulfite conversion technique, that DNA methylation analyses made a revolutionary step forward [44]. Sodium bisulfite has the property of converting unmethylated cytosine into uracil, whereas methylated cytosine remains unmodified. The combination of this chemical modification with genomic sequencing and methylation-specific PCR (MSP) made the study of DNA methylation changes widely available, and a large number of studies, especially dealing with CpG island hypermethylation of tumor-suppressor genes in cancer, were published in the late 1990s [45]. However, these PCR-based approaches are restricted to the study of few candidate genes and are not suitable as screening techniques to identify novel markers. To overcome this, techniques like Amplification of InterMethylated Sites (AIMS) and Restriction Landmark Genomic Scanning (RLGS), which combine the use of methylation-sensitive restriction endonucleases with one-dimensional or two-dimensional electrophoresis were established [46, 47]. These techniques are time consuming, and every new fragment identified as differentially methylated between a control and a test sample has to be cloned and sequenced.

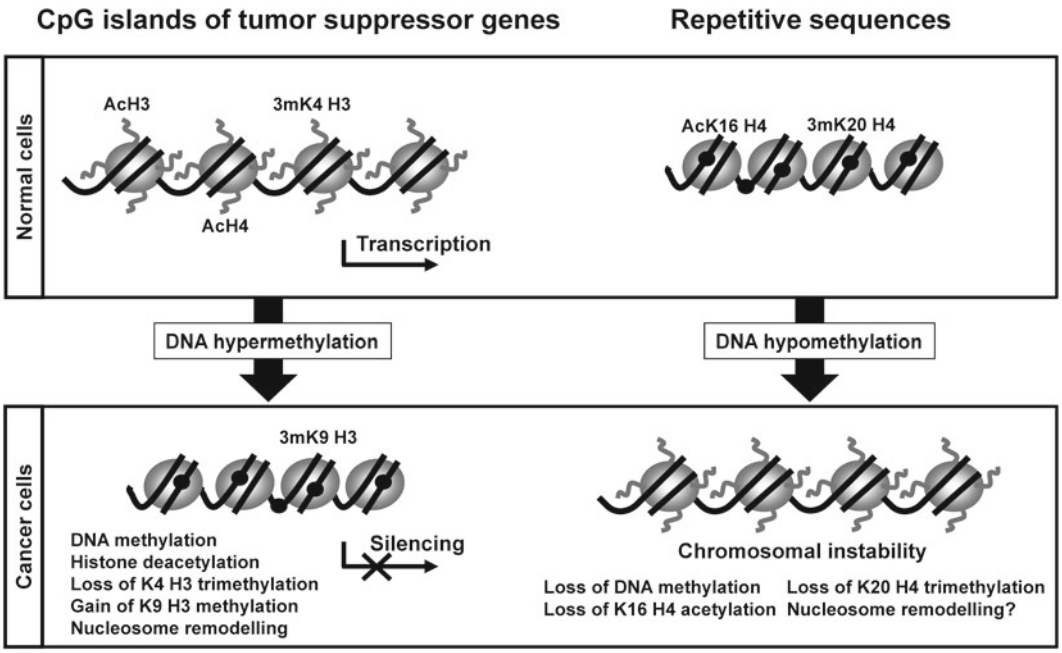

Fig. 16.1 Graphical display of the most common epigenetic changes in CpG islands of tumor suppressor genes and DNA repeats in normal and cancer cells.

An important step forward was been made in recent years with the introduction of the microarray technology [48], which allowed the simultaneous study of epigenetic changes of thousands of known sequences. Some of these array-based DNA methylation techniques are based on the enrichment of methylated DNA and sequential hybridization onto a dedicated microarray containing thousands of promoters or CpG islands, or even a tiling path array containing the whole genome [49–51]. Enrichment for methylated DNA can be achieved by immunoprecipitation of methylated sequences with an antibody specific for 5-methylcytosine (MeDIP) [52] or by methyl-CpG immunoprecipitation (MCIp), which uses a recombinant protein made of the methyl-CpG-binding domain of the MBD2 protein and the Fc fraction of the human IgG1 to directly isolate methylated DNA [53, 54]. Methylated DNA can also be isolated by digestion with methylation-specific endonucleases [50, 55, 56]. One of the limitations of these methods is that they only provide a blurry picture of the methylome and it is not possible to determine the methylation status of specific CpGs. This problem can be overcome by combining bisulfite treatment of the DNA and microarrays able to differentiate methylated and unmethylated alleles [57–59].

These methods allow a direct detection of DNA methylation patterns. However, there is an additional, but indirect, way for detecting hypermethylated genes. This method applies gene expression profiling before and after treatment with DNA demethylating agents like 5-aza-2′-deoxycytosine (5-AZA), so that hypermethylated genes become reactivated after treatment [60, 61]. Although this technique has allowed the detection of novel cancer-related hypermethylated genes, 5-AZA is highly toxic to the cells and can alter the expression levels of many genes regardless of their methylation status, leading to a high false-positive and false-negative rates, and a thorough and time-consuming data validation [43].

With regard to histone modifications, global alterations can be detected by isolating histone fractions by high-performance liquid chromatography (HPLC), and then analyzing them by high-performance capillary electrophoresis (HPCE) and liquid chromatography–electrospray mass spectrometry (LC–ES/MS) [62]. Specific modifications at each amino acid residue can also be characterized using antibodies in western blots, immunostaining [63], or tandem mass spectrometry (MS/MS) [62]. If the goal is to detect histone modifications at specific DNA stretches, a different strategy is necessary. DNA can be crosslinked to the associated histones and in a second step precipitated with antibodies specific to certain histone modifications (technique called chromatin immunoprecipitation or ChIP) so that a DNA fraction enriched for that specific histone modification can be isolated. Then, a PCR using primers for the region of interest can be used. Alternatively, a genome-wide picture of histone modifications can be obtained by the ChIP-on-chip technique. By means of this technique, the immunoprecipitated fraction can be labeled and directly hybridized onto a microarray or compared with the input DNA in two-color hybridization [64, 65].

In spite of the potential of microarrays to characterize DNA methylation and histone modifications across the genome, they are limited either by resolution, type and number of

sequences analyzed, or by their quantification accuracy. In any case, the complete characterization of the human epigenome of a given sample requires the quantification of the methylation status of each of the ~55 million CpG dinucleotides per diploid cell and the distribution of histone marks of every DNA region, and today this is far from possible using the current microarray platforms. The development of a new generation of sequencers is now revolutionizing both genomics and epigenomics [66–68]. These new sequencing technologies are based on pyrosequencing using millions of picoliter-scale reactions, sequencing by synthesis and sequencing by ligation [66], and are able to sequence up to 2 gigabases of DNA (and the human genome is made of ~3.1 gigabases) in a single experiment. The initial applications of these technologies in the epigenomics field have enabled profiling of the distribution of 20 different histone methylation marks in human CD4+ T-cells [69] or the chromatin state (using six different histone marks) of mouse embryonic stem cells and lineage-committed cells like neural progenitor cells and embryonic fibroblasts [70]. With regard to the detection of DNA methylation changes by high-throughput sequencers [71], the reduction from 4 to 3 bp of unmethylated sequences (C is transformed to U and then to T in the PCR reaction) after bisulfite treatment poses a methodological problem to identify the origin of the sequenced fragments. Therefore, current technologies do not yet allow direct sequencing of bisulfite-treated whole genomic DNA, although this will be most likely achieved in the near future.

16.4 DNA Hypomethylation in Cancer

One of the initial epigenetic changes discovered in cancer is that, in comparison to normal cells, cancer cells are characterized by a global hypomethylation of the DNA [18, 72, 73]. Such global loss of methylation mainly targets repetitive DNA sequences, coding regions, and introns. So far, it has been reported that DNA hypomethylation leads to generation of chromosomal instability, reactivation of transposable elements, and loss of imprinting in cancer [74]. Hypomethylation of DNA repeats results in a more open chromatin at those genomic regions and renders the DNA more susceptible to suffer DNA breaks by mitotic recombination of homologous repetitive sequences in different chromosomes (Fig. 16.1) [75–77]. This leads to chromosomal rearrangements, which represent one of the genetic hallmarks of cancer. Also, germline mutations in the *DNMT3B* gene in humans are associated with the ICF (immunodeficiency, centromeric region instability, facial abnormalities) syndrome, which displays DNA hypomethylation of repeats (like satellite 2), and chromosomal instability [78]. Hypomethylation of DNA in malignant cells can reactivate endoparasitic DNA, such as L1 (long interspersed nuclear elements), and Alu (recombinogenic sequence) repeats [79, 80]. These unmethylated transposons can be transcribed or translocated to other genomic regions so that chromosomal instability is further generated. The loss of methyl groups from DNA can also disrupt genomic imprinting. For instance, the hereditary Beckwith-Wiedemann syndrome shows loss of imprinting of *IGF2* (the insulin-like growth factor gene) and individuals affected with this syndrome have an increased risk of cancer [81].

16.5 Gene Silencing in Cancer by DNA Methylation

Tumor-suppressor gene silencing by CpG island hypermethylation is perhaps the best studied epigenetic change in cancer development (Fig. 16.1) [10, 11, 17, 82, 83]. The presence of CpG island promoter hypermethylation affects genes regulating virtually all important cellular functions, such as cell cycle (*p16INK4a*, *p15INK4b*, *RB1*, *p14ARF*), DNA repair (*BRCA1*, *hMLH1*, *MGMT*, *WRN*), cell adherence and invasion (*CDH1*, *CDH13*, *EXT1*, *SLIT2*, *EMP3*), apoptosis (*DAPK*, *TMS1*, *SFRP1*), carcinogen metabolism (*GSTP1*), hormonal response (*RARB2*, *ER*, *PRL*, *TSH* receptors), Ras signaling (*RASSF1A*, *NOREIA*), and microRNAs, among others [82]. Table 16.1 shows a summary of the most common hypermethylated genes in human cancer.

Some of the genes hypermethylated in cancer are common to most cancer subtypes, whereas others are considered to be cancer subtype specific [83, 84]. Hierarchical cluster analysis of DNA methylation profiles of tumor suppressor genes in different cancers leads to a classification according to diagnosis and therefore, each cancer type can be assigned a specific DNA *hypermethylome*. Such patterns of epigenetic inactivation occur not only in sporadic cancers but also in inherited cancer syndromes [85], in which hypermethylation can be the second lesion in the classical Knudson's two-hit model of cancer development [85, 86].

So far, most of epigenetic studies have targeted known tumor-suppressor genes. However, with the advent of the science of epigenomics, a more precise and less biased delineation of the cancer cell epigenome is becoming accessible. This strategy is already providing a complete new generation of epigenetic markers in cancer. An increasing number of microarray-based studies have focused on the detection of differentially methylated biomarkers associated with specific types of solid tumors like breast cancer [87–90], colorectal cancer [91–94], prostate cancer [95–97], lung cancer [98–101], head and neck cancer [49], oligodendroglioma [102], medulloblastoma [103], and Wilms tumors [104]. In hematological cancers, due to the large number of different cell types of the hematopoietic system, a wide range of different leukemias and lymphomas have been identified by means of morphological, immunohistochemical, and genetic features [105]. Now, several groups are using microarray-based DNA

Table 16.1 Summary of the best characterized genes silenced by CpG island promoter hypermethylation in human cancer (adapted from [3])

Gene	Function	Location	Cancer type
APC	Inhibitor of beta-catenin	5q21	Aerodigestive tract
AR	Androgen receptor	Xq11	Prostate
BRCA1	DNA repair, transcription	17q21	Breast, ovary
CDH1	E cadherin, cell adhesion	16q22.1	Breast, stomach
CDH13	H cadherin, cell adhesion	16q24	Breast, lung
COX2	Cyclooxygenase-2	1q25	Colon, stomach
CRBP1	Retinol-binding protein	3q23	Colon, stomach, lymphoma
DAPK	Pro-apoptotic	9q34.1	Lymphoma, lung, colon
DKK1	Extracellular Wnt inhibitor	10q11.2	Colon
ER	Estrogen receptor	6q25.1	Breast
EXT1	Heparan sulfate synthesis	8q24	Leukemia, skin
FAT	Cadherin, tumor suppressor	4q35	Colon
GATA4	Transcription factor	8p23	Colon, stomach
GATA5	Transcription factor	20q13	Colon, stomach
GSTP1	Conjugation to glutathione	11q13	Prostate, breast, kidney
HIC1	Transcription factor	17p13.3	Multiple types
HOXA9	Homeobox protein	7p15.2	Neuroblastoma
ID4	Transcription factor	6p22.3	Leukemia
IGFBP3	Growth-factor-binding protein	7p13	Lung, skin
Lamin A/C	Nuclear intermediate filament	1q21.2	Lymphoma, leukemia
LKB1/STK11	Serine–threonine kinase	19p13.3	Colon, breast, lung
MGMT	DNA repair of 06–alkyl-guanine	10q26	Multiple types
MLH1	DNA mismatch repair	3p21.3	Colon, endometrium, stomach
NORE1A	Ras effector homologue	1q32	Lung
p14ARF	MDM2 inhibitor	9p21	Colon, stomach, kidney
p15INK4B	Cyclin-dependent kinase inhibitor	9p21	Leukemia
p16INK4A	Cyclin-dependent kinase inhibitor	9p21	Multiple types
p73	p53 homologue	1p36	Lymphoma
PR	Progesterone receptor	11q22	Breast
PRLR	Prolactin receptor	5p13.2	Breast
RARB2	Retinoic acid receptor-beta2	3p24	Colon, lung, head, and neck
RASSF1A	Ras effector homologue	3p21.3	Multiple types
RB1	Cell-cycle inhibitor	13q14	Retinoblastoma
RIZ1	Histone/protein methyltransferase	1p36	Breast, liver
SFRP1	Secreted frizzled-related protein	18p11.21	Colon
SLC5A8	Sodium transporter	12q23	Glioma, colon
SOCS1	Inhibitor of JAK–STAT pathway	16p13.13	Liver, myeloma
SOCS3	Inhibitor of JAK–STAT pathway	17q25	Lung
SRBC	BRCA1-binding protein	1p15	Breast, lung
SYK	Tyrosine kinase	9q22	Breast
THBS1	Thrombospondin-1, anti-angiogenic	15q15	Glioma
TMS1	Pro-apoptotic	16p11	Breast
TPEF/HPP1	Transmembrane protein	2q33	Colon, bladder
TSHR	Thyroid-stimulating hormone receptor	14q31	Thyroid
VHL	Ubiquitin ligase component	3p25	Kidney, hemangioblastoma
WIF1	Wnt inhibitory factor	12q14.3	Colon, lung
WRN	DNA repair	8p12	Colon, stomach, sarcoma

methylation profiling to characterize the epigenome of this heterogeneous group of diseases and to identify diagnostic epigenetic marks. These studies have shown differential methylation profiles between mantle cell lymphoma (MCL) and follicular lymphoma (FL) [106], B-cell chronic lymphocytic leukemia (B-CLL), MCL, and FL [107, 108], cutaneous T-cell lymphoma and normal T-cells [109], acute lymphoblastic leukemia (ALL) and acute myeloid leukemia

(AML) [110], AML and normal monocytes [54], and ALL and normal peripheral blood [111]. Taken together, these studies and our recent unpublished experiments indicate that cancer cells, depending on the tumor subtype, might contain up to 2000 hypermethylated gene promoters.

The acquisition of differential methylation in cancer, like hypermethylation of tumor-suppressor genes, is thought to provide the tumor clone with a selective (e.g., proliferative) advantage. However, recent reports have proposed that there is an instructive mechanism behind aberrant DNA methylation in cancer [112, 113]. Keshet and colleagues performed a MeDIP-on-chip study in colon and prostate cancer and as well as identifying differentially methylated genes, they studied whether these genes show distinct biological features [112]. They discovered that genes differentially methylated in cancer are enriched for functional categories (e.g., cell adhesion, cell-cell signaling, signal transduction, and ion transport) and that the expression of some of them is already repressed (or expressed at low levels) in cells from matched normal tissues [112]. Furthermore, they detected a significant enrichment of sequence motifs and a significant clustering of such genes in chromosomal regions [112]. In line with this finding, another study has shown that large stretches of DNA containing several genes can become hypermethylated in cancer [114].

Various independent studies have recently provided further evidence for an instructive mechanism leading to selective methylation of certain groups of genes in most types of cancer. These studies took advantage of ChIP-on-chip data generated using antibodies against members of the polycomb repressor complex 2 in embryonic stem cells [115, 116]. The investigators found that a highly significant proportion of genes that become hypermethylated in cancer were already repressed at the embryonic stem cell stage by PcG marks [117–120]. These findings suggest that epigenetic changes of polycomb target genes occurring in a cell with stem cell features might represent the initial event in tumorigenesis (Fig. 16.2), which supports the cancer stem cell theory [10, 113, 121].

16.6 DNA Methylation also Targets miRNAs in Cancer

Aberrant repression of gene expression in cancer not only targets coding sequences but also small non-coding RNAs like miRNAs. These are made of short stretches of 22 nucleotides that are able to regulate gene transcription of target genes by sequence-specific base pairing in the 3′ UTR regions and subsequent degradation of the target mRNA or inhibition of translation. Target genes of miRNAs are involved in cellular functions like cell proliferation, differentiation, and apoptosis [122, 123]. Therefore, it is not surprising that miRNA expression has been found to be deregulated in cancer development [124, 125]. The role as tumor suppressors of miRNAs has been investigated in more detail for particular cases. For example, the downregulated let-7 and miR-15/miR-16, and miR-127 are known to target the oncogenic factors RAS and BCL-2, respectively [126, 127]. This may be explained by the failure of these miRNAs during post-transcriptional regulation in cancer cells [128], but additional mechanisms such as CpG island hypermethylation could also be involved. For instance, it has been observed that 5 % of human miRNAs are upregulated by treatment of bladder cancer cells with DNA demethylating agent and HDAC inhibitor [129]. In particular, miR-127 expression was induced by a decrease in DNA methylation levels around the promoter region of the miR-127 gene, and the proto-oncogene BCL6, a potential target of miR-127, was translationally downregulated after treatment [129]. Additionally, using a genetic approach that takes advantage of the genomic disruption of DNMT1 and DNMT3B in cancer cells, we have demonstrated that CpG island hypermethylation is a mechanism that can account for the downregulation of miRNAs in human cancer [130]. Most importantly, the epigenetic silencing of miR-124a, one of the DNA methylation-associated silenced miRNAs isolated using this approach, leads to activation of cyclin D kinase 6 (CDK6), a bona fide oncogenic factor, and phosphorylation of the retinoblastoma (RB1) tumor-suppressor protein [130].

16.7 Histone Modifications in Cancer Cells

Although less studied than DNA methylation, histone modification patterns are also highly disrupted in cancer. One of the best studied alterations is the acetylation of histone H4, which is globally hypoacetylated in esophageal squamous cell carcinoma [131, 132], gastric cancer [133], testicular cancer [134], and acute promyelocytic leukemia (APL) [135]. Furthermore, monoacetylated lysine 16 of H4 is globally reduced in various types of cancer [62], and lower levels of acetylated lysine 12 of H4 are an indicator of recurrence in prostate cancer [63]. Interestingly, exposure to the carcinogen Ni2+ induces a clear decrease in histone acetylation [136]. Another histone H4 modification, the trimethylation of lysine 20 of H4, which is enriched in differentiated cells [137] and increases with age [138], is commonly reduced in cancer cells [62, 139]. Global alterations of histone H3 modifications in cancer have been less thoroughly investigated. One study found that low levels of acetylation at lysines 9 and 18 of histone H3 are associated

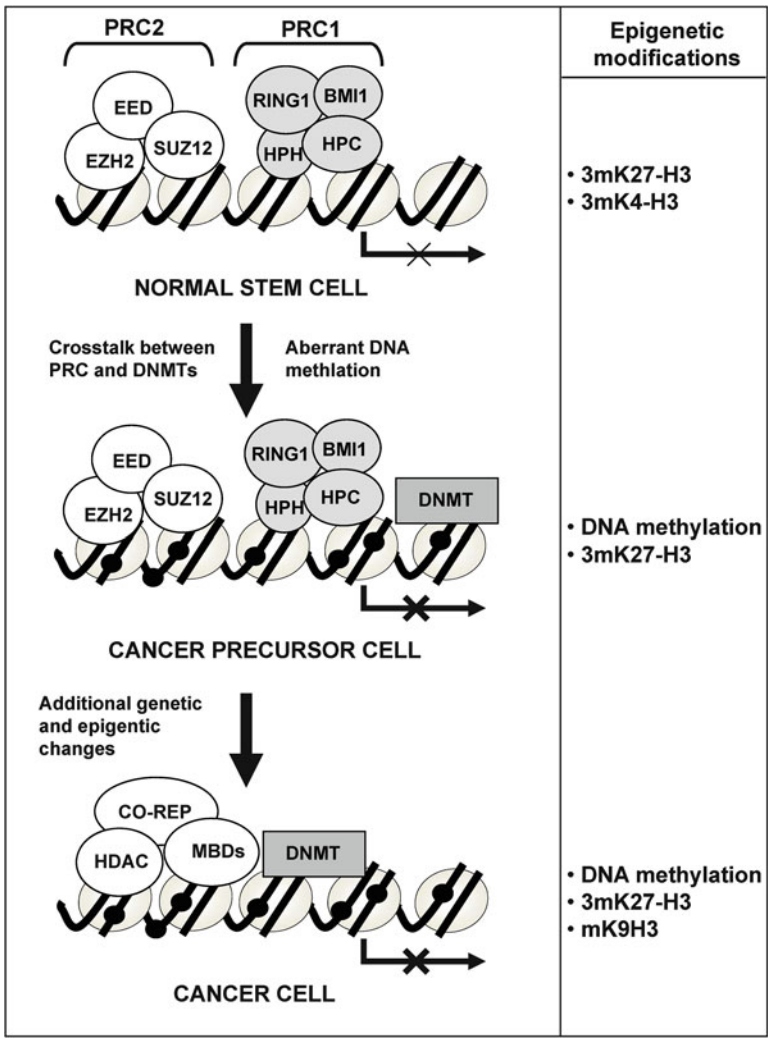

Fig. 16.2 Model of tumorigenesis by which cancer is initiated by a polycomb repressor complex (PRC)-associated DNA methylation followed by additional genetic and epigenetic changes (adapted from [120]).

with high recurrence of prostate cancer [63]. In two recent studies, H3 acetylation has been found to be reduced in colon cancer [140] and in several human colon cancer cell lines [141].

Hypermethylation of the CpG islands in the promoter regions of tumor-suppressor genes in cancer cells is associated with a particular combination of histone markers: deacetylation of histones H3 and H4, and methylation changes at various amino acid residues of H3, like loss of trimethylation at lysine 4, and gain of methylation at lysine 9 and trimethylation at lysine 27 [10, 30]. The presence of the hypo-acetylated and hypermethylated histones H3 and H4 silences certain genes with tumor-suppressor-like properties, such as p21WAF1, despite the absence of hypermethylation of the CpG island [142]. On the other hand, silencing of SIRT1, a histone deacetylase, leads to increased H3 and H4 acetylation of cancer genes that become reactivated despite full retention of DNA hypermethylation [143].

16.8 Interaction Between Genetic and Epigenetic Changes in Cancer Development

After decades of cancer research, it is now widely accepted that genetic and epigenetic mechanisms closely interact in carcinogenesis [144]. This interplay between genetic and epigenetic changes can be clearly observed in the case of tumor-suppressor gene inactivation, which can be caused by genetic (mutation, deletion) or epigenetic (DNA hypermethylation) means. For instance, the *VHL* gene is mutated in 60 % of the renal carcinomas and hypermethylated in 20 % of the remaining cases [145]. In the case of the *E-cadherin* gene, mutations and methylation are mutually exclusive in breast cancer [146]. Besides, genetic alterations (e.g., translocations and amplifications) targeting genes directly involved in the regulation of DNA methylation or histone

modification processes (e.g., *HDAC2*, *MML*, *MOZ*, *MORF*, *NSD*, and *GASC1*) are frequent in cancer [3]. Some genes of the polycomb repressor complex like *BMI1*, *EZH2*, and *SUZ12* are also targets of chromosomal changes [147, 148]. Interestingly, transgenic mice overexpressing the polycomb member Bmi1 develop lymphomas, and this process of lymphomagenesis is accelerated in double transgenic mice overexpressing Bmi1 and Myc, the latter is frequently translocated in lymphomas [149, 150]. The close interaction between genetic and epigenetic changes is also supported by the discovery of epigenetic changes that affect the stability of the genome. For instance, hypomethylation of DNA repeats leads to chromosomal changes by inducing chromosomal instability. Also, genes targeted by DNA hypermethylation are involved in DNA repair pathways like *BRCA1*, *hMLH1*, *MGMT*, and *WRN* [82]. In these cases, silencing of the DNA-repair gene blocks the repair of genetic mistakes, thereby opening the way to neoplastic transformation of the cell. Overall, these data show that (1) genetic and epigenetic changes represent alternative mechanisms targeting the same genes in cancer, (2) genetic changes of epigenetic genes can lead to epigenetic modifications (and vice versa), and (3) epigenetic changes of DNA repair genes can lead to genetic alterations.

16.9 The Origin of Cancer from a Genetic and Epigenetic Perspective

Two of the main issues in cancer research are (1) to discover the primary change triggering tumorigenesis and (2) to identify the type of cell that acquires such a change and clonally expands to give rise to an overt cancer.

With regard to the primary change, several lines of experimental evidence support the feasibility of an epigenetic origin of cancer [121]. For instance, epigenetic changes affecting genes known to be hypermethylated in cancer (e.g., *CDKN2A/p16*, *SFRP1/2/5*, *GATA4/5*, and *HIC1*) [10, 151, 152] can be observed in pre-neoplastic tissues, and in the case of *CDKN2A/p16*, its germline loss in transgenic mouse models increases the stem cell life span. Additionally, loss of imprinting by aberrant DNA methylation of the *IGF2* gene in Beckwith-Wiedemann syndrome is associated with cancer risk and expansion of progenitor cells [121].

There are also data clearly supporting a genetic origin of cancer. A strong argument for this hypothesis is that transgenic mice bearing chromosomal translocations and gene mutations develop cancer [153, 154]. Also, the existence of familial predisposition to cancer linked to the presence of inherited mutations provides strong evidence for a genetic origin of cancer [155].

With regard to the cell of origin, recent data on dysregulation of the polycomb system might indicate a relationship between cancer and stem cells. In stem cells, the polycomb group is involved in repressing genes involved in cell differentiation so these cells maintain their pluripotent state [148]. EZH2, a member of the polycomb group, is able to recruit DNMTs and direct DNA methylation towards specific genes [38]. Most interestingly, it was recently discovered that genes hypermethylated in cancer are indeed repressed in stem cells by proteins of the polycomb group, which strengthens the link between cancer, stem cells, and DNA methylation [117–120]. This finding strongly supports the concept that the initial cell acquiring epigenetic changes should have a kind of stem cell features [113, 121]. Further support for a stem cell involvement in the origin of cancer is that stem cells are more susceptible to genetic changes than differentiated cells [156].

The arguments shown above obviously indicate that genetic and epigenetic alterations collaborate in early stages of tumorigenesis and that cancer cells might originate from cells with stem-cell features (Fig. 16.2). In spite of all these findings, the order of the temporal occurrence of genetic or epigenetic changes is currently unknown. The available experimental data can support several possible temporal relationships, depending upon context. Interdisciplinary studies analyzing genetic, epigenetic, and transcriptional features in the same cancer biopsies and experimental models are required to shed light into the interaction between genetic and epigenetic factors in cancer.

16.10 Epigenetic Changes in the Clinical Management of Cancer Patients

The detection of epigenetic changes in cancer not only helps us to understand cancer biology but also have applications in the clinical management of patients. DNA hypermethylation markers can potentially be used as diagnostic tools, prognostic factors, and predictors of responses to treatment. For instance, the glutathione S-transferase gene (*GSTP1*) is de novo methylated in 80–90 % of patients with prostate cancer [157–159]. Thus, the detection of *GSTP1* methylation could help to distinguish between prostate cancer and a benign process. Analysis of CpG island hypermethylation has potential diagnostic applicability for carriers of high-penetrance mutations in tumor suppressor genes. For example, identification of DNA hypermethylation in a breast-biopsy specimen from a carrier of a *BRCA1* mutation could be useful when the pathological diagnosis is uncertain [85]. Analysis of several hypermethylated genes detects twice as many cancer cells in breast ductal fluids as conventional cytologic analysis [160], and hypermethylated genes can be found in exfoliated cells at different stages in the development of cervical cancer [161]. Aberrant DNA methylation of some genes is also associated with clinical outcome in patients with cancer. For instance hypermethylation of the death-associated protein

kinase (*DAPK*), *p16INK4a,* and epithelial membrane protein 3 (*EMP3*) has been linked to poor outcomes in lung, colorectal, and brain cancer, respectively [3]. Additionally, DNA methylation profiling by microarrays has also identified differential methylation patterns associated with prognosis in ovarian cancer [162] and breast cancer [163].

Hypermethylation of particular genes is potentially a predictor of the response to treatment. A classic example is the methylation-associated silencing of *MGMT*, coding for a DNA-repair protein, in gliomas [164]. MGMT reverses the addition of alkyl groups to the guanine base of DNA and is thus a point of attack for alkylating agents [165]. Two studies have shown that the hypermethylation of *MGMT* is an independent predictor of a favorable response of gliomas to carmustine (BCNU) [166] or temozolomide [167]. These findings have been confirmed by others [168]. Moreover, the hypermethylation of *MGMT* in untreated patients with low-grade astrocytoma and other cancer types is a marker of a poor prognosis [169], and it is probably related to the accumulation of mutations in these cancers [170]. The potential of the methylation status of *MGMT* and other DNA-repair genes to predict the response to chemotherapy has also been observed with cyclophosphamide (with the *MGMT* gene) [171], cisplatin (with the *hMLH1* gene) [172], methotrexate (with the reduced folate carrier gene *RFC*) [173], and irinotecan (with the *WRN* gene) [174].

16.11 Epigenetic Therapies of Cancer

One of the major promises of epigenetic changes in cancer is that, in contrast to genetic mutations, DNA methylation and histone modifications are reversible [10]. The fact that de novo DNA methylation is so prevalent in cancer has led to the development of novel therapeutic approaches aimed at reversing DNA methylation-induced gene silencing [175–177]. It is possible to re-express genes silenced by DNA methylation in cancer cell lines by using demethylating agents, like inhibitors of DNMTs, and to rescue their functionality [3, 178]. DNA-demethylating drugs in low doses have clinical activity against some tumors. Two such agents, 5-azacytidine (Vidaza) and 5-aza-2′-deoxycytidine (Decitabine), have been approved as treatments for myelodysplastic syndromes and acute myeloid leukemias [177, 179]. However, these demethylating agents have not yet been shown to have clinical activity against solid cancers [180]. Histone deacetylase (HDAC) inhibitors can induce differentiation, cell cycle arrest, and apoptosis in vitro [181], although it has not been possible to pinpoint a specific mechanism that explains these effects. In clinical trials, HDAC inhibitors are associated with a low incidence of adverse events [180]. The first drug of this type, suberoylanilide hydroxamic acid (Vorinostat), has been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma [182]. The efficacy of HDAC inhibitors in the treatment of other cancers is limited. The nonspecific effects of DNA-demethylating agents and HDAC inhibitors could have unintended consequences with regard to gene expression, and as a paradoxical result, they could have growth-promoting effects on a cancer by DNA hypomethylation-associated chromosomal instability. Interestingly, directed epigenetic-specific therapy using transcription factors that target particular gene promoters is under development [183]. For instance, the engineered zinc finger proteins target unique sequences in the *MASPIN* promoter; these proteins not only reactivate the epigenetically silenced gene but also inhibit tumor growth in vitro [184]. Until now, therapy with DNA-demethylating agents and HDAC inhibitors has been based on classic protein-coding tumor-suppressor genes, but the possibility of rescuing the growth-inhibitory effects of miRNAs by means of DNA-demethylation treatment [129, 130] suggests new epigenetic treatment strategies that are worthy of further exploration.

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MicroRNAs and Cancer

17

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17.1 Introduction

17.1.1 miRNAs Represent a Paradigm Shift in Our Understanding of Gene Expression

MicroRNAs are small noncoding RNAs, belonging to a novel class of regulatory molecules found in plants and animals that control gene expression by binding to complementary sites on target messenger RNA (mRNA) transcripts. Individual miRNAs are generated from large RNA precursors (termed pri-miRNAs) that are processed in the nucleus by the RNase III enzyme, Drosha, into ~70 nucleotide pre-miRNAs, which fold into imperfect stem-loop structures [1]. The pre-miRNAs undergo an additional processing step within the cytoplasm and mature miRNAs of 18–25 nucleotides in length are excised from one side of the pre-miRNA hairpin by another RNase III enzyme, Dicer [2, 3].

MicroRNAs have been shown to regulate gene expression in two ways. First, miRNAs that bind to protein-coding mRNA sequences that are exactly complementary to the miRNA induce the RNA-mediated interference (RNAi) pathway. Messenger RNA targets are then cleaved by ribonucleases in the RNA-induced silencing complex (RISC). This mechanism of miRNA-mediated gene silencing has been observed mainly in plants [4, 5], but an example is known from animals [6]. In the second mechanism, work from our laboratory and others has shown that miRNAs exert their effect by binding to imperfect complementary sites within the 3′-untranslated regions (3′UTRs) of their target protein-coding mRNAs, and repress the expression of these genes at the level of translation [7–13]. miRNAs identified in both plants and animals use this mechanism to exert translational control of their gene targets [14].

17.1.2 The First miRNAs Identified, lin-4 and let-7, Control Timing of Cell Differentiation

The founding members of the miRNA family, *lineage defective-4 (lin-4)* and *lethal-7* (*let-7)* [10], were identified in the nematode, *C. elegans* [10, 11], where they are members of the heterochronic pathway of genes regulating developmental timing. *C. elegans* growth and development is divided into three major stages called embryo, larva, and adult. Larval growth is subdivided into four larval stages (L1, L2, L3, and L4). Each larval stage ends in a molt and ultimately the animal matures into an adult. Mutations in the *lin-4* and *let-7* miRNAs result in inappropriate reiterations of the first larval stage (L1) and the fourth larval stage (L4) fates, respectively, and these defects lead to disruptions in cell cycle exit [10, 11]. For example, in wild-type animals, specialized skin cells, known as seam cells, divide with a stem cell pattern and terminally differentiate at the beginning of the adult stage. The seam cells fail to terminally differentiate in *lin-4* and *let-7* mutant animals, and instead reiterate the larval fate and continue to divide, a hallmark of cancer.

The expression patterns for *lin-4* and *let-7* correlate with their role in directing developmental timing. *lin-4* RNA accumulates during the L1 stage and is responsible for the

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_17

L1/L2 transition in nematodes by inhibiting the expression of *lin-14* and *lin-28,* repressors of post-L1 fates [11, 12, 15–17]. Likewise, *let-7* RNA accumulates during the L4 stage and is responsible for the L4/Adult transition by downregulating the expression of *lin-41*, *hbl-1*, and *RAS* [8, 9, 18, 19] through binding complementary sites in the 3′UTR.

17.1.3 miRNAs Are Abundant Throughout Animal Genomes

Hundreds of other miRNAs have recently been identified in the fly, worm, plant, and mammalian genomes. Because almost all of these were found through cloning and bioinformatic approaches [20–27] the biological role for the majority of miRNAs remains unknown, although those with known roles are all important regulatory molecules in a variety of developmental and metabolic processes. It is likely that the uncharacterized miRNAs also act as important gene regulators during development to coordinate proper organ formation, embryonic patterning, and body growth.

Our lab and others have found that *lin-4* and *let-7* miRNAs are evolutionarily conserved in higher animals, including humans and are also temporally expressed [28], indicating a universal role for these miRNAs during animal development [21, 29]. There are three exact copies of the mature *let-7* sequence in the human genome (referred to *as let-7a1, let-7a2, let-7a3*) under control of separate promoters and a variety of close homologues that differ from *let-7* at certain nucleotide positions (e.g., *let-7c*). The nematode, fly, and human *let-7* genes are processed from a precursor form (pre-*let-7*) that is predicted to form a stem-loop structure, which is also conserved.

17.1.4 miRNA Biogenesis and Cancer

Studies have shown that the miRNA and RNAi pathways may intersect [2, 3]. miRNAs copurify with components of the RNAi effector complex, RISC, suggesting a link between miRNAs and siRNAs involved in RNAi [30–32]. There is also an indication that some protein factors may play a role in both the miRNA ribonucleoprotein (miRNP) and RISC and that genes encoding these proteins are linked to cancer. For example, the Argonaute protein-coding genes *hAgo3, hAgo1,* and *hAgo4* reside in region 1p34–35, often lost in Wilms tumors, and *Hiwi* an additional Argonaut gene is located on chromosome 12q24.33, which has been linked to the development of testicular germ cell tumors [33]. In addition, *DICER*, the enzyme which processes miRNAs and siRNAs, is poorly expressed in lung cancers [34]. Expression levels of *DICER* and *DROSHA* in 67 non-small-cell lung cancer (NSCLC) samples were examined in the study and reduced expression of DICER was found to correlate with shortened postoperative survival. Kumar et al. [35] substantiated the indication that DICER might be able to prevent the transformation of lung tissue in a later work. This study showed that mouse lung cancer cells expressing short hairpin RNAs (shRNAs) targeting DICER had a decrease in the global miRNA levels and a more pronounced transformed phenotype [35]. Furthermore, deletion of DICER enhanced tumor development in both a xenograft model and a K-Ras-induced mouse model of lung cancer [35, 36].

17.2 miRNAs as Oncogenes and Tumor Suppressors

17.2.1 Evidence for the Role of miRNAs in Cancers

Recent studies from different laboratories have shown roles for the miRNAs themselves in human cancer (Table 17.1) [37]. The human miRNAs, *mir-15* and *mir-16*, are preferentially deleted or downregulated in patients with a common form of adult leukemia, B-cell chronic lymphocytic leukemia [38]. This observation suggests that miRNAs may function as tumor suppressor genes.

For years researchers were perplexed as to how the BIC RNA could consistently produce lymphomas and induce the overexpression of the MYC oncogene [39]. After analyzing the phylogenetically conserved region in BIC, Metzler et al. found that a high homology region in fact codes for the pri-miRNA of miR-155 [40]. Further studies by this group showed that miR-155 expression is upregulated 100-fold in pediatric Burkitt lymphoma, in addition to Hodgkin's lymphoma and primary mediastinal and certain subsets of large B-cell lymphoma [41, 42]. The etiological role of miR-155 in lymphoma development was further established by Costinean et al. from their work with transgenic mice carrying a miR-155 transgene with B-cell targeted expression [43]. The transgenic mice from this experiment developed preleukemic pre-B-cell proliferation at as early as 3 weeks of age and developed high-grade B-cell malignancies at 6 months of age. However, the oncogenic role of miR-155 is not limited to B-cell lymphoma as other studies have reported its upregulation in breast, lung, colon, and thyroid cancers [44, 45].

Another potentially oncogenic cluster of miRNAs is the polycistron miR-17-92, containing seven miRNAs: miR-17-5p, miR-17-3p, miR18a, miR-19a, miR-19b-1, and miR-92-1. This cluster was found to be overexpressed in samples of many kinds of lymphoma samples compared with normal tissues [46]. Using a mouse B-cell lymphoma model, He et al. found that the miR-17-92 cluster in conjunction with

Table 17.1 MiRNA expression and signature for various cancers

Tumor type	miRNAs	Comments	Reference(s)
Brain, GBM	miR-21, miR-221, miR-222, (up)	miR-21 upregulated in all high-grade tumors	[45, 47]
Breast cancer	miR-21, miR-155b (up) miR-125b, miR-145 (down)	Expression levels correlate with biopathological features and proliferation index	[59]
Cervical cancer	miR-21 (up) miR-143 (down)		[103]
Cholangiocarcinoma	miR-21, miR-141, miR-200b (up)		[103]
Chronic lymphocytic leukemia (CLL)	miR-15a, miR-16-1 (down) miR-29a-2, miR-29b-2, miR-29c (down)	Downregulated in 68 % of CLL patients	[59]
Colorectal cancer	miR-143, miR-145, miR133b (down) miR-31, miR-135b, miR-96, miR-183 (up)	miR-31 expression level correlates with cancer stage	[68]
Hepatocellular cancer	miR-18, miR-224 (up) miR-199a, miR-195, miR-200a, miR-125a, miR-122 (down)	Higher miR-18 expression level is inversely correlated with tumor differentiation	[104]
Lung, NSCLC	*let-7 (down)* *miR-21, miR-155 (up)*	Low *let-7* and high miR-155 expression levels correlate with poor survival	[18, 51, 52]
Lymphoma (B cells)	miR-155 (up)		[38, 39]
Pancreatic cancer	miR-21, miR-107 (up) miR-155, miR-103 (down)	Overexpression of miR-21 correlates with high proliferation index	[71]
Prostate cancer	let-7, miR-125b, miR-143, miR-145 (down)	Profile can separate between androgen positive versus androgen negative tumor	[72]
Testis, germ cell tumors	miR-372, miR-373 (up)		[105]
Thyroid, papillary ca (PTC)	miR-221, miR-222, miR-146b (up)		[41]
Thyroid, anaplastic ca (ATC)	miR-125b, miR-26a (down)	miRNA profile can separate ATC from normal and (PTC)	[67]

MYC accelerated tumor development [46]. At the same time, O'Donnell et al. independently identified the mir-17-92 cluster as a group of potentially cancer-related genes [47]. The group found that MYC activates the expression of this cluster and two miRNAs in this cluster, miR-17-5p and miR-20a, act to downregulate the transcription factor E2F1, which is also a MYC transcriptional target, promoting cell cycle progression. This feedback cycle allows MYC to control the accumulation of E2F1 and drive the cell toward proliferation, since E2F1 is known to sensitize cells to apoptosis at high concentrations.

Various studies reported that miRNAs are also aberrantly expressed in brain tumors. During the analysis of a global expression level of 245 miRNAs in glioblastoma multiforme (GBM), Ciafre et al. observed that miR-221 was highly upregulated [48]. In recent work, Gillies et al. reported that miR-221 and miR-222 directly target $p27^{Kip1}$, a key negative regulator of the cell cycle, in glioblastoma [49]. In another study, Chan et al. also observed that miR-21 was strongly overexpressed (5-fold to 100-fold) in highly malignant glioblastoma tumor tissues [50]. The investigators also found that knockdown of miR-21 in cultured glioblastoma cells activated caspases and resulted in more cell death by an apoptotic pathway. In a comprehensive analysis of miRNA expression signature of human solid tumors, Volinia et al. found that overexpression of miR-21 is shared by all six solid tumors studied, including breast, colon, lung, pancreas, prostate, and stomach [45]. This finding suggests that miR-21 plays an important regulatory role in a pathway shared by all of these cancer types. Despite the evidence that mutant miRNAs cause cancer, little is known about the mechanism of these miRNAs and how they affect cancer progression.

17.2.2 let-7 Regulates and Suppresses Activated RAS in *C. elegans*

Our lab recently showed that *let-7* regulates *let-60*, the *C. elegans RAS* oncogene homologue [18, 19]. *let-60/RAS* is best understood for its role in *C. elegans* vulval development [51]. During vulval development, a LIN-3/EGF signal from the anchor cell is received by the LET-23/EGFR receptor on

the vulval precursor cells (VPCs), P5.p, P6.p, and P7.p. The P6.p cell receives the most LIN-3 and activates a *RAS*/MAPK signal transduction pathway to adopt the primary induced (1°) fate. P5.p and P7.p receive less LIN-3 and also receive a second, lateral signal (involving LIN-12/Notch) from the 1° cell that induces them to the 2° fate. *let-60/RAS* contains multiple *let-7* complementary sites in its 3′UTR, and *let-60(lf)* suppresses *let-7* mutants. We found that the *let-60/RAS* 3′UTR is sufficient to restrict *let-60/RAS* expression only to the P6.p. In a normal animal, a *let-7* family member, *mir-84*, is expressed in all the VPCs except the primary induced cell, and represses *let-60/ras* expression in these cells. In *C. elegans* animals carrying activating *let-60/RAS* mutations more than one VPC is induced to differentiate into the 1° cell fate, leading to excess vulvae (thought of as vulval tumors), called a multivulva phenotype (Muv). We found that overexpression of *mir-84* suppressed the Muv phenotype of activating mutations in *let-60/RAS*.

17.2.3 let-7 Negatively Regulates Human RAS and Other Oncogenes

Many activating mutations in the human *NRAS, KRAS,* and *HRAS* genes alter the same amino acid affected by the *C. elegans let-60/RAS* activating mutation. Since *C. elegans RAS* is a target of *let-7*, and both human *RAS* [36, 52, 53] and *let-7* [18, 54] are implicated in lung cancer, our laboratory tested the hypothesis that human *RAS* is also a target of *let-7*. Our results show that all three *RAS* oncogenes have *let-7* complementary sites in their 3′UTRs and that *let-7* can reduce their expression in a 3′UTR-dependent manner [18] in vitro. We next evaluated the relationship between *let-7* and *RAS* in lung tumors and found that *let-7* was consistently downregulated in lung cancers relative to normal adjacent tissue (NAT), mirroring a recently published report about *let-7* [54]. We also found that in lung cancer tissue, where *let-7* levels are low, *RAS* protein levels are elevated relative to the NAT. Supporting the significance of our findings, a correlation was found where lung cancer patients with the least *let-7* expression were most likely to die from lung cancer [54, 55]. A similar correlation was seen when just the lung adenocarcinoma patients were followed [54].

let-7 has also been recently reported to be involved in the regulation of two other oncogenes, MYC and HMGA2. Sampson et al. showed that overexpression of pre-*let-7*a in a Burkitt lymphoma cell line led to downregulation of MYC expression and reverted MYC-induced growth in this cell line [56]. Interestingly, *let-7*a overexpression not only resulted in a decrease in MYC protein (75 %), but also its mRNA level (70 %), suggesting that *let-7*-mediated repression of MYC may act through the mRNA degradation pathway and not the translational inhibition pathway.

HMGA2 encodes a small, nonhistone chromatin-associated protein that plays a critical role in growth during embryonic development by altering chromatin architecture [57]. It is normally expressed at low levels in adult, but disruption of the gene by chromosomal translocation at 12q15 results in benign and malignant transformation in various tissues [58]. Several groups reported that this chromosomal translocation event removes the 3′UTR of HMG2A and results in the deletion of miRNA-binding sites [59–61]. Mayr et al. [59] and Lee et al. [60] showed that the HMG2A 3′UTR contains seven predicted conserved *let-7* binding sites, and a luciferase reporter assay using this 3′UTR confers repression by *let-7*. The two groups also demonstrated that overexpression of *let-7* inhibited proliferation in H1299 lung cancer cells and disruption of the pairing between *let-7* and HMGA2 led to enhanced tumor formation in a mouse xenograft model [59, 60].

These findings support the role of *let-7* as a tumor suppressor. It has been hypothesized that similar to its role in *C. elegans*, *let-7* functions during late stage embryonic development to maintain differentiated states by suppressing expression of embryonic genes (such as HMGA2) that are expressed in de-differentiated tissues [61]. When *let-7* repression is lost, embryonic genes are upregulated and trigger neoplastic transformation with characteristic of embryonic cells. Taken together, these studies suggest that *let-7* replacement may be a potentially useful cancer treatment, especially in lung cancer.

17.2.4 Mir-34 and the p53 Network

The *mir-34* family is an evolutionary conserved miRNA family first identified in *C. elegans*. It is located at a fragile site region at 1p36 and has been found to be frequently deleted in various cancers including breast and lung cancer [62]. Recently, numerous independent reports showed that miR-34 is a part of the p53 network and is a direct transcriptional target of p53 [63–67]. miR-34 is directly induced by p53 in response to DNA damage and oncogenic stress. miR-34 by itself has been shown to mimic p53 action to a certain extent, as evident by its anti-proliferation and apoptosis-inducing properties when overexpressed in primary fibroblast and some tumor cell lines [63, 65]. Microarray analysis showed that the induction of the miR-34 family led to the downregulation of hundreds of mRNAs. Some validated targets of miR-34 include CDK4, CDK6, cyclin E2, and E2F3. This was the first time that a miRNA was found to be an integral part of an established oncogene and tumor suppressor network, and suggests that there are other miRNAs belonging to these networks as well. These findings could partially explain why alterations affecting miRNAs are so prevalent in cancers.

17.3 miRNA Profiling: Diagnostics, Classification, Prognosis, and Treatment

miRNA expression profiling studies using microarray and other methods can differentiate normal from cancer tissues via a unique miRNA signature. These differences can classify different cancer types and cancer grades. Certain miRNA signatures are correlated with prognosis and could potentially be used someday to determine the specific course of treatment [44, 54, 55, 68–70].

Using a novel bead-based miRNA microarray assay, Lu et al. examined the expression profile of 217 miRNAs in a panel of 334 samples that included primary tumors, tumor-derived cell lines, and normal tissues [68]. The investigators found that miRNA profile can discern between normal and cancer tissues, separate different cancer types, stratify cancer differentiation state, and cluster sample groups according to their embryonic lineage. Moreover, miRNA profiles were more accurate than mRNA profiles in classifying cancer types and were able to categorize 17 poorly differentiated tumors of cancers of unknown primaries where histological appearance was not diagnostic, into their specific tissue lineages.

Studying colorectal cancer, Michael et al. were the first to recognize aberrant miRNA expression in solid tumors as the investigators identified 28 different miRNAs in colonic adenocarcinoma and normal mucosa, and found that miR-143 and miR145 were consistently downregulated in colon cancer [71].

Analyzing the miRNA expression in 104 pairs of primary lung cancers and corresponding noncancerous tissues, Yanaihara et al. found that a set of 43 unique miRNAs were able to discriminate normal from tumor tissues [55]. Among the miRNAs from the signature, high miR-155 and low *let-7*a-2 expression independently correlated with poor survival for adenocarcinoma patients. In a comparable study, Takamizawa et al. [54] found that *let-7* miRNA expression can classify 143 postoperative lung cancer patients into two prognosticator groups. Those with low levels of *let-7*a had significantly shorter survival after surgery [54].

In breast cancer, the first miRNA profiling study showed that a set of 15 miRNAs correctly predicted normal versus cancer tissues with 100 % accuracy, with the most significantly deregulated miRNAs being miR-125a, miR-125b, miR-145, miR-21, and miR-155 [72]. miR-21 and miR-155 were upregulated and the others were downregulated. miR-125a and miR-125b regulate the expression of the receptor tyrosine kinases ERBB2 and ERBB3 and their overexpression in SK-BR3 cells induced impaired anchorage-dependent growth and reduced invasion capacities [73]. Furthermore, miRNA expression was correlated with specific breast cancer pathological features, such as estrogen and progesterone receptor expression, tumor stage, and vascular invasion.

Papillary thyroid carcinoma (PTC) is the most common malignancy in thyroid tissue. Profiling of PTC by He et al. yielded a signature of five miRNAs (which included miR-221, miR222, and miR-146) that could separate PTC and normal thyroid tissue [44]. The investigators found that upregulation of miR-221, miR-222, and miR-146 corresponded to a dramatic decrease in KIT mRNA and protein levels. This suggests that these three miRNAs negatively regulate KIT and may contribute to PTC. Interestingly, in a separate study, Visone et al. [70] showed that another type of thyroid cancer, thyroid anaplastic carcinomas (ATC), has a completely different miRNA signature from PTC that clearly differentiates ATC from normal tissues and from PTC.

In the case of pancreas and prostate cancers, researches have also shown the presence of miRNA signatures that differentiate these cancers from normal tissues. Bloomton et al. showed that a signature of 21 upregulated and 4 downregulated miRNAs correctly differentiated pancreatic cancer from benign pancreatic tissue in 90 % of samples [74]. In the profile, two commonly malignancy-associated miRNAs, miR-21 and miR-155, were uniquely overexpressed in pancreatic cancer versus normal pancreas. Additionally, a subgroup of 6 miRNAs was able to predict long-term survival, in contrast to one, miR-196a-2, which predicted for poor survival. In the profiling of prostate cancer, Porkka et al. [75] found that there was differential expression of 51 individual miRNAs between benign prostatic hyperplasia (BPH) and prostrate carcinoma—37 miRNAs showed downregulation and 14 were upregulated in carcinoma samples [75]. Among the downregulated miRNAs are some familiar ones, including *let-7*a, miR-125b, miR-143, and miR-145. Furthermore, the miRNA expression patterns could classify the tumors according to their androgen dependence (hormone naïve versus hormone refractory). This finding indicates that miRNAs could be useful in diagnosis and perhaps in subsequent treatment decisions.

miRNA profiling is a powerful method that cannot only classify normal and cancer tissues, but also can discern between different cancer subtypes. It could potentially become an important tool for the pathologist with difficult cases such as poorly differentiated metastatic cancers of unknown primary.

17.4 The Cause of Aberrant miRNA Expression in Cancer

17.4.1 miRNAs at Chromosomal Fragile Sites

Several studies have evaluated the cause of altered expression of miRNAs in cancer. In one study, Calin et al. [62] identified the location of 186 miRNAs and compared this to the location of previously reported nonrandom genetic

alterations in cancer. The investigators found that overall, 98 of 186 (52.5 %) of miRNA genes are in cancer-associated chromosomal fragile sites, such as minimal regions of loss of heterozygosity, minimal regions of amplification, and common breakpoint regions. One example is miR-15a and miR-16-1 which are often deleted or downregulated in chronic lymphocytic leukemia (CLL) and are located within the intron of a noncoding RNA gene of unknown function, called deleted in lymphocytic leukemia 2 (DLEU2). Deletion of this 30 kb region within the 13q14 chromosomal locus is seen in more than 65 % of CLL cases, in 50 % of mantle cell lymphomas, 16–40 % of multiple myelomas, and 60 % of prostate cancers. The implication that miR-15a and miR-16-1 function as a tumor suppressor in CLL is further supported by Cimmino et al. [76] showing that these two miRNAs negatively regulate BCL2, an antiapoptotic gene that was reported to be overexpressed in many types of human cancers, including leukemias, lymphomas, and carcinomas. Additionally, *let-7g*, a miRNA that is often downregulated in lung cancer, maps to 3p21, a well-known region deleted early in the progression of lung cancer [77].

To examine the relationship between miRNA DNA copy number abnormalities and cancer, Zhang et al. employed a high-resolution array-based comparative genomic hybridization assay to study 283 known human miRNA genes in 227 human ovarian cancer, breast cancer, and melanoma specimens [78]. The results show that a significant number of genomic loci containing miRNA genes exhibit DNA copy number alterations in ovarian cancer (37.1 %), breast cancer (72.8 %), and melanoma (85.9 %). A total of 41 miRNA genes with gene copy number changes were shared among the three cancer types (26 with gains and 15 with losses). The same study also found high frequency copy number abnormalities of Dicer1, Argonaute2, and other miRNA associated genes in all three cancer types [78]. Findings from these studies support the notion that genetic instability resulting in copy number alterations of miRNAs and their regulatory genes is highly prevalent in cancer and could partially explain miRNA aberrant expression in cancer.

17.4.2 Epigenetic Regulation of miRNAs

It is well known that epigenetic control of gene expression by DNA methylation and covalent histone modification play an important role in tumorigenesis, as tumor cells can acquire epigenetic abnormalities that result in altered patterns of gene expression from normal cells [79]. miRNAs have been hypothesized to be susceptible to epigenetic control as one study showed that 155 out of 332 human miRNA genes analyzed were associated with CpG islands, which are targets of the DNA methylation machinery. Furthermore, the methylation frequency of miRNA genes seemed to be at least an order of magnitude higher than the methylation frequency of protein-coding genes [80]. Several recent studies have confirmed that this mechanism exists to regulate miRNA expression in various cancers.

Using miRNA array analysis, Scott et al. profiled miRNA levels in response to the histone deacetylase inhibitor (HDACi) LAQ824 in the breast cancer cell line SKBr3. After 5 h of exposure, significant changes were observed in 40 % of the >60 different miRNA species expressed in SKBr3 cells with 22 miRNA species downregulated and 5 miRNAs upregulated [81]. Additional antisense experiments targeting a downregulated miRNA, miR-27, showed that one of its potential targets is the proapoptotic protein RYBP [81].

In one of the first studies to clearly demonstrate epigenetic alterations of miRNA expression in human cancer cells, Saito et al. analyzed miRNA expression in T24 human bladder cancer cells and normal fibroblasts after treatment with the DNA-demethylating agent 5-aza-2′-deoxycytidine (DAC) and an HDAC inhibitor 4-phenylbutyric acid (PBA) [82]. Expression profiling revealed that 17 out of 313 human miRNAs studied showed more than threefold upregulation, with miR-127 showing the biggest change at a 49-fold increase [82]. MiR-127 is constitutively expressed in normal human fibroblasts and tissues as part of a miRNA cluster, but is downregulated or silenced in bladder, breast, cervix, pancreas, lung, and colon cancer cell lines, and prostate, bladder, and colon primary cancer tissues. miR-127 was found to be hypermethylated in both normal and cancer cells, and treatment with PBA and DAC led to specific induction of mir-127 within the cluster, with more pronounced upregulation of the miRNA observed in TP24 cancer cells. The investigators furthermore demonstrated that the proto-oncogene BCL6 is one of the targets of miR-127, as epigenetic reactivation of miR-127 resulted in a corresponding downregulation of the BCL6 protein level [82]. These results suggest that miR-127 may function as a tumor suppressor and that its epigenetic regulated gene expression is altered in various cancers.

In another study, Lujambio et al. showed that miR-124a is another potential tumor suppressor miRNA that is susceptible to altered epigenetic regulation in cancers [83]. In this study, miRNA expression profiling of the wild-type colon cancer cell line HCT-116 and a genetically engineered double knockout of DNA methyltransferase 1 (DNMT1) and DNMT3b HCT-116 cell line (DKO) showed 18 out of 320 miRNAs were upregulated more than threefold in DKO cell line. Of these miRNAs, miR-124a was analyzed further and found to be embedded in CpG islands, but was unmethylated in normal colon tissues. In contrast this miRNA is inactivated by promoter hypermethylated in various human cancer cells and tissues, including colon, breast, lung, leukemias, and lymphomas. Epigenetic silencing of miR-124a resulted in the upregulation of its target, CDK6, an oncogenic factor that targets the Rb (retinoblastoma) tumor suppressor protein

for phosphorylation and inactivation [83]. These studies confirm epigenetic regulation of miRNAs as another mechanism for control of miRNA gene expression and deregulation of this process could play a role in the etiology of cancer as a result of altered miRNA and cellular gene expression. This also presents an opportunity for therapeutic intervention, and indeed HDAC inhibitors are currently emerging as a promising class of anticancer drugs [84–86].

17.4.3 Single-Nucleotide Polymorphism of miRNAs

Base pairing between the mature miRNA and its target mRNA 3′ UTR is known to be highly conserved. Using genome-wide analysis to discover single-nucleotide polymorphisms (SNPs) located in the miRNA-binding sites of the 3′ UTR of various human genes, two studies concluded that miRNA-binding site SNPs are negatively selected in predicted conserved miRNA-binding seed sequences compared to other conserved motifs and the entire 3′ UTR [87, 88]. However, natural genetic variants do exist in the population and can predispose certain carrier groups to disease, as one study found 79 different SNPs within putative miRNA-binding sites present in the 3′ UTR of 129 genes involved in pathways commonly acknowledged as important in cancer [89]. In another example, when comparing the dbSNP database against human cancer specimens, Yu et al. found that 12 miRNA-binding site SNPs display an aberrant allele frequency in human cancers [88].

SNPs within the miRNA coding regions or within miRNAs binding sites are likely to be deleterious and can have an impact on an individual's risk to develop diseases such as cancer. In one of the first studies to illustrate this, He et al. observed that in 5 out of 10 thyroid cancer cases analyzed, where upregulation of miR-221, miR-222, and miR-146 was highest, there was a corresponding loss of KIT transcript and Kit protein in addition to germline SNPs in the two 3′UTR binding sites in KIT for these miRNAs [44]. Due to high familial incidence, these SNPs are hypothesized to predispose this group of carrier to thyroid cancer.

17.5 Therapeutic Applications of miRNAs

17.5.1 Strategies for miRNAs Inhibition

With miRNAs emerging as important oncogenic and tumor suppressive genes, therapy to inactivate or supplement specific miRNAs is a strategy to perhaps inhibit tumor progression [54, 76, 90]. Since pri-miRNAs, pre-miRNAs, and mature miRNAs are long and short oligonucleotides, all these molecules can be targeted by the antisense technology. Many groups have shown that anti-miRNA oligonucleotides (AMOs) with or without 2′ sugar modification (including 2′-O-methyl, 2′-O-methoxyethyl, and 2′-fluoro) can be effective inhibitors of miRNAs in cell culture and animal models [50, 91–94]. Si et al. showed that anti-miR-21 oligonucleotides can suppress both cell growth in vitro and tumor growth in the xenograft mouse model with MCF-7 breast cancer cells [94]. Furthermore, this anti-miR-21-mediated cell growth inhibition was associated with increased apoptosis and decreased cell proliferation, which is hypothesized to be the result of downregulation of the antiapoptotic Bcl-2 genes.

Locked nucleic acid (LNA)-based oligonucleotides have been shown to be more stable and less toxic in inhibiting endogenous miRNAs in *Drosophila melanogaster* cells, leading to upregulation of the cognate target protein [95]. Modified AMOs that are conjugated with cholesterol termed antagomirs have been shown to be effective in targeting miRNAs in vivo and can discriminate between single-nucleotide mismatches of the targeted miRNA [96, 97]. When antagomir targeting the liver-specific miR-122 was delivered intravenously in mice, the AMO efficiently inhibited the mature miRNA in various organs and increased levels of miR-122 target mRNAs were observed in the liver [97]. Additionally, since miR-122 plays a role in cholesterol biosynthesis, reduced levels of plasma cholesterol were also observed in treated mice. Most impressively, a single injection of 240 mg/kg of body weight conferred silencing for up to 23 days. As an alternative to AMOs, Ebert et al. recently developed microRNA inhibitors called microRNA sponges [98]. These molecules are transcripts expressed from strong promoters, containing multiple, tandem binding sites to specific miRNAs and competitively inhibit them. One advantage of this system is that one could construct sponges with combination of seed binding sites to inhibit an entire miRNA family or a miRNA cluster [98].

17.5.2 Strategies for miRNA Replacement

Strategies to overexpress miRNAs that function as tumor suppressors, such as the *let-7* and miR-34 families, could be employed to treat specific cancer types. Similar to siRNAs, modified matured miRNAs could be delivered in vivo by liposome or cholesterol conjugation. Kim et al. showed that by using a cholesterol conjugated siRNA their group was able to effectively inhibit tumor growth in colon adenocarcinoma [99]. Alternatively, a gene therapy approach can be used for miRNA replacement therapeutics. This involves expressing the pre-miRNA hairpin and flanking sequences from a polymerase II or III promoter in a viral vector, which is then processed by the endogenous miRNA machinery for proper target gene repression. One concern with this strategy is that while abundant expression ensures effective target

knockdown, it can overwhelm the miRNA pathway to the detriment of the host [100]. Thus, tissue-specific and control on/off expression of miRNAs would be the most prudent approach.

The use of miRNA therapy to complement traditional anticancer treatments appears to have great potential as reported for the first time from two groups that miRNAs can enhance response and suppress resistance to anticancer cytotoxic therapies. Meng et al. reported the use of anti-miR-21 and anti-miR-200b AMOs to increase the susceptibility of cholangiocarcinoma cells to the chemotherapy drug gemcitabine [101]. Recent work from our own laboratory showed that increasing levels of the *let-7* family of miRNAs can radiosensitize A549 lung cancer cells and a *C. elegans* in vivo model of radiation-induced cell death, while decreasing *let-7* levels causes radioresistance [102]. Significantly, these two papers also showed that chemotherapy and radiation treatment alter miRNA expression, perhaps as a part of the cellular damage repair pathway. As resistance to cytotoxic therapy accounts for the majority of failed cancer treatments and recurrences, it would be interesting to profile the miRNA differences between resistant and sensitive populations of cells to identify miRNAs that are involved in the resistant mechanisms or pathways, which then can be used to re-sensitize tumors to available treatments.

17.6 Conclusion

miRNAs are emerging as major players in the etiology of cancer. Some are authentic oncogenes or tumor suppressors as they function in controlling cell differentiation and apoptosis directly, while others are part of the existing oncogenic pathways. Beyond this miRNAs appear to play a role in the response to cancer therapy. Elucidating their regulation and targets will enhance our understanding of cancer biology and provide us with new targets for cancer diagnosis and therapy.

Acknowledgements PT was supported by NIH NRSA (F32CA130376), JBW and FJS by the CT Department of Public Health and Asuragen Inc.

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Erionite and Asbestos in the Pathogenesis of Human Malignant Mesotheliomas

18

Mutlay Sayan and Brooke T. Mossman

18.1 Introduction

Epidemiologic and animal studies indicate that exposure to asbestos or erionite fibers can result in pulmonary and pleural fibrosis, lung cancer, and malignant mesotheliomas (MM), unique tumors associated predominantly with human exposures to these fibers [1]. Recent studies have shown that erionite also causes these diseases, especially MM in families in Cappadocia, Turkey who are genetically predisposed to mineral fiber carcinogenesis [2]. In these circumstances, residents are exposed to high levels of erionite due to its incorporation into their homes. Soils in these areas also have erionite contamination, another source of environmental exposures. In contrast, asbestos-induced diseases have been usually associated with occupational exposures to these fibers in the past workplace before regulation of exposure levels. However, para-occupational MMs also have been detected in family members of asbestos workers and in individuals living in asbestos mining communities. Understanding the critical chemical and physical properties of asbestos and erionite fibers, including how fibers interact with target cells of disease, and how these interactions contribute to the pathogenesis of fibroproliferative and malignant diseases are critical to designing preventive and therapeutic approaches in high-risk individuals. This review focuses on similarities and differences among asbestos and erionite fibers, their effects on the pathogenesis of MM, and key pathways in cell signaling that are linked to fiber toxicity, cell proliferation, inflammation, and other features of carcinogenesis.

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18.2 Properties of Erionite and Asbestos

Erionite is a naturally occurring fibrous mineral belonging to a classification of minerals called zeolites that are often used industrially. [Note that a fiber is defined by most regulatory agencies as having ≥3:1 length-to-diameter ratio, whereas particles (<3:1 length-to-diameter ratio) can be spheroid, platy, or irregular in shape]. Zeolites usually occur in cavities in basic volcanic rocks and in other late stage hydrothermal environments such as those found in the Cappadocian region of Turkey and at other geological sites including parts of the western USA [3]. These minerals have a very large internal surface area resulting from the particular configuration of their crystalline lattice [4] and are able to exchange water molecules and cations without major changes to their structure. Most zeolites are non-fibrous. However, erionite is fibrous and occurs in needle-like bundles that resemble amphibole types of asbestos (Fig. 18.1) [5]. Fibrous morphology largely dictates their unique cancer-inducing properties.

Erionite has gas adsorption, ion exchange, and catalytic properties that are highly selective depending upon the molecular size of the adsorbed compounds [6]. The integral composition of erionite is made up of an interlocking tetrahedral of silicate aluminate which is negatively charged, allowing it to bind cations [7]. In addition, erionite adsorbs iron after inhalation and interacts with the respiratory epithelium. This adsorbed iron is associated with the toxicity of erionite [8].

Three types of erionite have been identified based upon differences in their levels of Ca, Na, and K (the predominant cations) and the amounts of Mg, Fe, Sr, and Ba in the structure. The general formula for the erionite-Ca type mineral is $(Ca_{3.56}K_{1.95}\ Na_{0.27}Mg_{0.30})\ (Si_{25.78}\ Al_{10.28}\ Fe_{0.01})\ O_{78}$, for the erionite-Na type mineral is $(Na_{4.00}\ K_{2.40}\ Ca_{1.13}Mg_{0.24})\ (Si_{26.69}\ A_{19.11}Fe_{0.22})\ O_{72}$, and for the erionite-K type mineral is $(K_{2.80}Na_{1.66}Ca_{1.03}Mg_{0.51})\ (Si_{28.21}\ A_{17.39}\ Fe_{0.41})\ O_{72}$ [9].

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_18

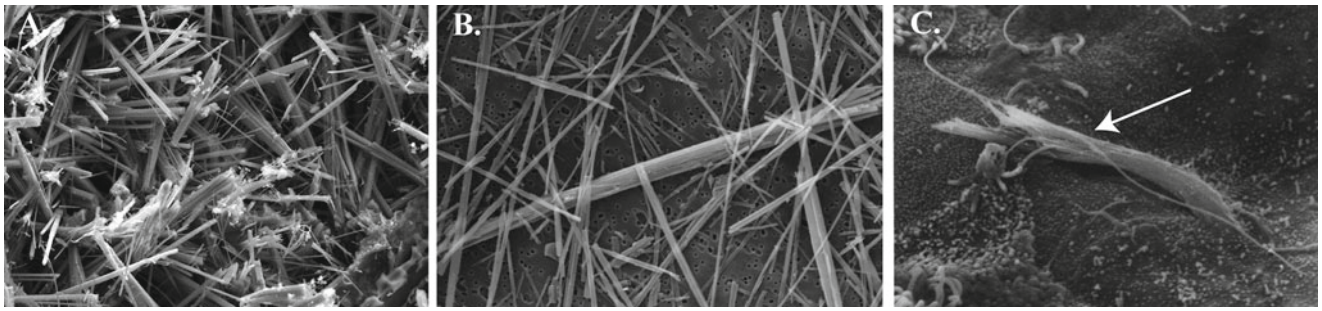

Fig. 18.1 Scanning electron microscopy (SEM) micrographs showing the morphology of erionite fibers, crocidolite asbestos, and chrysotile fibers. (**a**) Erionite fibers from Pine Valley, NV (courtesy of Dr. Ian Steele, Department of Geoscience, University of Chicago, Chicago, IL). (**b**) NIEHS reference sample of crocidolite asbestos. (**c**) A bundle of chrysotile fibers (*arrow*) interacting with the tracheal epithelial cell surface in organ culture (from [86]).

Asbestos is a commercial term for another group of naturally occurring crystalline fibers. Each group has its own distinctive structure and chemical composition. There are two subgroups: (1) a serpentine group, consisting of chrysotile [$Mg_6Si_4O_{10}$ $(OH)_8$]; and (2) the amphiboles, five groups of rod-like fibers including crocidolite [$Na_2(Fe^{3+})_2(Fe^{2+})_3$ $Si_8O_{22}(OH)_2$], amosite [$(Fe,Mg)_7Si_8O_{22}(OH)_2$], tremolite [$Ca_2Mg_5Si_8O_{22}(OH)_2$], anthophyllite [$(Mg,Fe)_7$ $Si_8O_{22}(OH)_2$], and actinolite [$Ca_2(Mg,Fe)_5Si_8O_{22}(OH)_2$] [10]. These fibers may exist in deposits with each other or other minerals and may adsorb other metals or organic compounds to their surfaces during mining or processing.

The potential of asbestos fibers to cause cancers and fibrosis has been linked primarily to their fibrous geometry and size—longer, thinner fibers being most bioreactive because of their incomplete phagocytosis, release of oxidants from cells, and their ability to act as a platform for cell proliferation [11, 12]. However, in addition to size, the chemical composition of asbestos fibers also plays an important role in determining the cytotoxicity, durability, and biopersistence of asbestos types [13]. Amphibole fibers, especially crocidolite and amosite asbestos, have the greatest carcinogenic potential in MMs in comparison to chrysotile in asbestos-exposed human populations. The increased pathogenicity of amosite and crocidolite asbestos may reflect their greater durability in the lung or pleura after inhalation as well as the high iron content of these amphiboles (20–30 % by weight) [13]. They also form ferruginous asbestos bodies in the lung in contrast to chrysotile fibers which may be iron-free or acquire iron naturally in trace amounts. Amphibole asbestos fibers also have a sharp, needle-like structure which causes their alignment with airways after inhalation and allows more effective penetration into the deep lung. In contrast, chrysotile fibers have a curly, snake-like structure (Fig. 18.1c) and break down into smaller fibrils over time. A number of studies have shown that smaller fibers or fragments (<5 μm) of asbestos, regardless of type, are less cytotoxic, carcinogenic, and fibrogenic than longer asbestos fibers [14]. This may be in part because they are more easily cleared from the lung over the long latency periods of asbestos-induced diseases.

On an equal weight basis, chrysotile fibers are more cytotoxic than amphibole fibers due primarily to their positive surface charge and greater surface area whereas amphiboles are neutral or slightly negative in charge [15]. Hydrated magnesium molecules are located on the apical oxygens of chrysotile accounting for its highly polar properties as well as dissolution of the mineral when magnesium is leached from the fiber. In nature, these structures are arranged as loose bundles that break down into smaller fibrils in tissue [1, 16].

For reasons that are unclear, erionite is more carcinogenic in rodents than asbestos, regardless of source [7]. Experimental studies indicate that erionite is 300–800 times more carcinogenic than chrysotile and 100–500 times more carcinogenic than crocidolite asbestos when administered through intrapleural routes. In intraperitoneal injection studies, erionite is 20–40 times more potent than chrysotile, and 7–20 times more potent than crocidolite in tumor induction [17]. These trends have also been observed in inhalation studies [18]. One theory is that erionite has a hexagonal structure with an internal surface of 200 m^2/g that is 20 times larger than crocidolite asbestos, thus allowing erionite to absorb many small molecules and to have a higher catalytic activity [19]. It was also found that on an equal weight basis, erionite induces more reactive oxygen species (ROS) than asbestos fibers [20].

18.3 Links Between Erionite, Amphibole Asbestos, and Malignant Mesothelioma

18.3.1 Features of Malignant Mesothelioma

Malignant mesothelioma (MM) is an aggressive tumor originating from the lining cells (mesothelium) of the pleural and

peritoneal cavities, as well as the pericardium and the tunica vaginalis. Although the main risk factor is exposure to asbestos or erionite, other factors such as Simian virus 40 (SV40) infection and inheritance of susceptibility genes likely play roles in initiation and progression of MM [21, 22]. MM may acquire many phenotypic forms (epithelioid, biphasic, and sarcomatoid) which makes them difficult to diagnose without multi-panel histochemical stains.

The combination of an accurate patient history, physical examination, radiology, and the confirmation of pathology is essential in the diagnosis of mesothelioma. Typical presenting features invariably occur in late stages of disease and include chest pain, dyspnea, and breathlessness due to pleural effusion [23, 24]. Treatment strategies include chemotherapy, radiotherapy, gene therapy, extrapleural pneumonectomy, surgical resection of tumors, or combined therapies. However, no effective therapies have been developed for MM, and because they are most often diagnosed at end stages of disease, clinicians often focus on palliation of symptoms and/or end-of-life care [25].

The latency period for development of MM is often 30–40+ years from initial exposure to asbestos fibers [13]. Because of difficulties in treatment and management, life expectancy in pleural MM is grim with median survival varying between 8 and 14 months [21]. However, peritoneal MM patients can survive 5 years or longer [26].

18.3.2 Malignant Mesothelioma in Turkey

18.3.2.1 Epidemiologic Studies

Annual asbestos-related mesothelioma cases in Southeast Turkey are reported to be 50 per million [27]. However, the annual incidence of mesothelioma related to environmental exposure to erionite was found to be from 2200 to 8000 cases per million in several villages such as Karain, Tuzkoy, and Sarihidir in the Cappadocia region of Turkey. This incidence rate is much higher than any other reported incidence of MM worldwide [28]. In 1987, Baris reported the epidemic of mesothelioma among residents of Karain, Tuzkoy, and Sarihidir [28]. The estimated incidence of pleural MM was indicated as 996 per 100,000 inhabitants [28]. It was noted that more than 50 % of deaths were caused by MM in these three villages [29]. A study based on 162 Turkish immigrants from Karain to Sweden also shows a strong correlation between erionite exposure and MM. Fourteen deaths were reported due to MM among the 18 (78 %) total deaths during the period between 1965 and 1997. The standardized incidence rates found in this study were 135 times higher among the males and 1336 times higher among the females compared to the general population of Sweden [30].

A mortality study between 1994 and 1997 suggested that MM might also occur in some of the other villages near the known three erionite villages in Cappadocia. These other villages are Cokek, Karlik, Karacaoren, Boyali, and Yesiloz. These investigators reported 53 deaths due to mesothelioma from the three erionite villages, Karain, Tuzkoy, and Sarihidir, and 11 deaths from the other four villages [31]. It was noted that MM cases were more prevalent in the erionite villages compared to the nearby villages despite the environmental contamination of erionite, which was often used in dwellings in the region [27].

In 2006, a study from 1979 to 2003 based on residents of two erionite-exposed, and one nearby control village, was published [32]. In this study, a total of 891 men and women, aged 20 years or older, were included, a total of 230 residents in villages without known exposure to erionite. Mortality data were obtained from hospital records and death certificates. During the 23-year follow-up, 119 deaths occurred from MM, the cause of 44.5 % of all deaths in the exposed villages. Seventeen patients had peritoneal mesothelioma; the remainder died of pleural mesothelioma. Only two cases of MM, one of each type, occurred in the control village, both in women born elsewhere [32].

18.3.2.2 Gene: Environmental Interactions

In Karain, Tuzkoy, and Sarihidir, MM occurs only in certain houses. Pedigree analyses revealed that in MM villages, MM was more frequent in certain families compared with others. However, in some families, MM developed among individuals who married into the affected family. For example, in one family, 17 of 30 members died of MM. Among the 17 MM, 5 occurred in individuals who married into this family [2]. This finding initially argued against a theory of genetic predisposition. However, it was found that those who married into a MM family and developed MM were also from MM families. When members of a family with high incidence of MM married into families with no history of MM, descendants developed MM [2].

To test the hypothesis that genetics alone could cause MM in certain families, 24 descendants from one family (ages 26–45 years) born and raised outside the village of Sarihidir were identified. No MM developed in this group. Instead, three MM cases were observed in the same age group among 29 members of a family born and raised in Sarihidir and exposed to erionite. These pedigree studies indicate that the MM epidemic in three Cappadocian villages is caused by genetic predisposition and may make certain individuals more susceptible to the carcinogenic effects of erionite exposures [2]. A team of US and Turkish investigators is now attempting to isolate the putative MM susceptibility gene(s). This gene(s) may be mutated or otherwise altered in

Cappadocian families and also may be affected in sporadic familial MMs in the Western world [32].

18.3.3 Epidemiology of Asbestos-Induced Malignant Mesothelioma

18.3.3.1 Historical Studies

Although adverse health effects of asbestos were first recognized by 1899 [33], dust control legislation for mines was not enacted in North America until 1971 by the Occupational Safety and Health Administration (OSHA). In 1935, the suggestion that mesothelioma resulted from occupational exposure to asbestos was first made by Gloyne [34], and the first clear evidence of a link between amphibole asbestos exposure and primary malignant tumors of the mesothelium was shown by Wagner in 1960 [35]. Wagner's observation was based on 33 cases of pleural mesothelioma in the Northwest Cape Province of South Africa, 28 in persons who had lived close to the crocidolite mines, mostly as children [35]. Confirmation was provided by eight case-control studies published in 1965–1975, based on a total of 657 cases, predominantly male, exposed to asbestos mainly in shipyards, heating trades, insulation, and factory work [36]. It was also shown that the hazards of asbestos dust were not confined to heavily exposed workers in asbestos factories but extended to insulation workers, other users of products containing asbestos, and people who lived close to asbestos factories [37–40].

The role of SV40, a polyomavirus, as a cofactor with asbestos fibers in the induction of malignant mesothelioma is controversial. A review by Gazdar indicates that some investigators have detected SV40 viral DNA sequences and oncoproteins in human pleural MMs [41]. Even though there are technical concerns about some of these findings [42], a role for SV40 as a carcinogen or co-carcinogen is biologically believable on much cellular data and results of animal models [43]. This suggestion is also supported by many of the molecular mechanisms of action of these viral oncoproteins [41].

18.3.3.2 Libby, Montana

Although approximately 2500 cases of MM are reported in the USA annually, a cluster has been noted in Libby, MT, a town with a population of ~2700, with nearly 12,000 people in the surrounding area. Libby had one of the world's largest vermiculite mines in operation from the 1920s to 1990. The Libby vermiculite had useful insulating and soil conditioning properties. However, ore from the mine was contaminated with fibrous and nonasbestiform amphiboles in veins throughout the deposit [44]. After 70 years of mining amphibole-contaminated vermiculite, amphibole asbestos related contamination was seen in areas surrounding the abandoned mine and in other areas throughout the town. For example, tree bark samples contain varying levels of amphibole contamination in Libby in the EPA-restricted mine area [45]. Amphibole fiber concentrations range from 41 to 530 million fibers per gram of bark while a bark sample collected ~11 km west of town along the railroad line had concentrations of 19 million fibers per gram [46]. This indicates that there is a potential exposure to asbestos during harvesting or disturbing contaminated woods in the Libby area. Libby was added to the Environmental Protection Agency's (EPA) National Priorities List in October 2002 for Superfund sites [47].

It is reported that nearly one million homes in the USA have used vermiculite-based insulation from the Libby mine [48]. Even though vermiculite is not classified as one of the six types of asbestos, Libby amphibole has been described as a tremolite-containing transition fiber, and exposure of residents and past workers at the Libby vermiculite mine has been associated with the development of pleural plaques and numerous asbestos-related diseases including asbestosis, pleural fibrosis, and MMs [49]. In this region, asbestos-related deaths are reported to be 40–80 times greater than reference populations in the USA [49]. Thus, Libby amphibole fibers may also be potent in the pathogenesis of MMs.

18.4 Mechanisms of Action of Asbestos and Erionite in the Development of Malignant Mesothelioma

18.4.1 Modern Concepts of Carcinogenesis

Several decades ago, it was thought that all carcinogens were mutagens, and a number of cell assays, including those using bacteria (the Ame's test), were developed with the hopes of identifying critical mutations and short-term testing of agents for carcinogenicity in lieu of expensive lifetime animal studies. Subsequently, it became clear in 2000 that multiple, sometimes common, mutations (i.e., in oncogenes) were required for the development of most cancers [50]. It also had become apparent that several of these mutations were spontaneously induced during aberrant cell replication when DNA repair was compromised, causing DNA damage and genome instability [51]. Carcinogens causing mutations in vitro by direct interaction with DNA or after metabolism and formation of DNA adducts are often termed initiators of cancer, although it is clear that genomic instability also occurs during the long latency periods (progression) of most cancers. Genetic instability is augmented by tumor promoters that induce cell proliferation and inflammation, chronic stimuli necessary for the development of cancers. In lung cancers, it is clear that asbestos fibers function primarily as co-carcinogens and tumor promoters, as mutations by asbestos are not observed in human bronchial epithelial cells or

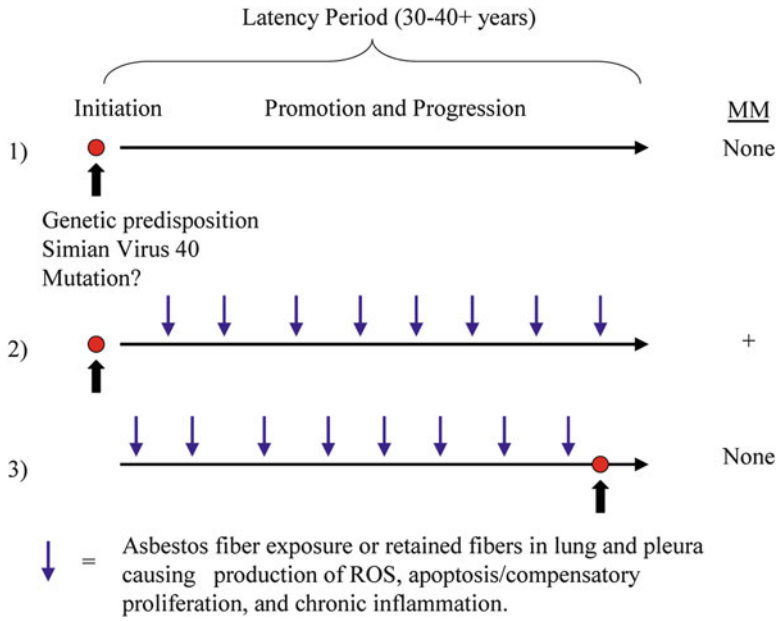

Fig. 18.2 A hypothetical schema for the development of human malignant mesotheliomas after exposures to amphibole asbestos fibers that are retained in the lung during the long latency period of these tumors. The sequence of events and outcomes of MM development in #1–3 are consistent with modern concepts of multi-stage carcinogenesis and the many observations that asbestos fibers play critical roles in tumor promotion and inflammation as opposed to initiation of cancers by mutations. Consistent with observations in mouse skin and other models of carcinogenesis, initiation alone does not cause tumors in the absence of tumor promotion that is characterized by chronic episodes of cell proliferation and inflammation. Moreover, initiation must be followed sequentially by these phenomena for MM to occur.

other epithelial cells in general [52]. In this scenario, polycyclic aromatic hydrocarbons (PAH) associated with smoking classically cause DNA adduct formation whereas asbestos fibers act as co-carcinogens to adsorb PAH and increase their delivery to lung epithelial cells [53]. On the other hand, asbestos fibers act on tracheal epithelial and mesothelial cells as tumor promoters (analogous to phorbol esters in mouse skin) in that they induce inflammation and IL-1B dependent cytokine production that is triggered by activation of the inflammasome [54]. Chronic cell proliferation is triggered by activation of growth factor receptors and protein kinases, increased cyclin D1 and induction of cycling cells [55], stimulation of ornithine decarboxylase [56], and hydrolysis of phosphatidylinositol, increases in diacylglycerol, and activation of protein kinase C at the plasma membrane [57–59]. Moreover, asbestos increases numbers of tumors at low, nontumorigenic concentrations of PAH in tracheal graft models [60]. None of these alterations are observed using nonfibrous, chemically similar analogs of asbestos. Moreover, longer fibers cause more striking alterations than shorter fibers or fragments.

Figure 18.2 depicts a hypothetical model of how amphibole asbestos fibers might promote the development of MMs initiated by tumor susceptibility gene alteration(s) or interaction with SV40, a DNA virus. This schema is supported by studies showing that mutations by asbestos fibers in vitro are large scale chromosomal deletions observed only at toxic amounts of fibers and dissimilar to clonal mutations detected in MM.

18.4.2 Methylation and Tumor Suppressor Genes

Asbestos fibers have not been detected in the nuclei of rodent or human mesothelial cells in situ nor are they mutagenic in a number of studies. An exciting recent development is the observation that gene locus-specific methylation alterations occur in human pleural MM, supporting the concept that epigenetic dysregulation is a crucial mechanism of action in the induction of MM [61]. Indeed, several putative tumor-suppressor genes have been reported in MM [61, 62]. It has also been reported that somatic mutations in the neurofibromatosis type 2 (Nf2) gene occur in 50 % of human MM [63]. After intraperitoneal injection of asbestos fibers, the susceptibility to induction of peritoneal MM increased in heterozygous Nf2-deficient mice [64]. Li-Fraumeni syndrome is a rare heritable cancer-susceptibility disorder characterized by carrying a mutant allele of the p53 gene. Even though mutations in the p53 tumor-suppressor gene are rare in human MMs, individuals with Li-Fraumeni syndrome show increased susceptibility for MM [65]. Susceptibility and accelerated progression of asbestos-induced mesotheliomas also are enhanced in heterozygous

p53-deficient mice [66]. These two models support a role of inactivation of the Nf2 and p53 tumor-suppressor gene pathways in the pathogenesis of asbestos-induced MM [67].

18.4.3 Cell Signaling Pathways Activated by Asbestos Fibers

Since asbestos fibers initially contact the cell membrane after addition to cell cultures, injection, or inhalation, work in our laboratory has focused on the pleiotropic effects of asbestos on cell signaling pathways that regulate cell injury, proliferation, and cell fate. As shown in Fig. 18.3, asbestos fibers may interact via the production of reactive oxygen or nitrogen species (ROS or RNS) [68] or directly with receptors such as the epidermal growth factor receptor (EGFR) [69] or tumor necrosis factor receptor (TNFR).

Direct interaction of asbestos fibers with the epidermal growth factor receptor (EGFR) activates Ras-Raf-extracellular signal-regulated kinase (ERK) pathways of the mitogen activated protein kinases (MAPKs). These pathways control expression and transcriptional activity of the Fos family members, including Fra-1, that comprise heterodimers with Jun family members to form the activator protein-1 (AP-1) transcription factor [70, 71]. Activation of ERK5 by asbestos may also regulate c-Jun family members that form homodimers or heterodimers with Fos proteins [72]. Both Fos and Jun proteins are encoded by early response proto-oncogenes that are induced transcriptionally by asbestos fibers in a dose-responsive fashion [73]. Even though linking increased expression of AP-1 subunits such as Fos and Jun to specific gene expression and phenotypic endpoints remains a challenge, it has been shown that increased expression of c-Jun by asbestos is critical to proliferation and transformation of tracheal epithelial cells [74].

Through possibly redundant phosphorylation cascades, the ERK1/2 and ERK5 pathways govern AP-1-dependent gene expression that regulates cell proliferation, cell migration, and aspects of neoplastic transformation. Activation of the phosphoinositol-3 kinase (PI3K)/AKT pathway by asbestos [75] may also promote cell survival through mTOR or the transcription factor, Nuclear Factor-KappaB (NF-kB) [76, 77] that is also classically linked to activation of TNFR and production of cytokines. Studies on isolated human MM cells and data from our laboratory using an SCID mouse xenograft model after injection of human MM [78, 79] sup-

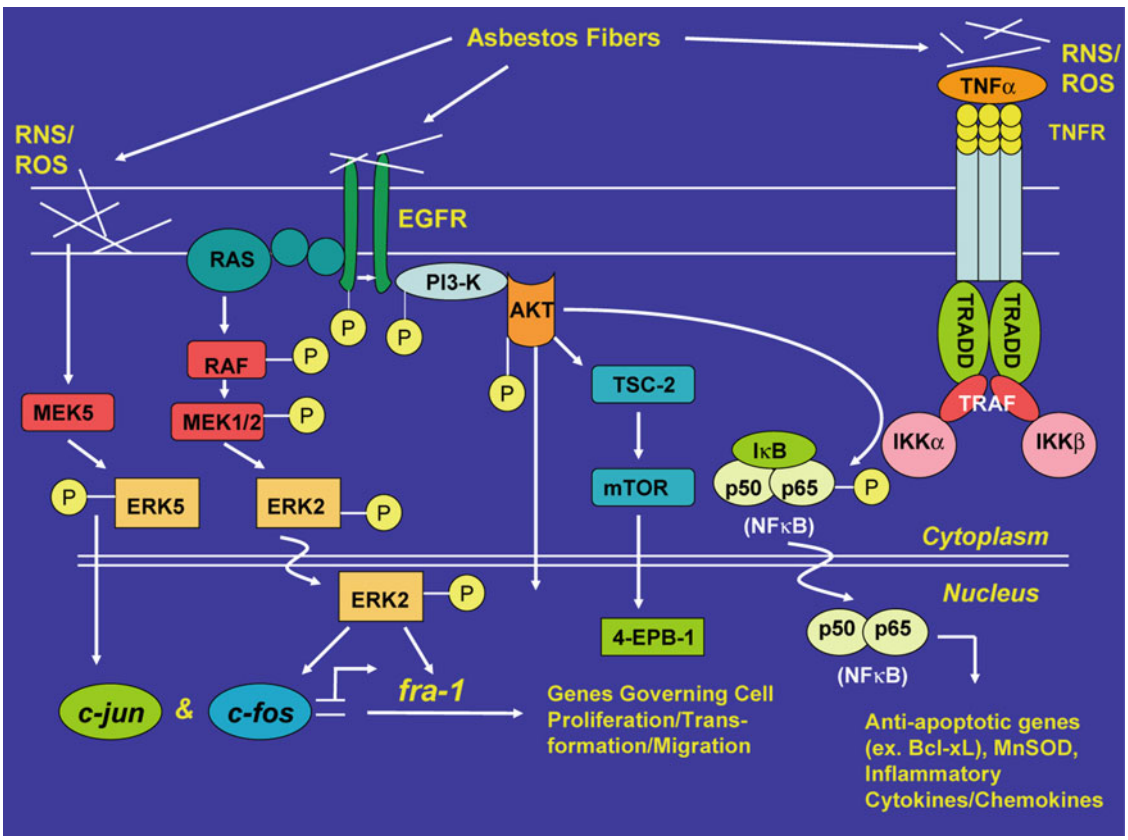

Fig. 18.3 A synopsis of known receptor activation and signaling cascades activated by asbestos fibers and leading to transcriptional activation of gene expression critical to cell proliferation, inflammation, and transformation of mesothelial and lung epithelial cells (modified from [67]).

port the concept that asbestos fibers via activation of NF-kB and other transcription factors cause autocrine production of inflammatory cytokines, growth factors, and angiogenic factors in human mesothelial cells that are recapitulated in a model of tumorigenesis.

After administration of asbestos, NF-kB is activated by asbestos fibers in rodent tracheal epithelial and mesothelial cells in vitro, and in the lungs of rats after inhalation [76, 77]. Furthermore, asbestos fibers cause transcriptional activation of a number of NF-kB-dependent genes, including *c-myc*, another early response proto-oncogene through an oxidant-dependent pathway [76]. Activation of NF-kB is critical in up-regulating the expression of many genes linked to proliferation, apoptosis, and chemokine/cytokine production, and is a critical transcription factor in modulation of chronic inflammation, a feature of asbestos-related diseases [67].

18.4.4 Mechanisms of Erionite-Induced Malignant Mesothelioma

Although studied less widely, it has been shown that the mechanisms of carcinogenesis by erionite are similar to mechanisms of carcinogenesis by amphibole asbestos fibers [59, 80]. For example, exposure to erionite fibers leads to generation of ROS from macrophages [59] and increases mRNA levels of the early response *c-fos* and *c-jun* proto-oncogenes in rodent mesothelial cells [80]. It has been recently shown that erionite is less cytotoxic in contrast to crocidolite asbestos, inducing proliferation and high growth rate in human mesothelial cells [81]. After short-term erionite exposure (24 h or 48 h), DNA synthesis is observed. In contrast, crocidolite asbestos causes initial apoptosis in rodent mesothelial cells that is followed by compensatory proliferation at these time points [82]. After long-term erionite exposure (30 days and 60 days), human mesothelial cells derived from foci of morphologic transformation express HGFR/Met, EGFR, and PDGFR to a higher extent than untreated human mesothelial cells. These growth factor receptors are known to be stimulated by crocidolite asbestos in mesothelial cells [69–71, 83, 84]. Analogous to our observations [67], erionite also induced AKT, ERK1/2, and NF-kB activities at higher levels in morphologically transformed as compared to untransformed human mesothelial cells [81].

18.5 Conclusions

In brief, the phenotypic and functional outcomes of asbestos or erionite exposures in mesothelial cells are related to fibrous geometry and fiber dose. Moreover, the multiple signaling and survival pathways activated by these diverse fibers are similar and may be induced by direct interaction of fibers with receptors or production of ROS or NOS. In general, both fiber types promote robust and persistent activation of signaling through ERK and other redox responsive kinase cascades in comparison to rapid and transient activation by growth factors or phorbol esters. The molecular mechanisms of carcinogenesis due to asbestos and erionite exposure are still under investigation in several laboratories and may yield unique pathways of erionite-induced tumorigenesis that explain its greater potency.

It is perplexing why erionite, a fibrous zeolite, has a higher carcinogenic potency relative to asbestos fibers based on both rodent studies and epidemiology. However, MM does not occur in all individuals exposed to erionite, but in the members of certain families. These findings suggest that MM may be caused by the combined effect of environmental exposure to erionite and susceptibility due to unknown genetic traits [2, 26, 32, 85]. Since familial MM occurs in some families in the US, and erionite and amphibole asbestos are environmental contaminants in some US soils, future research should focus on fiber exposures in these high-risk groups, specifically gene profiling and gender influences. A logical approach to future research should include strategies to reduce environmental exposures and mechanistic and clinical investigations to develop novel and effective therapies for MM.

Acknowledgements The authors appreciate the assistance of Maximilian MacPherson in the preparation of figures and the diligence of Jennifer Díaz in manuscript preparation. Research in Dr. Mossman's laboratory has been funded by grants from the NIH, ACS, and EPA.

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Viral Mechanisms in Human Carcinogenesis

19

Paul F. Lambert

19.1 General Principles of Virally Induced Cancer

Infectious agents, including viruses, bacteria, and parasites, are thought to be the etiologic agents in approximately 20 % of human cancers. Human oncogenic viruses include hepatitis B and hepatitis C viruses (associated with hepatocellular carcinoma), Epstein–Barr virus (associated with B-cell lymphomas, nasopharyngeal, and gastric carcinomas), human papillomaviruses (associated with cervical carcinoma, other anogenital cancers, and a subset of head and neck cancers), human T-cell lymphotropic virus I (HTLV-1; associated with Adult T-cell lymphomas), human herpesvirus type 8 (associated with Kaposi's sarcoma and primary effusion lymphomas), and the newest identified human tumor virus, Merkel cell carcinoma-associated polyomavirus. The initial recognition that viruses cause cancer arose from studies of animal viruses. In the early twentieth century, transmittable agents were demonstrated to cause tumors in chickens and in rabbits. The respective agents were later identified to be Rous sarcoma virus (RSV), the first studied RNA tumor virus, and Shope papillomavirus, the first studied DNA tumor virus. Much of our understanding of viral oncogenesis derived initially from the study of such animal viruses. In recent years, much attention has been focused on the study of human tumor viruses as etiological roles of such viruses in human cancers have been elucidated. The study of virally induced cancers in animals and in humans has provided many basic insights into cancer, the most important of which is the identification and functional characterization of two fundamental types of genes important in cancer: oncogenes and tumor-suppressor genes. From these collective studies several generalizable principles of virally induced cancer have arisen. First, the onset of virally associated cancer is characterized by long latent periods following initial infection. This suggests that the viruses alone are not sufficient to cause cancer. Also, given this long latency, oncogenic viruses must be able to persist in the host for long periods of time. Second, the cancers induced by viruses do not constitute a natural part of the viral life cycle. Rather, virally associated cancer appears to be a dead-end street for most viruses—by-products of the natural infection that provide no advantage to the virus evidenced by the fact that it is uncommon for progeny virus to be produced in the associated tumors. The third principle is that viral properties that contribute to the induction of tumors often play critical roles in the life cycle of the virus. In the following sections are described the basic properties of RNA and DNA tumor viruses and the major insights gained regarding how they contribute to cancer.

19.2 RNA Tumor Viruses

The field of tumor virology initially arose from the study of retroviruses that cause tumors in birds and mammals other than humans. The oncogenic retroviruses can be divided into three classes: (1) transducing retroviruses, (2) insertionally mutagenic retroviruses, and (3) true oncoretroviruses. Transducing retroviruses are ones that acquire genetic information from the host genome. RSV is one example. Most other examples also were isolated from avian hosts, although others have been isolated from feline, murine, and simian hosts. The cellular genetic information being transduced is a cellular proto-oncogene which, as present in and expressed from the transducing retrovirus, has oncogenic activity, i.e., it contributes to tumor formation in vivo and cellular transformation in tissue culture. The study of transducing retroviruses led to the discovery of more than a dozen oncogenes, including *src, ras,* and *myc*. The discovery of these virally encoded oncogenes, and their detailed study helped define numerous cellular signaling pathways that play fundamental roles in

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_19

normal cell physiology and when altered, in carcinogenesis. It was not recognized until the 1970s that these viral oncogenes actually had been picked up by the virus from the host organism through genetic recombination, which arises as a by-product of reverse transcription. This led to the recognition that animals encode oncogenes. The cellular copies of these genes were initially referred to as proto-oncogenes because it was recognized that the viral copies of these genes had undergone genetic changes that led to the increased expression and/or activity of the encoded gene product. These changes led to the oncogenic nature of the virally transduced gene. In contrast, the cellular homologs do not contribute to cancer in their wild-type state. For example, the virally encoded *src* and *ras* genes have amino acid substitutions at positions in these proteins that lead to their constitutive activity in signal transduction pathways, and the virally encoded *myc* gene is missing regions of the cellular gene that normally lead to a tight regulation of its expression at the transcriptional and post-transcriptional levels. No examples of transducing retroviruses have been found for human cancers; rather, most such viruses were identified from the evaluation of laboratory stocks of retroviruses that infect laboratory animal species. Generally, these transducing, oncogenic retroviruses are replication-defective because of the disruption of one or more essential viral gene by the insertion of the cellular proto-oncogene. The rare exception is RSV, which is a replication-competent virus in which the transduced cellular *src* gene did not disrupt function of any viral gene. How a transducing oncogenic retrovirus arises remains unclear. It likely results from a provirus that integrates close to a cellular proto-oncogene. This could result in the formation of a chimeric RNA of viral and cellular origin which, when packaged into progeny retrovirus and on reverse transcription, undergoes recombination with a co-packaged, intact viral transcript to produce the actual transducing viral genome.

Insertionally mutagenic retroviruses are ones that, as a consequence of insertion of the proviral genome into the host chromosomes, alter the structure and/ or expression of a cellular proto-oncogene or tumor suppressor gene. These retroviruses are also known as the weakly oncogenic retroviruses since they only rarely cause cancers in laboratory animals, reflective of the low likelihood that the provirus insertion occurs at or near a cellular proto-oncogene or tumor suppressor gene. There are several mechanisms by which insertion of provirus can lead to activation of a cellular proto-oncogene. First, the virus may integrate upstream of the cellular gene in the same sense orientation such that read-through transcription from the viral promoter leads to the generation of chimeric viral:cellular mRNAs that can encode the cellular proto-oncogene. Second, the viral transcriptional enhancer can upregulate the activity of the cellular gene's own transcriptional promoter. Finally, the virus can disrupt the integrity of the cellular mRNA leading to the loss of negative regulatory elements such as mRNA instability elements in the mRNA encoding the cellular protooncogene. Examples of each of these three cases exist in the literature. Many of the insertionally mutagenic retroviruses cause lymphomas/ leukemia in their natural host. In these tumors, the provirus is found nearby to the c-*myc* locus, leading to increased expression of the c-*myc* gene product. Insertionally mutagenic retroviruses can also integrate within alleles of tumor suppressor genes and disrupt their expression, thereby leading to cancer. The identification of such events is much less frequent than those that lead to the activation of cellular proto-oncogenes, presumably because of haplosufficiency.

True oncoretroviruses are ones that, through virally encoded activities, alter the host cell or the host cell environment, thereby making cells more susceptible to cancer development. There are only a few cases of true oncoretroviruses. One is HTLV-1, which causes T-cell lymphomas in humans.

19.3 DNA Tumor Viruses

Whereas the study of RNA tumor viruses was largely responsible for the discovery of oncogenes, the study of DNA tumor viruses led to the discovery and elucidation of the function of cellular tumor suppressor genes, most notably *p53* and *pRb*. Much of the initial knowledge about DNA tumor viruses came from the study of SV40, a simian virus. SV40 efficiently transforms cells in tissue culture and can cause tumors in hamsters, but not its natural host. Several viral gene products were found expressed in these transformed/tumorigenic cells and were identified because hamsters made antibodies against them. Most notable of these was the SV40 large tumor antigen (TAg). This protein is one of the most potent oncogenic factors known [1]. Its oncogenic activity is multifactorial, largely arising from its ability to bind and inactivate the cellular tumor-suppressor proteins p53 and pRb. p53 is now recognized to be functionally disrupted in most human cancers. The protein was originally identified by virtue of its association with SV40 TAg. Initial cloning and functional analysis of *p53* led to its designation not as a tumor suppressor gene but an oncogene. This was due to the fact that the initial *p53* clones were isolated from cell lines, i.e., populations of cells that had acquired an immortalized phenotype. In these cell lines the *p53* gene had undergone mutations that cause the encoded gene product to possess dominant-negative activity; that is, it could inactivate the normal p53 protein when put back into cells. It was not until the late 1980s that the wild-type *p53* gene was recognized to be a tumor-suppressor gene [2]. *p53* is thought to play an important role in orchestrating the cellular responses to certain forms of stress, including insult from DNA-damaging agents. Activation of *p53* leads either to growth arrest, which is believed to allow cells time to repair damaged DNA, or apoptosis (programmed

cell death). Without functional *p53,* cells have a greater propensity for undergoing genetic alterations, some of which can contribute to tumorigenesis. It is now understood that when SV40 TAg binds the wild-type p53 protein, it inactivates the latter's function [1]. SV40 TAg also can bind the gene product of the retinoblastoma tumor susceptibility locus (Rb) [3]. Rb and the related pocket proteins, p107 and p130, are critical regulators of the cell cycle. In the G1 stage of the cell cycle pRb binds to and inactivates a family of transcription factors, E2Fs, that regulate the activity of cellular genes involved in DNA synthesis and cell cycle control. Normally, pRb's activity is regulated by cyclin-associated kinases, which phosphorylate pRb and lead to its dissociation from E2Fs. TAg upon binding to pRb or its relatives p107 and p130 can also cause this dissociation and thereby lead to cell cycle deregulation.

The insights gained from the study of SV40 provided many insights about how human papillomaviruses cause carcinoma of the anogenital tract and head/neck region in humans, and potentially also shed light on the oncogenic activities of the recently discovered Merkel cell carcinoma associated polyomavirus. However, it would be an oversimplification to think that targeting p53 and pRb are the only means by which DNA tumor viruses transform cells or cause tumors in organisms. Much like what was learnt from the study of transducing oncoretroviruses that express mutated forms of cellular proto-oncogenes, there are many varied cellular pathways targeted by DNA tumor viruses that, when altered, contribute to transformation in tissue culture and tumorigenesis in vivo.

19.4 Human Tumor Viruses

19.4.1 Human Papillomaviruses

Papillomaviruses are small DNA viruses that infect the epithelial lining of their host and for the most part cause benign proliferative lesions such as warts that support production of progeny virus. There are over 100 defined human papillomavirus genotypes that can be subdivided into two classes, those that primarily infect cutaneous epithelia such as the epidermis lining the hands and feet, and those that primarily infect mucosal epithelia including that lining the anogenital tract and the oral cavity. The latter, mucosotropic HPVs, are the most common sexually transmitted pathogens in humans; greater than half the human population have been exposed to these HPVs in their lifetime. A subset of these mucosotropic HPVs that cause human cancers such as cervical cancer, anal cancer, and head and neck cancers. The recent generation of highly efficacious, prophylactic vaccines against two of the most common of these cancer-causing HPV genotypes, HPV16 and HPV18, provides the potential of reducing the incidence of human cancers caused by these viruses. However, their impact on global health is significantly limited by their availability, largely for socio-economical reasons.

Papillomaviruses are non-enveloped icosahedral viruses that contain a double-stranded DNA genome of about 8000 bp that encodes early and late genes. These viruses infect the poorly differentiated basal cells within stratified epithelium wherein the viral genome is established as a low copy, nuclear plasmid and a subset of viral genes are expressed The papillomavirus life cycle is intricately tied to the differentiation program of the host tissue. Progeny virus are only produced when the infected basal cell undergoes cell division and a daughter cell initiates terminal differentiation. This triggers amplification of the viral genome and expression of structural genes that encode the capsid proteins resulting in the production of progeny virus. The virus must manipulate the differentiating epithelia to facilitate its own life cycle. Many of these manipulations to the differentiating host cell are caused by the same viral genes that contribute to cancer.

Professor Harald zur Hausen and his colleagues at the German Cancer Center in Heidelberg are responsible for first uncovering the etiological role of HPVs in human cancer when they reported the presence of DNA of specific HPV genotypes in human cervical cancers [4]. This revelation earned Dr. zur Hausen the Nobel Prize in Medicine. A causal role of these same HPVs has since been established in other anogenital cancers including that of the vagina, vulva, penis, and anus, as well as the head and neck region, particularly the oropharynx. In many of these cancers, the viral DNA is found integrated into the cellular genome and no progeny virus is produced. The viral integration event almost always leads to a disruption in the E2 translational open reading frame (ORF). The E2 ORF encodes proteins involved in viral transcription and replication and its disruption leads to derepression of the viral transcriptional promoter that directs expression of the viral oncogenes, E6 and E7 [5]. Integration also disrupts an mRNA instability element, and this also facilitates increased expression of E6 and E7 [6]. Even in cancers where the genome remains extrachromosomal, E6 and E7 expression is reported.

E6 and E7 are directly implicated in oncogenesis. E7 can cooperate with E6 or the activated ras oncogene to transform fibroblasts in tissue culture, and E6 and E7 contribute to the immortalization of epithelial cells in tissue culture [7]. The expression of E6 and E7 was shown to be required for the continued proliferation of HPV-positive cervical and head/neck cancer cell lines; thus, E6 and E7 are thought to be required for the onset as well as persistence of HPV-associated cancers. When the expression of E6 or E7 is directed to stratified squamous epithelia of transgenic mice, including the epidermis, the mice exhibit thickening of the skin and spontaneously develop squamous cell carcinomas [8]. When treated persistently with exogenous estrogen,

these same mice, which also E6 and E7 in the epithelia lining the cervix and vagina, develop cervical and vaginal cancers (whereas nontransgenic animals do not) [9]. Furthermore, these mice are more susceptible than nontransgenic mice to head and neck cancers when treated with an oral carcinogen, again owing to the expression of viral oncogenes in the epithelia lining the oral cavity, oropharynx, and esophagus [10].

Even though E6 and E7 are well accepted to be the main oncogenes for HPV, more recent evidence implicates the E5 viral protein as a possible contributor to carcinogenesis, at least in the cancers where its expression is retained (integration of the viral genome in the host chromosome often leads to a reduced expression of E5). E5 is the main transforming oncogene of bovine papillomavirus (BPV). BPV E5's transforming activity correlates with its ability to activate the PDGF receptor in a ligand independent manner [11]. E5's role in HPV-associated cancers is less clear. In tissue culture HPV E5 acts as a weak oncogene. Nevertheless, a large fraction of cervical cancers still express E5. In recent studies, transgenic mice expressing E5 were found to be more susceptible to skin and cervical cancers implicating its role in HPV-associated human cancers [12]. The mechanism underlying HPV E5's role in cancers remains unclear.

As is the case with SV40 and adenoviruses, papillomaviruses have the ability to inactivate tumor suppressors. E7 binds to and induces the degradation of the tumor suppressor, pRb [7, 13]. The inactivation of pRb allows E2F transcription factors to be constitutively active, which likely accounts in large part for the increased proliferation seen in E7-expressing cells/tissues. The regions of E7 required for its inactivation of pRb have been mapped to conserved regions (CR) 1 and 2, so named because of their similarity to pRb-binding regions in the adenovirus oncoprotein E1A [7] . The CR1 and CR2 domains of E7 are both necessary for E7's induction of tumors in animals and transformation of cells. An exception is that when the CR1 and CR2 domains of E7 are mutated in the context of the whole papillomavirus genome, cells transfected with the genome still undergo transformation [14]. This suggests that E7 possesses transforming activities mediated by other activities besides inactivation of pRb. In vivo studies have confirmed that E7's carcinogenic phenotypes both in the cervix and the head and neck of the mouse cannot be attributed merely to its inactivation of pRb. E7 also inactivates proteins related to pRb, p107 and p130, which like pRB modulate the activities of E2F transcription factors. Studies now underway suggest that inactivation of both pRb and either p107 or p130 increases the susceptibility of mice to cervical and head/neck cancers in mice. E7 also can bind to and inactivate p21 and p27, inhibitors of cyclin-associated kinases that play critical roles in the control of the cell cycle [15, 16]. E7's inactivation of p21 has now been demonstrated to contribute to its oncogenic potential in the cervix [17]. Thus E7 causes cancer at least in part through its ability to disrupt the function of multiple cellular factors that are involved in the regulation of the cell cycle. E7 is known to associate with over 100 cellular factors in addition to the ones described above. Which of these interactions also contribute to E7's oncogenic potential remains unclear [18].

The discovery of the ability of E6 to inactivate the tumor suppressor, p53, was prompted by the oncogenic similarities between papillomaviruses and SV40 and adenovirus. E6 was found to form a tertiary complex with p53 and a cellular ubiquitin ligase, E6-AP [19, 20]. This leads to the ubiquitination of p53, which is then targeted for proteasomal degradation. The p53 tumor suppressor is involved in the G1 to S phase transition of the cell cycle and is induced in response to cellular stresses such as DNA damage. Although the importance of p53 in other cancers suggests that it is important in papillomavirus-associated cancers, inactivation of p53 cannot fully substitute for the activities of E6. For example, E6 was found to abrogate differentiation of skin epithelial cells in tissue culture. However, when the p53 in these cells was inactivated by overexpressing a dominant-negative form of p53, the cells could still differentiate. Also, p53-null mice differ from E6 transgenic animals in that they do not display thickened skin or spontaneously develop skin tumors. The alpha-helix-binding domain of E6, through which E6 associates with E6AP has been shown to be important for E6 oncogenic activities in mice, suggesting that the inactivation of p53 contributes to this process. Consistent with these results, E6 is unable to cause cervical cancers on an E6AP-null background [21].

E6 has been shown to bind a variety of protein partners, including E6-BP, paxillin, E6-TP, bak, and the human homolog of Drosophila disc large (DLG) tumor-suppressor gene [22]. The ability of E6 to associate with proteins correlates with its ability to induce aberrant DNA synthesis, transform cells in tissue culture, and contribute to carcinogenesis in mice. The binding partner responsible for those phenotypes has not been identified but candidates include Dlg, Scribble, and MUPP1, all of which have been reported to bind E6 through the PDZ domain. Interestingly, E6 causes destabilization of these PDZ proteins much like it does p53, and this is at least in part mediated by E6AP.

In addition, E6 can activate telomerase, the enzyme which extends the number of telomere repeats to the ends of chromosomes. This activity is thought to contribute at least in part to E6's ability to immortalize cells [23]. Thus, as with E7, E6 contributes to transformation in tissue culture and oncogenesis in vivo through multiple means. In addition to the cellular targets described above, E6 can associate with at least two dozen additional cellular factors. Their importance in E6-mediated oncogenesis remains to be unraveled.

19.4.2 Merkel Cell Polyomavirus

The most recently identified human tumor virus is a novel human polyomavirus identified in Merkel cell carcinomas, a rare cancer arising in a specialized cell of the skin involved in sensory transduction [24]. This polyomavirus encodes for two putative oncogenes, large tumor antigen and small tumor antigen, which bear similarities with the like genes of SV40 and murine polyomaviruses. The Merkel cell carcinoma associated polyomavirus is often found to be integrated into a human chromosome in the cancers, and because of frame-shift or nonsense mutations in its large tumor antigen gene, the virus is defective in its ability to replicate as a nuclear plasmid, its normal genomic state. These mutations lead to the expression of truncated forms of large tumor antigen gene product. Interestingly, the truncated large tumor antigens expressed in the cancers retain an ability to bind pRb and pRb-like proteins, leading to the prediction that this retained activity contributes to Merkel cell carcinoma development. Like other polyomaviruses, Merkel cell polyomavirus also encodes a small tumor antigen gene product that is predicted to modulate the activity of PP2A. In the case of SV40 and murine polyomavirus, small tumor antigen contributes to their transforming activity through their modulation of PP2A. As with HPVs, continued expression of Merkel cell polyomavirus-encoded tumor antigens is necessary for the continued growth and transformation properties of Merkel cell carcinoma derived cell lines in tissue culture [25]. Merkel cell polyomavirus represents an exciting new field of study in human tumor virus biology.

19.4.3 Epstein–Barr Virus

In the 1950s, while working as a missionary doctor in Africa, Denis Burkitt described the childhood tumor Burkitt's lymphoma (endemic BL) and postulated that an infectious agent could possibly act as the etiologic agent of BL [26]. In 1961, a collaboration among Denis Burkitt, Tony Epstein, and Yvonne Barr resulted in the establishment of BL-derived cell lines in culture. Electron microscopy examinations of the cell lines showed herpesvirus-like particles that are biologically and antigenically dissimilar to other human herpesviruses. Subsequent seroepidemiological studies indicated that the candidate human tumor virus named Epstein–Barr virus (EBV), also named human herpesvirus 4, presents ubiquitously throughout the human populations in two different types and is persistently infectious. The strong association between the virus and BL, its ability to transform primary human B lymphocytes into permanent lymphoblastoid cells, and a predominance of a single glycoprotein in the viral outer surface further differentiate EBV from other herpes members. All three subtypes of BLs—endemic, sporadic, and AIDS related—are thought to be associated with EBV infection. Early-onset endemic BL, the most common childhood cancer in equatorial Africa, occurs with an unusually high incidence of 5–10 cases per 100,000 individuals per year. Virtually all of the malignant cells of every analyzed endemic BL tumor are EBV genome positive. The sporadic BL, more commonly found in the United States and other Western countries, has a 50-fold to 100-fold lower incidence in comparison to the endemic BL with about 15–25 % EBV genome-positive tumors. Also, sporadic BL tumors have a slightly later age of onset, a different pattern of presentation, and frequently involves the abdomen and bone marrow instead of the jaw, as in the cases of the endemic BL. AIDS-related BL develops in approximately 10 % of AIDS patients in Western societies and reflects both early-onset and late-onset B-cell malignancies. However, even with such a high incidence of AIDS-related BL tumors, only 30–40 % of these are EBV genome positive. Remarkably, all known forms of BL have the t(8:14), t(2:8), and t(8:22) chromosomal translocations that are likely deleterious to B lymphocyte development. In addition to BL, EBV-associated malignancies also include immunoblastic B-cell lymphomas in immunosuppressed individuals, nasopharyngeal carcinoma, a subset of gastric carcinoma, Hodgkin's disease, and EBV-associated T-cell lymphomas.

Acute infection with EBV causes mononucleosis [27]. How EBV initially infects an individual is not clear, but it may do so through infection of epithelial cells lining the oral cavity. Usually, EBV infections become persistently latent, in which the viruses induce B-lymphocyte proliferation concurrently with viral DNA replication. Stable retention of EBV episomes is mandatory for the continual growth of EBV-positive BL cells. The viral protein EBNA1 is critical for the maintenance of the EBV replicon in infected cells and for the continued growth of BL cells [28, 29]. EBNA1 contributes both to the replication of the viral genome, and its efficient inheritance during cell division.

How EBV contributes to human cancer is not well understood. In tissue culture, EBV can efficiently induce the immortalization of B lymphocytes, a property that is likely to be related to its oncogenic properties in vivo [30]. The EBV genome is a 172 kbp linear, double-stranded DNA. In infected cells the EBV genome is maintained as a circular episome. Although the EBV genome can encode many genes, the fact that in EBV-immortalized B cells only a handful of viral genes are expressed helped researchers identify the genes relevant for immortalization. One viral gene, latent membrane protein 1 (LMP1), has been demonstrated to be critical for EBV's immortalization of B lymphocytes and in transgenic mice is sufficient to induce lymphomas. As its name implies, LMP1 is an integral membrane protein found in the cytoplasmic membrane of latently infected cells.

LMP1 has characteristics of a cell surface receptor; it displays a rapid turnover that is associated with endocytosis and it signals to NF-kappaB through association with TRAFFs and TRADDs [31]. This signaling capacity of EBV appears to be required for its capacity to induce B lymphocyte proliferation. In this regard, LMP1 is functionally analogous to CD40, a cellular receptor normally expressed in B lymphocytes which can induce B lymphocyte proliferation upon stimulation by its ligand. Unlike CD40, LMP1 is not known to have an associated ligand; consistent with this, LMP1 lacks a prominent extracellular domain to which an extracellular ligand could bind. Rather, LMP1's signal transduction activity appears to be constitutively active. In the past, LMP1 has been argued to cause the immortalization of B lymphocytes in part by inducing expression of the cellular anti-apoptotic factor Bcl-2. However, it is unclear whether the increased levels of Bcl-2 found in some EBV-infected cells are a direct consequence of LMP1 action or an acquired phenotype that correlates with improved survival of those cells in tissue culture. Another EBV-associated gene, EBNA2, has been shown to be critical for EBV's immortalization of B lymphocytes. However, its role is likely indirect through its ability to activate the transcription of LMP1.

With the recent advances in understanding the role of EBNA1 in the persistence of the EBV genome in human cells and the role of LMP1 in EBV's immortalization of B lymphocytes, scientists now have two relevant targets for developing novel antiviral therapies that could reduce the incidence of EBV-associated malignancy in the future.

19.4.4 Kaposi's Sarcoma-Associated Herpesvirus

Kaposi's sarcomas (KS) is a relatively rare disease that came into the limelight because of its prevalence amongst AIDs patients [32]. Chang, Moore, and colleagues used an enrichment procedure based on PCR to identify DNA sequences present in KS but absent in normal cells. They identified a new herpesvirus, Kaposi's sarcoma associated herpesvirus (KSHV, also termed human herpesvirus 8, HHV-8) present in these cancers [33] that is related to EBV, another human tumor virus. KSHV has also been detected in body-cavity-based lymphomas or primary effusion lymphomas (PEL) [34] and in an atypical lymphoproliferative disorder termed multicentric Castleman's disease (MCD) [35]. KSHV is now accepted as contributing causally to KS and PEL.

KSHV encodes genes with homology to cellular genes, some of which are candidates for contributing to viral oncogenesis. For example, KSHV encodes its own cyclin [36, 37]. KSHV cyclin can foster progression of nuclei isolated from cells in G1 to undergo DNA synthesis and induce cells to undergo apoptosis. Mice transgenic for KSHV cyclin and p53-null develop T-cell and B-cell lymphomas with a mean latency of 3 months, a latency shorter than that for the p53-null mice alone [38]. KSHV also encodes its own inhibitors of apoptosis, viral Bcl-2 and viral FLIP (an inhibitor of cellular FLICE, a protein-mediating Fas ligand-induced cell death). The viral Bcl-2 shares its limited sequence homology with cellular Bcl-2 in those regions critical for inhibiting apoptosis and fails to dimerize with cellular Bcl-2 and Bcl-XL thus avoiding regulation by potential cell-binding partners [39]. Viral FLIP inhibits apoptosis mediated by the Fas pathway and induced by cytotoxic T-lymphocytes [40]. Finally, KSHV encodes homologues of cytokines and cytokine receptors. Its viral IL-6 may promote proliferation of PEL cells, although they appear to be dependent on cellular IL-6 and not viral IL-6 for their continued growth [41]. KSHV also encodes a G protein-coupled receptor, viral GPCR, that, when expressed in endothelial cells in mice, leads to KS-like tumors [42].

19.4.5 Human T-Cell Leukotropic Virus Type 1

HTLV-1 is an oncogenic retrovirus associated with a variety of human diseases, including adult T-cell leukemia/lymphoma (ATL) [43]. The epidemiology of ATL suggests that cumulative genetic defects may be responsible for the acute T-cell malignancy. HTLV-1 transforms human T-cells in vivo and in cell culture. The development of T-cell transformation can be divided into two stages. In the early stage, the virus induces interleukin-2 (IL-2)-dependent T-cell proliferation, which mimics the action of antigens. In the normal T-cells, antigen-stimulated T-cells cease growth after a few weeks. However, HTLV-1-infected T-cells show infinite proliferation (i.e., immortalization). The uncontrolled cell proliferation is believed to facilitate secondary genetic changes. Therefore, the immortalized T-cells can progress to the second stage of transformation, which has a typical property of IL-2-independent growth. Recent evidence suggests that HTLV-1 not only induces the proliferation of host T-cells, but also protects the infected cells from undergoing apoptosis.

The HTLV-1 provirus genome is 9032 bp long and contains gag, pol, and env genes that encode the viral matrix (capsid and nucleocapsid proteins), enzymes (reverse transcriptase, integrase, and protease), and envelope protein (surface glycoprotein and transmembrane protein), respectively. The HTLV-1 genome contains unique regulatory genes, the Rex and Tax genes (analogous to the HIV rev and tat genes), at the 3′ end of the genome that are not common in other retroviruses. Rex is a 27-kDa nuclear phosphoprotein that regulates viral RNA processing. Rex enhances the expression of single-spliced mRNA and unspliced viral genomic RNAs encoding the gag-pol and env gene products but reduces the expression of double spliced tax/rex mRNA. At the early stage of viral gene expression in the viral life cycle, double-spliced mRNAs for Tax and Rex proteins are mainly

produced. Newly synthesized Tax protein transactivates the transcription of HTLV-1. However, accumulated Rex protein enhances the accumulation of unspliced and single-spliced viral RNAs and suppresses the accumulation of tax/rex mRNA. Therefore, Tax and regulate viral expression both positively and negatively. Transient expression of the viral genes may be one of the mechanisms to escape from immune surveillance of the host. Tax protein is a 40-kDa nuclear phosphoprotein and is considered an oncogenic viral protein that transactivates the transcription of HTLV-1 and also binds to cellular transcription factors or other cytoplasmic cellular molecules involved in the fundamental cell function such as IL-2, IL-2R, and c-fos and the parathyroid hormone related peptide. The oncogenic property of Tax protein is well documented in the development of ATL [43]. Indirect evidence showed that the 3′ end of the HTLV-1 genome (Tax gene) is necessary and sufficient for cell immortalization. In addition, co-transfection of Tax with Ras can also induce transformation of primary rat embryo fibroblasts. Finally, Tax transgenic mice develop several pathologies, including leukemia, mesenchymal tumor, and neuro-fibromas.

HTLV-1 is passed from male to female via semen, and from mother to child by breast milk. The latter route has been demonstrated prospectively. Encouraging carrier mothers to refrain from breast feeding has decreased transmission of HTLV-1 to their children by 80 % [44].

19.4.5.1 Hepatitis B Virus

Infection by hepatitis B virus (HBV) is strongly associated with the development of hepatocellular carcinoma (HCC), one of the ten most common carcinomas in the world [45]. This oncogenic association categorizes HBV as a tumor virus. HBV is a hepatotropic, circular, partially duplex DNA virus of 3.2 kb and replicates via an RNA intermediate and reverse transcription. Infection by HBV can lead to liver damage and such pathological diseases as chronic hepatic insufficiency and cirrhosis, leading to HCC. HCC is a difficult cancer to treat because symptoms occur late in the development of the carcinoma, often too late for clinical intervention. Unfortunately, the survival rate for individuals diagnosed with HCC is low. The association of HCC and HBV was determined from worldwide epidemiological studies [46]. In those areas of the world where a higher incidence of HCC was observed (e.g., sub-Saharan Africa and Southeast Asia), there was a higher rate of HBV. Though HBV-induced HCC is relatively rare in the USA and Europe where HBV infection rates are low, it is a leading cause of cancer in those areas where HBV infection rates are high. Studies in animal systems have also demonstrated a correlation between infection with HBV and the occurrence of HCC. For example, 100 % of woodchucks infected with woodchuck hepatitis virus (WHV) develop HCC. There is a long latency period of at least 30 years between infection by HBV and the development of HCC in humans. During this time, the virus integrates into the host genome and viral protein expression is ablated. Infection by HBV can lead to either acute or chronic hepatitis. Acute infection involves symptoms such as fever, fatigue, anorexia, and nausea, which are soon followed by jaundice. In adults, the disease is usually cleared by an immune system response to viral surface antigen. However, in 1–8 % of infected adults infection persists and the virus undergoes active replication. Chronic infection rates are much higher (approximately 90 %) in the case of perinatal and early childhood infections. It is these chronically or persistently infected patients that are at highest risk for developing HBV-associated HCC. Persistently infected individuals may be asymptomatic for years, although the HBsAg surface antigen can be detected during this time. In other patients, chronic hepatitis B occurs, with symptoms including fatigue, anxiety, and anorexia, associated with hepatocellular necrosis and inflammation. A fraction of those patients with chronic hepatitis will develop cirrhosis of the liver. Cirrhosis can then lead to HCC. How HBV causes HCC is a matter of debate, although there are two main pathways by which HBV might be acting: directly through a viral protein or integration or indirectly, as an consequence of chronic liver damage due to viral infection and host-immune responses to it. HBV may directly induce HCC either in trans, by an activity of one of its gene products early on in cancer development, or by a cis, as a result of viral integration (i.e., akin to insertionally mutagenic retroviruses). For HBV to cause oncogenesis through a trans-acting mechanism, it must be actively undergoing transcription. Since most advanced HCCs display no viral gene expression, this putative pathway must act primarily at the initial stages of cancer cell growth while viral genes are still being expressed. There are multiple viral gene products which have been argued to play a role in promoting uncontrolled cell growth. It is common to find HBV genomes integrated into the host chromosome in HCC. These integrations commonly lead to the retention of a portion of the viral genome, including the X and preS/S regions [47]. The X gene product of HBV has transcriptional activation activity. The activity of X in the context of the in vivo viral life cycle is unclear, although X has been shown to activate many promoters in reporter gene assays, including the herpes simplex virus (HSV) tk promoter, the HIV long-terminal repeat (LTR), and the HBV Enl. Cellular promoters activated by X include those for MHC class I, c-myc, and RNA polymerase III. It is possible that X activates cellular oncogenes, leading to uncontrolled cell growth. X has also been shown to inhibit nucleotide excision repair, leading to accumulated mutations. This could cause an increased mutation rate induced by chemical carcinogens such as aflatoxin B1. X has also been found to bind to the p53 tumor suppressor protein and may therefore interfere with the latter's role in DNA damage response [48]. Some mice transgenic for X driven by the X promoter develop HCC. However, this effect is not seen using heterologous promoters or subgenomic or genomic HBV DNAs containing the X region.

HBV alternatively may lead to cancer via a *cis*-acting mechanism, whereby the integration of the virus into the host genome can cause interruption of a tumor suppressor gene or activation of an oncogene. An example of insertional activation is observed in WHV-induced HCC. Using Southern blotting analysis, WHV has been found to be integrated near to the N-myc loci in 40 % of HCCs. Using the more broad-scanning technique of pulse-field gel analysis, an even higher percentage of tumors have been found to be integrated near N-myc. However, insertional activation by HBV has not been regularly demonstrated in human HCCs using either technique. However, this does not mean that this phenomenon is not occurring because current techniques allow detection only within a few kilobases of a gene of interest.

Another major school of thought is that HBV can lead to HCC via an indirect pathway. Infection of the liver by HBV can cause liver damage, which leads to a chronic proliferative response in the liver to replace lost cells. The resulting increase in DNA synthesis increases the probability of mutations occurring, leading to uncontrolled cell growth. This theory is supported by the fact that most diseases of the liver leading to liver damage, such as HCV infection, alcoholic cirrhosis, and Wilson's disease, are associated with an increased risk for the development of HCC. The Large S (L) protein product of HBV, when expressed, is toxic to cells and can lead to liver damage. Transgenic mice engineered to overexpress the HBV L protein display hepatocellular necrosis and regeneration, which eventually leads to HCC [49]. Although this result is potentially informative, it should be interpreted carefully because the levels of L protein in these mice are much higher than those in an HBV infection. HBV induced liver damage alternatively could be the result of host immune responses to viral gene products.

Although the association of HBV infection with the development of HCC is high, the mechanism by which the virus causes cancer clearly is still a matter of debate. It is possible that a combination of pathways described above are contributing to the oncogenic activity of HBV. Further clarification of this effect will pave the way for more effective treatments of HCC associated with HBV. Meanwhile, efforts are underway to eradicate HBV infections through mass immunizations.

19.4.6 Hepatitis C Virus

Human hepatitis C virus (HCV) is another etiological agent for hepatocellular carcinoma (HCC). Approximately half of the cases in the USA are ascribed to HCV [50]. HCV represents the most common chronic viral infection among bloodborne pathogens in the U.S., where the rate of infection during the period from 1988 to 1994 was estimated to be 1.8 % [51]. The World Health organization estimates the worldwide infection rate at 3 %. Infection is thought to arise from contaminated blood, use of shared needles among intravenous drug users, organ transplantation, hemodialysis, sexual transmission, and vertical transmission. As with people chronically infected with HBV, HCC in HCV-positive individuals correlates with chronic hepatitis and cirrhosis. 2–7 % of chronically infected people develop HCC [52]. The hyperproliferative state induced by chronic hepatitis and cirrhosis is argued to lead to an accumulation of genetic changes and contribute to the onset of liver cancer. How HCV contributes to liver carcinogenesis is not known.

HCV is a flavivirus. As such it is unique among the human tumor viruses for having RNA as its only genetic material. HCV contains a 9.6 kb, positive-stranded RNA as its genome, which encodes a single translation product of approximately 3000 amino acids. This polyprotein is cleaved by proteases to yield at least ten proteins [53]. The study of HCV has been daunting for at least two reasons. Its sequence varies in infected people such that there are six recognized genotypes with multiple subtypes among patients and a spectrum of quasi-species within any one infected individual. Members of these quasi-species within one patient vary by 1–2 % in their sequence [54]. In addition, there is no available cell culture for HCV. Researchers have made heroic efforts to overcome this latter hurdle and have met with partial success. In 1999 Bartenschlager and colleagues described the replication of a subgenomic derivative of HCV in a cell line derived from a human HCC [55]. These subgenomic replicons were difficult to establish, but once established exhibited bona fide characteristics of flaviviral nucleic acid replication. One to five thousand molecules of plus-strand RNA were present in each cell; minus-strand RNA was present at 10–20 % of the level of the plus strand; and this viral RNA replication was insensitive to treatment of cells with actinomycin D [55]. This mode of nucleic acid replication in cells will allow the elucidation of the cis and trans elements it requires, but with an unexpected twist. The efficient replication of the subgenomic derivatives of HCV requires mutations in at least two viral genes that enhance replication in cells but abrogate infectivity of intact HCV RNA [56].

19.4.6.1 Evidence for a Direct Role of HCV in HCC from Studies of the HCV Core Protein

The HCV core protein can cooperate with an activated form of the ras oncogene to transform primary rodent cells in tissue culture and mice transgenic for the HCV core protein develop HCC. Several potential mechanisms have been invoked to explain the transforming potential of HCV Core protein. It has been found to enhance cell proliferation through the stimulation of the mitogen-activated protein kinase [57, 58]. More recently, the core protein has been found to activate STAT3, and this activation may contribute

to its transforming potential [59]. Whether the HCV core protein contributes directly to human HCC is uncertain. Neither replication of subgenomic replicons in cell culture nor infection of chimeric mice populated with human hepatocytes will allow ready testing of the possible role of the core protein in HCV's oncogenesis.

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Hormonal Carcinogenesis: The Role of Estrogens

20

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20.1 Background: Estrogens

In animals 17β-estradiol (E2) is typically thought of as a female sex hormone. The importance of estrogens in females is unambiguous. However, in males estrogens are also important steroid hormones, which target a variety of reproductive organs, including the prostate. In the female estradiol is primarily produced in the ovaries and acts to stimulate growth and development of the reproductive tract and mammary gland. In the male, estrogen is produced by adipose tissue, the adrenals, testicles, and even the prostate. The role of estrogen/estrogen receptor (ER) signaling in the prostate gland is poorly understood.

Steroid hormones including estrogen are derived from cholesterol. Cholesterol is subsequently converted to pregnenolone, progesterone, and then to testosterone (T) via several different cytochrome P450 enzymes. Testosterone is then converted to E2 by the P450 aromatase/CYP19 gene product. Therefore, aromatase is a key controller of the ratio of androgen to estradiol. This may be important because as men age the ratio of serum E2:T increases [1–4]. Since aromatase is expressed in the prostate, it is likely that local estradiol production plays an important role in prostatic function [5–7].

Circulating estrogens in premenopausal women originate from ovarian steroidogenesis and are in constant flux depending upon the menstrual cycle and pregnancy. However, after menopause, local aromatization of androgens and *de novo* biosynthesis [8] of E2 provide a source of estrogen to responsive tissues despite low circulating levels of estrogens. The adrenals in both men and women produce androgen which can be converted to estrogens via the action of aromatase. In males serum testosterone levels decrease steadily after the age of 39 [9] while estrogen levels remain steady resulting in a change in the androgen:estrogen ratio [10].

20.2 Hormonal Control of Prostate Growth and Development

The prostate develops from the embryonic endodermal urogenital sinus (UGS) in response to androgens, which act via the androgen receptor [11]. During prostatic development androgens mediate their effects via mesenchymal-epithelial interactions [11]. Although the prostate is primarily considered to be an androgen target, it is also a target for estrogen. Estrogens control prostatic development and function both directly and indirectly. Estrogens can elicit their effects directly on the prostate gland which causes squamous metaplasia when unopposed by androgens [12]. Transcriptional regulation by estrogens has been observed in certain genes having putative estrogen response elements (EREs) within their promoters [13–15]. These genes include members of the insulin-like growth factor, fibroblast growth factor, hepatocyte growth factor, nerve growth factor, and transforming growth factor beta families.

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_20

Importantly, these potent growth factor pathways have been implicated in human carcinogenesis of many organs including the prostate [16–20].

Organismal physiology of estrogens is extremely complicated because exogenous and endogenous estrogens can indirectly affect target tissues via systemic modulation of other hormones. For example, estrogens can have indirect systemic effects on the prostate gland by acting on the hypothalamus-pituitary-gonadal axis. Estrogens inhibit the release of gonadotropin-releasing hormone from the hypothalamus, which prevents the release of luteinizing hormone (LH) from the pituitary. Interstitial cells of the testicle (Leydig cells) are dependent upon LH for production and secretion of T. Thus, exogenous estrogens affect the prostate by inducing a chemical castration or androgen deprivation, which elicits prostatic epithelial apoptosis and generalized regression of the gland. Additionally, estrogen can cause the pituitary release of prolactin, which is a mitogen for the prostate.

Estrogens can act genomically in classical or nonclassical manners or they can act non-genomically [21–23]. It is thought that estrogens mediate their effects primarily through ER-α and/or ER-β in the prostate [7, 22, 24–26]. The hydrophobic estrogen molecule can diffuse through plasma membranes and bind to ERs found within the cytoplasm or nucleus where cofactors bind to the estrogen-ER complex. The estrogen-ER complex binds to EREs within promoters of genes either via direct protein-DNA interactions or indirectly via interactions with AP1 and SP1 sites within the target DNA (typically promoter regions) of estrogen-regulated genes. Stimulators or corepressors may bind to the estrogen-ER complex to improve or inhibit transcription. Signaling via this genomic route (i.e., estrogen/ER signaling) may take hours to facilitate a response.

ERα and ERβ are widely accepted as estrogen nuclear receptors based on their genomic effects on gene expression. Contrasting genomic ER function, membrane forms of ER exist as ERα and ERβ as well as a truncated form of ER-α termed ERα-36 or ERα-46 [21]. Both are associated with rapid non-genomic signaling. Alternative membrane-bound ERs were investigated when estrogens mediated a rapid (within minutes) induction of signaling. These investigations led to the discovery of intracellular G-protein-coupled ER (GPER) also known as GPR30 [27]. GPER is a member of the G-protein-coupled receptor (GPCR) superfamily whose activation regulates many biological responses of both normal and cancer cells [28]. Estrogen binds GPER, ER-α, and/or ERα-36/46 which stimulates transactivation of epidermal growth factor receptor (EGFR), leading to rapid phosphorylation of mitogen-activated protein kinase (MAPKs), and mobilization of intracellular calcium through synthesis of phosphatidylinostol 3,4,5-triphosphate (PI3K) in the cytoplasm.

In addition to estrogen GPER also binds selective estrogen receptor modulators (SERMS), selective estrogen receptor degraders (SERDS, and environmental ER activators [29]. Cross talk between GPER and growth factor receptors including receptor tyrosine kinases (RTKs), EGF receptor, and IGF receptors have been introduced as a possible signaling mechanism associated with cancer and metastasis [28]. Dimerization of growth factors in the RTK family causes activation of the MAPK cascade, which leads to gene transcription, cell proliferation, and PI3-K/Akt signaling, which promotes cell survival. Additionally deregulated RTK signaling is critical in cancer development and associated acquired resistance to endocrine therapy and reduced cell survival [2]. Overexpression of GPER is associated with tumor progression, increased metastasis, and acquired tamoxifen resistance [28]. The relationship between ERα, ERα-36/46 and GPER signaling is still poorly understood. However current research has focused on the relationship of ERα/variants and GPER using compounds that act as differential antagonists and agonists of the two. Understanding this relationship could provide therapeutic targets for drug therapy in hormone-induced cancers involving estrogen response signaling. Recently, a new mouse model of loss of membrane ERα function was developed [30]. Future studies using new models to decipher estrogen hormone action will lead to a better understanding of how estrogens elicit non-genomic signaling during pathogenesis.

20.3 Estrogens Are Genotoxic Carcinogens

Oxidation of steroidal estrogens and synthetic estrogens [31, 32] are capable of creating toxic agents that react with DNA inducing mutations and mutagenic reactive oxygen species (ROS). The next section will explore how estrogen/estrogen metabolites affect carcinogenesis.

20.3.1 Conversion of Estrogen to Catecholestrogens

Estradiol (E_2) and estrone (E_1) can be converted to catecholestrogens, 2 and 4 hydroxyestrogens [2-OHE1(E2) 4-OHE1(E2)], in both hormone-dependent and -independent cells. P450 1A enzymes are the predominant extrahepatic enzyme that converts steroid hormones to 2-hydroxyestrogens [33–36], but due to their lack of specificity, up to 20% of estrogen is converted to the more potent 4-hydroxyestrogens [37]. Specific estrogen 4-hydroxylase(s) have been identified in various organs including kidney, uterus, and pituitary [37–39]. In humans P450 1B1, an estrogen 4-hydroxylase is present in the breast, ovary, adrenal, and uterus [40, 41]. The tissue specific expression of these P450 isoforms contributes to the differential toxicity of estrogen in different tissues [42].

The hepatic P450s likewise convert estrogen to 4-OHE1 (E2) and may contribute to hepatic tumors in women who used oral contraceptives [43] especially contraceptives that were lacking progesterone.

Not all estrogen metabolites are carcinogenic; indeed some such as 2-methyl-estrogen may be protective prompting the question is estrogen-induced cancer driven by lack of these metabolites, and are their levels dependent upon polymorphisms present within the P450 1A gene [44] that may be specific to susceptible populations.

4-OHE1 (E2) has been shown to be oxidized to a semiquinone or a 3,4-quinone, which can generate reactive oxygen species (ROS) [45]. These molecules can react with the deoxyribonucleotides adenine and guanine within the genomic DNA [46]. Attempts to repair such damage may result in mis-match repair or base pair deletion leading to cancer-promoting mutations and/or cell transformation [8, 47–49]. Evidence that estrogen is indeed a genotoxic carcinogen exerting a mutagenic effect at the level of DNA is rapidly accumulating [45, 50–59].

2-OHE1, another estrogen metabolite, is capable of inhibiting HPV-mediated cellular proliferation by interacting with exons 6 and 7. Interestingly, 16α-OHE1, another estrogen metabolite, promotes proliferation via the same mechanism [60]. HPV and the estrogen metabolite 16α-OHEI may be capable of synergistically interacting to initiate cancers that are known to be HPV related such as cervical cancer [61]. Ratios of 2-OHE1 to 16α-OHE1 are lower in prostate and breast cancer compared to normal [62]. The transforming activities of estrogens and their metabolites were ranked as follows: 4-OHE1 (E2) >16alpha-OHE1 > E2 = 2-OHE1 (E2) [49, 63].

20.3.2 Estrogen DNA Adducts

DNA adducts are formed when chemical carcinogens bind covalently to DNA. There are two types of binding, stable, and depurinating. The differences between the two types depend on where the estrogen binds to the DNA.

20.3.3 Synthesis and Deactivation of Estrogen and Its Metabolites

The estrogens E_1 and E_2 are formed as are all steroids from cholesterol. They are formed by aromatization of 4-androsten-3,17-dione and testosterone, respectively, in an enzymatic reaction catalyzed by aromatase (P450 CYP19). They can be interconverted by 17β-estradiol dehydrogenase. The metabolism of estrogens in the liver and other tissues results in 2-4-hydroxylated estrogens that are then inactivated by conjoining reactions such as glucuronidation and sulfation that prevent excretion into the urine and feces; see Fig. 20.1. Quinines and semiquinones formed by estrogen oxidation are neutralized via conjugation with glutathione or reduced by quinone reductase.

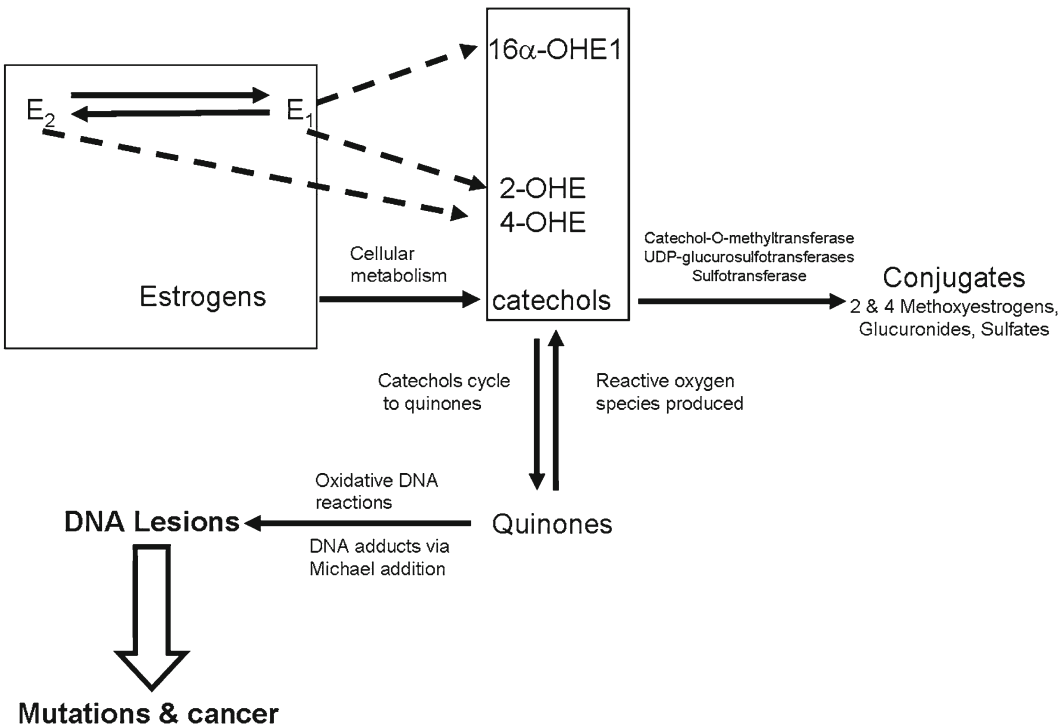

Fig. 20.1 Schematic of the production of estrogen metabolites, conjugates, and DNA adducts.

20.4 Induction of DNA Mutations by Estrogens

20.4.1 Mutations at Level of Chromosome

Chromosome gain and loss (aneuploidy) without gene mutation were observed in Syrian hamster embryo (SHE) cells transformed with E_2 or diethylstilbestrol (DES) suggesting that non-disjunction is responsible [64–66]. E_2, DES, and their quinone metabolites bind microtubules disrupting their polymerization in vitro [67–71] independent of estrogen receptor status [67]. Aneuploidy correlates with estrogen-induced cell transformation of SHE cells and may contribute to hormonal carcinogenesis [72, 73] and appearance of micronuclei [74].

20.4.2 Gene Mutations

Stable binding of DNA adjuncts to the exocyclic N^6 amino group of Adenine (A) or the N2 group of guanine (G) remain in the DNA unless they are detected and restored to normal by the DNA repair machinery. Unless the cell has faulty repair machinery these modifications should not be mutagenic. Depurinating DNA adducts bind N-3 /N-7 of adenine and/or N-7/N-8 of guanine. Depurination leads to apurinic sites that can generate mutations throughout the genome [75]. The formation of DNA adducts is independent of estrogen receptor status as shown by studies in the ERαKO/Wnt-1 mouse which displayed DNA adducts in the mammary gland [76].

20.4.3 Estrogen Receptors

There are two distinct nuclear ERs, ER-α and ER-β, and both have different ligand binding preferences [77, 78] and target genes. The molecular structures of ER-α and ER-β are similar and contain the six domains found in most steroid hormone receptors. The DNA-binding domains are nearly identical between ER-α and ER-β, while the A/B domain and the N-terminal domain share little homology. The N-terminal ligand-binding domains of ER-α and ER-β are approximately 50 % homologous, but the C-terminals are distinct between ER-α and ER-β. The genomic location for ER-α gene is on the q-arm of chromosome 6, whereas ER-β is located on the q-arm of chromosome 14. Both genes have multiple isoforms, ER-α has at least two [79], whereas ER-β has at least 5 [80]. The roles of ER-α and ER-β and their respective isoforms during prostate development, maturation, and carcinogenesis remain to be elucidated. Such structural and regulatory differences confer unique biological responses adding to the complexity of estrogen/ER signaling in estrogen target organs such as the prostate, mammary gland, and bone. Little is known about specific ER target genes and their function during normal growth, development, and carcinogenesis of the prostate and other estrogen target organs. Determining the molecular, cellular, and physiological functions of ER-α and ER-β will most certainly improve our understanding of prostate biology and carcinogenesis.

ER-β is highly expressed within the prostatic epithelium of the adult [81], while ER-α is predominantly expressed within the adult prostatic stroma and to a lesser extent within the adult prostatic epithelium. During prostate pathogenesis, such as benign prostatic hyperplasia (BPH), ER-α-positive cells and protein levels do not change in total numbers; however, stroma ER-α-positive cells significantly decrease in numbers whereas the number of ER α-positive epithelial cells increase. This tissue-specific change in ER-α positivity may play an important role in estrogen signaling and pathogenesis. The tissue distribution of the multiple isoforms of ER-α [80] and ER-β [79] change throughout prostatic development, maturation, and carcinogenesis, and their function is not fully understood.

The use of ER knockout (ERKO) mice null for either ER-α or ER-β has been helpful in elucidating the roles of these receptors in the prostate and other estrogen target organs [82]. In general the prostate develops normally in estrogen receptor-α knockout (αERKO) mice, and prostates of adult αERKO mice are basically normal suggesting that ER-α has a minor role in normal prostatic development and growth [24, 81]. More recently, it has been demonstrated that a subtle, but perhaps important prostatic phenotype exists in αERKO mice. These mice contain a decreased number of ductal branches, and individual ducts appear to be larger in size [81].

In prostate cancer and BPH, ER-α appears to be essential, because αERKO mice do not undergo disease progression [7, 83]. Estrogen receptor-β was initially cloned from the prostate and was subsequently shown to be highly expressed in adult prostatic epithelium. Subsequent studies have focused on determining the function of ER-β in the prostate. Surprisingly, prostates of ER-β knockout mice are also normal. It was reported that aged prostates from βERKO mice exhibited basal epithelial cell hyperplasia and reduced apoptosis [84]. However, these observations could not be verified by other laboratories [85]. It was also proposed that ER-β may act as a tumor suppressor during prostatic carcinogenesis as prostatic carcinomas have decreased levels of ER-β protein during tumor progression. Definitive proof of ER-β acting as a tumor suppressor remains to be established, but is an area of intense investigation.

One area in which use of αERKO and βERKO mice has been informative regarding estrogen action in the prostate concerns the induction of squamous metaplasia of prostate. A well-recognized effect on the prostate of chronic exposure to estrogens is squamous metaplasia of the epithelium [12]. Squamous metaplasia of the prostate is a benign condition and is the consequence of estrogen induction of proliferation

and squamous differentiation in prostatic basal epithelial cells. Studies using wild-type (wt) and αERKO and βERKO mice indicate that estrogen induction of prostatic squamous metaplasia requires estrogen signaling through ER-α but not ER-β [86]. For example, long-term treatment of wt and βERKO mice with diethylstilbestrol (DES) elicited pronounced prostatic squamous metaplasia, while similar treatment of αERKO mice had no such effect.

It is well established that signaling through the androgen receptor is required for prostatic development as mice lacking functional androgen receptors are devoid of a prostate as well as other male reproductive organs. The lack of prostate development in AR-null mice was shown to be associated with the absence androgen signaling in stromal cells [87]. Stromal control of prostatic development may also be important in estrogen-induced dysmorphogenesis, perinatal dysplasia, squamous metaplasia, and carcinogenesis following exposure to exogenous estrogens in various model systems. In this regard, immunohistochemical and steroid autoradiographic methods have superbly demonstrated the presence of ER-α in prostatic stromal cells during prostatic development and in prostatic pathogenesis [88–91].

To determine the respective roles of ER-α in the epithelium versus stroma during estrogen-induced squamous metaplasia of the prostate, tissue recombinants were prepared with prostatic epithelium (PrE) and prostatic stroma (PrS) from wt and α−ERKO mice: wt-PrS + wt-PrE, α−ERKO-PrS + α−ERKO-PrE, wt-PrS + α−ERKO-PrE, and α−ERKO-PrS + wt-PrE. Diethylstilbestrol (DES), a synthetic estrogen, induced squamous metaplasia only in tissue recombinants composed of wt-PrS + wt-PrE (both ER-α-positive). Tissue recombinants having α−ERKO tissues (α−ERKO-PrS+ α−ERKO-PrE, wt-PrS+ α−ERKO-PrE, and α−ERKO-PrS + wt-PrE) did not develop squamous metaplasia. Therefore, estrogen induction of squamous metaplasia of the prostate appears to require the binding of estrogen to stromal ER-α (paracrine mechanism) as well as direct action of estrogen via epithelial ER-α. Similarly, estrogen-induced cornification of vaginal epithelium requires the presence of ER-α in both the epithelium and stroma [92]. The importance of stromal-epithelial interactions mediated via ER-α signaling in the prostate has also been suggested in studies evaluating the effects of estrogenic exposure on neonates [82, 90, 93].

20.5 Role of Estrogens in Breast Cancer

Steroidal estrogens are known to be human carcinogens [94] as reported in The 11th Report on Carcinogens (RoC), a report compiled by both Federal and non-governmental scientific groups. Unlike the majority of human cancers which may present at early and late life the hormonally regulated cancers in women have a different risk pattern with very low risks before 20 years that rise until about 50 years of age when the rate of increase slows but incidence rate rises reflecting the total lifetime risk of breast cancer standing at present at 1:8. These data mirror the rise in hormones particularly E_2 up until 50 years of age when menopause results in changing hormone spectra in the tissue and serum [95–97].

Major risk factors for breast cancer are early age of first menses, late age at menopause [98], null parity [99], lack of exercise, obesity [100, 101], and hormone therapy [102, 103], all of which are estrogen linked [104]. Cancer-preventative trials using tamoxifen have reported favorable outcomes [105–110]; however tamoxifen is a selective estrogen receptor modulator (SERM) and is contraindicated in women who retain their uterus due to increased uterine cancer risks with tamoxifen treatment [105]. Interestingly tamoxifen can protect the rodent (but not human) uterus [110], kidney [107, 111], and breast from E_2-induced cancers [107, 112], but due to conversion into a genotoxic agent tamoxifen can cause liver tumors [105, 113, 114] and transforms SHE cells [115].

20.6 Role of Estrogens in Prostate Cancer

Epidemiologic and experimental studies have shown a pivotal role of steroid hormones in prostate cancer. The evidence emerges from four lines of research. (1) In animals susceptible to prostate cancer (men and dogs), the E2-to-androgen ratio increases during aging when prostate cancer manifests and is diagnosed [116, 117]. Indeed, increase in the E2:T ratio is temporally related with the development of both benign prostatic hyperplasia and prostate cancer [116, 117]. (2) African-American men, who have the highest incidence of prostatic cancer, have elevated levels of both free plasma E2 and T [118]. Caucasian-American, Latino-American, and Asian-Americans (male populations with lower incidences of prostate cancer) have lower levels of serum E2 and T [2]. (3) Testosterone in combination with estrogens induces prostatic hyperplasia in dogs and induces prostate cancer in rats and human tissues [116, 117, 119–122]. (4) The incidence of prostate cancer is highest in elderly men (across all ethnic backgrounds) who have an increased E2:T ratio [123–127]. Systemically, the change in the E2:T ratio is attributed to a decline in testicular function (reduced androgen production) and increased aromatase activity (E2 production). At the cellular level increased prostatic estrogen exposure occurs through high aromatase activity derived primarily from the prostatic stroma [6, 128]. The role of aromatase and hence estrogen/ER signaling in prostate cancer are currently being investigated. Use of aromatase knockout mice (ArKO) and inhibitors of aromatase activity have proven to be useful in understanding the importance of local prostatic E2 production and its relation to carcinogenesis.

20.7 Carcinogenicity of Estrogen in Humans

Estrogen exposure and subsequent cancer risks have been extensively examined in the human female population. Oophorectomy, and subsequent lowering of steroid levels, protects against tumor formation. In utero exposure to exogenous estrogens correlates with future breast cancer risk with low exposure being protective [129]. DES exposure during this time frame is linked to vaginal and cervical clear cell carcinoma, in the so-called DES daughters [130–133].

Testosterone has been both positively [134–137] and negatively [138, 139] associated with breast cancer in a process thought to involve its conversion to estradiol [140, 141]. Androgenic levels are high in postmenopausal women when the incidence of breast cancer is highest [135]. Furthermore treatment of cystic breast disease with androgens increased the risk of breast cancer [142]. The role of aromatase in the conversion of androgens to estrogens is currently being evaluated in breast carcinogenesis.

20.8 Models of Estrogen-Induced Prostate Cancer

Prostate cancer occurs with high frequency in men, and rarely occurs spontaneously in rats, mice, and other laboratory animals. Estrogen-induced tumors are primarily observed in rodent (rat and mouse) reproductive or endocrine tissues including the mammary gland (both male and female), uterus, cervix, vagina, testis, pituitary, and bone [143–148]. However, prostate cancer can be induced experimentally with E2 + T treatment (which mimics the hormonal milieu of men as they age) in rodent, canine, and human models [120–122, 149–151]. Three mechanisms have been proposed to explain estrogen-associated carcinogenesis: (1) Receptor-mediated estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) are steroid hormone receptors that upon binding steroid initiate transcription due to their transcription factor properties; (2) oxidative metabolism of estrogen, see above; and (3) induction of aneuploidy.

20.8.1 Estrogen and Perinatal Prostate Development

The embryonic and neonatal prostate is particularly susceptible to the pathogenic effects of exogenous estrogens, a phenomenon referred to as estrogen imprinting [152–154]. Estrogen imprinting signifies an irreversible usually deleterious morphogenesis, altered function, biochemical change, and/or pathogenesis elicited by exogenous estrogens administered experimentally during perinatal and early postnatal development. Perinatal exposure of embryos and neonates to exogenous estrogens elicit a multitude of deleterious effects on the prostate, which may lead to carcinogenesis [155]. During gestation the embryo/fetus is naturally exposed to high levels of maternal, placental, and fetal estrogens. In this regard, pregnant African-American females have the highest circulating levels of estrogen [156], and African-American men develop the highest incidence of prostate cancer [157]. Thus, it is possible that African-American men may have an increased incidence of prostate cancer due to their in utero exposure to high levels of estrogen. Neonatal or embryonic rodents (mice or rats) exposed to DES develop increased rates of prostatic carcinoma or premalignant pathologies in adulthood [152, 158–160]. In a similar manner more than one million humans were exposed in utero to the potent estrogen, DES, from the mid-1940s to the early 1970s when massive doses of this synthetic estrogen were prescribed to pregnant women for prevention of miscarriage. These so-called DES sons, men who were exposed to DES in utero, are now approaching an age in which prostate cancer is diagnosed [161]. Therefore, over the next decade more research on this cohort may elucidate the relevance of exogenous estrogen-imprinting in humans and its possible relationship to prostate pathology.

20.8.2 Carcinogenesis Induced by Estrogen Plus Testosterone

Noble was the first to report that chronic exposure to E2- and T-induced prostate cancer in rats [119]. Long-term treatment of Noble rats with E2 and T increased the incidence of epithelial dysplasia, which in turn progressed to adenocarcinoma of the prostate which metastasized [119, 162–165]. Similar studies have subsequently been performed utilizing Sprague-Dawley rats and have verified that E2 plus T induces and promotes prostate cancer [162, 164, 165]. The significance of these observations are underscored by the fact that these hormones are found naturally in men, and the E2:T ratio increases in aging men coincident with the development and diagnosis of prostate cancer. The drawbacks of the Noble rat model are long cancer latency, difficulty in altering genetics (i.e., gain or loss of function), and tumors are of rodent and not human origin. Moreover, it is difficult to study the role of estrogen/ER signaling via stromal-epithelial interactions in the rat models.

The seminal work of Noble has been integral to our current understanding of prostatic carcinogenesis and has led to the development of newer prostate cancer models which take advantage of human cells, mouse genetics, and stroma-epithelial interactions [120–122]. The use of tissue recombination technology, and thus the ability to alter the genetics of the epithelium and stroma, has yielded evidence demonstrating the important role of estrogens in carcinogenesis.

Wang and colleagues developed a model of prostatic carcinogenesis based upon tissue recombinants composed of urogenital sinus mesenchyme (UGM; fetal prostatic stroma) recombined with mouse prostatic epithelium (mPrE) derived from retinoblastoma deficient (Rb−/−) mice [166]. When UGM + mPrE-Rb−/− tissue recombinants were grafted and grown in untreated male athymic mouse hosts, no cancer was observed. However prostatic carcinogenesis rapidly occurred when tissue recombinants were grown in E2 + T-treated hosts. The cancer incidence in this model was 25 % at 2 months of hormone treatment. This finding underscored the importance of both E2 and T in the development of prostate cancer in a mouse model.

Another tissue recombination model utilizing E2 plus T to induce prostate cancer is the UGM + human prostatic epithelia (hPrE) models. Tissue recombinants composed of UGM + BPH-1 or RWPE-1 (BPH-1 and RWPE-1 are both non-tumorigenic human prostate epithelial cell lines) grown in untreated male athymic mouse hosts develop into benign prostate tissue. However, malignant human cells developed when UGM + BPH-1 or UGM + RWPE-1 tissue recombinants were grown in E2 + T-treated hosts. Non-tumorigenic human BPH-1 epithelial cells undergo irreversible malignant transformation in this UGM + BPH-1 E2 + T treatment model, because when BPH-1 cells were purified from UGM + BPH-1 E2 + T-induced tumors, they developed malignant tumors that metastasized when re-grafted into new hosts without stroma or hormones. Moreover, human (BPH-1-derived) malignant cells were found in the circulation and metastases were observed in lymph nodes, lung, and liver [120]. In contrast, UGM + BPH-1 tissue recombinants grown in untreated hosts or with T or E2 separately, rarely formed tumors but instead developed benign and ductal structures (untreated or T) or squamous metaplasia (E2). When BPH-1 cells were re-derived from such UGM + BPH-1 grafts grown in untreated hosts, the re-derived BPH-1 cells did not form tumors when grown in new host animals [120]. Steroid hormone levels in the E2 + T-treated hosts were normal for T and nearly physiologic (2–4 times higher than normal) for E2. Importantly, these hormonal profiles were dynamic and changed over the 4-month period of tumor induction similar to the changes observed in aging men (increasing E2:T ratio with time) [120]. A unique feature of the UGM + BPH-1 or UGM + RWPE-1 hormonal carcinogenesis models is that the epithelia are of human origin, and thus genetic and epigenetic aspects which may be unique to human cells are easily evaluated and should be highly relevant to human disease. Lastly, UGM + hPrE tissue recombination models are able to easily test gain and loss of stromal function through use of UGM derived from mutant mice (e.g., αERKO-stroma or ER-α-expression vectors) which allows for determination of their roles in hormonal carcinogenesis.

A major drawback of rat models is their lack of gene-gain or loss of function abilities. To this end, mouse models (transgenic and knockouts) and cell line (expression vectors and siRNA) models are unmatched for their experimental flexibility. For example, we have extended the hormonal carcinogenesis protocol of Noble and found that wt mice of different strain backgrounds (C57Bl/6 or CD-1 strains), when treated with E2 + T, developed prostaic carcinoma in situ by 4 months of age with high efficiency (40 %). The resultant dysplastic lesions exhibited reduced and diffuse E-cadherin staining and increased cellular proliferation accompanied by loss of prostatic basal cells and loss of smooth muscle in the adjacent stroma. Interestingly, C57Bl/6 mice were more sensitive to E2 + T treatment compared to CD-1 mice as evidenced by an increased incidence of prostatic intraepithelial neoplasia (PIN) in C57Bl/6 mice, which coincides with known differences in responsiveness to estrogen in these two mouse backgrounds [167]. One of the most important aspects of this new mouse model is the ability to use genetically engineered mutant mice to evaluate hormone action and estrogen-regulated downstream pathways involved in carcinogenesis.

In these aforesaid models of hormonal carcinogenesis, molecular and cellular changes induced either directly or indirectly by estrogens lead to increased proliferation, enhanced growth factor signaling pathways, phosphorylation of intracellular signaling molecules, cell survival, oxidative stress, invasion, epigenetic change, malignant transformation, and/or metastatic spread. Importantly, all animal models of prostatic hormonal carcinogenesis require estrogens for carcinogenesis. Androgens acting alone are inefficient inducers of prostatic carcinogenesis [119]. The significance of estrogen in hormonal carcinogenesis of the prostate was further emphasized in our previous report in which E2 and androgen levels were titrated to reveal an estrogen threshold for carcinogenesis below which carcinogenesis dropped abruptly [120].

20.9 Aromatase and Prostatic Pathogenesis

Estrogens produced from various tissues diffuse into the systemic circulation and hence act in an endocrine manner on cells of target tissues. While most cells are exposed to serum levels of estrogens, in the prostate local production of E2 by means of aromatase leads to increased intraprostatic estrogen levels presumably exceeding that in the circulation (systemic E2 plus local E2). Such increased E2 exposure due to local E2 synthesis via aromatase is thought to contribute to progression of breast cancer [168, 169], and such studies on breast cancer may serve as a conceptual model for determining the function of aromatase/ER signaling during carcinogenesis. Interestingly, the aromatizable androgen, T, by itself can

stimulate prostatic carcinogenesis in Noble and Lobound-Wistar rats albeit with low efficiency relative to that of E2+T [119, 170]. Since prostates express aromatase, it is likely that T may act as a substrate for local estrogen production within the prostate. Thus, administration of exogenous T results in exposure of the prostate to both androgen and locally produced E2. This interpretation is supported by studies with dihydrotestosterone (DHT), an androgen which cannot be aromatized to estradiol. In this regard when DHT plus E2 (instead of T+E2) was administered, the incidence of prostatic carcinogenesis was greatly reduced in Noble and Lobound-Wistar rats [150, 157, 164, 170]. One interpretation of these findings is that generation of prostatic carcinogenesis by E2+T only works provided that T can be locally converted into E2 by aromatase. Collectively, these experiments support the pro-oncogenic role of aromatase, and hence estrogen/ER signaling in carcinogenesis.

20.9.1 Perinatal Exposure to Estrogen Induces Prostatic Pathogenesis

To identify the mechanism of hormone action in perinatal estrogen imprinting, Prins and colleagues utilized αERKO and βERKO mice [90]. They demonstrated that adult prostate dysplasia was induced by neonatal estrogenization (estrogen-imprinting) and that estrogen action was a consequence of signaling through ER-α and not through ER-β. This is because when neonatal αERKO mice were treated with exogenous estrogen they did not develop dysplasia later in adulthood, while βERKO and wt mice developed prostatic dysplasia later in life following neonatal estrogen treatment. Since ER-α is expressed predominantly in the stroma of untreated mice, it was concluded that stromal ER-α mediates prostate dysplasia, even though this hypothesis has not been tested through analysis of the appropriate tissue specific loss of function experiments.

Estrogen imprinting elicits permanent alterations in androgen receptor levels, interacinar stroma volume, inflammatory reactions, epithelial hyperplasia, and development of dysplastic lesions in the prostate [89, 153, 159, 171, 172]. Increased expression of ER-α, c-myc, and c-fos as well as decreased expression of AR and ER-β occurred following perinatal exposure to exogenous estrogens and may be part of the downstream mechanisms associated with development of prostatic lesions later in life [89, 171, 173–175].

Another potential source of exogenous estrogens is the environment. Estrogenic compounds are found in numerous products including dental sealants, water treatments, insecticides, and plastics (used to store food and beverages). The effects of environmental estrogens on reproductive status have been known for years. The identification of these estrogens has led to the term endocrine disruptor [176]. One compound currently under scrutiny is bisphenol-A (BPA), a weak estrogen and a component of many plastics. It has been suggested that BPA and other endocrine disrupters may have pathogenic effects in humans, although this is controversial and hotly debated [177]. Recently estrogenic endocrine disrupters, specifically bisphenol A, have been found to induce prostate dysplasia when administered neonatally [155, 178]. A downstream pathway from BPA/ER signaling involved in the process of prostatic dysplasia, appears to be phosphodiesterase type 4 variant 4 (PDE4D4), an enzyme involved in regulating levels of cellular cAMP, which is epigenetically altered via hypomethylation during estrogen imprinting [155]. Constitutive activity of this enzyme is likely a contributing factor to early stages of carcinogenesis. BPA does bind and activate both ER-α and ER-β albeit with lower efficiency than E2 [77, 78]. However, environmental estrogens, such as BPA, are known to elicit their biological activity at much lower concentrations during development than in adulthood [172]. Determining which environmental estrogens are involved in prostatic abnormalities and their mechanisms of action will be important for our understanding of how carcinogenesis is initiated and/or stimulated.

20.9.2 Induction of Carcinogenesis in Adults

Prostates of adult C57Bl/6 mice treated with E2+T implants were significantly enlarged relative to control untreated animals and contained areas of high grade prostatic intraepithelial neoplasia (PIN) after 4 months of E2+T exposure [7]. Prostates from hormone treated mice exhibited biochemical changes consistent with prostate cancer progression. These changes included increased PCNA-positive cells (cellular proliferation), diffuse and reduced expression of E-cadherin, and phosphorylation of AKT. Additionally, areas of high-grade PIN exhibited marked decreases in prostatic basal cells and loss of smooth muscle in the stroma. As with the Noble and Lobound-Wistar rat models, this new mouse model suggests that E2+T induces both initiation and promotion of malignant lesions. Therefore, determination of the role of steroid hormones, especially estrogens, in prostate carcinogenesis may lead to the identification of both initiating factors and those involved in the promotion of cancer.

In prostates from E2+T treated wt mice, ER-α was detected in epithelial as well as stromal cells in areas of PIN, which further adds to the complexity ER-α signaling during carcinogenesis. As seen with human carcinogenesis, ER-α was expressed in the stroma, while epithelial ER-β was reduced or absent in PIN-lesions of wt mice treated with E2+T. Therefore, it is reasonable to assume that increased stromal ER-α signaling mediates the carcinogenic effects of estrogen.

Use of αERKO and βERKO mice have provided additional information on the respective role of these receptors in

normal development and carcinogenesis of the prostate. In αERKO mice, E2 + T failed to induce dysplasia or high grade PIN, suggesting a key role of ER-α in hormonal carcinogenesis [7]. In contrast, ER-β knockout mice (which express ER-α) developed prostatic hyperplasia, dysplasia and high grade PIN comparable to that seen in wild-type mice when treated with E2 + T. This mechanism is also observed for BPH [83]. Several markers indicative of carcinogenesis including E-cadherin, cellular proliferation (PCNA labeling index), and presence of both basal epithelial cells and smooth muscle cells were all within normal parameters in E2 + T-treated αERKO mice. However, in βERKO mice the expression profile of these markers deviated from normal and were consistent with a cancer progression. Together these data highlight the importance of signaling through ER-α in the induction of prostatic carcinogenesis. Future experimentation will determine the specific gain and loss of function of ER-α and ER-β in stromal and/or epithelial tissue compartments and will allow dissection of the mechanism of hormone action and the relevant downstream mediators of hormone-induced prostatic carcinogenesis. See Fig. 20.2 for schematic of estrogen action in the prostate.

The existence of both ER-α and ER-β in the benign and malignant prostate suggests multiple roles for these receptors. It is not known whether ER-α and ER-β interact directly with each other (transcriptional cooperativity) or indirectly (via paracrine factors) during prostate cancer progression. Given the principal expression of ER-β is within the epithelium and the predominant stromal expression of ER-α, this makes the task of determining the role estrogen/ER signaling in pathogenesis very difficult. In this regard, it has been reported that ER-α levels increased with prostate cancer progression [179], even though consensus on this point is still needed [149, 180]. In contrast, ER-β expression decreases during prostate cancer progression, but may reappear in later stages [179–182]. Re-expression of ER-β has been associated with metastases and an increased rate of prostate cancer reoccurrence [180, 183]. These findings suggest that ER-α and ER-β may play separate and pivotal roles in prostatic carcinogenesis. Additionally, the function of ER-α versus ER-β are likely to be different depending on the stage of carcinogenesis.

Estrogen receptor-β has been dubbed a brake for cancer [184] as demonstrated by the ability of ER-β-specific agonists to decrease normal adult prostate cell proliferation [185]. Additionally, βERKO mice have been observed to have increased prostatic basal cell hyperplasia [186]. Finally, ER-β has been shown to increase the expression of detoxifying agents such as glutathione S-transferase-π which acts to prevent oxidative genomic damage and thus stabilizes genomic integrity [187–189]. These data suggest a potential therapeutic/preventative role for ER-β-signaling in prostate cancer progression.

While the significance of decreased ER-β expression during malignancy is unknown, the mechanism of this loss has been attributed to methylation of the ER-β promoter and hence silencing of ER-β transcription [190, 191]. The mechanism for re-expression of ER-β in metastatic cancer may involve demethylation of a unique CpG island residing in the promoter of ER-β, which has been shown to be inversely related to the stage of carcinogenesis [191, 192]. Determination of the precise roles of ER-β as well as their downstream targets will aid in our understanding of this process.

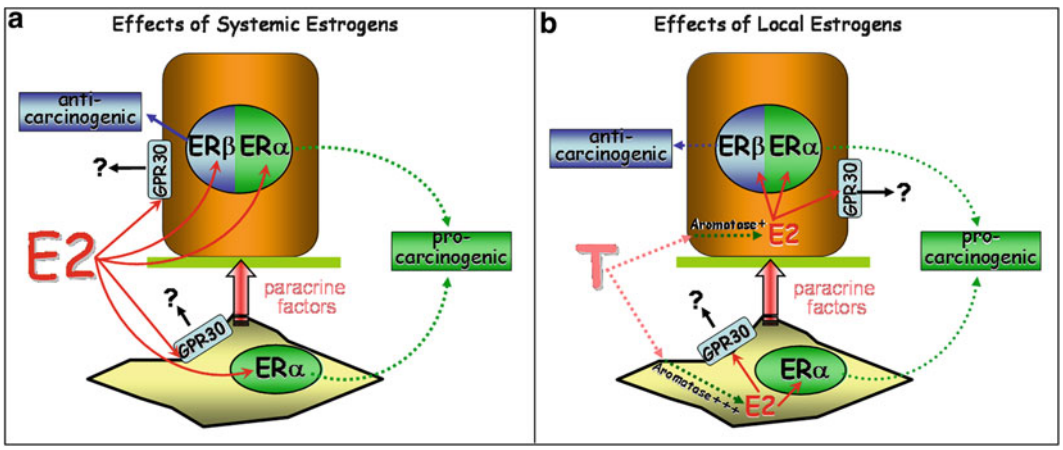

Fig. 20.2 The systemic and local effects of estrogens in stromal-epithelial interactions of the prostate. (**a**) Circulating estradiol (E2) enters the prostate and can bind and activate stromal and/or epithelial estrogen receptors (ER-α or ER-β). E2 binding to stromal and/or epithelial ER-α is likely to be involved in the promotion of carcinogenesis. E2 activation of stromal ER-α stimulates the secretion of putative E2-regulated paracrine factors which stimulate carcinogenesis. Unlike ER-α, ER-β when occupied by ligand slows prostatic epithelial proliferation and hence may be anti-carcinogenic. It is currently unknown if the E2 binding to membrane-bound GPR-30 (or ERα, ERα-36, or ERα-46) is involved in prostate biology or pathogenesis. (**b**) T can be converted to E2 via the P450 enzyme aromatase and thus increase local prostatic concentrations of E2, which may enhance estrogenic effects via ER-mediated events.

Furthermore, understanding the effect of estrogens on ER-β promoter methylation during carcinogenesis may give us additional insights for therapeutic targeting using selective estrogen receptor modulators (SERMs). Ultimately the appropriate timing of therapy as well as use of demethylating agents will aid in our understanding of how these compounds mediate their affects and elucidate of the underlying mechanisms.

20.10 Chemo-Prevention of Carcinogenesis

The involvement of estrogen in prostate cancer suggests that it should be possible to prevent prostate cancer through manipulation of estrogen pathways. The SERM, tamoxifen, has been successfully used in breast cancer studies and is the standard of care in many hospitals. Additionally, there are several models of prostatic carcinogenesis that suggest that anti-estrogens may be chemo-preventative or chemo-therapeutic [193, 194]. Antiestrogens inhibit cell proliferation and induce cell death in breast cancer cells [195, 196]. In regard to the prostate, the SERM, toremifene, decreased the rate of progression of cancer as well as inhibited the presence of high-grade PIN in TRAMP mice [197]. Interestingly these effects were not associated with changes in serum levels of E2 and T, which suggests that the therapeutic effect may have been direct. Toremifene binds and inhibits both ER-α and ER-β. Estrogen receptor-β has been determined to be a suppressor of prostatic growth [84, 91, 183, 185], while prostatic ER-α appears to enhance prostate epithelial growth [86, 90, 198]. Thus, anti-estrogenic activities of tamoxifen/toremifene may exert their actions through ER-α or ER-β or both. However, since estrogen-induced metaplasia, dysplasia, and high grade PIN doesn't occur in αERKO mice [7, 86, 90], it is likely that ER-α mediates the carcinogenic effects of estrogen, and thus chemo-preventative effects of SERMs may also operate via ER-α. Relevance of these studies to human disease is seen within a recent clinical trial of the effects of anti-estrogens in human prostatic carcinogenesis. Men with high grade PIN were treated with toremifene for 1 year, which significantly decreased the rate of prostate cancer progression [199]. In this study men tolerated the SERM well with few side effects. However, these studies have not been resilient [200]. Nonetheless, other SERMs (e.g., raloxifene, tamoxifen) which may recruit different cofactors and thus may lead to more efficacious effects and hence prevent disease progression. Further studies will be needed to establish the efficacy of this therapy and to identify which ER and which tissue layer is affected by the SERM. Moreover, determination of ER-downstream effectors will help dissect the mechanism of estrogen/anti-estrogen action in prostatic carcinogenesis.

Chemoprevention may also be provided through dietary means. Several epidemiological studies linked one's diet to prostate cancer [201–203]. The high fat/low fiber Western diet, as opposed to the low fat high fiber Asian diet, has been inferred to be a risk factor in prostate cancer. In this regard, there is at least one dietary factor related to estrogen/ER signaling: phytoestrogens. High amounts of phytoestrogens in the diet have been determined to be inversely correlated with prostate cancer risk [204]. The isoflavanoid phytoestrogen, genistein, is found in soybeans and soybean products. Genistein binds and activates ER-α and ER-β, although it is a better ligand for ER-β. Thus, genistein selectively promotes ER-β signaling (although ER-α signaling still occurs) in the prostate. If ER-β-signaling is important in preventing cellular proliferation, this may be a mechanism of action for genistein and other phytoestrogens. In support of phytoestrogens as inhibitors of prostatic carcinogenesis, prostatic dysplasia was reduced in DES-treated mice (perinatal model) fed a soybean rich diet [205]. Genistein also reduced rates of prostatic carcinogenesis observed in carcinogen-treated Lobound-Wistar rats [206]. Finally, TRAMP (a transgenic mouse model of prostate cancer) mice had fewer poorly differentiated cancers on a genistein-rich diet [207]. Collectively, these data support the role of phytoestrogens/genistein and ER-β signaling in prevention of prostatic carcinogenesis.

20.11 Chemotherapy

Historically, administration of exogenous estrogens have been used in prostate cancer therapy as chemical castration agents. In this case, the mechanism of estrogen action was indirect, having its' direct effect on the hypothalamus-pituitary-gonadal axis and thus inhibiting androgen secretion, leading to prostatic epithelial apoptosis. However, since estrogens modulate initiation and progression of cancer, anti-estrogens or SERMs have been used experimentally to treat and prevent different stages of prostate cancer. A considerable amount of data has been demonstrated that SERMs may be effective therapies for prostate cancer. The capacity of SERMs to halt cancer cell growth is attributed to a number of different estrogen-regulated pathways and mechanisms including: cytotoxicity [208], microtubule destabilization [209], angiogenesis, anti-metastatic [210], DNA synthesis inhibition [211], cell cycle disruption [212, 213], induction of apoptosis [212–214], and dysregulation or activation of apoptosis machinery [215–217]. These data support the notion that anti-estrogens such as tamoxifen prevent prostate cancer progression, even though SERM therapy in later stages of prostate cancer have not been particularly effective [218–222]. Due to the different ERs/ER-pathways and their tissue specificity and temporal characteristics, each stage of carcinogenesis is expected to respond to SERMS differently. For example, since ER-α (a putative enhancer of carcinogenesis) is present and abundant during the benign/premalignant stage of carcinogenesis, it may be useful to antagonize ER-α in early rather than in later

stages of the disease when it's expression is lower. Likewise, since ER-β (a negative regulator of epithelial growth) is lost during cancer progression, it may be futile to treat prostate cancer using ER-β agonists at this stage. Treatment with ER-β agonists may be most effective in early or late (metastatic) stages of carcinogenesis at times when ER-β is strongly expressed. To emphasize this point, the success of a clinical Phase 2b trial using the anti-estrogen, toremifene, in men with high-grade PIN has provided new insight into the timing of when to treat prostatic carcinogenesis [199].

20.12 Conclusion

Epidemiological and experimental data create a clear link between estrogens and prostatic carcinogenesis. Since estrogens can affect the prostate in many ways and at different times throughout life, it is likely that targeting therapies directed toward ER-α or ER-β selectively and or collectively may be a successful chemo-preventative strategy for prostatic carcinogenesis. Moreover, anti-estrogen treatment directed towards specific tissue compartments (e.g., stromal versus epithelial) and specific ERs at different times of carcinogenesis may also prove beneficial in preventing carcinogenesis. In order to determine mechanisms associated with ER-signaling in the prostate, it is likely that the elucidation of how estrogens mediate their effects temporally and within specific tissue types is needed. The success of anti-estrogens in preventing early stages of carcinogenesis bodes well for the prevention of prostate cancer.

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Sporadic Cancers

21

Clemens B. Tempfer and Marc-André Reymond

21.1 General Aspects

Cancer may occur in hereditary and sporadic forms. Clearly, the majority of cancers develop sporadically. For example, in breast cancer, over 90 % of cases are sporadic and only 5–10 % of cases are considered hereditary, with mutations in the tumor suppressor genes BRCA1 and BRCA2 accounting for most of these cases [1]. Among women with synchronous endometrial and ovarian cancer, 7 % meet clinical and/or molecular criteria of hereditary non-polyposis colon cancer (HNPCC) syndrome [2]. Other cancer entities are entirely sporadic and have almost no proportion of hereditary cases, e.g., cervical cancer [3]. In contrast to hereditary cancers with a single genetic factor of high penetrance, the reason why sporadic cancer develops seems to be multifold. In a minority of sporadic cancers, a single etiologic factor can be identified. For example, up to 98 % of cervical cancer cases are caused by malignant transformation of cervical epithelial cells via genomic incorporation of the E5, E6, and E7 proto-oncogenes and subsequent targeting of the p53 and Rb pathways [3]. However, in most sporadic cancers an unknown number of factors, which are alone neither necessary nor sufficient to cause malignant transformation, act together and increase the susceptibility to one or more specific malignancies. This may be achieved by direct interaction such as DNA damage through ionizing radiation or genotoxic compounds or by indirect interaction such as reduced immunocompetence and subsequently limited immunoclearance of malignant cells or cancerogenic viruses such as the Human Papilloma Virus (HPV). In the end, all of these factors influence malignant transformation by genetic and/or epigenetic events and subsequently cause loss of cell proliferation control. In this respect, gatekeeping genes, i.e., those genes that are directly regulating the growth of cells, are frequently mutated in sporadic cancers through somatic mutations, as well as through germline mutations in predisposed individuals [4]. Thus, there seems to be a gradual, but not a principal difference between cancerogenic pathways in sporadic and hereditary cancers.

A wide variety of risk factors for the development of sporadic cancer have been identified, among them physical and chemical agents including radiation and exogenous toxins, and endogenous factors such as hormones and dietary contents. In addition, numerous individual biologic and socioeconomic variables such as age, immune status, viral infections, education, and ethnicity have been associated with cancer risk. Some of these have an obvious and well described etiologic link to cancer such as infection with HPV or exposure to ultraviolet radiation. In other cases, the pathophysiologic reason for the observed association with cancer is less clear, as with oral contraceptive use and cervical cancer or breast feeding and breast cancer. In this chapter we present and discuss an overview of factors found to be associated with the development of sporadic cancer.

21.2 Risk Factors and Associations

21.2.1 Age

The incidence of most cancers increases with age. The reason behind this phenomenon may be the fact that accumulation of mutations is a common driver of both aging and cancer. These mutations may be caused by physical and chemical noxious agents as well as endogenous genetic and epigenetic factors, such as telomere biology, gene silencing, apoptosis, and loss of heterozygosity [5]. Studies conducted in yeast, worms, flies, and mice confirmed substantial changes in gene expression during the natural aging process. Among them, the most important was silencing of tumor

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_21

suppressors and other genes involved in the control of cell cycle, apoptosis, detoxification, and the cholesterol metabolism. In addition, age-dependent gene expression of oncogenes typically associated with diseases of advanced age has been documented [6].

Cancer epidemiology shows age-specific distribution patterns. Most cancers have one or more characteristic age-specific incidence peaks. In an overview of 600,000 cases of breast cancer in the US between 1950 and 2003, for example, a bimodal incidence peak at 50 and 70 years was described before the introduction of widespread screening mammography in the early 1980s [7]. Thereafter, age distribution shifted to older ages at diagnosis and later returned to a bimodal distribution similar to patterns before screening within the last decade. These data most likely reflect a complex interaction between age-related biologic factors, risk factors, and interventional phenomena. Specifically, patterns of mammography screening and use of hormone replacement therapy have been shown to parallel breast cancer incidence rates from 1980 to 2006 in a study of a large US health care plan [8]. These two factors are therefore thought to be the major contributors to changes in sporadic breast cancer incidence. Clearly, the association between age and cancer incidence is influenced by many factors and may be more reflective of general characteristics of a given society than of basic cancer biology principles.

Another example of the influence of environmental factors on age-specific incidence peaks is sexual activity and age-specific incidence rates of cervical cancer. Exposure to the DNA damaging effects of the E5, E6, and E7 protein gene sequences of human papilloma virus (HPV) high-risk subtypes such as HPV 16 and 18 is dependent on sexual activity. In addition, a latency period of chronic infection as well as the maturity of the immune system influence the age at onset of cervical intraepithelial neoplasia. Interaction of these factors results in two incidence peaks of cervical intraepithelial neoplasia and invasive cervical cancer at 33 and 51 years of age, respectively [9]. In this case, age is both a surrogate marker for sexual behavior and a biologic factor, because immunological HPV clearance rates are age-dependent.

Also, age may be a risk factor of cancer as a consequence of specific birth cohorts being associated with cancer incidence. This can be due to a known carcinogen exerting its influence in utero or in early childhood. Diethylstilbestrol exposure in utero, for example, has been shown to result in a subsequently increased risk of adenocarcinoma of the vagina in specific birth cohorts [10]. After identification and elimination of the causative agent, this rare form of cancer is no longer seen among other birth cohorts. Associations between cancer incidence and birth cohorts may also be due to the exposure to an unknown risk factor. In a study of testicular cancer incidence patterns in the Netherlands between 1970 and 2004, the incidence rate of testicular cancer remained stable for all age groups at 3/100,000 person years until 1989, but increased annually thereafter by 4 % to 6/100,000 until 2004 [11]. It has been hypothesized that changes in lifestyle factors such as increased maternal age, genetic, and environmental factors, i.e., endocrine disruptors, are responsible for this pattern of testicular cancer incidence.

21.2.2 Obesity

Obesity, defined as a body mass index (body weight in kilograms/body height in meters) of >30, is associated with a significantly increased risk of cancer. Obese individuals have a higher risk of esophageal, colorectal, liver, gallbladder, pancreatic, renal, endometrial, ovarian, prostate, and breast cancer, as well as hematologic malignancies such as non-Hodgkin lymphoma and multiple myeloma. Obesity accounts for 20 % of all cancer deaths [12].

It is believed today that obesity affects several pathophysiologic systems promoting malignant transformation. Obesity is associated with increased sex hormone concentrations due to increased aromatase activity in adipocytes, increased ovarian androgen production, reduced sex hormone binding globulin production, and reduced progesterone production. Other potentially carcinogenic hormones and cytokines such as insulin, insulin-like growth factor-1, leptin, tumor necrosis factor-alpha, plasminogen activator-inhibitor, and angiotensinogen have been found to be increased in the serum and tissue of obese individuals [13]. The contribution of individual hormones and cytokines to malignant transformation is unclear and specific combinations of substances seem to exert a pro-carcinogenic effect, while others do not. For example, evidence from large prospective-randomized intervention trials such as the Women's Health Initiative showed that 5 years of treatment with conjugated equine estrogens and medroxyprogesterone acetate significantly increased breast cancer risk, whereas 8 years of treatment with conjugated equine estrogens alone significantly reduced breast cancer incidence among women with at least 80 % treatment adherence [14].

Obesity also affects sporadic cancer recurrence risk and overall survival. In breast cancer survivors, obesity is an independent predictor of disease-free and overall survival [15]. From a technical perspective, obesity may also adversely affect surgical cancer treatment. Laparoscopic lymph node sampling, for example, is less often successful in obese women with pelvic malignancies compared to nonobese controls. Also, completion of a state-of-the-art surgical staging is achieved significantly less often among obese patients. Erkanli et al. cite a successful surgical staging rate of 93 % in women with a BMI < 30 compared to 81 % in women with a BMI > 40. The technically demanding paraaortic lymph node staging procedure was performed in 74 % of eligible patients

with a BMI < 30 compared to 48 % of eligible patients with a BMI > 40 [16]. Another factor which negatively affects cancer treatment and prognosis of obese individuals is screening compliance. Both cervical cancer screening by cervical PAP smear and breast cancer screening by mammography is underused by obese women. The reason for this is unclear, but socioeconomic status, embarrassment, and lack of mobility due to associated morbidity have been cited [17].

21.2.3 Ethnicity

Ethnic background plays a major role in sporadic cancer incidence, diagnosis, treatment, and prognosis. This is believed to be due to many reasons, among them cultural phenomena, life style patterns, socioeconomic factors such as education level and access to health care as well as genetic factors. Knowledge of ethnicity-specific cancer characteristics has implications for cancer screening programs among ethnic minorities. Tiwari et al. investigated 256 British colorectal cancer patients and found that Afro-Caribbeans, Asians, and Mediterraneans presented at significantly younger ages compared to Caucasians [18]. Also, Afro-Caribbeans had significantly more right-sided and Mediterraneans more left-sided colorectal cancer. Afro-Caribbean and Caucasian patients presented with more advanced stages compared to Mediterraneans. These data indicate that culture-specific dietary patterns, access to health care interventions such as colorectal screening, and genetic factors may influence cancer incidence and cancer biology in different ethnic groups. In another study of ethnic differences in incidence and outcome of 156,570 US women with sporadic breast cancer, all ethnic minority groups including African-American, Hispanic, American Indian/Alaskan Natives, and Asian/Pacific Islanders had significantly lower incidence rates compared to Caucasian women [19]. These differences were explained by a differential distribution of breast cancer risk factors such as mammography screening participation, age, number of first degree relatives with breast cancer, prior breast biopsy, alcohol intake, education, as well as age at menarche, age at first birth, and age at menopause in all investigated ethnic groups. Breast cancer in African-Americans also had significantly more often unfavorable characteristics and a higher mortality compared to Caucasian women, independent of body mass index, hormone therapy use, and socioeconomic characteristics pointing to a strong genetic background for this phenomenon.

In a study of 13 US cancer registries with cervical cancer incidence data collected between 1992 and 2003, significant differences between ethnic groups were identified [20]. Hispanic whites, for example, had the highest cervical cancer incidence rates, whereas non-Hispanic whites had the lowest incidence rates. African-Americans, on the other hand, had the lowest incidence rates of cervical adenocarcinoma. It is of note, that in this study most of the variation among ethnic groups was explained by differences in screening participation and socioeconomic characteristics. Also supporting the importance of socioeconomic factors for cancer incidence, diagnosis, and treatment, a Swedish study analyzed a national cancer database over a period of 14 years and found that University graduates were significantly more likely to be diagnosed with breast cancer compared to women without a University education, but had the highest survival rates after a diagnosis of breast cancer [21]. These data indicate that most variations in cancer incidence among ethnic groups are due to differential distribution of socioeconomic risk factors. However, among selected ethnic groups such as African-Americans genetic factors also seem to play a significant role.

21.2.4 Immunosuppression

Immunologic aspects play an important role in the biology of sporadic cancer. For example, markers of immunologic activity such as local inflammatory reaction at the cancer site and increased production of C-reactive protein are independent prognostic factors for overall and disease-free survival in cervical, ovarian, and endometrial cancer [22]. A state of chronic inflammation has been described as an etiologic factor of cancerogenesis in gastric, pancreatic, and prostate cancer [23, 24]. On the other hand, immunosuppression also seems to increase the risk of malignant transformation.

In individuals with rheumatoid arthritis, treatment with cyclophosphamide, methotrexate, and azathioprine is associated with an increased cancer risk [25–27]. Also, the use of immunomodulatory biologic therapies such as infliximab and adalimumab increased the risk of malignancy threefold in a meta-analysis of randomized trials [28]. In a US study of 13,001 individuals with rheumatoid arthritis, exposure to biologic therapies increased the risk of non-melanoma skin cancer and melanoma, but not solid tumors and lymphoproliferative malignancies [29].

The effect of immunosuppressive antirejection drugs after organ transplantion on the occurrence of cancer was first described in the 1970s. The excess risk of malignancies among immunosuppressed people is accounted for by non-Hodgkin lymphoma, Kaposi's sarcoma, non-melanoma skin cancer, and anogenital cancers. These malignancies are also characteristic of Human Immunodeficiency Virus (HIV)-infected individuals. For example, in a study of 8074 HIV-infected individuals and 2875 transplant recipients undergoing chronic immunosuppressive therapy, the cumulative probability of cancer after 15 years was 13.3 % and 14.7 %, respectively. The increased overall risk of cancer at any site was 2.2-fold increased for transplant recipients and 9.8-fold increased for HIV-infected individuals [30].

The most common entities were Kaposi's sarcoma, non-Hodgkin lymphoma, liver, lung, and cervical cancer. In a long-term study of patients with membraneous nephropathy undergoing chronic cytotoxic and immunosuppressive therapy, a doubling of cancer incidence was observed during 15 years after diagnosis [31]. Clearly, chronic immunosuppression favors the development of virally induced cancers, but cancer types not associated with infectious agents also seem to develop more often among individuals with a compromised immune system.

Altering the immune system after cancer surgery adversely affects prognosis and outcome. For example, in a randomized trial of 847 women with ovarian cancer, the addition of interferon gamma 1-b to standard carboplatin–paclitaxel chemotherapy significantly reduced overall survival [32]. In summary, the available data indicate that deviations in the immunologic control both in the form of increased immunoactivity and immunosuppression may contribute to the process of malignant transformation.

21.3 Exogenous Factors

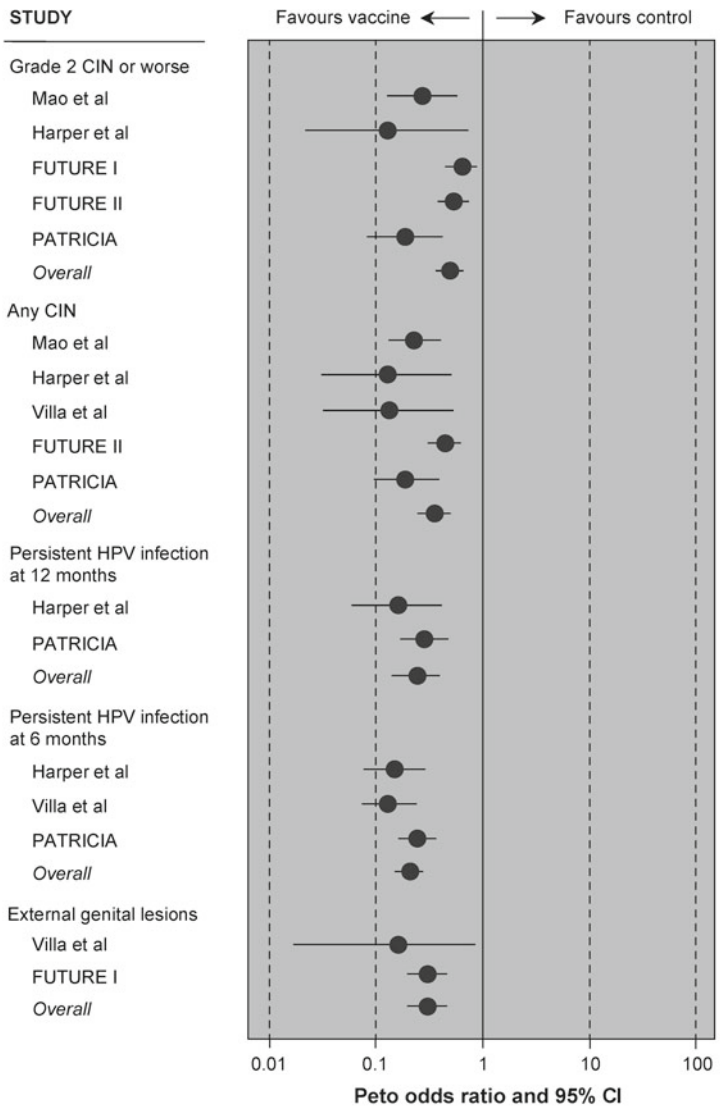
21.3.1 Infectious Agents

Cancer is a disease initiated by genetic events and infectious agents can cause or promote malignant transformation by indirectly or directly damaging DNA. It has been estimated that 15 % of all cancers are etiologically linked to viral infection. Specific cancers with an established viral etiology are T-cell leukemia, hepatocellular carcinoma, and cervical cancer, being associated with human T-cell leukemia virus type-I, hepatitis B virus, and high-risk HPV, respectively [33]. Virally triggered carcinogenesis may be achieved by the integration of proto-oncogenic viral DNA sequences into the host genome. These DNA sequences then function as oncogenes causing expression of oncoproteins such as Tax, HBx, and E6/E7, capable of inducing genome instability. Cervical cancer, for example, is believed to be almost exclusively due to an infection with HPV high-risk subtypes such as HPV 16 and HPV 18. Experimental evidence in neural progenitor cells demonstrates that integration of the HPV E5, E6, and E7 proto-oncogenes leads to the degradation of the tumor suppressor proteins p53 and pRB involving MEK-ERK signalling pathways [34]. Consequently, it has been successfully demonstrated that prophylactic vaccination against specific epitopes of HPV high-risk subtypes such as HPV 16 and HPV 18 efficiently prevents cervical dysplasia. In a meta analysis of six randomized controlled trials and three follow-up reports of non-randomized vaccination trials, HPV vaccination reduced the frequency of high-grade cervical dysplasia by 86 % (Odds Ratio 0.14; 95 % Confidence Interval 0.09–0.21) among HPV naïve women aged 15–25 years [35]. Figure 21.1 shows the clinical outcomes of HPV vaccination trials in an intention-to-treat meta-analysis. Bivalent and quadrivalent HPV vaccines have been proven to effectively prevent the development of cervical cancer and cervical cancer predecessors such as cervical intraepithelial neoplasia (CIN). These vaccines prevent up to 50 % of cervical cancers. Next generation nonavalent HPV vaccines are designed to cover a higher range of the HPV spectrum and may be able to prevent up to 90 % of cervical cancers [36]. In theory, highly effective vaccines may be able to eradicate cervical cancer. Other cancers are HPV-associated, but to a lesser extent then cervical cancer. Anal, vulvar, vaginal, and head-and-neck cancers are associated with HPV high-risk type infections in 10–70 % of cases [37, 38].

Another form of cancer with an infectious etiology is gastric cancer. Helicobacter pylori (H. pylori) infection is the leading cause of gastric cancer worldwide. Infection with this ubiquitous infectious agent is harbored by approximately half of the population worldwide [24]. Malignant transformation is achieved through a multistep cascade including chronic inflammation, atrophy, metaplasia, dysplasia, and invasive cancer. Interestingly, the target cells of malignant transformation in H. pylori caused gastric cancer are bone marrow derived tissue stem cells. These cells are infected in bone marrow and recruited into gastric tissue after local atrophy due to chronic inflammation acting then locally as cancer stem cells. In view of the high prevalence of H. pylori infection and gastric cancer, it is clear that powerful modulatory factors exert their influence. However, the exact nature of these modulatory effects is unknown. Immunologic competence and genetic susceptibility of individuals as well as genetic variation of H. pylori DNA, for example, may play a role in the etiology of both atrophic gastritis and gastric cancer. In a study of 104 affected individuals from Costa Rica, one of the countries with the highest gastric cancer incidence and mortality rates in the world, expression of the H. pylori genes vacA s1b and vacA m1 was associated with gastric cancer and the expression of vacA m1 was associated with atrophic gastritis [39].

21.3.2 Diet

The role of dietary factors in the development of cancer is controversial. While many researchers and lay people instinctively believe that diet—being one of the most basic aspects of life and affecting all organ systems—is very likely to affect cancerogenesis, study data are controversial. For example, a prospective Swedish cohort study of 10,564 men with a mean follow-up of 11 years found no association between dietary intake of total, saturated, or monounsaturated fat, and risk of prostate cancer [40]. A nationwide study from Sweden found no reduced risk of breast cancer in women with regular fish consumption [41]. The prospective National Institutes of Health-AARP Diet and Health Study



Modified intention-to-treat meta-analysis of clinically important outcomes in selected studies of prophylactic vaccination against human papillomavirus (HPV)-related infection and disease. (Modified intention-to-treat analyses included study participants who received at least 1 dose of vaccine and who either were negative for relevant HPV types at enrolment [Harper, Villa, PATRICIA] or were randomly assigned to study group irrespective of their baseline HPV status [Mao, FUTURE I, FUTURE II].) CIN = cervical intraepithelial neoplasia.

Fig. 21.1 Clinical outcomes of HPV vaccination trials in an intention-to-treat meta-analysis. Adapted from [35].

investigated grain and fiber intake of 291,988 men and 197,623 women aged 50–71 years. Total dietary fiber intake was not associated with colorectal cancer. However, whole-grain intake was inversely associated with a moderately reduced colorectal cancer risk (Relative Risk 0.79; 95 % Confidence Interval 0.70–0.89) [42]. Other studies found an association between monounsaturated and polyunsaturated fatty acid intake and breast cancer. A population-based prospective cohort study including 61,471 women reported an inverse association with monounsaturated fat intake and a positive association between polyunsaturated fat intake and breast cancer. The reduction of relative risk for each 10-g increment in the daily intake of monounsaturated fat was 0.45 (95 % Confidence Interval 0.22–0.9) [43]. In accordance, a prospective cohort study of 90,655 premenopausal women, recruited within the Nurses' Health Study, found that the dietary intake of animal fat, mainly from red meat and high-fat dairy foods, during the premenopausal years is associated with an increased risk of breast cancer [44]. Unfortunately, dietary modification aimed at reducing dietary fat has not led to a reduced incidence of breast cancer and colon cancer in a large randomized trial of 48,835 postmenopausal women [45, 46]. In accordance, a controlled dietary modification was also insufficient to significantly

reduce the recurrence risk in a randomized trial of 3088 early breast cancer survivors [47]. Specifically, adoption of a diet that was very high in vegetables, fruit, and fiber, and low in fat did not reduce additional breast cancer events or mortality during a 7.3-year follow-up period. On the other hand, the randomized Women's Intervention Nutrition Study, launched in 1987, found a reduction of breast cancer recurrences among estrogen receptor-negative disease [48]. This randomized trial of 2437 women with early-stage breast cancer determined that low-fat dietary interventions can influence body weight and decrease breast cancer recurrence. Dietary fat intake was lower in the intervention than in the control group and relapse rates were 12.4 % in the control group versus 9.8 % women in the dietary group. However, this effect was restricted to estrogen receptor-negative disease. In summary, no clear and concise dietary strategy has emerged for the primary or secondary prevention of breast cancer.

Other dietary compounds potentially affecting cancer development are phytoestrogens found for example in soy. The sustained consumption of phytoestrogen-rich food correlates with a reduced incidence of breast cancer among Asians. Of note, this association has not been found in European and US populations [49]. Human dietary intervention trials have noted a direct relationship between phytoestrogen intake and a favorable hormonal profile associated with a decreased breast cancer risk. According to a systematic review, 22 case–control and cohort studies examined the incidence of breast cancer among women with and without a diet high in phytoestrogens. A meta-analysis of 21 of these studies among Asian populations found a significantly reduced incidence of breast cancer among past phytoestrogen users. Some, but not all randomized controlled trials document a beneficial effects of phytoestrogens on surrogate parameters such as bone mineral density, vasodilation, platelet aggregation, insulin resistance, and serum concentrations of triglycerides, high-density lipoprotein, and low-density lipoprotein. However, none of the available randomized controlled trials documents a protective effect of phytoestrogens for the clinical end point of site-specific cancer or overall cancer incidence [50]. No consistent evidence of an inverse association between phytoestrogen consumption and breast cancer incidence has been documented among US and European women [49, 50]. Epidemiologic and rodent studies suggest that breast cancer chemoprevention by dietary phytoestrogen compounds may be dependent on the ingestion of large amounts of phytoestrogens before puberty, when the mammary gland is relatively immature [49].

Some dietary components such as polyphenols in tea beverages may affect cancerogenesis, but the evidence supporting this assumption is controversial. Indirect evidence from epidemiologic studies suggests that the incidence of prostate cancer may be lower in populations with regular tea consumption [51].

Adherence to a Mediterranean diet has been associated in observational studies with a reduced incidence and mortality of some cancers. In a meta-analysis of 21 cohort studies including 1,368,736 subjects and 12 case–control studies with 62,725 subjects, adherence to a Mediterranean diet resulted in a significant reduction of overall cancer mortality (10 % reduction) and colorectal cancer (14 % reduction), prostate cancer (4 % reduction), and aerodigestive cancer (56 % reduction) incidence. Nonsignificant changes could be observed for breast cancer, gastric cancer, and pancreatic cancer [52]. However, there is no controlled evidence regarding a cancer-preventing effect of the Mediterranean diet. Thus, it remains unclear, what the exact dietary strategy for cancer prevention should be and whether or not diet is an independent factor or works only within certain cultural, genetic, or climate conditions.

21.3.3 Alcohol

Alcohol features prominently among dietary factors associated with sporadic cancer incidence. However, the biologic effect of alcohol on cancerogenesis is unclear. It has been hypothesized that one mechanism implicated in alcohol-related carcinogenesis may be oxidative stress from alcohol metabolism, inflammation, and increased iron storage. Ethanol-induced cytochrome P-450 2E1 produces various reactive oxygen species, leading to the formation of lipid peroxides such as 4-hydroxy-nonenal. Furthermore, alcohol impairs the antioxidant defense system, resulting in mitochondrial damage and apoptosis. Chronic alcohol exposure elicits tissue hyperregeneration due to the activation of tissue-specific survival factors and interference with the retinoid metabolism. In addition, direct DNA damage may result from acetaldehyde, which can bind to DNA, inhibit DNA repair systems, and lead to the formation of carcinogenic exocyclic DNA adducts. Finally, chronic alcohol abuse interferes with methyl group transfer mechanisms and may thereby alter gene expression [53].

A wide variety of cancers are found at an increased incidence among chronic alcohol users. In case–control studies, the association is dose-dependent, but varies with different cancer sites. Associations between alcohol consumption and an increased risk of cancer have been reported for breast, lung, and ovarian cancer in case–control and prospective cohort studies.

For example, consumption of 20 g or more of alcohol per day increases the risk of breast cancer by 70 % compared to nondrinkers. It is of note that even moderate levels of alcohol intake, i.e., 8 g per day, are sufficient to increase the risk of breast cancer by 50 % [54]. In a large prospective Danish cohort of 13,074 women, an alcohol intake of more than 27 drinks per week increased breast cancer risk more than threefold among premenopausal women irrespective of

alcohol type. In postmenopausal women, an intake of more than six spirits per week increased breast cancer risk over twofold [55]. This effect may be limited to estrogen receptor positive breast cancers and may also be enhanced by the concomitant use of hormone replacement therapy [56]. Carpenter et al. described a statistically significant 1.8-fold increased risk of lung cancer for one or more drinks of hard liquor per day from ages 30 to 40 years after adjusting for other risk factors such as smoking [57]. Serous invasive ovarian cancer has also been associated with alcohol consumption. Interestingly, this effect was restricted to early adult regular drinking suggesting an increased susceptibility of ovarian epithelial tissue to alcohol-induced genetic damage during the early reproductive phase [58].

21.3.4 Radiation

The cancerogenic effect of ionizing radiation based on increased mutagenesis, DNA adducts, and chromosome strand breaks has been established since the early twentieth century. Chronic low-dose exposure, as well as acute high-dose exposure, to ionizing radiation increases the incidence of various forms of malignancies including solid cancers. In a long-term study of 105,448 survivors of the Hiroshima and Nagasaki nuclear bomb explosions, 17,448 first primary cancers were diagnosed between 1958 and 1998 [59]. Solid cancer rates increased by 35 % per Gray for men, and by 58 % per Gray for women at age 70, after exposure at age 30. Significant radiation-associated increases in risk were seen for most sites including oral cavity, esophagus, stomach, colon, liver, lung, non-melanoma skin, breast, ovary, bladder, nervous system, and thyroid, as well as all histologic subgroups of these cancers.

Nuclear power plant accidents such as those in Three Mile Island, Chernobyl, and Fukushima are associated with different cancer risks compared to atomic bomb explosions. In the region around Chernobyl, for example, more than five million people may have been exposed to excess radiation, mainly through contamination by iodine-131 and cesium isotopes. Interestingly, studies evaluating leukemia and non-thyroid solid cancers have not shown consistently elevated risks in the regions around Chernobyl. In the population around Three Mile Island, there was a notable temporary increase in cancer diagnoses in the years immediately after the accident, but this increase may have been the result of intensified cancer screening in the area. Long-term follow-up has shown no increases in cancer mortality [60].

Low-dose long-term exposure also leads to a significant increase in solid cancer risk, as demonstrated by the Techa River cohort study [61]. This investigation of 446,588 person-years of individuals exposed to environmental radiation releases associated with a Soviet nuclear weapons facility in the Southern Urals found a significant dose response with 3 % of solid cancers attributable to radiation exposure.

Another form of electromagnetic waves, i.e., ultraviolet (UV) radiation, has also been associated with sporadic cancer risk. Chronic exposure to solar UV radiation is the most important risk factor of non-melanoma skin cancer [62]. The typical UV-induced DNA damage consists of the generation of dimeric photoproducts between adjacent pyrimidine bases. Both UV-A and UV-B radiation induce pyrimidine dimers and promote the production of oxygen and nitrogen species with subsequent damage to DNA, proteins, and lipids. One of the typical genetic targets of UV radiation-induced mutations is the tumor suppressor gene p53. In addition, UV radiation has an immunosuppressive effect adding to its carcinogenic activity [62]. UV radiation also promotes the development of cutaneous malignant melanoma, but the mechanisms of this effect are less clear.

Surprisingly, UV radiation also has cancer protective effects. Of note, nearly 20 types of cancer have been found to be inversely associated with solar UV-B exposure. In a meta-analysis of second cancers after non-melanoma skin cancer, the risk of subsequent colon, gastric, rectal, cervical, and esophageal cancers was significantly reduced [63]. These data suggest that UV-B exposure may be protective against many internal cancers possibly due to increased vitamin D production.

21.3.5 Chemical Agents and Smoking

Exposure to chemical agents is associated with a wide variety of sporadic cancers. Polycyclic aromatic hydrocarbons and benzopyrenes, for example, induce cancer and sarcoma in murine models [64, 65]. These chemical agents may enter the body directly via environmental exposure or via specific vehicles such as cigarette smoke. Cigarette smoke contains potent carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, benzopyrene, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine [66]. In a case–control study of nine cancer centers from six European countries, smoking cigarettes at any time was associated with a fivefold increase in lung cancer risk and current smoking increased the risk ninefold. The association was dose-dependent and time-dependent. A significant excess risk of 70 % was associated with every ten pack-years smoked. After 10 years of smoking cessation, the relative risk decreased to 20 % compared to current smokers. Adverse characteristics were inhalation of smoke, smoking non-filter cigarettes, smoking dark-type cigarettes, and starting smoking at young age. The typical histologic type of lung cancer associated with cigarette smoke was small-cell lung cancer. In absolute terms, the proportion of lung cancer cases in the population attributable to cigarette smoking varied strongly, ranging from 14 % to 85 % [67].

Active and passive cigarette smoking is associated with a significantly increased risk of lung cancer as well as other forms of cancer, e.g., bladder cancer and head and neck cancer. In a prospective cohort study of 120,852 adult subjects, current cigarette smokers had a threefold higher bladder cancer risk compared to nonsmokers. Ex-smokers also experienced a twofold increased risk. In absolute terms, half of all cases of male bladder cancer and one-fifth of female bladder cancer were attributable to cigarette smoking [68]. In a prospective cohort study of 476,211 individuals, cigarette smoking was a strong risk factor of head and neck cancer with hazard ratios of 12.9 in women and 5.4 in men. Ever-smoking accounted for 45 % of head and neck cancers in men and 75 % in women, suggesting causality [69].

Asbestos is a chemical carcinogen prominent due to its past widespread use in fire protection devices in industry and construction. One of the malignancies associated with asbestos exposure is mesothelioma. In a South East English study of 5753 affected individuals, the highest incidence rates of mesothelioma were found along the Thames river and its estuaries reflecting areas of asbestos use in shipbuilding and industry in the past [70].

Cadmium is a toxic metallic trace element which is capable of blocking oxidative phosphorylation. Exposure to environmental cadmium increases cancer incidence and cancer-specific mortality. In a 20-year longitudinal study of Japanese living in a cadmium-polluted area, cancer-specific mortality was more than doubled (Rate Ratio 2.58; 95 % Confidence Interval 1.25–5.36) [71].

Chemical agents may influence carcinogenesis by inducing chronic inflammation. In an Australian case–control study of 1576 women with invasive and low malignant potential ovarian cancers and 1509 population-based controls, the use of talcum powder in the pelvic region was associated with a small, but statistically significantly increased risk [72].

21.3.6 Hormonal Factors

Hormonal factors play a major role in the development of sporadic cancer. Both cancer-promoting and cancer-preventing effects have been described for a variety of hormones and a variety of cancers. One of the most widely studied cancers with respect to the etiologic role of hormones is breast cancer. Epidemiologic studies link several factors related to estrogen production to an increased risk of breast cancer. These include early menarche, late menopause, obesity, use of postmenopausal hormone therapy, and plasma estradiol levels. Estrogens and estrogen metabolites may mediate breast cancer via estrogen receptor-mediated stimulation of breast cell proliferation with a concomitant enhanced rate of mutations or via genotoxic estradiol metabolites with a resulting increase in DNA mutations. Estradiol metabolites can cause DNA damage by formation of estradiol-adenine-guanine adducts which are released from the DNA backbone leaving depurinated sites which undergo error prone DNA repair. In addition, mutations and oxygen free radicals may be caused by redox cycling of 4-OH estradiol to the 3–4 estradiol quinone and back conversion to 4-OH estradiol. If one or both pathways are operative, sufficient numbers of mutations accumulate over a long period of time to induce neoplastic transformation [73]. From a clinical perspective combined estrogen/progestogen supplementation use has been demonstrated to significantly increase the incidence of breast cancer in a large randomized trial, the WHI study [74]. Of note, this was not the case when estrogen, i.e., conjugated equine estrogens, was used as a monotherapy [14]. Thus, the combination of exogenous estrogens and progestogens is cancerogenic, a fact that has been attributed to interactions between progestogens and the RANKL (receptor activator of NF-κB ligand)/RANK system [75]. It has been demonstrated that the in vivo administration of the progestogen medroxyprogesterone acetate triggers induction of the key osteoclast differentiation factor RANKL in mammary-gland epithelial cells. Genetic inactivation of the RANKL receptor RANK in mammary-gland epithelial cells prevented MPA-induced epithelial proliferation.

Antiestrogens such as tamoxifen and raloxifen have been shown to effectively prevent breast cancer in randomized trials [76]. Estrogens and antiestrogens also appear to play a role in other cancers, for example in lung cancer. In a Canadian case–control study, exposure to antiestrogens was associated with a significantly decreased mortality in those exposed both before and after the diagnosis of non-small-cell lung cancer [77].

Another line of evidence highlights an emerging role of estrogens in the initiation and progression of different malignancies through their interaction with stem cells [78]. Estrogens are involved in increasing hematopoietic stem cell self-renewal in female subjects and more specifically during pregnancy. It is likely that normal and tumor thyroid tissues, which express estrogen receptors, could be subject to the same mechanism of estrogen action [79].

Other hormonal compounds which have been associated with cancer development include growth hormone and endocrine disruptors. Growth hormone treatment has been found to be associated with an increased risk of central nervous system malignomas in some studies [80]. However, the etiologic role of growth hormone with regard to central nervous system malignomas is controversial, since other studies did not confirm an increased cancer risk among recipients of growth hormone therapy. Several epidemiological, cellular, and molecular studies demonstrate a role of environmental chemicals with endocrine disrupting activities in the pathogenesis of numerous diseases including cancer [81], but their exact role has yet to be defined.

Hormones can act as powerful protectors against some cancers and at the same time as promoters of other cancers. This pleiotropic role of hormones is best illustrated by oral contraceptives. These compounds consist of the synthetic estrogen 17-beta-ethinyl-estradiol and a progestogen (e.g., levonorgestrel, dienogest, cyproterone acetate, chlormadinone acetate, etc.). Oral contraceptives are the most widely used hormones among women. Thus, epidemiologic data are available demonstrating clear cancer-promoting and cancer-preventing effects.

In a systematic review to estimate associations between oral contraceptive use and breast, cervical, colorectal, and endometrial cancer incidence, 44 breast, 12 cervical, 11 colorectal, and nine endometrial cancers studies were included [82]. Breast cancer incidence was slightly but significantly increased in users and the risk of cervical cancer was increased with duration of oral contraceptive use in women with human papillomavirus infection. Compared with never-use, ever-use of oral contraceptives was significantly associated with decreases in colorectal and endometrial cancers.

21.4 Genetic Susceptibility

21.4.1 Genetic Susceptibility and Sporadic Cancers

The number of reports investigating sporadic cancer susceptibility based on the carriage of low-penetrance, high-frequency polymorphisms has steadily increased over the last years. Evidence based on meta-analyses of individual case–control studies is accumulating defining specific individual variations in cancer susceptibility. Polymorphisms and mutations leading to functionally relevant biochemical consequences are capable of influencing the development and clinical course of cancer. It has become clear that sporadic cancer as well as many other diseases is the result of a complex interaction between environmental factors and various different genes as well as gene–gene interactions. Consequently, typical phenotypes do not exhibit classic Mendelian inheritance patterns attributable to a single gene locus. Moreover, different genes seem to act in various combinations, thus causing individual degrees of susceptibility in a given person. A polygenetic background has been proposed for many quantitative traits such as hypertension and diabetes, and more generally, for complex traits such as cancer.

21.4.2 Methodological Issues

Besides whole genome searches and gene expression profiling, genetic association studies are used to identify candidate genes with a possible etiologic role in a given disease. Furthermore, genetic association studies can identify specific genetic variations which may be used clinically to describe an increased or decreased risk to develop the condition as well as to describe an altered response to a treatment intervention. In these studies, single nucleotide polymorphisms, i.e., nucleotide deletions, insertions, and substitutions, are used. In addition, microsatellite markers, i.e., replications of specific nucleotide combinations, may be used. Genetic association studies do not prove an etiologic link between the polymorphism and/or the gene in question and the investigated disease. Methodological reasons account for that shortcoming, e.g., low sample size, chance findings, multiple comparisons and subsequent type I error inflation, ethnic background of the study subjects, varying disease definitions and inclusion/exclusion criteria, and ascertainment bias. Therefore, interpretation of studies with positive associations between genetic polymorphisms and endometriosis need to be interpreted with caution.

21.4.3 Polymorphisms and Specific Cancers

The amount of evidence with respect to genotype–phenotype correlations has increased substantially over the last years. These data indicate that a number of polymorphisms in genes associated with estrogen metabolism, cell cycle control, and xenobiotic metabolism are markers of overall cancer risk with the effects of all of these polymorphisms strongly depending on ethnic background. A meta-analysis of seven case–control studies and 4284 individuals established a variant of the Type I Transforming Growth Factor Beta Receptor (TGFBR) gene (TGFBR1*6A) as a risk factor of a variety of cancers [83]. Specifically, the analysis shows that homozygous carriers of this polymorphism have a more than twofold (Odds Ratio 2.53; 95 % Confidence Interval 1.39–4.61) increased risk of cancer. Analysis of specific cancer types showed that TGFBR1*6A carriers are at significantly increased risk of developing breast, ovarian, and colon cancer as well as hematological malignancies. Carriage of specific polymorphisms also modulates response to therapy and survival of cancer patients. A polymorphism of the mannose binding lectin 2 gene (MBL2), for example, is associated with lung cancer survival, dependent on ethnic background and smoking history [84]. Rylander-Rudqvist et al. performed a case–control study of 1521 cases and 1498 controls, and reported that a variant of the CYP1B1 gene (CYP1B1*3) is a genetic risk modifier of long-time hormone replacement therapy. Women who had used menopausal hormone replacement therapy for 4 years or longer and carried the CYP1B1*3/*3 genotype had a twofold increased risk of developing breast cancer [85].

One of the most intensely studies cancer types with respect to polymorphisms is breast cancer. Breast cancer has a strong genetic component, but most cases of breast

cancer are sporadic and do not occur in families with a characteristic pedigree compatible with BRCA-mutations. On the other hand, a first-degree relative of a woman with breast cancer has a twofold increased risk of developing breast cancer herself. Therefore, it is reasonable to speculate that low-penetrance susceptibility genes may play a role in the biological history of sporadic breast cancer. A number of candidate genes have been identified in this respect, most of which are involved in estrogen metabolism and cell growth regulation.

Over 40 association studies investigating polymorphisms of estrogen metabolizing genes and genes involved in cell cycle control and metabolism of xenobiotics have been published. Dunning et al. investigated most of the studies published until 1999 and combined the results of 46 of these individual studies investigating over 10,000 women [86]. They found that statistically significant differences in genotype frequencies were present for a polymorphic marker of the aromatase gene [CYP19 $(TTTA)_{10}$ microsatellite], the GSTP Ile105Val and GSTM1 gene deletion polymorphisms, and the TP53 Arg72Pro polymorphism. In addition, meta-analyses of case–control studies indicate that TGFBR1*6A, HRAS1, GSTP Ile105Val, and GSTM1 SNPs are low-penetrance genetic risk factors of sporadic breast cancer. Nested case–control studies within the prospective Nurses' Health Study found seven polymorphisms—hPRB +331G/A, AR CAG repeat, CYP19 (TTTA)10, CYP1A1 MspI, VDR FOK1, XRCC1 Arg194Trp, and XRCC2 Arg188His—to be small but significant risk factors for sporadic breast cancer [87]. As demonstrated by all of these studies, a wide variety of polymorphisms seems to confer a small, but statistically significant risk of breast cancer. However, common genetic variants associated with breast cancer only modestly improve the performance of traditional risk models for breast cancer. This has been demonstrated in a study using traditional risk factors and ten common genetic variants associated with breast cancer in 5590 case subjects and 5998 control subjects, 50–79 years of age, from four US cohort studies and one case–control study from Poland [88]. Next-generation sequencing has led to a further explosion of discoveries of novel genetic mutations that reveal a promiscuity of genotype–phenotype relationships. Although such discoveries may ultimately revolutionize clinical care in the future, at this time, genetic susceptibility testing based on mutation/polymorphism panels is not recommended [89].

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Hereditary Cancer

22

Zoran Gatalica, Anatole Ghazalpour, Stan L. Lilleberg, Jeffrey Swensen, and Henry T. Lynch

22.1 Introduction

The first systematic documentation of a hereditary cancer family was reported over 100 years ago [1] by Aldred Scott Warthin, M.D., Ph.D. Dr. Warthin (Fig. 22.1) was truly a Renaissance man of his time. At age 21, he graduated from the Cincinnati Conservatory of Music. He was a musician, teacher, writer, editor, poet, and linguist. As a pathologist he was a remarkably creative physician-scientist. In 1895, he completed postgraduate work in pathology in Germany, and joined the pathology department at the University of Michigan. While he was setting up his household in Ann Arbor, he wondered why his seamstress was so depressed. She told him that her depression was due to the fact that she knew she would die of cancer of the bowels or female organs, since "… *everyone in my family dies of those cancers* …" Warthin listened carefully and began compiling her family history, in particular recording the pathology, and in 1913, he published [1] the pedigree of what he called Family G (Fig. 22.2). Finally, just as his seamstress had predicted, she died at an early age of metastatic endometrial carcinoma.

Half a century later, Lynch et al. [2] described two Midwestern families with clinical features strikingly similar to Warthin's Family G. Individuals in these families had early-onset colorectal cancers and a litany of other cancer types, particularly gynecologic and gastric. Warthin's successor as chairman of pathology in Ann Arbor, Michigan, invited Lynch to take possession of Warthin's documents, which included detailed scrolls of the pedigree of Family G (Fig. 22.2), slides and cancer tissue blocks. An update of Family G was published in 1971 [3]. The family's mismatch repair (MMR) gene mutation was finally identified in 2000 in a splice site of the *MSH2* gene [4]. In 2005, a description of over a century of work on Family G was published [5]. Importantly, the pattern of cancer has continued relentlessly in Family G in accord with an autosomal dominant mode of genetic transmission.

With the continued efforts of many scientists it was finally accepted that cancer was a genetic disease. In fact, to reflect the recent revolution in cancer research and the discoveries made, it should be stated that cancer is a genomic disease, or a disease of the genome [6]. Over the last 10–15 years, breakthroughs in the identification of many important genes have significantly improved our understanding of cancer pathogenesis. At the foundation of this discovery era was the wealth of information provided by the Human Genome Project.

The intent of this chapter is to review the genes known to be associated with hereditary forms of cancer/cancer syndromes, and highlight examples of pathways in which the products of these genes interact. We also indicate where current knowledge is limited and where further investigation may be warranted.

22.2 Cancer Development

According to current models, tumor development is a continuous process of mutation accumulation that ultimately leads to cellular autonomy, unlimited growth and metastasis [7]. One of the most important events in this process is the initial destabilization of homeostasis that allows mutations to accumulate in genes that regulate cell proliferation, development, and differentiation. The genetic mechanisms involved in the earliest stages are not clear, but certain phenotypic features are notable. The cancer cell phenotype can be described by six hallmark features: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, avoidance of programmed cell death, limitless replicative

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_22

potential, sustained angiogenesis, tissue invasion, and metastasis [7]. Generally it has been thought that three to seven successive mutations are required for the malignant conversion [8–10]. Various sources of spontaneous DNA damage are estimated to alter about 25,000 bases per human genome per cell per day out of the 3×10^9 base pairs in the genome [11]. However, cells are endowed with specific mechanisms for repairing the damage and for maintaining mutations at reasonable levels. Consequently, the mutational load at any given time in both germ-line and somatic cells can be viewed as a dynamic equilibrium between the extent of DNA damage and the efficiency of DNA repair [12]. In principle, the likelihood that a single cell will acquire enough independent mutations necessary for malignant cancer development is quite low and statistically unlikely, given that the normal mutation rate is approximately 1.4×10^{-10} mutations per base pair per cell generation [13]. However, it is now well recognized that this theory is contradicted by the rate of incidence of various cancers. Not surprisingly, cancer biology and genetics have found a way around these statistical predictions. It has been suggested that two major mechanisms could result in higher mutation rates that would make cancer development more probable. First, highly selective mutations could occur that enhance cell proliferation, creating an expanded target cell population for the next mutation [14]. Second, mutations could occur that affect the stability of the entire genome at the DNA and/or the chromosomal level, thereby increasing the overall mutation rate [13, 15].

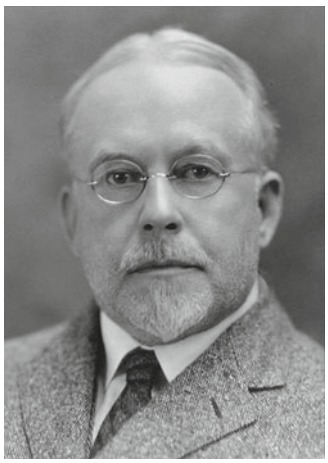

Fig. 22.1 Aldred Scott Warthin, M.D., Ph.D., (1866–1931) pioneer in hereditary cancer research.

Tumorigenesis is associated with alterations (mutations) in two main classes of genes—oncogenes and tumor-suppressor genes—each of which can drive neoplasia by increasing the tumor cell burden through stimulating cell division or inhibiting cell death or cell-cycle arrest.

Less than 10 % of patients with cancer have highly penetrant inherited (germ-line) mutations that predispose to cancer. Germ-line mutations of genes known to be responsible for inherited forms of cancer and their syndromes are summarized in Table 22.1. A mutation within this context is defined as any change in the sequence of the gene, including single base pair substitutions, large or small deletions/insertions and amplifications or translocations of chromosomal segments. In the germ line, the most common mutations are subtle (point mutations or small deletions/insertions), whereas all types of mutation can be found in tumor cells.

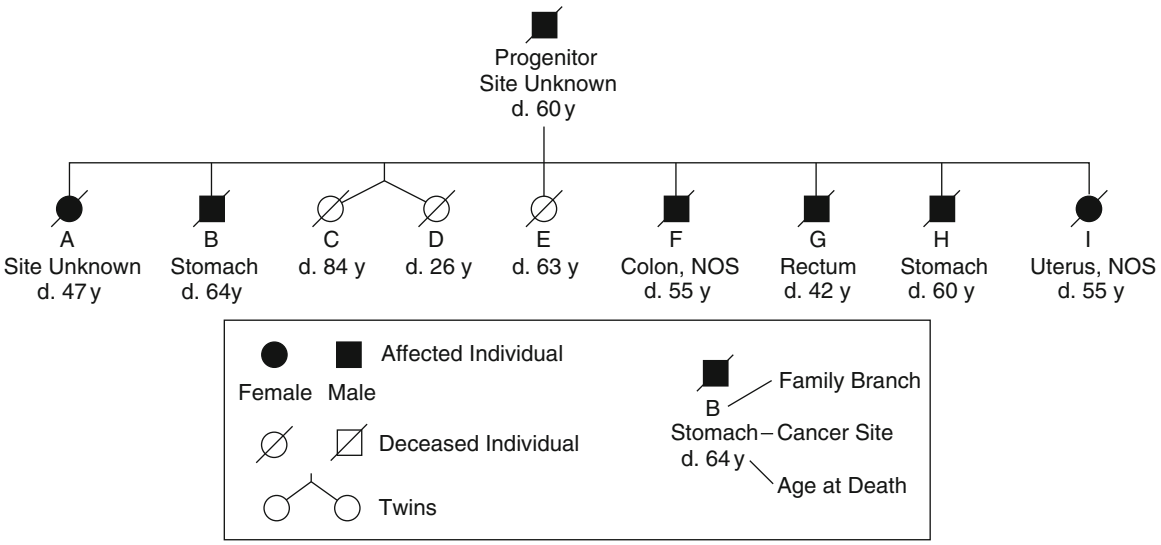

Fig. 22.2 Pedigree family G. This family was described in the first published systematic evaluation of a hereditary cancer syndrome, now known to fit criteria for Lynch syndrome. This is an abridged version of the pedigree since the updated publication contained an extremely large number of individuals. In this figure, each individual (A to I) represents a branch of the family; blackened symbol represents affected branches.

Table 22.1 Overview of inherited cancer syndromes

Cancer gene category	Syndrome	Gene (alias)	Gene location	OMIM ID	Major tumor types
Tumor-suppressor genes (genomic stability and repair genes)					
	Ataxia telangiectasia	*ATM*	11q22.3	208900	Leukemias, non-Hodgkin lymphomas
	Bloom syndrome	*BLM*	15q26.1	210900	Leukemia, lymphoma
	Hereditary breast and ovarian cancer	*BRCA1, BRCA2*	17q21, 13q12.3	113705, 600185	Carcinomas of breast and ovary
	Fanconi anemia	*FANCA, C or G in 90% cases*	16q24.3, 9q22.32, 9p13.3	607139, 613899, 602956	Acute myeloid leukemia, solid cancers
	Lynch syndromes (HNPCC, Lynch I, Lynch II, Lynch III, Turcot and Muir–Torre syndromes)	*MLH1, MSH2, MSH6, PMS2*	3p21.3, 2p22-p21, 2p16, 7p22	120436, 120435, 600678, 600259, 609309, 609310, 614350, 614337	Colon adenocarcinoma, uterus endometrial adenocarcinoma; skin sebaceous tumors (Muir-Torre); biallelic inheritance results in pediatric lymphomas and brain tumors (Lynch III)
	Attenuated polyposis (MAP)	*MUTYH*	1p34.3-p32.1	608456	Colon adenomas and adenocarcinomas
	Nijmegen breakage syndrome	*NBS1*	8q21	251260; 613065, 609135	Lymphoma, leukemia
	Rothmund–Thomson syndrome	*RECQL4*	8q24.3	268400	Bone osteosarcoma
	Werner syndrome	*WRN*	8p12-p11.2	277700	Meningiomas, soft tissue sarcomas
	Xeroderma pigmentosum	*XPA, XPC, ERCC2, ERCC3, ERCC4, ERCC5, DDB2, POLH*	9q22.3, 3p25, 19q13.2-q13.3, 2q21, 16p13.3-p13.13, 13q33, 11p12-p11, 6p21.1	278700, 278720, 126340, 133510, 133520, 133530, 600811, 603968	Non-melanoma skin cancer (basal cell and squamous cell carcinomas) and cutaneous melanoma
Tumor-suppressor genes (other)					
	Familial adenomatous polyposis (FAP), desmoid disease, Turcot syndrome	*APC*	5q21-q22	175100; 135290	Adenomas and adenocarcinomas of colon, small intestine (and CNS for Turcot syndrome)
	Oligodontia–colorectal cancer syndrome	*AXIN2*	17q23-q24	604025;608615	Colon adenomas and adenocarcinomas
	Juvenile polyposis	*BMPR1A, SMAD4*	10q22.3, 18q21.2	174900; 601299, 600993	Juvenile polyps in gastrointestinal tract
	Familial gastric carcinoma	*CDH1 (E-cadherin)*	16q22.1	192090; 137215	Diffuse gastric, and lobular breast carcinoma
	Familial atypical multiple mole melanoma	*CDKN2A (P16/INK4A, p14/ARF)*	9p21.3	600160; 155601	Cutaneous melanoma, pancreatic adenocarcinoma
	Familial cylindromatosis (Brooke–Spiegler syndrome)	*CYLD*	16q12.1	605041; 605018	Cylindromas of skin
	Hereditary multiple exostoses	*EXT1, EXT2, EXT3*	8q24.11-q24.13, 11p12-p11, 19p	133700; 608177; 133701; 608210; 600209	Osteochondromas
	Hereditary leiomyomatosis and renal cell carcinoma (HLRCC)	*FH*	1q43	136850; 150800	Leiomyomas of skin and uterus; renal carcinoma
	Birt–Hogg–Dube syndrome	*FLCN (BHD)*	17p11.2	135150; 607273	Diverse renal tumors, skin fibrofolliculoma

(continued)

Table 22.1 (continued)

Cancer gene category	Syndrome	Gene (alias)	Gene location	OMIM ID	Major tumor types
	Simpson–Golabi–Behmel syndrome	*GPC3*	Xq26	300037; 312870	Wilms tumor, hepatoblastoma
	Hyperparathyroidism jaw-tumor syndrome	*HRPT2 (CDC73)*	1q31.2	145001; 607393	Parathyroid adenoma and carcinoma, ossifying fibroma of the jaw
	Multiple endocrine neoplasia type 1	*MEN1 (menin)*	11q13.1	131100; 613733	Parathryoid and pituitary adenomas, pancreatic islet cell tumors
	Neurofibromatosis type 1, Neurofibromatosis–Noonan syndrome, Watson syndrome, Juvenile myelomonocytic leukemia (JMML)	*NF1 (neurofibromin)*	17q11.2	162200; 613113; 601321;162210; 193520; 607785	Neurofibroma
	Neurofibromatosis type 2	*NF2*	22q12.2	101000; 607379	Bilateral vestibular schwannomas; meningiomas
	Basal cell nevus syndrome (Gorlin syndrome)	*PTCH1, PTCH2, SUFU*	9q22.3, 1p34.1, 10q24.32	109400; 601309; 603673; 607035	Basal cell carcinomas of skin
	Cowden disease (CD)/ Bannayan–Riley–Ruvalcaba syndrome (BRRS)	*PTEN*	10q23.31	153480; 158350; 601728	Breast, thyroid and endometrial carcinomas; skin trichilemmoma GI hamartomas
	Carney complex type I	*PRKAR1A*	17q24.2	160980; 188830	Myxomas of the skin, heart and breast
	Hereditary retinoblastoma	*RB1*	13q14.1-q14.2	180200; 614041	Retinoblastoma (usually involving both eyes)
	Familial paraganglioma–pheochromocytoma syndromes caused by SDHB, SDHC, and SDHD mutations	*SDHB, SDHC, SDHD*	1p35-36.1, 1q21-23, 11q23	606864; 115310; 171300; 185470; 602413; 602690; 168000	Multiple bilateral paragangliomas of the neck
	Peutz-Jeghers syndrome	*STK11 (LKB1)*	19p13.3	175200; 602216	Stomach and intestinal hamartomatous polyps and adenocarcinomas
	Medulloblastoma predisposition	*SUFU*	10q24.32	607035; 155255	Medulloblastoma
	Li–Fraumeni syndrome, Hereditary Adrenocortical Carcinoma	*TP53 (p53)*	17p13.1	191170; 151623; 202300	Breast cancer, bone and soft tissue sarcomas
	Tuberous sclerosis, lymphangioleiomyomatosis (LAM)	*TSC1 (hamartin), TSC2 (tuberin)*	9q34.13, 16p13.3	191092; 605284; 606690	CNS cortical hamartomas, skin subungual keratomas, renal angiomyolipomas
	von Hippel–Lindau syndrome	*VHL*	3p25.3	608537; 193300	Renal clear cell carcinoma, CNS capillary hemangioblastoma
	WAGR, Denys–Drasch, Frasier syndrome, and non-syndromic hereditary Wilms' tumor	*WT1*	11p13	607102; 194070; 194080; 136680	Wilms' tumor (nephroblastoma)
Oncogenes					
	Familial malignant melanoma	*CDK4*	12q14.1	123829; 609048	Cutaneous melanoma
	Familial gastrointestinal stromal tumors	*KIT*	4q12	164920; 606764; 273300	Multiple gastrointestinal stromal tumors (GIST)

(continued)

Table 22.1 (continued)

Cancer gene category	Syndrome	Gene (alias)	Gene location	OMIM ID	Major tumor types
	Hereditary papillary renal cell carcinoma (HPRCC)	*MET*	7q31	164860; 605074	Multiple bilateral type I papillary renal cell carcinoma
	Familial gastrointestinal stromal tumors	*PDGFRA*	4q12	173490; 606764	Multiple gastrointestinal stromal tumors (GIST)
	Multiple endocrine neoplasia type II (A and B)	*RET*	10q11.2	164761; 171400; 162300; 142623; 188550	Medullary thyroid carcinoma, parathyroid hyperplasia, adrenal pheochromocytoma

Neoplasia is initiated by a mutation in a cancer gene that results in a clonal expansion, with subsequent mutations in additional genes resulting in further rounds of expansion as part of the process of tumor progression [16, 17]. An individual with a germ-line mutation in a cancer predisposition gene has an early start on tumor development, because a mutation that can contribute to cancer is already present in every cell. As such, these individuals may readily develop multiple tumors that are detectable at an early age. In many dominantly inherited cancer syndromes, the acquired first somatic mutation affects the normal copy of the gene inherited from the unaffected parent [18].

Under normal conditions, proto-oncogenes (the normal, non-mutated form of an oncogene) stimulate cell proliferation and differentiation. These genes mainly encode proteins involved in signal transduction pathways and include growth factors, growth factor receptors, signal transducers, and transcription factors (e.g., the *RET* and *MET* genes). When mutated (activated) these genes become oncogenes and gain the ability to dominantly promote neoplastic processes. In contrast, tumor suppressors are genes whose loss of function promotes malignancy [19, 20]. They are usually negative regulators of growth or other functions that may affect invasive and metastatic potential [21]. Tumor suppressor genes can be divided into two broad functional classes: (1) gatekeepers which directly regulate cell proliferation and are rate limiting for tumorigenesis; (2) caretakers which maintain the integrity of the genome and promote cancer growth indirectly by causing an increased mutation rate [22]. Interestingly, the most common forms of hereditary cancer predisposition are caused by inherited mutations of caretaker genes.

The contribution of tumor suppressors to tumorigenesis was initially proposed by Alfred Knudson in 1971, based upon epidemiological studies of retinoblastoma [23]. According to Knudson's two-hit model, both copies of a tumor suppressor gene need to be inactivated for tumor formation. In the familial form of a disorder associated with a tumor suppressor gene, one mutated allele is inherited from one of the parents and the second allele is somatically inactivated. When the second hit involves a large deletion or mitotic recombination (reduces the mutation to homozygosity), it is referred to as loss of heterozygosity (LOH). Knudson's model has later been applied to other forms of familial cancer as well [18, 24]. However, not all loss-of-function mutations in tumor suppressor genes are truly recessive [25]. Evidence suggests that haploinsufficiency (functional loss of one allele) of some genes might provide a growth advantage [26]. Thus, lower gene-dosage, rather than biallelic inactivation of a tumor suppressor gene may be sufficient to exert a cellular phenotype that leads to tumorigenesis. This may be particularly true for caretaker genes, where haploinsufficiency could result in mildly increased genomic instability [27].

In contrast to classical monogenic Mendelian disorders, such as Duchenne muscular dystrophy or cystic fibrosis, wherein mutations in a single gene cause disease, a single gene defect or alteration does not cause cancer. The vast majority of genetic alterations in cancer are somatic and found only in tumor cells. In an individual with a dominant cancer predisposition syndrome, all cells in the body are *a* priori heterozygous, carrying one mutated and one wild-type allele [19]. The germ-line mutation can be considered as the first hit required for tumorigenesis. The next genetic change in a cell often involves the loss of the remaining wild-type allele (second step in the Knudson's two hit hypothesis) [18]. Even though additional somatic changes are still needed, the presence of a constitutional mutation greatly quickens the process of malignant conversion, and individuals with such mutations are at higher risk of developing cancer. Mutations with strong effects are likely to cause early onset of the disease and development of multiple primary tumors; they are inherited in simple Mendelian fashion, tend to cause familial clustering of disease and show dominant pattern of inheritance [28, 29]. However, the cancers develop only after additional somatic genetic mutations occur. Importantly, clinicians must also consider the pattern of these multiple primary cancers (e.g., breast and ovarian cancers in carriers of *BRCA1* and

BRCA2 mutations; medullary thyroid carcinoma and pheochromocytoma in the carriers of a *RET* mutation, etc.) which could then guide them toward the correct diagnosis.

Most cancer susceptibility syndromes result from inherited mutations in tumor suppressor genes rather than proto-oncogenes [19]. Germ-line mutations in only a limited number of proto-oncogenes (e.g., *RET*, *MET, KIT, PDGFRA,* and *CDK4*) (Table 22.1), have been shown to be involved in hereditary cancer predisposition [30–32]. Although they are rare, these syndromes are of vast biological importance. The study of genes responsible for such disorders, and the cellular pathways disrupted by the mutated proteins, provides significant insights into the molecular origin and pathogenesis of both inherited and sporadic forms of cancer [28].

A subclass of tumor suppressor genes, known as caretakers or stability genes, promotes tumorigenesis in a completely different way when mutated. This group includes the mismatch repair (MMR), nucleotide-excision repair (NER), base-excision repair (BER), and homologous recombination repair genes responsible for correcting subtle errors made during normal DNA replication or induced by mutagenic exposure. In normal cells, genetic alterations are kept to a manageable minimum by stability genes, and mutation rates are much higher when these genes are inactivated [33]. As a result, all genes are potentially at risk for mutations, but only mutations in oncogenes and tumor suppressor genes will provide a selective advantage to the cancer cell.

From this, it is best to picture mutated cancer genes as contributors to cancers, rather than the sole cause. In most cases involving genes associated with hereditary cancer syndromes, the individual with the mutation faces a high probability of developing cancer, but not 100 % certainty. Penetrance is defined as the probability that a person who has the genotype will manifest the expected phenotype. For example, a carrier of a mutation in the *BRCA1* gene (breast and ovarian cancer susceptibility) has an approximate 70–85 % chance of developing breast cancer during their lifetime. Although this is a very high risk, the penetrance is incomplete and not everyone with a mutation will develop the disease. There are some important exceptions to incomplete penetrance; virtually all carriers of a pathogenic mutation in the *TP53* gene (Li–Fraumeni syndrome) or the *APC* gene (familial adenomatous polyposis coli) develop cancer. Differences in penetrance between carriers of a germ-line mutation can be associated with environmental exposures and an individual's genetic makeup (modifier genes).

22.3 Oncogenes

Oncogenes are altered (mutated) in ways that cause the gene product to be constitutively activated or active under conditions in which the normal gene (proto-oncogene) is not active. Activation of an oncogene can result from chromosomal translocation (*BCR-ABL)*, gene amplification (*MYC)*, or intragenic mutations (*RET*) affecting key functional moieties that regulate the activity of the gene product. Activation of an oncogene through the alteration of a single allele is usually sufficient to bestow a cell with a selective growth advantage. Examples of oncogenes associated with inherited cancer syndromes and the associated tumors are included in Table 22.1. Such genes include *RET* and *MET*, which encode receptor tyrosine kinases involved in essential processes such as cell cycle progression, proliferation, differentiation, motility, and survival [34]. Many oncogenic tyrosine kinases are constitutively activated due to specific gain-of-function mutations [34, 35].

22.3.1 RET Oncogene and Multiple Endocrine Neoplasia Type 2 (MEN 2)

One oncogene that has been studied extensively within the context of hereditary cancers is the REarranged during Transfection (*RET*) gene, which encodes a transmembrane receptor tyrosine kinase (RTK) [36, 37]. Normal *RET* is expressed mainly in developing and adult neural ectoderm. *RET* mutations are oncogenic owing to an intrinsic gain-of-function in the receptor tyrosine kinase activity. Germ-line mutations in *RET* are known to cause multiple endocrine neoplasia type II (MEN2), which consists of three distinguishable clinical phenotypes (MEN 2A, MEN 2B, and familial medullary thyroid carcinoma FMTC) [38–42]. MEN 2A is characterized by specific endocrine neoplasms (medullary thyroid carcinoma, pheochromocytoma and parathyroid adenoma); MEN 2B is characterized by neuromas of the lips and tongue, marfanoid habitus, medullary thyroid carcinoma, and pheochromocytoma; FMTC is characterized by medullary thyroid carcinoma in the absence of other tumors.

Almost all of the germ-line *RET* mutations that cause MEN2 are missense, but a small fraction represent small deletions or insertions that preserve the open reading frame. The mutations are concentrated in a fraction of the open reading frame. Most mutations occur in the cysteine-rich portion of the extracellular domain. Specific missense mutations with a highly focused distribution are characteristic of mutations that cause oncogenic gain-of-function, in this case in Ret RTK activity [43–45]. This contrasts with the *RET* mutations that cause familial Hirschsprung disease; such mutations cause loss of protein function and have a much broader distribution across the gene [46, 47]. Hirschsprung disease represents the main genetic cause of functional intestinal obstruction with an incidence of 1/5,000 live births. This developmental disorder is a neurocristopathy and is characterized by the absence of the enteric ganglia along a variable length of the intestine [48, 49]. Occasional MEN2 families with specific mutations express Hirschsprung disease.

22.3.2 RET Activation and Signaling Pathways

One of the two *RET* mutations commonly associated with MEN 2B results in the substitution of threonine for methionine at codon 918 (M918T) in the tyrosine kinase domain [50–52]. *RET* M918T, which also occurs somatically in some sporadic medullary thyroid carcinomas (MTC), leads to a Ret receptor that autophosphorylates in the absence of ligand stimulation [53]. The constitutive activation of the variant Ret RTK results in aberrant cell signaling through a series of cytoplasmic kinases, including JUN kinase, p38 mitogen activated protein kinase, Ras/extracellular signal-regulated kinase, phosphatidylinositol 3-kinase, and Akt [54].

The mechanisms of Ret signaling in tumorigenesis are known in considerable detail, since it is part of a well-studied family of RTKs. The Ret protein is a subunit in a plasma-membrane signaling complex that includes four ligands and four co-receptors [55]. The Ret intracellular domain has at least 12 autophosphorylation sites, and phosphotyrosine 1062 in particular is a binding site for many different docking proteins and is important for the oncogenic activity of Ret [56–59].

22.3.3 Genotype:Phenotype Correlations: RET:MEN 2

Activating mutations in *RET* can be inherited or somatic. Germ-line point mutations are causal for the dominantly inherited disorders MEN 2A, MEN 2B and FMTC [50, 51]. Mutations of cysteine residues in the extracellular domain of RET are frequently associated with MEN 2A (MTC, pheochromocytoma, and parathyroid hyperplasia). MEN 2B (MTC, pheochromocytoma, ganglioneuromas, and marfanoid habitus) is associated almost exclusively with mutations of codons 918 or 883 in the intracellular domain of RET. Genetic testing to identify patients at risk for MEN2 and FMTC has led to clinical screening and prophylactic thyroidectomy in mutation carriers [45, 60, 61].

More than ten somatic rearrangements of *RET* have been identified in papillary thyroid carcinomas [61]. These oncogenic mutations activate the Ret tyrosine kinase domain via different mechanisms, making Ret an excellent candidate for the design of molecular targeted therapy. Recently, various therapeutic approaches, such as tyrosine kinase inhibition, gene therapy with dominant negative *RET* mutants, monoclonal antibodies against oncogene products, and nuclease-resistant aptamers that recognize and inhibit Ret have been developed [62]. Two small molecule kinase inhibitors, vandetanib and cabozantinib, are approved for the treatment of locally advanced and metastatic medullary thyroid carcinoma [59].

22.3.4 MET Oncogene and Hereditary Papillary Renal Cell Carcinoma (HPRCC)

Recent studies have shown that renal cell carcinomas (RCC) are a clinically and genotypically heterogeneous group, consisting of different histologic types with different clinical courses, different responses to therapy, and different associated gene defects [63–65]. Clear cell RCC is the most common type (75 %), papillary RCC is 10 %, chromophobe RCC is 5 %, and the remainder is composed of rare histopathologic and molecular entities. Kidney cancer occurs in both sporadic (non-inherited) and hereditary forms. Most of what is known about the genetic basis of kidney cancer has been learned from study of the hereditary forms [66]. There are four well-defined hereditary syndromes associated with kidney cancer: (1) von Hippel–Lindau (VHL), (2) hereditary papillary renal cell carcinoma (HPRCC), (3) Birt Hogg Dubé (BHD), and (4) hereditary leiomyomatosis and renal cell carcinoma (HLRCC) [66, 67]. This discussion will focus on HPRCC, a cancer syndrome caused by mutations in the *MET* gene that are inherited in an autosomal dominant fashion [68]. HPRCC is highly penetrant and tends to have a late onset of clinical disease. However, an early-onset HPRCC phenotype has also been described. Affected individuals are at-risk of developing bilateral, multifocal, type 1 papillary renal carcinoma [68, 69]. Macroscopically, the tumors contain areas of hemorrhage, necrosis, and cystic degeneration (Fig. 22.3). Histologically, Delahunt and Eble have [70] classified papillary RCCs into type 1 (papillae covered by small cells, single layer) and type 2 (large cells with eosinophilic cytoplasm and pseudostratification). In chromosomal analysis, characteristic gains of chromosomes 7, and 17, and the loss of Y chromosome are frequently found in type 1 papillary RCCs, but the chromosomal aberration of type 2 papillary RCCs seems to be more heterogenous than that of type 1 papillary RCCs. Through genetic linkage analysis on affected families, mutations that activate the *MET* proto-oncogene were found to be the causal for HPRCC [68]. In addition, somatic missense mutations were found in the tyrosine kinase domain of the *MET* gene in the subset of sporadic (non-inherited) papillary renal carcinomas [69]. Three mutations were located in codons homologous to those in which activating mutations occur in the *KIT* and *RET* proto-oncogenes.

22.3.5 MET Activation and Signaling Pathways

Met receptor is activated through gene mutations or by binding of its natural ligand, hepatocyte growth factor/scatter factor (HGF/SF). Activation leads to a plethora of biological and biochemical effects in the cell (cell motility and

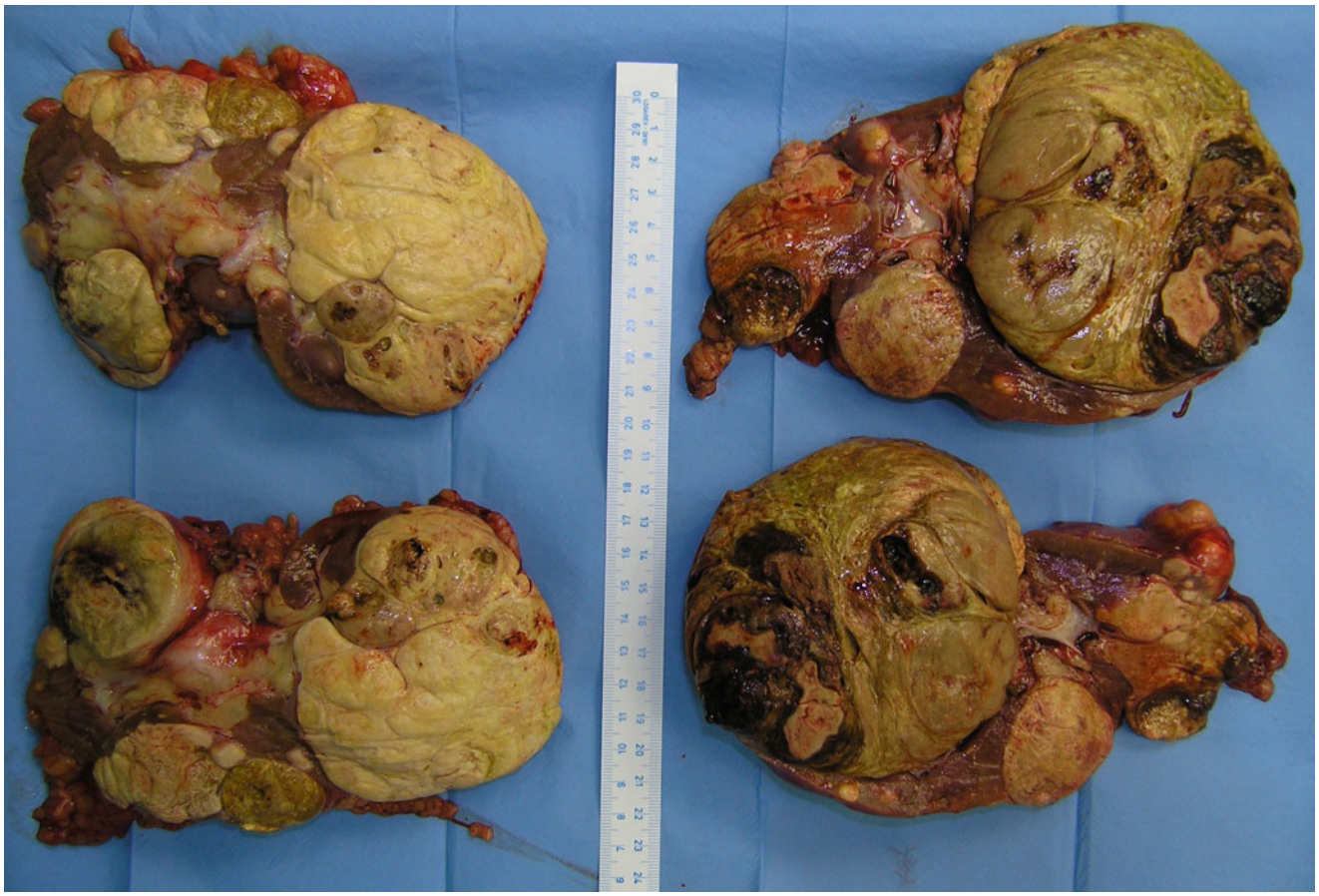

Fig. 22.3 Bilateral renal tumors in a young person with clinicopathologic characteristics of hereditary papillary renal carcinoma. Courtesy of Professor Milan Hora, M.D., Ph.D., (Pilsen, Czech Republic).

scattering, proliferation, branching morphogenesis, angiogenesis, invasion, and eventual metastasis) [71]. The unique biochemical and biological effect of these *MET* mutants has been investigated in several model systems, confirming their suspected oncogenic potential [72–77]. Upon HGF/SF binding, Met autophosphorylation occurs on two tyrosine residues (Y1234 and Y1235) within the activation loop of the tyrosine kinase (TK) domain, which regulates kinase activity. Phosphorylation on two tyrosine residues near the COOH terminus (Y1349 and Y1356) forms a multifunctional docking site that recruits intracellular adapters via Src homology-2 (SH2) domains and other recognition motifs, leading to downstream signaling [78, 79]. An intact multifunctional docking site is required to mediate transformation and induce a metastatic phenotype [80]. Among the many genes up-regulated by pathway activation relevant to cancer are those encoding proteases that regulate HGF/SF and Met processing and extracellular matrix remodeling, as well as *MET* itself, creating the potential for Met overexpression in otherwise normal target cells through persistent ligand stimulation [78].

Inappropriate Met signaling in disease can resemble developmental transitions between epithelial and mesenchymal cell types normally regulated by HGF/SF. While epithelial malignancies (carcinomas) frequently overexpress Met (Fig. 22.4) and paracrine delivery of HGF/SF results in dysregulated signaling, tumors of mesenchymal origin (sarcomas) arising from cells that normally express HGF/SF often acquire Met expression resulting in autocrine Met signaling [79, 80].

Beyond missense mutations, it is important to note that trisomy of chromosome 7, which contains both the *MET* and *HGF/SF* genes, occurs in the majority of sporadic papillary RCC [81]. A detailed study of trisomy 7 in hereditary papillary RCC revealed nonrandom duplication of the mutant *MET* allele in 100 % of tumor samples [82]. Overall, *MET* mutation occurs at a much lower frequency than most other mechanisms of pathway activation in human cancers. Nonetheless, mutations provide strong direct evidence of the pathway's oncogenic potential and a means to identify patient subpopulations that might benefit most from Met-targeted therapy. The current understanding of Met-mediated

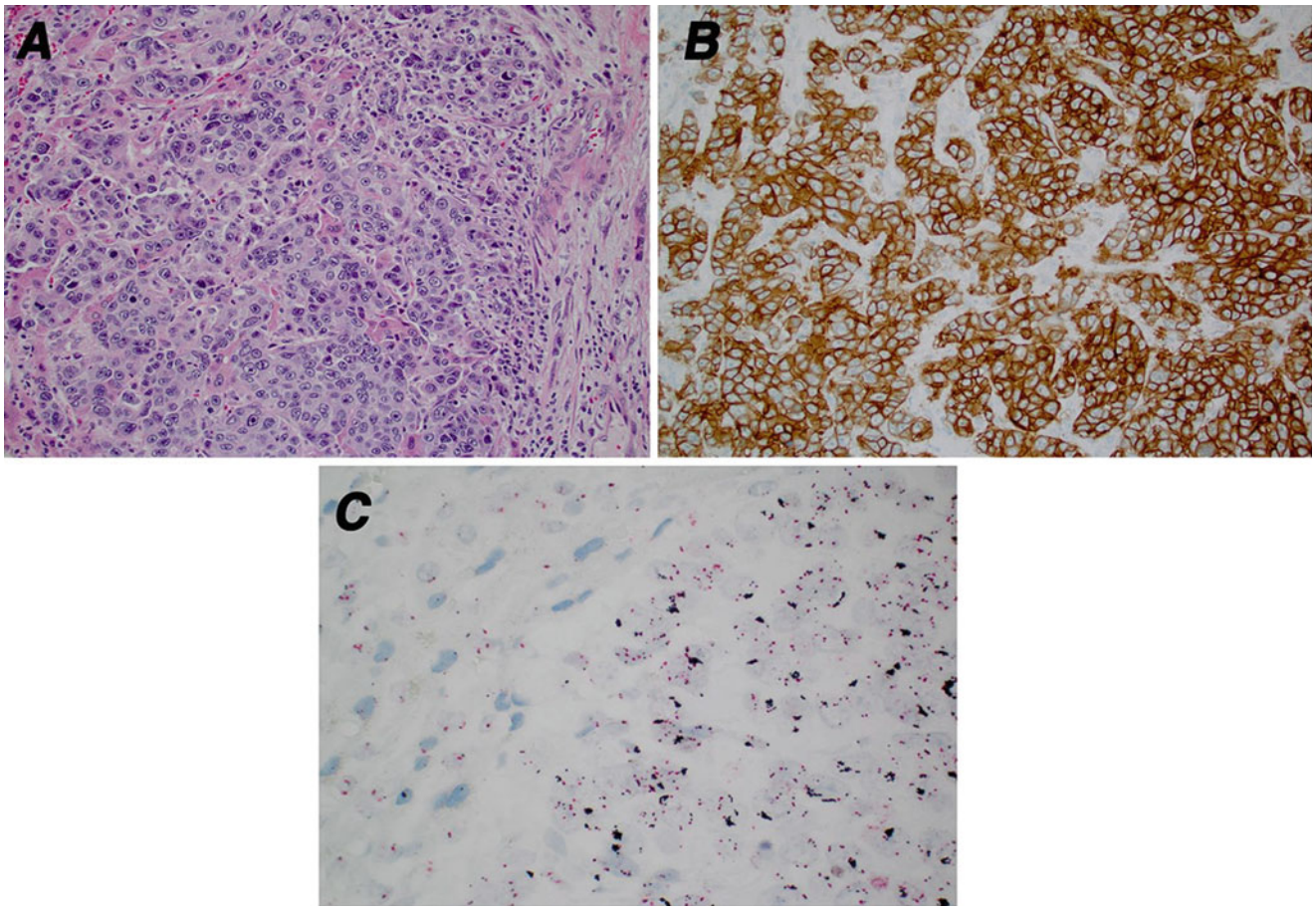

Fig. 22.4 Carcinoma of esophagus. (**a**) Hematoxylin and eosin stained section of the tumor. (**b**) The tumor is overexpressing Met (immunohistochemical stain showing Met expression brown). (**c**) *MET* gene amplification (dual chromogenic DNA in situ hybridization with clusters of black *MET* and variable red chromosome 7 centromeric signals) in the tumor (*right*) but not in the normal tissue (*left*).

oncogenesis supports at least three directions for cancer drug development: (1) antagonism of ligand/receptor interactions, (2) inhibition of TK catalytic activity, and (3) blockade of receptor/effector interactions.

22.4 Tumor Suppressors

In 1971, Alfred George Knudson, Jr., a medical geneticist specializing in cancer genetics, studied 48 cases of bilateral and unilateral retinoblastoma aiming to understand the genetic basis of cancer in these two apparently similar but subtly different subgroups of patients. By utilizing statistical modeling, Knudson was able to show that the presence and the frequency of tumors in retinoblastoma patients were consistent with two mutational events [23]. By fitting the data into this mathematical model, Knudson was also able to demonstrate why a small subset of carriers (i.e., cases in which only one mutation is present) do not develop tumors in the eye. This seminal work provided the basis for further research that collectively has led to the identification of the most common class of hereditary cancer predisposition genes: the tumor suppressor genes.

Classic tumor suppressor genes are autosomal recessive at the cellular level, meaning that within a cell both alleles of the gene have to harbor inactivating mutations for the phenotype to manifest. However, cancer predisposition syndromes caused by germ-line mutations in tumor suppressor genes, are inherited in an autosomal dominant fashion. For cancer to occur, the second allele of the tumor suppressor gene is somatically mutated in a susceptible cell type.

The distinction between tumor suppressor genes and oncogenes is mechanistic by nature. For a gene to be classified as a tumor suppressor, loss of function mutations in that gene must be shown to promote tumorigenesis. This contrasts with oncogenes which require activating mutations to drive tumorigenesis. Based on this broad definition, a wide variety of genes can act as tumor suppressors. As such, these genes are functionally grouped into two main categories: (1) Gatekeepers that directly regulate cell cycle progression, and (2) Caretakers that maintain the integrity of the genetic material in the cell. However, at the molecular and cellular level

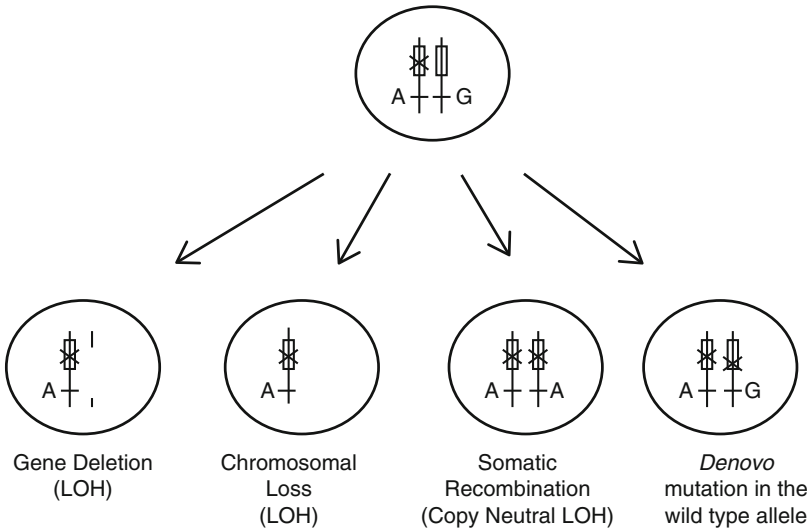

Fig. 22.5 Four alternatives to generate second hit in a cell with a germ-line mutation. From the four mechanisms, three will generate a pattern consistent with loss of heterozygosity. *LOH* loss of heterozygosity. Copy-neutral LOH is a special case of LOH where the wild-type allele is replaced by the mutant allele. This is generally achieved by somatic recombination.

tumor suppressor genes span a wide range of biological functions including transcription factors (*PAX5*), transcription turnover (*DICER1*), protein turnover (*FBXW7*), protein modification (*PTPN12*), and metabolism (*FH*), and their functions cannot always be neatly grouped into gatekeeper and caretaker categories.

22.4.1 Knudson's Hypothesis: The Two-Hit Model

Knudson's mathematical modeling led to what is known as Knudson's two-hit hypothesis. This states that tumors develop as a result of two events that affect separate alleles of the same gene. Consistent with this hypothesis was the observation that familial cases of retinoblastoma had younger age at diagnosis and were more likely to have bilateral tumors than individuals with sporadic retinoblastoma. Accordingly, Knudson proposed that familial cases were born with one mutated allele of the gene (accounting for the first hit) and they only needed to acquire a second mutation (second hit) to develop the cancer. In contrast, three non-mutually exclusive requirements needed to be met in sporadic cases: (1) de novo occurrence of two mutations, (2) occurence of both mutations in the same cell, and (3) occurence of the mutations in different alleles of the same gene. This model accounted for the infrequent nature of sporadic retinoblastoma as the co-occurrence of all three requirements was unlikely given the low rate of spontaneous mutation in a normal cell.

Mechanistically, the second hit described by Knudson could be accounted for via alternative molecular events. One possibility is that an inactivating point mutation (e.g., nucleotide substitution or frameshift) occurs in the remaining wild-type allele. A second possibility is that the wild-type gene is entirely deleted (leaving the cell with no functional copy of *RB1* gene), referred to as loss of heterozygosity (LOH). A third possibility, termed copy-neutral LOH, occurs when the wild-type allele is replaced with the mutant allele via somatic recombination. Figure 22.5 illustrates the various events which could account for a second hit. The term loss of heterozygosity was coined when Cavenee et al. [83] utilized RFLP techniques to look for somatic events that occurred during tumorigenesis. In these experiments, the germ-line cell was shown to be heterozygous for a linked marker residing outside the *RB1* gene. However, when the wild-type allele was lost through a large deletion or somatic recombination, the linked marker appeared homozygous (i.e., heterozygosity was lost). Intragenic mutations (substitutions or small insertions/deletions) did not result in LOH for the linked marker.

22.4.2 Haploinsufficiency

Knudson's two-hit model emphasizes a second hit in a tumor suppressor gene is the requirement for the development of cancer. If true, this hypothesis predicts that the presence of one mutation (the first hit) is benign (without effect) at the cellular level. In fact soon after Knudson proposed the two-hit model, mouse models were created to test this prediction [84]. In these experiments one of the two functional alleles of the tumor suppressor gene was knocked out leaving the animal with single functional allele. One such experiment performed with a gene called *Ptc* (also known as *Ptch1*) confirmed this prediction [85]. However, for some mouse models the results suggested that the first hit was not completely benign and a single mutation increased the chance for the organism to develop a tumor [26]. One such model was for the *TP53* gene which is estimated to be mutated in half of all

human cancers. In these experiments, half of the mice carrying the heterozygous null mutation of *Tp53* developed cancer after 550 days which was in sharp contrast to the 20% frequency of tumor development in the wild-type mice with two functional copies of *Tp53* [86]. Interestingly, when the *Tp53* gene was sequenced in tumors from the heterozygous-null mice, no second hit was detected in the majority of the cases. Additionally, all tumors expressed the wild-type p53 protein (assessed by western blot) and were capable of carrying out normal p53 function in biochemical assays. These observations provided evidence that the first hit was not a completely benign event and put the organism at increased risk of developing tumor. These experiments also showed that tumor development in the *Tp53* heterozygous-null mice was delayed compared to the homozygous-null mice. Similar results in regard to the frequency of tumor and the age of onset were obtained when yet another tumor suppressor gene (*CDKN1B* also known as p27) was tested in similar fashion [87]. In these studies it was shown that mice heterozygous for an inactivating mutation in *CDKN1B* developed cancer more frequently than wild-type mice and less frequently than homozygous mutant mice. In addition these mice were more sensitive to tumor formation when irradiated with X-rays compared to wild-type mice. These and similar observations with other tumor suppressor genes [26] led to the concept of haploinsufficiency in tumor suppressor genes. This term, which was originally coined by Curt Stern while studying gene dosage effects in Drosophila [88], was used to explain the lack of a wild-type phenotype in an organism carrying only one functional (wild-type) allele of a gene. Accordingly, a haploinsufficient tumor suppressor gene is a gene that alters the wild-type phenotype of the organism when it is present in a hemizygous state. The intermediate phenotype of heterozygous *Tp53* null animals suggests there is a gradient dependency on gene function. This contrasts with the more traditional and simplistic threshold model that predicts the presence or absence of a phenotype above or below a certain threshold for either gene expression or gene activity/function [89]. This new paradigm has been demonstrated for the *PTEN* gene where subtle fluctuation of expression was shown to dictate the extent of disease progression in prostate cancer [90] and in breast cancer [91]. In order to both accommodate the concept of haploinsufficiency in Knudson's model and to highlight the significance of tumor suppressor dosage sensitivity, Berger and colleagues [89] proposed a shift to a continuum model for tumor suppression. In this new paradigm, tumor suppressor genes exhibit various degrees of sensitivity to dosage change. Some genes, such as *PTEN* or *TP53* are more sensitive to subtle changes in activity or expression and others, such as *RB1*, are less prone to this dosage sensitivity. Accordingly, the latter group is more likely to conform to the classical two-hit model.

22.4.3 Tumor Suppressor Functional Classification

Motivated by two papers describing the molecular mechanism of *BRCA1* and *BRCA2* function, Kinzler and Vogelstein used the terms Gatekeepers and Caretakers to describe the cellular functions of tumor suppressor genes. Gatekeepers were defined as genes that directly regulated the growth of tumors by inhibiting growth or promoting death. In contrast, the term caretakers was used to describe genes directly involved in maintaining the stability and integrity of the genome [22]. Mutations in gatekeeper genes provide unregulated proliferation opportunities leading to selective expansion of cells carrying mutations. In probabilistic terms, the clonal expansion of mutated cells provides a higher likelihood for subsequent mutations to accumulate, furthering the neoplastic transformation and cancer progression. In contrast, mutations in caretakers result in unrecognized DNA damage and errors in DNA replication leading to accumulation of mutations within the cell. As such, cells with mutations in a caretaker gene will have a higher mutation rate, increasing the likelihood of malignant transformation. Neoplasia is a multistep process that begins by a single driver mutation that allows clonal expansion of a mutated cell. This is followed by subsequent mutations, resulting in further rounds of clonal expansion and cell transformation.

22.4.4 Genotype:Phenotype Correlations: Tumor Suppressor Gene Example: PTEN

PTEN (phosphatase and tensing homolog) is a tumor suppressor gene which encodes a dual-specificity phosphatase that can dephosphorylate both protein and phospholipid substrates. The tumor-suppressor function of Pten is primarily mediated through its lipid phosphatase activity, with its main phospholipid substrate being phosphatidylinositol triphosphate (PIP3) [92].

By regulating intracellular levels of PIP3, Pten is able to function as a negative regulator of the oncogenic Akt pathway [93, 94]. This pathway is closely linked to the new blood vessel development, deregulated cell growth, and resistance to apoptosis that are seen in cancer. When Pten function is lost or aberrant, a major consequence is increased levels of phosphorylated Akt (p-Akt), the activated form of Akt. Thus, p-Akt levels are an important marker of Pten status in terms of its lipid phosphatase function. The protein phosphatase activity of Pten is not as well understood. However, it does appear to mediate arrest of the cell cycle in G1 phase and control spreading and migration of cells through the mitogen activated protein kinase (MAPK) pathway [95], cyclin D1 [96], and focal adhesion kinase (*FAK*) [97]. Recent studies

have demonstrated that the sublocalization or partitioning of Pten between the cytoplasm and the nucleus has a role in the regulation of the Akt and MAPK pathways [98, 99]. Abnormal Pten localization, as a result of *PTEN* gene mutations, is speculated to be a mechanism linked to cancer predisposition.

The clinical spectrum of disorders that are known to be associated with germ-line *PTEN* mutations (known collectively as the *PTEN* hamartoma tumor syndrome or PHTS) encompasses Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba (BRR) syndrome, Proteus-like syndrome, autism spectrum disorder with macrocephaly, and macrocephaly and VATER (vertebral, anal, tracheal, esophageal, and radial/renal anomalies) association [100, 101]. To date, the majority of studies on *PTEN* mutations and clinical phenotypes have focused on defining predictive parameters for patients presenting signs of Cowden syndrome [102]. Clinical manifestations of CS include hamartomatous tumors in multiple organs, notably multifocal and bilateral benign breast lesions, thyroid lesions, and hamartomatous polyps of the stomach and colon.

Cancer-specific phenotypes associated with PHTS have become a very significant area of clinical interest, noting that the lifetime risk of breast cancer in females has been reported to be as high as 50 %, compared to 11 % for the general population [103, 104]. In addition, the gene expression profile of the PHTS breast cancers shows considerable overlap with a characteristic breast cancer subgroup known as molecular apocrine breast carcinoma (Fig. 22.6) [105]. It is now also apparent that endometrial cancer and thyroid carcinomas are frequent [106, 107]. Other cancer types that might occur at a higher frequency include renal cell carcinoma, colorectal cancers, and melanoma.

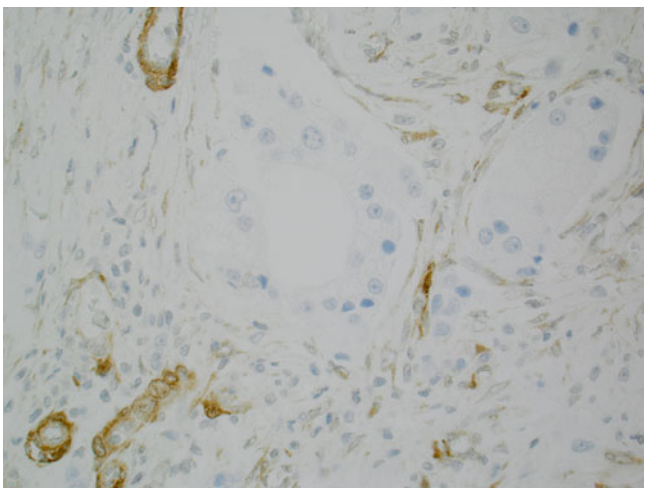

Fig. 22.6 Apocrine breast carcinoma lacking Pten. This section of an apocrine breast carcinoma is stained with antibodies against Pten (Immunohistochemistry). Normal expression of the protein (*brown*) can be seen in the nonneoplastic endothelium and loss of Pten expression in the neoplastic glands (center).

The phenotypic spectrum of germ-line *PTEN* mutations has provided substantial evidence that all patients with *PTEN* mutations should be routinely monitored annually as recommended by the NCCN (The National Comprehensive Cancer Network; http://www.nccn.org/).

22.5 Genomic Stability and Repair Genes (Caretaker Genes)

In contrast to gatekeeper tumor suppressor genes, caretaker genes do not directly regulate proliferation, but act to prevent genomic instability. Thus, mutation of caretaker genes allows for mutations in other genes, leading to accelerated conversion of a normal cell to a neoplastic cell. Many caretaker genes are required for the maintenance of genome integrity. Caretaker genes that play a role, directly or indirectly, in the repair of DNA double-strand breaks through homologous recombination are associated with cancer-predisposition syndromes that include hereditary breast and ovarian cancer, Fanconi anemia, and ataxia telangiectasia [108].

Nucleotide excision repair (NER) is of particular importance for correction of UV-light induced DNA damage (cross linking of pyrimidine residues). Germ-line mutations in any of several NER genes give rise to xeroderma pigmentosum (XP). XP is an autosomal recessive disorder characterized by severe actinic changes leading to early onset of skin cancers, various ocular manifestations, and occasional neurological abnormalities [109].

DNA mismatch repair (MMR) is another important genome maintenance system. MMR ensures genomic stability by correcting mismatches generated during DNA replication and recombination and by triggering apoptosis of cells with large amounts of DNA damage [110, 111].

22.5.1 Genotype-Phenotype Correlations: Lynch Syndrome

Lynch syndrome (OMIM No. 120435–6) is a hereditary cancer syndrome with relatively high incidence (approximately 2–5 % of all newly diagnosed colorectal carcinomas, CRC) caused by a mutation in one of the DNA mismatch repair (MMR) genes. It is a subgroup of hereditary non-polyposis colorectal cancer (HNPCC) which also includes familial colorectal cancer type-X (FCCTX) and familial serrated neoplasia associated with the colorectal cancer somatic BRAF mutation [112]. The molecular genetic *conditio sine qua non* for Lynch syndrome diagnosis is a germ-line mutation in one of the DNA mismatch repair (MMR) genes [111, 113]. The major MMR genes that harbor causal mutations are *MSH2*, *MLH1*, *MSH6*, and *PMS2*. Lynch syndrome is characterized by early age at onset of CRC, excess of synchronous and

Table 22.2 Cardinal features of Lynch syndrome

Mode of inheritance	Autosomal dominant
Average age for the onset of colorectal carcinoma (CRC)	45 (vs. 63 in the general population)
Progression time from adenoma to carcinoma	Approximately 3 years (vs. 8–10 for sporadic adenoma–carcinoma sequence)
Location of CRC	70–85 % proximal to splenic flexure
Risk of additional colorectal carcinoma	25–30 % develop second primary CRC within 10 years since subtotal colectomy
Extracolonic malignancies	Endometrium (40–60 % for female mutation carriers) Ovary (12–15 % lifetime risk for carriers) Stomach, small bowel, hepatobiliary tract, pancreas, upper urinary tract (renal pelvis and ureter) Brain (Turcot syndrome) Skin neoplasms (Muir–Torre syndrome: sebaceous adenomas, carcinoma, and multiple keratoacanthomas)
Pathomorphologic features	Poorly differentiated CRC with excess of mucinous and signet ring type of carcinomas; excess of tumor infiltrating lymphocytes
Survival	Increased in comparison to sporadic CRC
Confirmation of diagnosis	Identification of germ-line mutation in one of the mismatch repair genes

metachronous CRCs, a predilection to the proximal colon, accelerated carcinogenesis, and an excess risk for extracolonic cancers (including endometrium and, to a lesser extent, ovary, stomach, hepatobiliary tract, small bowel, pancreas, brain, transitional cell carcinoma of the ureter and renal pelvis, and sebaceous tumors in the Muir–Torre variant of the Lynch syndrome) (Table 22.2). No significant increase in breast, lung, or prostate cancer has been reported indicating that MMR mutations do not put every organ/cell type at an increased risk for cancer [114, 115]. Penetrance by the age of 70 for colorectal carcinoma in men varies from 22 to 100 %, based on the study population and the affected MMR gene. Similarly, penetrance for endometrial cancer in women varies from 16 to 71 % [116]. The best-known recurrent mutation causing Lynch syndrome is an A → T transversion in the donor splice site of intron 5 of *MSH2* (c.942 + 3A > T). This mutation apparently arises repeatedly because the region is vulnerable to slippage during the DNA replication process. The mutated nucleotide is the first in a tract of 26 adenines. It occurs worldwide and may account for as much as 5–10 % of all Lynch syndrome cases. In contrast, a genomic deletion of exon 16 of *MLH1* accounts for >50 % of all Lynch syndrome in Finns [117]. Molecular genetic heterogeneity must be considered when assessing the Lynch syndrome cancer phenotype. While more evidence will be required to substantiate differences in phenotype, current data suggest that *MLH1* mutations have a higher frequency of CRC than do *MSH2* mutations, which have more extracolonic cancers and Muir–Torre features (variant of Lynch syndrome with sebaceous tumors). Deletions of the last exon of the gene immediately upstream of *MSH2, EPCAM* (also known as *TACSTD1*), lead to epigenetic silencing of *MSH2* and a Lynch syndrome-like phenotype [118]. *MSH6* mutations, when compared with those of *MLH1* and *MSH2*, show a lower frequency of CRC but an excess of endometrial cancer. The phenotypic consequences of *PMS2* mutations are highly variable. Notably, individuals who inherit mutations in both alleles of the same MMR gene present with constitutional mismatch repair deficiency syndrome, characterized by childhood onset of cancers (e.g., glioblastoma, gastrointestinal malignancies, hematologic malignancies) and café-au-lait spots.

Mutations causing functional deficiency in mismatch repair proteins frequently result in microsatellite instability (MSI). Microsatellites are genomic regions in which a short DNA segment or a single nucleotide is repeated. There are hundreds of thousands of microsatellites in the human genome. During DNA replication, mutations occur in some microsatellites owing to frequent misalignment of their repetitive subunits and result in contraction or elongation (instability) of the repeats. These abnormalities are usually repaired by the MMR proteins. However, repair is inefficient in tumors with a deficiency of these proteins. Typically, in cancer cells, half or more of all microsatellites have mutations (contraction or elongation), so MSI testing serves as an excellent marker of MMR deficiency. Although most microsatellites occur in noncoding DNA where contractions or elongations have little or no effect on protein function, there are genes that have repeat tracts in their coding regions where alterations can impact gene function (e.g., *TGFβRII, IGFIIR, MSH3, MSH6, BAX, E2F4*, and *BLM*) [119]. MMR deficiency also results in increased numbers of nucleotide substitution mutations.

A high level of microsatellite instability (MSI-H) is observed in about 15 % of sporadic colorectal carcinomas where it is associated with hypermethylation of the promoter region of *MLH1*. MSI-H underlies a distinctive tumorigenic pathway because cancers with MSI-H exhibit many differences in genotype and phenotype relative to cancers without MSI-H, irrespective of their hereditary or sporadic origins. Genetic, epigenetic, and transcriptomic differences exist between cancers with and without MSI-H. The *BRAF* V600E mutation is associated with sporadic MSI-H colorectal cancers harboring *MLH1* methylation (CpG island methylator phenotype) but not Lynch syndrome-related colorectal cancers. The differences in genotype and phenotype between cancers with and without

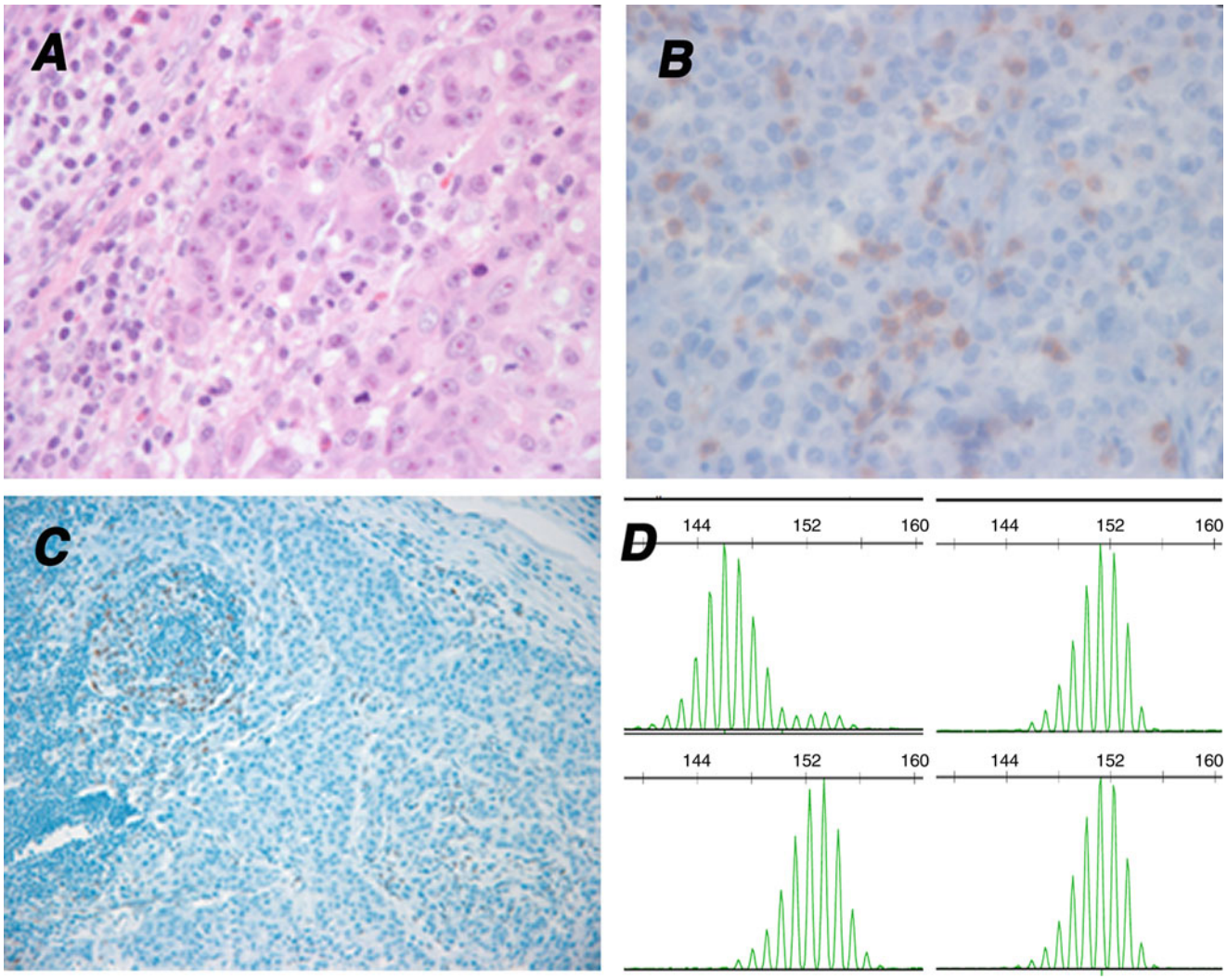

Fig. 22.7 Lynch syndrome, microsatellite instability-high colorectal adenocarcinoma. (**a**) Colorectal carcinomas in Lynch syndrome are characteristically high grade with medullary morphology (high grade carcinoma with malignant cells showing vesicular nuclei, prominent nucleoli, and abundant pink cytoplasm on H&E stain) with prominent intratumoral lymphocytes (small cells with darkly staining nuclei). (**b**) Tumor infiltrating lymphocytes are CD3 positive T-cells (*brown* color, immunohistochemical stain). (**c**) Loss of expression of MSH2 mismatch repair protein in malignant cells of colon carcinoma contrast to the preserved expression (*brown* staining nuclei) of MSH2 in mature lymphocytes (immunohistochemical stain). (**d**) Microsatellite instability: DNA isolated from both normal tissue (*bottom*) and colon cancer tissue (*top half*) from two patients (*left* and *right*). The patient on the left shows changes in the amplification pattern as a consequence of a defect in the mismatch repair. The patient on the *right* shows no change in amplification pattern supporting the absence mismatch repair defect.

MSI-H are likely to be causally linked to their differences in biological and clinical features. Therefore, the diagnosis of MSI-H in cancers is considered to be of increasing relevance. MSI-H is also a useful screening marker for identifying patients with Lynch syndrome, which could affect the efficacy of chemotherapy and allow identification of at-risk unaffected family members [120, 121].

Pathological features of colorectal cancers in Lynch syndrome are shared with MSI-H sporadic CRC and characteristically consist of poor differentiation (medullary carcinoma), mucinous, and signet-ring cell features (Fig. 22.7). Strong lymphoid reaction in the form of a Crohn's-like reaction (lymphoid nodules, including germinal centers, located at the periphery of infiltrating colorectal carcinomas), and particularly the presence of infiltrating lymphocytes within the tumor show strong predictive association with MSI-H CRC [122].

22.6 Mitochondrial Tumor Suppressor Genes

Although mitochondrial dysfunction is a common acquired feature of various cancers, germ-line heterozygous mutations in the autosomally encoded mitochondrial enzymes have only been described recently [123, 124]. There are mul-

tiple familial paraganglioma syndromes associated with germ-line mutations in genes encoding the four subunits of succinate dehydrogenase (*SDHA, SDHB, SDHC,* and *SDHD*) or mitochondrial complex II. Recent discoveries show that these genes are also implicated in the sporadic forms of the disease. A syndrome of multiple cutaneous and uterine leiomyomata (MCUL) is associated with germ-line mutations in fumarate hydratase (*FH*). If there is also a component of renal carcinoma then the syndrome is called hereditary leiomyomatosis and renal cell cancer (HLRCC).

Paragangliomas are tumors of neuroendocrine cells that arise in the parasympathetic and sympathetic nervous systems. Sympathetic paragangliomas arise from the adrenal medulla (where they are called pheochromocytomas) or from the paravertebral sympathetic chain from neck to abdomen, and they commonly secrete catecholamines. Parasympathetic paragangliomas arise from cell nests (paraganglia) adjacent to blood vessels. Symptoms associated with these tumors are related to their sizes and locations. Morbidity and mortality is due to the local and regional spread to adjacent nervous and vascular structures, major cardiovascular complications associated with secretion of catecholamines and the risk of recurrences and metastases. Hereditary paragangliomas are more often multifocal, recurrent, sometimes malignant, have an earlier age of occurrence than sporadic cases. Since early detection of paragangliomas reduces the incidence of morbidity and mortality, genotypic analysis in the search of *SDHB, SDHC,* and *SDHD* (and rarely *SDHA*) mutations in families of affected patients plays a front-line diagnostic role, leading to more efficient patient management. Additional genes (e.g., *SDHAF2*, *MAX*, *TMEM127*) are also associated with hereditary paraganglioma and/or pheochromocytoma syndromes.

Leiomyomas are tumors of the smooth muscle cells arising almost in any organ. In HLRCC both skin and uterine leiomyomas are present. Risk of developing renal carcinoma is estimated at 2–6 %. Renal cell carcinoma is often of papillary type II phenotype. Both sexes can be affected by often painful, clustered skin leiomyomas, and women affected with MCUL present with symptomatic uterine leiomyomas (fibroids) requiring hysterectomy for the control of symptoms.

22.6.1 Mechanisms Linking Loss of SDH and FH to Development of Cancer

SDHs and *FH* are housekeeping genes coding for enzymes in Krebs cycle. Functional losses of SDH and FH lead to a pseudohypoxic state in which succinate and fumarate accumulate and leak from mitochondria into the cytoplasm where they inhibit prolyl hydroxylation of hypoxia inducible factor (HIF1α). HIF1α plays a crucial role in regulation of genes important in cell function under various oxygen tension conditions. Under normal oxygenation HIF1α is hydroxylated and slated for degradation via the VHL-dependent ubiquitination pathway. However, under hypoxic or pseudohypoxic conditions (increased cytosolic succinate and/or fumarate) prolyl hydroxylase enzymes (PHD) are inhibited and HIF1α escapes degradation. Stable HIF1α then translocates to the nucleus where it can heterodimerize with HIFβ to form an active HIF transcription factor leading to expression of genes that can enhance survival in hypoxic conditions or reduce apoptosis (cell death) [125, 126].

22.7 Genetic Polymorphisms and Susceptibility to Cancer

Population data indicate that there are significant differences in individual susceptibility to cancer among humans. Inherited deficiencies in host defense mechanisms against DNA damage can modify cancer susceptibility as well as therapy response. It is believed that these differences arise from inherited genetic predispositions which in turn may be related to the genetic polymorphism (primarily single nucleotide polymorphisms or SNPs) of several enzymes involved in DNA repair, detoxification, and xenobiotic metabolism.

Functional polymorphism in the *MDM2* gene has been extensively studied in association with cancer risks in various populations [127]. The Mdm2 protein is an important part of the p53 tumor suppressor pathway. This pathway is activated upon cellular stress, such as DNA damage, and it elicits cell cycle arrest, senescence, and apoptosis, crucial for prevention of the propagation of DNA damage that could lead to tumor formation. Inactivation of p53 (through acquired mutation) is the most common genetic event in cancer seen in at least 50 % of all cancers. Mdm2 protein plays a crucial role in the p53 pathway where it is the primary E3 ubiquitin ligase for the p53 protein targeting it for proteasome degradation. Hence, Mdm2 provides negative regulation of the most important cellular tumor suppressor. It is known that a subset of human tumors show overexpression of Mdm2 and in experimental models even a modest change in Mdm2 cellular levels can affect cancer formation through the weakening of the p53 pathway.

The G-allele of the single nucleotide polymorphism (SNP309, T/G) in the promoter of *MDM2* increases the binding affinity of the transcriptional activator Sp1 which results in increased cellular concentration of *MDM2* mRNA and protein. Individuals with G/G genotype have up to 2.5-fold higher Mdm2 levels than individuals with T/T genotype. Additionally, carriers of a germ-line mutation in the *TP53* gene (Li–Fraumeni syndrome) who also possess the G-allele of SNP309 (either heterozygous or homozygous state)

develop cancer on average 9 years earlier than those with T/T genotype and also more commonly develop multiple tumors in their lifetime. Similar trends are seen in cancer patients without known germ line mutation in *TP53* (sporadic cancer cases) [128–130].

Genome-wide association studies (GWAS) using high-density SNP genotyping arrays identified common genetic variations contributing to many normal and pathologic traits. The vast majority of GWAS identified SNPs lie in intergenic or intronic regions, and therefore, are likely to influence gene regulation, while only a small fraction of variants affect protein coding regions. There are approximately 4.5 million SNPs in the human genome, which are at the core of inherited genetic variation in humans. However, at this moment no more than weak associations have been found between them and various cancers. These data suggest that the modulation of cancer risk depends not only on a single gene/SNP, but also on a joint effect of multiple polymorphisms (or haplotypes) within different genes or pathways, in close interaction with environmental factors. Virtually all responses to environmental carcinogenesis reflect the contributions of dozens of enzymes, and only simultaneous genotyping and combined analyses of different polymorphisms in larger numbers of patients and controls, as well as stratification of results by ethnicity, gender, and tumor characteristics may uncover the role of these SNPs in sporadic cancer.

22.8 Genetic Counseling

Prior to gene testing, high-risk individuals must receive genetic counseling, so they fully understand the pros and cons of cancer genetic testing, allowing them to provide fully informed written consent. Specifically, the patient must be able to determine whether such DNA testing is acceptable emotionally; he/she must also feel secure regarding issues of confidentiality, which may relate to the patient's perception of insurance or employment discrimination. They must also consider how their close relatives, particularly their children, will react to the presence of the mutation. Will this result in alienation with such questions as "… *Why did you have children, knowing that you'd pass on to us a high cancer risk?* …" Given these concerns, the physician must also carefully appraise, in so far as possible, the individual's emotional state in terms of his or her ability to accept a positive or negative DNA mutation test result. Multiple concerns may arise, such as the perception of insurance discrimination, which in some circumstances may deter mutation testing. When the physician lacks expertise in these matters, the patient should be referred to a medical geneticist and/or a medical genetics center experienced in all facets of hereditary cancer.

In conclusion, recent advances in our knowledge of cancer genetics are beginning to pay translational dividends in the management of this common clinical problem. Individuals with inherited cancer syndromes are at significant risk of developing both benign and malignant tumors as a result of a germ-line mutation in a specific tumor suppressor gene or proto-oncogene. We are now able to accurately screen and counsel individuals at risk for rare inherited cancer syndromes. Some neoplasia risk can be reduced by various preventive surgery measures, e.g., prophylactic bilateral mastectomy and/or bilateral salpingo-oophorectomy in *BRCA1* or *BRCA2* mutation carriers. Finally, our advancing knowledge has led to significant inroads into understanding what genetic alterations define prognosis and predict response to specific chemotherapeutic agents, and we are beginning to explore the utility of this knowledge in mass genetic-based clinical screening efforts. Enthusiasm must be tempered, however, by the extraordinary cost that often accompanies relatively modest gains. Finally, although genetic-based therapy often receives the greatest attention, molecular genetics, will likely have the greatest cost-effective impact in primary prevention and early diagnosis.

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Retinoblastoma: Clinical and Molecular Perspectives

23

J. William Harbour

23.1 Introduction

Retinoblastoma is the most common childhood eye cancer, affecting approximately 1 in 250,000 children in the USA [1], and it is one of the clinical success stories in pediatric oncology. A century ago, retinoblastoma was uniformly fatal, but today the survival rates are greater than 95 % in developed countries. This dramatic improvement in clinical outcomes is the result of advances in diagnostic techniques and treatment modalities [2, 3]. In addition, retinoblastoma has had a far-reaching impact that on cancer research that far exceeds its clinical prevalence. The retinoblastoma (RB) gene encodes a protein that is central to cell cycle regulation and is the namesake of a tumor suppressor pathway that is disrupted in virtually all human cancers.

23.2 Clinical Perspectives

23.2.1 Epidemiology and Clinical Genetics

Retinoblastoma is the most common intraocular malignancy in the pediatric population. There are no significant gender or race predilections. No specific environmental risk factors have been identified. Retinoblastoma accounts for approximately 3 % of all registered cancers in children [4].

About 93 % of new cases of retinoblastoma are sporadic with no family history. Among sporadic cases, about 60 % will have the nonheritable form and 40 % will have the heritable form of retinoblastoma. In patients with nonheritable disease, the eye cancer is unilateral and typically diagnosed between 1 and 2 years of age, although it can be diagnosed in older individuals. There is no increased risk of other cancers, and if the primary cancer is treated effectively, these patients have a normal life expectancy [5].

Patients with the heritable disease are usually diagnosed at an earlier age, typically within the first year of life. About 85 % of these patients will have multiple, bilateral eye tumors, and about 10 % will develop a midline intracranial tumor similar in embryonic origin and histologic appearance to retinoblastoma [6, 7]. Patients with heritable retinoblastoma are also at risk of many other types of second primary cancers throughout life. The risk of all of second primary cancer is particularly elevated if the patient has received radiation therapy [8–10].

The inheritance pattern of retinoblastoma was instrumental in the discovery of the RB tumor suppressor gene [11]. From a clinical standpoint, retinoblastoma is inherited in an autosomal dominant fashion, and for many years it was assumed that the gene causing retinoblastoma would operate in a dominant fashion [12]. In 1971, Alfred Knudson proposed the landmark two-hit hypothesis suggesting that the RB gene is, in fact, recessive at the molecular level [13]. The hypothesis suggested that a germ line mutation was present in most or all cells of the body in heritable cases, and that a second somatic mutation was rate-limiting in cancer formation. In the nonheritable cases, both mutations occurred as somatic events, thereby explaining why the disease was always unilateral and did not predispose to second primary cancers.

23.2.2 Clinical Features

Since most patients are young children at initial diagnosis, it is rare for them to complain of ocular symptoms. The most common presenting signs are leukocoria (white pupil reflex) and strabismus (misaligned eyes). Family history is important, but is usually negative. On clinical examination, retinoblastoma typically involves the posterior segment of the eye

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_23

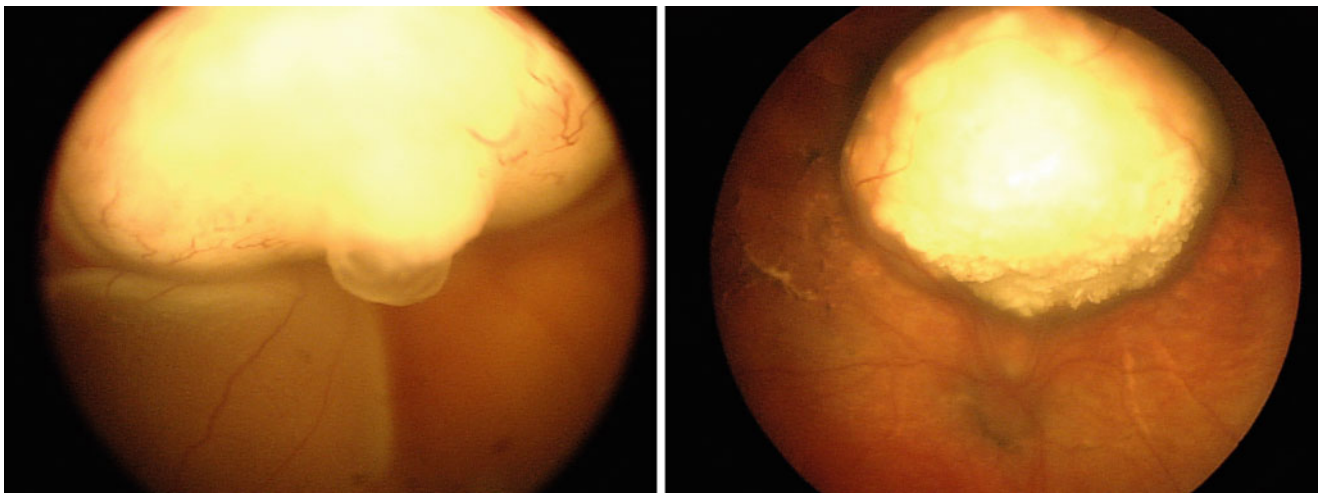

Fig. 23.1 Clinical appearance of retinoblastoma. A large retinoblastoma before (*left*) and after (*right*) chemotherapy. Note the marked reduction in tumor size after chemotherapy. This view was obtained with a fundus camera that simulates the view with indirect ophthalmoscopy.

and is best visualized with indirect ophthalmoscopy (Fig. 23.1). Retinoblastomas usually appear grey to white in color and may grow beneath the retina (exophytic pattern) or into the vitreous cavity (endophytic pattern). Accompanying findings can include retinal detachment, vitreous seeding, iris neovascularization, glaucoma, and invasion of the choroidal, optic nerve, and anterior segment. Ultrasonography and computed tomography are useful in confirming intratumoral calcifications, which are almost pathognomonic for retinoblastoma in children under 5 years old.

The differential diagnosis of retinoblastoma includes persistent hyperplastic primary vitreous, Coats' disease (congenital retinal telangiectasis), ocular toxocariasis, endophthalmitis, retinopathy of prematurity and other conditions that can result in the appearance of a white pupil reflex.

The benign variant of retinoblastoma is referred to as retinoma or retinocytoma [14, 15]. These benign retinal tumors can be distinguished from retinoblastoma clinically by features such as calcification and surrounding chorioretinal atrophy, and histopathologically by the presence of well-differentiated retinal-like cells forming fleurettes and rosettes. The molecular pathogenesis of these lesions remains uncertain, but they can be found in the parents of children with sporadic heritable retinoblastoma and in patients with low-penetrance forms of retinoblastoma.

23.2.3 Histopathology

The cell of origin for retinoblastoma remains unclear but is most likely a retinal progenitor cell that has the capacity to differentiate along the lineage of photoreceptors and Mueller cells [16–18]. Retinoblastomas are composed of small round cells with large nuclei and minimal cytoplasm (Fig. 23.2). Tumor cells vary in the degree of differentiation, with Homer-Wright rosettes, Flexner-Wintersteiner rosettes, and fleurettes representing attempts at photoreceptor differentiation. However, unlike most cancers the degree of differentiation in retinoblastoma has no prognostic significance. Histopathologic features with indisputable prognostic significance include optic nerve and choroidal invasion [19].

23.2.4 Second Primary Tumors

Patients with heritable retinoblastoma have a systemic predisposition to cancer resulting from the germ line RB gene mutation present in most or all cells in the body. Multiple types of second primary cancers can be seen, including osteosarcomas, soft tissue sarcomas, melanomas, and epithelial cancers [8, 20]. The incidence of second primary tumors is roughly 25 % at 40 years after diagnosis of retinoblastoma [9]. If a patient receives radiation therapy, the risk of second malignancies increases significantly [21]. The finding of biallelic RB gene mutations in osteosarcomas and other second primary cancers lends support to the idea that the two-hit hypothesis of tumorigenesis also applies to these tumors [22].

23.2.5 Treatment and Prognosis

Over the past century there have been steady advances in the treatment of retinoblastoma [2]. Enucleation (eye removal) remains an important treatment option in certain cases (e.g., unilateral cases, very large tumors and invasion of the optic nerve or anterior segment). External beam radiotherapy (EBRT) was a mainstay of treatment for many years. However, the increased risk of second primary tumors and facial bone deformities

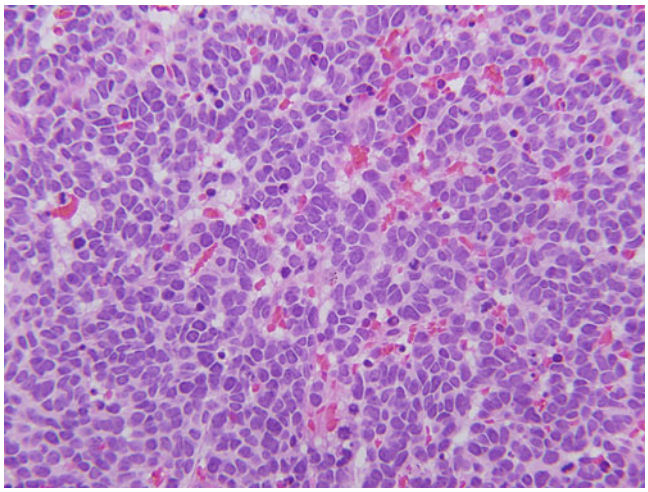

Fig. 23.2 Histopathologic appearance of retinoblastoma. Hematoxylin and eosin staining reveals small round blue cells with large nuclei and scant cytoplasm. This example is poorly differentiated.

associated with radiation has tempered enthusiasm for this treatment, particularly in patients younger than 12 months of age, who are at highest risk for these complications [23].

As an alternative to EBRT, several groups pioneered the use of chemotherapy in the 1990s [24, 25]. It was known from previous work several decades before that retinoblastoma responds to chemotherapy but frequently recurs due to the emergence of multidrug resistance. Therefore, the new approach combined chemotherapy with local consolidative techniques, such as laser and cryotherapy, to ablate the residual tumor masses after they had first been reduced in size by chemotherapy [24, 25]. Currently, the most frequently used agents are carboplatin, vincristine, and etoposide, given in 6–9 cycles. The survival rate in most developed countries is now greater than 95 %, but there are still many deficiencies that need to be addressed in the management of retinoblastoma. The risk of second primary tumors with current chemotherapy regimens remains unclear. Eyes with significant vitreous seeding generally do not do well with chemotherapy and often require radiation to salvage the eye. Intensive research efforts continue to identify new therapeutic options.

23.3 Molecular Perspectives

23.3.1 Molecular Genetics and Background

Retinoblastoma has played a pivotal role in cancer research that far outweighs its epidemiologic significance. Despite its rarity, retinoblastoma has provided unique and far-reaching insights into the genetics and molecular biology of cancer. The autosomal dominant inheritance of retinoblastoma has attracted the attention of cancer researchers for many years. The pivotal breakthrough occurred in 1971, when Alfred Knudson proposed his two-hit hypothesis that proposed that retinoblastoma results from the mutational inactivation of both alleles of a putative recessive oncogene [13]. The retinoblastoma locus was mapped to chromosome 13q through its linkage to esterase D, an enzyme that had previously been mapped to this region and which could be assayed for its enzymatic activity [26]. Esterase D activity was found to be about half the normal level in retinoblastoma patients with karyotypically visible deletions on chromosome 13q [27]. In another study, esterase D activity was 50 % of normal in healthy tissues of retinoblastoma patients but was undetectable in tumor tissue [28], providing further support for Knudson's hypothesis. The recessive nature of the retinoblastoma locus was conclusively demonstrated by loss of heterozygosity in tumor tissue [29], a technique that subsequently has become a standard for studying tumor suppressor genes. Based on the proximity to the esterase D gene, several groups independently identified and characterized a nearby gene that proved to be the retinoblastoma gene [22, 30, 31]. Reintroduction of this gene into retinoblastoma cells suppressed the tumor phenotype, providing functional evidence for its role as a tumor suppressor [32].

23.3.2 The RB Gene

The retinoblastoma gene (RB) spans over 200 kilobases of DNA and contains 27 exons [33, 34]. Germ line mutations tend to cluster at CpG areas but are also spread throughout the gene with no mutational hot-spot [35, 36]. Germ line mutations can include deletions, frameshift and nonsense mutations, splicing mutations, and mutations in introns resulting in a truncated protein product [35]. The second hit is usually a nondisjunction or recombination event during mitosis, resulting in reduction to hemizygosity of the mutant allele [37]. Most RB mutations result in >90 % penetrance, but some mutations have been associated with low penetrance retinoblastoma. These low penetrance mutations can be divided into those that cause a reduction in the amount of normal Rb protein (usually promoter mutations) and those that result in a partially functional Rb protein (usually missense mutations) [38].

23.3.3 The Rb Protein

The retinoblastoma protein (Rb) is composed of 928 amino acids and has three functional domains: the N-terminus, the central pocket domain, and the C-terminus. The pocket, which is formed by the highly conserved A and B boxes, is required for binding to E2F, viral oncoproteins and chromatin remodeling proteins, and it is required for tumor suppression [39, 40]. Most RB mutations affect the pocket [35]. The C-terminus is also required for efficient binding to

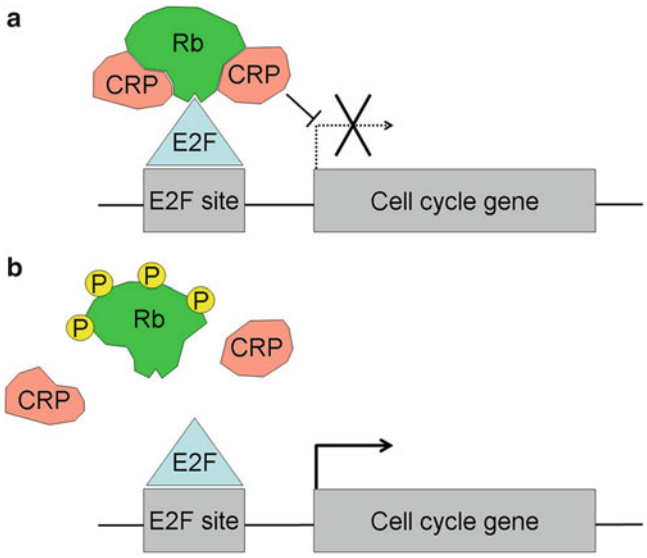

Fig. 23.3 The molecular mechanism of action of Rb. (**a**) When Rb is hypophosphorylated and active, it binds to E2F transcription factors that bind to E2F sites in the promoter regions of genes involved in cell cycle progression and apoptosis. Rb directly inhibits E2Fs by binding and masking the transactivation domain, and it also recruits chromatin remodeling proteins (CRPs) that alter local chromatin structure into a conformation that is not permissive for transcription. (**b**) When Rb is hyperphosphorylated, it does not bind efficiently to chromatin remodeling proteins or E2Fs, which allows E2Fs to transactivate cell cycle genes.

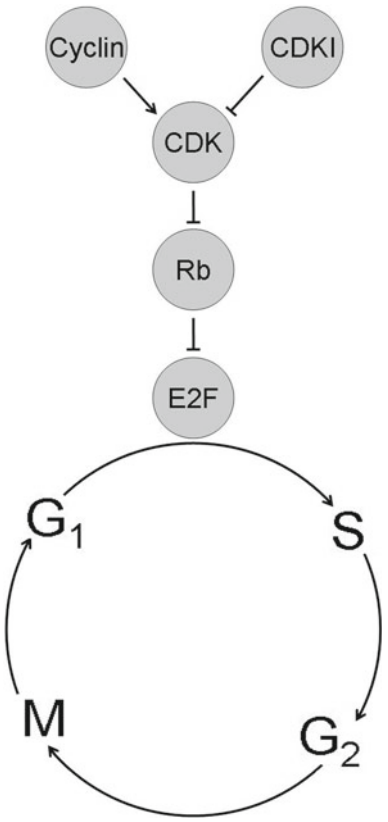

Fig. 23.4 The retinoblastoma tumor suppressor pathway.)E2F transcription factors promote cell cycle progression in part by transactivating genes involved in DNA synthesis (S) phase of the cell cycle. Hypophosphorylated, active Rb inhibits cell cycle progression from G_1 phase into S phase by directly inhibiting E2Fs and by actively repressing transcription of cell cycle genes. Rb can be inhibited by hyperphosphorylation catalyzed by cyclin dependent kinases (CDK). CDKs, in turn, require activation by cyclins, and they can be inhibited by CDK inhibitors (CDKIs) such as p16. Cancers tend to upregulate cyclins and CDKs and to downregulate CDKIs through genetic mutation or functional perturbation.

E2Fs, and it has binding sites for the oncoproteins MDM2 and c-abl [41–44]. The function of the N-terminus is less clear, and it does not appear to be required for tumor suppression [45].

A major function of Rb is to inhibit the G_1–S phase transition of the cell cycle [46]. Protein activity is modulated during the cell cycle by phosphorylation, which can occur on 16 cyclin-dependent kinase Ser/Thr-Pro phosphoacceptor sites. Rb phosphorylation varies throughout the cell cycle, with hypophosphorylated Rb predominating in G_0 and G_1, and hyperphosphorylated Rb in S-phase and G_2/M [47–49]. Rb usually becomes hypophosphorylated during differentiation and senescence, and it becomes hyperphosphorylated in cells reentering the cell cycle [50–52]. The Rb protein can also be modulated by viral oncoproteins, such as SV40 large T antigen, adenovirus E1a protein and herpes virus E7 protein, which bind and inhibit Rb, thereby stimulating cell cycle progression [53–55] (Fig. 23.4).

A major binding partner of Rb is the E2F family of transcription factors (E2Fs) [56]. Rb does not have a DNA binding domain but is brought to specific promoters through its interaction with E2Fs. E2F-responsive genes contain one or more E2F binding sites in their promoters, and these genes play an important role in cell cycle, apoptosis and other cellular functions [57]. In addition to a DNA binding domain, E2Fs 1–5 also have a transactivation domain by which they promote the transcription of specific genes. Rb inhibits transcription in part by binding and masking the E2F transactivation domain [58, 59]. In addition, Rb actively represses promoter and enhancer elements independently of its effect on the E2F transactivation domain [60, 61]. This so-called active repression is now thought to result from the recruitment by Rb of chromatin remodeling proteins that reconfigure local chromatin into a nonpermissive state for transcription [62]. These proteins include histone deacetylases, DNA methyltransferases, and polycomb complexes [63–68]. The functional significance of these two independent mechanisms of transcriptional repression by Rb remains controversial (Fig. 23.3). However, it seems clear that active repression by Rb is required for the full tumor suppressor activity of Rb, including its inhibition of the G_1–S phase transition [69] (Fig. 23.4).

In addition to its role as a cell cycle inhibitor, Rb also inhibits apoptosis [46]. These seemingly opposing roles of Rb have not been satisfactorily reconciled. The widely accepted view of Rb as an on-off regulator of the G1–S transition that is completely inactivated every cell cycle does not explain why cells do not undergo apoptosis with every attempt at cell division. The most straightforward explanation is that Rb is not completely inactivated every cell cycle but that the degree of Rb phosphorylation and inactivation can be graded, depending on the cellular context. In support for this idea, Rb is phosphorylated in several hierarchical steps, and the phosphorylation events regulating the cell cycle can be differentiated from those regulating apoptosis [70, 71]. It seems likely that Rb is only partially phosphorylated and inhibited during normal cell division, and that this incomplete inactivation is sufficient to block its cell cycle inhibitory function and to allow cell cycle progression without triggering apoptosis [72]. This residual anti-apoptotic activity may persist unless Rb is more completely phosphorylated (or unless the RB gene is mutated), suggesting that Rb may serve as a buffer against apoptosis during normal cell division and a checkpoint for triggering apoptosis under abnormal stress conditions such as excessive mitogenic stimulation, DNA damage, and hypoxia in which apoptosis may be favored for survival of the organism.

23.3.4 The Rb Pathway

Although RB gene mutations are common in only a few types of cancer, such as retinoblastoma and small cell lung cancer, the RB pathway is functionally disrupted in virtually all human cancers [73, 74]. The RB pathway is composed of proteins that modulate Rb phosphorylation state. Rb is phosphorylated by cyclin-dependent kinases (CDKs), which are activated by interaction with cyclins [74, 75]. Therefore, overactivity of cyclins or CDKs can lead to functional inactivation of Rb. For example, cyclin D1 is a bona fide oncogene that is overexpressed and functionally inhibits Rb in many cancer types [76, 77]. A constitutively active CDK4 mutant that maintains Rb in a phosphorylated state has been identified in some cancers [78]. On the other hand, CDKs are inhibited by proteins such as $p16^{INK4a}$, which is a tumor suppressor that is mutationally inactivated in some cancers [79]. Interestingly, the cancers associated with complete Rb inactivation by genetic deletion (e.g., retinoblastoma and small cell lung cancer) have a different phenotype than those associated with partial Rb inhibition by loss of p16Ink4a or overexpression of cyclin D (e.g., melanoma, most solid tumors). The former tend to exhibit rapid proliferation, high apoptotic rates and sensitivity to chemotherapy, whereas the latter tend to have slow rates of proliferation and apoptosis and tend to be resistant to chemotherapy [80].

23.4 Conclusions

Retinoblastoma continues to provide important new insights into cancer biology and treatment. New discoveries will continue to emerge from the study of retinoblastoma and the RB pathway, and these will likely play an important role in the continued search for better cancer therapies.

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Xeroderma Pigmentosum and the DNA Damage Response to Ultraviolet Light

24

James E. Cleaver

24.1 Xeroderma Pigmentosum: Clinical and Genetic Aspects of Disease

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease with sun sensitivity and progressive degenerative changes of sun-exposed portions of the skin and the anterior portion of the eyes. Patients develop non-melanoma skin cancer and melanoma; about 20 % of patients show additional developmental disorders and progressive neurological degeneration [1, 2]. XP occurs at a frequency of about 1 per million in the USA and Europe, but is considerably more common in Japan (1 in 100,000) and North Africa. The frequency of heterozygotes in Japan is as high as about 1 % [3]. The median age of onset of symptoms is between 1 and 2 years and can include severe sunburn with blistering and persistent erythema after short sun exposure, and development of freckling within a few years of life. The skin symptoms are directly related to the failure of nucleotide excision repair (NER) of damage to DNA of the cells of the skin and eyes caused by solar exposure in the UVB range of the sun's spectrum (280–310 nm) [4–6]. Defective DNA repair leads to cancer development with a median age of onset of 8 years, nearly 50 years younger than that in the general population of the United States [7]. Heterozygotes (parents) are generally without symptoms of solar sensitivity, but in several mouse knockouts of XP genes heterozygous abnormalities have been observed [8–11].

Several other recessively inherited diseases are associated with XP and involve related pathways of DNA repair or rare mutations that present distinctive symptoms. Cockayne syndrome (CS) is a wasting disease with progressive neurological deficits including demyelination, ataxia, cerebellar atrophy, and calcification in the brain, particularly in the basal ganglia, but with no increased risk of cancer [12, 13]. Symptoms may be present at birth or during childhood and the average lifespan is 12 years [12, 13]. CS encompasses a wide range of clinical symptoms from the very mild UV^{s} syndrome that shows virtually no neurological symptoms and only mild skin sensitivity to solar exposure [14–18], through varying degrees of severity of CS symptoms [12, 13], to the extremely severe neonatal lethal disorder cranial oculo-facio-skeletal syndrome (COFS) that has been associated with CSB and other repair genes [19–21]. CS individuals are deficient in a sub-pathway of NER known as transcription-coupled repair (TCR) [22–24]. Trichothiodystrophy (TTD) is a developmental disorder in which patients have sulfur-deficient brittle hair with characteristic tiger tail banding when examined in polarized light, skin photosensitivity without increased pigmentation or cancer, and immune deficiency caused by mutations in components of the transcription factor TFIIH that contains NER gene products [2, 25]. Although CS and TTD do not exhibit cancer in humans, the corresponding mouse knockout strains all show increased skin cancer incidence when exposed to UVB or skin carcinogens [8, 26, 27].

The molecular basis of XP, CS, and TTD is a series of defects in components of the DNA damage response (DDR) to UV damage involving the NER system that removes and replaces DNA damage in DNA or the low fidelity polymerase Pol eta (η) that replicates past DNA damage. Genetic analysis of XP showed that there are 8 different groups: XP-A through XP-G, that represent different steps in damage recognition and excision and XP-V that corresponds to the bypass polymerase Pol eta. Other repair deficient disease genes include *CSA*, *CSB*, and *TTDA*. Many components of the NER system, estimated to be about 30 [22], have not been identified among human patients either because mutations would be lethal or have not been clinically recognized due to extreme rarity. Mouse knockouts have revealed the essential nature of some of these genes and can present with symptoms significantly different from the corresponding

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_24

human patients, either more severe or less. XP-A patients for example have severe progressive neurodegeneration, but the mouse knockout is without neurological disorder [28, 29]. XP-B and XP-D patients have severe neurodegenerative symptoms, but the mouse knockouts are lethal in very early embryo [30]. XP-E and XP-F patients are generally only mildly affected, but the mouse knockouts have severe disorders [9, 31]. One patient was identified with a severe COFS-like disorder due to mutations in *ERCC1*, a cofactor for the XP-F gene [21]. These differences between human and mouse have several explanations, in addition to those associated with broad mouse and human differences. Differences arise because mutations may cause different phenotypes from complete gene loss, as well as because of the selective screen by which only a subset of mutations give rise to viable patients.

24.2 The DNA Damage Response: Oncogenes, Tumor Suppressors, Recombination, and Signal Transduction

In recent years there has been a convergence in understanding that many endogenous and exogenous factors, including oncogenes, tumor suppressors, radiation, chemical carcinogens, and viral infections can all impact a common, but complex, set of pathways that regulate cellular survival, apoptosis, and genomic stability: the DNA damage response (DDR) and cell cycle checkpoints (Fig. 24.1) [32, 33]. The DDR involves a cascade of damage recognition, repair, and signal transduction that coordinates the response of the cell cycle to DNA damage. The DDR activates checkpoints that delay the cell cycle, provides time for repair, and directs damaged cells into senescent or apoptotic pathways. The DDR is significantly different in primary normal cells (fibroblasts, keratinocytes) as compared to immortalized (telomerase-expressing) or transformed cells, due to significant changes in p53 expression, kinase activities, and signal transduction. Associated with these changes in the DDR, the spontaneous mutation rate is low in primary cells but increases by orders of magnitude in transformed, malignant cells [34]. Consequently tumors contain large numbers of mutations and other genomic changes [35, 36]. The emerging complete picture of the interacting signal transduction pathways remains unclear, but is increasingly recognized as an important part of the overall process of carcinogenesis [32, 33].

The DDR has been most extensively studied as a response to DNA double strand breaks (DSBs), usually caused directly by X-rays [37]. Common assumptions assert that DNA double strand breaks activate the kinases ATM and Chk2, and replication stress (e.g., hydroxyurea, UV light) activates

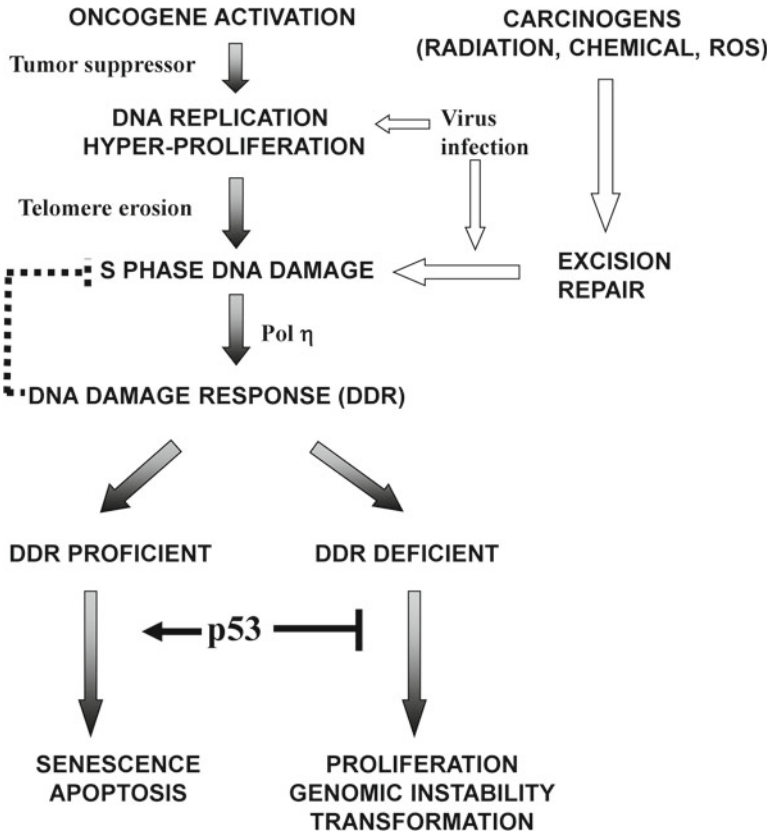

Fig. 24.1 Nucleotide excision repair and the DNA damage response from radiation and chemicals, oncogene driven replication and virus infections. Adapted with modifications from ref. [33].

ATR and Chk1 [37–46]. ATM can also be activated by oxidative stress without necessarily involving DSBs [47]. The Chk1 and Chk2 kinases phosphorylate p53, cause nuclear translocation of the Cdc25 phosphatases, and increase expression of p21. Increased p21 results in G1/S, S, and G2/M cell cycle arrest, during which DNA repair can occur. Both ATM and ATR kinases are involved in the DSB and replication stress responses with considerable variation according to the damage and cell type [48]. At least 700 different protein substrates have been identified as targets for phosphorylation by these kinases [49]. There will be substantial differences in the structure of DNA and chromatin adjacent to DSBs compared with that resulting from replication arrest which lead to different signaling cascades and checkpoint activation [50–52]. The ATR pathway is essential for viability, ATR and Chk1 knockout strains are lethal [53]. ATM and Chk2 mutants are viable even though cells lacking these kinases display genomic instability and a predisposition to cancer [54]. The long history of sensitization of cells to UV damage by growth in caffeine [55] now appears to be explained at least in part by the sensitivity of the ATR-CHK1 pathway to caffeine [56, 57].

A major early signal of DNA damage and repair is the phosphorylation of the minor histone H2AX which occurs within minutes of DSB induction. H2AX is one of several nonallelic isoforms of histone H2A [58]. H2AX is ubiquitously distributed throughout the genome and has phosphorylation sites within the conserved C-terminal region at serine 139 and tyrosine 142 [59, 60]. Induction of DNA DSBs triggers H2AX phosphorylation (γH2AX) over megabases adjacent to DNA DSBs [60, 61]. After exposure to ionizing radiation, H2AX phosphorylation promotes chromatin condensation, which is detectable by immunofluorescence microscopy as nuclear γH2AX foci [60]. Initial recruitment of DNA repair and checkpoint factors, e.g., Mre11, Rad50, Nbs1, Brca1, and 53BP1, to DNA DSBs is not affected in H2AX knockout mice but repair and checkpoint factors subsequently fail to form foci, indicating that γH2AX functions to concentrate repair and checkpoint proteins in the vicinity of DNA lesions [62, 63].

The DDR from UV-induced photoproducts is more complex, and has distinct G1 and S phase responses. NER in G1, for example, excises UV photoproducts before onset of DNA replication and thereby eliminates mutagenic lesions completely [64]. NER is required during this phase for activation of the ATR-Chk1-p53 signal pathway [65]. Cells defective in the global branch of NER (XP-A through XP-G), but not the TCR branch (CSA, CSB), show reduced ATR-dependent signaling in G1 [65] and less γH2AX formation [66]. H2AX-knockout mice are cancer-prone and the knockout cells are sensitive to X-rays and etoposide that make DSBs [67], but show little sensitivity to UV light or cisplatin that do not produce direct DNA DSBs (Cleaver, unpublished observations). Therefore despite extensive H2AX phosphorylation after most forms of DNA damage, γH2Ax is only functionally important for DSBs but is only a biomarker for other forms of damage.

Damage that persists in S elicits a stronger ATR-Chk1-p53 response than in G1 [65], and contributes to recovery of DNA replication [68]. Stalled replication forks form long (kilobase) stretches of single-stranded DNA [44, 69–73]. These become coated with RPA that activates ATR through ATRIP [44]. The bypass polymerase Pol η is recruited through reversible ubiquitylation of PCNA [74–77]. A small fraction of arrested replication forks degrade to DSBs [78] or are resolved by nonhomologous or homologous recombination [79]. Replication fork breakage may be an active enzymatic process, and by analogy with DNA cross-links could involve either MUS81 or the NER nuclease XPF/ERCC1 [80]. UV-induced replication arrest results in large increases in γH2AX formation associated with coordination of the numerous replication and recombination factors associated with the arrested forks [66, 81, 82], but the absence of H2AX does not confer any increased UV sensitivity (Cleaver, unpublished observations). Resolution of the arrested replication forks also depends on p53, and cells lacking both p53 and Pol η are extremely sensitive to cell killing by UV light [83].

Resolution of arrested forks can result either in complete restoration of normal DNA replication, or large changes in genomic stability leading to mutagenesis or malignant transformation (Fig. 24.1).

24.3 Two Branches to Damage Removal

The main substrates for NER relevant to XP are the UV photoproducts between adjacent pyrimidines: the cyclobutane pyrimidine dimers and the [6-4] pyrimidinone dimers [84]. These are produced in DNA in skin over the whole UV wavelength range (UVA–UVB–UVC), although the most important wavelengths from solar exposure, from a practical standpoint, are UVB, approximately 280 to 320 nm [85]. The precise proportions of these photoproducts, and their pyrimidine constitution (T=T, C=C, T=C, and C=T) vary with wavelength, but in general they are the archetype large DNA adduct that is the main class of substrates for NER [86–89].

NER can remove DNA damage before DNA replication begins (Fig. 24.2), and consequently plays a major role in reducing the amount of damage that becomes fixed as mutations during replication [64, 90], but there is also evidence for a direct role in the UV damage response during the S phase, especially the ATR/Chk1 pathway [91]. The NER process has two main branches distinguished by the mechanisms used for initial recognition of DNA damage [92]:

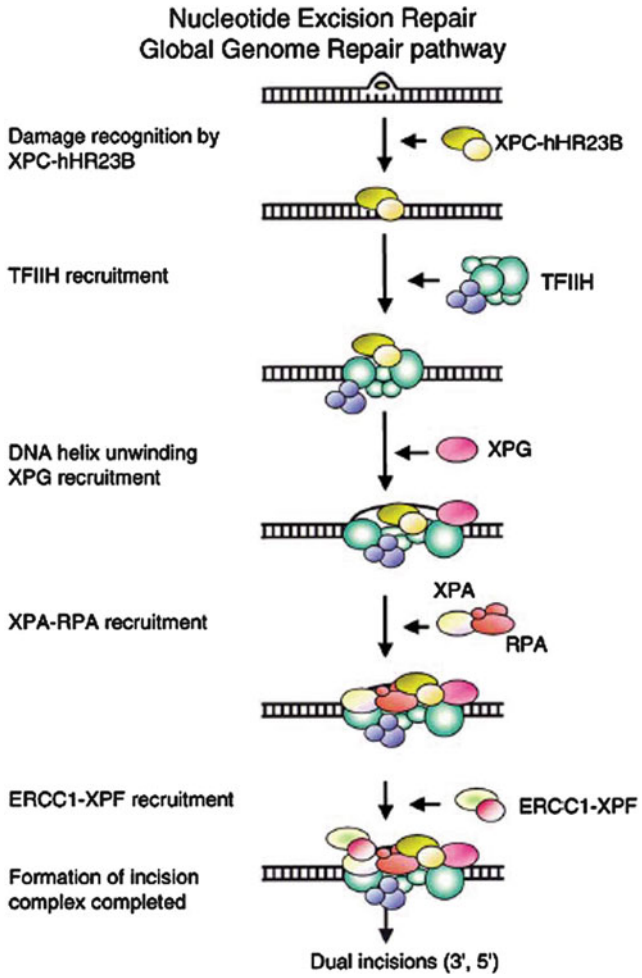

Fig. 24.2 Sequence of action of nucleotide excision repair (global genomic repair) on untranscribed DNA. Transcription coupled repair employs the arrest of RNA Pol II at DNA photoproducts as the damage recognition step, bypassing XPE and XPC, and entering the excision process at the TFIIH step. Reproduced from ref. [107] with permission from Elsevier.

transcription coupled repair (TCR) by which actively transcribed genes are repaired more rapidly than the rest of the genome [93–97], and global genomic repair (GGR), by which non-transcribed regions of the genome are repaired [6]. Damage in transcribed genes is recognized through the arrest of RNA Pol II that is relieved through the action of two proteins, CSA and CSB. Damage in the non-transcribed or global regions of the genome is recognized by the binding of two heterodimeric XP proteins, XPE (DDB1/DDB2) and XPC/HR23B, which may act in concert to bind to damaged DNA [6]. TCR and GGR converge on a common pathway through which the damage is verified (XPA/RPA), the DNA is unwound by the XPB and XPD helicase components of the transcription factor TFIIH, cleaved by structure-specific nucleases (XPG, XPF/ERCC1), and the damage excised and replaced.

The complete excision process on metabolically inactive DNA can be carried out in vitro with a minimal set of recombinant proteins: RPA, XPA, TFIIH (6 subunit core of XPB, XPD, p44, p34, p52, p62, and 3 subunit kinase CAK), XPC, hHR23B, XPG, ERCC1, and XPF [98–101]. Both TCR [102, 103] and GGR [104–106] are regulated by p53.

The sequence of action of the damage recognition, unwinding, and excision steps are interwoven rather than proceeding in a strictly sequential process [107]. The individual factors of NER associate independently on UV photoproducts without a necessary preassembly of a repairosome complex, although many of the components exist as heterodimers or within the larger 10-member TFIIH complex [100, 107–109]. The individual damage-binding proteins have relatively low affinity for damage as separate factors, but cooperatively XPA, XPC, and RPA, with cooperation from TFIIH, form a high specificity complex [110].

24.4 Global Genomic Repair

The initial damage recognition factors uniquely required for GGR are the XPC and XPE (DDB2) DNA binding proteins (Figs. 24.2 and 24.3), both of which function as heterodimers: DDB1/DDB2 and XPC/HR23B. Naturally occurring mutations in XPE (DDB2) have only a small effect on the levels of NER [111], whereas mutations in XPC cause a large reduction in NER and increased UV sensitivity. For this reason, XPC appears to be the major DNA damage recognition protein and XPE may play a lesser role, but this conclusion may be misleading. Mouse knockouts in *Ddb2* [9] and virally induced downregulation of DDB1 [112] have a large influence on the excision process.

The XPE group includes patients who are mildly to moderately affected and whose cells carry out near normal levels of NER [113]. The XPE gene product is the smaller partner of a dimeric protein with subunits of 127 kDa (DDB1) and 48 kDa (DDB2) [104, 113–118]. Mutations that are diagnostic for XPE are located in the DDB2 subunit (see http://www.xpmutations.org for a summary of XP and CS mutations). DDB2 and p53 regulate each others' expression [119, 120], with the consequence that XPE cells and those from mouse knockouts are resistant to p53-dependent UV induced apoptosis [9].

The DDB1/2 heterodimer is involved in the recognition of damaged DNA and has the capacity to bind to UV-damaged DNA with a chemically defined footprint [114, 121–124]. Binding appears specific for most cyclobutane and [6-4] photoproducts, except for the T[cis,syn]T photoproduct which is marginally discriminated from normal undamaged DNA [121]. DDB1 (p127) is present in excess over its partner, and is predominantly cytoplasmic but translocates to the nucleus after UV irradiation [125], a function that requires

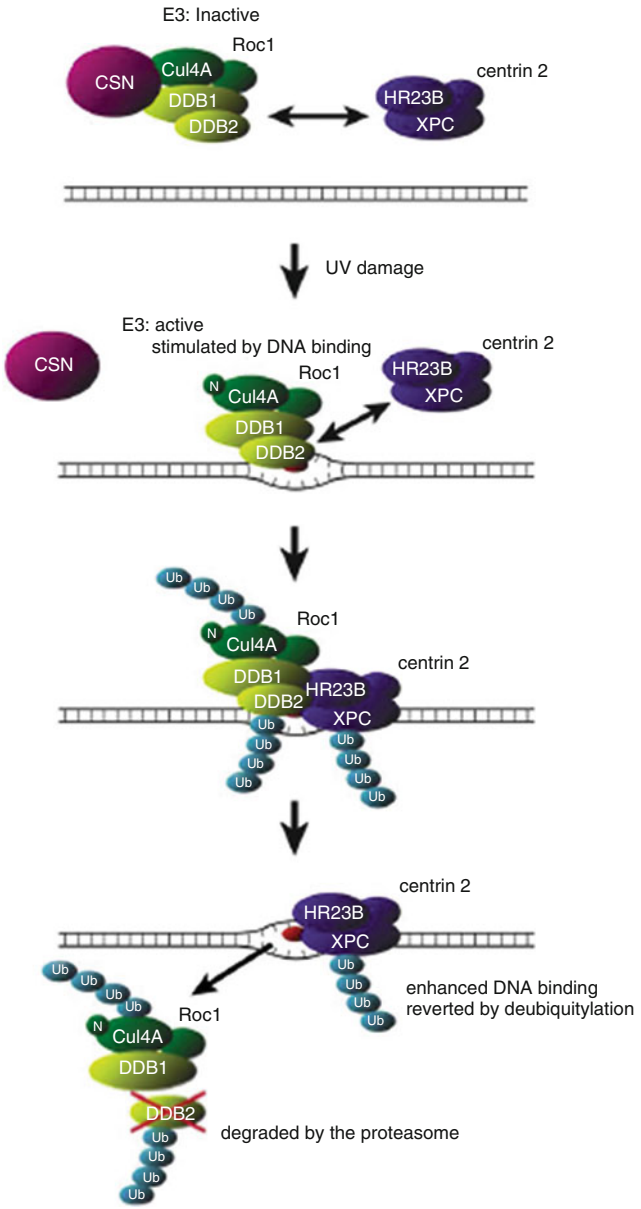

Fig. 24.3 Model for UV-Induced DDB1/2 dependent ubiquitylation of XPC. In unirradiated cells, DDB1/2-associated E3 is inactivated by its interaction with the COP9 signalosome (CSN). Thus, XPC is not ubiquitylated, despite its interaction with DDB1/2. Upon UV irradiation, UV-DDB translocates onto the damaged chromatin by binding to lesions, particularly [6-4] photoproducts. Its dissociation from the CSN and the neddylation of cullin 4A (indicated by "N") activate E3. The binding of UV-DDB to the lesion further stimulates E3 activity. The activated DDB1/2-E3 then recruits XPC via protein–protein interaction, and XPC, DDB2, and cullin 4A are ubiquitylated at the lesion site. Polyubiquitylated UV-DDB1/2 loses its damaged DNA binding activity, whereas the DNA binding of XPC is potentiated by its ubiquitylation. This results in the displacement of UV-DDB by XPC on the lesion. The ubiquitylated DDB2 is degraded by the proteasome. The ubiquitylated form of XPC reverts to the unmodified form through deubiquitylation. Reproduced from ref. [285] with permission from Elsevier.

DDB2 [126], and has a large number of other functions apparently unrelated to NER.

XPC is the major damage binding protein that initiates NER [127]. XPC binds tightly with a cofactor HR23B, one of the two human homologs of the yeast Rad23 gene product, and both are located on chromosome 3p25, a region deleted frequently in human squamous cell carcinomas [128]. The XPC/HR23B complex [129, 130] binds to damage in non-transcribed DNA, acting to stabilize subsequent XPA/RPA binding to the damaged site, with a high affinity [92, 127]. XPC is displaced through ubiquitylation by the E3 ligase activity of DDB1/DDB2. XPC has sequence similarity to single strand binding proteins and DNA binding appears to be critically dependent on one tryptophan residue (Tryp690) [131]. The mechanism appears to involve binding to the undamaged strand depending on the relaxation of base-pairing in the damaged region, giving the recognition process great versatility and independence from the actual chemistry of the damaged site [131].

Residual repair in XP-C cells is confined to small transcriptionally active islands of the genome, where repair occurs at normal rates, surrounded by oceans of unrepaired transcriptionally silent DNA [132–135]. The XPC/HR23B complex is associated with CEN2, a protein that stabilizes XPC and which is also involved in centrosome duplication, suggesting a direct link between GGR and cell division [136]. The constitutive level of expression of XPC is controlled by p53, and expression can be induced by UV and ionizing radiation [137, 138].

Several of the UV repair components, including XPC are also involved in repair of base damage or double strand breaks from ionizing radiation [139–141], which may represent a minor function of the ability of XPC to bind to small distortions with single strand properties [131].

24.5 Transcription-Coupled Repair

Pyrimidine dimers are excised more rapidly from actively transcribed genes, especially from the DNA strand used as the template for transcription [93–97]. A detailed base by base analysis of excision in the promoter and early region of the *PGKI* gene, using ligation-mediated PCR, revealed that excision is slow where binding proteins interact with the promoter but increases immediately after the ATG start site for transcription [142]. The initial damage recognition mechanism for TCR may be the stalled RNA Pol II, itself, although a potentially large number of gene products play a role in mediating the increased rate of repair in transcriptionally active genes [96, 97]. During transcription on a damaged template the pyrimidine dimer causes mis-incorporation of

uridine into the messenger RNA, which blocks further transcription [143].

The arrest of RNA Pol II at sites of DNA damage during transcription elongation plays a central role for damage recognition in TCR [144]. The blocked RNA Pol II masks damaged sites and must be removed, or backed off, to alleviate the arrested transcription and provide access for repair enzymes. Recent evidence suggests that the CSB protein prevents Pol II back-tracking induced by TFIIS, to produce a stable stalled complex [143]. This complex provides time for the assembly of the NER excision complex and expansion of the transcription bubble by TFIIH. Excision then removes the damaged strand, the Pol II enzyme and the nascent transcript.

Cells from patients suffering from the neurodegenerative and sun-sensitive disease Cockayne syndrome are specifically defective in TCR [12, 145]. The excision of DNA photoproducts from total genomic DNA of CS cells is normal, but repair of transcriptionally active genes is reduced [145], and DNA and RNA synthesis fail to recover to normal levels after UV irradiation [146]. The neurodegenerative symptoms of CS could be a direct result of neuronal loss through apoptosis of non-dividing cells that experience natural oxidative damage in the brain. Two CS genes, *CSA* and *CSB*, are involved specifically in TCR. CSA contains WD-repeat motifs that are important for protein–protein interactions [147]. CSB contains an ATPase, helicase motifs, and a nucleotide binding domain, but only the latter is essential for TCR and CSB does not function as a helicase [148, 149] The CSB protein is associated in the RNA Pol II elongation complex with DNA and nascent RNA [150–152] and can actively wrap DNA [153]. The CSA protein is neither part of this complex, nor forms a stable complex with CSB [150], but is still required for TCR during transcription elongation [151, 154]. Cells lacking either CSA or CSB are unable to ubiquitinate the CTD of RNA pol II [155].

24.6 Damage Verification and Unwinding

After damage recognition through the specific GGR or TCR proteins, a process of damage verification appears to occur in which the region containing the damage is expanded into a single stranded bubble by the 3′-5′ (XPB) and 5′-3′ (XPD) helicases contained in the transcription factor TFIIH [109, 156]. The initial DNA binding proteins are replaced by another damage binding complex, XPA and RPA, that stabilizes the region and has a larger binding affinity [157, 158]. XPA binds to both the damaged and the undamaged strand, but RPA binds mainly to the undamaged strand [157]. XPA binds to the p34 subunit of RPA through its N-terminal region and to the p70 subunit through a central portion in exon IV; to ERCC1 through a small region in exon II; to DNA through its central region covering approximately exons III to V and a Zn finger in exon III; and TFIIH through its N-terminal region [159].

The transcription factor TFIIH plays a central role in unwinding the damaged region and downloading several of the other NER components. TFIIH can interact with all of the components of the core excision complex, individually, but its strongest binding under physiological conditions is with XPC and XPG [109]. Mutations in the XPB and XPD components can also affect the promoter opening function of TFIIH [160, 161]. The phenotypes of mutations in *XPD* are associated with three different clinical disorders: XP group D, TTD, or a rare combination of XP and CS. XP-B is similarly associated with a combined XP and CS disorder.

The XPB protein is required for both repair and basal transcription, whereas XPD is dispensable for basal transcription, which may explain why fewer surviving patients with mutations in XPB are reported than XPD [65]. Most TTD patients exhibiting UV sensitivity fall into the XP-D complementation group [30]. Human mutations that are relevant to the disease phenotype in the *XPB* and *XPD* genes are all mis-sense because loss-of-function mutations are lethal, although the severity can be influenced by gene dosage [30]. XPD is an essential protein for transcription and cell viability, and no patient can have two null alleles [30]. Several cases of XP-D and TTD patients have an allele in common, suggesting that the second alleles determine the clinical presentation, which is supported by studies in *S. pombe* showing the shared alleles behave as null mutations [162] and would play no direct role in the disease. The distribution of mutations within the XPD protein reveals that essentially all XP-D mutations fall within one of the conserved helicase domains [163]. This pattern indicates that these mutations can be expected to reduce the helicase activity of the protein [160]. In contrast, the TTD mutations usually fall outside of the helicase domains and show significant clustering at the C-terminus of the protein. The structure of the *Archeal* homolog of XPD has elucidated the functional consequences of many mutations by placing them within the structure of the protein [164, 165]. A mouse strain with a mutation that copies a human *TTD* mutation in *XPD* has similar symptoms to those seen in human TTD patients, together with an aging and cancer-prone phenotype [26].

TTD-specific mutations may subtly interfere with the ability of XPD to interact with its partner proteins within the TFIIH complex and thereby destabilize the complex [160, 166]. Evidence in support of this idea comes from the identification of a component of the transcription factor TFIIH corresponding to TTD-A patients that have no mutations in *XPB* or *XPD* but have low levels of TFIIH [167]. This component has a small size, only 8 kDa [168, 169] and is mutated in three patients with TTD, and appears to be required for maintenance of normal levels of TFIIH.

24.7 Excision Nucleases

The final stable complex that binds and remains at the site of DNA damage acts as an assembly point for the XPG and ERCC1/XPF nucleases that cut the damaged strand 3′ and 5′ to the damaged site. The excision process removes a 27–29 nucleotide oligonucleotide containing the photoproduct by cleavages 5 nucleotides on the 3′ side of the photoproduct, and 24 nucleotides on the 5′ side [170]. The structure-specific cleavage pattern is determined by binding of RPA to the unwound damaged site, and the excised fragment is close in size to the footprint of RPA on the DNA [171]. Slight variations in the precise sites of cleavage result in the removal of variable fragments between 27 and 29 nucleotides in size. The nucleases also function in the repair of DNA-DNA crosslinks [172], recombinational repair [173], chromatid exchange aberrations [174], and telomere maintenance [175, 176]. These nucleases a have sequence similarities to the FEN-1 class of nucleases and interact with PCNA and hence play a role in DNA replication [177, 178].

The 5′ cleavage occurs first and is carried out by a heterodimer of XPF and ERCC1 that incises DNA on the 5′ side of the damaged site [108, 123, 179, 180]. The *XPF* and *ERCC1* genes may represent an ancient duplication and are unstable unless in a heterodimer complex [181]. The enzymatic activity resides in the XPF partner of the complex [182]. XPA serves as an anchor for the 5′ nuclease through binding to ERCC1 [183]. Patients with mutations in XPF are rare and most cases have been found in Japan with relatively mild symptoms [184]. Only one patient has been identified with mutations in ERCC1, which is associated with extremely severe symptoms [21]. In contrast knockout mice for both the *XPF* and *ERCC1* genes are very severely affected and are neonatal lethal [31, 185, 186].

The XPG nuclease cleaves on the 3′ side of the damage and interacts with the XPC-hHR23B complex and TFIIH [109, 187]. The XPG nuclease has a complex range of activities, and mutations in XPG are associated with both XP and CS diseases [12, 188, 189]. XPG is an endonuclease in the FEN-1 family that is capable of strand-specific cleavage of a range of DNA substrates that may arise during DNA replication, repair, and recombination [173]. The enzyme binds to the single strand/double strand junction at the NER bubble but remains inactive until unwinding by TFIIH from the XPF/ERCC1 cleavage causes its activation. The protein not only cleaves the 3′ side of photoproducts during NER [190, 191], but it also resolves recombination intermediates by strand specific cleavage at double strand-single strand junctions [173, 192]. XPG is also a cofactor for thymine glycosylase through which it stimulates repair of oxidative DNA damage (thymine glycols, 8-oxoguanine, etc.) that may also be important in maintaining neural functions that fail in CS patients [191, 193, 194].

The XPG gene product interacts with RNA Pol II by interaction with the elongation factor TFIIS, independently of CSB and facilitates efficient transcription elongation [195], which provides an explanation for the complex symptoms of some XPG patients that show both XP and CS symptoms. The excised region is replaced by the action of a complex similar to that involved in normal DNA replication [101]. PCNA is loaded onto the DNA by the 5 subunit RFC complex, which then anchors the replicative polymerases Pol delta or Pol epsilon [196]. The cell cycle regulator p21 is upregulated after damage and slows S phase progression through binding to PCNA, but it appears to be specifically degraded during the repair polymerization. The final closure of the repaired site occurs with DNA ligase, most probably ligase I [196].

24.8 Nucleotide Excision Repair: Polymorphisms and Heterozygote Risk

The disease alleles of the NER genes represented in the human diseases XP, CS, TTD, and COFS represent high penetrance mutations that cause disease when homozygous [197]. There are in addition susceptibility alleles that may give rise to disease at lower penetrance according to lifestyle, diet, hormonal balance, and exposure to environmental chemicals, radiation, and infectious agents. A large number of alleles present at low frequency individually could combine to create variation in disease frequency in the overall population. Heterozygosity in XP genes has also been of concern, especially in parents of affected children.

Polymorphisms have been found in most of the XP genes and many disease associations have been claimed [198–202]. Many of these polymorphisms result in nonconservative amino acid changes that could have an impact on NER function and disease [203]. The reported associations between repair gene polymorphisms and disease suffer in many cases from problems of statistical power. A detailed reexamination suggests that as yet few of the reported associations are on sufficiently large data sets to be firmly established [199].

Heterozygosity in NER genes has not been clearly associated with significant disease among XP parents, although the evidence is largely anecdotal. Detection of small increases in skin cancer among XP parents is likely compromised by the small numbers of subjects, and low solar dose rate, especially if they share their children's restricted sun exposure. In contrast several mouse strains with NER genes knocked out have shown increased cancer in the heterozygotes. These include the XPE (Ddb2) mouse [9], the Xpc mouse [10], and

the Xpv (Pol eta) mouse [11]. These may represent increased ease of detection of an heterozygous UV sensitivity by the high UVB dose rates used in mouse cancer studies, as well as potential unique properties of the repair genes on mouse backgrounds as compared to human.

24.9 Replication of Large DNA Adducts: Class Y Bypass Polymerases

DNA photoproducts are blocks to the replicative DNA polymerases, alpha, delta, and epsilon that cannot accommodate large distortions such as DNA photoproducts or adducts in their active sites [204, 205]. Replicative bypass of these photoproducts is achieved instead by damage-specific polymerases with relaxed substrate specificity, now defined as class Y polymerases [206, 207]. Class Y polymerases have larger active sites that allow them to read through noninformative sequence information resulting from DNA damage [208]. As a consequence these polymerases have high error rates on the order of 1 % when assayed in vitro, and this property must be restricted in vivo to prevent catastrophic levels of mutation [209, 210].

Three class Y polymerases have been identified in the mammalian genome, Pol eta, iota, and kappa. The genes *Pol eta* and *Pol iota* are close homologs, unique to mammalian cells, and only a single *Pol eta* (*yRad30*) gene is found in yeast [206]. Pol iota has a poorer capacity for replication of UV damage and Pol kappa seems completely unable to replicate UV damage. Pol eta has a greater capacity for replicating T-containing photoproducts and Pol iota a preference for C-containing photoproducts. Pol eta can therefore insert adenines across from thymine-containing photoproducts accurately replicating a T=T pyrimidine dimer. Pol iota can insert guanines across from cytosine-containing photoproducts, resulting in accurate replication of a T=C pyrimidine dimer or [6-4] photoproduct.

Pol eta is regulated by several mechanisms that prevent extensive replication and restricts its subcellular location. Pol eta acts distributively, and extends nascent DNA chains by one or two bases across from the photoproducts, after which replication switches to Pol zeta or kappa [211]. Pol eta is uniformly distributed in the nucleus, and excluded from the replication fork until replication is stalled by UV damage, whereupon it relocates with other proteins in microscopically visible foci coincident with PCNA at the replication fork [74, 212]. Relocation requires specific sequence motifs at the C-terminal end of the protein for translocation and for binding to ubiquitylated PCNA [212, 213].

The error-free DNA damage replication pathway involving Pol eta and other polymerases is part of an overall DNA replication pathway for UV damage defined in yeast as the RAD 6 epistasis group [214]. Several of the components of this pathway are ubiquitinating enzymes that target PCNA, which functions as a major control point for the overall process [215]. PCNA is modified by UBC9 that is a small ubiquitin-related modifying enzyme (SUMO), and by Rad6 and the MMS2-UBC13 heterodimer that are ubiquitin-conjugating enzymes [215]. Multiple modifications compete for the same lysine (K164) residue on PCNA and redirect subsequent pathways through SUMO, monoubiquitination, and multiubiquitination [215]. Recruitment of Pol eta is regulated by reversible ubiquitylation of PCNA by the Rad6/Rad18 ligase and the USP1 deubiquitylating protease [77]. The monoubiquitylation favors recruitment of Pol eta, whereas Ub(K63) favors error-free bypass or recombination, and sumoylation favors normal DNA synthesis [76]. Delays in replication fork progression leads to autodegradation of USP1 and increased PCNA ubiquitylation which recruits Pol eta [77], as well as Pol iota and Pol kappa [216]. Pol eta is consequently recruited to the replication fork in response to more general cellular stresses than just its specific role in bypass of bulky UV DNA lesions.

Pol eta plays a large number of roles in situations where replication presents significant obstacles to normal replication polymerases as well as during important physiological processes. Pol eta is important, for example, during immunoglobulin hypermutation and contributes to generating the strand bias of A–T base pair substitution errors [217–225], and in homologous recombination during immunoglobulin conversion [226]. Pol eta also plays a role in the extension of invading strands in D-loops during homologous recombination to prevent replication fork degradation [227], although in contrast Pol eta deficiency in vivo increases homologous recombination [79]. Immunoglobulin hypermutation is also enhanced by other polymerases such as Pol iota [228] and Pol theta [229]. Deficiencies in nucleotide pools, such as caused by the inhibitor hydroxyurea, are also sufficient to recruit Pol eta to replication forks [82]. Pol eta activity is enhanced by interaction with other components of the damage response that accumulate at replication forks, such as the mismatch proteins MSH2-MSH6 [230], although mismatch repair generates a different mutation spectrum than Pol eta [223].

In the absence of the low fidelity polymerases, a complex set of overlapping intra-S phase checkpoints are activated that prevent gross chromosome rearrangements [81, 231, 232]. The extended regions of single stranded DNA remaining after the fork progresses without Pol eta increase signaling by the ATRIP-ATR kinase pathway and enhance S phase arrest [44]. Blocked replication forks are further stabilized in UV damaged cells by p53, and its inactivation by viral transformation allows fork breakage and DNA rearrangements to occur [74, 81, 83, 231, 233]. The actual frequency of fork breakage is low [78] and most arrested forks are resolved without breaks [68]. Central players in these rearrangements

are the protein complex hMre11/hRad50/Nbs1 and Rad51 that are involved in nonhomologous and homologous recombination, DNA replication arrest, and telomere maintenance [81, 231, 232, 234].

24.10 Systemic Impacts of Nucleotide Excision Repair

24.10.1 Disorders of the Central Nervous System

Patients with mutations in the NER genes associated with TCR, both XP and CS, often exhibit progressive neurological abnormalities in addition to skin photosensitivity [235] and this is presumed to be caused by endogenous sources of DNA damage. Consistent with this attribution, mouse embryo fibroblasts derived from *Csb−/−* mice are sensitive to X-rays and oxidative damage [236–238], but in both species the sensitivity to oxidative damage is greater for mutations in CSB than CSA [236, 239].

The neurological symptoms include progressive mental deterioration, diminished deep tendon reflexes, and deafness sometimes beginning early in life [12]. Patients may have microcephaly, intellectual deterioration with loss of the ability to talk, and increasing spasticity with loss of ability to walk, leading to quadriparesis, in addition to dwarfism, and immature sexual development. The major histological feature is primary neuronal degeneration with dysmyelination, particularly in the cerebral cortex and cerebellum. CS patients show extensive cerebellar atrophy including a marked reduction of granule cells and the loss of Purkinje cells [13, 240].

The neurological symptoms of XP and CS patients have been ascribed to defective repair in the brain of endogenous oxidative damage that blocks transcription [191, 193, 237, 241–243]. The precise origin of oxidative damage is not known, but presumed to be mitochondrial leakage. Cerebellar granule cells are vulnerable to a variety of toxins that decrease glutathione levels and make the cells more vulnerable to cellular damage from reactive oxygen species [244, 245]. Purkinje cells are susceptible to ischemic death due to their reduced capacity to sequester glutamate and reduced ability to generate energy during anoxia [246]. Tissues that degenerate in XP and CS appear to be sensitive to oxygen levels, including the Purkinje cells, retina, and oligodendrocytes [247–250]. TUNEL staining and oxidative stress has been observed in cerebellar granule cells from clinical samples of CS and XP-A patients [251–254].

No consistent correlation has yet been established between sites of mutation and the severity of the clinical symptoms. Null mutations in the early region of the CSB gene have been found in a mildly UV sensitive patient with minimal neurodegenerative symptoms [14], and in quite severe patients [255, 256]. The presence of the PiggyBac transposon in intron 5 is flanked by *CSB* mutations and its contribution to the symptoms remains to be established [255, 257].

Mouse knockouts of XP and CS genes generally present a weaker neurological phenotype than the corresponding human disorder but greater cancer predisposition [8, 258, 259]. *Xpa−/−* mice, for example, have no neurological deficit, and only a slight reduction in lifespan [28, 29], in comparison to human XP-A patients who are usually severely affected. Cs-a and Cs-b mice, unlike the corresponding human CS disorder, are predisposed to UVB-induced and chemically induced skin cancer [259–262].

The severity of the neurological symptoms in mice has been increased by crossing strains of Cs-b or Xp-g knockouts with Xp-a or Xp-c knockouts to increase the extent of NER deficiency. These include *Csb−/−* mice crossed with *Xpa−/−* mice [28], Ttd mice crossed with *Xpa−/−* [263], and *Xpg−/−* crossed with *Xpa−/−* [264]. Loss of both Purkinje cells and granule cells occurred in *Csb−/−/Xpc−/−* mice [265]. The compound knockout *Csb−/−/Xpa−/−* mice had stunted dendritic trees of Purkinje cells, along with decreased thickness of the molecular layer and internal granule layer [28]. These observations suggest that the cerebellum is particularly susceptible to DNA repair deficiencies. A dramatic increase in p53 accumulation and apoptosis was identified in the external granule cells of the cerebella of *Csb−/−/Xpc−/−* mice, suggesting that the loss of both Csb and Xpc rendered these cells vulnerable to cell death. Other NER deficient mouse strains exhibit increased cell death and/or increased p53 staining in the brain. *Ercc1−/−* mice show increased p53 levels in the brain and liver [185]. In *Xpg−/−* mice, pyknotic figures and reduced calbindin staining were observed in the Purkinje layer, while TUNEL staining was strongest in the granule layer, and rare in the Purkinje layer [266]. In *Csb−/−/Xpa−/−* mice, TUNEL staining was increased in the external granule layer at day 8 [28].

The most obvious source of endogenous DNA damage would be the high level of oxidative metabolism in the brain [267], combined with its relative deficiency of antioxidative protective mechanisms [268–270]. DNA lesions are repaired more slowly in neurons relative to dividing cells suggesting that lesions are likely to accumulate in neurons over time [270]. The relevant DNA damage must be of the kind that is a significant substrate for the NER system, rather than the commonly oxidized bases such as 8-OH-dG because neurodegeneration is greater when strains are crossed to reduce overall NER capacity, but not when crossed with strains specifically defective in repair of oxidative damage such as *Ogg1* [265]. Even though loss of *Ogg1* increases the mutation rate in vivo for the oxidative lesion 8-OH-dG [271], the neurological status of *Ogg1*-knockout mice seem normal,

indicating that the major classes of oxidative damage are not the proximal cause of neurodegeneration.

Xpc and Csb are both components of the endogenous proteolytic system: Xpc through the proteasome [272], and Csb and Csa as cofactors of an E3 ubiquitylation ligase [273]. Consequently XP and CS could, like many other neurodegenerative disorders, be caused by defective clearance of denatured proteins [274].

24.10.2 The Somatotroph Axis (GH/IGF-1)

Interactions between DNA repair deficiencies and hormone-dependent pathways contribute to the complexity of many of the DNA repair deficient human disorders [2]. Developmental disorders involving reduced stature and weight gain and liver abnormalities were seen in the first mouse knockout targeting the *Ercc1* gene [185], and in subsequent knockouts of *Xpg, Xpf*, and combined strains [26, 28, 263, 264, 275, 276]. This pathology has been identified as the result of accumulation of DNA damage, increased levels of p53, and cellular apoptosis in the liver [31, 276]. The damage affects major hormonal pathways of growth regulation and aging: the growth hormone (GH) and insulin-like growth factor-1 (IGF-1) [31, 276].

Mutations in the XPD component of the transcription factor TFIIH, with which CS proteins interact, also result in many transcriptional defects including: reduced responses to nuclear receptors [277, 278], dysregulation of peroxisome proliferator-activated target genes [279], vitamin D receptor-responsive genes [280], and thyroid hormone-responsive genes leading to hypomyelination [281]. Nuclear receptors that mediate hormone-dependent gene activation require phosphorylation by the cdk7 component of TFIIH, and the *XPD* repair gene influences signal transduction by retinoids (RAR and RXR), vitamin D, steroids (ER, AR, and GR), and thyroid hormone [278].

24.10.3 DDB1 and DDB2: Essential Functions and Viral Infection

The XPE complementation group of XP represents a mildly affected group of patients whose cells carry out near normal levels of NER [111, 113], and for this reason XPE was thought to have a relatively minor role in NER. The discovery that DDB1 is a cofactor in a DDB1-Cul4A-ROC1 E3 ubiquitin ligase [273] indicated that the heterodimer DDB1/DDB2 could play a wider role in many cellular functions depending on the interacting substrates for ubiquitylation. At least 30 target proteins of the E3 ubiquitin ligase have been identified including DDB2, CSA, CSB, XPC, histone H1, histone 2a, and histone 4, PCNA, and transcriptional activation through E2F1 [126, 282, 283]. DDB2 exists in at least four forms: (1) free DDB2, (2) the DDB1/DDB2 heterodimer, and two complexes associated with protein degradation, (3) the CUL4, and (4) the COP9 signalosome [284]. The ubiquitylation of XPC plays an important part of the sequential loading of XPC onto damaged sites and its subsequent displacement by the XPA/RPA binding complex (Fig. 24.3) [285].

The role of DDB1/2 in DNA repair in vivo is still unclear because, despite its affinity for damaged DNA [121], it is dispensable for in vitro excision experiments where the damage is in pure DNA [284]; the repair deficiency is more explicit when the substrate is chromatin bound. Suggested functions [286] include: DDB1/2 binds to damaged DNA, ubiquitylates DDB2 to release it and download XPC/HR23B, which in turn is ubiquitylated to increase it binding affinity [285]; DDB1/2 ubiquitylates histones to make regions of DNA more accessible to repair; DDB1/2 plays accessory roles in the cell cycle, signal transduction, and mutation.

DDB2 expression is induced by UV light through p53 transactivation in human cells, but not mouse or hamster cells [104–106], and is anti-apoptotic [9]. A consensus DNA-binding site for p53 is present in the human p48 promoter region but has either diverged or is absent from the mouse homolog [287]. These observations provide a partial explanation for early observations that excision repair can be low in some mouse cell strains, especially for cyclobutane dimers but not [6-4] photoproducts [288–290].

Targeted knockdown of DDB1 efficiently reduces excision repair of pyrimidine dimers, but not [6-4] photoproducts [291]. UV irradiation converts DDB1 from cytoplasmic into a tightly bound chromatin form that recruits DDB2 and CUL 4A to chromatin-bound functions [284]. The cytoplasmic DDB1 protein also interacts with a range of viral proteins [112, 292, 293] and the amyloid precursor protein involved with Alzheimer's [294]. The Vpr protein of HIV downregulates NER through interaction with DDB1, stimulating an ATM/ATR damage response, indicating common pathways for cellular attack by DNA damaging agents and viral infection (Fig. 24.1) [112].

Elimination of *Ddb1* in mouse knockouts is lethal in the early embryo, being essential for normal development of the epidermis and neuronal precursor cells which are eliminated by p53-dependent apoptosis [295, 296]. This resembles the neuronal loss in TCR defective Cockayne mice and humans [265].

Acknowledgements We are grateful to the Luke O'Brien Foundation and the XP Society whose continued support and encouragement have been instrumental in furthering our work on XP and Cockayne Syndrome. The work described here was supported in part by grants from the National Institutes of Neurological Disorders and Stroke grant 1R01NS052781 (JEC), P01 AR050440-01 (PI: E. Epstein). This chapter draws upon and updates several previous reviews [84, 235, 297].

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Hereditary Colorectal Cancer

25

Sonia S. Kupfer and Nathan A. Ellis

25.1 Introduction

Colorectal cancer (CRC) is one of the most significant causes of cancer morbidity and mortality in the United States. It is estimated that approximately 142,500 men and women will have been diagnosed with CRC and 51,370 persons will have died of this disease in 2010 [1]. CRC incidence rates increase with age with greater than 90 % of cases occurring in persons over the age of 50. The age-adjusted standardized incidence and mortality rates are higher in males than in females, and incidence and mortality also vary between different populations (Table 25.1).

Twin [2] and family [3–5] studies have shown that genetic risk factors influence CRC incidence in up to 35 % of cases, but within that 35 % the level of risk conferred by genetic factors that predispose to CRC varies enormously. In comparison to the risk for a person with no relatives with CRC (Fig. 25.1), the risk for a person who has a single first-degree relative with CRC is threefold greater (6 % lifetime risk), and it is eightfold greater for a person who has two first-degree relatives with CRC (17 % lifetime risk). Moreover, risk is increased fivefold if the affected first-degree relative was diagnosed before age 45 [6, 7]. There is also increased risk of developing CRC in the first-degree relative of a person who has been diagnosed with one or more adenomatous colonic polyps [8, 9]. These correlations between family history and CRC susceptibility are propelled by a range of genetic mechanisms, from high-risk, disease-causing mutations that ablate gene function to low-risk genetic variants that perturb gene function in subtler ways. In addition, genetic risk factors can interact with each other and with nongenetic factors to increase or decrease CRC risk. However, the impact of interaction on risk is poorly understood.

The correlations between family history and CRC risk are also reflected in the types of family histories in which CRC occurs and the proportions of these types. Approximately 70 % of CRCs occur in persons with no known family history of CRC, so-called sporadic CRC (Fig. 25.2). In ~25 % of CRC cases, the person with CRC has up to two first-degree or second-degree relatives with CRC, which is often termed familial CRC [10, 11]. In ~5 % of cases, there is a dramatic family history (three or more CRCs in multiple generations in the family). Cases with strong family histories are often associated with a disease-causing mutation in a known hereditary CRC gene. For example, in ~3 % of all CRC cases, a mutation in one of the mismatch repair (MMR) genes is present. When a disease-causing MMR gene mutation is present, the CRC predisposition syndrome is referred to as Lynch syndrome or hereditary non-polyposis colorectal cancer (HNPCC). In ~1 % of CRC cases, a mutation in the adenomatous polyposis coli (*APC*) gene is present, which is strongly associated with colonic polyposis. When a mutation in *APC* is present, the syndrome is referred to as familial adenomatous polyposis (FAP). In <1 % of CRC cases, a mutation is present in a gene that is associated with a hamartomatous syndrome, including familial juvenile polyposis, Cowden's disease, and Peutz-Jeghers syndrome (PJS).

Given the range of risks associated with known genetic risk factors and the distribution of family histories of CRC, what do we mean by hereditary CRC? Certainly, syndromes such as Lynch syndrome and FAP, which are associated with single-gene mutations and found in multiple family members, are hereditary in the classical Mendelian sense. When a disease-causing mutation is known to be present in a person, the quantitative information regarding cancer risk is available. However, family history is an imprecise tool for predicting the presence of a disease-causing mutation. In the

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_25

Table 25.1 Colorectal cancer (a) incidence and (b) mortality by gender and race 2002–2006

	White	African Americans	Asian American and Pacific Islander	American Indian and Alaska Native	Hispanic/Latino
(a) Incidence					
Males	58.2	68.4	44.1	38.1	50
Females	42.6	51.7	33.1	30.7	35.1
(b) Mortality					
Males	21.4	31.4	13.8	20	16.1
Females	14.9	21.6	10	13.7	10.7

Rates per 100,000 age adjusted to 2000 US standard population
Adapted from ACS Cancer Statistics

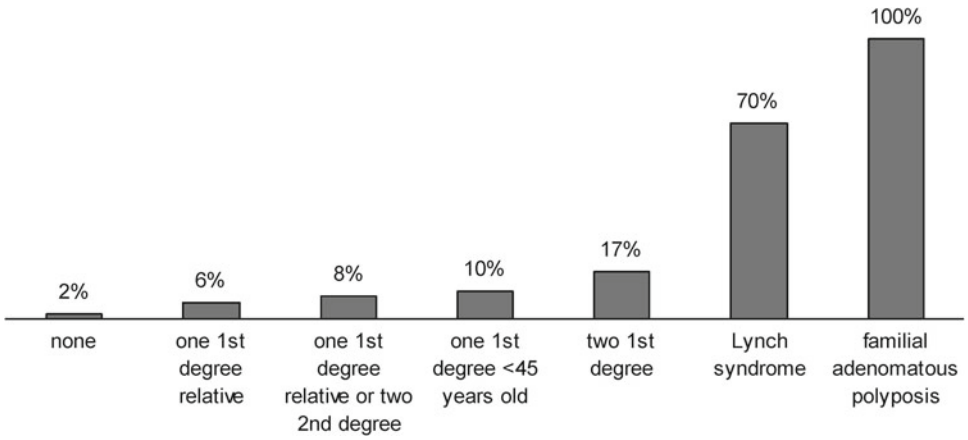

Fig. 25.1 Familial colorectal cancer risk. Estimates of the absolute lifetime risk of colorectal cancer as a function of family history of colorectal cancer. Adapted from Burt [10].

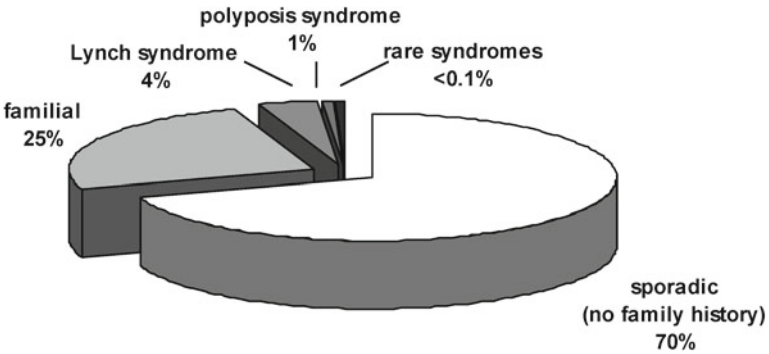

Fig. 25.2 Genetic factors in colorectal cancer. Percentage of all colorectal cancers that develop in the context of hereditary, familial, or sporadic colorectal cancer settings. Adapted from de la Chapelle [22] and Burt [10].

following examples, we present some of the reasons for this lack of predictive power. These examples show how family history can be a poor predictor of CRC risk, and they underline the importance of developing more precise and reliable genetic tools to identify persons at high risk of CRC.

25.1.1 Variable Penetrance

One and the same MMR gene mutation can be found in families with a range of family histories of CRC from very strong to relatively weak. One reason for this range of family histories is that penetrance is variable (penetrance is defined as the probability of developing the disease given the presence of the mutation). On average, penetrance of MMR mutations is less than 50 %. The reasons for variable penetrance are not well understood. Probabilistic events (family size, ascertainment bias, etc.) may explain some of the variation in family history. Deterministic factors also probably play an important role, including genetic factors that modify the risk conferred by the cancer-causing mutation (these genetic factors are called modifiers), exposure to environmental factors, or both.

25.1.2 New Mutations

Up to 30 % of *APC* mutations are new mutations that are not associated with previous family history of CRC, but which

are every bit as dangerous to the present and subsequent generations. Clearly, in these cases, there is no family history to help predict disease occurrence.

25.1.3 Recessive Inheritance

Late-onset Mendelian syndromes associated with increased susceptibility that are caused by recessive genes are difficult to identify and characterize because family sizes are generally small and penetrance is dependent on age. Neither parent is likely to have been diagnosed with CRC and the probability for each son or daughter of having both disease alleles is 25 %. A perfect example of this scenario is the recently described CRC syndrome *MYH*-associated polyposis (MAP), which is associated with biallelic recessively inherited mutations in *MUTYH* (also referred to as *MYH*). Many more recessive CRC syndromes may explain CRC incidence in persons with no previous family history or in familial CRC.

25.1.4 Polygenic Inheritance

Strong family histories of CRC may not be associated with classical single-gene, Mendelian mutations, but rather with the presence of multiple genetic risk factors that combine to create high risk. This scenario can resemble recessive inheritance in that each parent may be at low risk of CRC because he or she carries one or two low-risk or moderate-risk genetic factors. Segregation of genetic factors to a son or daughter from both parents is thus a relatively low-frequency event.

25.1.5 Hereditary Colorectal Cancer

For the purposes of this chapter, we define hereditary CRC as a syndrome caused by one or more genetic risk factors that are associated with a strong family history of CRC, which can be established statistically through tests of genetic segregation in families. Consequently, the main focus here is on the syndromes defined by disease-causing mutations in single-gene Mendelian disorders. There is a general consensus now that the known single-gene disorders do not account for all the genetic variation that influences CRC susceptibility and that many low-risk or moderate-risk genetic variants need to be identified in order to explain the remaining heritability of CRC. We note that numerous reports describing low-risk CRC susceptibility factors have recently been published and elucidation of the role and impact of the major low-penetrance risk alleles is an active area of current genetic investigation [12]. These low-risk genetic factors explain but a small fraction of the yet-to-be-determined CRC susceptibility factors. Consequently, the search goes on to identify genetic risk factors that explain the heritability of CRC with the hope that these factors will some day allow a more precise and reliable prediction of CRC risk.

25.2 Lynch Syndrome

The non-polyposis disorders are so called to distinguish them from those syndromes that lead to the formation of numerous polyps of the lower gastrointestinal tract. Foremost among the non-polyposis disorders is Lynch syndrome (hereditary non-polyposis colorectal cancer). This syndrome arises from mutations in the MMR pathway and results, to varying degrees, in a phenotype of early-onset CRC and extracolonic malignancies. The MMR genes function in a specific type of DNA repair that ensures high fidelity of DNA replication. When MMR function is deficient in a cell, it is associated with a high mutation rate, which drives carcinogenesis. The high mutation rate can be detected using a simple polymerase chain reaction (PCR) assay that tests for microsatellite instability (MSI). Tumors that exhibit MSI have the hypermutation phenotype that is associated with loss of MMR gene function.

Historically, the earliest descriptions of Lynch syndrome came from the pathologist Aldred S. Warthin at the University of Michigan who, in 1913, published a review of several cancer-prone families [13]. Approximately 50 years later, Henry T. Lynch and colleagues collected data from these same families and recognized that CRC was the most prominent cancer in this syndrome. Initially, this familial syndrome was given the name hereditary non-polyposis colorectal cancer (HNPCC) to distinguish it from hereditary conditions in which cancers arise in a milieu of carpeting colonic polyps. In 1984, the name Lynch syndrome was proposed in honor of Dr. H.T. Lynch's seminal observations. Moreover, HNPCC was thought to be a misleading name because polyps are still encountered albeit not to the degree seen in polyposis conditions. Arguably, the definition of polyposis is somewhat arbitrary. In 1993, the field of Lynch syndrome genetics was energized by the identification of *MSH2* and, shortly thereafter, *MLH1* as genes the mutation of which results in Lynch syndrome. In the 1990s, clinical criteria and guidelines were established including the Amsterdam criteria and Bethesda guidelines that are used to guide molecular genetic testing in individuals who might be affected by the syndrome (Table 25.2). The Amsterdam criteria were devised to enrich for families with mutations to help in the identification of the culprit genes; the Bethesda guidelines were an attempt to devise a more inclusive set of rules to identify mutation carriers. These guidelines nonetheless cannot be used to identify all persons with disease-causing mutations or families in which MMR gene mutations are segregating,

Table 25.2 Amsterdam II criteria and revised Bethesda criteria

Amsterdam II	Revised Bethesda Patient who meets Amsterdam II criteria
Three or more relatives with a Lynch syndrome- associated cancer	CRC diagnosed in a patient less than 50 years old
Two or more successive generations affected	presence of synchronous or metachronous CRC or other LS-associated tumor regardless of age
One or more relatives diagnosed before age 50	CRC with microsatellite instability in patient less than 60 years old
One should be a first-degree relative of the other two	CRC diagnosed in one or more first-degree relative with a LS-associated tumor, with one of the cancer diagnosed less than 50 years old
Exclude familial adenomatous polyposis	CRC diagnosed in two or more first- or second-degree relatives with LS-associated tumors, regardless of age

CRC colorectal cancer, *LS* Lynch syndrome

because age of disease onset is variable, penetrance is variable, and family sizes are often small. Universal screening—MSI testing, immunohistochemistry (IHC) for MLH1, MSH2, MSH6, and PMS2, or both—on all CRCs has been recommended, which obviates the need for guidelines, and it has slowly gained widespread acceptance. As whole-genome DNA sequencing becomes more efficient and lower cost, we can foresee a day in which genetic screening using whole-genome sequencing would help overcome the limitations of clinical guidelines.

25.2.1 Natural History of Lynch Syndrome

Lynch syndrome is the most common type of hereditary CRC and is estimated, depending on the study, to represent 1–6 % of all CRC cases. It is inherited in an autosomal dominant fashion. It is highly penetrant with approximately 50 % of mutation carriers developing CRC and approximately 80 % developing CRC or a malignancy associated with Lynch syndrome [14].

The clinical features of Lynch syndrome include CRC in multiple family members in several generations. The mean age of cancer diagnosis is 45 years. Approximately 70 % of colon cancers are proximal to the splenic flexure, though it should not be overlooked that up to 30 % of Lynch syndrome-associated tumors are found in the distal colon. The histologic features of these tumors include tumor-infiltrating lymphocytes, mucinous and signet-ring features, and medullary growth patterns. In addition, progression of colonic adenomas to carcinomas is accelerated compared to the rate in sporadic CRC. Compared to an 8–10 year progression interval, in Lynch syndrome, adenomas become carcinomas in 2–3 years [15].

Table 25.3 Lifetime risk of extracolonic tumors reported in families with a mismatch repair mutation

Endometrial cancer	27–71 %
Ovarian cancer	3–13 %
Gastric cancer	2–13 %
Urinary tract cancer	1–12 %
Brain tumor	1–4 %
Biliary cancer	2 %
Small bowel cancer	4–7 %

From Vasen HF et al. J Med Gen 2007;44:353–362 [22]

Although CRC is the predominant tumor, extracolonic tumors also can develop. Lynch-associated tumors are listed in Table 25.3. Endometrial cancer is the second most frequent malignancy in Lynch syndrome. Additional tumors include ovary, stomach, small bowel, pancreas, hepatobiliary, brain, and urothelial tract [16]. A subset of patients with MMR gene mutations develop cutaneous lesions, a syndrome known as Muir-Torre (see Sect. 25.6). Given the number and diversity of related tumors, clinicians need to be especially astute and query patients' family histories carefully in order to facilitate diagnosis.

25.2.2 Genetics of Lynch Syndrome

25.2.2.1 Inheritance

Lynch syndrome is inherited as an autosomal dominant disorder. Carriers inherit one mutated MMR gene from one parent and one un-mutated gene from the other parent. The heterozygous cell has sufficient MMR function to carry out normal DNA repair and protect the cell from excessive mutation. However, when, in a somatic precursor cell, the un-mutated gene is lost by normal mutagenic processes, which inevitably happens in the many cell divisions that occur during human development and adult life, then this cell loses all MMR function and excessive mutation ensues in the subsequent generations of this cell's lineage. A striking hypermutability develops and increases the probability that transforming mutations will accumulate in oncogenes or tumor suppressor genes. The second hit that leads to loss of the un-mutated MMR gene results from random mutational processes, including large gene deletion, chromosome loss and re-duplication, homologous recombination proximal on the chromosome to the mutated MMR gene followed by segregation of the two chromosomes carrying the same mutated allele, nucleotide substitution, or small insertion or deletion. There are rare reports of children with biallelic *MLH1* and *MSH2* mutations who are prone to develop cancers at a young age. These cancers include leukemia, lymphoma, brain tumors,

and gastrointestinal malignancies. There are also café au lait spots reminiscent of neurofibromatosis. This syndrome is referred to as the Childhood Cancer Syndrome (CCS) [17, 18].

25.2.2.2 MSH2, MLH1, and MSH6

In 1993, *MSH2* was identified as the first Lynch syndrome-associated gene. Linkage analysis performed in two large Lynch syndrome families mapped a susceptibility locus to chromosome 2p16. Subsequent work identified the *MSH2* gene in this region as a candidate locus [19]. Germline mutations of *MSH2* were subsequently identified in Lynch syndrome patients. *MSH2* mutations constitute the largest percentage of mutations found in Lynch syndrome. Linkage methods were also utilized to identify the *MLH1* gene in Scandinavian Lynch syndrome families [20]. The gene was localized to chromosome 3p21 and subsequent work revealed germline *MLH1* mutations in Lynch syndrome patients. Mutations in *MSH2* and *MLH1* account for the majority (over 85 %) of MMR gene mutations in patients with Lynch syndrome. In 1997, disease-causing mutations were identified in Lynch syndrome families in the *MSH6* gene, which is localized within 500 kb of *MSH2* on chromosome 2p21 [21]. *MSH6* mutations account for approximately 10 % of all mutations in Lynch syndrome.

A multi-national database maintained by the International Collaborative Group on HNPCC (ICG-HNPCC now called InSight) was established to catalog Lynch syndrome-associated mutations. The group's website (http://www.insight-group.org/) provides regular updates on mutations reported worldwide in MMR genes. To date, there are over 500 reported mutations primarily involving *MLH1, MSH2,* and *MSH6*. Most of the mutations cause loss of MSH2 function. The majority of these null mutations result in premature translation-termination of the protein through large multi-exon deletions or small insertions or deletions that cause reading frame shifts, nucleotide substitutions that cause stop codons or aberrations in RNA splicing. In addition, a significant proportion of the putatively disease-causing mutations cause amino acid substitutions that produce a mutant protein that has no catalytic activity. These missense mutations can pose a serious challenge in the clinical interpretation of the mutation because (1) biochemical information of the amino acid change is usually unavailable, (2) it is difficult to distinguish rare disease-causing variants from rare benign variants, and (3), if the affected is in a small family, segregation analysis lacks power.

The most common recurrent mutation in *MSH2* is an A→T transversion at c.942+3 in the donor splice site of intron 5. The substitution disrupts normal splicing so that the mutant gene produces an abnormal mature mRNA in which exon 4 is joined to exon 6. The abnormal mRNA encodes a functionally inactive, mutant protein with an in-frame deletion of amino acids 265–314. *MSH2c.942+3* is a hot spot for mutation. The adenine at this site is the first in a run of 26 adenines, which presumably causes the hypermutability. However, the mechanism is not understood. It is estimated that this mutation accounts for 5–10 % of all Lynch syndrome mutations and it has arisen independently multiple times on different chromosomal backgrounds. *MSH2c.942+3A>T* is the most common mutation in Newfoundland, and it occurs on one and the same chromosomal background, indicating that in this population the mutation is inherited identical by descent from a founder individual who introduced the mutation into the population many generations ago. Numerous other MMR gene mutations are known to be shared by unrelated individuals due to founder mutation in an ancestral individual, as the mutation is found on the same chromosomal background [22].

25.2.2.3 PMS2

The *PMS2* gene is localized to chromosome 7p22, originally identified based on its homology with bacterial and yeast MMR genes [23]. Early work on *PMS2* was complicated by the existence of a family of *PMS2* related genes and a partial *PMS2* pseudogene, confounding investigators who thereby missed disease-causing variants in PMS2. Mutations in *PMS2* appear to predispose to Turcot syndrome and early-onset CRC (see Sect. 25.6). However, this finding is controversial as a large European group could not replicate the findings [24]. Similar to the situation for *MLH1* and *MSH2*, biallelic *PMS2* mutations cause a childhood cancer syndrome [25].

25.2.2.4 EPCAM

In 2009, two groups studied cancers that were deficient for MSH2 and MSH6 protein by immunohistochemistry or that exhibited MSI but in which no germline mutation had been identified in *MSH2, MSH6,* or *MLH1* [26, 27]. Diagnostic multiplex ligation-dependent probe amplification (MLPA) analysis (a method that allows detection of large genomic deletions) showed a decreased signal for exon 9 of the *EPCAM* gene (also referred to as *TACSTD1*), which is the last exon in the gene that is located 16 kb upstream of *MSH2*, but the deletion did not extend into the *MSH2* gene. Further analysis of Dutch and Chinese families showed that deletions of the 3′ end of *EPCAM*, which includes the polyadenylation-signal, abolish transcription termination. Failure to appropriately terminate *EPCAM* transcription, in turn, leads to transcription read-through into the *MSH2* promoter across its CpG-rich promoter region. Inappropriate transcription of the *MSH2* promoter results in induction of methylation of the *MSH2* promoter and transcriptional silencing of *MSH2* in cells expressing *EPCAM*. This unusual mechanism explains both the *MSH2* methylation mosaicism (cells that express *EPCAM* exhibit the methylation, whereas cells that do not express *EPCAM* do not exhibit methylation) and the allele-specific methylation of the *MSH2* promoter (*MSH2* methylation occurs

in *cis* on the chromosome that carries the *MSH2* deletion and not on the intact chromosome). Moreover, this mechanism also explains why the abnormal pattern of mosaic somatic methylation segregated with the mutation in families.

A subsequent clinical study of 194 carriers of 3′ *EPCAM* polyadenylation-signal deletions was noted to have similar ages of colorectal cancer onset as *MLH1* and *MSH2* carriers [28]. In contrast, only three women with *EPCAM* deletions were found to have endometrial cancer leading to a lower cumulative lifetime risk compared to other MMR gene mutation carriers. *EPCAM* is expressed at lower levels in uterine tissue. Consequently, *MSH2* silencing as result of *EPCAM* polyadenylation-signal deletion may occur less frequently in the uterus. Higher rates of duodenal and pancreatic cancers were noted in *EPCAM* polyadenylation-signal deletion carriers compared to other Lynch syndrome families. Whether screening recommendations should be different for *EPCAM* polyadenylation-signal deletion carriers remains to be determined. Molecular genetic testing for *EPCAM* deletions is recommended for the subset of families in whom germline mutations in MMR genes are suspected but have not been identified.

25.2.2.5 Genotype-Phenotype Correlations

Clinically, Lynch syndrome cancer phenotypes vary as a function of the mutated MMR gene involved. Mutations in *MLH1* have been associated with higher incidence of CRC, whereas *MSH2* mutations have been associated with higher rates of extracolonic tumors. *MSH6* mutations have been associated with lower rates of CRC with higher rates of endometrial cancer [29], while the phenotypic pattern associated with *PMS2* mutations is still unclear. Furthermore, while the mean age of colorectal cancer diagnosis in *MSH2* and *MLH1* mutation carriers is 43–46 years, in *MSH6* mutation carriers, the mean age of cancer diagnosis is 10 years older [29]. *EPCAM* deletion carriers appear to have lower rates of endometrial cancer but possibly higher rates of duodenal and pancreatic tumors [28].

The reasons for these gene-specific clinical phenotypes are poorly understood and may be due to different functions of MMR proteins, modifier genes and/or environmental risk factors. Data on modifier genes in Lynch syndrome are conflicting. Candidate genes such as *TP53* [30], *NAT2* [31–33], and *CCND1* (the gene that encodes cyclin D1) [34, 35] have been tested for modifier effects but the data are not conclusive. Environmental risk factors have also not been extensively examined, though a multicenter American study found tobacco use to be associated with higher risk of CRC development [36]. More recent data has suggested that higher body mass indices (BMIs) greatly increase the risk of colorectal adenomas, a finding that was noted only in men [37]. Further studies with large, collaborative study design are needed to elucidate the role of modifier genes and environmental factors in Lynch syndrome.

25.2.3 Management of Lynch Syndrome

25.2.3.1 Diagnosis

Diagnosis is defined by the presence of a mutation in one of the MMR genes. The diagnosis can be supported by the presence of MSI in the cancer, absence of MMR protein by immunohistochemistry (IHC), or a variety of other laboratory tests (biochemical or mRNA analysis, methylation testing, etc). Statistical models that take into account all available cancer family history and laboratory testing have been developed to estimate the probability that a person carries a mutation [38, 39] and universal testing of all incident CRC cases helps to identify mutation carriers, who are then referred to the genetics clinic. Unaffected relatives can then learn their mutation status and take preventive measures [38, 39].

There are two approaches to molecular testing for Lynch syndrome. The first is to test for a mutation in an MMR gene directly by PCR-based direct DNA sequencing and some form of analysis for large deletions (MLPA or hybridization-based assays). The sensitivity of PCR-based direct sequence analysis is 80–90 %. However, the high frequency of amino acid substitutions of unknown clinical significance reduces the specificity of this testing and additional testing (e.g., MSI testing) would be required in these cases. The other approach is to test the tumor for MSI, MMR protein staining by IHC, or both, and then perform mutational analysis on cases in which MSI is found and/or an MMR protein is absent. MSI is more sensitive than IHC because as noted above missense mutations constitute a substantial proportion of Lynch syndrome mutations. The sensitivity of MSI testing is ~90 %. Consequently, in families with compelling family history, both approaches may be used. If no germline MMR mutation is identified, a missed mutation (e.g., methylation aberration transmitted through the germline) or mutation in one of the less-often-sequenced MMR genes (e.g., *PMS2*) is possible. Testing of additional tumors in the family for MSI or loss of expression of MMR protein or linkage analysis can help rule out missed mutation. Since MSI or loss of expression of MMR protein can result from non-hereditary causes, namely, by somatic mutation or somatic gene silencing by methylation of the *MLH1* promoter, methylation studies and/or *BRAF* mutation assessment can be performed to help identify such tumors [40]. Finally, advances in DNA sequencing technologies have led to the development of methods to sequence many culprit genes at one time, which provides a simplified approach to mutation identification in presumptive carriers. Indeed, it is now feasible to sequence the entire genome on a clinical basis, and the day is coming when people will have their whole genome sequenced in order to learn their genetic risks and take appropriate medical precautions.

25.2.3.2 CRC Treatment and Survival

There has been conflicting evidence about response to chemotherapy and survival in tumors with MSI (including Lynch syndrome and non-hereditary cases) compared to tumors without MSI. Survival studies do not suggest major differences in Lynch syndrome patients with CRC compared with sporadic CRC [41, 42]. In contrast, a systematic meta-analysis of MSI status and prognosis in CRC (including both Lynch syndrome-associated and non-hereditary tumors) suggested that patients with tumors that exhibit MSI had a 15 % better outcome compared with those with tumors that did not exhibit MSI [43]. As result of these studies, most oncologists withhold chemotherapy for Stage II CRCs with MSI, because the outcomes are the same if not better without treatment.

Regarding responsiveness to chemotherapeutic agents, in vitro studies have suggested that MMR deficiency reduces the cytotoxicity of 5-fluorouracil (5-FU), which is a commonly used chemotherapeutic agent for CRC [44, 45]. To date, studies on response to chemotherapy stratified by MSI status have yielded mixed results and, therefore, MSI status is not yet recommended as a predictive marker of response to chemotherapy.

Finally, immune checkpoint inhibitor therapy is now being used now for metastatic MSI-H colon cancer.

25.2.3.3 Screening

Colonoscopy remains the preferred method for screening in established or suspected Lynch mutation carriers. The current recommended screening regimen is colonoscopy every 1–2 years starting at age 20–25 years or 10 years younger than the youngest age at diagnosis in the family [46]. The recommendation for short intervals between colonoscopies is based on evidence of accelerated adenoma-to-carcinoma progression in MMR mutation carriers [47]. The relative risk reduction with colonoscopic screening was 62 % in two Finnish studies [48, 49]. This translates into a 72 % reduction in mortality attributed to colonoscopic screening with polypectomy [50, 51].

Data on screening for extracolonic cancer is less clear. Endometrial sampling for endometrial cancer, transvaginal ultrasound for endometrial and ovarian cancer, and urinalysis with cytology for urothelial malignancies are often recommended. Similarly, prophylactic colectomy, hysterectomy, or oophorectomy may be pursued but solid prospective data on the effectiveness of these surgical options is not currently available [46].

25.2.3.4 Prevention

The effect of nonsteroidal anti-inflammatory drugs (NSAIDs) as a chemopreventive agent in CRC development in MMR mutation carriers is unknown. A recent study investigating the effect of sulindac on surrogate endpoints for cancer showed increased epithelial proliferation in the proximal colon of Lynch syndrome patients taking the NSAID, which does not support a chemopreventive role for this NSAID [52]. The first large-scale, randomized control trial of aspirin and starch in MMR mutation carriers did not show differences in CRC incidence between the treatment and standard-care arms at 5 years [53]. However, subsequent follow-up data suggested a delayed benefit in aspirin users compared to placebo (Burn, unpublished data). Further studies would need to be done to elucidate the potential role of NSAIDs, aspirin, and other chemopreventive agents in Lynch syndrome.

25.3 Mismatch Repair

The MMR system recognizes and corrects incorporation errors that occur during DNA replication, some chemically induced or spontaneous DNA lesions, and mismatches produced during homologous recombination. Components of the system are also utilized during somatic hypermutation in the maturation of B-cells. MMR repairs both base-base mismatches and insertion/deletion loops (IDLs). Base-base mismatches can be generated by DNA polymerase incorporation errors that escape the proofreading activity of the DNA polymerases. IDLs occur when primer and template strands dissociate and re-anneal incorrectly. Slipped strand mispairings, as these are called, occur preferentially in repetitive DNA sequences of one-nucleotide to five-nucleotide repeats, referred to as microsatellites. Slipped strand mispairings in microsatellite regions can be of varying lengths depending on where loops form in the double helix. The high frequency of unrepaired IDLs plays an important role in development of MSI. MSI is one part of the mutator phenotype associated with MMR gene mutations, in which somatic mutations accumulate at a higher rate as a result of loss of MMR function.

MMR pathways are conserved across all domains of life. Initially, investigation of prokaryotic MMR provided major insights into the existence and functions of genes involved in MMR. The genes *mutH*, *mutL,* and *mutS* were identified in *E. coli,* and subsequently homologues for *mutL* and *mutS* were found in yeasts, mammals, and other species. In Fig. 25.3, we depict MMR mechanisms in eukaryotes schematically. MMR involves four stages: (1) mismatch recognition, (2) incision of the strand that contains the erroneous base(s), (3) strand excision to remove the base(s), and (4) restoration of the correct base by DNA replication using the undamaged DNA template. While MMR mechanisms have been more precisely defined in prokaryotes, the focus in this chapter is on eukaryotic MMR mechanisms. Recent review articles provide in-depth discussions of MMR biochemistry in both prokaryotes and eukaryotes [54–56].

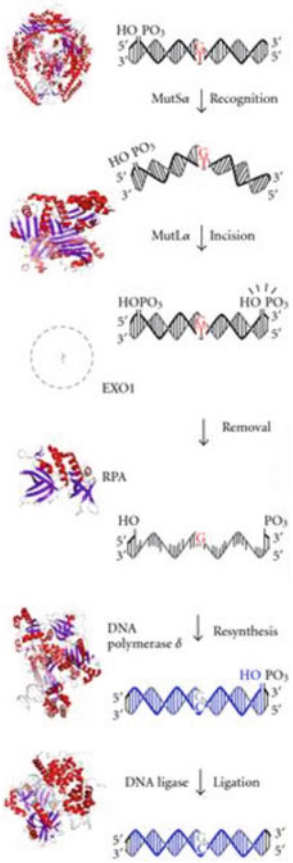

Fig. 25.3 Mismatch repair (MMR) mechanisms in eukaryotes. MMR is a mechanism to repair mismatched bases and small insertion/deletion loops. MMR involves four stages: (1) mismatch recognition, (2) incision of the strand that contains the erroneous base(s), (3) strand excision to remove the base(s), and (4) resynthesis by DNA replication to restore the correct base using the undamaged DNA template followed by ligation. The figure depicts the main steps of MMR along with structural models of the human proteins based on crystallography data. In the first stage, a DNA substrate with a mismatch is shown along with a nick 5′ of the mismatch that represents a discontinuity in the duplex that is usually associated with RNA replication; the discontinuity is required for recognition of the nascent strand on which the mismatch arose through replication error. MutSα (depicted to the left of the substrate) recognizes the mismatch. In the second stage, MutSα binds to the mismatched bases and recruits MutLα (left of the substrate) to the repair site. In the third stage, MutLα nicks 3′ of site of the mismatched base; MutLα can also nick 5′ of the site. In the fourth stage, EXO1, for which a crystal structure is not available, excises the mismatched DNA segment by its 3′ to 5′ exonuclease activity, and single-stranded DNA-binding protein RPA (depicted to the left of the substrate) binds the single-stranded DNA as it is exposed by EXO1. In the fifth stage, DNA polymerase δ (depicted at *left*) resynthesize DNA across the gap, and in the sixth stage DNA ligase I (depicted at *left*) seals the remaining nick by ligation to complete mismatch repair. Adapted from [56] and used by permission.

25.3.1 Eukaryotic MMR

Eukaryotic MMR involves homologues of bacterial components MutS and MutL but not MutH. MutS homologues include MSH2 (*M*ut*S* *H*omologue), MSH3, and MSH6. MutL homologues include MLH1, MLH3, PMS1 (*p*ost-*m*eiotic *s*egregation-1), and PMS2. In contrast to MutS and MutL, which are homodimers in prokaryotes, eukaryotes have evolved heterodimers composed of subunits that are homologous but that have different structures and functions. One such heterodimer is MutSα, which is composed of MSH2 and MSH6. MutSα recognizes mismatched bases and initiates repair of base-base mismatches and small IDLs. 80–90 % of the cellular MSH2 is found in the MutSα complex. MutSβ, composed of MSH2 and MSH3, repairs larger IDLs. MutLα is a heterodimer composed of MLH1 and PMS2; approximately 90 % of MLH1 in humans is found in the MutLα complex. The MutLβ complex is composed of MLH1 and PMS1 and the MutLγ is composed of MLH1 and MLH3. MutL homologues are responsible for strand incision.

25.3.2 MutS Homologue Function

Studies in *E. coli* have elucidated the molecular mechanisms of MutS and provide insight into function of MutS homologues in eukaryotes. Crystallography has revealed that eukaryotic MutS complexes bind double-stranded DNA as heterodimers [56]. Mismatch recognition appears to occur via non-specific DNA binding and bending to search for mismatched bases in an ATP-dependent manner.

MutS mismatch recognition occurs by stacking the mismatched base with a phenylalanine and adjacent glutamate residue intercalated from the minor groove side. These phenylalanine and glutamate residues are highly conserved among bacterial and eukaryotic MutS homologues (specifically MSH6 in humans). In the *E. coli* MutS-DNA complex, the DNA is kinked and unbent at a mismatched site forming a stable recognition complex. Upon mismatch recognition, MutS exchanges ADP to ATP and undergoes a conformational change forming a sliding clamp. This sliding clamp has also been confirmed in eukaryotic MutS complexes [56].

Subsequent activation of downstream reactions of the MutS sliding clamp remains controversial. The moving or cis model postulates that the sliding clamp diffuses along the DNA to activate downstream reactions. The stationary or trans model proposes that MutS remains bound to the mismatched base after initial recognition and the ATPase activity is required for verification of mismatch recognition [56].

25.3.3 MutL Homologue Function

Of the eukaryotic MutL complexes (MutLα, MutLβ, and MutLγ), the majority of MMR appears to be performed by MutLα (composed of MLH1 and PMS2 in humans). MutL and MutLα complexes have an N-terminal ATPase/DNA-binding domain and a C-terminal endonuclease/DNA-

binding domain. MutL proteins are ATPases of the gyrase/Hsp90/histidine-kinase/MutL family (GHKL family) which undergo conformational changes with ATP binding and hydrolysis required for MutL endonuclease activity.

In *E. coli,* MutL interacts with the MutS-DNA complex that activates MutH restriction endonuclease activity and strand incision. MutH is an endonuclease found in Gram-negative bacteria that is bound to hemimethylated GATC sites to the left or right of the mismatch. Activated MutH carries out incision of the newly synthesized DNA strand by recognizing the hemimethylated GATC site. MutH nicks the unmethylated strand at this site, which serves as a point of entry for the subsequent excision reaction. Hence, MMR in *E. coli* is known as methyl-directed MMR.

Eukaryotes, however, do not have MutH homologues, raising the question of how strand discrimination takes place in 5′ and 3′-directed MMR. Recent work has indicated that human and yeast MutLα have latent endonuclease activity that nicks the nascent DNA strand (as opposed to the template strand) proximal to the mismatched base duplex. MutLα nicks 5′ or 3′ of a mismatch, which serves as an entry for exonucleases. MutLα may recognize the nascent DNA strand through interactions with proliferating cell nuclear antigen (PCNA).

25.3.4 Additional Factors in Eukaryotic MMR

Excision events are catalyzed by factors with exonucleolytic activities from strand nicks either 5′ or 3′ of the mismatch lesion. EXO1, which has a 5′ to 3′ exonuclease activity, plays a central role in the excision reaction in eukaryotic MMR; however, a factor(s) with 3′ to 5′ exonuclease activity must also come into play. Moreover, genetic evidence indicates additional exonucleases are involved because *exo1* null alleles have a less severe phenotype than *msh2* null alleles in yeast and in mice [57, 58]. Single-stranded DNA-binding protein replication protein A (RPA) functions to stabilize the gap left by exonuclease degradation of the site of the base-base mismatch, and it inhibits EXO1 activity. The gap is filled by DNA Polymerase δ and the nick is sealed by DNA ligase 1 [55].

25.4 Tumorigenesis

25.4.1 MSI

The discovery of MSI as a marker in Lynch syndrome proved important in defining the mechanism of tumorigenesis in Lynch-related cancers as well as in some non-hereditary cancers. In general, microsatellites, repeats of one to five nucleotides, have a higher mutation rate than other DNA sequences, because DNA polymerases tends to stall or slip on these sequences, the nascent replicated strand loops out, and, if the loop is not corrected to the normal state, the loop can be processed to a gain or loss of repeat units. The DNA repair mechanism that repairs loops to their normal state is the MMR pathway [59].

In contrast to Lynch syndrome, in which a germline mutation in an MMR gene is present and MMR function is lost by somatic mutation of the un-mutated gene copy, in about 15 % of non-hereditary CRC cases, MMR gene function is lost by biallelic methylation of the *MLH1* promoter, resulting in MSI and tumorigenesis. Methylation of the *MLH1* promoter results in reduced gene expression without changing the nucleotide sequence, a phenomenon known as an epigenetic change. In mammals, DNA methylation only occurs in the dinucleotide sequence CpG. Certain gene promoters, predominantly the promoters of housekeeping genes, contain a high proportion of CpG dinucleotides [60]. In general, promoter methylation down-regulates gene transcription, effectively silencing the gene. In 1998, two groups independently reported hypermethylation of the *MLH1* promoter and MSI in sporadic colorectal tumors. In these studies, 84–100 % of tumors with MSI had a hypermethylated *MLH1* promoter and protein expression was absent by immunohistochemistry [61, 62]. These tumors appear to have similar clinicopathological features as the tumors associated with Lynch syndrome, which nearly always exhibit MSI.

Colorectal tumors (cancers and adenomas, as well as other Lynch-related tumors such as endometrial cancers) can be tested by PCR amplification of microsatellites or by immunohistochemistry (IHC). In the PCR-based MSI assay, DNA from microdissected tumor and normal tissue is prepared and five to ten microsatellite loci are amplified by PCR. By fragment analysis with DNA electrophoresis, the lengths of DNA fragments at each locus are determined; the appearances of novel fragments in the tumor in contrast to the normal tissue are indicators of the hypermutation phenotype. Currently, there are several commercially available panels of microsatellite repeat loci that are routinely used in clinical testing. A panel of markers that consist of long mononucleotide runs in the adenine base are most reliable efficient in distinguishing MSI from non-MSI tumors [63]. In the IHC-based test, a tumor specimen is stained with antibodies to the major MMR proteins, MLH1, MSH2, MSH6, and PMS2. Normally, these proteins can be found in the nucleus of dividing cells. If the tumor specimen fails to stain with one of these antibodies, then the tumor is scored MMR protein absent (a positive result), and this is an indication that MMR function is deficient. False-negative IHC results can arise when protein expression is present but the MMR protein is catalytically dysfunctional due to missense mutations; hence, in the hereditary situation, as noted above the MSI test is more sensitive than the IHC test.

25.4.2 Mutator Carcinogenic Pathway

Analysis of tumors with MSI has revealed different mechanisms of CRC carcinogenesis, referred to as the mutator pathway, which is partially distinct from the dominant mechanisms that operate in the classic adenoma-to-carcinoma sequence referred to as the chromosomal instability pathway [64]. Genes targeted in the mutator pathway have mononucleotide runs that are susceptible to mutation when MMR is not functional. *TGFBR2* (also referred to as *TGFβ-RII*) is one of the most commonly targeted genes in the mutator pathway. Loss of responsiveness of the inhibitory effects of TGFβ contributes to the development of many cancers but is particularly prominent in CRC. TGFβ sends an inhibitory signal for cell proliferation that is mediated through two receptors: TGF*β*-RI and TGF*β*-RII. TGF*β*-RII is susceptible to replication errors because of a run of ten adenines in the part of the gene that encodes the signal sequence. Mutations in TGF*β*-RII cause failure of binding and response to TGFβ. Moreover, experimental introduction of a normal TGF*β*-RII gene into receptor-negative colon cancer cell lines restores TGF*β*-RII function and suppresses cancer-cell tumorigenicity [65].

Other genes that are mutated in the mutator pathway include those involved in signal transduction (IGFII, PTEN), apoptosis and inflammation (BAX, caspase-5), transcription regulation (E2F4, TCF-4), and DNA repair (MSH6, MSH3, MLH3, MED-1, RAD50, DNA-PKcs, BLM). Functional studies are ongoing to determine which genes play a role in major tumor initiation and progression and to distinguish them from alterations that are merely bystanders in the milieu of increased mutagenesis.

25.5 Familial Adenomatous Polyposis (FAP)

In contrast to Lynch syndrome, patients with polyposis syndromes are less challenging to identify clinically because of the presence of many adenomatous polyps in the colon and rectum. Adenomatous polyps are precursor lesions of CRC. The prototypical polyposis syndrome is FAP, and it is caused by either inherited or *de novo* germline mutations in the *APC* gene. There is marked heterogeneity in expression of *APC* mutations leading to a spectrum of disease rather than a single phenotype. This spectrum includes typical FAP, attenuated FAP (AFAP), profuse polyposis, and Gardner's syndrome. In the last 10 years, a distinct polyposis syndrome MAP was identified due to mutations in the *MUTYH* gene.

Histologic confirmation of a condition of multiple colonic polyps was recognized over 120 years ago when Sklifasowski published a case of polyadenoma tractus intestinalis. An inherited version of this condition was reported a year later by Cripps who described two siblings each with multiple adenomas. In the 1920s, a polyposis registry was begun at St. Mark's Hospital in England from which Lockhart-Mummery and Dukes published the first series of ten families with familial intestinal polyposis. In 1951, Gardner recognized a polyposis condition which was associated with desmoids, bone and cyst-like surface tumors. In 1987, the *APC* gene was localized to 5q21-22. The last decade has seen advances in understanding of genetics, pathophysiology, and management of polyposis syndromes ranging from recognition of attenuated FAP and MAP, genotype-phenotype correlation, surgical colectomy techniques, chemoprophylaxis, and improved screening strategies [66].

25.5.1 Clinical Aspects of FAP

25.5.1.1 Natural History

FAP accounts for less than 1 % of all CRC [67]. The incidence is thought to be 1 in 13,000 to 1 in 18,000 in Northern European populations [68, 69]. It is inherited in an autosomal dominant fashion. *De novo* germline mutations account for up to 25 % of cases [70]. The penetrance of polyposis approaches 100 % in individuals with *APC* mutations, although there is substantial variation in disease presentation as will be discussed. Without intervention, malignant transformation of one or more polyps is highly likely to occur on average by the fourth decade of life.

Patients present with hundreds of adenomatous polyps that carpet the colon and rectum starting in adolescence. Cancer develops by age 35–40 with 70–80 % of the tumors being localized distally [69, 71]. Additional clinical features include desmoids tumors, upper intestinal (gastric and duodenal) polyps and cancer, thyroid cancer, hepatoblastoma, and congenital hypertrophy of the retinal pigment epithelium (CHRPE). Although a minor part of the syndrome and still relatively infrequent, an increased frequency of pancreatic adenocarcinomas is associated with FAP. Relative and absolute lifetime risk of extracolonic cancers associated with FAP is presented in Table 25.4.

Table 25.4 Risks of extracolonic tumors in familial adenomatous polyposis (FAP)

Tumor	Relative risk (%)	Absolute lifetime risk (%)
Desmoids	852	15
Duodenal cancer	330.8	3.0–5.0
Thyroid	7.6	2
Brain	7	2
Ampullary	123.7	1.7
Pancreatic	4.5	1.7
Hepatoblastoma	847	1.6
Gastric		0.6

From Galiatsatos *Am J Gastroenterol* 2006 [72]

The clinical spectrum of FAP includes Gardner syndrome, AFAP, profuse polyposis, and Turcot syndrome. All of these clinical variants result from *APC* mutations and are associated with mutations at different regions of *APC* (the allelic series). Gardner syndrome is a condition of gastrointestinal polyposis associated with osteomas as well as epidermal cysts, desmoids, and thyroid tumors. In these patients, the extraintestinal manifestations are particularly prominent compared to typical FAP. Dental abnormalities including supernumerary and impacted teeth are seen in one quarter to one third of patients with this variant of the syndrome. In AFAP, there are between 10 and 100 colorectal adenomas. These polyps are more proximally located and the age of CRC onset is 15 years later than individuals with FAP. AFAP patients may not have a family history and often do not exhibit extracolonic tumors. In contrast, profuse polyposis describes individuals with more than 5000 colonic adenomas.

25.5.1.2 Diagnosis

FAP is usually diagnosed when patients present with carpeting adenomatous colonic polyps or when a relative is diagnosed with FAP. Algorithms have been developed to aid in the clinical diagnosis and management of FAP in affected individuals and their first-degree family members [72]. For an individual in whom FAP is suspected, full DNA sequencing of the *APC* gene is recommended. For first-degree relatives, the recommendation is to test for the mutation found in the proband beginning at age 10–12. Embryonic testing is also available.

25.5.1.3 Prophylaxis

If an *APC* mutation is identified, patients should be referred for proctocolectomy. In addition, patients should have upper endoscopy every 1–3 years, annual thyroid examination, and periodic abdominal ultrasound for pancreatic cancer and desmoids tumor screening. If a mutation is identified in a family member, colonoscopy should be performed yearly from age until the onset of polyps at which time prophylactic colectomy is recommended. If genetic testing is not desired or possible in a first-degree relative or no mutation is identified in the proband, flexible sigmoidoscopy is recommended annually starting at age 10–12. If polyps are found, individuals should consider colectomy. If no polyps are found by age 50, general screening guidelines can be followed [72].

As a result of screening and appropriate therapy, there has been a 55 % reduction in incidence of CRC at time of FAP diagnosis (that is, diagnosis of FAP prior to CRC diagnosis) as well as an improvement in survival for all FAP patients [73, 74]. Colectomy dramatically reduces the risk of CRC development. Surgical options are either subtotal colectomy with ileorectal anastomosis (IRA) and sigmoidoscopy every 6 months or total colectomy with ileal-pouch anal anastomosis (IPAA) with periodic sigmoidoscopy of the pouch. Presently, IPAA, which removes all colorectal mucosa, has become the preferred surgical approach. There is still a small, but not negligible risk of polyps and cancer in both the remnant rectum (with IRA) and the pouch (with IPAA). The option of IRA is most appropriate in young patients with few rectal adenomas and a family history of a mild phenotype as well as in AFAP [75].

25.5.1.4 Chemoprevention

Over the past decade, chemoprevention in FAP has gained attention. Clinically investigated agents include NSAIDs (predominantly sulindac), COX-2 inhibitors (both celecoxib and rofecoxib) as well as nutritional, micronutrient, and combination therapies. The prophylactic role of COX-2 inhibitors has been questioned due to important cardiovascular risks. Presently, these preventive medical therapies are not considered alternatives to surgical colectomy. Most studies have been performed for polyp prevention post-colectomy. Chemoprevention is most appropriate in young patients with adenomas before surgery, post-IRA patients for prevention of rectal adenomas, individuals with duodenal adenomas, and those with abdominal wall or intra-abdominal desmoids [76].

25.5.2 Genetics of FAP

25.5.2.1 APC Gene

APC was localized to chromosome 5q by the identification of a deletion of chromosome 5q in an individual with Gardner syndrome [77]. In 1991, linkage analysis and conventional positional cloning strategies were used to identify mutations in the *APC* gene in families with FAP, confirming this gene's causative role in the syndrome [78]. The gene has an 8538 bp open reading frame with 15 transcribed exons (Fig. 25.4). Exon 15 is the largest exon and accounts for 75 % of the coding sequence and is the location of most germline and somatic *APC* mutations [79]. Most germline and somatic *APC* mutations result in premature chain-termination. Truncated protein products can be detected by a molecular genetic assay referred to as in vitro synthesized protein (IVSP), and this analysis can be carried out on exon 15 directly from genomic DNA or on *APC* cDNA preparations from cellular RNA.

More than 800 different germline and somatic *APC* mutations have been reported. The region located at the 5′ end of exon 15 roughly between codons for amino acid residues 1286 and 1513 comprises the mutation cluster region (MCR). The most common germline mutations are 5-bp deletions that occur between codons 1061 and 1309; these mutations

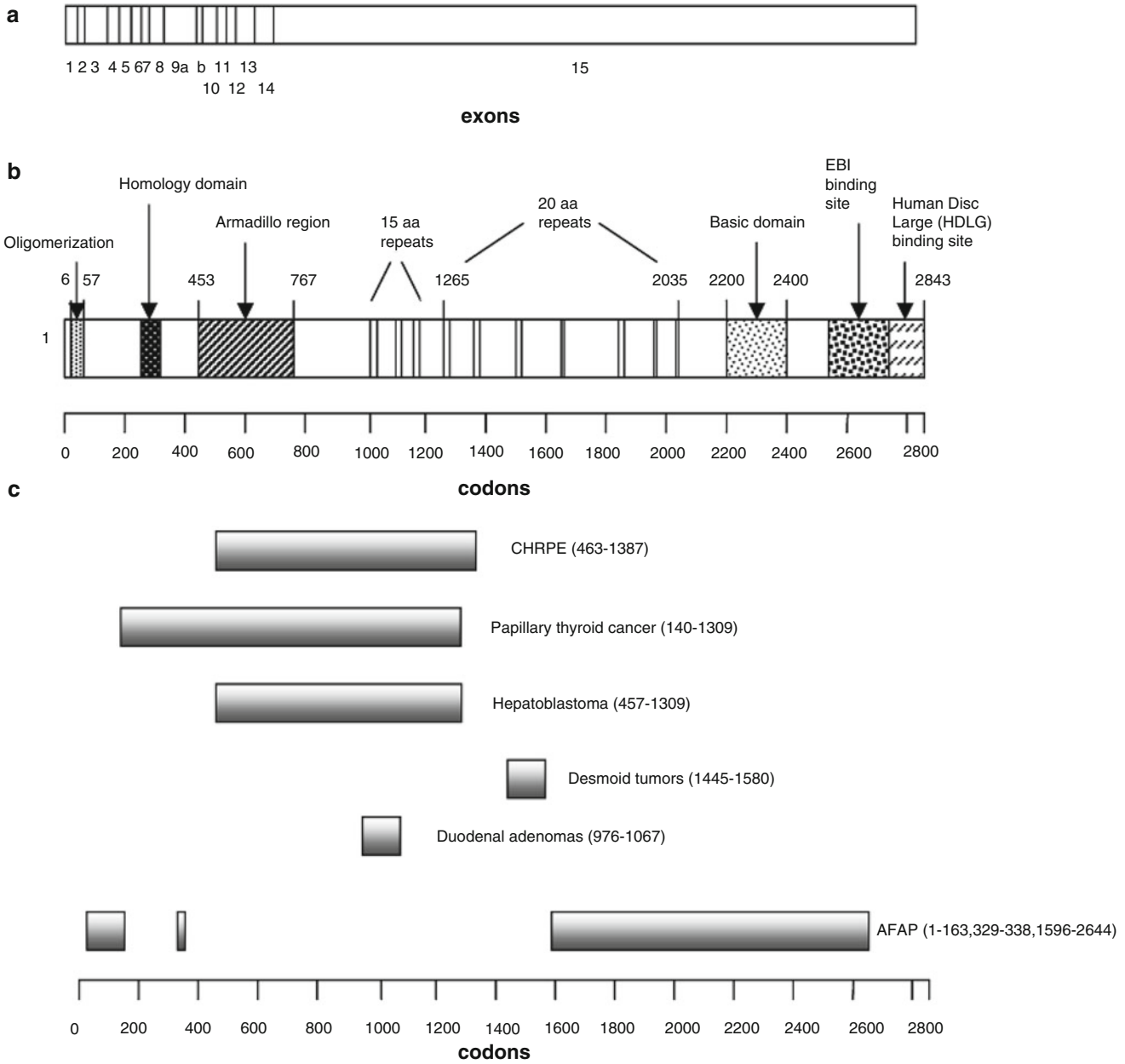

Fig. 25.4 APC gene and protein. (**a**) The *APC* gene encodes 15 exons. The drawing shows the relative sizes of each coding segment in the genome. (**b**) The APC protein contains numerous structural motifs and binding domains. See text for more details on selected regions of the protein. (**c**) *APC* mutations in different regions of the gene, as depicted by the shaded rectangles, are associated with different clinical susceptibilities. CHRPE, congenital hypertrophy of the retinal pigmented epithelium; AFAP, attenuated familial adenomatous polyposis. Adapted from Galiatsatos [72] and used by permission.

account for 1/3 of all germline mutations [80]. Somatic *APC* mutations occur most frequently between codons 1286 and 1513 with hotspots at codons 1309 and 1450 [79].

Inactivating somatic mutation of *APC* is the predominant first step in mutational pathway of colorectal carcinogenesis occurring in over 85 % of all CRCs, consistent with its role as a tumor suppressor. In order for *APC*-associated disease to develop, two hits are required. Loss of *APC* function in a precursor colonocyte leads to increased proliferation of the mutant cell. In FAP, the first hit is usually inherited from a parent, but the somatic mutation that constitutes the second hit may not be entirely random. More advantageous mutations are selected for during tumor initiation. These advantageous somatic mutations are not functionally null, instead they retain some APC activity in down-regulating Wnt signaling rather than allowing for excessive β-catenin accumu-

lation that would induce apoptosis. This is known as the just-right signaling model [81].

25.5.2.2 Genotype-Phenotype Correlations

Location of germline mutations in *APC* partially predicts the varying polyposis phenotypes, although the underlying mechanisms for these genotype-phenotype correlations have not been fully elucidated. Mutations between codons 1250 and 1464 in the MCR have been associated with profuse polyposis [82]. Age of CRC onset in this sub-type is about 8 years earlier compared to typical FAP. In contrast, the milder phenotype of AFAP is associated with mutations at the very 3′ end of the *APC* gene, nonsense mutations at exon 9, as well as nonsense and frameshift mutations at exons 3, 4, and 5 [83–87].

Certain extracolonic manifestations of FAP also appear to have specific genotype-phenotype correlations. First, CHRPE can be seen in families with a mutation anywhere between codons 311 and 1465. Desmoid tumors have been associated with mutations at the 3′ end of the gene downstream of codon 1400. While no large study has been done to confirm the association between upper intestinal polyps and *APC* mutations, it has been suggested that mutations from codons 976 to 1067 increase risk of duodenal adenomas. The evidence for associations with desmoid and upper gastrointestinal tumors is weaker than it is for CHRPE. Additional correlations are shown in Fig. 25.4c.

25.5.2.3 Min Mouse Model

The *m*ultiple *i*ntestinal *n*eoplasia (*Min*) ($Apc^{Min/+}$) mouse is the best-characterized model of FAP. The *ApcMin/+* mouse has a mutation at codon 850 and develops multiple adenomas of the small intestine as well as a more limited number of colonic lesions [88]. This mouse model has been used to characterize genetic and epigenetic modifiers of FAP. It has also been useful for testing putative chemotherapeutic regimens including NSAIDs.

25.5.2.4 Modifier Genes

Modifier genes have been implicated in the variable expression of FAP phenotypes [89]. In 1993, Dietrich reported a modifier gene on chromosome 4 in the Min mouse model [90]. The *m*odifier *o*f *M*in 1 (Mom1) strongly influences tumor number in the animal model. A candidate gene in this region is secretory phospholipase A2 (Pla2s) or its human equivalent locus *PLA2G2A* [91]. Variants in *PLA2G2A* have been associated with severity of duodenal polyposis in FAP patients [92]. In the last decade, two further modifiers (Mom2 and Mom7) [93, 94] have been described in the Min model, but investigators have yet to study these loci in humans. Another candidate modifier gene is N-acetyltransferase (both NAT1 and NAT2), which has been associated with a twofold increase in the number of polyps in FAP patients [95].

25.5.3 APC Protein and Wnt Signaling Pathway

25.5.3.1 Canonical Wnt Signaling

Wnts (the name is derived from the homologous genes identified by the *Drosophila melanogaster* mutant *wingless* and the mouse mutant *int1*) are a family of conserved signaling proteins that allow cells to converse with one another in order to coordinate a variety of cellular processes in development, homeostasis, and wound healing. There are several Wnt signaling pathways (referred to as canonical and non-canonical). While the canonical pathway is the best-characterized, non-canonical pathways include the planar cell polarity pathway, the Wnt/Ca pathway, and the protein kinase A pathway.

The *canonical* pathway refers to Wnt signaling that ultimately leads to β-catenin translocation to the nucleus (Fig. 25.5). Normally, in the off state of Wnt signaling, β-catenin is maintained in the cytoplasm by a protein complex known as the destruction complex composed of AXIN1, APC, and glycogen synthase kinase-3β (GSK-3β). In the on state, Wnt proteins bind to a receptor complex made up of Frizzleds and low-density lipoprotein receptor-related protein (Fz/LRP) leading to phosphorylation of a downstream protein Disheveled (Dvl). Phosphorylated Dvl inhibits GSK-3β leading to cytoplasmic accumulation of β-catenin which is not degraded and which translocates to the nucleus. Nuclear β-catenin complexes with T-cell transcription factor (TCF) and lymphoid enhancer-binding factor (LEF), which, upon recruitment of coactivators, leads to transcription of target genes. A recent review provides additional detail on APC and Wnt signaling in CRC [96].

25.5.3.2 APC Protein

APC is expressed in most cell types. In the colon, APC is a regulatory protein of colonic epithelial cell homeostasis. The protein is comprised of 2843 amino acids with several functional domains (Fig. 25.4). These domains have been implicated primarily in Wnt signal transduction but also in cell migration, adhesion, and chromosomal segregation. In the N-terminus, there is an oligomerization domain and a region consisting of armadillo repeats. The central portion consists of several 15- and 20-amino acid repeats. The C-terminus contains a basic domain as well as binding sites for EB1, microtubules, actin, human disc large (HDLG) protein, and scribble. β-catenin binding occurs at three of the 15 amino acid repeats and seven of the 20 amino acid repeats in APC, with the latter being essential for β-catenin downregulation. Three so-called serine-alanine-methionine-proline (SAMP) repeats also are crucial to regulate β-catenin degradation via binding to AXIN1. Inactivating *APC* mutations generally result in truncation of the C-terminus of APC thereby eliminating the SAMP repeats and all but one of the

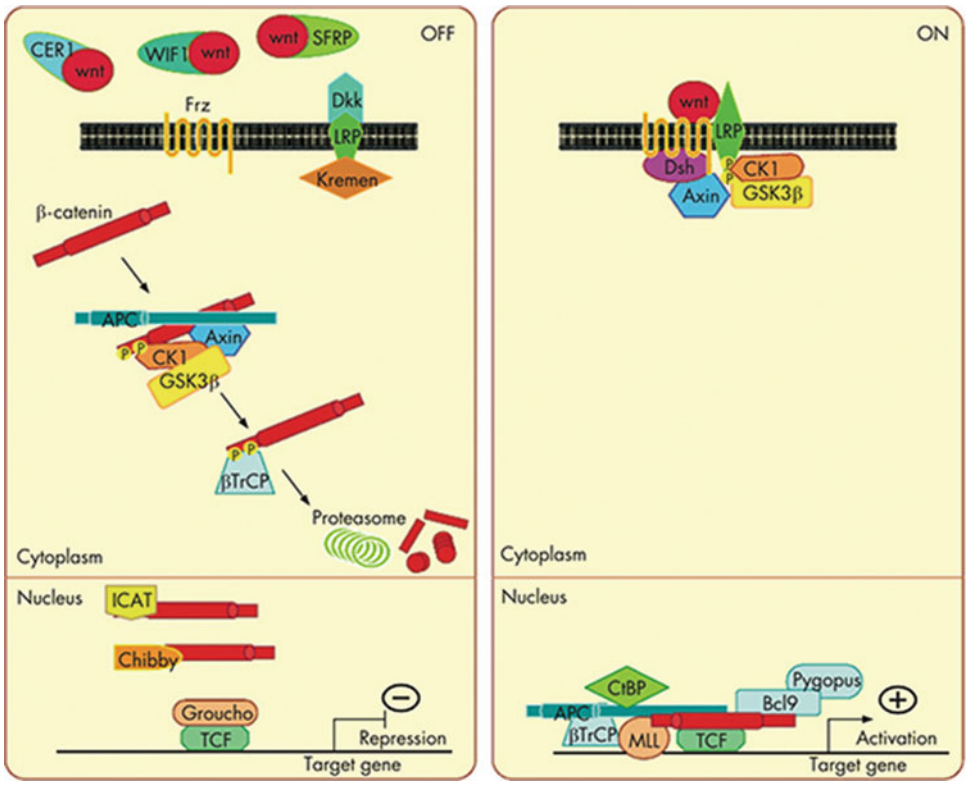

Fig. 25.5 The canonical Wnt signaling pathway. (**a**) Off state. The Wnt signaling pathway is in the off state either when Wnt is not present or when Wnt binding to its membrane-bound receptors, including Frizzled (Frz) proteins and co-receptors low-density lipoprotein receptor-related protein (LRP), is prevented by Wnt binding to other factors, including CER1, WIF1, and SFRP. In the cytoplasm, a complex composed of APC, Axin, casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β), aptly named the destruction complex, phosphorylates β-catenin and targets it for destruction. In the destruction complex, GSK3β and CK1 act to phosporylate β-catenin, while Axin and APC function as scaffold proteins that assemble substrate (β-catenin) and enzymes (GSK3β and CK1). β-Transducin repeat-containing protein (βTrCP) shuttles phosphorylated β-catenin to the proteosome for degradation. In the nucleus, Wnt target genes are silenced by a repressor known as Groucho that interacts with DNA-bound T-cell factor (TCF). In addition, the inhibitor of β-catenin (ICAT) and Chibby further suppress β-catenin nuclear activity. (**b**) On state. In the on state, Wnt binding to Frz and LRP results in phosphorylation of the LRP cytoplasmic tail by CK1 and GSK3β as well as recruitment of axin to phosphorylated LRP by dishevelled (Dsh) to inactivate the destruction complex. In the nucleus, Wnt target genes can be transcribed by displacement of Groucho, TCF interaction with β-catenin and recruitment of B cell lymphoma 9 (Bcl9), Pygopus, mixed-lineage leukemia (MLL) methyltransferases, APC, C-terminal-binding protein (CtBP), and βTrCP. Several target genes known to be regulated by β-catenin and Wnt signaling have been identified including *c-myc, cyclin D1, CDH1, Tcf-1, c-jun, Fra-1, PPARd, Gastrin, uPAR, MMP7, Survivan, Axin2/Conductin, CD44, Id2,* among others. Figure used by permission from Schneikert and Behrens [96].

20 amino acid repeats. The truncations result in removal of an interaction site with AXIN and consequent dysregulation of β-catenin degradation. In addition, the truncated APC protein lacks nuclear export sequences (NESs), which appears to be important in normal shuttling of β-catenin from the nucleus to the cytoplasm [97].

While APC has been primarily characterized as a scaffold protein in Wnt signaling as discussed above, there is emerging evidence of its role in Wnt pathway-independent activities which may contribute to carcinogenesis. There are data that APC is involved in apical-basal and front-rear polarity, migration, differentiation, DNA replication, mitosis, DNA repair, and apoptosis. Appreciation of APC's Wnt-independent activities has important implications especially as Wnt inhibitors are being investigated as cancer therapeutics. For a comprehensive discussion of APC and Wnt-independent pathways, readers are directed to a review by Prosperi and Goss [98].

25.5.3.3 Wnt Pathway Inhibitors

Given evidence of dysregulated Wnt signaling in CRC as well as other tumor types, there has been interest in developing agents that can inhibit the pathway and prevent nuclear transcriptional functions of β-catenin. There are two classes of Wnt pathway inhibitors: small-molecule and biological inhibitors [99]. Small-molecule inhibitors of the Wnt pathway include agents such as nonsteroidal anti-inflammatory drugs (NSAIDs), vitamins (such as A and D), and polyphenols (such as curcumin and resveratrol). NSAIDs, such as aspirin, sulindac, and cyclooxygenase (COX) inhibitors,

have been studied for prevention of adenomas in FAP and in patients with CRC. Their mechanism of action might be through inhibition of Wnt signaling via prostaglandin suppression of β-catenin degradation [100]. Additional small-molecule inhibitors have been identified through high-throughput screening studies and are classified into four groups: β-catenin/TCF antagonists, transcriptional co-activator modulators, PDZ domain of Dvl binders and alternative mechanism-based inhibitors. These agents are largely still in discovery or preclinical development; though, in 2010, a transcriptional co-activator modulator targeting CBP is in phase I testing. Biological inhibitors include monoclonal antibodies and small interfering RNA (siRNA) against Wnt-1 and/or Wnt-2 have shown promise as possible therapeutic agents and are currently in preclinical development. While Wnt pathway inhibitors hold promise as therapeutic agents, there are concerns about specificity given the role of Wnt signaling in normal cell homeostasis, in particular, stem cell homeostasis. The future of Wnt pathway inhibitors as therapeutic agents depends on specifically targeting tumor cells with aberrant Wnt signaling. The role of newer Wnt pathway inhibitors in FAP remains to be determined.

25.6 Muir-Torre and Turcot Syndrome

Muir-Torre and Turcot syndrome are rare variants of hereditary CRC syndromes. Individuals with Muir-Torre present with uncommon sebaceous skin tumors and internal malignancies. Most patients have mutations in *MSH2* though there have been reports of mutations in *MLH1* as well. Interestingly, some of *MSH2* mutations in this variant are the same as those in classic Lynch syndrome perhaps due to underreporting of personal or family histories of skin conditions. A review paper provides additional detail on Muir-Torre syndrome [101].

Turcot syndrome (TS) describes patients with hereditary CRC associated with CNS tumors. The molecular basis of TS can result from germline defects in *APC* or MMR genes. TS due to mutations in MMR genes results in CRC and gliomas in both children and adults. In contrast, TS associated with *APC* mutations leads to CRC and childhood cerebellar medulloblastoma. Reported mutations in MMR genes associated with TS include *MSH2, MLH1,* and rarely, *PMS2.*

25.6.1 MYH-Associated Polyposis (MAP) Syndrome

Since 2002, a distinct hereditary polyposis syndrome associated with mutations in the gene *MUTYH*, which is the human ortholog of the base-excision repair gene *MutY* of *Escherichia coli*. MUTYH is a DNA glycosylase that initiates the base-excision repair of specific lesions in oxidized DNA; consequently, mutations in *MUTYH* lead to accumulation of mutations due to oxidative DNA damage. The 8-oxo-guanine (GO) lesion is the major oxidative lesion in DNA, which results in a GO:C mismatch in the DNA [102]. The mismatch is repaired by the product of the *OGG1* gene and the base-excision repair pathway (Fig. 25.6). However, if the GO base is not repaired prior to DNA replication, a GO:A base pair will arise after DNA replication. The mismatched A base pair is specifically excised by MUTYH. Following excision of the A base pair, a C base is inserted opposite the GO base and the GO:C mismatch is subsequently resolved to a G:C base pair by *OGG1* and the base-excision repair pathway.

The initial discovery of *MUTYH* in cancer susceptibility came through a study of a family of three sibs with *APC* mutation-negative FAP [103]. By sequencing the *APC* gene in the colonic adenomas, investigators discovered a skew in the types of somatic mutations that had arisen in the *APC* gene. The skew in mutation types was consistent with a defect in the base-excision repair of oxidative damage. All three siblings were genetic compounds for the *MUTYH* mutations Y165C and G382D, whereas unaffected family members were either homozygous for the normal allele or heterozygous suggesting autosomal recessive inheritance. These *MUTYH* mutations greatly reduced the activity of the protein when experimentally introduced at the orthologous position in the *E. coli* MutY protein. Further genetic epidemiologic studies have confirmed that Y165C and G382D are disease-causing mutations associated with MAP. These mutations account for the majority of Caucasian cases of MAP [104, 105]. Biallelic *MUTYH* mutations have been detected in approximately 0.5 % of consecutive CRC cases. This frequency is approximately 50–100 times greater than the expected frequency of biallelic *MUTYH* mutations carriers in the general population. In addition, 36 % of the CRCs arising in biallelic *MUTYH* mutation carriers occur in the absence of detectable polyp development. There is evidence for a small increase in CRC risk in carriers of mono-allelic *MUTYH* mutations [106].

Clinically, MAP may be difficult to distinguish from FAP or AFAP in that numbers of adenomas can range from none to hundreds. Furthermore, polyp histology appears to be heterogeneous in MAP with hyperplastic and sessile serrated adenomas being common [107]. This finding suggests that aberrant base-excision repair targets both *KRAS* as well as *APC* in MAP. In contrast to FAP, MAP presents at a later age with a mean age of CRC diagnosis at age 50 and there is rarely a significant family history of CRC. Genetic testing

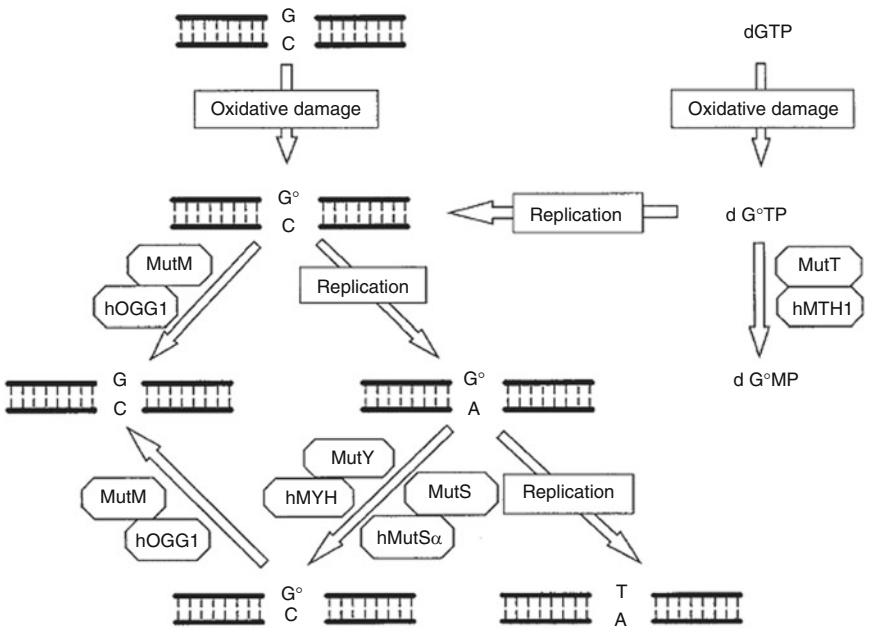

Fig. 25.6 Base-excision repair. In *E.coli,* MutT, MutM, MutY, and MutS and their respective homologues in humans, hMTH1, hOGG1, hMYH, and hMutSα repair oxidative DNA damage. Specifically, this mechanism protects against the mutagenic effects of 8-oxo-guanine (G°). MutT (hMTH1) prevents 8-oxo-dGMP incorporation into DNA. MutM (hOGG1) is a DNA glycosylase that removes the oxidized base. MutY (hMYH) is also a DNA glycosylase that excises the A residue that is misincorporated opposite the 8-oxoG residue during replication. MutS (hMutSα) is an alternative pathway for this excision step. Cells defective in base-excision repair accumulate G:C to T:A transversions. In MYH-polyposis, patients have defective hMYH function due to biallelic mutations. Used by permission from Lu [117].

for MAP is done when a patient with polyposis tests negative for *APC* mutations. Currently, affected individuals should undergo regular colonoscopy and probably also upper endoscopy to screen for duodenal polyposis. Unfortunately, due to its recessive inheritance, MAP patients may not be diagnosed until multiple polyps and/or CRC is already present.

25.6.2 Hamartomatous Polyposis Syndromes

A distinct group of hereditary polyp conditions result in formation of hamartomas, which are an overgrowth of mesenchymal, stromal, endodermal, and ectodermal tissues. Together, these conditions represent <1 % of all CRC. However, given the substantial risk of cancer in affected individuals and the possibility of genetic testing/counseling in family members, understanding these syndromes is important. A number of syndromes fall into the hamartomatous polyposis group, including familial juvenile polyposis, Cowden's disease, Peutz-Jeghers, Bannayan-Riley-Ruvalcaba syndrome, Basal nevus syndrome, Neurofibromatosis I, MEN2B, and mixed polyposis. A summary of clinical, genetic, and pathophysiologic characteristics is presented in Table 25.5. An in-depth discussion of hamartomatous syndrome can be found in a comprehensive review article [108]. Here, we briefly discuss familial juvenile polyposis, Cowden's disease, and Peutz-Jeghers.

25.6.3 Familial Juvenile Polyposis

Familial juvenile polyposis is estimated to have an incidence of 1:100,000 live births and is the most common of the hamartomatous syndromes. It is inherited in an autosomal dominant fashion. It is characterized by multiple hamartomas in the colon and rectum. The syndrome can co-occur with Osler-Weber-Rendu syndrome. Risks of colon, gastric, small intestinal, and pancreatic cancer are increased. By age 60, the risk of CRC is estimated at 68 % and of gastric cancer at 20 %. Screening with colonoscopy and upper endoscopy is important with referral for colectomy in cases of diffuse polyposis. The genes involved include SMAD4, on chromosome 18q21.1, which encodes a protein involved in signal transduction in response to TGF-β, and *BMPR1A*, on 10q22.3, which encodes a receptor for BMP1.

Table 25.5 Hamartomatous syndromes

Syndrome	Gene (location)	Cancers	Select clinical features
Juvenile polyposis	*SMAD4*(18q21.1) *BMPR1A*(10q22.3)	Colon Gastric small intestine Pancreatic	Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu) especially with *SMAD4* mutations
Peutz-Jeghers	*STK11*(18p13.3)	Thyroid Ovarian Lung Pancreatic Breast	Predominantly small intestinal hamartomas that may obstruct or bleed; hyperpigmented macules mouth, eyes, nostrils, perianal, buccal mucosa
Cowden's	*PTEN*(10q23.31)	Thyroid Breast Endometrial Brain Renal cell Colon	Mucocutaneous features (present in up to 80 %) Macrocephaly Mixed colonic polyposis

Adapted from Schreibman *Am J Gastroenterol* 2005 [108]

25.6.4 Peutz-Jeghers Syndrome

PJS is a hamartomatous polyposis associated with mucocutaneous hyperpigmentation. The incidence is similar to Cowden's syndrome at 1:200,000 live births. It is inherited in an autosomal dominant fashion. There is variable penetrance with some affected family members developing polyps and oral hyperpigmentation and some only with skin changes. In contrast to familial juvenile polyposis and Cowden's syndrome, polyps are more often seen in the small intestine, although they do occur in the colon and stomach. Polyps usually form in early adolescence. Besides oral mucosal hyperpigmentation, PJS can be associated with genitourinary tumors. There is a risk of malignant transformation in the intestinal polyps as well as in breast, ovarian, and pancreatic tissue. One gene associated with PJS is *STK11*, a serine-threonine protein kinase 11 (*LKB1*), located on 19p13.3. This gene product is a tumor suppressor gene. A quarter of PJS mutations are thought to be de novo or result from low-penetrance variants. Aggressive screening is recommended given the possibility of multiple cancers.

25.6.5 Cowden's Syndrome

Cowden's syndrome has an incidence estimated at 1:200,000 live births. It also has an autosomal dominant inheritance pattern. Mutations in the phosphatase and tensin homolog gene (*PTEN)* on chromosome 10q23 have been associated with individuals with Cowden's syndrome. Cowden's syndrome may involve the skin, breast, and thyroid. Lifetime risk of breast cancer is estimated at 36 % and of thyroid cancer at 10 %. The risk of CRC is controversial, though screening with colonoscopy is advocated. A study of 127 *PTEN* mutation carriers undergoing routine colonoscopic screening showed that mixed polyposis especially hyperplastic polyps was common and that the risk of early-onset colorectal cancer was greatly elevated compared to controls [109].

25.7 Conclusion

Mendelian CRC hereditary syndromes reviewed in this chapter have provided great insight into molecular mechanisms of colorectal cancer overall. Moreover, timely identification of mutation carriers and at-risk family members allows for implementation of surveillance programs and surgical treatments that save lives. While there are clearly unanswered questions remaining in hereditary CRC syndromes, great progress has been made in this field since Warthin's original description of cancer-prone families in the early 1900s.

Returning to the question posed in this chapter's introduction "…*what is hereditary CRC?*...," there is clearly more to CRC heritability than the Mendelian syndromes reviewed here. A few notable examples of developments in the field warrant comment. Recent work has identified a likely new rare CRC susceptibility gene *GALNT12* which is involved in O-glycosylation leading to aberrant glycosylation [110]. A study using familial CRC kindreds identified a linkage peak at chromosome 9q22-31 [111], which has been validated in multiple, independent studies [112, 113]. Further fine-mapping localized this linkage signal to a five SNP haplotype at 98.15 Mb on chromosome 9q which harbors a number of candidate genes [114]. Another study has identified germline structural variants in the tumor suppressor gene *PTPRJ* in early-onset CRC that leads to hypermethylation and gene inactivation [115].

Finally, much effort has been invested in identifying novel genetic variants that increase susceptibility to CRC using genome-wide technologies. Genome-wide association studies have identified common genetic variants (with minor allele frequencies over 5 %) using familial and/or early-onset cases [116]. These studies have yielded important results. In particular, they have highlighted the TGFβ/SMAD signaling pathway in CRC susceptibility and many novel associations have opened new avenues in genetic risk factor research. More work is needed to identify likely causal variants in these genes/chromosomal regions and test their functional consequences. With the advent of next-generation sequencing,

the field is moving toward whole-exome and whole-genome sequencing to discover common as well as rare susceptibility variants. While these approaches have substantial bioinformatics challenges, the future is bright in our efforts to further elucidate molecular mechanisms of CRC.

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Hereditary Breast and Ovarian Cancer

26

Michael P. Lux, Mayada R. Bani, Peter A. Fasching, and Matthias W. Beckmann

BRCA1/BRCA2-associated BC has special pathological characteristics. The lesions have an increased mitotic rate greater pleomorphism, higher proliferation rates, a lower grade of differentiation, and are more often grade III. *BRCA1*-associated carcinoma is more frequently estrogen-receptor-negative and progesterone-receptor-negative. Hereditary and sporadic OC show similar pathological characteristics, but the affected women have a longer relapse-free interval after initial chemotherapy and a longer overall survival.

Clinical options require information regarding the risks of the disease and its mutation status. Chemoprevention is currently a controversial topic. The use of oral contraceptives can be regarded as reducing the risk of OC. Prophylactic mastectomy and bilateral ovariectomy are the only options that lead to a demonstrable reduction in risk but they do of course affect the patient's physical integrity. It is not currently known whether intensified early cancer detection is individually beneficial, but this is currently the option that is least invasive and least burdensome to the patient. Although hereditary BC has different pathological characteristics and the *BRCA* mutation is an independent negative prognostic factor, there are currently no special treatment guidelines. Without adjuvant hormone therapy or chemotherapy, the overall survival in *BRCA* mutation carriers is reduced. Chemotherapy regimens involving platinum are particularly beneficial in the treatment of hereditary BC.

26.1 Introduction

Breast cancer (BC) is the most frequent form of carcinoma in women. The incidence of BC is still increasing, particularly in women under the age of 50 [1]. Most cases of BC affect older women and have a sporadic pathogenesis. Approximately 10–15 % of all cases of BC and 25–40 % of cases in patients under the age of 35 involve a genetic predisposition. *BRCA1/BRCA2* mutations are responsible for 3–8 % of all cases of BC and 15–20 % of familial cases (but are found in over 80 % of families in which six or more cases occur) [2, 3]. In addition, the familial BC risk is associated with an increased risk for ovarian cancer (OC). Ten percent of all cases of OC involve a predisposition with autosomal-dominant inheritance, particularly with *BRCA1/BRCA2* mutations [4]. The cumulative risk for disease is 39 % in carriers of the *BRCA1* mutation and 11 % in carriers of the *BRCA2* mutation, up to the age of 80 [5]. Most cases of OC are diagnosed at an advanced stage [6]. The prognosis is still poor, despite the enormous efforts that have been made in the field of therapy. It is essential to identify and provide genetic consultation for women who are at high risk, in order to assess the individual disease risk, calculate the probability of mutation carrier status, offer genetic analysis, advise women on the need for intensified early detection of cancer, provide preventive care and prophylactic surgery, and offer psychological support [1, 7, 8].

26.2 Epidemiology

26.2.1 Carcinoma Incidence and Risk for Disease by the Hereditary Breast and Ovarian Cancer Syndrome

The incidence of BC is still increasing. However, current studies suggest that the mortality is declining due to improved diagnostic methods, adequate early cancer detection programs,

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_26

and increased awareness regarding BC in the general population [9]. The cumulative risk of developing BC is 2 % up to the age of 50, and it increases to 10 % up to the age of 80 [10]. There are many risk factors for BC (Table 26.1) [3, 11]. In addition, factors such as age, a lack of previous pregnancies, and a family history of BC increase the risk [12, 13]. Nutrition, lack of exercise, environmental factors, use of oral contraceptives, and hormonal therapy can also affect the risk [12].

A family history of BC is an important risk factor. The disease has a hereditary pathogenesis in approximately 5–10 % of all patients with BC and 25–40 % of those under the age of 35. The risk of developing BC increases with the number of affected family members (Table 26.1)—for example, it is doubled when there is a first-degree relative with BC [14].

Mutations in the *BRCA1* and *BRCA2* genes are responsible for 3–8 % of all cases of BC and 40 % of familial cases of BC [2, 3]. The prevalence of germline *BRCA1* or *BRCA2* mutations in the general population is 0.1–0.2 % [15]. The risk of BC up to the age of 70 is 65 % (95 % CI, 44–78) for *BRCA1* mutation carriers and 45 % (95 % CI, 31–56) for *BRCA2* mutation carriers [5]. In addition, mutation carriers have an increased risk of developing a relapse after BC. Haffty et al. [16] reported a relapse rate of 49 % during a follow-up period of 12 years (with a rate of sporadic carcinoma of 21 %).

Women with a familial risk, particularly those with *BRCA1/BRCA2* mutations, develop the disease at a younger age than in sporadic cases. *BRCA1* mutation carriers have an 18 % risk (15 % for *BRCA2* mutation carriers) of developing BC up to the age of 39, and the risk increases to 59 % (34 % for *BRCA2* mutation carriers) in the age group of 40-49 years [17]. Malone et al. [18] detected a *BRCA* mutation in 9.4 % of women with BC under the age of 35 and in 12.0 % of women with BC under the age of 45.

Table 26.1 Risk factors for breast cancer [3, 11]

Risk factor	Relative risk (RR)
Family history	
First-degree relative with BC	2.40
Mother with BC before the age of 50	2.41
Sister with BC before the age of 50	3.18
Two first-degree relatives with BC	2.93
Three or more first-degree relatives with BC	3.90
History of breast biopsy	
Atypical ductal hyperplasia	4.30
Lobular carcinoma in situ	6.90
Reproductive history	
Age at first childbirth 35 years versus 20 years	1.32
Age at menarche 11 years versus 13 years	1.20
Age at menopause 54 years versus 50 years	1.31

BC breast cancer

OC is the sixth most frequent cancer in women, and there has been a noticeable increase in the incidence of the disease over the last century. The highest incidences in the world are found in developed Western countries, although stabilization in the incidence has been noted during the last 30 years. The use of oral contraceptives (reducing ovulation) and the practice of carrying out bilateral ovariectomy in patients undergoing hysterectomy are partly responsible for this trend [1].

The risk of OC rises with increasing age and involves hormonal interactions. Table 26.2 provides an overview of the risk factors for OC [19]. A considerable increase in the risk is observed in patients with a family history of OC. At least 10 % of all epithelial ovarian cancers are hereditary, with mutations in the BRCA genes accounting for approximately 90 % of cases and most of the remaining 10 % being attributable to hereditary nonpolyposis colorectal cancer [20]. Women with a *BRCA1* mutation have a cumulative lifetime risk of 39 % (95 % CI, 18–54) of developing OC, while in those with a *BRCA2* mutation the cumulative lifetime risk is 11 % (95 % CI, 2.4–19) [5]. The risk increases with age. King et al. [21] reported a risk of 3 % (2 % in *BRCA2* mutation carriers) of developing OC under the age of 40. The risk increased to 21 % (2 % in *BRCA2* mutation carriers) up to the age of 50, 40 % (6 % in *BRCA2* mutation carriers) up to the age of 60, and 46 % (12 % in *BRCA2* mutation carriers) up to the age of 70. The lifetime risk was 54 % (23 % in *BRCA2* mutation carriers). Other studies have reported risk levels of 29 % (in *BRCA1* mutation carriers) and 0.9 % (in *BRCA2* mutation carriers) up to the age of 50 [3, 22].

Table 26.2 Risk factors for ovarian cancer [19]

Risk factors	Relative risk (RR)
Family history	
First-degree relative diseased	3.1–3.6
Second-degree relative diseased	2.9
Two or more relatives diseased	4.6
Hereditary syndrome (HBOC/HNPCC)	25–30
Additional risk factors	
History of infertility	2.0–5.0
No pregnancies	2.0–3.0
Early menarche	1.5
Late menopause	1.5–2.0
Women from North America/Europe	2.0–5.0
Caucasian	1.5
Exposure to talc	1.0–2.5
Socially advantaged living standard	1.5–3.0
Older age	3.0
Nutrition (animal fibers, galactose)	1.5–2.0

HBOC hereditary breast-ovarian cancer, *HNPCC* hereditary nonpolyposis colorectal cancer

26.2.2 BRCA1/BRCA2 and Other Hereditary Breast and Ovarian Cancer Syndromes

BRCA mutations are responsible for a large proportion of cases of familial BC with autosomal-dominant inheritance. However, these only represent a small proportion of all cases of hereditary BC. *BRCA1* and *BRCA2* are each responsible for 20 % of cases of familial carcinoma [23]. In recent years, a number of other genes have been identified that are associated with a predisposition to develop BC. *CHEK2* was detected in 1999. *CHEK2* is a tumor-suppressor gene that encodes a protein kinase required for DNA repair and replication [24]. *CHEK2* mutations can be identified in 5 % of patients with familial BC, and are also associated with a disposition to develop sarcoma and brain tumors. The individual lifetime risk of developing BC is less than 20 % [23]. The Li–Fraumeni syndrome is caused by mutations in *TP53*. It is responsible for approximately 1 % of cases of familial BC. The lifetime risk of BC is 90 %. *PTEN* mutations cause a predisposition to develop thyroid carcinoma, BC, and benign hamartoma (Cowden's disease), but are only responsible for a small proportion of familial BC syndromes [23]. The type I Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC) involves the development of colorectal carcinoma at a young age. Type II also involves extra-colorectal carcinoma, particularly in the female reproductive tract [25]. Women in HNPCC families have a tenfold increase in the risk of developing OC [26]. Table 26.3 presents an overview of common hereditary BC and OC syndromes [27].

Fifty-four percent of cases of familial BC are caused by genes that have not yet been identified, with a variable level of associated risk [23]. Identifying these genes is challenging due to their genetic heterogeneity, reduced penetrance, and different frequencies of mutation. Numerous approaches have been tested in the effort to identify other high-penetrance and low-penetrance genes. Case-control studies are used to identify polymorphisms with an associated risk. Candidate genes are selected on the basis of biological plausibility—usually genes involved in cellular processes such as detoxification, DNA repair, immune system control, and steroid hormone metabolism. A large number of genes have already been described [28].

Women with *BRCA* mutations show a high degree of variability in the site of BC and in age at diagnosis. This can be explained by different types or locations of the mutations. On the other hand, exogenic factors or risk-modifying genes might also be responsible for the variation. Another approach has attempted to describe risk modifiers of this type [29]. Hereditary BC and OC syndrome is a complex phenomenon that involves multiple factors, with numerous different approaches needed to understand the genesis.

26.2.3 The Hereditary Breast and Ovarian Cancer Syndrome and Other Associated Carcinomas

Women with two BC and/or OC lesions are at increased risk of developing other types of cancer. Evans and colleagues [30] analyzed the data for 2813 women in England who had at least two carcinomas of the breast and/or ovaries (2274 women with two BCs, follow-up 4.9 years; 25 women with two OCs, follow-up 2.7 years; and 514 women with BC and OC, follow-up 2.7 years). An increased risk was found for four different types of carcinoma. The standard incidence ratio (SIR) was 14.7 (95 % CI, 1.73–51.6) for oropharyngeal carcinoma, 4.68 (95 % CI, 2.02–9.22) for malignant melanoma, 3.07 (95 % CI, 1.72–5.06) for endometrial carcinoma, and 5.04 (95 % CI, 1.8–11.0) for myeloid leukemia. The SIR for colon carcinoma was 1.60, but the figure did not reach statistical significance (95 % CI, 0.93–2.54). These observations are almost identical with the published data for

Table 26.3 Hereditary carcinoma syndromes including BC or OC [27]

Syndrome	Chromosome, gene	Primary carcinoma	Secondary carcinoma
Familial BC/OC syndrome	17 g21 *BRCA1*	BC, OC	Colon carcinoma, prostate carcinoma
Familial BC/OC syndrome	13q12 *BRCA2*	BC, OC	Male BC, endometrial carcinoma, prostate carcinoma, oropharyngeal carcinoma, pancreatic carcinoma
Cowden's disease	10q23 *PTEN*	BC, thyroid carcinoma	Intestinal hamartoma, cutaneous lesions
Li–Fraumeni syndrome	17p13 *TP53*	Sarcoma, BC	Brain tumor, leukemia
HNPCC	2p15 *MSH2, 3, 6* 3p21 *MLH1* 2p32 *PMS1* 2p32 *PMS1* 7p22 *PMS2*	Colorectal carcinoma	Endometrial carcinoma, OC, Muir–Torre syndrome, hepatobiliary carcinoma, genitourinary carcinoma, glioblastoma
Louis–Bar syndrome	11 g22 *ATM*	Lymphoma	Cerebellar ataxia, immune deficiency, glioma, medulloblastoma, BC (heterozygous)
CHEK2	13q21 *CHEK2*	BC, sarcoma, brain tumor	

BC breast cancer, *HNPCC*, hereditary nonpolyposis colorectal cancer, *OC* ovarian cancer

BRCA-linked carcinomas. The Breast Cancer Linkage Consortium [22] detected an increase in the relative risk of developing oropharyngeal cancer (RR 2.26) and malignant melanoma (RR 2.58). In addition, there was an increased risk for gastric cancer (RR 2.59), carcinoma of the gallbladder (RR 4.97), and pancreatic cancer (RR 3.51). *BRCA2* mutation carriers have no increased risk for colorectal carcinoma, but *BRCA1* mutation carriers have a relative risk of 4.11. The risk of prostate carcinoma is also increased (RR 1.82) [31]. Women aged 15–34 whose mother had two BCs were found to be at increased risk in a Swedish study (SIR 2.26; 95 % CI, 1.04–4.34) [32]. Together with the data published by Evans et al. (showing a fivefold increase in the risk) [30], these results underline the hypothesis that the lesions have a similar origin. Evans et al. [30] did not detect a higher risk of developing cervical cancer, but this might be explained by the average age of the group of patients studied (56 years). Thompson et al. [31] reported a relative risk of 3.72 (95 % CI, 2.26–6.10) on the basis of a study including 699 *BRCA1* mutation carriers and 11,847 relatives. The risk of endometrial cancer was also increased (RR 2.65; 95 % CI, 1.69–4.16). The Hereditary Ovarian Cancer Clinical Study Group [33] diagnosed six of 857 women with *BRCA1* or *BRCA2* mutations with endometrial cancer after an average follow-up period of 3.3 years, in comparison with 1.13 cancers expected (SIR 5.3; $P=0.0011$). Four of the six patients had used tamoxifen in the past. The risk among women who had never been exposed to tamoxifen treatment was not significantly elevated (SIR 2.7; $P=0.17$), but among the 226 participants who had used tamoxifen (220 as treatment and six for the primary prevention), the relative risk for endometrial cancer was 11.6 ($P=0.0004$). The main factor contributing to the increased risk for endometrial cancer among *BRCA* carriers is tamoxifen treatment for a previous breast cancer.

26.3 Genetics

Modern molecular–biological techniques have made it possible to obtain new insights into the genetic basis for carcinogenesis. The genes that play a key role in carcinogenesis can be classified into different categories (e.g., oncogenes, tumor-suppressor genes). *BRCA1* and *BRCA2* are tumor-suppressor genes with autosomal-dominant inheritance.

The existence of a familial disposition to develop BC and OC has been recognized for many years, but the genetic basis for this was unclear until 1990. Families with large numbers of individuals who developed BC and OC were recorded for linkage analyses, and a locus was detected on chromosome 17 [34]. Tumors in family members almost always showed a loss of heterozygosity at 17q, suggesting that the associated gene, named *BRCA1*, was a tumor-suppressor gene. Further studies confirmed the location of *BRCA1*, and an 8 cM-long candidate region in men and a 17 cM-long region in women was identified within the following 2 years. Finally, mutations in the *BRCA1* gene were detected in five independent cases [35]. Subsequently, missense mutations were detected throughout the opening reading frame, which suggested that the full-length gene product is a major effector of tumor suppression. At the same time, a further susceptibility gene, *BRCA2*, was described on 13q12 [36].

In 1971, Knudson observed that two consecutive mutations in a single tumor-suppressor gene are necessary to transform a normal cell into a tumor cell [37]. The identification of homozygous tumor cells in heterozygous patients led to the conclusion that one mutation is inherited, while the second mutation—with loss of the remaining functional copy of the tumor-suppressor gene—is acquired during an individual's lifetime [38]. It is assumed that a two-hit mechanism of this type is responsible for many hereditary cancer syndromes involving Mendelian inheritance. Normally, the first inherited mutation is a point mutation, while the second mutation is caused by loss of part of the chromosome by nondisjunction, mitotic recombination, or de novo deletion in familial and also sporadic cases. The mechanism involved in *BRCA1* and *BRCA2* differs from that in Knudson's model, which was originally proposed to explain both familial and sporadic cases of retinoblastoma due to mutation of a single tumor-suppressor gene. Loss of heterozygosity can be seen at the *BRCA1* and *BRCA2* locus in sporadic BC, but the remaining allele almost never mutates [39]. *BRCA*-linked disease is therefore reserved for the hereditary syndrome, and *BRCA*-associated BC must be regarded as a different entity from sporadic BC. This view is supported by differences in the pathology, prognosis, and therapeutic management.

Although little is known regarding the functions of *BRCA1* and *BRCA2*, some similarities between the two genes have been described. It is now clear that the normal protein products of *BRCA1* and *BRCA2* are involved in the fundamental cellular processes of maintaining genomic integrity and transcriptional regulation. Both genes have a comparable length of genomic DNA and are activated in most tissues. *BRCA1* consists of 24 exons, 22 of which encode for the protein [40]. The ATG start codon is on exon 2. Exon 11, which consists of 3427 base pairs, is exceptionally long and covers more than half of the encoding regions of *BRCA1*. The complete sequence is known.

BRCA2 has 27 exons and approximately 80 kb. Like *BRCA1*, *BRCA2* has large encoding exons. Exon 10 consists of 1116 bp and exon 11 of 4933 bp. Both genes encode for large proteins. The *BRCA1* protein is a polypeptide with 3418 amino acids, with a mass of 384 kDa (albumin has a mass of 69 kDa). A list of known mutations is available in the Breast Information Core databases (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic).

26.3.1 BRCA Expression

It is thought that the expression and regulation of the two genes are similar. Expression can be detected in almost all human tissues. The highest mRNA levels can be found in the thymus [41]. Intensive studies have been carried out in mice. Chodosh's laboratory detected the highest level of expression in tissues with high proliferation rates [42]. *BRCA2* also has an effect on almost all tissues [43, 44]. Both genes are activated in murine breast tissue, and expression increases during pregnancy [43]. The coordinated regulation of *BRCA1* and *BRCA2* suggests that the genes are induced by, and may function in, overlapping regulatory pathways involved in the control of cell proliferation and differentiation. Their levels are highest during the S phase, which suggests a function in DNA replication. The two genes are located in the nucleus of somatic cells, where they coexist in characteristic subnuclear foci that are redistributed after DNA damage [45]. On the basis of the genes' upregulated expression during puberty and pregnancy, it has been suggested that estrogen may stimulate the expression of *BRCA1* and/or *BRCA2* [46].

26.3.2 BRCA Protein Motifs

In many cases, the function of a gene can be understood through comparison with that of similar known genes, but the sequences of *BRCA1* and *BRCA2* have no significant similarities with other genes.

BRCA1 has an N-terminal zinc-binding RING motif, which is defined by a series of precisely positioned cysteine and histidine amino acids and has been described in several genes [47, 48]. The RING motif plays a key role in numerous pathways. Protein–protein interactions show that *BRCA1* and *BARD1* form a heterodimer complex. *BARD1* has a RING motif similar to that in *BRCA1*, and this is responsible for the interaction. Somatic mutations of *BARD1* have been detected in breast, ovarian, and endometrial carcinoma [49].

The carboxyl end of the Brca1 protein carries two tandem-repeat globular domains, termed Brct (standing for *BRCA1* C-terminal) [50]. This domain has been described in several other proteins and is responsible for p53 linkage; p53 is a tumor-suppressor gene that is mutated in numerous cancer entities. In addition, it is thought that Brct binds other proteins such as Rad9, Xrcc1, Rad4, Rap1, and Ect2, which are involved in cell cycle regulation and DNA damage response [51]. Detection of missense mutations in the encoding regions has demonstrated that intact functioning of these domains is necessary for tumor suppression [35]. The Brct and RING motifs have not been detected in *BRCA2*.

In 1996, Chen et al. [52] described a direct interaction between the Brca1 protein and importin, a component of the core membrane. In addition, Brca2 binds directly to Rad51, a human homologue of the *Escherichia coli* RecA gene, which plays an important role in recombination and double-strand break repair [53]. The Brca1 protein interacts indirectly with Rad51.

26.3.3 BRCA as Caretaker of Genomic Integrity

Biochemical, genetic, and cytological studies have revealed multiple functions for the *BRCA1* and *BRCA2* genes. Brca1 and Brca2 interact with Rad51. All three proteins are located in nuclear foci during the S phase after exposure to ionizing radiation [54–58]. It is thought that the Brca1 and Brca2 proteins form a DNA double-strand breakage repair system with Rad51 [59]. Brca proteins appear to be responsible for the maintenance of genomic integrity and control of homologous recombination. If DNA synthesis is arrested due to external agents, Brca1 is rapidly phosphorylated in sites of DNA synthesis together with Brca2, Rad51, and Bard1 [56, 57]. This underlines the function of the corresponding genes for homologous recombination during the S phase. Expression of the three genes is upregulated during development. In addition, the proteins are located in meiotic chromosomes during the formation of synaptinemal complex, a structure that is associated with the regulation of meiotic recombination [39, 55, 56]. These observations indicate that *BRCA1* and *BRCA2* cooperate in a single pathway.

Support for these observations has been obtained from gene-targeting experiments. *BRCA1*+/– mice are normal and fertile and lack tumors at the age of 11 months. Homozygous *BRCA1* mutant mice die before day 7.5 of embryogenesis, and homozygous *BRCA2* mutant mice die at around day 8.5 [55, 60]. In both cases, cells show activation of the p53 pathway and p21, a DNA damage-dependent cell cycle checkpoint effector. Subsequently, fulminant chromosome breakage can be observed [61]. This underlines the major role of Brca protein–Rad51 complexes in recombination during the S phase [39]. In addition, homozygous *BRCA* mutant cells show centrosome amplification mitotic checkpoint defects and defective transcription-coupled repair of oxidative DNA damage [62, 63].

Several different biochemical fractionations of *BRCA1* have been published that potentially implicate *BRCA1* in many processes, including chromatin remodeling [64], transcription regulation [65, 66], mismatch repair, and recombination control [67].

26.3.4 Structure–Function Relationships of Brca Proteins

The RING domain of Brca1 is able to mediate ubiquitin-conjugating enzyme-dependent ubiquitination in vitro. This

is enhanced by Brca1–Bard1 RING–RING heterodimers [68]. If missense mutations arise affecting the RING domain, the in vitro ubiquitination ligase function is lost [68]. Similar mutations failed to reverse hypersensitivity to ionizing radiation and caused increased sensitivity to DNA-damaging agents [69, 70]. This suggests that the ubiquitination ligase function is required for tumor suppression. Brca1 also interacts with Bap1, a ubiquitin hydrolase [71]. There appears to be an intriguing interplay between these proteins with regard to the promotion (Bard1) or prevention (Bap1) of ubiquitin-mediated protein degradation [70].

The C-terminal Brct domains of Brca1 appear to be multifunctional. The Brct region can stimulate transactivation of reporter genes, interact with transcriptional corepressor CtIP, and is necessary for interaction between the full-length Brca1 protein and the RNA polymerase II holoenzyme [39]. These observations suggest that the Brct domain has an important function as regulator of transcription. Mutations affecting the Brct region also affect the function of double-strand breakage repair. This part of the polypeptide therefore has at least a dual function [72].

The functions of many regions of the Brca1 polypeptide are not known, although a large number of protein–protein interaction domains have been identified [73]. It has been shown that a central region of the polypeptide has DNA-binding activity. Deletion of part of this region abrogates the genome integrity maintenance function, and missense mutations affecting this region disrupt the double-strand repair function [72].

Analysis of Brca2 proteins has focused on BRC repeats, which have been shown to mediate the Brca2–Rad51 interaction [74, 75]. It appears that Brca2 may play a more direct role in DNA repair than Brca1. Davies et al. [76] produced synthetic BRC-repeat peptides. Binding of the Brca peptide to Rad51 prevented the formation of a Rad51 DNA nucleoprotein filament. This suggests that Brca2 modifies the DNA-binding activity of Rad51 and prevents the formation of multimeric Rad51 complexes. Rad51 can be kept in an inactivated state by binding to Brca2 to prevent unplanned interactions within the nucleus. Rad51 is thus maintained in a state of readiness for relocation to sites of DNA damage, where activation allows repair by homology-directed gene conversion [76]. These observations are underlined by the fact that human and mouse cells that are deficient in Brca2 fail to develop Rad51 nuclear foci after induction of DNA damage [77].

26.3.5 Models of BRCA-Linked Cancer Predisposition and Tumor Suppression

It is not known why *BRCA*-linked cancer predisposition manifests in particular epithelial tissues such as the breast and ovaries, although several hypotheses have been developed to explain this. *BRCA* mutations may have tissue-specific effects that promote transformation and make breast and ovarian cells more sensitive to local mutagens, such as estrogen metabolites [78].

The recombination functions of *BRCA1* and *BRCA2* may not be their only functions. The function of tumor suppression may involve some multifunctionality. Inactivation of *BRCA1* or *BRCA2* might produce cancer-predisposing defects in multiple biochemical pathways. There is already some evidence that *BRCA1* is essential for ductal morphology in the developing breast [79], and that *BRCA1* and *BRCA2* regulate the growth response to estrogen-receptor signaling [80]. Further studies are needed to reveal the way in which Brca proteins are able to function as tissue-specific tumor suppressors. An alternative hypothesis is that the frequency with which loss of the second *BRCA* allele occurs may effectively be higher in tissues with prolonged proliferation, such as the breast and ovaries [77].

BRCA-linked tumors show differences in the age of cancer onset, the degree of penetrance, and even the type of tumor to which individuals are predisposed. Identifying the genetic and environmental factors that influence phenotypic effects of mutations may provide new insights into the functioning of *BRCA1* and *BRCA2*.

26.4 Pathological Characteristics

BRCA1/BRCA2-linked BC has special clinical and pathological features (Table 26.4) [81–83]. The prevalence of ductal and lobular carcinoma in situ (DCIS/LCIS) is lower than in sporadic cases (DCIS 45 % versus 55 %, LCIS 3 % versus 6 %) [84, 85]. In addition, DCIS can be detected more often

Table 26.4 Clinicopathological features of hereditary breast cancer [81–83]

	Sporadic BC (%)	*BRCA1* (%)	*BRCA2* (%)
Grade 3	35	67	62
DCIS	55	38	53
LCIS	6	2	3
ER-positive	65	10	50
PR-positive	59	21	45
erbB2-positive	15	3	3
p53-positive	80	60	74
Medullary BC	2–4	13	
PFC	7	25	
Mitotic count		+	
Pleomorphism		+	
Lymphoplasmacytic infiltrates		+	
Tubular formations		–	–
Lobular formations		+	

BC breast cancer, *DCIS*, ductal carcinoma in situ, *ER* estrogen receptor, *LCIS* lobular carcinoma in situ, *PFC* proliferative fibrocystic changes, *PR* progesterone receptor, +, higher frequency than sporadic breast cancer; –, lower frequency than sporadic breast cancer

in *BRCA2*-linked BC than in *BRCA1*-linked BC (53 % versus 38 %) [1]. Analysis of the clinical features of the lesions has shown that medullary tumors are more frequent (13 % versus 2–4 % in sporadic cases) and that the prevalence of invasive lobular and tubular carcinomas is reduced in *BRCA1* mutation carriers [81, 82]. These lesions were associated with dense lymphocyte infiltration. *BRCA1*-linked BCs are characterized by high rates of mitosis, increased pleomorphism, high proliferation rates, low differentiation, and an increased prevalence of grade III tumors [81, 82, 84]. This association is not observed in *BRCA2*-linked carcinomas. However, the latter develop tubular formations less often than sporadic carcinoma [81, 82]. In comparison with sporadic carcinoma, *BRCA1*-linked carcinoma shows reduced estrogen-receptor and progesterone-receptor expressions. While only 10 % of *BRCA1*-linked carcinomas are estrogen-receptor-positive (65 % in sporadic carcinomas), *BRCA2*-linked carcinomas have normal estrogen-receptor expression [81, 82]. In addition, estrogen-receptor expression in *BRCA1* mutation carriers shows a clear degree of age dependency. While mutation carriers under the age of 45 only show expression in 19.0 % of cases, among women aged 45–55 the figure is 31.1 % and among women aged 55–65 it is 38.0 % ($P=0.20$) [86, 87]. In carriers of *BRCA2* mutations, the age distribution is homogeneous ($P=0.418$).

Lakhani et al. [82] reported that 21 % of *BRCA1*-associated carcinomas express the progesterone receptor (59 % in control individuals). While 15 % of the control individuals were Her2-positive, only 3 % of *BRCA1/BRCA2*-linked carcinomas showed Her2 expression. Adem et al. [83] found that *BRCA1/BRCA2*-associated carcinomas showed proliferative fibrocystic changes less frequently. These changes were seen in 25 % of the control tumors in patients without a family history and in 7 % of *BRCA1*-linked carcinomas ($P=0.075$). In addition, mutation carriers show increased MIB-1-Ag expression in comparison with patients with sporadic carcinoma. This is associated with an increased proliferation rate. At Garber et al. [86] summed up the typical features of *BRCA* tumors as follows:

- *BRCA1* breast cancers:
  Are ER-negative, PR-negative, and Her2-negative in 80 % of cases.
  Are of the basal-like type on microarray analysis in 80 % of cases.
  Show low expression of cyclin E, p27, and AKT.
  Are often positive for CK5, CK6, CK17 by IHC.
  Show a high frequency of p53 mutations.
  Show frequent amplification of myc and EGFR.
- *BRCA2* breast cancers:
  Do not show a typical phenotype (unlike the features observed with BRCA1-associated cancers).
  Tend to be of higher grade and have less tubule formation in comparison to sporadic cancers.
  Have estrogen and progesterone receptor profiles similar to those of sporadic cancers (most are ER-positive).

A comparison between sporadic and *BRCA*-linked OCs did not reveal any differences in type, grading, or stage [88]. Although most of the clinical and pathological characteristics of familial and sporadic carcinoma are similar, women with *BRCA*-linked carcinoma have a longer relapse-free interval and a longer overall survival after initial chemotherapy [88].

BRCA mutation is an independent prognostic factor [89]. By analyzing Ki-67-positive cell nuclei, Levine et al. [90] showed that familial OCs have a significantly higher proliferation rate than sporadic carcinomas ($P=0.017$). This may explain the better response to chemotherapy. There were no differences between *BRCA1*-linked and *BRCA2*-linked carcinomas. Familial OCs showed a reduced frequency of mucin formations (2 % versus 12 % in sporadic cases) and are more often at an advanced stage at the initial diagnosis [88]. In addition, *BRCA*-linked carcinoma is associated with p53 mutations [91] and peritoneal carcinosis (24 % versus 14 % in sporadic cases) [92].

In ovaries removed from patients undergoing prophylactic ovariectomy for a known family history of ovarian cancer, there are more surface epithelial inclusion cysts and surface micropapillae than in ovaries removed from women lacking such a history [20]. Precursor lesions such as dysplasia and atypical hyperplasia have been reported in fallopian tubes removed prophylactically. Rare cases of unexpected microscopic carcinomas of the ovary and fallopian tube have been discovered [93, 94].

It can be assumed that familial OC has a different pathogenesis and is more aggressive than sporadic cases [95]. Comparison of *BRCA1*-linked and *BRCA2*-linked OCs showed that *BRCA2*-associated carcinomas are more often diploid (13 versus six of 60 carcinomas analyzed) [96].

26.5 Counseling and Risk Calculation

26.5.1 Interdisciplinary Genetic Counseling

Current insights regarding the genetic disposition to develop carcinoma have raised requirements for all physicians. It is not possible for all physicians to keep up with all the changing insights into diagnosis, early cancer detection, DNA analysis, and support for women who have a familial risk. However, all physicians should be encouraged to refer patients who have a family history of BC and/or OC to interdisciplinary genetic care centers. Collaboration between physicians in the fields of gynecology, histopathology, human genetics, psychosomatic medicine, molecular biology, and radiology is the appropriate approach in order to identify women who are at familial risk, to calculate the risk

of the disease and mutation carrier status, to inform affected individuals about early cancer detection, chemoprevention, and prophylactic surgery, and to provide analysis of the *BRCA1/BRCA2* genes [1, 97, 98]. Psychological support can also be offered. A psychological counseling session is a prerequisite for analysis of a patient's *BRCA1* and *BRCA2* genes [97]. The decision on whether to carry out genetic testing is based on inclusion criteria, which can be checked in relation to pedigrees. If the inclusion criteria are met, blood samples can be requested from family members who already have disease. Detection of a mutation is probable if an affected family member is analyzed. If a mutation is detected, the consulter can also be analyzed to confirm or exclude the identified mutation. Analysis of the *BRCA1/BRCA2* genes is carried out by direct sequencing of the gene or denaturing high-performance liquid chromatography analysis of frequent mutations [99, 100]. Actual, also next generation sequencing can be done in validated laboratories. Founder effects can be observed for specific mutations in different regions and countries.

26.5.2 Determining and Calculating Risk

Calculation of the lifetime risk of disease and of the probability of mutation is required by patients and physicians in clinical practice, particularly in the setting of genetic care centers. The certainty with which a known mutation in a defined gene location (e.g., in the *BRCA1* or *BRCA2* genes) can be diagnosed is greater than 97 %. However, as the remaining predisposing genes that are responsible for the other 50 % of hereditary breast carcinomas have not yet been identified, testing is not possible. In concrete terms, this means that it can only be established or excluded with a certainty of 50 % whether a mutation is present in a given individual [101]. If the findings are negative, the test is regarded as being uninformative—i.e., the individual has to be treated as if no testing had been carried out. Risk calculation is extremely important here in order to support clinical decision-making. The threshold values for the risk situation have been set internationally at between 15 % and 30 % [101].

Different calculation models are available. The Gail model is a widely accepted calculation model [102]. Age, age at menarche, number of breast biopsies, age at first childbirth, and number of first-degree relatives with BC are used to calculate the lifetime risk for women without risk factors. A model updated with data from the Surveillance, Epidemiology, and End Results (SEER) study and with death statistics is available online (http://bcra.nci.nih.gov/brc/).

The risk calculation in the Claus model is based on the number of affected first-degree and second-degree relatives and their age at the onset of disease [103]. The original version consisted of tables providing information about the risk in 10-year intervals (from 29 to 79 years of age) relative to age and the age of affected relatives. Various software programs have incorporated this model, such as CancerGene (http://www3.utsouthwestern.edu/cancergene/files/download.htm).

BRCAPRO [104, 105] uses information about male and female relatives, unilateral and bilateral BC, and OC cases in the family. The model also incorporates published gene frequencies and data on the penetrance of *BRCA1* and *BRCA2* [17, 106, 107]. The calculation can be used for different frequencies and penetrance functions. The program can be downloaded free of charge as element of the CancerGene program, or can be purchased in context of a pedigree program (www.cherwell.com).

This program postulates the existence of another gene linked with BC in addition to *BRCA1* and *BRCA2*. The probability of a predisposing mutation and the lifetime risk can be calculated on the basis of family history. Reproductive and hormonal risk factors are incorporated into the model (use of hormone replacement therapy, age at menarche, age at menopause, body mass index, and history of atypical hyperplasia) [107].

In clinical practice, it needs to be taken into account that all of these models are based on different statistical methods and different epidemiological data sets and that they use different patient history parameters. Programs such as CancerGene include several calculation models. The Gail model is the one most widely used in the USA, and it can be used for women with no family history of BC and/or OC. The Claus model and BRCAPRO should be used for patients with a positive family history. The European model (Tyrer–Cuzick) includes family history and the patient's individual medical history and appears to be an adequate model for European women with risk factors [108]. Calculation models can provide support for decision-making regarding genetic testing. Consulters should be informed about the consequences of risk calculation and options should be offered.

26.6 Options

The choice of clinical options requires information regarding the patient's risk of disease and mutation status. The options include primary, secondary, and tertiary prevention.

26.6.1 Early Cancer Detection

26.6.1.1 Breast

Table 26.5 shows the recommended intensified early cancer detection program for women with a high familial risk who are *BRCA1/BRCA2* mutation carriers [1, 97]. It is not currently known whether there is any individual benefit of an

Table 26.5 Intensified early cancer detection program [1, 97, 98]

Age	Examination
	Start at the recommended age, or 5 years before the youngest affected relative
25	Monthly self-palpation of the breast
25	Biannual palpation by gynecologist
25	Biannual ultrasonography of the breast (7.5–13 MHz)
40	Annual ultrasonography of the breast
30	Annual mammography
25	Annual magnetic resonance imaging
25	Biannual vaginal palpation
25	Biannual transvaginal ultrasound

intensified early cancer detection program for *BRCA1/BRCA2* mutation carriers. However, this option is less invasive and less burdensome for the patient in comparison with chemoprevention or prophylactic surgery, and it should be offered to women at risk [98].

Self-palpation of the breast should be started at the age of 25 to ensure regular examinations and an ability to recognize the normal characteristics of breast tissue [109]. Women should carry out palpation every month at the same time (if possible on the second to 6th day of the cycle) in order to assess tissue changes. An analysis of several studies reported a reduction in BC <2 cm in size and in lesions with positive lymph nodes in patients carrying out regular self-palpation of the breast. Engel et al. [110] detected an age-related reduction in mortality of 25 %. Hackshaw and Paul [111] analyzed 20 observational studies and three clinical trials. They did not find a significant reduction in the mortality rate associated with self-palpation (RR 0.90; 95 % CI, 0.72–1.12). Nor did providing teaching and training in palpation lead to any reduction (RR 1.01; 95 % CI, 0.92–1.12). Self-palpation was associated with increased numbers of breast biopsies. However, women with a family history of BC and/or OC might be able to benefit from this method more than the general population. Confidence in the method and the accuracy of the assessment can be improved with practice and medical instruction.

Clinical examination of the breast can detect palpable BCs. In addition, it is capable of detecting tumors that are overlooked at mammography or occur during the screening interval. The estimated sensitivity of the method is reported as 17–89 %, depending on the examiner's experience and the size of the tumor [112]. Clinical examination can be beneficial for young women with a familial risk, in view of the reduced sensitivity of mammography in dense tissue [113].

Mammography should be started with a baseline examination at the age of 30 and if possible, examinations should be conducted in a setting in which images from previous examinations are available. The efficacy of this approach has been demonstrated for women aged 50–69. Mammography can achieve a 30 % reduction in the mortality rate [114]. The provision of screening in patients under the age of 50 is currently a matter of controversy. However, its effectiveness (a 20 % reduction in the mortality rate) can be regarded as proved in women aged 40–49. The U.S. Preventive Services Task Force [115] has stated that the current results do not argue either for or against mammographic screening for young women. The high risk of BC in women with a family history of BC and/or OC suggests that they are able to benefit from this approach, although the currently available data do not clearly prove this. There have only been a few studies analyzing the effectiveness of screening in high-risk women. False-positive results and a potential increase in the risk as a result of early and repeated radiation exposure are often mentioned in relation to mammography. The risk increases when mammographic screening is started early and continues at regular intervals [114]. A higher radiation dosage is needed, as tissue is dense in the young breast. It is estimated that BC cases caused by mammography are low in comparison with the numbers of BCs detected by mammographic screening. The benefits of early cancer detection may predominate, so that an early start is justifiable for women at familial risk. Progression proceeds faster in the premenopausal period, underlining the need for short screening intervals. A 1-year interval should be offered to young women with a familial risk [109, 116].

High-resolution ultrasonography of the breast (7.5–13 MHz) should be carried out biannually and started at the age of 25. In view of the reduced sensitivity of mammography in young women and the potential increase in the risk of hereditary cancer due to mammography, ultrasonography of the breast should be incorporated into early cancer detection programs and women should be encouraged to increase their participation [117, 118].

Warner et al. [119] carried out surveillance of 196 *BRCA* mutation carriers using clinical examinations, ultrasonography, magnetic resonance imaging (MRI), and mammography. Although mammography was able to detect invasive carcinomas in areas of low density, the detection rate was reduced in high-density areas. Ultrasonography of the breast had a sensitivity of 60 % and a specificity of 93 %. Ultrasound may be beneficial in combination with other methods in women with a familial risk [119]. The results presented by Kuhl et al. [120] support the use of a combination of ultrasonography, MRI, and mammography for early cancer detection.

MRI of the breast is the method with the highest sensitivity for detecting local relapses in radiographically dense breast tissue (with a sensitivity of 92 %, in comparison with 86 % for ultrasound and 66 % for mammography). It is therefore an important element of maintenance after breast-preserving surgery [121]. It is possible to detect small carcinomas (>4 mm) if angiogenesis and multicoated BC occur, and to carry out MRI-guided intervention [122]. In a prospective study including 192 asymptomatic women with a family history of BC and/or OC, Kuhl et al. [120] reported

on the efficacy of this method in young women at familial risk. Surveillance was carried out with clinical examinations, ultrasonography, mammography, and MRI. Nine BCs were detected. MRI was the most sensitive and specific method. Several other studies have provided further support for these results [119, 123–126]. MRI was found to be superior to ultrasound and mammography in a study including 235 *BRCA* mutation carriers [127]. The highest efficacy was obtained with a combination of all of the methods. Kriege et al. [128] carried out surveillance in 358 mutation carriers and 1052 women at high risk (with a follow-up period of 2.9 years). Forty-five BCs occurred; 49 % of the cases were capable of being diagnosed by MRI alone; 77 % of the lesions were detected in stage N0, and 46 % were smaller than 1 cm. In two recent studies [126, 129], the sensitivity of MRI was 77–91 %, while mammography only had a sensitivity of 33–40 %. The low sensitivity of mammography is explained by two factors. In addition to the density of the glandular parenchyma in young women, *BRCA*-associated breast carcinomas show atypical characteristics with all imaging methods. They express microcalcifications more rarely. In addition, these carcinomas (not only with medullary differentiation, but also with ductal differentiation) often show morphological characteristics that would really be typical of benign tumors (fibroadenomas) or even cysts — smooth delimitation, apparently expansive growth, a hypoechoic or almost anechoic internal structure, and dorsal signal enhancement [101]. In an update of their surveillance study, Kuhl et al. report that 86 % of stage 0 or stage I carcinomas were detected [129]. This mainly applied to lesions detected using MRI, which were all diagnosed at stage pTis or pT1, N0. With purely mammographic early detection, the rate of interval carcinomas is up to 36–56 % [125]. Using MRI early detection, the rate was 2 % (one of 43) [129]. These results underline the importance and efficacy of the MRI. However, only 5.4 % of women at familial risk use this method for early cancer detection [130, 131]. This can be explained by the high cost of the method, its limited availability, and the limited information about its efficacy. Annual MRI screening examinations can be offered to high-risk women in specialized centers.

26.6.1.2 Ovaries

Early cancer detection is a potential option for *BRCA1/BRCA2* mutation carriers and high-risk women at familial risk. At present, there is no evidence for a reduction in the mortality rate as a result of early cancer detection [113]. There is also a lack of detailed guidelines [113]. The NIH Consensus Statement on Ovarian Cancer recommends transvaginal ultrasonography and CA-125 assessment for screening of *BRCA* mutation carriers every 6–12 months, starting at the age of 35 [132]. The Cancer Genetics Studies Consortium Task Force recommends these methods starting at the age of 25–35 [133]. In interdisciplinary genetic clinics in Germany, biannual transvaginal ultrasonography and pelvic examination is recommended starting at the age of 25 [1, 97].

The recommendation that CA-125 assessment should be carried out is based on the association of OC with elevated CA-125 levels. However, only 50 % of patients with early-stage disease have elevated CA-125 values. In addition, non-malignant conditions (e.g., endometriosis or benign ovarian changes) and nongynecological cancer can also lead to elevated CA-125 values. The low predictive value of the finding and the fact that raised values can be caused by benign conditions exclude the use of this test for general screening and it should be regarded with reservations in the screening of high-risk women [1, 97].

Transvaginal ultrasonography is capable of delineating the size and morphology of a lesion. The method can assess whether a lesion is malignant or benign on the basis of morphological criteria (thickness of the septum, size of cystic findings, papillary structures) [113]. Transvaginal ultrasonography is a low-cost, noninvasive method that is well tolerated by most patients. However, the quality of the examination depends on the examiner's level of experience and the quality of the equipment. Scoring systems using color duplex sonography can objectify the assessment. Sensitivity levels of nearly 100 % and a specificity of 94.9 % can be achieved [134]. Normal physiological changes in advance of ovulation can simulate cancer-linked values in duplex sonography. The examination should therefore not be conducted around the time of ovulation in premenopausal women [135].

The results of ongoing studies are awaited in order to establish the appropriate use and screening intervals with this method. Its efficacy needs to be reexamined in view of recent technological improvements and the higher resolution available with modern equipment. At present, consulters should be informed regarding the lack of evidence available for the efficacy of screening with this technology.

26.6.2 Chemoprevention

26.6.2.1 Breast Cancer

Estrogen may modulate the risk of BC in women with *BRCA* mutations, since premenopausal ovariectomy has a preventive effect, tamoxifen decreases the risk of a second carcinoma and improves overall survival, and two main estrogen tissues (breast and ovarian tissue) are at high risk for carcinoma in *BRCA1* and *BRCA2* mutation carriers. Estrogens increase the probability of mutation due to enhanced proliferation and the direct genotoxic effects of estrogen metabolites. The combined effect of estrogen-promoted cell proliferation and the loss of the *BRCA* DNA repair pathway may increase unwanted events and mutations

that lead to carcinogenesis in breast cells [136]. Chemoprevention focuses on a variety of endocrine, paracrine, and autocrine factors [95, 98]. Aromatase inhibitors, retinoic acid, isoflavonoids, gonadotropin-releasing hormones, low-dose contraceptives, and hormone replacement treatment have been tested.

The concept of using tamoxifen for BC prevention was based on experiments conducted in the early 1970s. A 50 % reduction in the risk of contralateral BC was demonstrated in studies of adjuvant therapy with tamoxifen. Subsequently, the results of four studies (the National Surgical Adjuvant Breast and Bowel Project, the Breast Cancer Prevention Trial, Phase 1 (BCPT-P1), the Royal Marsden Hospital Randomized Chemoprevention Trial, and the Italian Randomized Trial among Hysterectomized Women) were published, which analyzed the administration of tamoxifen in comparison with a placebo for the prevention of BC. The BCPT-P1 study included 13,388 women with a 5-year BC risk of at least 1.7 % (calculated using the Gail model; average risk 3.2 %; 5 years administration of tamoxifen 20 mg/days versus placebo). After a follow-up period of 4 years, the risk of estrogen-receptor-positive BC was reduced by 49 % in patients treated with tamoxifen [117, 137]. By contrast, studies in Europe did not observe a reduced risk with tamoxifen [138, 139]. The differences might be explained by the use of different variables in the different studies (age, compliance, follow-up, additional administration of hormone replacement treatment) and differences in the inclusion criteria and in the way in which risks were estimated and calculated [117]. In the Italian study, the lack of any observed effect of tamoxifen might have been due to the size of the group of patients studied ($n = 5408$), the low risk (48 % with bilateral ovariectomy and no BC risk as a requirement for participation in the study) and limited compliance (149 participants did not complete the 5th year). The patients in the British study were much younger than those in the BCPT-P1 study (62 % versus 39 % of the patients were younger than 50) and had a higher level of familial risk (96 % versus 76 % with an affected first-degree relative). Differences in these factors might have led to a higher risk level in participants in the British study and could explain the observed failure of tamoxifen [140]. The studies show that 6600 women with a low or moderate risk of BC would need to be treated with tamoxifen to prevent 82 early lymph node-negative, estrogen-receptor-positive cases of BC. The use of tamoxifen for prevention is currently a matter of controversy. King et al. [141] analyzed the 288 cases of BC in the BCPT-P1 study; 6.6 % ($n = 19$) of the BC cases were associated with *BRCA1/BRCA2* mutations. The use of tamoxifen reduced the incidence of BC by 62 % in *BRCA2* mutation carriers. There was no influence on the risk in *BRCA1* mutation carriers. The patients has started tamoxifen at the age of 35. Whether an earlier start might reduce the incidence of BC requires further investigation. In addition, the short treatment period might have led to an early therapeutic effect. Narod et al. [142] reported that the use of tamoxifen is capable of reducing the risk of contralateral BC in women who are at familial risk and are *BRCA* mutation carriers. The data from these studies should be discussed with women at risk.

The selective estrogen-receptor modulator raloxifene is currently the subject of ongoing studies. The Multiple Outcomes of Raloxifene Evaluation (MORE) study analyzed the reduction in the numbers of bone fractures associated with the use of raloxifene (120 mg/days versus a placebo) and reported a 60 % reduction in the incidence of BC as a side effect in a group of 7505 postmenopausal women [143, 144]. Further studies have analyzed the effectiveness of aromatase inhibitors such as anastrozole in comparison with a placebo (IBIS II), or exemestane versus a placebo, for the prevention of BC [144, 145]. Regarding the IBIS-II-trial, 1920 women were randomly assigned to receive anastrozole and 1944 to placebo. After a median follow-up of 5·0 years, 40 women in the anastrozole group (2%) and 85 in the placebo group (4%) had developed breast cancer (hazard ratio 0·47, 95% CI 0·32-0·68, p<0·0001). The predicted cumulative incidence of all breast cancers after 7 years was 5.6% in the placebo group and 2.8% in the anastrozole group. The GISS study is analyzing the preventive effect of screening and the use of gonadotropin-releasing hormone (GnRH) analogues and ibandronate in comparison with screening alone in premenopausal women with a high level of familial risk [146–148]. Pilot studies have reported a risk reduction of 50 % with 10 years of goserelin administration and 70 % after 15 years. Administration of the drug is combined with bisphosphonates to avoid reductions in bone density.

26.6.2.2 Ovarian Cancer

The genesis of OC is associated with rapid changes in and repair of epithelial cells after ovulation. The long-term use of oral contraceptives can reduce the risk of OC by approximately 50 % and the mortality rate by approximately 80 % [149–151]. In low-risk women, oral contraceptive use for at least 5 years leads to a reduction in the relative risk to 0.68 (95 % CI, 0.5–0.9) and 10–14 years use is associated with a relative risk of 0.54 [19]. Comparable results have been reported for the mortality rate, with a reduction in the relative risk to 0.2 (95 % CI, 0.0–1.3) with 10 years intake of oral contraceptives [19, 152].

The use of contraceptives, particularly monophase compounds, is an option for the prevention of OC in high-risk women. Narod et al. [153, 154] reported a significant reduction in the risk of OC in two studies with *BRCA1/BRCA2* mutation carriers. The odds ratio was 0.44 (95 % CI, 0.28–0.68), with a risk reduction of 4.4 % per year of intake ($P = 0.056$). However, it should be noted that the risk reduction is not yet evident after 10 years of use.

26.6.3 Prophylactic Surgery

26.6.3.1 Prophylactic Mastectomy

At present, the view that bilateral prophylactic mastectomy and ovariectomy are effective is based on the results of retrospective studies. Detailed guidelines for the indication and technique are lacking. Numerous retrospective studies have been conducted to analyze the efficacy of prophylactic mastectomy. The studies show major differences in the inclusion criteria, the proportion of high-risk women or mutation carriers included, and follow-up periods (Table 26.6) [155–160]. An important study is the retrospective analysis carried out by Hartmann et al. [159]. The incidence of BC was reduced by 90 %. Afterwards, the women were screened for *BRCA1* and *BRCA2* mutations. In 26 identified mutation carriers, no BC developed during a follow-up period of 13.4 years [159]. Seventy-six mutation carriers with prophylactic mastectomy and 63 mutation carriers without prophylactic mastectomy are receiving surveillance in the first prospective study to be conducted [123]. At present, no BC has been observed in the group with prophylactic surgery, while eight cases have been detected in the control group (Table 26.6). These preliminary results support the published data from retrospective studies.

A significant portion of breast tissue remains in the area of the areola after subcutaneous mastectomy. In 11 patients with relapses, three of the relapses were observed in the area of the areola and eight in the conserved tissue lobe [161]. In addition, there is still a need for regular mammography. False-positive results may increase due to scar tissue and postoperative calcifications, which require additional biopsies. Total mastectomy is capable of reducing breast tissue by 90–95 %; only total mastectomy with a thin skin flap provides the maximum prevention [161, 162]. A breast reconstruction (primary or secundary) and a psychological support should be offered to all patients with prophylactic surgery.

26.6.3.2 Prophylactic Ovariectomy

Prophylactic ovariectomy is the most commonly used method of reducing the risk of OC. The benefits of this option decline with increasing age at surgery. The completion of family planning or a minimum age of 35–40 is recommended, due to the young average age of onset in this disease [132].

The efficacy of this option has been confirmed by numerous studies (Table 26.7) [163–167]. Three studies on the topic analyzed a total of 396 high-risk women who underwent prophylactic ovariectomy [163–165]. Seventeen cases of OC were observed. The highest risk appears to be the development of peritoneal carcinosis, which was diagnosed in 11 patients (3 %). These observations might be explained by the presence of an occult OC at the time of surgery, malignant ectopic ovarian tissue, or transformation of the peritoneum, which has the same embryonic origin as the surface epithelium of the ovaries. Two retrospective studies have reported that this method is effective in *BRCA1* and *BRCA2* mutation carriers (Table 26.7). Kauff et al. [167] reported a risk reduction of 85 %. Rebbeck et al. [166] observed a risk reduction of 96 %. These results support the recommendation that this option should be chosen after the completion of family planning, as the median age of onset was 50.8 years in the control group (range 30–73 years). The average age at onset was 50.0 years in *BRCA1* mutation carriers and 55.0 years in *BRCA2* mutation carriers in the German group included in the Hereditary Breast and Ovarian Carcinoma Consortium (*Konsortium hereditäres Mamma- und Ovarialkarzinom*) study [8]. Both of the above studies also reported a reduction in the risk of BC with prophylactic ovariectomy (68 and 53 %). The possibility of reducing the risk of relapses and of contralateral BC was mentioned above.

In a study of *BRCA* mutation carriers and those with a high-risk family pattern, an occult malignancy was identified during prophylactic ovariectomy in 13.3 % (four of 30) [168], with three of the occult carcinomas being found in the area of the fallopian tubes. Deligdisch et al. [169] identified dysplasias in 77.6 % of ovaries removed prophylactically from *BRCA* mutation carriers; dysplasias were only found in 33.3 % of women with negative tests. Due to the high probability of an occult carcinoma and the risk of tubal carcinoma or primary peritoneal carcinoma, a standardized

Table 26.6 Overview of studies on prophylactic mastectomy

	Humphrey et al. [155]	Woods et al. [156]	Bohmert et al. [157]	Pennisi and Capozzi [158]	Hartmann et al. [159]	Meijers-Heijboer et al. [160]
Study design	Retrospective	Retrospective	Retrospective	Retrospective	Retrospective	Prospective
Total no. of patients (*controls*)[a]	285	1400	155	1500	639	76 (63)
No. of high-risk patients (*controls*)[b]	16 (6 %)	n/a	90 (58 %)	735 (49 %)	214 (33 %)	76 (100 %)[c] *63 (100 %)*[c]
Mastectomy	Subcutaneous	Subcutaneous	Simple	Subcutaneous	Subcutaneous	Simple
Median follow-up period (*y*)	n/a	16–20	2	9	14	3
No. of patients with BC (*controls*)	3	3	n/a	6	7	0 (8)
No. of high-risk patients with BC (*controls*)	19	n/a	n/a	0.8	1.4	0 (8)

[a]Early cancer detection alone
[b]Patients with a family history of breast cancer or ovarian cancer, or ductal or lobular carcinoma in situ, or contralateral breast cancer
[c]*BRCA* mutation identified

Table 26.7 Overview of studies on prophylactic ovariectomy

	Tobacman et al. [163]	Piver et al. [164]	Struewing et al. [165]	Rebbeck et al. [166]	Kauff et al. [167]
Study design	Retrospective	Retrospective	Retrospective	Prospective	Prospective
Total no. of patients (*controls*)[a]	28	324	44	259 (*292*)	98 (72)
No. of high-risk patients (*controls*)	28[b]	324[b]	44[b]	219 (*240*) BRCA1 42 (52) BRCA2	56 (48) BRCA1 42 (24) BRCA2
Median follow-up period (*y*) (*controls*)	1–10	1–27	460 Person-years	8.2 (*8.8*)	2.0 (*2.1*)
Total no. of patients with OC (*controls*)	3	6	2	2 (58)	1 (5)
% of high-risk patients with OC (*controls*)	10.7	1.9	4.5	0.8 (*19.9*)	1.0 (*6.9*)

[a] Early cancer detection alone
[b]Patients with two affected first-degree relatives, or identified *BRCA* mutation, or hysterectomy due to hereditary nonpolyposis colorectal cancer

Table 26.8 Recommendations for conducting prophylactic ovariectomy, in collaboration between the patient, human geneticist, gynecologist, surgeon, and pathologist [170]

1	Bilateral adnexectomy with complete removal of the fallopian tubes (open or laparoscopic)
2	Cytological examination of the peritoneal rinse fluid (procedure: instillation of 50 mL sterile saline into the abdominal cavity and removal of the fluid after 10 min)
3	Multiple peritoneal biopsies
4	Multiple biopsies from the greater omentum
5	Serial sections of the entire fallopian tubes and ovaries at 2-mm intervals and microscopic examination of all sections

procedure should be used in prophylactic ovariectomy, which should include removal of the tubes completely along with the adjacent peritoneum, rinse fluid cytology, and biopsies from the peritoneum and greater omentum (Table 26.8) [8, 20]. Using this protocol, Powell et al. [170] were able to identify seven occult carcinomas in 41 *BRCA* mutation carriers (four tubal carcinomas and three ovarian carcinomas).

Prophylactic ovariectomy can lead to significant psychological stress for patients in relation to their physiological integrity and an increased risk of various diseases may result from the induced menopause. Patients need to be informed about the consequences—e.g., the climacteric syndrome with hot flushes, sweating, palpitations, tachycardia, insomnia and depressive mood, osteoporosis, and cardiac and cerebrovascular diseases [171].

26.6.4 Therapy

Although there are significant differences in the pathological characteristics of hereditary and sporadic BC, no specific guidelines are available on the treatment of patients with hereditary BC. In most cases, there is a lack of adequate risk perception, consultation, and genetic analysis of the *BRCA1/BRCA2* genes at the time of the primary diagnosis. Subsequently, the familial risk and the associated risk of a second BC or OC does not influence treatment decision-making. High-risk women are treated on the basis of general guidelines (www.ago-online.de).

Goffin et al. [172] reported that the overall survival is particularly reduced if *BRCA1* mutation carriers do not receive chemotherapy (278 women with BC, 30 mutation carriers, RR 3.3; 95 % CI, 1.2–8.8; $P=0.01$). There is a statistically significant reduction in the survival in mutation carriers with negative lymph-node status (RR 5.6; $P<0.001$). This could be explained by the reduced frequency of chemotherapy in patients found to have a negative lymph-node status (24 % versus 79 %; $P=0.01$). In view of these results, the possible administration of chemotherapy may be discussed with patients in whom there is no lymph-node involvement who have a positive family history, or in whom a *BRCA* mutation has been identified. Only 10 % of *BRCA1*-linked tumors show estrogen-receptor expression. As endocrine therapy is not possible in this group of patients, survival is significantly impaired (RR 2.3; 95 % CI, 1.2–4.7; $P=0.02$). Narod et al. [142] analyzed the possibility of reducing the risk of contralateral BC with tamoxifen administration in 476 *BRCA1* and 117 *BRCA2* mutation carriers. Thirteen percent ($n=64$) of the *BRCA1* mutation carriers and 33 % ($n=39$) of the *BRCA2* mutation carriers were treated with tamoxifen. The risk was reduced to a statistically significant extent in both groups (OR 0.38; 95 % CI, 0.19–0.74 and OR 0.63; 95 % CI, 0.20–1.59, respectively). The protective effect of tamoxifen administration increased continuously up to 4 years. The authors consider that tamoxifen administration is capable of reducing the risk of contralateral BC by 50 %, and this view is supported by the results of a published meta-analysis. Tamoxifen prevents the development of contralateral carcinoma independently of the hormone-receptor status of the primary BC [173].

A few studies have analyzed the use of different chemotherapeutic agents in patients with hereditary BC. *BRCA*-linked BC lesions have different levels of sensitivity to

chemotherapy regimens. An in vitro analysis reported a reduced sensitivity to taxanes and doxorubicin, while the sensitivity to cisplatin was increased [174]. Although anthracyclines and taxanes are frequently used, their efficacy may be reduced in patients with hereditary BC. Tumors arising in carriers of heterozygous germline mutations in the *BRCA1* or *BRCA2* gene have generally lost the wild-type allele and therefore do not express functional protein. Although a haploinsufficiency phenotype is still a theoretical possibility for *BRCA1* or *BRCA2*, the majority of the evidence suggests that this is not the case, at least for DNA repair functions. This provides an ideal target for treatment [175, 176]. Sensitivity to cisplatin may be increased as a result of DNA damage [174, 177]. To investigate whether the in vitro evidence can translate into improved clinical efficacy, studies randomly assigning patients with metastatic *BRCA1* or *BRCA2* familial breast cancers to either carboplatin or docetaxel chemotherapy have been conducted [176, 178–180]. A clinical trial has presented that patients with a BRCA mutation associated triple negative breast cancer can extremely benefit from combinations with platinum and taxane and achieve a pathologic complete response rate of up to 90 %. This has been confirmed by the phase-III-trial Geparquinto.

Single-strand breaks are usually repaired via the base excision pathway; inhibition of this pathway therefore substantially increases the number of unrepaired single-strand breaks, subsequently leading to collapsed replication forks and double-strand breaks at replication forks. Poly-ADP ribose polymerase (PARP) plays an important role in DNA repair (excision of DNA base pairs) by binding directly to the damaged DNA. Inhibition of this enzyme by RNA interference, or using chemical inhibitors, leads to severe, highly selective toxicity in *BRCA1*-defective and *BRCA2*-defective cells, with a selectivity that is several times higher than that of conventional chemotherapy drugs [181, 182]. The use of PARP inhibitors thus represents a targeted approach to the treatment of *BRCA1/BRCA2*-associated breast carcinomas [86]. The PARP inhibitor olaparib is already approved for patients with a platin-sensitive relapse of ovarian cancer and proven BRCA mutation (germline or tumour). Trials of PARP inhibitors in *BRCA1/BRCA2* mutation carriers with breast cancer are currently ongoing.

With regard to surgical treatment, two important aspects need to be considered. The risk of a contralateral BC is approximately 40 % [142]. Haffty et al. [16] compared the results of surgery in *BRCA1/BRCA2* mutation carriers ($n=22$) and women with sporadic BC ($n=105$). Breast-preserving therapy was carried out in both groups. Relapses and cases of contralateral BC were found to be significantly more frequent in the group of mutation carriers after a follow-up period of 12 years (49 % versus 21 %, $P=0.007$; and 42 % versus 9 %, $P=0.001$, respectively). Although endocrine therapy with tamoxifen and prophylactic ovariectomy are capable of reducing the risk of relapse by 50 and 56 %, respectively [142], bilateral mastectomy needs to be discussed with mutation carriers and high-risk women who have primary disease. In addition, the sensitivity of mammographic surveillance is reduced in mutation carriers. In a study of early cancer detection, Goffin et al. [183] detected six of 13 BCs in *BRCA1* mutation carriers using mammography, while mammography identified 96 of 108 patients with sporadic BC (46 % versus 89 %; $P<0.001$). In the group studied by Meijers-Heijboer et al., 75 % of women with identified *BRCA* mutations and BC opted for bilateral mastectomy with reconstruction [160].

These results support the view that it is necessary to carry out genetic testing when primary disease is diagnosed. Prophylactic ovariectomy needs to be discussed with mutation carriers and women who are at high risk. With regard to the reduction in the risk of contralateral BC associated with prophylactic ovariectomy, Narod et al. [142] reported an odds ratio of 0.31 (95 % CI, 0.15–0.67) on the basis of 185 mutation carriers with BC under the age of 50 who underwent bilateral ovariectomy. The odds ratio was 0.85 (95 % CI, 0.22–2.36) if the age of onset was above 50 years. The risk reduction was persistent. Møller et al. [184] followed up 36 *BRCA1* mutation carriers with BC (21 of whom underwent prophylactic ovariectomy). The 5-year survival rate was 67 % versus 44 %. If mutation carriers with ovariectomy had a negative lymph-node status, the 5-year survival rate was 100 %, compared with 42 % in women who did not undergo ovariectomy ($P=0.009$).

In contrast to hereditary BC, the overall survival is longer in women with hereditary OC in comparison with those with sporadic OC, independently of pathological differences and different stages at the primary diagnosis [88, 92, 185]. *BRCA*-linked tumors show a higher response to platinum-containing chemotherapy agents. Cass et al. [92] analyzed the response, overall survival, and in vitro sensitivity on the basis of 35 mutation carriers (22 with *BRCA1* and 12 with *BRCA2* mutations) and 37 women with sporadic OC. Both groups received a chemotherapy regimen containing platinum. The median survival period was longer in mutation carriers (91 months versus 54 months; $P=0.046$). In addition, in vitro analysis was able to predict the response in the group of mutation carriers ($P=0.0096$). Increased sensitivity to agents containing platinum has been demonstrated in several in vitro studies [186, 187]. This may be explained by the DNA damage induced by platinum and the reduced ability to repair such damage due to loss of function of the *BRCA* gene [92]. On the other hand, the results might also be explained by a higher proliferation rate, with the associated sensitivity to chemotherapy regimens [90].

26.6.5 Follow-Up

Follow-up is becoming increasingly important in patients with hereditary BC and/or OC. General guidelines should be used, owing to the lack of prospective studies [171]. The family history should be analyzed during the first follow-up visit in order to detect the individual risk of contralateral BC, relapse, and OC. Genetic testing should be offered if required. The risk level in the patient's relatives (e.g., daughter, sister, and mother) should also be identified and appropriate options should be offered if needed. The main focus should of course be on early cancer detection in the associated organ—e.g., the ovaries in women with BC. Ovariectomy and prophylactic tamoxifen administration should be discussed. Prophylactic mastectomy must be offered to women with OC. MRI appears to be superior to mammography [119, 120, 123, 124, 127, 128], and the use of MRI should be considered in the context of follow-up examinations.

26.7 Prognosis

Although *BRCA*-linked BC is associated with higher proliferation rates and lower grading, the results of survival analyses are controversial. Only one study was able to detect improved survival among mutation carriers [188]. Further studies reported no differences, or a poorer prognosis. Several studies detected a clearly reduced survival period in comparison with patients with sporadic BC [189, 190]. The authors considered that the poorer survival might be explained by lower grading, more advanced stages at the primary diagnosis, and reduced estrogen-receptor and progesterone-receptor expression. Comparison with sporadic BCs with similar stages and pathological characteristics should show comparable survival rates. Stoppa-Lyonnet et al. [191] identified the *BRCA1* mutation as an independent prognostic factor (Table 26.9). Mutation carriers had a reduced survival period and a shorter metastasis-free interval. *BRCA1* mutation continued to be an independent prognostic factor after adjustment for pathological characteristic (death, RR 3.5, 95 % CI, 1.3–9.7; metastasis, RR 2.6, 95 % CI, 1.0–6.5). Goffin et al. [172] identified the *BRCA1* mutation as an independent prognostic factor in a study including 287 Ashkenazi Jewish women. A univariate analysis of 141 patients with a negative lymph-node status showed that the *BRCA1* mutation status (RR 5.6) was significantly associated with a higher mortality rate ($P > 0.001$). In the multivariate analysis, the mutation status (RR 3.5) was still significant ($P = 0.003$). This might be explained by the absence of chemotherapy for an aggressive tumor.

In contrast to BC, patients with *BRCA*-linked OC have a better survival (Table 26.10). This has been confirmed by several studies. Cass et al. [92] analyzed the survival in 71 Ashkenazi Jewish women with OC, including 22 with *BRCA1* mutations and 12 with *BRCA2* mutations. *BRCA* mutation carriers with advanced disease survived longer (91 months versus 54 months; $P = 0.046$) and had a longer relapse-free interval (49 months versus 19 months; $P = 0.16$). David et al. [192] observed an improved median overall survival period (53.3 months versus 37.8 months) and 3-year survival rate (65.8 % versus 51.9 %), independent of age and the stage at diagnosis of the primary disease (896 patients, 243 mutation carriers).

26.8 Conclusion

Familial risk is an important risk factor in BC. A hereditary origin is present in 5–10 % of patients with BC. Ten percent of all cases of OC are associated with a familial predisposition. Some 15–20 % of all cases of familial BC are linked to *BRCA1* and/or *BRCA2*. In addition, an association with *BRCA1* mutations is found in 80 % of families with familial

Table 26.9 Prognosis in *BRCA*-linked breast cancer

	BRCA1	Sporadic BC	95 % Confidence interval	*P*
5-Year survival rate[a]	49 %	85 %		0.0001
Local recurrence-free interval (5 years)[a]	54 %	79 %		0.11
Metastasis-free interval (5 years)[a]	18 %	84 %		0.0001
RR of death with mutation, univariate (*multivariate*) analysis[b]	1.9 (*1.4*)	1	0.99–3.6 (*0.7–2.9*)	0.052 (*0.3*)
Adjusted for N[a]	3.5	1	1.3–9.7	0.02
N-negative, univariate (*multivariate*)[b]	5.6 (*3.5*)	1	2.3–14.0 (*1.1–11.0*)	0.0001 (*0.03*)
N-positive, univariate (*multivariate*)[b]	0.8 (*0.8*)	1	0.2–2.3 (*0.2–2.5*)	0.6 (*0.6*)
RR of metastasis with mutation, univariate (*multivariate*) analysis[b]	1.6 (*1.2*)	1	0.9–2.9 (*0.7–2.4*)	0.1 (*0.5*)
Adjusted for N/ER[a]	2.6	1		0.05

BC, breast cancer, *N*, node, *RR* relative risk
[a]Stoppa-Lyonnet et al. [191] (*BRCA1* $n = 19$; sporadic carcinoma $n = 91$)
[b]Goffin et al. [172] (*BRCA1* $n = 30$; sporadic carcinoma $n = 248$

Table 26.10 Prognosis in *BRCA*-linked ovarian cancer

	BRCA	Sporadic OC	*P*
Survival (median)[a]	91 Months	54 Months	0.046
Survival (median)[b]	53.43 Months	33.1 Months	–
Survival (median, stages III + IV)[c]	77 Months	29 Months	<0.001
Survival (median, stages III + IV)[b]	51.2 Months	33.1 Months	–
Local recurrence-free interval (median, stages III + IV)[a]	49 Months	19 Months	0.16
Local recurrence-free interval[c]	14 Months	7 Months	<0.001
Recurrence rate[a]	72 %	84 %	n/a
2-Year survival rate[a]	100 %	83 %	–
3-Year survival rate[b]	65.8 %	51.9 %	<0.001
3-Year survival rate (stages III + IV)[b]	60.3 %	44.5 %	–
5-Year survival rate[a]	65 %	48 %	–

[a]Cass et al. [92] (*BRCA* $n=29$; sporadic carcinoma $n=25$)
[b]David et al. [192] (*BRCA* $n=229$; sporadic carcinoma $n=549$)
[c]Rubin et al. [185] (BRCA $n=43$; sporadic carcinoma $n=43$)

OC and an association with *BRCA2* mutations in 15 %. *BRCA* mutations also increase the risk of oropharyngeal carcinoma, malignant melanoma, endometrial carcinoma, cervical cancer, colon cancer, prostate cancer, gastric cancer, cancer of the gallbladder, and pancreatic cancer. Early cancer detection with screening at regular intervals appears to be adequate for the associated carcinomas. *BRCA1*-linked and *BRCA2*-linked carcinomas have special pathological characteristics, which can be relevant for the treatment and prognosis. An increased understanding of the roles played by *BRCA1* and *BRCA2* in DNA repair is beginning to suggest potential therapeutic strategies. Tumors that are deficient in *BRCA1* or *BRCA2* may be highly sensitive to DNA cross-linking agents.

With the growing knowledge available regarding the genetic basis for carcinogenesis and familial disposition, the requirements for all physicians have become more complex. Specialized interdisciplinary genetic cancer clinics are able to identify familial risk, the risk of disease, and the probability of positive mutation status and can provide patients with information regarding the need for intensified early cancer detection, chemoprevention, and genetic testing. Genetic testing can be offered to patients if the inclusion criteria are fulfilled.

It is mainly young women with BC, and women with disease who have numerous affected relatives, who take advantage of the opportunity for genetic testing. Receiving information regarding the level risk in their children and relatives, as well as the risk of relapse, contralateral BC, and OC affects decision-making in women who have already developed the disease.

Risk calculation programs can establish the level of risk without the need for genetic testing and can help patients decide whether to undergo genetic testing of the *BRCA1* and *BRCA2* genes. The available calculation models are based on differing statistical methods, epidemiological data, and different patient history parameters. The Claus model and BRCAPRO should be used if there is a positive family history. The European model (Tyrer–Cuzick) may be the best model for calculating the risk in a European group with several risk factors.

Providing information on the risk of BC and OC and the probability of a *BRCA* mutation is a basic prerequisite for discussing the need for, and availability of, early cancer detection, specific therapy, chemoprevention, and prophylactic surgery with the patients concerned.

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The Molecular Basis of Prostate Carcinogenesis

27

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27.1 Introduction

This review on prostate cancer is an attempt to update the reader on the current information we have on the disease. There are no definite cures yet for prostate cancer and the exact mechanism of development is far from clear. However, over the years, the information obtained on the disease has increased due to the unceasing flow of research data. In the following pages, you will be briefed on the basic characteristics of prostate cancer, the characterization of the genomic regions affected, the candidate genes involved, the secondary sites of metastases such as bone and the models that have been developed to investigate the disease in detail.

27.2 Epidemiology of Prostate Cancer

Langstaff reported the first case of prostate cancer in 1817 followed by J. Adams, a surgeon in London, who examined the cancer histologically [1, 2]. Prostate cancer is a non-skin cancer that is the cause of the second largest number of male-related deaths in the countries of the West [3]. It is known as the cancer of the elderly as older men over the age of 65 are diagnosed with cancer of the prostate [4]. This is probably why many men die with the cancer rather than due to it [4, 5]. This is observed in the case of asymptomatic sporadic prostate cancer which account for >90 % of the disease [6]. Hereditary and familial prostate cancer, on the other hand, is observed in younger males and is dependent on family history [5]. In 2007, the statistics in CA Cancer Journal for Physicians predict that prostate cancer accounts for ~218,885 new cases and ~27,350 deaths [7, 8].

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27.3 Risk Factors for Prostate Cancer

There is no single cause of prostate cancer, but it primarily arises due to aging, discrepancies in sex hormone levels, genetic factors, and environmental agents [4, 9].

27.3.1 Aging

Prostate cancer is known as a cancer of the elderly for the main purpose that tumors arise with increase in age of men [10]. There is a logarithmic increase in malignant potential with increase in age. Prostate cancer has a long natural history and develops very slowly which might account for the delayed appearance of the tumor in the life of an individual. In most cases, carcinoma of the prostate rarely sees the light of day during the lifetime of an individual and is observed during an autopsy. Latent cancers of the prostate and benign prostatic hyperplasia (BPH) are observed more frequently, and differ from the few cancers that develop malignant potential. Surgery is the most common form of treatment to remove BPH [10, 11]. Another hypothesis for the contribution of aging to prostate cancer is the shortening of telomere sequences and a subsequent increase in the enzyme telomerase which can be observed in prostatic intraepithelial neoplasia (PIN) and carcinomas [9]. Carcinoma arises predominantly from the peripheral zone compared to the central zone of the prostate [11].

27.3.2 Hormones

Hormones are an integral part of the prostate as they regulate its growth, differentiation, and maintenance. Androgenic hormones such as dihydrotestosterone are found in the prostate. Testosterone diffuses into the prostate and is converted to dihydrotestosterone in the prostate which is the major form of androgen metabolized in the prostate. Prostate cancer shows high levels of androgens when first diagnosed and androgen

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_27

ablation therapy is commonly used to counter the growth of the cancer which results in the blockade of the androgen supply. However, it has not been confirmed that androgenic hormones are the cause of prostate cancer [4, 12, 13].

27.3.3 Genetic Factors

Hereditary and familial cancer account for ~9 % of prostate cancer cases [4, 6, 9]. Prostate cancer is hereditary when there are at least three first-degree relatives or three consecutive generations or two or more relatives (at a young age) affected by the disease. The loci and the candidate genes affected in hereditary prostate cancer differ from those affected in sporadic cancer [6]. Linkage analysis of the hereditary prostate cancer families identified 6 loci that are associated with the disease. HPC1 or hereditary prostate cancer gene 1 is the name given to one of the loci, 1q24–25, and the gene that maps to the HPC1 locus is called *RNASEL*. In addition the other loci affected are 1p36 (gene *CAPB*), 1q42.2–43 (*PCAP*), 8p22–23 (*MSR1*), 17p11 (*HPC2*), 20q13 (*HPC20*), and Xp27–28 (*HPCX*) [6, 14, 15]. The two main candidate genes in the list are *RNASEL* and *MSR1*. *RNASEL* encodes an endoribonuclease involved in the proapoptotic activity of the interferon induced 2-5A system. Mutation in *RNASEL* reduces the enzymatic activity. *MSR1* is a macrophage specific receptor binding to Gram-positive and Gram-negative bacteria. It is difficult to distinguish hereditary cancer from sporadic cancer especially when dealing with families that have a high penetrance of prostate cancer. The detection of hereditary cancer only in older patients prevents the simultaneous identification of the cancer in a younger generation to confirm the hereditary aspects of the disease [15]. Sporadic prostate cancer accounts for >90 % of prostate cancer. The chromosomal loci involved in sporadic prostate cancer differ from those affecting hereditary cases. The loss of chromosomal loci indicates the loss of potential candidate genes which may be tumor suppressor genes. These genes encode proteins that function as hormone receptors, cell-signaling molecules, and cell adhesion factors. Some of the loci that are affected are 3p22–26, 5q22, 10q23, 13q14, 18q21, and others.

27.3.4 Diet

Dietary factors are known to affect the incidence of prostate cancer. It has been shown that Asian men are less likely to develop prostate cancer compared to their counterparts in the Western countries [1, 4]. The incidence of prostate cancer in African-American males is 15 % compared to 10 % in Caucasian-American males. The incidence of prostate cancer is 120 times lower in Asian men [16]. This is due to differences in diet in the two parts of the world. In addition, it has also been observed that when Asian men migrate to the west, there is an increase in prostate cancer incidence in the first and second generations of these men. Selenium and Vitamin E levels are inversely correlated to prostate cancer incidence [1, 4, 10, 16].

27.4 Diagnostic and Screening Tests

Langstaff reported the first case of prostate cancer in 1817. Since then the reported cases of incidence of prostate cancer have steadily increased due to the screening methods that have been employed to detect prostate cancer. Digital rectal examination (DRE), Prostate-Specific Antigen (PSA) test, Transrectal UltraSound (TRUS), Needle Biopsy are some of the tests used to diagnose prostate cancer. DRE detects palpable tumors which however can result in false positives if the growth is benign prostatic hyperplasia. The PSA test is the universal test used to detect prostate cancer or the initiation of the disease by measuring the serum concentrations of PSA. The normal concentration range for PSA is 0–4 ng/ml. TRUS is an imaging technique where a handheld transducer is used with a frequency between 6 and 7 MHz and rotated around in the prostate to give an idea of any potential outgrowths and their size range. Needle Biopsy is used to confirm a diagnosis of prostate cancer. The diagnosis obtained from either of the above three methods warrants confirmation by performing a biopsy. While each method identifies prostate cancer, the sensitivity is higher when two or more methods are combined to detect the cancer [4].

ABCD and TNM staging are the popular forms of staging of prostate cancer. In the ABCD system, early stage prostate cancers are referred to as stage A cancers and subsequent increases in tumor growth are labeled stages B, C, and D. The TNM classification identifies the cancer based on initial growth, presence of positive lymph nodes, and burden of metastasis. DRE, PSA, TRUS, and DNA ploidy are used to identify the stage of prostate cancer. Computed tomography (CT), magnetic resonance imaging (MRI), and radionuclide bone scans confirm the clinical staging results. Biopsies are histologically graded as per the Gleason grading system which is the most popular form of cancer grading. For most solid tumors, the histopathological indicators of biological aggressiveness have been determined through the comparison of tumor morphology with corresponding clinical outcome. Surgical staging is done by radical prostatectomy or lymphadenectomy [4].

The histological grade of the primary prostate cancer is evaluated using the Gleason grading system [17]. In this system, grading is based upon the degree of glandular differentiation and growth pattern of the tumor as it relates to the prostatic stroma. The pattern may vary from well differentiated (grade 1) to poorly differentiated (grade 5). This system

takes into account tumor heterogeneity by summing the score of both the primary and secondary tumor growth patterns. For example, if the majority of the tumor is well differentiated (grade 1) and the secondary growth pattern is poorly differentiated (grade 5), the combined Gleason sum would be a 6. Low (i.e., score = 5) Gleason sum prostate cancers predictably have minimal aggressive behavior whereas very high (score = 8–10) Gleason sum tumors are usually highly aggressive [18]. Unfortunately, the intermediate (score = 5–7) Gleason sum tumors are highly unpredictable in their clinical aggressiveness [18]. This limitation is of particular importance as the majority of tumors (76 %) fall into this intermediate Gleason sum category [19]. Thus, predicting the biologic potential of the majority of prostate cancer in asymptomatic patients based upon histology alone is problematic.

27.5 Anatomy and Histology of the Prostate

To understand the molecular aspects of prostate cancer, it is essential to understand the anatomy and histology of the prostate gland. The prostate gland arises from different segments of the urethra and is located at the base of the bladder. It is a fusion of glandular and non-glandular regions [9, 20]. The prostate gland can be divided into three main glandular zones: (1) the peripheral zone, (2) the central zone, and (3) the transition zone, and in addition, a smaller periurethral gland region [9, 20, 21]. The basic function of the prostate is to secrete proteins to the seminal fluid [9]. The prostate develops from the urogenital sinus during embryogenesis and the differentiation process requires the interaction of the epithelial cells with the mesenchyme. This interaction is essential since absence of either component fails to generate the prostate and this has been elegantly shown through the recombination experiments of the laboratory of Cunha [22]. Androgen signaling also plays an important role in this interaction by acting on the mesenchymal component for the initial development of the prostate and subsequently on the epithelial component instructing the cells to secrete proteins.

The prostate epithelium consists of three types of cells: luminal, basal and neuroendocrine.

27.5.1 Luminal Cells

The luminal cells are secretory cells which secrete the seminal fluid. These cells also exhibit the androgen receptor and other proteins such as cell surface marker CD57 and prostate-specific antigen (PSA) [9]. The secretory cells of the peripheral, transition, and periurethral gland zones are more uniform in structure compared to the irregular luminal secretory cells [21].

27.5.2 Basal Cells

The basal cells lie between the basal lamina and the luminal cells. These cells do not have a secretory function, possess the androgen receptor in very low quantities, and cannot be detected immunohistochemically by staining for PSA. They express anti-apoptotic genes such as *Bcl2*, DNA damage protecting genes, for example, *Gst-π, c-Met,* p53-related protein *p63*, and prostate stem-cell antigen (*PSCA*) [20, 23]. The basal cells are made up of stem cells and the transit-amplifying cells which are intermediate cells of the prostate. These transit-amplifying cells are postulated to act as the precursors of prostatic inflammatory atrophic cells (PIA) which go on to become prostatic intraepithelial neoplasia followed by carcinoma [23].

27.5.3 Neuroendocrine Cells

In all the zones of the glandular prostate, a small population of endocrine–paracrine cells is located on the basal cell layer but do not grow into the lumen. These are called neuroendocrine cells and were first described by Pretl [24]. These cells have no specific function but may play a regulatory role in the development of the prostate as well as in the secretory aspect of the luminal cells. Somatostatin, bombesin, serotonin, and calcitonin are some of the peptides secreted by the neuropeptide cells and can be used as markers to identify these cells [24]. During androgen ablation therapy, prostate cancer cells regress and subsequently regrow in the absence of androgen. Neuroendocrine cells are observed during the stage when the prostate cells start to develop in the absence of androgen [24].

27.5.4 Glandular Tissues of the Prostate

The urethra is the base around which the zones of the prostate develop. The urethral wall forms the backdrop for the development of the ducts of the peripheral and central zones. The prostatic urethra is divided equally into the distal peripheral and proximal central halves divided at the point of the verumontanum by an angular (35°) wall between the apex and the bladder neck. The ejaculatory ducts lie along the proximal part of the urethra ending at its base [20].

27.5.5 Peripheral Zone

The peripheral zone forms approximately 70 % of the prostate. The ducts protrude from the lateral side and posteriorly from the urethral wall. Most carcinomas arise in the peripheral region.

27.5.6 Central Zone

The central zone covers 25 % of the prostate and the ducts surround the ejaculatory ducts which lie in the distal urethra. Carcinomas rarely arise in the central zone.

27.5.7 Transition Zone

Benign prostatic hyperplasia (BPH) arises in the transition zone which lies in the angular region of the urethra between the verumontanum and the bladder neck. This zone consists of 5 % of the prostate.

27.5.8 The Periurethral Gland Region

This region consists of small ducts and acini that lies within the proximal urethral segment and is the site where BPH arises [20, 21]. There are ducts and acini in all the glandular regions of the prostate which terminate in either the capsule or the anterior fibromuscular stroma. The ducts and acini stain for PSA and prostatic acid phosphatase (PAP) [20, 21].

27.5.9 Non-glandular Tissues of Prostate

Preprostatic sphincter, striated sphincter, anterior fibromuscular stroma, and prostatic capsule are the non-glandular tissues of the prostate. The preprostatic sphincter lies posterior to the urethra and its fibers spread and connect with the ducts of the transition zone. Anteriorly, the fibers end in the anterior fibromuscular stroma. The anterior fibromuscular stroma extends up to the prostate apex and into the capsule laterally. It is also in contact with the preprostatic sphincter and indirectly with the transition zone. The prostate is covered with striated sphincter muscles that end incompletely at both the distal and proximal ends. The prostatic capsule encloses a major part of the prostate and the terminal acini of the peripheral and central zones are in contact with the capsule. The capsule does not extend over the ejaculatory ducts or the bladder neck. Neurovascular bundles innervate the lateral portion of the capsule.

27.6 Prostate Cancer Progression Pathway

How is the morphology of the prostate and prostatic cells interconnected with the tumorigenic process? The three major processes affecting the prostate are prostatitis, BPH, and prostatic adenocarcinoma [25].

27.6.1 Prostatitis

Inflammation of the prostate, prostatitis, affects 2–10 % of men who are under the age of 50. The National Institutes of Health (NIH) classified prostatitis into four categories: (1) acute bacterial prostatitis, (2) chronic bacterial prostatitis, (3) non-bacterial prostatitis, and (4) prostatodynia. Acute and chronic bacterial prostatitis are rare occurrences affecting 2–5 % of men. They are caused by infection from bacteria such as *Escherichia coli* and *Serratia marcescens*. Local symptoms are urinary frequency and pelvic pain and systemic symptoms are fever and chills. Local and systemic symptoms are seen in acute prostatitis while intermittent local symptoms are observed in chronic prostatitis. Treatment normally involves anti-microbial agents and drainage of urine. The common form of prostatitis is category III which is chronic non-bacterial prostatitis. It affects 90–95 % of men. Infectious cause is not seen in non-bacterial prostatitis. The National Institutes of Health Chronic Prostatitis Symptom Index Questionnaire (NIH-CPSI) is used to assess the condition. There are two subcategories of non-bacterial prostatitis which are inflammatory (IIIA) and non-inflammatory (IIIB). Treatment involves the use of anti-inflammatory drugs, thermal therapies. However, specific direct treatment of the condition has not been resolved. Category IV or asymptomatic inflammatory prostatitis does not require any treatment. It is observed in some cases where the PSA levels are high but there is no correlation to prostate cancer [26–28].

27.6.2 Benign Prostatic Hyperplasia (BPH)

BPH and prostate cancer are observed with a high frequency in men of advanced ages. How does BPH differ from prostate cancer? BPH develops in the region of the prostate that is adjacent to the urethra. BPH nodules, unlike prostate cancer, arise in the transition epithelial zone of the prostate. BPH involves an increase in the size of the prostate from its normal size of 15–20 g to three times the size of approximately 33–52 g during autopsy and surgery, respectively [29]. BPH accounts for a large percentage of surgeries in the Western world due to improved screening procedures. The prevalence of BPH is low in men of approximately 30 years of age. However, the prevalence of BPH increases linearly with increasing age and is maximum in men who are in their seventh decade of their lives [29, 30]. There are two stages in BPH: microscopic and macroscopic. The microscopic stage is found to be present in almost all men of the world during their lifetime. This consists of the development of the BPH nodules. One-half of the microscopic nodules develop further resulting in a macroscopic enlargement of the prostate

now termed as the macroscopic stage. The macroscopic stage consists of the pathological phase and the clinical phase [30]. Detection of the clinical stage of BPH or dysuria involves surgical intervention. A couple of factors resulting in the development of BPH are a functioning testis providing a steady supply of testosterone coupled with a steady increase in age of the individual. The influence of hormones is very obvious since BPH fails to develop in castrated men [29, 30]. However, the changes that occur within the cells of the prostate also contribute to the development of BPH. There are three theories that have been proposed which may explain the etiology of BPH. The first theory deals with the influence of hormones for e.g. DHT. The DHT hypothesis states that a functioning testis, supplying androgens, is involved in the development of the prostate. With aging the androgen balance shifts resulting in an increase in androgen levels which may explain the hyperplastic growth of the prostate leading to BPH. The second theory involves the stem-cell-epithelial cell interaction. This was proposed by Reischauer in 1925 and restated by McNeal where they observed that BPH results from an initial glandular budding and branching of the stromal cells of the prostate evolving into alveoli consisting of epithelial cells [30]. In subsequent analyses on animal models, this induction of epithelial cells from stromal cells could be demonstrated. Cunha et al. have shown the induction of bladder epithelial cells to form prostate cells by mouse embryonic urogenital sinus mesenchyme which essentially are prostate stromal cells [22]. The induction is sensitive to androgens as castrated animals fail to show the stromal-epithelial induction. The third hypothesis is the stem-cell hypothesis. Stem cells are regenerative cells that can grow in conditions where the cell number is very low due to extreme physiological conditions or environmental influences. The prostate is a self-renewing tissue comprised of equal numbers of proliferating cells and cells undergoing apoptosis. This equilibrium in the tissue allows the prostate to maintain its size in an individual. Originating from the stem cells, the amplifying cells exhibit limited proliferation and are active in the regeneration of tissue within the prostate. The limited proliferation results in an amplification of the number of cells added to the regenerating prostate. The transit cells are a subset of the amplifying cells and have very limited clonal expansion. Unlike the amplifying cells, are dependent on the supply of androgens. The possible explanation for the development of BPH can be attributed to the clonal expansion of these stem cells which results in the overgrowth of the prostate. However, with androgen ablation, the transit cells which, make up majority of the prostate, die to cause involution of the prostate. This stem cell theory explains the regeneration of the prostate when androgen therapy is given to castrated men resulting in the prostate regaining its maximal size. Other factors also play a role in the development of BPH. The changes observed in BPH cells compared to normal are androgen metabolism and receptor differences, variable degrees of cytosine residue methylation and variations in levels of secretory proteins [30]. The incidence of BPH is similar to prostate cancer exhibiting racial differences with blacks showing a large frequency of the disease and Asians show the least [31].

Latent prostate cancers are most often observed during screening tests. Some of the latent cancers develop into malignant metastatic cancers. The prostate cancer progression pathway has been shown to consist of four predominant molecular chromosomal aberrations. The normal epithelium of the prostate cell develops into prostatic intraepithelial neoplasia (PIN) when the basal cells are lost. A candidate gene that is a cause of this transformation is NKX3.1, which is a result of loss of the locus 8p21. LOH is observed in *PTEN* and *Rb* which lie on loci 10q and 13q resulting in loss of the basal lamina. Invasive carcinoma is generated with the breach of the basal lamina. Eventually with the loss of 17p containing the candidate gene p53, the cancer cells metastasize to distant sites such as bone [25, 30, 32].

Understanding the molecular aspects of cancer is vital to understand the underlying pathways actively playing a role in tumorigenesis. The tumorigenic process results in the transformation of the cells in the prostate resulting in the following stages. Inflammation due to damage to the tissue results in damage to the cells and subsequently the genome. It also triggers the production of cytokines and other DNA repair mechanisms. Oxidative radicals such as hydrogen peroxide, singlet oxygen, superoxide, and nitric oxide are produced to counter the infectious agents. Their action affects the infectious agents as well as the bystander host cells. The damage to the host cells can result in genomic damage and mutations which may initiate the tumorigenic process. GSTP1 hypermethylation or the reduced effect of the DNA glycosylase/AP lyase-OGG1 reduces the ability of normal cells to counter the damage of oxidants [25]. Besides inflammation, other factors such as unequal levels of androgens and dietary factors can initiate the tumorigenic process.

27.6.3 Prostatic Inflammatory Atrophy (PIA)

DeMarzo in 1999 proposed the development of PIN from prostatic inflammatory atrophy or PIA through the atrophy of the luminal secretory cells with surrounding inflammation and is observed adjacent to cells that have undergone PIN and carcinoma. Such cells are found in the peripheral zone of the prostate and very rarely in the central zone of the prostate [23, 33]. The epithelial cells undergoing atrophy are associated with acute or chronic inflammation. Such cells undergo morphological changes to resemble prostatic intraepithelial

neoplasia (PIN) or may resemble minute carcinoma cells directly without a precursor lesion of PIN. Chromosome 8 is amplified in PIA cells and show other changes similar to PIN and cancer such as GSTP1 promoter methylation and p53 mutations. Certain cells that do not show inactivation of GSTP1 activity are able to counter the effects of oxidative damage [15, 33]. Cells undergoing PIA are proliferative, expressing AR and bcl-2 and rarely undergo apoptosis [23]. They also exhibit low levels of the cyclin-dependent inhibitor $p27^{Kip1}$ which may be responsible for the initiation and proliferation of cells [33]. High levels of COX-2 and glutathione S-transferase A1 (GSTA1) are observed in cells undergoing PIA [30, 32, 33]. PIA may give rise to PIN in some cases while in other cases this is not observed. Hence, PIA can be considered as a precursor or intermediate lesion in the tumorigenic process of the prostate.

27.6.4 Prostatic Intraepithelial Neoplasia (PIN)

When the luminal cells undergo proliferation with dysplasia along the ducts, it results in prostatic intraepithelial neoplasia or PIN. PIN has two forms, low-grade (LGPIN) and high grade (HGPIN). In 1986, McNeal and Bostwick proposed diagnostic criteria to detect PIN in the prostate which resulted in the three grade system [34]. However in 1989, the American Cancer Society and National Cancer Institute converted this into a two grade system where Grade 1 is LGPIN and Grades 2 and 3 together are HGPIN. In 1987, Bostwick and Brawer coined the term prostatic intraepithelial neoplasia. In general, HGPIN is considered to be a precursor of invasive carcinoma, but some studies have implicated the possible involvement of LGPIN as a precursor as well [16]. It is possible that the changes accumulating in LGPIN and HGPIN ultimately evolve into carcinoma despite the long latency period. LGPIN have enlarged nuclei, small nucleoli, normal chromatin content which may be slightly increased as well. The basal cell layer is intact in LGPIN. HGPIN on the other hand, have large uniform nuclei and nucleoli which are similar to carcinoma cells. The basal cell layer is disrupted in HGPIN and is absent in carcinoma [35]. A key marker protein to indicate the presence or absence of the basal cell layer is the anti-keratin stain 34βE12 through immunohistochemical analysis. Some of the marker proteins, amplified in the transition of HGPIN to carcinoma, are bcl-2, C-erbB-2, C-erbB-3 oncoproteins, racemase, c-Met protooncogene, telomerase, AMACR, and PSCA [35, 36]. Secretory marker proteins such as prostatic acid phosphatase (PAP) and PSA are downregulated with advanced PIN and carcinoma. Common allelic loss is observed in PIN and invasive carcinoma in loci such as 8p, 10q, and 16q. Epigenetic regulation of GSTP1 where the promoter is hypermethylated is observed in both PIN and carcinoma [37]. Digital rectal examination, TRUS, and PSA tests may be used to detect PIN. Repeat biopsies for cancer detection have reduced following a diagnosis of HGPIN. This is due to screening of younger males and as a result a reduction in tumor volume. Androgen deprivation therapy reduces the number of cases of PIN due to apoptosis in these cells [35].

27.6.5 Intraductal Prostatic Adenocarcinoma (IDC-P) and Invasive Carcinoma

Melicow and Pachter identified the differences between IDC-P and HGPIN. IDC-P is the next stage after HGPIN and coexists with adjacent invasive carcinoma [20, 38]. IDC-P is a late event in prostate cancer found adjacent to tumors with sizes greater than 4 cm^3. IDC-P comprises 0.4–0.8 % of prostatic adenocarcinomas and is characterized histologically by Gleason grade 3 and 4 cribriform carcinoma [20, 38–40]. This histology of the prostate is seen in the ducts and acini where the lumen is filled with malignant cells [41]. Endometrioid carcinoma is another term given to IDC-P due to the observation of ductal outgrowths near the verumontanum of the prostate resulting in an enlarged palpable prostate. Distant metastases were observed in the bone, brain, lymph nodes, and lungs [39]. Histologically, the tumor cells show pseudostratification with elongated nuclei and ample amounts of amphophilic cytoplasm. Other histologies include papillary form cells, glandular gland like cells with slit-like lumen spaces and central necrosis (comedonecrosis). Invasive carcinoma is classified as Gleason grade 5 where the cells lost the basal cell layer as compared to HGPIN and IDC-P. Invasive lesions contain tiny blood vessels and can be detected by immunostaining for basal cell-specific keratin. While IDC-P is restricted to the ducts and acini of the prostate, invasive carcinoma can metastasize [42]. The tumors involving IDC-P originated from the periphery of the prostate or secondary prostatic ducts [40]. Ultrastructurally, IDC-P cells reveal the following features of gland like cells with distinct basal lamina, microvilli in the lumen, large nuclei, and nucleoli. In addition, prostatic origin is confirmed in IDC-P cells due to the presence of prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA). There are a few variables that are strongly correlated with tumor volume. The volume of IDC-P tumor is strongly correlated with the extent of penetration of the capsule in terms of its thickness and width [42]. IDC-P tumors have much in common with invasive carcinomas in terms of genomic instability with higher frequencies of allelic imbalance (60 %) observed compared to HGPIN or BPH. Allelic imbalance in the form of loss of heterozygosity is seen in IDC-P and invasive carcinoma. LOH in certain loci are shared between IDC-P and invasive carcinoma while there are areas of LOH unique to each of them. If IDC-P evolves

into invasive carcinoma, the unique areas of LOH in IDC-P can only be explained as the result of selective clonal growth in IDC-P which gives rise to invasive carcinoma. The LOH observed in IDC-P indicates its correlation with a poor prognosis of prostate cancer. Gene amplification of *c-myc* on 8q24 and gene fusion of *TMPRSS2-ERG* are seen commonly in IDC-P as well as invasive carcinoma [38, 41]. McNeal et al. [42] observed that rather than being a precursor to invasive carcinoma, IDC-P was found as large foci within invasive carcinoma which was larger than 2–4 cm^3. When IDC-P arises within invasive carcinoma, it generates a malignant potential within the invasive tumor. IDC-P that develops as foci within invasive carcinoma can be distinguished based on its cribriform cells which still retain the basal cell layer [42]. IDC-P possesses two types of cells, tall pleomorphic, mitotically active cells staining poorly for prostate-specific antigen (PSA) and cuboidal, cells containing a secretory layer along with abundant cytoplasm expressing large amounts of PSA [41]. The treatment regimen for IDC-P is similar to that followed for hormone dependent prostate cancer. Transurethral resection, hormone ablation using diethylstilbestrol or orchiectomy, and radiation therapy are some of the techniques utilized [43].

27.6.6 Androgen-Independent Prostate Cancer

It is well known that hormone ablation treatment of prostate cancer initially causes the cancer to regress. Most patients are disease free for approximately 1.5–3 years before the cancer reappears with intense ferocity and gains the capability to proliferate independent of the supply of androgens. Androgens maintain the ratio of cells within the prostate where there is a balance between dividing proliferating cells and the cells undergoing death. On hormone deprivation, the cells undergoing apoptosis increases and the equal ratio of cells is affected leading to cancer cell death and the regression of prostate cancer. Charles Huggins and Clarence V. Hodges showed the effect of castration on acid phosphatase and alkaline phosphatase. Acid phosphatase levels decreased sharply on castration while alkaline phosphatase levels decreased gradually. Alkaline phosphatase is an enzyme found in bone and cartilage and is indicative of activity of cells outside the prostate gland. Acid phosphatase is found in the cells of the prostate and is an effective indicator of the growth of prostate cells [44].

Androgens comprise testosterone, dihydroepiandrosterone (DHEA), androstenediol, and androstenedione. Testosterone makes up 90 % of the androgens and freely diffuses into prostate cells. Testosterone which is synthesized in the testes is found in blood in the bound form where albumin or sex-hormone-binding-globulin binds the hormone. On entering prostate cells, the enzyme 5α-reductase (SRD5A2) converts testosterone to the active hormone, dihydrotestosterone (DHT), which binds to the androgen receptor (AR). This causes a conformational change in the receptor which then dissociates from the heat shock proteins and is translocated into the nucleus where it binds to androgen response elements in the promoter regions of genes to activate their transcription [45–47]. So what causes the cells in the prostate to get recharged after castration and to proliferate resulting in a more aggressive androgen-independent cancer? There have been many theories proposed on the causes of recurrence of prostate cancer. Five main theories are: (1) Hypersensitive pathway, (2) Outlaw pathway, (3) Promiscuous pathway, (4) Coactivators and Corepressors, and (5) Bypass pathway [45–47]. These theories deal with the androgen receptor and the role it plays in the development of the hormone refractory or androgen-independent cancer. In the hypersensitive pathway, the androgen receptor becomes extremely sensitive to very low amounts of androgens that may be present even after castration or medical blockade of androgens. The androgen receptor through mutations or overamplification or increased 5α-reductase enzyme levels may become sensitive to low levels of androgens. 30 % of androgen-independent cancers show amplification of androgen receptor in cells which may be a result of selective outgrowth following death of cells during castration [46, 48]. Increased 5α-reductase enzyme levels result from a polymorphism where there is a substitution of valine with leucine at codon 89. This is commonly observed in African men indicating the ethnic influence in prostate cancer [46, 47]. Androgen-independent prostate cancer, though independent of the supply of androgens tend to express the androgen receptor at high levels in most cases. The outlaw pathway is functional when the AR pathway is activated by growth factors and receptor tyrosine kinases. In these cases, growth factor pathways such as insulin growth factor (IGF), keratinocyte growth factor (KGF) can bind and activate the androgen receptor in the castrated state as they are overexpressed. Receptor tyrosine kinases such as HER-2/neu are overexpressed in androgen-independent cancers that results in the activation of the androgen receptor. IL-4 and IL-6 are also activators of the androgen receptor pathway in the hormone refractory state [45, 46]. The cross talk between NF-kappa B and AR have indicated the role of the NF-kappa B signaling pathway in the development of androgen-independent cancer. The promiscuous pathway results in the androgen receptor (AR) being receptive to ligands other than DHT. Non-androgenic steroids, anti-androgens can activate the AR due to missense and other mutations which may expand the ligand-binding specificity of the AR. LNCaP cells possess an AR where threonine is substituted with alanine at codon 877 which is sensitive to a wide range of steroid ligands. CWR22 cells also show a substitution of

histidine with tyrosine at codon 874 in the sequence that encodes AR. Mutations in the AR are observed in TRAMP mice in three regions: (1) the signature loop of the receptor, (2) the flanking region where p160 coactivators bind, and (3) the ligand-binding domain [46]. Coactivator levels are increased in androgen-independent cancers which enhances the sensitivity of the androgen receptor to various ligands other than androgens. Coactivators such as ARA70, ARA55, SRC-1, P/CAF, and GRIP1/TIF2 are overexpressed in prostate cancer [45, 46, 49]. Bypass pathway, as the name suggests, circumvents the AR pathway and utilizes other pathways in stimulating the prostate cells to proliferate in an androgen-independent environment. The activation of *bcl2* in PIN lesions can cause the cells to avoid utilizing the androgen receptor pathway. Similarly, the activation of oncogenes or inactivation of tumor suppressor genes may result in other bypass pathways. Neuroendocrine cells secrete neuropeptides such as bombesin which enhance the proliferative rate of cells in a cancerous environment in the absence of hormones [45, 46, 49]. Shi et al. [50] have examined the alterations involved in the transition of LNCaP cells into androgen-independent cells and have observed upregulation of growth factors, the Bcl-2 protein and the Akt pathway. The three sublines generated were representative of the theories proposed of development of androgen-independence [50]. Chen et al. [51] also performed similar analyses using LNCaP and the androgen-independent C4-2 cells and analyzed genes such as TMEFF2, NKX3.1, and AMACR to compare the gene expression differences. In addition, these genes were further analyzed in xenografts of these cells to observe consistency in expression. The study analyzed 51 candidate genes and compared the results to human prostate cancer tissues and showed a correlation. The study by Shi et al., as well as Chen et al., showed the difference in gene expression in androgen-dependent and androgen-independent prostate cancer [51].

Besides the androgen receptor, the microenvironment plays a role in the development of androgen-independent cells. Studies by Cunha et al. have shown the effect of the stroma on the epithelial cells of the prostate. Recombination of mice epithelial cells with inactive AR with stromal cells of mice containing functional AR showed that the mice epithelial cells developed normal prostatic cells yet the reverse combination failed to yield functional epithelial cells despite the addition of androgens. This indicated the importance of the stroma in prostate cell development [48]. The stromal cells can activate the epithelial cells through paracrine signals while the growth of prostate cells in an androgen-independent environment can also occur in an autocrine manner. This was observed in experiments where prostate cancer cells could proliferate in nude mice that possessed either wild type AR or where the AR was inactive. The proliferation of prostate cells in an AR-null environment indicated autocrine signaling of cells to activate pathways besides the AR signaling pathway to survive in a hormone refractory environment [48]. Akakura et al. [52] have demonstrated the effect of intermittent androgen therapy on androgen-dependent tumors where it was observed that androgen-dependent tumors transplanted in castrated mice showed a regression. When the tumors had regressed 30 %, they were transferred to intact mice. The tumors started to develop only to be transplanted back into castrated mice. This intermittent procedure was conducted to observe the rate at which the tumors became androgen independent. The time taken for the tumors to develop into androgen-independent tumors was threefold. Such intermittent therapy could delay the development of the aggressive tumor that is hormone refractory [52]. Research has been carried out on androgen-dependent prostate cancer cells to observe their growth into androgen-independent cells and to analyze the changes that occur with this transition. Hendriksen et al. [53] have analyzed the androgen receptor pathway in LNCaP cells as well as xenografts from intact and castrated mice to observe the genes that are upregulated or downregulated in the transition to androgen independence. It was observed that androgen receptor genes that were not stroma-associated were upregulated in primary prostate cancer with low levels of stromal cells. In low grade carcinoma, genes that were involved in cell growth, proliferation, and apoptosis were expressed to higher levels compared to high-grade carcinoma where differentiation is lowered. The genes that are upregulated in low and high-grade carcinomas were involved in metabolism, exocytosis, and protein folding. *SIM2* and *AMACR* were upregulated in prostate carcinoma. The study showed that stress response genes, *HERPUD1* and *STK39* were downregulated and were good indicators of metastasis [53].

27.7 Epigenetic Regulation in Prostate Cancer

The term epigenetics refers to the external modification of DNA bases which are inherited but do not cause a change in the DNA sequence. Epigenetic modifications vary from methylation of DNA to acetylation, phosphorylation, and methylation of histones [54]. DNA methylation occurs when a methyl group is added to the 5′ carbon of cytosine. Such modifications are generally observed in CpG islands that are 0.5–2 kb long and found in the promoter regions of genes. CpGs comprise 1 % of the genome. DNA methyltransferases or DNMTs are enzymes that catalyze the methylation of cytosines where S-adenosylmethionine (SAM) is the methyl donor for the reaction [54, 55]. In the cancer context, DNA methylation can be of two types, hypermethylation

and hypomethylation. Hypermethylation results in the silencing of genes by methylation of the promoters of genes. Thus, loss of heterozygosity (LOH) observed in many cancers may be the result of methylation of an allele resulting in the loss. Hypomethylation on the other hand results when there is a loss of methylation leading to activation of genes. Genes such as *GSTP1* and *14-3-3* are methylated and silenced in prostate cancer [55]. Yegnasubramanian et al. [56] analyzed the hypermethylation patterns in 73 primary and 91 metastatic human prostate cancer and compared the changes in methylation observed in the progression of cancer. They identified a unique methylation pattern involving the genes *GSTP1*, *APC*, *PTGS2*, *MDR1*, and *RASSF1a*. Hypermethylation of the CpG islands at the promoter region of the above genes was found at a high frequency in prostate cancer and was a distinguishing characteristic from other cancers. Recurrence in prostate cancer was correlated independently with high Gleason grade, pathological stage as well as methylation of the *PTGS2* gene which encodes a cyclooxygenase gene that converts arachidonic acid to prostaglandins which are involved in inflammation. The methylation pattern did not differ between primary and metastatic carcinomas which indicated that methylation arose early in the progression of prostate cancer. There was a striking difference in the number and frequency of genes that were methylated between BPH and primary prostate cancers [56]. Lodygin et al. [57] in a similar study identified genes that were silenced in prostate cancer and analyzed 50 genes that were induced by treating prostate cancer cell lines (LNCaP, PC3, DU145) with the demethylating and inhibition of deacetylase agents 5-aza-2′deoxycytidine and trichostatin A (TSA). These genes were selected based on their functions and can be considered as potential candidates for therapy in prostate cancer. *SFRP1* and *DKK3* were genes that downregulated the Wnt pathway, *p57/KIP2* regulates the cell cycle negatively, Glutathione peroxidase 3 (*GPX3*) protects cells against oxidative stress. These genes were shown to be methylated in prostate cancer which explains the release of the prostate cells from regulating factors and the development of dedifferentiation [57]. Histone modifications such as acetylation, methylation, ubiquitinylation regulate the transcriptional activities of genes. Histone acetyltransferases (HAT) catalyze the transfer of acetyl groups to histones. The androgen receptor is regulated by p300, PCAF that are histone acetyltransferases and upregulate AR gene expression. Similarly, histone deacetylation of the vitamin D receptor is observed in prostate cancer cells which inhibit the anti-proliferative activity of the receptor. Deacetylation is promoted by the histone deacetylase enzyme (HDAC). Histones are additionally modified by methylation of lysine and arginine residues. Global hypomethylation which involves a reduction in the overall CpG methylation content of the genome is observed in benign as well as malignant prostate cancer. Gene-specific hypomethylation in prostate cancer is observed in genes such as *PLAU* which encodes a tumor invasive gene urokinase plasminogen activator and heparanase [54, 55]. Epigenetic mechanisms contribute to the process of tumorigenesis in prostate cancer along with mutations and are also involved in the progression to androgen-independence. As in the study by Murillo et al. [58], it was observed that methylation of specific genes can be observed in androgen refractory prostate cancer cell lines. LNCaP and its derivative androgen-independent cell lines, Rf and RfL were analyzed for genetic changes as well as epigenetic alterations. The locus 6q24-q26 was studied since it is known to have high allelic loss in cancer. The gene PLAG1 was identified in this locus and was seen to be downregulated in the androgen refractory cell lines. On further analysis, this downregulation and loss of one allele of the 6q locus was attributed to methylation [58]. Treatment of epigenetic modifications is universal irrespective of the cancer. Methyltransferase inhibitors such as 5-aza-2′deoxycytidine and decitabine (DAC) restore expression of genes such as GSTP1 and histone deacetylase inhibitors such as MS-275, SAHA can be used to reverse the deacetylation of genes resulting in their activation. These treatments are effective for short periods of time and do not completely shrink the tumors. Combination therapies involving demethylase and HDAC inhibitors are used [54, 55].

27.8 Genome-Wide Allelotyping of Prostate Cancer

Allelotyping of the genome with the help of techniques such as loss of heterozygosity (LOH), microsatellite instability (MSI), fluorescence *in situ* hybridization (FISH), spectral karyotyping (SKY), and comparative genomic hybridization (CGH), informs us of the regions within the genome that exhibit loss, amplifications, or duplications. Most often, regions of loss within the genome are interpreted to harbor tumor suppressor genes that function as the caretakers of the genome. Loss of a tumor suppressor initiates changes within the genome that may trigger the tumorigenic pathway. Many laboratories have attempted to analyze the prostate cancer genome utilizing the above mentioned techniques. The difficulty in procuring prostate cancer samples hampers the large scale analysis of the genome. Allelotyping the different stages of cancer gives an indication of the genomic changes that occur in the process of developing a tumor or in other words elucidate the tumor progression pathway.

Allelic loss in prostate adenocarcinoma has been reported predominantly in chromosomal arms, 8p, 10q, and 16q [59–62]. Kunimi et al. [62] showed 50 % loss on chromosome 8, 60 % on chromosome 16, and 30–55 % on the short and long

arms of chromosome 10, respectively, in 18 prostate adenocarcinoma samples. Similarly, Carter et al. [60] showed in 28 prostate cancer samples, approximately 30 % loss on chromosomal arms 10q and 16q. Other chromosomal arms were lost at a lower frequency for e.g. gains in the long arms of chromosomes 7 and X. Gains were observed on chromosomes 2p, 3p, 12p and 12q, 13q, 17q, 8q, 9q, 16p, 20, and 22 [61]. Losses have been recorded at 18q which is lost only if another allele is lost indicating a minimal role in the initiation of cancer. This high frequency of 18q loss was reported by Joos et al. and Kunimi et al. [61, 62]. Tumor histological grade is directly correlated to the frequency of allelic loss. The greater the number of losses, the higher the tumor grade [62]. The number of samples analyzed by the above researchers was small ranging from 10 to 28 samples. However, the results were obtained by techniques such as LOH and CGH. The use of different techniques to arrive at the same result shows that the loss is real on the chromosomes. In addition to identifying the allelic loss patterns, it was also essential to narrow down the specific regions that were lost within the loci.

The Kunimi study showed ~50 % loss of the 8p locus [62]. High density mapping of the 8p locus revealed losses in the 8p22 region where the MSR gene resides. However, the losses were not restricted to 8p22 alone. There was heterogeneity in the region of loss on the 8p arm with some tumors showing complete loss of the 8p arm or intermittent losses either distal or proximal to the 8p12-8p21 region [63]. Similar LOH mapping was carried out for chromosomes 8, 10, and 16 in another study by [59]. Allelic loss was observed across both the arms of chromosome 10 using ten markers spread across the entire chromosome. Similarly nine markers used for chromosome 16 were localized to the long arm and confirmed the region proximal to the centromere to be frequently lost [59]. A comparative CGH analysis carried out on ten prostate adenocarcinoma cases confirmed the LOH on 8p, 10q, and 16q. Gains were observed on 7q the X chromosome, 8q, 9q, and 16p. LOH analysis did not detect the gains on 8q, 9q, and 16p. Loss of chromosome 12p was reported only in the CGH data [61]. MSI allelotyping of 100 prostate adenocarcinoma samples provided clues to the presence of three genetic progression pathways that may operate in the development of prostate cancer. The first pathway results when there is a loss of 8p and 13q and these may be early events since the losses were recorded in >70 % of the cases. An alternative pathway involves the loss of 7q, 17p, and 18q which also eventually acquire losses in 8p and 13q. This accounts for 13 % of the prostate cancer cases. A third pathway is observed in advanced states of prostate cancer where losses are observed on 5q, 9p, and 17p. Loss of 5q is predominantly associated with lymph node metastasis of prostate cancer. These observations were obtained from pure tumor samples which had been procured through the highly specific laser capture microdissection technique [64].

27.9 Candidate Genes in Hereditary and Sporadic Prostate Cancer

Genes implicated in hereditary prostate cancer differ from those affecting sporadic cancer. In hereditary cancer, based on linkage analysis studies, loci implicated in disease progression have been analyzed to identify candidate genes. Some of the genes identified in hereditary cancer are HPC1, HPCX, PCaP, HPC2, and CaPB.

27.9.1 HPC1

Hereditary prostate cancer gene 1 (HPC1) was identified through linkage analysis of hereditary prostate cancer families. The region 1q24–25 was identified as a candidate region in families with high risk of hereditary prostate cancer. *RNASEL* is a gene that has been mapped to the *HPC1* locus. RNASEL contains somatic mutations in hereditary prostate cancer and is regulates antiviral and anti-apoptotic effects in the cells which are induced by interferons [65].

27.9.2 HPCX

HPCX is found on the X chromosome indicating sex chromosome linkage in hereditary prostate cancer [14]. *HPCX* is found in the region between Xq27–28 which spans approximately 10 Mb. Evidence of association between HPC1 and HPCX has been observed in some hereditary prostate cases though this is not observed in all the cases.

PCAP: PCAP was detected at locus 1q42–43 through combined fine mapping and a genome screening technique [14]. It is observed in hereditary families with early onset of the disease [65].

27.9.3 HPC2/ELAC

HPC2 is found at the locus 17p11. Truncating mutations such as missense and presence of a premature stop codon were found at this locus in high-risk hereditary prostate cancer families. These mutations modify the risk to prostate cancer. HPC2 encodes a hydrolase which acts like DNA repair enzymes [65].

27.9.4 CaPB

CaPB has been reported to be linked to the locus at 1p36. There is a linkage between this locus and brain cancer since families with no history of brain cancer that have prostate cancer do not show this locus to be affected [14].

Genome-wide allelotyping of sporadic prostate cancer has identified regions that are consistently lost in disease progression. The genes of interest that lie within these regions have been identified and analyzed further for their relevance to sporadic cancer development.

27.9.5 NKX3.1

The 8p21 locus showed nearly 50 % allelic loss in prostate cancer. *NKX3.1* is a candidate prostate-specific homeobox gene at this locus and maps within the critical region of 8p12–21 [9, 65, 66]. The protein is expressed largely in the prostate and expression is lost with the progression to prostate cancer. This indicates that NKX3.1 acts as a tumor suppressor gene. Somatic mutations have not been observed in this gene despite allelic loss and so its role in sporadic prostate cancer is debated though the presence of loss and haploinsufficiency may be the cause of disease progression. Haploinsufficiency of NKX3.1 in mice gives rise to lesions similar to PIN in humans [9, 65]. NKX3.1 is a transcription factor and activates as well as represses genes [65]. PSA is repressed by NKX3.1 by binding to the promoter region of the DNA (Nelson WG). Although protein expression is lost in cancer, NKX3.1 mRNA is still expressed [32, 65]. NKX3.1 expression has been found to be ~20 % in PIN, 6 % in low stage, 22 % in high stage prostate cancers, 34 % in hormone refractory cancer and 78 % in prostate metastases [33, 65].

27.9.6 PTEN

Allelic loss at 10q24 has been observed in prostate cancer. *PTEN* (phosphatase and tensin homologue) was discovered in gliomas which showed the same allelic loss and is considered a candidate tumor suppressor gene. PTEN encodes a lipid phosphatase that negatively regulates phosphoinositol-3-kinase (PI3K/Akt) signaling and cleaves 3-phosphorylated phosphoinositides [32, 33, 65]. The PI3K/Akt pathway regulates cell cycle and apoptosis by phosphorylation of p27 and BAD, respectively. Thus, PTEN is considered to regulate cell proliferation and cell death [65]. Similar to NKX3.1, somatic mutations have not been observed in PTEN. However, haploinsufficient PTEN appears to be associated with later stages of prostate cancer. These studies have been carried out in mice which have led to the development of prostatic hyperplasia and dysplasia lesions [9, 65]. Haploinsufficiency observed in PTEN is not observed universally in all the prostatic cells though its importance in prostate cancer progression cannot be underscored with observations of decreased expression in primary tumors and xenografts [9]. Studies in mice have also shown that when NKX3.1 and PTEN are haploinsufficient, prostatic hyperplasia arises. PTEN also modifies the androgen receptor pathway by affecting the PI3K/AKT signaling pathway. This interaction will be described in the section on the androgen receptor signaling pathway.

27.9.7 CDKN1B

A cyclin-dependent kinase inhibitor, CDKN1B/p27^{kip1}, is downregulated frequently in prostate cancers [9, 32]. p27^{kip1} maps to the 12p12–13 locus. Loss has been observed at this locus although p27^{kip1} itself is not mutated. Phosphorylation or ubiquitinylation alters the localization of p27^{kip1} [9]. PI3K/AKT signaling pathway negatively regulates the levels of p27^{kip1}. PTEN which negatively regulates the PI3K/AKT pathway increases the levels of p27^{kip1}. Mice, where p27 has been targeted for disruption develop prostatic hyperplasia while those that have heterozygous alleles of PTEN and loss of both alleles of p27 develop prostate cancer [25, 32]. p27 loss is correlated to tumor grade with no loss observed in BPH and maximum loss observed in prostate cancer [9].

27.9.8 Rb

Allelic loss has been reported on 13q, a locus that contains the retinoblastoma gene (*Rb*). Mutations as well as loss of protein expression have been detected in localized and metastatic prostate cancer. *Rb* regulates apoptosis of prostate cells in the presence of androgen. Tissue recombination studies have shown that loss of Rb can cause dysplasia and invasive carcinoma in the prostate yet many studies have failed to show the similar association [9].

27.9.9 GSTP1

Hypermethylation of glutathione S-transferase (GSTP1) is a common event observed in sporadic prostate cancer. GSTP1 protein is observed in basal epithelial cells and in cells that have undergone prostatic inflammatory atrophy (PIA). However, absence of the GSTP1 protein is observed in cells that have undergone prostatic intraepithelial neoplasia (PIN). This loss of protein expression is attributed to hypermethylation of the promoter sequence of GSTP1. GSTP1 is a detoxifying enzyme that protects epithelial cells from genomic damage by carcinogens [25]. GSTP1 is generally not expressed in the luminal secretory cells. The role of GSTP1 in cancer is that of a caretaker gene rather than a tumor suppressor gene. GSTP1 may be required to detoxify PhIP-DNA adducts which form during the consumption of red meat or oxidating radicals-DNA adducts that form during oxidative stress [32].

27.9.10 AMACR

α-Methylacyl-CoA racemase (AMACR) is a mitochondrial and peroxisomal enzyme and influences metabolism of substrates by β-oxidation. The expression of the enzyme is increased with increasing grade of prostate cancer with normal prostate cells showing low frequency of enzyme expression. It has been found to be overexpressed in all prostate cancers [25].

27.9.11 AR

The androgen receptor found on the X chromosome is subjected to mutations or changes in protein expression in prostate cancer. Changes in the androgen receptor gene sequence affect the signaling pathway and other interconnecting pathways. More details will be given on the androgen receptor in the section on androgen receptor signaling.

27.10 Signaling Pathways in Prostate Cancer

Signaling pathways are activated in the process of development of prostate cancer. In prostate cancer, the signaling pathways are interconnected and ligands affect more than the immediate downstream target in their respective signaling pathway. This can be observed in the NF-kappaB and Stat3 pathway or the PI3K/AKT and AR pathway.

27.10.1 Androgen Receptor Signaling

The androgen receptor is a transcription factor, a 110 kDa phosphoprotein, and plays an important role in the development of the prostate. The AR belongs to the steroid hormone receptor superfamily [45, 49]. The androgen receptor mediates the activity of androgens such as testosterone and dihydrotestosterone (DHT) which bind to the androgen receptor elements in the promoter sequences of genes responsive to androgens and influence their transcription [45, 49, 67, 68]. The androgen receptor signaling pathway is essential for the development of prostate cancer and its influence can be observed in prostatic hyperplasia and prostatic adenocarcinoma [67–69]. The androgen receptor maintains the homeostasis in the prostate. Lowered androgen receptor activity results in cell death while increased activity induces the proliferative capacity of the prostate cells [70]. Despite androgen ablation therapy, an active androgen receptor signaling pathway is observed in hormone refractory prostate cancers. The cause for the activation of this receptor pathway is due to myriad reasons. To understand the full potential of the androgen receptor signaling pathway, it is necessary to start at the basic level of the androgen receptor structure.

The androgen receptor is found on the X chromosome between Xq11 and Xq12. It comprises 8 exons and is 110 kDa in size. Exon 1 is the largest and encodes the amino-terminal domain (NTD), exons 2 and 3 encode the DNA-binding domain (DBD), part of exon 4 encodes the hinge region while the remaining part of exon 4 to exon 8 encode the ligand-binding domain (LBD) [68, 71]. The NTD contains the polymorphic trinucleotide repeat sequences, CAG and GGC, which are 6–39 and 7–20 repeats, respectively and encode polyglutamine and polyglycine, respectively. Ethnic differences observed in repeat sequences are linked to the incidence of prostate cancer. African-Americans have shorter CAG repeat sequences, Caucasians have intermediate repeat length sequences and Asians have the longest CAG repeat sequences. The highest incidence of prostate cancer is seen in African-Americans compared to Asians and Caucasians. CAG repeat length of greater than 40 results in neuromuscular atrophy termed as Kennedy's disease [12, 68, 70, 71]. The correlation between the CAG repeats and the incidence of prostate cancer risk is fluid with few studies indicating a positive correlation while others have shown a negative correlation [12, 71]. Contrary to the distribution of the CAG repeats, African-Americans show the least frequency of the 16-GGC repeats while Asians show the highest. It may be assumed that the GGC repeats play a role in preventing the development of prostate cancer. In prostate cancer, an association of short CAG repeats and low frequency of GGC repeats have been found [71]. Besides the repeat sequences, two forms of polymorphisms are associated with the risk of prostate cancer. The R726L polymorphism, observed at codon 726 in the hinge region, occurs in linkage disequilibrium with the long CAG repeats in prostate cancer patients. Similarly, a single nucleotide polymorphism results in either an allele that can be digested by the Stu1 restriction enzyme or an allele that cannot. All these polymorphisms give rise to male baldness which is an initiator of prostate cancer.

The AR when translated is complexed with heat shock proteins to form a heterocomplex. Hsp40, Hsp70, Hsp90, and co-chaperones p23 and Hop bind to the AR when it is not bound to any ligand and stabilize the receptor protein. This multiprotein complex enables the androgen receptor to bind androgens. The binding of androgens releases the heat shock protein complex from the androgen receptor and causes the translocation of the androgen receptor into the nucleus. Within the nucleus, the androgen receptor dimerizes and binds DNA at sequences containing the androgen response elements. This assembly recruits additional transcription factors and coactivators or corepressors to regulate gene expression. Following the dissociation of the ligand, AR is shuttled back to the cytoplasm where heat shock proteins bind to form the multiprotein complex [68, 71].

27.10.2 AR Gene Alterations

Prostate cancer initially develops in the presence of androgens and a functioning androgen receptor signaling pathway. Hormone ablation therapy, in the form of surgical or medical castration, results in remission of prostate cancer. However, this sets a stage for the development of mutations and adaptive changes to the androgen receptor. AR gene amplification, mutations in the androgen receptor have been observed as adaptations to hormone deprivation. Approximately 25–30 % of prostate cancer patients show overamplification of the androgen receptor following hormone deprivation. Overamplification of the receptor enables it to be responsive to extremely low levels of androgens found in conditions of androgen deprivation. Overamplification develops only in response to the hormone ablation therapy as patient samples that showed this phenotype did not show the same prior to the treatment. Such patients showed a better response to hormone treatment, a longer survival rate, increased response to second-line hormone therapies [67, 68]. AR gene overexpression may result either due to amplification of the gene, increased gene transcription, enhanced stability of the transcript or protein [45].

AR gene mutations cause the AR to undergo changes that improve the transactivation of the AR and enhance its activity in the presence of coactivators [67]. The gene mutations cause androgen-insensitivity syndrome (AIS) which downregulates the development of internal and external male organs [45]. The first AR gene mutation was studied in the LNCaP cell line where it was found the cell line became responsive to anti-androgens. The mutation T877A causes a change in threonine to alanine in the androgen receptor and broadens the ligand specificity of the receptor [67]. AR point mutations occur in the range of 20–40 % in the male population [68]. Point mutations rarely arise in the receptor in primary prostate cancers but occur with increasing frequency in patients undergoing hormone ablation therapy. AR mutations are observed in three regions of the receptor spanning approximately 15 % of the receptor [45, 71]. The mutations are observed in the ligand-binding domain (LBD), the DNA-binding domain (DBD), and amino-terminal transactivation domain. In the LBD, the mutations are observed in a 20 amino acid signature sequence which is required for ligand specificity. Mutations are also observed in the boundary of the hinge region with the LBD and the AF-2 binding site for p160 cofactors. These changes in the AR sequence affect the transactivation of the AR and its specificity to ligands. Mutations have been observed at the carboxy terminal of the DBD which affect transactivation and transrepression of the AR and also it's DNA-binding capability. Signaling pathways are activated or repressed due to the broader specificity of AR with mutations for ligands [71]. Amino-terminal transactivation domain mutants are observed in the NTD of the receptor. Mutations are observed at the region of the polyglutamine tract or at a region amino terminal to the DBD which affects the transactivation of the receptor either in a ligand-dependent or ligand-independent manner. The carboxy terminal region of the NTD is also subjected to mutations which affect the receptor activation [71].

The p160 and p300/CBP coactivators interact with the NTD at a glutamine rich region and are affected by mutations in the region. Coactivators can increase transactivation of variant AR and thus raise the frequency of incidence of prostate cancer. In the presence of coactivators such as ARA70 and ARA160, the activity of agonists such as 17β-estradiol and antagonists such as hydroxyflutamide are enhanced. P160 coactivators can enhance the transactivation activity of AR in the presence of unconventional ligands such as estradiol and progesterone. Similarly, the coactivator Tat interactive protein 60 kDa (Tip60) is found to be increased in expression in hormone refractory prostate cancer [67, 71].

Mutations in the NTD of AR are more common in prostate cancer that has developed after androgen ablation when compared to mutations of the C-terminal which are more common in prostate cancer before hormone deprivation [69].

27.10.3 Signaling Pathways Interacting with AR

There are a number of growth factors and coactivators that interact with the AR and influence the AR signaling pathway in androgen-independent prostate cancer. They are growth factors such as epidermal growth factor (EGF), interleukin-4 and interleukin-6, cell cycle proteins, Ras-the Raf-MAPK pathway, and the PI(3)K/Akt pathway [68, 70, 72].

27.10.3.1 Growth Factors

Epidermal growth factor (EGF), insulin growth factor (IGF), and keratinocyte growth factor (KGF) activate AR. The EGF receptor family member ErbB2/Her-2/neu has a tyrosine kinase activity which is active in the absence of ligand. It activates AR and causes an increase in PSA expression even in the absence of ligand. The expression of Her2 is linked to the activation of MAP kinase and PI3K/Akt pathways which are active in the hormone refractory state of prostate cancer. Such cells have an added growth advantage [68, 70, 71].

27.10.3.2 Interleukin-4 and Interleukin-6

The interleukins are cytokines which increase the expression of AR in the androgen refractory state. PSA expression is increased in the presence of IL-4 while IL-6 activates the STAT3-AR interactions. STAT3 interacts with AR at a specific region on the AR and disruption of this direct interaction affects the activity of AR [68, 70]. IL-6 causes a signal transduction effector in the IL6 receptor, gp130, to homodimerize

resulting in the activation of the Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. STAT3 is phosphorylated by JAK. IL-6 converts an androgen-dependent prostate cell into an androgen-independent cell by enhancing androgen receptor activity as well as the STAT3 and MAPK signaling pathways [73]. IL-6 prevents the apoptosis in prostate cells which is mediated by the STAT3 pathway [74]. The PI3K/AKT pathway is not affected by IL-6 overexpression. PSA expression is increased in the presence of IL-6 overexpression [73, 74]. A recent publication has shown that IL-6 mediates its growth stimulatory effect on androgen-deprived prostate cancer cells by acting in an autocrine manner. Studies carried out on LNCaP cells which are androgen-dependent show inhibition of growth in the presence of IL-6 with reduced AR expression while prolonged exposure to IL-6 causes the LNCaP cells to develop into androgen-independent cells. This shows two phases of activation of IL-6 on prostate cells, a paracrine effect in the initial stage and an autocrine effect in the androgen-independent growth of prostate cancer cells. It is at this stage that the AR signaling pathway is activated [73, 74].

27.10.3.3 Cell Cycle Proteins

AR is either activated or repressed by different cell cycle proteins. Cyclin D1 represses AR transactivation and P/CAF competes with this interaction and prevents the inhibition of AR. Cyclin E is an activator of AR and is not inhibited by any other protein and may be responsible for the androgen-independent growth of prostate cancer cells. pRB also activates AR but in an androgen-dependent manner. The corepressors are c-Myc, cyclin-dependent kinases (CDKs), Bcl-2, NK-kB, and others [70].

27.10.3.4 Ras-Raf-MAPK Pathway

Guanine nucleotide proteins such as Ras are activated during growth factor mediated signaling. Cell cycle proteins are activated by phosphorylation by the Ras-Raf-MAPK pathway. This pathway plays a role in the progression of cells from Go/G1 to the S phase. Overexpression of mutated Ras leads to proliferation of cells. This pathway is activated in both hormone-sensitive and hormone-deprived prostate cells.

27.10.3.5 Protein Kinases

Protein kinases A, B, and C activate AR and are induced by growth factors mentioned above.

27.10.3.6 Calcium and Calmodulin

Calmodulin binds to AR in the presence of calcium and stabilizes it. Calmodulin activates a number of kinases including Akt which interact with AR and form a complex and influences the stability.

27.10.3.7 Proteasome

The 26S proteasome ubiquitinates AR and the proteins that form a complex with AR cause timely degradation of the complex and enable cell cycle progression. The proteasome pathway activates NF-kB which leads to AR transcription and translation into protein [68, 70, 72].

27.10.4 NF-Kappa B Signaling

Nuclear factor-kappa B (NF-kappa B) signaling consists of the canonical and the non-canonical pathways. NF-kappa B is a complex of proteins that are present as dimers. These proteins fall in two classes. The first class consists of RELA (p65), RELB, and c-REL, and the second class consists of NF-kB1 (p105) and NF-kB2 (p100). The REL proteins dimerize with the second class to activate the expression of genes. p105 is a precursor protein which is proteolytically processed into the mature protein p50 while p100 is processed into p52. The canonical pathway consisting of dimers containing RELA, NF-kappa B1/p50, and c-REL are present in the inactive form in the cytoplasm by the inhibitors of kappa B proteins (IkB). IkB proteins are phosphorylated by the IkB kinase (IKK) complex specifically IKKβ that inactivates IkB through ubiquitinylated degradation. This releases the NF-kappa B complex which translocate to the nucleus and activates targets of inflammation, cell proliferation, anti-apoptosis, and negative regulators of NF-kappa B proteins. The second non-canonical pathway consists of RELB and NF-kappa B2/p52. Members of the tumor-necrosis factor (TNF) cytokine family activate IKKα along with a second protein kinase NIK which in turn induces the proteolytic processing of NF-kappa B2 resulting in the translocation of RELB/p52 dimers to the nucleus [75, 76].

At which stage is NF-kappa B activated in the tumorigenic process of the prostate? NF-kappa B is expressed variably in different stages and is present in different locations based on the tumor stage of the prostate. Domingo-Domenech et al. [77] have shown in their study that NF-kappa B is localized in the cytoplasm in luminal cells and in the nucleus in basal cells. Cytoplasmic NF-kappa B is observed in PIN lesions while cytoplasmic as well as nuclear NF-kappa B staining is seen in prostatic adenocarcinoma. It was also observed in the analysis of patients that nuclear NF-kappa B could be considered as an indicator of biochemical relapse. The risk of biochemical recurrence increased when nuclear NF-kappa B was present along with high PSA levels and high Gleason score [77]. Sweeney et al. showed the similar result of increased NF-kappa B staining in advanced stages of prostate cancer compared to the benign state. Increased NF-kappa B expression was seen in hormone-independent cell lines [78].

Chen and Sawyers [98] have shown the role of the canonical pathway, RELB/p50 dimers, in activating PSA by binding to four sites in the enhancer region of the PSA gene. AR also binds to the enhancer region of PSA indicating a cross talk between the NF-kappa B and the AR pathways. Studies in the androgen-independent cell line, LAPC-4, have shown that NF-kappa B to be present in high levels and the activation is observed in the nucleus with lower levels of IKKβ. This provides a role for NF-kappa B in androgen-independent tumors [51]. In another study by Catz [79], the activation of *bcl-2* expression by NF-kappa B has been shown. NF-kappa B binds to two consensus site on the bcl-2 promoter sequence p2. TNF-α regulates the activation of *bcl-2* by NF-kappa B. *bcl-2* expression is reduced in the presence of hormones which is indicative of its role in hormone-independent prostate cancer [79]. Suyun Huang et al. have shown that blocking the NF-kappa B pathway in prostate cancer cells inhibits the expression of proteins related to the angiogenic, invasive, and metastatic pathways. The androgen-independent prostate cancer cell line PC-3 was utilized in this study where stable cell lines transfected with control vector or with a dominant negative mutant form of IkBα (IkBαM) were generated. Though the cell viability is not affected, the expression of angiogenic factor, VEGF, invasion protein, IL-8, metastasis protein, MMP9 was reduced at the transcriptional and translational levels. The vascularization of cells transfected with IkBαM was significantly lowered compared to the normal cells and the cells transfected with the control vector indicating the effect of downregulating the NF-kappa B pathway [80]. A similar result was shown by Andela [81] by suggesting a role for NF-kappa B in prostate cancer metastasizing to the bone. In this study, the expression levels of MMP9 and IL-6 were markedly reduced when PC-3 cells were transfected with a mutant form of IkBα. The role of NF-kappa B is reinforced in metastasis and bone resorption through the expression differences in MMP9 and IL-6. The study also showed a subsequent increase in the anti-metastatic factor, TIMP1, in the stable cell line transfected with the mutated IkBα [81].

Our laboratory has shown the processing of NF-kappa B2/p100 is mediated by Stat3 and involves the acetylation of cyclic AMP-response element binding protein (CBP/p300). Stat3 when activated involves the processing of p100 to p52. Activation of Stat3 occurs through acetylation mediated by CBP/p300. Active Stat3 was shown to mediate the anti-apoptotic activity of p52. This interaction of Stat3 with the noncanonical NF-kappa B pathway regulated anti-apoptosis [82]. Stat3 regulation of NF-kappa B is mediated by LIGHT. LIGHT induces phosphorylation of Stat3 with the help of NIK which is phosphorylated. Only in the presence of NIK, can LIGHT mediate Stat3 phosphorylation and this phosphorylation increases with increase in LIGHT dosage. These results indicate the NF-kappa B and the Stat3 pathways are interlinked in prostate cancer [83].

27.10.5 STAT3 Signaling

Signal Transducers and Activators of Transcription (STAT) are a group of seven genes named as STATs 1, 2, 3, 4, 5A, 5B, and 6 [84]. The STAT signaling cascade originates through growth factors and cytokines in the plasma membrane of the cell resulting in STAT proteins being phosphorylated in the cytoplasm, at the tyrosine and serine residues, dimerizing of the phosphorylated STAT proteins and its subsequent translocation to the nucleus [84, 85]. Members of the Janus Kinase family (JAK) such as JAK1, JAK2, and Tyk2 or other kinases such as Src and BCR-Abl kinases phosphorylate the STAT proteins. The STAT signaling controls cell differentiation, development, proliferation among other functions [84]. There is a significant role played by the STAT signaling pathway in prostate cancer when it is upregulated and is involved in cross talk with other signaling pathways such as NF-kappa B pathway. Huang et al. [85] have shown the effect of constitutively active STAT3 on prostate cancer progression. It was observed that benign prostate epithelial cells that expressed constitutively active STAT3 did not require JAK kinases to phosphorylate the STAT3 proteins. However, inhibition of the STAT3 pathway in the STAT3 stable cell lines compromised the cell viability as these cells showed a significantly higher growth rate in unsupplemented medium. In addition these cells gained androgen insensitivity indicating self-sufficiency in the presence of STAT3 signaling [85]. In a similar study by Tam et al. [86], hormone sensitive tumor samples were compared to those that were hormone insensitive for the levels of IL-6 and phosphorylated STAT3. Immunohistochemical staining was performed for IL-6 and phosphorylated STAT3 to identify the risk of obtaining prostate cancer. Increased IL-6 was associated with hormone insensitive patients and also indicated shorter time to relapse. Increased phosphorylated STAT3 was observed in hormone insensitive patients and indicated a greater survival rate [86]. IL-6 activation of STAT3 was enhanced by AR in the presence of DHT. DHT could influence the activation of STAT3 in the presence of IL-6 which was inhibited by flutamide, the anti-androgen. Conversely, inactive STAT3 inhibited AR activation by IL-6 in the presence of DHT [87]. Azare et al. [88] showed the effect of fibronectin in the transition of epithelial to mesenchymal phenotype in prostate cells which contain constitutively active STAT3. The cell motility is enhanced in the presence of integrin β6 and fibronectin with a reduction in the expression of E-cadherin. The significance of this finding however could not be observed in the immunohistochemical staining of human prostate tissues [88]. EGF signaling activates STAT3 signaling in prostate cells and enhances migration and invasiveness of cells [89]. EGF can enhance AR activation by increasing STAT3 levels. STAT3 levels are increased in the presence of IL-6 which increases the interactions between STAT3 and AR. The increase in EGF signaling causes AR signaling to be dependent on STAT3 [90].

27.10.6 PI3-K/Akt Signaling

Akt or protein kinase B is a serine/threonine kinase family of proteins and is a proto-oncogene. Akt1, Akt2, and Akt3 are members of the Akt family. It derives its name protein kinase B from its homology in the catalytic domain of the protein structure to protein kinase A and C. It plays a role in cancer through the phosphatidylinositol 3-kinase PI3-K/Akt pathway. The Akt protein structure consists of an N-terminal pleckstrin homology (PH) domain and a C-terminal regulatory domain. Akt is located in the cytoplasm in its inactive state. On activation by ligands such as growth factors, cytokines, T-cell receptors, PI3-K, a lipid kinase, is activated by receptors, such as tyrosine kinase receptors. This results in second messengers including phosphatidyl3,4,5-trisphosphate (PI-3,4,5-P_3) or phosphatidylinositol 3,4-bisphosphate (PI-3,4-P_2) to recruit Akt to the plasma membrane by binding to the PH domain. Akt undergoes a conformational change which results in the phosphorylation of the kinase activation domain at Thr308. A second phosphorylation site is at Ser473. On getting phosphorylated, Akt translocates into the nucleus to regulate the proteins involved in angiogenesis, cell survival, and proliferation. The levels of PI-3,4,5-P_3 and PI-3,4-P_2 are regulated by PI3-K and phosphatases. One of the phosphatases is the phosphatase and tensin homologue (PTEN) which converts PI-3,4,5-P_3 to PI-4,5-P_2. The SHIP phosphatases, SHIP1 and SHIP2, convert PI-3,4,5-P_3 to PI-3,4-P_2. Similarly, Akt is negatively regulated by protein phosphatases and positively regulated by proteins such as caveolin-1 (cav-1). Serine/threonine protein phosphatases, PP1 and PP2A, dephosphorylate Akt at Thr308 and Ser473. PP1 and PP2A regulate processes such as glycogen metabolism, protein synthesis. Caveolin-1 positively regulates Akt by inactivating PP1 and PP2A. Caveolin-1 is upregulated in prostate cancer. Akt promotes anti-apoptotic pathways by regulating genes involved in apoptosis such as forkhead receptor (FKHR). Akt phosphorylates FKHR which results in its sequestration in the cytoplasm by 14-3-3 proteins preventing it from activating proapoptotic proteins such as BIM and FAS ligands [91].

Yamaguchi et al. [92] have demonstrated the anti-apoptotic activity of Akt through techniques ranging from flow cytometry to fluorescence in situ hybridization (FISH) in lymphoid cells. In experiments designed to study a range of apoptotic proteins, the study showed using FISH that Akt prevented Bax from translocating to the mitochondria even when the cell survival factor, IL-3, was absent. It was observed that the active phosphorylated form of Akt inhibited caspase activation and maintained the membrane potential of the mitochondria. Contrary results would have been observed if the proapoptotic factor, Bax, was active [92]. Akt can activate NF-kappa B signaling by phosphorylating the IkB kinases which inactivate its interaction with NF-kappa B proteins. This leads to the translocation of the NF-kappa B proteins into the nucleus to activate the transcription of downstream target genes. Similarly, the AR signaling pathway is regulated by Akt. The androgen receptor mediated apoptotic pathway is negatively regulated by Akt. In a study by Lin [93], it was observed that Akt phosphorylates AR at two distinct sites, Ser-210 and Ser-790. The specificity of Akt for the AR signaling pathway was observed when constitutive Akt was transfected into DU145 cells. It was observed that in the presence of constitutive Akt, the androgen receptor is phosphorylated. Subsequent to AR phosphorylation, the interaction between AR and its coactivators are inhibited by Akt. The use of inhibitors of PI3-K/Akt and PI3-K/p70S6K showed that the PI3-K/Akt signaling pathway was effective in regulating the AR signaling pathway. The rates of apoptosis were significantly lower in prostate cancer cells that were treated with inhibitors to PI3-K. Stable cell lines of LNCaP treated with a dominant negative mutant of Akt showed an increase in p21 protein expression which is a proapoptotic factor and this was specific to prostate cancer cells [93]. Akt may influence the progression of prostate cancer to hormone-independent prostate cancer by inhibiting apoptosis and promoting G1 cell cycle progression [94].

Brunet et al. [95] have shown the interaction of Akt with the forkhead receptor ligand 1 (FHRL1) which results in its phosphorylation and inactivation. The FKHRL1 functions in the nucleus where it stimulates apoptosis in the cell. In the cancer arena, Akt is activated which then phosphorylates FKHRL at three sites causing FKHRL to get sequestered in the cytoplasm. Immunolocalization confirmed the subcellular staining of FKHRL. The protein that cooperates with Akt in the sequestration is the 14-3-3 protein which is a cytoplasmic phospho-serine binding protein. FKHRL binds to a consensus sequence similar to the insulin response sequence (IRS). The activation of apoptosis by FKHRL is through the consensus sequence termed FH-responsive element (FHRE). This sequence can be found on ligands such as Fas. The interaction of FKHRL with Fas which induced apoptosis was confirmed by determining the percentage of apoptotic cells in cell lines that were transfected with the Fas ligand and the FKHRL. This study elegantly showed the specificity of the interaction of Akt with FKHRL in regulating the apoptotic pathway.

Another cellular aspect that is controlled by the PI3-K/Akt pathway is prostate cancer cell invasion [95, 96]. In a study by Shukla et al. [97] the effect of the PI3-K/Akt pathway on the invasive properties of prostate cancer cell lines, LNCaP, 22RV1, PC-3, and DU145 were analyzed. The levels of PTEN and activated Akt were inversely correlated in prostate cancer cells. PI3-K protein levels were lowered in androgen-dependent cells in the presence increasing concentrations of the PI3-K inhibitor while they were minimally affected in androgen-independent cell lines, DU145 and PC-3. With the lowering of PI3-K, the levels of p-Akt were lowered resulting in decreased invasive capacities. When the levels of p-Akt were high, the levels of invasion proteins matrix metalloproteinase (MMP-9) and urokinase plasminogen activator (uPA)

were increased. When human prostate tissue samples were analyzed, the PI3-K levels were found to be high in high Gleason grade tumors. With the increase in PI3-K levels, active Akt was increased and PTEN levels were lowered. This seems to indicate the role of PI3-K/Akt in prostate cancer progression and cell invasion [97]. A study in mice showed that PTEN deficient mice were tumorigenic. This tumorigenic capacity was seen to be inhibited by Akt deficiency and haploinsufficient Akt also inhibited the development of prostate cancer [98]. Another aspect of Akt signaling is in indicating the risk of recurrence of prostate cancer. It is unknown how prostate cancer recurs despite hormone ablation therapy. Initially, prostate cancer is dependent on androgen and on removal of androgens from the environment, prostate cancer regresses. Yet, 18 months later, cancer reappears. In order to determine the factors that may cause prostate cancer to recur, Bedolla et al. [99] looked at the effect of PTEN expression and the presence of active Akt or p-Akt in prostate cancer tissue. Paraffin-embedded samples were analyzed through immunohistochemistry for the status of PTEN and p-Akt. It was observed that PTEN negative samples showed prostate cancer recurrence that was close to twice the rate for samples that were positive for PTEN expression. Conversely, high p-Akt levels give rise to five times the frequency of recurrence as compared to samples that have low levels of p-Akt. Univariate analysis indicates p-Akt and PTEN are predictors of recurrent disease in converse ways. A multivariate analysis has shown the combination of Gleason score, p-Akt and PTEN levels are important predictors of recurrent prostate cancer [99]. Besides interacting with the AR signaling pathway, PI3-K/Akt also interacts with NF-kappa B and this has been demonstrated in TRAMP mice. It was observed with increase in age of TRAMP mice, the levels of NF-kappa B increased in both the nucleus and cytosol. Simultaneously, the inhibitory kinase, IKKα, is decreased and IkBα is increased in TRAMP mice while no significant change was observed in the non-transgenic controls. Invasion proteins such as MMP-9, cell cycle proteins such as cyclin D, angiogenesis proteins such as VEGF all showed high rates of expression in TRAMP mice. PTEN levels were measured in TRAMP mice compared to non-transgenic mice and the non-phosphorylated and phosphorylated forms of PTEN were found to increase as the age of the transgenic mice increased. This study showed the connection between the tumor suppressor gene PTEN and the PI3-K/Akt pathway [100].

27.11 Prostate Cancer Metastasis

Distant site metastases are seen in the metastatic stage of prostate cancer. Some of the sites of metastases are bone, lymph nodes, lung, liver, and brain. Metastases to the lymph nodes and bone are the most frequent and predominant. Lymph node metastases account for 70 % while bone account for 90 % of prostate cancer metastases. This review will focus on bone metastases and the genes that regulate the development of the cancer.

27.11.1 Bone Metastases

Prostate cancer that becomes androgen independent is very aggressive resulting in highly invasive cancers. The bone is the primary site of invasion of aggressive prostate cancer and accounts for ~90 % of distant site metastases. Skeletal bone cancer results in severe pain, hypercalcemia, and frequent fractures [101, 102]. Bone metastases are seen in cancers of the lung, breast, and kidney. However, the unique feature of bone metastases in prostate cancer is that the cells are predominantly osteoblastic. Breast, kidney, and lung cancer bone metastases are mainly osteolytic [103]. Bone cells are made up of osteoblasts and osteoclasts. Osteoblasts are bone-forming cells while osteoclasts are bone-resorbing cells. Once bone metastasis sets in, it signifies the terminal stage for patients diagnosed with prostate cancer. There is no cure for these skeletal metastases [102, 104].

The bone comprises of a homeostatic balance between osteoblasts and osteoclasts. The basic function of osteoblasts is to produce the bone matrix which becomes mineralized. Growth factors such as bone morphogenetic protein (BMP), transforming growth factor-β (TGFβ), fibroblast growth factor (FGF), endothelin-1 (ET-1), serine proteases such as urokinase and prostate-specific antigen (PSA) activate osteoblast proliferation or differentiation [105, 106]. Alkaline phosphatase is an enzyme found on the surface of osteoblasts and is involved in bone mineralization [106]. Osteoclasts on the other hand are bone-resorbing cells and differentiate after osteoblasts develop [107]. Osteoblasts activate osteoclast precursors by expressing osteoprotegerin (OPG) and receptor activator of nuclear factor-kB ligand (RANKL). RANKL binds RANK on the osteoclast precursor cell causing it to differentiate into an osteoclast. OPG is an alternate receptor for RANKL which acts as an internal control for the development of osteoclasts [108]. When imbalance sets in between the osteoblasts and osteoclasts, cancer is initiated [105].

Bone metastases develop in two stages. Prostate cancer cells initially home into the bone followed by tumor development. The exact method of development of bone metastases has not been elucidated. However, work done by Sikes et al. [109] has shown that cells migrate toward bone based on their invasive capacity compared to their adhesive potential. In the study, the highly invasive cell line C4-2 was compared to LNCaP and the non-tumorigenic cell line P69 for their adhesive and invasive properties. It was observed that the tropism for bone by prostate cancer cells was inversely proportional to the tumorigenicity of cells with C4-2 cells showing the least tropism toward bone cells. Conversely, the

invasive capacity of C4-2 cells was higher in bone cells compared to P69. This study indicated the possible form of interaction of prostate cells with bone which depends not just on cell surface proteins to initiate contact and expansion but also on the bone microenvironment which influences different cells in varying ways. LNCaP cells though invasive toward bone are not as metastatic and this can be explained by the varying interactions of the bone extracellular matrix with the cells [109]. The androgen-independent C4-2 cells are derived from LNCaP by coculturing LNCaP cells with bone stromal cell line MS. Cell lines were derived from the bone metastases developed from the C4-2 cell line. Comparison of the invasive capacity of the C4-2 cell line and the derivative bone cell lines showed higher invasive potential compared to the parent LNCaP cell line. The progression of the LNCaP cell line into an androgen-independent cell metastasizing toward the bone imitates the situation in vivo in humans [110]. The clonal nature of bone metastatic cells is obvious when PSA levels are measured. PSA protein is expressed at high levels in the cells although one of the cells failed to show sufficient RNA. On growing out the cell line, some of the cells showed sufficient RNA levels of PSA. This indicates the heterogeneity of prostatic bone tumors [110].

A study of the factors responsible for prostate cancer death due to metastases to the bone analyzed 68 prostate carcinoma patients and clinical features such as Gleason score, time of survival between diagnosis, and surgical treatment, levels of PSA, staining for biomarkers (including chromogranin A) showed three features to be responsible for increased death due to metastasis of prostate carcinoma in the bone. It was observed that a high Gleason score of 8 or more is responsible for bone metastases and greater frequency of death. The time of survival reduces with increasing time difference between time of diagnosis and surgical treatment. This is due to the fact that the longer it takes to detect the metastasis the more developed the cancer, resulting in a greater likelihood of death. In addition, staining of chromogranin proved to be an indicator of decreased survival in prostate cancer patients with bone metastasis. However, the sample set of this study is small to consider the observations to be entirely representative of prostate cancer bone metastases in the population. A larger sample study is required to confirm the above results. It was also observed in the sample set that androgen receptor was positive in the bone metastatic cells [111].

Vascular endothelial growth factor (VEGF) induces osteoblast differentiation but not mineralization. A study of PC-3, LNCaP, and C4-2B cells showed VEGF levels are present at high levels. C4-2B conditioned medium stimulates osteoblast differentiation and was used in culturing cells such as PC3 and LNCaP in addition to C4-2 itself. An inhibitor to the receptor of VEGF showed that VEGF when inhibited reduces the capacity of osteoblast differentiation. Alkaline phosphatase and osteocalcin levels are reduced concomitantly with the reduction of VEGF levels. C4-2B cells when injected into mice tibia show increase in bone marrow density as compared to injected C4-2B cells that are exposed to VEGF inhibitor clearly indicating the role of VEGF in the ossification process [101].

Prostate cancer that metastasizes to the bone shows an increased activity of osteoblasts compared to osteoclasts and thus increased bone formation. However, this bone formation does not improve the strength of the skeletal structure because the internal bone structure is weaker than that formed in a normal process. The collagen bundles formed in the normal lamellar bone are arranged in straight long bundles which are tightly packed giving the bone its strength. However, when osteoblasts are activated by prostate cancer cells, they induce bone formation where the collagen is loosely packed in a random manner resulting in weak bones. This is the reason for bone fractures in such a condition. The combination of weak bone structure coupled with osteolytic activity further reduces bone strength [108]. Berruti et al. [112] have surveyed the various skeletal damages that can occur due to prostate cancer metastasizing to the bone. Some of these skeletal damages in order of greater prevalence are spinal instability, spinal compression, hypercalcemia, bone fractures. Alkaline phosphatase is the predominant marker that correlated significantly with bone formation and deoxypyridinoline is a significant marker of bone resorption. Deoxypyridinoline correlated significantly with skeletal damages in the study of 112 patients [112].

Parathyroid hormone-related peptide (PTHrP) released by the tumor cell induces osteoblasts to overexpress RANKL and downregulate OPG. RANKL then acts on osteoclast precursors to develop into osteoclasts which then perform bone resorption. The process of bone resorption releases various growth factors such as TGF-β, IGF1, and calcium. SMAD and MAPK are activated as separate signaling pathways which lead to the release of PTHrP resulting in the repetition of the cycle of osteoblast-osteoclast interactions [106].

Prostate cancer can metastasize to the bone with the help of the Wnt proteins. It has been shown that the Wnt signaling pathway is activated in prostate cancer where Wnt1 and β-catenin are upregulated. Wnts 3a and 11 are present in androgen-independent cancer. Wnt3a activates AR signaling pathway in the absence of androgen and regulate the level of β-catenin [103]. Hall et al. have shown that prostate cancer promotes osteoblast differentiation through Wnt proteins [104]. DKK1 is an antagonist of Wnt and binds to the lipoprotein receptor-related proteins (LRP) and causing it to internalize to be degraded by lysosomes [103]. Prostate cancer cells that express Wnt proteins show metastasis to the bone. However, when DKK1 is expressed in the cells, the activity of Wnts is reduced resulting in reduced bone formation. The in vivo study showed a decrease in bone density

within the tibia of mice bones. There is a destruction of the lamellar bone when DKK1 is overexpressed in the prostate cancer cells. Mineralization is observed in vitro in prostate cancer cells that downregulate DKK1 expression indicating the osteolytic effect of DKK1 [104].

Current therapies related to bone metastases seek to identify the proteins that are involved in the differentiation pathways of bone cells. Bisphosphonates are pyrophosphate compounds that bind to mineralized cell surfaces and prevent the action of osteoclasts in bone resorption. It is a drug that is currently marketed. Some of the drugs that are in phase II or III trials are osteoprotegerin and PTHrP antibodies. In addition, there are endothelin receptor antagonists (atrasentan) that target osteoblast differentiation and bone morphogenetic antagonists (BMPs such as Noggin and anti-BMP-6). There are radioactive drugs such as strontium-89, Samarium-153, and Rhenium-186 that are used to prevent the development of the bone matrix and osteoblast differentiation [102, 106].

27.12 Animal Models of Prostate Cancer

The Dunning and Noble mouse models of prostate cancer initiated the study of prostate cancer in vivo [113, 114]. Greenberg at al. [115] developed a transgenic mouse model in 1995 that recapitulated the process of intraepithelial neoplasia to invasive carcinoma in humans. Transgenic adenocarcinoma mouse prostate or TRAMP is the term given to the mouse model developed by Greenberg et al. [115]. The concept involved using the rat probasin promoter to drive the expression of genes of interest. The probasin gene is a prostate-specific gene and, the probasin promoter drives the expression of genes specifically in the prostate. In the TRAMP model, the SV40 large T antigen was placed under the influence of the probasin promoter since the effect of the T antigen on initiating tumorigenesis was observed in the pancreas and mammary gland [115]. The tumors that developed were mainly in the dorsal and ventral sections of the mouse prostate and resembled the human invasive carcinoma of the prostate. The cells were pleomorphic with angular nuclei and small amounts of cytoplasm. The progression to hormone independence was an important point to study in prostate cancer. When AR immunostaining was performed in TRAMP mice, it was observed that increased loss of epithelial differentiation was inversely proportional to the level of AR staining. The time taken for the tumors to progress to cancer in the mice was approximately 20–40 weeks with hyperplasia observed around 10 weeks of age while invasive carcinoma was observed in the mice at around 20 weeks of age. This rapid progression was observed when a matrix attachment region (MAR), intended to enhance the probasin based gene expression, was added to the probasin-Tag construct. In the absence of the MAR, the rate of tumor progression though lower, still occurred in the transgenic mice. The advantage of TRAMP is that the tumors develop specifically only in the prostate making it an ideal model for a focused analysis of prostate cancer. The molecular events that occur in the development of prostate tumors as well as the transition from hormone dependence to hormone independence as observed in the reduction of AR staining can be studied in the TRAMP model [115].

In the study by Gingrich et al. [116], the progression to androgen independence was studied in the TRAMP model. The basis for the study was to study if the androgen-independent growth in cells was induced due to castration or androgen-insensitive cells were already present in a heterogeneous prostate which were activated on castration. The genitourinary weight was compared between normal mice and TRAMP mice. The GU weight was larger in the TRAMP mice but a timescale progression of weight difference between castrated TRAMP mice showed that the GU weight of castrated TRAMP mice at 12 weeks was lower than those that were castrated at 18 weeks. This increase in GU weight with increase in time following castration indicated a subsequent growth of the prostate cells. This experiment showed the presence of androgen-independent cells that were present in the prostate but were activated only under conditions of castration [116].

Besides the TRAMP mouse model, there have been mouse models that look at the effect of growth factors on the development of the prostate and the effect of drugs on the prostate. The mouse model, Tag/Ghr$^{-/-}$ was generated by crossing a GH receptor knockout mouse with the C3[1]/Tag mouse. The resulting Tag/Ghdr$^{-/-}$ mouse failed to develop PIN lesions as compared to Tag/Gh$^{+/+}$ mice despite the presence of the T antigen, and androgen receptor expression indicating the importance of growth factors for prostate cancer progression [117]. A transgenic mouse model termed ARR2 Pb-Lux expresses luciferase specifically in the prostate under the influence of the androgen receptor and provides a useful model to test the effect of androgen receptor inhibiting drugs. The luciferase signal is diminished when the androgen levels are reduced due to the use of bicalutamide, an androgen receptor inhibiting drug, or due to castration. This transgenic mouse provides a useful method of analyzing histological and molecular changes in the prostate in a noninvasive manner [118].

Prostate tumor cell lines are widely used to study the tumorigenic process in the prostate. Commonly used prostate cell lines are LNCaP, CWR-22, PC-3, DU145, RWPE-2, TSU-PRI, RWPE-2, and ALVA101 [119]. LNCaP is an androgen-dependent cell line that was derived in 1977 from a metastatic lymph node of a 50 year-old Caucasian male. This cell line is most commonly used to study the properties of prostate cells in the presence of androgen. In addition,

LNCaP cells are used to develop androgen-independent cell lines in an effort to study the progression of androgen-dependent cells to an androgen-independent state. Our laboratory has generated the androgen deprivation induced androgen suppressed cell line, LNCS from LNCaP [120]. LNCaP cells were cultured in a charcoal stripped medium which was devoid of hormones and after approximately 20 passages, the cells changed in morphology and androgen-dependence. While LNCaP cells have filamentous processes and a functional androgen receptor, LNCS cells grow in clumps and do not possess a functional androgen receptor. LNCS cells when grown in FBS medium are growth suppressed and resume normal growth in charcoal stripped medium. PSA levels are found to be insignificant in LNCS compared to LNCaP. The LNCS cell provides a useful model to analyze the progression of androgen-dependent cells to an androgen deprivation induced androgen suppressed state [120]. A Q640X mutation in AR in LNCaP cells exhibits a paracrine effect on neighboring cells which have normal AR resulting in a ligand-independent increase in overall AR and PSA expression of cells that do not possess the mutated AR. This is another mechanism by which cells can develop in the absence of androgen [121]. LN-REC4, LNCaP-SF, C4-2 cells are derived from LNCaP cells and are all androgen independent [110, 122]. LNCaP-CR is a cell line derived from LNCaP which is cytokine resistant [123].

The PC-3 cell line was established in 1979 from a lumbar vertebra metastasis in a 62-year-old Caucasian male. The PC-3 cell line is an androgen-independent cell line where the androgen receptor is absent. High levels of transforming growth factor (TGF-α) and epidermal growth factor-receptor (EGF-R) are observed in the PC-3 cell line indicating growth factors and not androgen are responsible for the growth of the prostate cells. PC-3 cells are in general unresponsive to androgens. DU145 cells are androgen-independent cells derived from a metastatic central nervous system lesion. The growth factors that stimulate the growth of DU145 are TGF-α, fibroblast growth factor (FGF), and insulin growth factor (IGF-1) [119, 124].

The ALVA101 cell line is derived from a bone metastasis and expresses the androgen receptor, PSA as well as 5α-reductase activity. These cells are consequently androgen responsive [119]. Cell lines are a vital in vitro tool to analyze the events that occur in the tumorigenic process in vivo. Most cell line studies provide the basis for in vivo analyses of tumorigenic progression and enable drug studies.

27.13 Conclusion

This review has covered the broad aspects of the tumorigenic process of prostate cancer. No doubt, there are many discoveries that may not have been touched upon in the article. However, one can observe the different angles with which prostate cancer has been studied. There have been advances made in the androgen refractory state of prostate cancer where a number of cell line models and xenograft models are providing us with clues to the functioning of the prostate cell in a hormone-free environment. Genome-wide allelotyping studies have provided us with a base on which further exploration of candidate genes can be conducted. One can observe that the study of cancer is ultimately a combined analysis using different techniques, cell lines, animal models, tissue analyses to give a more comprehensive overview of the disease. The heterogeneity of prostate cancer also increases with age and environment. To this end, the results reviewed in this chapter emphasize the need for the use of different techniques in analyzing prostate cancer.

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Molecular Basis of Lung Carcinogenesis

28

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28.1 Introduction

Lung cancer is the leading cause of cancer deaths in the Western world, estimated to account for over a quarter of the cancer deaths in both men and women [1]. The World Health Organization has estimated that perhaps about one million people die from lung cancer worldwide each year. Lung cancer has surpassed breast cancer as the leading cause of cancer death in women in several western countries. Changes in smoking habits have meant that the incidence and death rates due to lung cancer are decreasing in men, but increasing in women.

Clinically, lung cancer is usually classified into non-small-cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with the latter representing 15–20 % of cases (Table 28.1). This classification relies on differences in histological and neuroendocrine (NE) features. A characteristic neuroendocrine phenotype is observed in the pulmonary NE cells of normal lung and in a subset of lung malignancies including SCLC, bronchopulmonary carcinoids, and NSCLC with neuroendocrine features, which includes the presence of cytoplasmic dense core granules [2, 3], and expression of L-dopa decarboxylase, chromogranin A, synaptophysin A, neuron specific enolase (NSE) and neural cell adhesion molecule (NCAM). In contrast, most NSCLC lack these properties and are comprised of three major subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. In vitro, NSCLC and SCLC subtypes also tend to have different biological properties such as the adhesion of NSCLC cells to substrate which is generally absent in SCLC cell lines. This is relevant to molecular pathogenesis as the CD44 adhesion molecule which is involved in intercellular interactions was found to be predominantly associated with the NSCLC phenotype, indicating that it may be a useful differentiation marker [4]. Nonetheless, there are some lung cancers with features of both NSCLC and SCLC.

Although it is still uncertain whether SCLC and NSCLC are derived from the same or different cell lineage, much progress has been made towards understanding the molecular basis of lung cancer, particularly involving classic oncogenes and tumor suppressor genes (TSGs or recessive oncogenes). The current paradigm suggests that human epithelial tumors, such as lung cancer, arise as a result of the accumulation of multiple independent molecular events that target critical genetic pathways in key cells. These events appear distinct from the random background genetic damage that is often seen in advanced neoplasms. Specific oncogenes and tumor suppressor genes (TSGs) are the likely targets of somatic aberrations resulting from the genotoxicity of tobacco smoke carcinogens.

The critical cellular pathways affected directly or indirectly by these somatically acquired aberrations are becoming increasingly recognized as biochemical functions of the proteins encoded by mutated genes are unraveled. Cancer is not only caused by abnormal cell proliferation with loss of the usual cellular growth control mechanisms but is also influenced by abnormalities in apoptosis (programmed cell death). Indeed, solid tumors ensue from a balance between cell proliferation and cell death. The regulation of these processes is complex. There are positive factors such as cytokines, hormones, growth factors, and their specific receptors which, upon ligand binding, then signal effector genes via

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_28

Table 28.1 Selected characteristics of small cell and non-small-cell lung cancer

	SCLC	NSCLC
Frequency	~20–25 %	~75–80 %
Histology	Scant cytoplasm, small hyperchromatic nuclei, fine chromatin, indistinct nucleoli, tumor in sheets	Abundant cytoplasm, pleomorphic nuclei, coarse chromatin, prominent nucleoli, squamous/glandular architecture
Neuroendocrine phenotype	~100 %	Large cell neuroendocrine carcinomas and carcinoids
Peptide secretion	ACTH, AVP, calcitonin, ANF	PTH
Radiation-sensitivity	80–90 % Shrinkage	30–50 % Shrinkage
Chemo-sensitivity	High	Low
Oncogenes		
RAS mutation	<1 %	~15–20 %
MYC amplification/overexpression	~15–30 %	~5–10 %
ERBB2 overexpression	<10 %	~30 %
EGFR mutation	Rare	~10 % (Higher in Asians, nonsmokers, adenocarcinomas, females)
EML4/ALK fusion		~5 %
ROS1 fusion		~1–2 %
LKB1 mutation		~9-
HER2 mutation/amplification		~2–4 %
PIK3CA mutation/amplification		~2–18 %
TTF1 amplification		~15 %
BRAF		~2–3 %
MET mutation/amplification	? Rare	~1–20 %
FGFR1 amplification		~22 % SCC
SOX2 amplification		~23 %
Putative autocrine loops	GRP/GRP receptor, SCF/KIT	HGF/MET, neuregulin/ERBB
Tumor suppressor genes		
CDKN2A mutation	<1 %	~10–40 %
TP53 mutation	~75–100 %	~50 %
17p LOH	~80–90 %	~70 %
Abnormal TP53 expression (immunohistochemistry)	~40–70 %	~40–60 %
Absent RB1 expression	~90 %	~15–30 %
13q LOH	~75 %	~40–60 %
3p allele loss	>90 %	~50–80 %
9p LOH	~20–50 %	~50–75 %
Absent CDKN2A expression (immunohistochemistry)	~0–10 %	~30–70 %
Other genetic deletions, e.g., 5q, 8p, 11p, 18q	Variable	Variable
Telomerase expression	~100 %	80–85 %

various signal transduction cascades, as well as negative regulators of cell growth and proliferation.

Karyotypic and molecular analyses have demonstrated that lung cancer cells have accumulated several genetic lesions, with perhaps ten or more such events required for the development of an overt lung cancer. Knowledge of the temporal sequence and timing of these genetic lesions in the multistep process of lung carcinogenesis is increasing with improvements in techniques allowing examination of DNA from tiny preneoplastic bronchial lesions, such as with laser capture microdissection. Nonetheless, there appears considerable inter-individual heterogeneity in the timing and number of genetic lesions that occur during lung carcinogenesis; with various alternate pathways leading to bronchogenic carcinoma. While some of the molecular events are commonly found in both SCLC and NSCLC, others show greater specificity for one or other of the subtypes (Table 28.1). These events can largely be categorized into the original and emerging hallmarks of cancer: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis (programmed cell death), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, reprogramming of energy metabolism, and evasion of immune destruction [5].

28.2 Molecular Changes in Overt Lung Cancers

28.2.1 Aneuploidy and Copy Number Variations

Aneuploidy, an abnormal number of chromosomes representing an abnormal total DNA content, is another common characteristic of tumor cells. In solid tumors, aneuploidy is linked to genomic instability, which includes chromosome instability (CIN). With the onset of genomic studies came reports of gene expression signatures associated with aneuploidy and cell proliferation. Many solid tumors, including lung cancers, often have marked aneuploidy, ranging from hypoploidy to hyperploidy [6, 7]. Several studies have assessed the value of aneuploidy as a marker of biological aggressiveness, but the prognostic value of aneuploidy in lung cancer is controversial [8, 9]. Newer techniques have allowed the identification of extensive areas of aneuploidy in the respiratory epithelium of lung cancer patients, a finding consistent with the field cancerization theory [10].

Early pivotal cytogenetic studies in tumors demonstrated karyotypic features of double-minute chromosomes (DMs) and novel staining regions called homogenously staining regions (HSRs), corresponding to amplified DNA sequences which may contain between twenty and several hundred copies of a specific chromosomal sequence. As the amplified sequences often involve several hundred thousand base pairs, it is possible that more than one oncogene is contained within the amplified region. Chromosomal comparative genomic hybridization (CGH) and array CGH are molecular cytogenetic techniques that can detect gains and losses of DNA in a tumor genome. CGH techniques led to the identification of several new chromosomal regions affected by either deletions or increased DNA copy number in lung cancer genomes [11–15] and include specific allelic loss at 3p, 4q, 9p, and 17p and gain at 1q, 3q, 5p, and 17q [16–20]. More recently, array CGH was developed for high-throughput, high-resolution DNA dosage analysis, where microarrays are spotted with unique genomic probes. Early array CGH microarrays consisted of large genomic clones, such as bacterial or P1 artificial chromosomes (BACs and PACs) or cosmids, robotically spotted onto glass slides, whereas later platforms used short, 25–70-mer oligonucleotides. Moreover, bioinformatic advances have also facilitated the use of single nucleotide polymorphism (SNP) arrays initially developed for genotyping to be used to detect DNA copy number variations [21]. A number of studies have reported microarray-based CGH analyses to detect genomic aberrations in lung cancers, but few in primary SCLC because of its rare resectability (Table 28.2).

Likewise, loss-of-heterozygosity (LOH) events as indicators of inactivation on one allele of TSGs were more finely mapped using these high-throughput technologies. Nonetheless, the earlier polymorphic DNA marker-based technologies of Southern Blot and repetitive sequence PCR (e.g., microsatellite) contributed greatly to the localization and identification of candidate TSGs by identifying allele loss in tumor cell lines, primary tumor cells and preneoplastic cells associated with invasive cancers.

With the advent of newer technologies such as digital PCR and massively parallel sequencing (commonly referred to as next generation sequencing or NGS), comes even more capacity to detect and identify copy number variation in lung cancer. Rapid technological advances mean that NGS can be applied to the study of formalin-fixed, paraffin-embedded (FFPE) samples with relatively small quantities of DNA starting template [22], using next-generation sequencing for high resolution multiplex analysis of copy number variation from nanogram quantities of DNA from formalin-fixed, paraffin-embedded specimens. Indeed, NGS has largely superseded older techniques for detecting tumor CNVs.

28.3 Oncogenes and Growth Stimulation

Proto-oncogenes encode proteins that are positive effectors of the transformed phenotype and may be considered positive growth regulators. The normal cellular counterparts of these proto-oncogenes are often components of normal growth signaling pathways. Activation of these proto-oncogenes usually occurs via aberration of a single allele, including by gene amplification, point mutation and constitutive overexpression, leading to a gain in function or dominant effect. These proto-oncogene products include various growth factors, receptor tyrosine kinases, non-receptor tyrosine kinases, membrane-associated G proteins, cytoplasmic serine/threonine kinases, and nuclear transcription factors.

28.3.1 ERBB Receptor Tyrosine Kinases

The epidermal growth factor receptor or ErbB family of tyrosine kinases consists of four members—ErbB1 (EGFR), ErbB2, ErbB3, and ErbB4 [72]. The family of tyrosine kinases is important for many developmental and physiological processes. ERRB receptors are activated in response to peptide binding resulting in receptor hetero- or homo dimerization [73]. The peptide growth factor ligands that interact with the ERBB family of transmembrane receptor tyrosine kinases are called neuregulins, neu differentiation factors, or heregulins [74]. The neuregulin and ERBB (ERBB2, ERBB3, and ERBB4) families may be considered to be potential growth stimulatory loops involved in the development of lung cancer [75]. On binding neuregulin, ERBB receptors homodimerize or heterodimerize,

Table 28.2 Published array CGH in lung cancer showing copy number variations (DNA gains and losses)

Study	Platform (probes)[a]	Resolution (kb)	Samples[b,c]
NSCLC			
[23]	BAC (348)		37 NSCLC (21 SCC, 16AC)
[24]	SNP (1494)		33 Lung cancer cell lines (19 NSCLC, 14 SCLC)
[25]	cDNA (8000)	376	14 NSCLC (8AC, 6 SCC), 14 NL
[26]	BAC (3014)	1000	50 NSCLC (29 SCC, 21AC)
[27]	cDNA (~10,000)	1000	20 NSCLC cell lines (11AC, 7 SCC, 2 LCC), 9 SCLC cell lines
[28]	BAC (800)		27 NSCLC cell lines (11 SCC, 10AC, 6 LCC)
[29]	Oligo (22,500)	54.8	44 NSCLC (18AC, 26 SCC)
[30]	cDNA (12,814)		8 NSCLC
[31]	SNP (115,593)		51 NSCLC (37AC, 10 SCC, 4 BrAC); 26 NSCLC cell lines (14AC, 4 SCC, 4 LCC, 2 NSCLC, 2 BrAC, 1 AdSq); 19 SCLC tissues; 5 SCLC cell lines
[32]	BAC (32,433)	<1000	28 NSCLC cell lines (18AC, 9 SCC, 1 LCC)
[33]	BAC/PAC (4523)	~700	20 NSCLC cell lines (7 SCC, 7AC, 6 LCC)
[34]	cDNA (12,814)		20 (8AC, 6 SCC, 5 LCC, 1 AdSq)
[35]	cDNA (11,367)	564	23 NSCLC (7AC, 15 SCC, 1 AdSq); 3 metastases; 10 NL
[36]	BAC		32 NSCLC
[37]	BAC (4046)	1000	36 NSCLC (22 SCC, 14AC)
[38]	cDNA (~22,000)	~30	76 NSCLC (40 SCC, 36AC), 56 NSCLC cell lines
[39]	BAC (2464)		75 NSCLC (43AC, 32 SCC) (Current, former and never smokers)
[40]	BAC (4030)	1000	24 NSCLC (12AC, 12 SCC) (with PBMC)
[41]	BAC (32,000)		85 NSCLC (56AC, 20 LCC, 9 Other)
[42]	BAC (>26,000)	<1000	1AC, 1 SCLC, 1 LCNEC (from same patient)
[43]	BAC (>26,000)	<1000	112 LC cell lines (77 NSCLC, 32 SCLC) (with and without EGFR TKI treatment)
[44]	Oligo (244,000)	2.1	42 NSCLC (from 20 patients: 6 synchronous, 14 metachronous)
[45]	BAC (4046)	1000	36 NSCLC (22 SCC, 14AC) [37]
[46]			76 NSCLC (40 SCC, 36AC), 56 NSCLC cell lines [38]
[47]	BAC (>26,000)	<1000	161 NSCLC (103AC, 58 SCC) (correlated with mRNA microarray profiles) [38]
[48]	BAC (4362)	1000	20 NSCLC (11AC, 9 SCC)
[49]	Multiple		>3400 NSCLC tumors and cell lines (from public repositories for 20 studies)
[50]	Oligo (244,000)	2.1	123 Paired normal and NSCLC (57AC, 50 SCC, 13 LCC, 3 unclassified)
Adenocarcinoma			
[51]	BAC (800)		55AC
[52]	BAC (1440)	2300	15AC
[53]	SNP (500,000)		575AC
[54]	BAC (2621)	1000	3AC cell lines
[55]	BAC (>26,000)	400	26AC (non-mucinous BrAC, invasive AC with BrAC features)
[56]	BAC (1440)	2300	21AC (11 early response, 10 non-relapse)
[57]	Oligo (385,806)	6	138AC
Squamous cell carcinoma			
[58]	BAC (1440)	2300	14 SCC
[59]	BAC (6500)		35 SCC (with and without COPD)
[60]	Oligo	1000	22 SCC
[61]	BAC (>26,000)	<1000	52 SCC (with and without arsenic exposure)
[62]	Oligo (244,000)	2.1	62 SCC
SCLC/neuroendocrine			
[63]	SNP (1494)		17 SCLC
[24]	SNP (1494)		33 Lung cancer cell lines (19 NSCLC, 14 SCLC)
[64]	5p BAC (491)	100	15 SCLC cell lines
[65]	1p BAC (642)		15 SCLC cell lines
[66]	cDNA (39,632)	70	24 SCLC cell lines
[27]	cDNA (~10,000)		20 NSCLC cell lines (11AC, 7 SCC, 2 LCC), 9 SCLC cell lines

(continued)

Table 28.2 (continued)

Study	Platform (probes)[a]	Resolution (kb)	Samples[b,c]
[67]	BAC (800)		10 SCLC, 31 LCNEC
[31]	SNP (115,593)		51 NSCLC (37AC, 10 SCC, 4 BrAC); 26 NSCLC cell lines (14AC, 4 SCC, 4 LCC, 2 NSCLC, 2 BrAC, 1 AdSq); 19 SCLC tissues; 5 SCLC cell lines
[68]	BAC (32,433)		14 SCLC cell lines, 6 normal cell lines
[69]	SNP (114,000)	23.6	23 SCLC cell lines
[42]	BAC (>26,000)	<1000	1AC, 1 SCLC, 1 LCNEC (from same patient)
[43]	BAC (>26,000)	<1000	112 LC cell lines (77 NSCLC, 32 SCLC) (with and without EGFR TKI treatment)
[70]	BAC (2464)		46 SCLC, 5 SCLC cell lines
[71]	Oligo (180,000 and 105,000)		13 SCLC cell lines

[a]*BAC* bacterial artificial chromosome microarray, *cDNA* cDNA microarray, *Oligo* oligo microarray, *SNP* single nucleotide polymorphism microarray

[b]Studies used primary tumor tissue except where specified

[c]*SCLC* small cell lung carcinoma, NSCLC non-small-cell lung carcinoma, *AC* adenocarcinoma, *SCC* squamous cell carcinoma, *LCC* large cell carcinoma, *BrAC* bronchioloalveolar carcinoma, *LCNEC* large cell neuroendocrine carcinoma, *NL* normal lung, *PBMC* peripheral blood mononuclear cell

subsequently inducing intrinsic kinase activities that initiate intracellular signal transduction cascades such as the MAP kinase pathway. Although ERBB2 (also commonly known as ERBB2/neu) by itself lacks ligand binding ability, it plays a major coordinating role by enhancing and stabilizing dimerization. Each directly liganded receptor appears to dimerize preferentially with ERBB2, and resulting ERBB2-containing heterodimers have very high signaling potency. Activation of ERBB2 plays an important role in the development of many human cancers. The three major mechanisms leading to ERBB activation in cancer are gene amplification, altered ligand expression, and mutations in the receptor kinase or extracellular domain. The ERBB2 gene maps to chromosome 17q21, and its amplification and overexpression has been implicated in the development of several human cancers. Although amplification appears uncommon in lung cancer, ERBB2 is highly expressed in over a third of NSCLCs, especially in the adenocarcinoma subtype [76–78]. Experiments with transfected ERBB2 suggested that ERBB2 overexpression on its own was insufficient, but did contribute, to tumor induction in immortalized human bronchial epithelial cells [79]. Additionally, an anti-ERBB2 monoclonal antibody has been developed which can inhibit the in vitro growth of NSCLC cell lines expressing ERBB2 [80]. Some but not all studies have suggested that ERBB2 overexpression correlates with shorter survival in lung cancer [81–83]. In any case, other observations including the enhanced metastatic potential resulting from transfection of the ERBB2 gene into a NSCLC cell line in a xenograft model [84] support the idea that ERBB2 overexpression may be an adverse clinical indicator in some lung cancer patients. Transfection and overexpression of the ERBB2 gene in a constitutively low ERBB2 expressing NSCLC cell line also led to the induction of a drug resistant phenotype [85]. In vitro assays further suggest that ERBB2 overexpression may be associated with intrinsic multidrug resistance to chemotherapy agents in NSCLCs [86]. ERRB2 mutations in themselves are fairly rare in NSCLC [87, 88], with mutations being in-frame insertions situated in exon 20 (reminiscent of EGFR mutations). It is well recognized that increased copy numbers of ERBB2 in breast cancer predict response to trastuzumab [89]. Interestingly it appears that this may also be important for a group of non-small-cell lung cancer patients. ERBB2 amplification is found in 11 % of NSCLC specimens [90]. Clinical studies demonstrated that the subgroup of NSCLC patients with ERBB2 amplification achieve clinical benefit when trastuzumab is added to chemotherapy regimens [91].

28.3.2 EGFR

Another ERBB member, ERBB1 (better known as epidermal growth factor receptor, EGFR), has a role in regulating epithelial proliferation and differentiation. Epidermal growth factor (EGF) and transforming growth factor-α (TGFα) are prominent amongst the six characterized mammalian ligands that bind to the EGFR receptor. While no ligand has yet been identified for ERBB2, it is the most frequently used partner in heterodimerization with EGFR. The production of EGFR ligands, especially TGFα by lung cancer cells expressing cognate receptors, has led to suggestions that this system represents an important autocrine loop in lung cancer [75, 92,,93]. Ligand binding to EGFR results in homodimerization or heterodimerization which initiates autophosphorylation of tyrosine residues in the intracellular domain [94]. This initial phosphorylation event is thought to trigger a phosphorylation mediated signal transduction cascade through two major pathways—the Ras/Raf/MAP kinase and the PI3K-Akt pathway. Ras/Raf/MAPK activates MAPK3 and MAPK1 transcription factors resulting in increased cell

proliferation, survival, and transformation, while PI3K-Akt inactivates BCL2 family proteins and caspase 9, thus promoting survival in the face of apoptotic stimuli including radiation and DNA damage. Both pathways are implicated in development of malignancy [95]. Transgenic mice lacking EGFR have been generated. These mice develop abnormal epithelia in several organs, including the lung, in which there was impaired branching, deficient alveolization and septation [96, 97].

Activation of EGFR in lung cancer cells generally occurs by overexpression, with mutations and gene amplification being mechanistically implicated. Activation appears to be more common in NSCLC than in SCLC and may be related to tumor stage and differentiation [92, 98]. In NSCLC, amplification of EGFR is a common event resulting in overexpression in 60 % of metastatic NSCLC and is associated with an adverse prognosis [99–101]. In addition, in NSCLC there is increased expression of EGFR ligands (EGF and TGFα), which establish an autocrine loop of hyperactivation [102]. EGFR mutations of the tyrosine kinase domain are of two main types—either small in-frame deletions or missense substitutions clustered within the ATP binding pocket encoded by exons 18–21 [103–105]. Mutations occur more often in individuals with adenocarcinoma histology, East Asian origin, female gender, and never smoker status. In addition, they appear to be inversely correlated with KRAS mutations [106]. Mutations associated with tyrosine kinase inhibitor (TKI) responsiveness occur in the ATP-binding pocket of the tyrosine kinase domain of EGFR and are referred to as activating mutations [107]. In the presence of an activating mutation there is decreased affinity of this binding site for ATP and increased affinity for TKIs [108]. Interestingly, transgenic mice models have demonstrated that induction of activating EGFR mutations results in the development of adenocarcinoma like lung cancer [109]. Activating mutations are categorized into three classes: (1) class I mutations consist of deletions (in exon 19), (2) class II mutations consist of single nucleotide substitutions causing an amino acid change in exon 21, and (3) class III mutations consist of in-frame deletions or insertions (in exon 20). A 15 bp deletion involving amino acid residues leucine 747 to glutamic acid 749 (ΔLRE) in exon 19 and a point mutation substituting arginine for leucine at codon 858 (L858R) in exon 21, account for 44 and 41 % respectively of all NSCLC EGFR mutations [107, 110]. Preliminary evidence suggests that patients with the exon 19 mutation (ΔLRE) respond better to TKIs than patients with the exon 21 point mutation, L858R [111–113]. Further, there are polymorphisms associated with increased EGFR protein production (shorter CA-SSR1 length and variant forms of SNPs-216 and -191). These were found to be rare in East Asians as compared to other ethnicities, but in tumors from East Asian patients, EGFR mutations were found to favor the shorter CA-SSR1 allele, and selective amplification of the shorter allele of CA-SSR1 occurred frequently in tumors harboring a mutation [114]. Interestingly, another specific EGFR mutation, T790M, has been linked to acquired TKI resistance, and also to familial NSCLC [115–117] and was found to display a growth advantage over wild-type EGFR in a human bronchial epithelial (HBEC) cell line [118]. This immortalized HBEC model system which can be easily manipulated is likely to gain increasing prominence in the functional testing of candidate molecules [119].

Both types of activation mutations are associated with increased autophosphorylation of Tyr1068 at the C-terminal indicating increased EGF-dependent receptor activation [104]. Consistent with the association found between tumor p-Akt expression and gefitinib response, is mechanistic evidence that signaling through the antiapoptotic STAT/Akt may be an important downstream pathway of activation for mutated EGFR [120]. Parallels may be drawn with the clinical effectiveness of another TKI, imatinib mesylate, in chronic myeloid leukemia and gastrointestinal stromal tumors expressing activating c-Kit mutations, in which constitutive STAT activation is frequent. The story in lung cancer may be more complex, since several EGFR signaling pathway genes have been found to be mutated in NSCLC. EGFR and KRAS mutations are detected in ~10 % and 15–20 % of NSCLCs, respectively. Somatic mutations are much less common in ERBB2 (~2 %; exons 19 and 20) and HER4 (~2 %, exons 20, 23), the lipid kinase PIK3CA (~4 %; exon 9), and the serine/threonine kinase BRAF (~2 %; exons 11 and 15) [121]. Other rare mutations remain to be discovered for example in FGFR4 [121]. Most of these alterations have been found to be gain-of-function mutations and are not usually associated with co-mutations in the other genes apart from PIK3CA mutations.

There is evidence that some cancers may become dependent upon certain growth pathways or upon specific components of the pathway, possibly to the extent of requiring their signaling to maintain the malignant phenotype. This phenomenon has been described as a state of oncogene addiction of cancer cells. Thus, lung cancers most dependent upon signaling through EGFR and its downstream cascade may be more susceptible to drugs targeted to this pathway. Clinical responses to the EGFR TKIs, gefitinib and erlotinib, have been shown to be largely independent of expression of EGFR, but were discovered to be strongly associated with the presence of mutations in the EGFR tyrosine kinase domain [103–105] and possibly gene amplification [122–124]. Second-generation agents, such as afatinib, are also effective GFR TKIs, and have distinguishing properties such as being irreversible inhibitors.

Some experimental molecular studies provide evidence that oncogenic kinases produce both pro-survival and pro-apoptotic signals that decay at different rates upon

oncogene inactivation; pro-survival signals are rapidly attenuated, whereas pro-apoptotic signals are relatively longer-lived. This differential signal decay creates a temporal window during which pro-apoptotic outputs from the oncogenic kinase predominate to actively promote tumor cell death upon kinase inhibition, known as oncogenic shock [101, 125].

28.3.3 EML4/ALK Fusion Genes

Chromosomal rearrangements involving the tyrosine kinase anaplastic lymphoma kinase (*ALK*) occur in cancers including NSCLC. *EML4-ALK* fusion was first discovered by screening a cDNA library derived from the tumor of a Japanese male patient with adenocarcinoma of the lung [126]. The *EML4-ALK* fusion represents an inversion on the short arm of chromosome 2 (Inv(2)(p21p23)) that joins exons 1–13 of EML4 (echinoderm microtubule associated protein-like 4) to exons 20–29 of ALK [126], resulting in a chimeric protein consisting of an EML4 N-terminus and an ALK C-terminus.

Since then, multiple other variants of EML-ALK have been reported, all of which encode the same cytoplasmic portion of *ALK* but contain different truncations of EML4 as well as rarer fusions of *ALK* with other partners such as *TFG* and *KIF5B* [127]. The aberrant activation of ALK signaling is consistent with the notion of oncogene addiction as it is associated with marked sensitivity to ALK inhibitors such as crizotinib [128, 129]. Understanding the role ALK fusion genes play in lung carcinogenesis has meant a relatively rapid translation of ALK-fusion targeted treatment to the clinic for the genetically definable subset of affected patients representing a few percent of NSCLC tumors and more common in female never smokers. Like EGFR mutations, these aberrations are the so-called actionable mutations which enable the concept of precision medicine to be realized.

28.3.4 ROS1 Fusion Genes

Another gene that has recently been discovered to be rearranged in a subset of NSCLC is ROS1, an orphan receptor tyrosine kinase which is phylogenetically related to ALK [130]. Like tumors with ALK1 fusion gene drivers, tumors with ROS1 fusions also appear more common in never smokers and are also sensitive to crizotinib [131].

28.3.5 RAS Signal Transduction Pathway

The RAS gene family represents oncogenes which are important in a subset of lung cancers. RAS genes encode 21 kDa proteins, members of a large family of proteins including rho, rac, rab that regulate cytoskeletal changes, vesicular and nuclear transport, and proliferation. Mutations in the *RAS* genes are common in several cancers, including lung cancer [132]. The RAS proto-oncogene family (KRAS, HRAS, and NRAS) is usually activated by point mutations at codons 12, 13, or 61. Mutations affect 15–20 % of all NSCLC and approximately 20–30 % of lung adenocarcinomas but are very uncommon in SCLC [133]. Mutations in KRAS account for approximately 90 % of the RAS mutations in lung adenocarcinomas, and 85 % of KRAS changes involve codon 12. In unstimulated cells, RAS is inactive. Following ligand binding, receptor tyrosine kinases signal the RAS protein by interaction with downstream molecules such as GRB2 and the guanine nucleotide exchange factor, SOS. Wild-type CDKN1A-RAS protein is able to bind to guanosine triphosphate (GTP) but also has intrinsic GTPase activity which can hydrolyze bound GTP to guanosine diphosphate (GDP). Active GTP-bound RAS stimulates a downstream cascade ending with MAP kinase which migrates to the cell nucleus and subsequently activates various transcription factors. When GTP is hydrolyzed to GDP, the molecule assumes its inactive configuration and the signal transduction pathway returns to its inactive state. When a RAS gene undergoes oncogenic missense mutations, the mutant CDKN1A-RAS oncoprotein loses its capability to hydrolyze GTP and the molecule can no longer switch back to its inactive configuration. The resultant inappropriate growth signal to the cell nucleus is thought to contribute to unrestrained cellular proliferation; effectively causing a gain of function. This model underscores the general concept that proto-oncogenes encode proteins with important regulatory functions and that oncogenic activation results in mutant proteins with altered function.

The majority (70 %) of KRAS codon 12 mutations are G-T transversions, with either cysteine (TGT) or valine (GTT) replacing the wild-type glycine (GGT). This type of mutation also affects the TP53 gene in lung cancer cells, and represents the type of DNA damage expected from bulky DNA adducts caused by the polycyclic hydrocarbons and nitrosamines in tobacco smoke [134]. The correlation of KRAS mutations with a smoking history further implicates a causative role of tobacco smoke carcinogens in the acquisition of these mutations [135]. KRAS mutations are associated with distinct clinical characteristics in patients with NSCLC. They occur more frequently in lung adenocarcinomas from former or current smokers [136]. Furthermore in early stage lung cancer, the presence of RAS mutations is predictive of early relapse [137]. The relationship of KRAS mutations to a poor prognosis in both early and late stage NSCLC is debated and may be technique related [138–146]. Although it has also been suggested that RAS mutations induce resistance to chemotherapy and radiation, no association between RAS mutation and in vitro resistance against a

range of chemotherapeutic agents was found in a panel of NSCLC cell lines [147]. In lung adenocarcinomas the presence of KRAS mutations predict resistance to anti-EGFR therapies (gefitinib and erlotinib) [148]. Consequently in many centers routine testing for KRAS mutations in lung adenocarcinomas is performed to predict response to anti-EGFR therapy [149]. The reason for the predilection of RAS mutations for the adenocarcinoma histological subtype of lung cancers is unclear. One possible explanation is that the activation of certain oncogenes may result in disparate tumor differentiation pathways. To study this, various oncogenes have been introduced into a non-tumorigenic cell line BEAS-2B, derived from normal bronchial epithelial cells transfected with SV40. Overexpression of c-myc and c-raf-1 for instance resulted in the development of large tumor cells with certain neuroendocrine markers [150]. Indeed, the subtype specificity may be even more intriguing as a study has reported that KRAS mutations were seen in parenchymal, but not in bronchial adenocarcinomas, indicating genetic heterogeneity [151]. Furthermore, the goblet-cell subtype appears to have the highest frequency of KRAS mutations compared with other adenocarcinoma subtypes [152]. Some investigators have also suggested that rare alleles of the HRAS minisatellite locus represent a major risk factor for common types of cancer, including lung cancers, but this possibility has not been firmly established [153]. Considerable attention has been directed towards the development of RAS inhibitors, however to date none has achieved clinical utility [149]. Consequently, novel approaches to block posttranslational activation of RAS have been pursued, such as farnesyl transferase inhibitors and other inhibitors of downstream RAS effectors [149].

Another proto-oncogene, BRAF, which encodes a direct downstream effector of RAS may also be relevant to lung carcinogenesis. BRAF somatic missense mutations are common in malignant melanomas and are occasionally found in a range of human cancers. In lung cancer perhaps 3 % of NSCLC may have mutated BRAF1 [154–156]. Most melanoma BRAF mutations involve codon 599, but NSCLC BRAF mutations are different, most being non-V599. This may result in therapeutic differences between lung cancer and melanoma in response to RAF inhibitors, which are now subject to clinical trial.

The other signal transduction molecules downstream of RAF in this pathway include MEK (MAP kinase/ERK-activating kinase), and ERK (extracellular signal-regulated kinase) as well as their regulatory phosphatases (such as PP2A). Although constitutively active mutants of MEK were shown to be capable of transforming cells suggesting that MEK can function as a dominant oncogene, studies have shown that the MAP2K1 and MAP2K2 genes are only rarely mutated in lung cancer [157]. On the other hand, the mitogen-activated protein kinase kinase 4 (MKK4) gene, located approximately 10 cM centromeric of TP53 on 17p, has been found to be homozygously deleted in a NSCLC cell line, leading to speculation that it may be a candidate tumor suppressor [158].

28.3.6 FGFR1

Overall, unlike adenocarcinomas which have a number of actionable mutations (EGFR mutations, ALK and ROS1 fusions), these potential targets appear less common in the other major NSCLC subtype, SCC. Recently, focal fibroblast growth factor receptor 1 (FGFR1) amplification was discovered in squamous cell lung cancer ($n = 155$), and appears to be a relatively frequent finding (up to 22 % in an independent cohort) [159].

28.3.7 MYC

Stimulation of the RAS signal transduction cascade ultimately activates nuclear proto-oncogene products, including MYC which belongs to the basic helix–loop–helix leucine zipper (bHLH-LZ) class of transcription factors. MYC has been implicated in normal cell growth and proliferation through interaction with genes involved in DNA synthesis, RNA metabolism, and cell-cycle progression [160]. MYC proto-oncogenes are the cellular homologs of a gene present in several highly oncogenic avian retroviruses. Of the well-characterized myc genes, MYC is the most frequently activated in SCLC and NSCLC. On the other hand, its closely related cellular homologues, MYCN and MYCL1, are usually only activated in the SCLC subtype. In fact, MYCL1 was initially isolated from the DNA of a SCLC [161]. Activation of the myc genes has been observed by gene amplification or transcriptional dysregulation, leading to protein overexpression [162]. These genes may be amplified to 20–115 copies per cell, and in most cases, only one member of the MYC family is amplified. Gene amplification is generally associated with enhanced mRNA expression and increased protein production. A review of 17 different studies calculated that 36 of 200 (18 %) SCLC tumors and 38 of 122 (31 %) SCLC cell lines had gene amplification of one member of the MYC family [133]. In comparison, 25 of 320 (8 %) NSCLC tumors and 3 of 15 (20 %) NSCLC cell lines had MYC amplification. Thus, MYC family activation in general appears to be more frequent in SCLC than NSCLC. MYC amplification appeared to occur more frequently in cell lines which are often derived from metastatic lesions than in primary tumors, in patients previously treated with chemotherapy, and in the variant subtype SCLC [163]. These observations may help explain why MYC amplification has been reported to correlate with adverse survival.

Rather than the translocation and point mutations seen in lymphomas, there have been reports of MYCL1 amplification with rearrangement in which MYCL1 fuses to the RLF gene, thereby resulting in a chimeric protein [164, 165]. In some cases, MYCL1 expression may be associated with neuroendocrine differentiation. For instance, all-trans-retinoic acid mediated growth inhibition in a SCLC cell line was associated with increased neuroendocrine differentiation and MYCL1 expression but decreased MYC expression [166].

28.3.8 Other Nuclear Proto-oncogenes

MYB, JUN, and FOS have also been implicated in lung cancer although their precise functional importance and biological role is still being investigated. JUN and FOS are heterodimeric proteins that function as immediate early transcription factors regulating cellular proliferation. There is conflicting data in lung cancer regarding their role in lung carcinogenesis. Some investigators have described higher expression of these genes in normal lung tissue adjacent to tumor than in the tumor itself. Conversely, other studies have reported higher expression in tumors with lack of expression in normal epithelium [167–170].

28.3.9 Oncogenes and Growth Stimulatory Loops

The hepatocyte growth factor/scatter factor (HGF/SF) stimulates epithelial cells to proliferate, move and also carry out complex differentiation programs, such as morphogenesis and angiogenesis. HGF appears be a potent mitogen for normal and neoplastic bronchial epithelium [171]. During lung development, HGF levels increase during postnatal lung maturation and its receptors are expressed on bronchial and alveolar type II cells. HGF is involved in embryonal lung budding and branching, and stimulates mitogenesis and/or motogenesis of human bronchial epithelial, and alveolar type II, and SCLC cells in vitro. HGF is expressed at very low levels in normal lung but these levels increase in response to local lung or distant injury [172]. The MET proto-oncogene which encodes the HGF receptor was generally expressed in normal lung, as well as in SCLC and NSCLC. On the other hand, HGF was expressed in many NSCLCs but not in SCLCs, thereby indicative of an autocrine loop in the former [173–175]. A study of resected lung cancer tissue showed MET expression in 34 of 47 adenocarcinomas and 20 of 52 squamous carcinomas by western blotting and immunohistochemistry [176]. This study also suggested a poorer prognosis for tumors expressing the receptor especially for adenocarcinomas. Western blotting of proteins extracted from 56 NSCLCs (predominantly adenocarcinomas) using a polyclonal anti-HGF showed that high levels of immunoreactive HGF were associated with poorer survival for stage I tumors [177]. Recently, a gefitinib-sensitive lung cancer cell line that developed resistance to gefitinib as a result of focal amplification of the MET proto-oncogene was reported, also MET amplification was detected in 4 of 18 (22 %) lung cancer specimens that had developed resistance to gefitinib or erlotinib [178]. This suggests that MET amplification causes gefitinib resistance by driving ERBB3 (HER3)–dependent activation of PI3K, a pathway thought to be specific to EGFR/ERBB family receptors.

Another autocrine growth loop may involve the insulin-like growth factors, IGF-I and IGF-2, and the type I IGF receptor, IGF-R, which are frequently co-expressed in both SCLC and NSCLC [179]. Of the insulin-like growth factor family, it appears that IGF-2 may be the predominant member involved with the autocrine growth stimulation of lung cancer. The KIT proto-oncogene which encodes a tyrosine kinase receptor and its ligand, stem cell factor (SCF), are co-expressed in many SCLCs, and may thus represent another autocrine loop for lung cancers [180, 181]. In SCLC, activation of this putative SCF/KIT autocrine loop could conceivably provide a growth advantage or mediate chemoattraction. Unlike gastrointestinal stromal tumors, mutations in KIT are rare, although KIT expression can be demonstrated in SCLCs [182–184]. Platelet-derived growth factor (PDGFB), which is the proto-oncogene counterpart of v-sis, and its receptor (PDGFBR) were also found to be co-expressed in lung cancer, generating another potential autocrine loop [185].

In essence, many proto-oncogenes encode growth factors, regulatory peptides or their receptors and are expressed by lung cancer cells or adjacent normal cells, thus providing a number of autocrine or paracrine growth stimulatory loops [186]. Other autocrine systems not obviously involving established proto-oncogenes also exist. In fact, the autocrine loop comprising gastrin-releasing peptide, other bombesin-like peptides (GRP/BN) and their receptors is arguably the best characterized growth stimulatory loop in lung cancer. GRP/BN has been associated with many physiologic effects including regulation of secretion, growth, and neuromodulation. There are three human GRP/BN receptor subtypes which belong to the G-protein coupled receptor superfamily with seven predicted transmembrane domains [187]. The cellular responses of SCLC to GRP/BN stimulation have been extensively studied [188]. Preliminary data also suggests that GRP/BN may regulate the MAP kinase Cascade, at least in certain tumor cells [189]. IHC studies showed that approximately 20–60 % of SCLC cancers expressed GRP, while NSCLCs expressed GRP less frequently [133]. In comparison, expression of the three GRP/BN receptors is widespread in both SCLC and NSCLC cell lines, with most cell lines expressing at least one of the three receptors and many cell lines expressing more than one receptor [187].

28.3.10 BCL2 Proto-oncogene and Apoptosis

Tumor cells can acquire the ability to escape apoptotic pathway by which normal cells would usually undergo programmed cell death (apoptosis) in response to appropriate conditions such as DNA damage. A large and growing number of apoptosis regulatory gene products are classifiable into cell death agonists (Bax, Bak, Bcl-XS, Bad, Bid, Bik, Hrk) or antagonists (BCL2, Bcl-XL, Bcl-w, Bfl-1, Brag-1, Mcl-1 and A1) [190]. Two key members of the apoptotic pathway are the BCL2 proto-oncogene product and the TP53 tumor suppressor gene product. BCL2 antagonizes the induction of programmed cell death by TP53. By protecting cells from the apoptotic process, BCL2 probably plays a role in determining the chemotherapy response through repression of apoptosis in cancer cells. A BCL2-transfected human SCLC cell line showed higher resistance to some anticancer agents by inhibiting apoptosis [191], and SCLC cells transfected with antisense oligodeoxynucleotides to BCL2 mRNA showed reduced cell viability with decreased BCL2 levels facilitating apoptosis [192]. BCL2 expression may correlate with neuroendocrine differentiation [193]. BCL2 expression is also relatively higher in squamous cell carcinoma (25–35 %) than in adenocarcinoma (~10 %) [193–196]. An inverse relationship between immunohistochemical BCL2 expression and abnormal TP53 expression in resected NSCLCs has been reported [197, 198]. These results suggest the hypothesis that either TP53 mutation or upregulation of BCL2 expression is sufficient to modify the apoptotic pathway in NSCLC, and that BCL2 positive tumors may show less aggressive behavior [197]. In contrast, SCLCs usually have both TP53 mutations and BCL2 overexpression. Previous IHC studies of BCL2 in lung cancer have shown highest expression in small cell cancers. For instance BCL2 protein is immunohistochemically expressed in most SCLCs (75–95 %) [193, 199, 200]. This observation initially seems inconsistent with the finding that SCLCs are often much more sensitive than NSCLCs to chemotherapy; a situation in which tumor death usually occurs by apoptosis. In addition, there was a paradoxical trend, albeit nonstatistically significant, towards longer survival in patients whose SCLC tumors express BCL2 [200]. Likewise, a better survival of BCL2 positive lung cancer cases was observed in NSCLC [194–197]. Thus the role of BCL2 in lung cancer is complex and likely to further unfold. Notably, the suggestion that BCL2 may be converted to BAX-like death effectors by the caspase family of cysteine proteases may be relevant [201]. Alternatively, increased BCL2 immunoreactivity may possibly indicate reduced rather than enhanced function, similar to the situation of overexpression of nonfunctional mutated TP53. A BCL2 related protein called BAX promotes apoptosis and may act as a tumor suppressor [202]. Furthermore, BAX may be a downstream transcription target of the TP53 pathway. BAX complexes with BCL2 to form homodimers or heterodimers, and it has been suggested that the BCL2–BAX ratio determines cellular apoptotic susceptibility. For instance, the immunohistochemical staining of BAX and BCL2 were inversely related in 121 neuroendocrine (NE) lung cancers. Most carcinoids showed low BCL2 and high BAX expression in contrast to the inverse situation in most SCLCs and large cell NE cancers [203]. This would potentially lead to a higher degree of apoptosis in carcinoids compared to SCLCs, correlating with clinical behavior where carcinoids are markedly less metastatic than SCLC.

28.4 Tumor Suppressor Genes and Growth Inhibition

28.4.1 Genetic Loss in Lung Cancer

Apart from aneuploidy, lung cancer cells are also characterized by many other structural cytogenetic abnormalities including deletions and nonreciprocal translocations. Frequent deletions of chromosome region 3p14-23 was one of the initial cytogenetic observations made in SCLC, this finding was later found to be also applicable to NSCLC [204]. Subsequently, many specific deletions at a range of chromosomal regions have been revealed by cytogenetics, molecular allelotyping, and chromosomal and array CGH, data which suggests the presence of underlying tumor suppressor genes in lung cancers.

TSG (e.g., TP53, RB1) products are negative growth regulators. It is their loss of function, classically by loss of one allele and point mutation of the other allele, which contributes to malignant transformation. The requirement for the mutations to affect both alleles of the tumor suppressor gene represents the two-hit hypothesis initially proposed by Knudson for retinoblastomas. Using allelotyping, chromosomal regions 1p, 1q, 3p (several sites), 5q (APC/MCC cluster), 8p, 9p21 (CDKN2A), 11p13, 11p15, 13q14 (RB1), 17p13 (TP53), and 22q as well as several other sites have been found to be frequently involved in lung cancer cells or cell lines derived from lung cancer tissues [205]. This leads to the notion that if most of these sites encode tumor suppressor genes, then individual tumors must have acquired inactivation of multiple genes to become clinically evident. Some of these are common to both SCLC and NSCLC, and some are more frequent in a given histologic type. The best characterized of these deletions appear to target genes which are now accepted as classical tumor suppressor genes such as TP53, RB1, and CDKN2A. On the other hand, there is also mounting evidence that there may be other closely situated genes that are also affected by the chromosomal deletions, particularly as some of these deletions may be quite large and even involve the whole chromosomal arm or chromosome.

28.4.2 TP53

Mutations in the TP53 gene are the most common genetic alteration found in human cancers. The TP53 gene encodes a protein that functions as a transcription factor, particularly in response to DNA damage by γ or ultraviolet irradiation and carcinogens [206]. It has been called the guardian of the genome and the guardian of the G1 checkpoint. TP53 is believed to play a major role in maintaining the integrity of the genome since loss of TP53 function allows inappropriate survival of genetically damaged cells, leading to the evolution of a cancer cell. DNA damage is a major upstream event in TP53 activation and results in a rapid increase in the level of TP53 protein, and activation of TP53 as a sequence specific transcription factor regulating expression of downstream genes. The net effect is either a stop to cell cycle progression to permit repair, or apoptosis if the damage is too great. TP53 can itself detect and bind sites of primary DNA damage, using its C-terminal domain. Hypoxia is also able to stimulate TP53 levels and lead to apoptotic cell death [207]. Oxygen delivery and blood supply becomes rate-limiting when a tumor reaches a critical size and tumor. Hypoxia may thus act as a physiological selective agent against apoptosis-competent cells in tumors. On the other hand, such selection may allow for the expansion of clones with acquired defects in their apoptotic program genes. The genes downstream of TP53 include CDKN1A (also called WAF1/CIP1), MDM2, GADD45A (growth arrest and DNA damage-inducible), BAX, IGF-BP2, and cyclin G, which participate in controlling cell cycle arrest at the G1/S phase transition and apoptosis. A link between mutant TP53 and aneuploidy has been revealed by studies implicating TP53 as an active component of a mitotic spindle checkpoint and as a regulator of centrosome function. Thus, TP53 appears to participate in the DNA damage checkpoints of the cell cycle at both the G1/S transition and at the G2/M boundary.

The TP53 gene plays a critical role in lung cancer as well as in many other types of cancers, and genomic interrogation indicates consistent features across tumor types [208]. Many somatic TP53 mutations in human tumors and cell lines have been published, and compiled into large databases. In both SCLC and NSCLC, one copy of the chromosomal region 17p13 which contains TP53 is frequently deleted; one hit. Structural abnormalities in TP53 and also p16 occur commonly in lung cancer, as shown by structural aberrations on FISH analysis (especially breaks and loss) [209]. Mutational inactivation of the remaining allele, the second hit, occurs in 75–100 % of SCLCs and ~50 % of NSCLCs [134], leading to loss of TP53 function. Although found throughout the entire coding region, TP53 mutations in lung cancer are most common in the evolutionarily conserved exons 5–8. The types of TP53 mutations include missense and nonsense mutations, splicing abnormalities, as well as larger deletions. Evidence supporting a causative role for tobacco smoke in inducing TP53 mutations comes from observing that TP53 mutations in lung tumors correlate with cigarette smoking, and that the most common TP53 mutations in lung cancer are the G-T transversions expected from tobacco smoke carcinogens [134]. Additional evidence for a pulmonary oncogenic role for TP53 dysfunction comes from the finding that transgenic mutant TP53 mice develop lung cancers in addition to bone and lymphoid tumors [210]. Furthermore, reintroducing a wild-type TP53 gene into lung cancer cells dramatically blocked tumor cell growth due to apoptosis (not G1 arrest) despite concurrent abnormalities of several other tumor suppressor genes and oncogenes [211, 212].

Many TP53 mutations are missense mutations which prolong the half-life of the TP53 protein to several hours, leading to increased protein levels which can be detected by immunohistochemistry as a surrogate for molecular analysis [213]. IHC studies have shown abnormal TP53 expression in 40–70 % of SCLCs and 40–60 % of NSCLCs [203, 214–217]. Most studies have shown that the frequency of TP53 overexpression is higher in squamous cell carcinomas than in adenocarcinomas. The predictive value of TP53 mutations for survival, whether assayed by immunohistochemistry or by molecular analysis, is controversial. A summary of 14 studies of the prognostic importance of TP53 mutations or overexpression in NSCLC (mutational analysis (4 studies); immunostaining (8 studies) and both techniques (2 studies) yielded controversial results [218]. TP53 mutations predicted shortened survival in half of the four reported mutational analyses, whereas the other two found no such difference. Of the 10 IHC studies, aberrant TP53 expression was associated with a shortened survival in five studies, an improved survival in three studies, and no survival effect in two studies. In one study that simultaneously analyzed both mutations and protein expression, TP53 overexpression but not gene mutation predicted shortened survival. Perhaps the various TP53 mutants or types of wild-type TP53 overexpression have different effects on lung cancer behavior. Alternatively, wild-type TP53 expression may be immunohistochemically detectable in certain tumors. Finally, different antibodies which may not be strictly comparable for detecting aberrant TP53 expression have often been used.

In certain cancers, such as that of the uterine cervix, TP53 can be alternatively inactivated through binding of the oncogenic E6 protein of human papilloma virus (HPV) to the TP53 protein; a process which inactivates its tumor suppressor activity by promoting TP53 degradation. The epitheliotropic HPV may also be involved in some respiratory tract lesions, for example, HPV subtypes 6 and 11 have been associated with most cases of tracheal and bronchial papillomatosis. While there are reports of neoplastic transformation of these benign papilloma, it has also been suggested that HPV may also play a part in the development of de novo

bronchogenic carcinomas [219–221]. Morphological studies have shown occasional presence of HPV-suggestive lesions in primary squamous cell carcinomas, and DNA hybridization studies to detect HPV DNA in lung cancers show conflicting results, ranging from 0 to 40 % [222–224]. Most PCR studies looking for HPV sequences suggest that any potential involvement of HPV in primary lung cancer is likely to be limited [225–228] although some investigators have found more frequent involvement using in-situ hybridization and PCR [229].

CDKN1A, also known as WAF1 (wild-type TP53-activated fragment 1), CIP1, or p21, is a TP53-responsive gene, which inhibits cyclin/cyclin dependent kinase complexes in the G1 phase of the cell cycle as well as proliferating cell nuclear antigen (PCNA). Although not somatically mutated in lung cancer [230], CDKN1A RNA and protein overexpression was seen in ~65 % of NSCLC cases, especially in well differentiated tumors. This high frequency suggests that CDKN1A can be expressed independently of TP53 gene/protein alterations which are so frequent in lung cancers [231]. A case control study has suggested that a C to A codon 31 polymorphism (ser->arg) in CDKN1A is associated with the development of lung cancer [232].

MDM2 is an oncoprotein that can inhibit both TP53 and RB1. By binding its transcriptional activation domain, MDM blocks the ability of TP53 to regulate target genes. It also causes rapid reduction of TP53 levels through enhanced proteasome-dependent degradation. Conversely, TP53 activates the expression of the MDM2 gene in an autoregulatory feedback loop. However, phosphorylation of TP53 by DNA-dependent protein kinase (DNA-PK) after DNA damage leads to reduced interaction of TP53 with MDM2, most likely due to a TP53 conformational change [233]. In some human sarcomas and brain tumors, the chromosome 12q MDM2 gene is amplified and its protein overexpressed. In lung cancer MDM2 gene amplification was only detected in 2 of 30 NSCLCs. Nonetheless, these investigators also found MDM2 protein expression in 48 of 201 NSCLCs by immunohistochemistry and suggested that MDM2 expression without abnormal TP53 expression was a favorable prognostic factor [234]. Sp1 can further activate MDM2 and repress TP53, and this is associated with overexpression of DNA 5′-cytosine-methyltransferase1 (DNMT1), which can epigenetically dysregulate tumor suppressor genes [235].

A gene encoding TP73, a protein that shares considerable homology with TP53 was mapped to 1p36 [236]. This is of interest as 1p36 is also a site of frequent allelic deletion in lung and other cancer cells [205]. Although TP73 mutations were not detected in neuroblastomas despite frequent LOH, TP73 can activate the transcription of TP53-responsive genes and inhibit cell growth in a TP53-like manner by inducing apoptosis [237].

28.4.3 The RB1/Cyclin D1/CDK4/CDKN2A Pathway

28.4.3.1 The Retinoblastoma (RB1) Gene

The retinoblastoma (RB1) gene located in chromosomal region 13q14 encodes a nuclear phosphoprotein that was initially identified as a tumor suppressor gene in retinoblastomas. The RB1/cyclin D1/CDK4/CDKN2A pathway is central to the regulation of the G1 to S phase transition of the cell cycle. Hypophosphorylated RB1 binds and controls other cellular proteins including the transcription factor E2F which is essential for the G1/S phase transition when bound to E2F sites in cooperation with the DP family of transcription factors. Transcriptional activation is mediated by free E2F whereas hypophosphorylated RB1 antagonizes heterodimers formed by E2F and DP, thereby resulting in inhibition of S phase entry. Cyclin D1/cyclin dependent kinase 4 (CDK4) and other cyclin/CDK complexes phosphorylate RB1 with subsequent loss of its binding pocket activity that is needed to sequester the transcription factors, thereby releasing E2F and allowing entry into S phase. It is becoming more apparent that one of the four genes responsible for RB1/cyclin D1/CDK4/CDKN2A pathway is mutated or functionally altered in many cancers including lung cancers. It has also been shown that the RAS signaling pathway may functionally link to cell cycle regulation by RB1 [238]. Furthermore, RB1 also appears to have other functions; it can for instance repress transcription of all three nuclear RNA polymerases classes (Pol I, Pol II, and Pol III) [239]. RB1 also appears to inhibit apoptosis and may be actively involved in induction of differentiation. In terms of interacting partners for RB1, overexpression of E2F1 or E2F1/TFDP1 cooperates with activated ras in fibroblast transformation assays, and these transformed cells can form tumors in nude mice. Studies of E2F1 deficient transgenic mice have however suggested that E2F1 may also have a tumor-suppression function since mice lacking E2F1 developed a broad spectrum of tumors including highly invasive lung adenocarcinomas [240]. Nonetheless, studies of E2F1 in lung cancer cells have not yet been reported.

RB1 mutations together with loss of the wild-type allele have been consistently demonstrated in lung cancers [241, 242]. The RB1 protein is abnormal in over 90 % of SCLCs and 15–30 % of NSCLCs [243–245]. RB1 mutations in lung cancers include truncation by deletions, nonsense mutations, or splicing abnormalities. There are only a few studies of RB1 point mutations in lung cancer at least partly due to its 200 kb genomic size and 27 exon structure. However, from these limited studies, most mutations result in RB1 truncation [246], although a rare missense mutation in the RB1 pocket domain has been shown to cause defective RB1 phosphorylation and binding to oncoproteins [247]. The

sensitivity for detecting RB1 abnormalities in SCLCs varies by detection method: ~20 % by Southern blot analysis detecting band loss; ~60 % by Northern blot analysis detecting absent or abnormal RNA; and ~90 % by protein or immunohistochemical (IHC) analysis. Thus RB1 abnormalities are very frequent in SCLC, particularly in comparison to NSCLC. In NSCLC, a large study showed RB1 abnormalities in 2/219 (0.9 %) by Southern analysis, 22/219 (10 %) by Northern analysis, and 53/163 (32 %) by IHC [243]. The absence of RB1 expression was associated with poor prognosis in NSCLCs, particularly stage I and II disease, in some but not all studies [245, 248–250]. On the other hand, the observation of frequent LOH on chromosome 13q but relatively less frequent RB1 inactivation in NSCLCs, have prompted the notion that the allele loss on 13q targets other tumor suppressor genes on this chromosome apart from RB1 [251].

The relatives of retinoblastoma patients who are also germ line carriers of an RB1 mutation, are about 15 times more likely to die from lung cancer than the general population [252]. Furthermore, it has been shown that reintroduction of a wild-type RB1 gene led to growth suppression of SCLC cells [253]. There are two other members of the RB1 gene family, p107 and pRB2/p130, which are structurally and functionally related genes. They have also been implicated in lung cancer to a limited extent [254]. One SCLC cell line was shown to have a point mutation of p130 in a splice acceptor site leading to loss of exon 2 and production of a truncated p130 protein [255].

28.4.3.2 Cyclin D1 and CDK4

The relatively infrequent involvement of RB1 in NSCLC compared to SCLC suggested that alternative members of this growth suppressive pathway might be affected. As cyclin D1 inhibits the activity of RB1 by stimulating its phosphorylation by CDK4, cyclin D1 overexpression was an attractive candidate for disrupting the RB1 growth control pathway. In keeping with this, cyclin D1 was found to be overexpressed in some NSCLCs with normal RB1 protein expression [256, 257]. Cyclin D1 is encoded by the CCND1 (also known as PRAD1 or BCL1) proto-oncogene which is situated on chromosome 11q13. Compared with an immortalized bronchoepithelial cell line, cyclin D1 was overexpressed by several to 100-fold in 11/12 NSCLC cell lines [256]. Amplification of cyclin D1 was detected in 15 % and overexpression in 47 % of 53 primary NSCLC tumors [257]. Cyclin D1 immunohistochemical overexpression has been reported to correlate with Ki67 labeling and with patient survival [258, 259]. Abnormal immunostaining of CCND1 and RB1 has also been frequently seen in epithelial cells from the resection margin of lung cancers, raising speculation that these changes may be relatively early events in lung carcinogenesis [260]. Although gene amplification of CDK4 has been reported in 10–15 % of malignant gliomas and certain other malignancies, its role in lung cancer has not yet been reported. Interestingly, the chromosome 12q13-15 region which harbors the CDK4 gene also contains the MDM2 locus. While CDK4 and MDM2 often show co-amplification in sarcomas and glioblastomas, some tumors show only CDK4 amplification but not MDM2 amplification, and vice versa, indicating that each gene is an independent amplification target [261].

28.4.3.3 CDKN2A and Other CDK Inhibitor Genes

The CDKN2A protein functions as a cell cycle modulator which appears to regulate RB1 function by inhibiting CDK4:cyclin D1 kinase activity, and represents another important genetic target for disrupting the RB1/cyclin D1/CDK4/CDKN2A pathway. The CDKN2A (or MTS1) tumor suppressor gene is situated at chromosome 9CDKN1A. The short arm of chromosome 9, including 9CDKN1A frequently undergoes allele loss and mutation in a variety of human cancers including lung cancer [262–265]. A summary of a wide variety of cancers identified several mutational hot spots (point and other mutations including deletions, insertions and splice mutations), including some at conserved residues within the ankyrin domains of CDKN2A [266].

CDKN2A abnormalities have been extensively reported in lung cancer and are found frequently in NSCLC but rarely in SCLC. Homozygous deletion or point mutations have been observed in 10–40 % of NSCLCs [267–275]. Absent expression of CDKN2A was detected by Northern blot, Western blot or IHC analyses in 30–70 % of NSCLC. Epigenetic hypermethylation of 5′ CpG islands cause the functional downregulation of CDKN2A in lung cancers carrying no genetic mutations of CDKN2A [276, 277]. The multiple mechanisms of CDKN2A inactivation may account for the relatively low rates of inactivating deletions and point mutations seen in the earlier genetic studies [278]. Since higher frequencies of deletions or mutations have been observed in cultured cell lines and metastatic sites compared to primary lesions, it has been suggested that CDKN2A mutations may be associated with tumor progression and more advanced lung cancer [279]. While an IHC study of primary NSCLCs demonstrated an association of CDKN2A -negativity with more advanced with clinical stage [250], this finding was not confirmed in another study [280]. Nevertheless, both studies showed that about 30–40 % of early stage primary NSCLCs had absent CDKN2A expression. Finally, there is conflicting data as to whether the absence of CDKN2A expression is a predictor of adverse survival in NSCLC [250, 281]. CDKN2A abnormalities are perhaps the most common mechanism for inactivating the RB1/cyclin D1/CDK4/CDKN2A cell cycle control pathway in NSCLC. Conversely, direct RB1 inactivation appears to be the preferred mechanism in SCLC. Consequently, lung cancers are in general character-

ized by either RB1 inactivation (~90 % of SCLC and 15–30 % of NSCLC) or CDKN2A inactivation (30–70 % of NSCLC); either scenario leading to loss of this growth inhibitory pathway. The apparently mutually exclusive inactivation of either RB1 or CDKN2A has been well documented in NSCLC case series [250, 280, 282–286]. Furthermore, the simultaneous inactivation of both RB1 and CDKN2A is uncommon, but cyclin D1 overexpression can coexist with each of these abnormalities [278]. It is also noteworthy that a significant proportion of NSCLCs (10–30 %) appear to be normal for both RB1 and CDKN2A, thereby implicating cyclin D1 and CDK4 alterations.

The CDKN2A locus also encodes a second protein product which originates from an unrelated exon of CDKN2A (exon 1b) spliced onto exon 2 in an alternate reading frame (human p16b, murine p19ARF) [287]. Thus, exon 2 which is often deleted or mutated in NSCLC, is common to both CDKN2A and p19ARF. Intriguingly, mice lacking p19ARF but expressing functional CDKN2A were prone to tumor development, possibly through the TP53 pathway as TP53-negative cell lines were resistant to p19ARF-induced growth arrest [288]. Thus the extent of p19ARF damage through deletions and mutations to the CDKN2A locus, and its possible contribution to human lung carcinogenesis requires further investigation.

There are a number of other CDK inhibitor genes including CDKN2B, CDKN2C, CDKN2D, CDKN1A, CDKN1B, and CDKN1C. However, apart from CDKN2B, mutational analyses of these genes have not detected significant genetic changes in lung cancer. CDKN2B (also called CDKN2B or MTS2) shares ~70 % amino acid similarity and is situated immediately centromeric to CDKN2A. It also functions to restrain cell growth, probably by acting as an effector of TGFβ-mediated cell cycle arrest [289]. Co-deletion of CDKN2B and CDKN2A frequently occurs in NSCLC but point mutations targeting CDKN2B itself appear to be uncommon. The CDKN2C and CDKN2D genes have not been shown to be mutated in lung cancer [290]. Similarly, CDKN1B has not been found to be mutated in lung cancers [291], but low levels of CDKN1B protein were associated with a poor outcome in NSCLC patients [292]. The CDKN1C CDK inhibitor which maps to 11p15, is usually imprinted with expression of the maternal allele only. Thus, CDKN1C expression can be downregulated by selective loss of the maternal alleles, and this was found to occur in 11/13 lung cancer cases with LOH of 11p15 [293]. As point mutations have not been described in lung cancer, one could speculate that loss of a single allele may nonetheless represent the second hit needed to inactivate the imprinted CDKN1C gene.

28.4.3.4 Candidate 3p Tumor Suppressor Genes

It has long been known that chromosome 3p deletions occur commonly in cancers, notably lung and renal cancers. The very frequent deletion of one copy of the short arm of chromosome 3 in both SCLC (>90 %) and NSCLC (>80 %) has provided a strong basis for the hypothesis that one or more lung cancer tumor suppressor genes exist on this chromosomal arm. The karyotypes of most SCLC and many NSCLC have a del(3p) in addition to other often complex changes. These cytogenetic 3p deletions were subsequently confirmed by allelotyping which showed allelic loss not only in invasive cancers, but also in preneoplastic respiratory epithelial lesions associated with NSCLC [204, 294, 295]. The three distinct 3p regions have been identified by allelotyping are 3p25-26, 3CDKN1A.3-22, and 3p14-cen, consistent with the notion that there are probably three (or more) different tumor suppressor genes located on 3p [296]. In addition, as loss of both alleles resulting in homozygous deletions are thought to be strong markers for the locations of tumor suppressor genes, it is notable that five separate homozygously deleted regions have also been found in several lung cancer cell lines. There is one in the 3p14.2 region (FHIT gene location), one at 3p12-13 (U2020 cell line deletion) [297, 298], and three at the 3CDKN1A region.

The FHIT gene comprising 10 exons encoding a 1.1-kb transcript, maps to 3p14.2 and encompasses approximately 1 Mb of genomic DNA which includes the human common fragile site (FRA3B) and the t(3:8) translocation breakpoint of familial renal cell carcinoma. FRA3B is the most frequent of the common fragile sites which are chromosomal sites prone to breakages under stress, such as aphidicolin treatment. FHIT is a candidate tumor suppressor gene for lung cancers on the basis of frequent loss of heterozygosity in lung cancer and homozygous deletion in several lung cancer cell lines; the latter particularly affecting NSCLC [299–301]. Although reverse transcriptase PCR showed that 40–80 % of lung cancers demonstrated aberrant FHIT transcripts, these were nearly always also accompanied by wild-type FHIT transcripts [299–301]. As in other cancers, point mutations of FHIT appear to be rare in lung [299, 300], and there were initial concerns that the deletions noted merely represented the susceptibility of the FRA3B fragile site to breakages. Nevertheless, although FHIT abnormalities differ from the mutations and loss of wild-type transcript expression expected of classic tumor suppressor genes, absence of FHIT protein in primary lung tumors and cell lines correlating to DNA and/or RNA abnormalities has been detected by Western blot and immunocytochemical analyses [302]. Additionally reintroduction of exogenous wild-type FHIT suppressed tumorigenicity in nude mice of human cancer cell lines including a NSCLC cell line [303]. Furthermore, the much more frequent FHIT allele loss in lung cancers from smokers (80 %) compared to nonsmokers (22 %) suggests that this chromosomal region is selectively targeted by the carcinogens in tobacco smoke [304].

The 3CDKN1A.3 region has been extensively examined for putative tumor suppressor genes by several groups. The

finding of several homozygously deleted lung cancer cell lines suggested a role for two distinct 3CDKN1A.3 regions. One region is defined by three distinct homozygous deleted SCLC cell lines with a minimum common deleted region mapped to span 370 kb [305–307]. The other 3CDKN1A region illustrated by several homozygous deletions has an estimated ~800 kb deletion [308]. Apart from these regions, hMLH1 (the human homolog of the yeast mutL gene) also resides on chromosome 3CDKN1A and while mutations of the hMLH1 gene have been found in hereditary colon cancer, lung cancer studies have not been reported [309]. Other well characterized candidate 3CDKN1A TSGs are SEMA3B and SEMAF, RASSF1A, and FUS1 [310]. SEMA3B and SEMAF are close to the other genes and part of the semaphorin axonal guidance gene family, both encode secreted proteins [311]. RASSF1A is frequently hypermethylated in lung cancer but rarely mutated. RASSF1A is part of a complex similar to the Drosophila Hippo/Salvador/Lats tumor-suppressor network, is conserved in mammalian cells, and may be involved in controlling mitotic exit [312]. Fus1 is next to RSSF1A and while mutation of FUS1 is infrequent in lung cancers, protein under expression has been reported and exogenous overexpression of Fus1 protein inhibited colon formation in some NSCLC cell lines [313].

Other candidate 3p lung cancer suppressor genes include the von Hippel–Lindau (VHL) tumor suppressor gene at 3p25 which is frequently mutated in renal cell carcinoma but only uncommonly involved in lung cancers [314]. Abnormalities of retinoic acid receptors (RARs) have also been implicated in lung cancer pathogenesis. Several studies have indicated abnormalities of the expression or function of the RARB gene which maps to chromosome region 3p24, another site of frequent allele loss in lung cancer [315–320]. Mutational analysis however has failed to demonstrate mutations in RARB. Additionally, the TGFβ-type II receptor (TGFβ RII) gene at 3p22 is another candidate tumor suppressor gene as discussed below.

28.4.3.5 LKB1/STK11

Frequent losses of chromosome 19p in lung adenocarcinomas led to the fine mapping of the short arm of chromosome 19 and the discovery of the LKB1/STK11 gene which mapped in the minimal-deleted region [321]. Germ-line mutations at LKB1/STK11 result in the Peutz–Jeghers syndrome and an increased risk of cancer, and there is a relatively high frequency of somatic alterations (mainly nonsense mutations) in primary lung adenocarcinomas and in lung cancer cell lines [321]. These mutations may be linked to KRAS mutations and smoking and male gender in a subset of poorly differentiated lung adenocarcinomas, and are associated with transcriptional deregulation of molecules involved in signal transduction (e.g., FRAP1/mTOR, ARAF1, and ROCK2), cytoskeleton (e.g., MPP1), transcription factors (e.g., MEIS2, ATF5), metabolism of AMP (AMPD3 and APRT), and ubiquitination (e.g., USP16 and UBE2L3).

28.4.3.6 Other Candidate Tumor Suppressor Gene Locations

Apart from the known and candidate tumor suppressor gene locations discussed above, cytogenetic and allelotyping studies have shown allelic loss of many other chromosomal regions in lung cancer, thereby implicating involvement of other tumor suppressor genes. The chromosomal regions include 1p, 1q, 2q, 5q, 6p, 6q, 8p, 8q, 10q, 11p, 11q, 14q, 17q, 18q, and 22q. CGH and array CGH are helping to more precisely define these regions (Table 28.1).

Several of these chromosomal regions contain known or candidate tumor suppressor genes (such as WT1 at 11p13, DCC at 18q21, NF2 at 22q12), but these genes have not been found to be mutated in lung cancer [322, 323]. Regions on chromosome 5q, around the APC and MCC gene cluster are also frequently deleted in both subtypes of lung cancer. However, APC mutations have not been detected in lung cancer [323, 324]. In addition, others have reported high rates of allelic loss as well as a homozygous deletion in the 5p13-12 region [325, 326]. There has also been cytogenetic and molecular evidence of frequent allele loss of parts of chromosome 11p in lung cancer [327–330]. Apart from allele loss in the 11p13 region, refined mapping of the telomeric 11p15.5 region has suggested the location of two distinct tumor suppressor genes [331]. Loss of genetic material from 11q including the chromosomal region which houses the ATM tumor suppressor gene (11q23) is seen in a number of human cancers including lung [332], breast, ovary, cervix, colon, and skin. Nonetheless, ATM mutations have not been reported in lung cancer.

In addition, the presence of homozygously deleted chromosomal regions 2q33, 8, and X/Y in lung cancer imply yet other unidentified tumor suppressor genes [333]. The comparative genomic hybridization (CGH) technique also detected deletions at 1p, 2q, 3p, 4p, 4q, 5q, 6q, 8p, 9p, 10q, 13q, 17p, 18p, 18q, 21q, and 22q, and characterized the different deletion patterns between SCLC and NSCLC, as well as between adenocarcinoma and squamous cell carcinoma subtypes [11–13, 15].

At 10q23, a candidate tumor suppressor gene, PTEN, is somatically mutated in various tumors including glioblastoma, and prostate, kidney, and breast cancers [334]. PTEN mutations appear infrequent in lung cancers [335] but PTEN expression can be downregulated in lung cancers [336]. PTEN is a part of the PI3K/AKT pathway, and like other examples in lung cancer, other members of this pathway can be involved. Phosphatidylinositol 3-kinase (PI3K) activity is implicated in diverse cellular responses triggered by mammalian cell surface receptors, such as cellular proliferation,

growth, apoptosis, and the cytoskeleton, and which are activated in multiple advanced cancers. Unlike other tumors such as colorectal cancers, PIK3CA (encoding the catalytic subunit of PIK3) is only mutated in a small subset of lung cancers [337, 338]. A downstream effector of PI3K is the protein kinase AKT. AKT is negatively regulated by PTEN. AKT appears to be activated in NSCLC lines and seemed to promote survival [339]. Furthermore, nicotine or the tobacco-specific carcinogen, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), could rapidly and potently activate AKT in both NSCLC and SCLC cell lines. Nicotinic activation of AKT increased phosphorylation of multiple downstream AKT substrates including GSK-3, FKHR, tuberin, mTOR, and S6K1 [340].

28.5 Molecular Genetic Changes in Preneoplasia

Much of our knowledge about the series of preneoplastic changes in bronchial epithelium has been based on the histological appearances of bronchial epithelial cells. Before the appearance of a clinically overt lung cancer, a sequence of morphologically distinct changes (hyperplasia, metaplasia, dysplasia and carcinoma in situ) can be observed in bronchial epithelium. The sequential changes for cancers which arise from the proximal airways and bronchi (predominantly squamous cell carcinomas) have long been recognized, whereas changes in peripheral bronchioles and alveoli in evolution to cancers (adenocarcinomas and large cell carcinomas) have been recognized more recently. A number of studies have shown that the preneoplastic cells and bronchial epithelium adjacent to cancers contain a number of genetic abnormalities which are identical to some of the abnormalities found in overt cancer cells. These include abnormalities of MYC and RAS upregulation, cyclin D1 expression, TP53 immunoreactivity, and DNA aneuploidy [10, 260, 341–346]. A follow-up longitudinal study of ex-chromate workers confirmed that some TP53 immuno-positive dysplasias progress to subsequent squamous cell carcinoma [345]. Based on the observation that bronchial dysplastic lesions with elevated telomerase levels, increased Ki-67 index, and expression of TP53 do not regress, it has been suggested that lesions with these molecular changes may progress to invasive squamous carcinomas [347].

In elucidating the temporal sequence of molecular changes, allelotyping analysis of precisely microdissected foci of preneoplastic cells suggests that 3p allele loss is the earliest change followed by 9p allele loss, 17p allele loss (and TP53 mutation), 5q allele loss, and RAS mutations [341, 348–351]. It has been hypothesized that one or more 3p tumor suppressor gene(s) may be critical for proximal squamous lung cancer pathogenesis as abnormalities of 3p appear to be amongst the earliest detectable genetic lesions.

For the adenocarcinoma subtype, based on topographical analysis, it is speculated that KRAS activation may occur early during adenocarcinoma tumorigenesis [346]. In this context, KRAS mutations can be found in atypical adenomatous hyperplasia (AAH), postulated by some to be the precursor lesion for adenocarcinomas [151, 352]. In addition, with laser capture microdissection, EGFR mutations can be demonstrated in apparently morphologically normal appearing bronchial epithelial cells adjacent to tumors known to harbor EGFR mutations [353]. A study of EGFR and KRAS gene mutations in synchronous pulmonary lesions including AAH, carcinomas in-situ (non-mucinous bronchoalveolar carcinoma—BAC), minimally invasive adenocarcinomas, and overtly invasive adenocarcinomas indicated different mutation rates for each gene along this presumed temporal sequence of carcinogenesis [354]. KRAS was mutated in 33 % of AAH, 12 % of carcinomas in situ, 8 % of minimally invasive adenocarcinomas, and 0 % of well-differentiated adenocarcinomas, compared with EGFR mutation rates of 25, 51, 36, 86, and 67 %, respectively. EGFR mutations are found in normal bronchial epithelium and non-malignant epithelium adjacent to tumors in a proportion of patients with AAH and bronchoalveolar carcinoma (BAC), and in 40 % of patients with EGFR mutated adenocarcinomas [353]. In contrast, EGFR mutations are rare in squamous cell carcinomas and therefore EGFR is not implicated in squamous carcinogenesis.

These observations are consistent with the multistep model of carcinogenesis as well as the field cancerization theory proposed by Slaughter. This theory suggests that as the whole aerodigestive tract is repeatedly exposed to carcinogenic damage (tobacco smoke), it is at risk for developing multiple, separate foci of neoplasia [355]. Interestingly, the 3p, 9p, and 17p deletions showed allele-specific loss, defined as deletion of the same allele in preneoplastic tissues as in the primary tumor, even when these lesions were geographically and morphologically distinct [349]. Possible explanations for these allele specific changes include: clonal spread of mutated cells throughout the lung; inherited differences in alleles predisposing to their loss; or some event occurring during lung embryogenesis affecting a particular set of alleles. In support of the clonal spread theory, an identical TP53 point mutation was identified in multiple dysplastic lesions from both lungs of a smoker with chronic obstructive pulmonary disease, thereby indicating an unusual field cancerization mechanism and clonal spread [356].

28.6 Lung Cancer Genome

The combination of the Human Genome Project characterization of human genome sequence and very rapid technological improvements in sequencing tools and bioinformatic analyses has enabled genome-wide investigation of

somatic mutations in human cancers. Several large scale sequencing projects have been reported that aim to identify genes that contain driver somatic mutations in tumor samples compared to passenger mutations that do not contribute to carcinogenesis. For instance in one study, more than 1000 somatic mutations were found in 274 megabases (Mb) of DNA corresponding to the coding exons of 518 protein kinase genes in 210 diverse human cancers, of which perhaps 120 genes were thought to harbor driver mutations [357].

In lung cancer, an earlier study used SNP arrays to identify homozygous deletions and chromosome amplifications in primary lung carcinoma and cell lines. Two homozygous deletion regions were identified, one near PTPRD on 9p23 and another in 3q25. High-level amplifications were identified within 8q12-13 in two SCLC specimens, 12p11 in two NSCLC specimens, and 22q11 in four NSCLC specimens. Tyrosine kinase genes which showed high-level amplification included EGFR (3 NSCLC), FGFR1 (2 NSCLC), ERBB2 (1 NSCLC), and MET (1 NSCLC) [31].

Some studies have focused on candidate genes. DNA sequencing of 623 candidate genes in 188 lung adenocarcinomas revealed more than 1000 somatic mutations including in 26 genes that are mutated at significantly high frequencies, such as tyrosine kinase genes; ephrin receptor genes, vascular endothelial growth factor receptor KDR and NTRK genes. These data also provided evidence of somatic mutations in primary lung adenocarcinoma for tumor suppressor genes implicated in other cancers including NF1, APC, RB1, and ATM and for sequence changes in PTPRD as well as the frequently deleted gene LR 1B [358]. Another study searched for somatic mutations in 1507 coding genes from 441 tumors (breast, lung, ovarian, and prostate). These investigators discovered 2576 mutations, with heterogeneity in rates and mutated genes across tumor types and subtypes. The 77 statistical significantly mutated genes included protein kinases, G-protein-coupled receptors such as GRM8, BAI3, AGTRL1, and LPHN3. Another 35 altered genes including GNAS were identified by the integrated evaluation of somatic mutations and copy number alterations [359].

Unbiased studies analyzing the entire genome have recently been reported. Massively parallel sequencing of a SCLC line, NCI-H209 demonstrated 22,910 somatic substitutions, including 134 in coding exons, evidence of different mutation signatures, as well a tandem duplication affecting CHD7 [360]. A comparison of the complete sequences of a primary lung tumor (60× coverage) and adjacent normal tissue (46×) revealed >50,000 high-confidence single nucleotide variants including KRAS [361]. The investigators estimated a 17.7 per megabase genome-wide somatic mutation rate; with a distinct pattern of selection against mutations within expressed genes compared to non-expressed genes and in promoter regions up to 5 kb upstream of all protein-coding genes, as well as a higher rate of amino acid-changing mutations in kinase genes.

Since then, the field is rapidly progressing and an increasing number of NGS studies are now reported, summarized in Table 28.3. Perhaps the most striking findings include identification of novel lung cancer genes, increasing recognition of actionable mutations in adenocarcinoma [362], and emerging molecular themes in cancers arising in different organs [208, 363].

28.6.1 Gene Expression Profiling of Lung Cancer

The expression of regulatory genes of a cell determines its phenotype. Expression can be assessed by measuring its end product, protein, or its intermediate product, mRNA. Microarray technology allows the simultaneous analysis of expression of thousands of genes, generating gene expression patterns that may characterize a disease state. Tumor gene expression data or profiles/signatures can identify unique expression patterns, with implications for predicting cause, i.e., etiology; source, i.e., tissue of origin, and behavior such as prognosis (prognosticator) and response to therapy (predictor). Whilst mRNA levels do not necessarily correlate with protein concentrations in the cell, the efficiency by which mRNA microarrays provide genome wide quantitative information of gene expression data means that expression-based classification of many cancers has now been reported (Table 28.4).

In lung cancer, most of the work has been performed in NSCLC, where gene expression profiling is often correlated with diagnosis and histology, survival and prognosis, treatment response including sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors [377–384], and smoking [385, 386]. In SCLC, gene expression profiling studies have likewise been used to correlate with diagnosis and the identification of novel markers [387–392], survival and prognosis [383], and treatment response and sensitivity [382, 383, 390]. These analyses provide critical insight into the different pathways which may be involved in different phenotypes. The publication requirement that microarray data be made publicly available (through facilities such as Gene Expression Omnibus) which can then be mined using associated resources at the NCBI, Oncomine [393], Connectivity Map [394] and many others will mean that there will be significant ability for researchers to use the collective data sets for testing and formulation of new hypotheses. It also provides the opportunity for peer review and independent replication—a crucial step if genomic profiling is to advance into the clinic.

Table 28.3 Next-generation sequencing studies of lung cancer

First author	Year	Project design	Principal findings
Campbell [364]	2008	Illumina genome analyzer 2 Lung cancer cell lines WGS	306 germ line structural changes; 103 somatic rearrangements
Pleasance [360]	2010	SOLiD; 1 SCLC cell line WGS	22,910 somatic mutations; 134 in coding regions. 58 structural changes. Tobacco associated mutation signatures
Lee [361]	2010	cPAL; 1 NSCLC/normal lung pair WGS	>50,000 SNPs, 392 in coding regions; 43 structural variants
Ju [365]	2011	Illumina HiSeq 2000 and genome analyzer IIx; 1 NSCLC/normal lung pair WGS	10,724 SNPs, 334 in coding regions. Novel KIF5B-RET fusion
Imielinski [366]	2012	Illumina HiSeq: 183 ACs and matched normal tissue; 159 WES, 23 WES + WGS, 1 WGS	Novel recurrently mutated genes: ARID1A, RBM10, U2AF1. In-frame exonic alterations in EGFR and SIK2
The Caner Genome Atlas Research Network [367]	2012	Illumina HiSeq: Integrated analysis of 178 SCCs and matched germ line; 19 WGS, 178 WES	Novel mutation in HLA-A; significantly altered pathways: NFE2L2/KEAP1 (34 %), squamous differentiation (44 %), PI3K/AKT (47 %); CDKN2A/RB1 (72 %)
Peifer	2012	Illumina genome analyzer IIx. 29 SCLC exomes, 2 genomes and 15 transcriptomes	High mutation rate: 7.4 ± 1 protein changing mutations per 106; TP53 and RB1 inactivation in all; recurrent mutations in histone modifiers CREBBP, EP300, and MLL
Govindan [368]	2012	Illumina genome analyzer II; 17 WGS; 16AC and 1 large cell carcinoma	Novel mutations in chromatin modification and DNA repair pathways; 14 fusions including ROS, ALK and metabolic enzymes. Possible roles of EGFR and KRAS as tumor initiators
The Pan-Cancer Project [369]			
Ciriello [370]	2013	Hierarchical classification of genetic (WES, SNP array, and epigenetic (methylation array) events 3229 tumors of 12 cancer types including 229 lung AC and 182 SCC.	AC are characterized by either mutations or copy number alterations, SCC are characterized by primarily copy number alterations
Lawrence [371]	2013	WGS and WES of 3083 tumors of 12 cancer types including 514 lung cancers: 179 SCC (14 WGS and 165 WES) and 335AC (WES)	Lung AC and SCC have high rates of somatic mutations relative to other tumors: C → A mutations predominate
Zack [372]	2013	Somatic copy number alteration (SCNA) profiling of 357 AC, 344 SCC using SNP array and WGS	Lung AC and SCC have high rates of whole genome duplication relative to other tumor types (59 % AC and 64 % SCC); recurrent focal SCNAs seen in lung SCC and head and neck SCC
Kandoth [373]	2013	Mutational analysis of 3281 tumors from 12 cancer types including 230 lung AC and 178 SCC	The highest mutation rates of all cancers studied were seen in Lung AC and SCC; this was related to *TP53* mutations; *KEAP1* mutations predominate in lung AC and SCC; *EPHA*, *SETBP1*, and *STK11* mutations predominate in Lung AC
Tamborero [374]	2013	Mutational analysis of 3205 tumors from 12 tumor types including 226AC and 174 SCC	Lung AC and SCC have high rates of protein activating mutations in high confidence driver mutations relative to other tumor types (median of 9 per tumor)
Gonzalez-Perez [375]	2013	Resequencing data from 4623 exomes from 13 tumor types including 390AC, 31 NSCLC, 174 SCC, 69 SCLC	Int0Gen-mutations platform is a Web based analysis pipeline which is able to summarize genomic data systematically
Lawrence [376]	2014	Predominantly WES of 405AC tumor-normal pairs and 178 SCC tumor-normal pairs	Estimates suggest the number of driver mutations detected in SCC may more than double given sufficient sample size. In excess of 3000 tumor-normal pairs required to detect alterations in 90 % of genes mutated at 2 % above background with 90 % power

WGS whole genome sequencing, *WES* whole exome sequencing, *CNV* copy number variation, *SNP* single nucleotide polymorphism, *AC* adenocarcinoma, *SCC* squamous cell carcinoma, *SCLC* small cell lung cancer, *cPAL* combinatorial probe anchor ligation

Moreover, a gene expression study of human airway epithelial cells obtained bronchoscopically in smokers and never smoker controls has contributed to knowledge of the smoking transcriptome, including persistent gene deregulation even after smoking cessation (information deposited in the SIEGE (smoking induced epithelial gene expression) database) [448, 449]. These investigators have also identified an 80-gene biomarker in histologically normal large-airway epithelial cells obtained at bronchoscopy that distinguishes smokers with and without lung cancer [450], paving the way for potential development of a diagnostic tool.

Table 28.4 A selection of gene expression microarray studies in lung cancer (N.B. Some studies appear more than once)

Analysis type	Reference	Platform[a]	No. of probes/ elements	Samples in training set (TNM stage)[b]	Samples in test set (TNM stage)[b]	Analysis and findings[c]
Diagnosis/subtype comparison						
	[388]	Affymetrix U95Av2	20,951	127AC, 21 SCC, 20 carcinoid, 6 SCLC, 12 metastases, 17 NL	–	Unsupervised clustering found histology subclasses
	[387]	cDNA	>23,000	41AC (6 pairs), 16 SCC (5 pairs), 5 LCC, 5 SCLC, 5 NL	–	Unsupervised clustering separated tumors histologically
	[389]	cDNA	2400	10 SCC, 10AC, 2 SCLC, 5 SCLC cell lines, 1 carcinoid, 2 colon Ca, 1 NL	–	209 Genes overexpressed in SCLC
	[395]	Affymetrix HuGeneFL	>7000	86AC (I,III), 10 NL	–	Unsupervised clustering found subclasses associated with cancer/ non-cancer, stage and differentiation
	[396]	cDNA	47,650	19AC, 14 SCC, 4 LCC, 2 carcinoid	–	Genes overexpressed in cancer vs non-cancer
	[397]	cDNA	1185	14AC (I–III), 4 NL	–	DE genes between AC and NL
	[398]	Oligo		32 NSCLC	–	Expression profiles correlated with histology
	[384]	cDNA	23,040	22AC, 14 SCC, 1 AdSq	–	Unsupervised clustering found subclasses associated with histology and ± lymph node metastasis
	[399]	cDNA	425	10 NSCLC (I–III) (7AC, 3 SCC) and matched NL	–	DE genes in tumor compared to normal lung. DE genes in stage IA ($n=5$) compared to advanced stage ($n=5$)
	[392]	Affymetrix U95Av2	20,951	21 SCLC cell lines, 18 NL, 8 xenografts	–	Identified DE genes between the two variants of SCLC
	[400]	cDNA	5184	11 SCC, 9AC, 5 non-SCLC, 3 met LC, 3 NL	9 NSCLC (4AC, 4 SCC, 1 LCC), 4 met LC	Gene expression predicted tumor (vs. non-tumor), and histology
	[401]	cDNA	2400	12AC, 3 SCC (I, III)	–	75 Genes DE between no metastasis, micrometastasis, and overt metastasis
	[402]	cDNA	1185	13 SCC (I–III), 13AC [397], 4 NL	–	DE genes in SCC and AC compared to NL. DE genes in SCC compared to AC
	[391]	cDNA	1185	13AC [397], 13 SCC [402], 2 LCC, 1 AdSq, 1 LCNEC, 7 SCLC	–	Identified genes DE between high-grade neuroendocrine carcinomas (SCLC and LCNEC) and other lung cancers
	[403]	cDNA	10,750	42 NSCLC (I–III)	–	62 DE genes between angiogenic compared with non-angiogenic tumors
	[404]	cDNA	2305	6 SCC, 4 NL	–	26 DE genes between SCC and NL
	[52]	Oligo	10,416	79 NSCLC (I–III) (30 pN-, 49 pN+)	33 NSCLC (I–III) (21 pN0, 12 pN+)	33-gene signature to predict lymph node metastasis
	[379]	Affymetrix U133A	>22,000	36 Cell lines: 18AC, 4 SCC, 14 SCLC	–	Compared NSCLC to SCLC to identify subtype specific differences. Correlated expression with copy-number differences

(continued)

Table 28.4 (continued)

Analysis type	Reference	Platform[a]	No. of probes/ elements	Samples in training set (TNM stage)[b]	Samples in test set (TNM stage)[b]	Analysis and findings[c]
	[405]	Affymetrix U95Av2 and HuGeneFL, cDNA	2848	231AC (I–IV) 31 [387], 72 [395], 128 [388]	–	Bronchoid, squamoid and magnoid subtypes within AC
	[406]	Affymetrix Custom	59,000	89 NSCLC (I–IV) (49 SCC, 40AC), 15 NL	–	344 DE genes between NSCLC and NL. 72 DE genes between AC and SCC
	[407]	Oligo	21,619	149 NSCLC (90AC, 35 SCC, 18 LCC, 4 AdSq, 2 LCNEC)	–	293 DE genes between TRU and non-TRU type AC
	[390]	cDNA	32,256	15 SCLC, 37 NSCLC [384], 14AC [381]	–	DE in SCLC vs. NL and NSCLC
	[408]	Affymetrix U95A and U95Av2	20,951	(1) 48 NSCLC (27 SCC, 21AC), 17 NL; (2) 45 NSCLC (25AC, 20 SCC), 33 NL	(1) 160 NSCLC (139AC, 21 SCC), 6 SCLC, 17 NL; (2) 17 NSCLC (10 SCC, 7AC), 20 NL	162 DE genes comparing AC, SCC and NL (training set 1). 96 % classification accuracy in 183 samples (test set 1). 20-gene signature (training set 2) had better accuracy (97 %) (test set 2)
	[409]	cDNA	7237	69 NSCLC (36 SCC, 30AC, 3 LCC)	75 NSCLC (39AC, 29 SCC, 7 LCC) (IHC validation)	Identified genes correlating with histopathology and EGFR mutation
	[410]	Affymetrix U133A	>22,000	100AC (I–III)	–	Identified clusters that correlated with histological subtypes and KRAS and EGFR mutation status
	[411]	Affymetrix U133Plus2	>54,000	46 NSCLC (32 SCC, 14AC) and paired NL	48 NSCLC, 22 NL	Signatures for SCC and AC histology
Prognosis						
	[388]	Affymetrix U95Av2	20,951	127AC, 21 SCC, 20 carcinoid, 6 SCLC, 12 metastases, 17 NL	–	Unsupervised clustering found subgroups with significantly poorer survival
	[387]	cDNA	>23,000	41AC (6 pairs), 16 SCC (5 pairs), 5 LCC, 5 SCLC, 5 NL	–	Unsupervised clusters in AC with significant survival differences
	[395]	Affymetrix HuGeneFL	>7000	86AC (I,III), 10 NL	84AC (stage I–III)	Correlating expression with survival identified 50-gene signature
	[385]	cDNA	18,432	19AC (I–II)	–	27 DE genes between survivors and non-survivors (at 5 years)
	[412]	Affymetrix HU6800	4578	63AC (I)	–	303 DE genes between ± active lymphocytic response. *RANTES* genes was a predictor of survival
	[413]	cDNA	19,200	19AC, 14 SCC, 4 other (I–III)	–	22 Genes correlating with to early recurrence
	[414]	Affymetrix HuGeneFL	>7000	36AC (I) [395]	60 Stage I AC	5 DE genes between recurrence vs. no-recurrence. Three gene-ratio tests could predict outcome
	[415]	Affymetrix Hu6800 and Hu35KsubA	16,063	64 Primary AC, 12 metastatic AC (lung, breast, prostate, colorectal, uterus, ovary)	62AC (I–II) [388]	128-gene signature to predict metastasis, refined to 17-gene signature
	[383]	cDNA	1185	29 NSCLC (II–IV), 18 SCLC	–	3 Genes correlated with survival

(continued)

Table 28.4 (continued)

Analysis type	Reference	Platform[a]	No. of probes/ elements	Samples in training set (TNM stage)[b]	Samples in test set (TNM stage)[b]	Analysis and findings[c]
	[416]	Affymetrix U95Av2 and HuGeneFL	6124	79AC, 10 NL [395]	83AC, 16 NL [388]	16-gene signature for survival
	[417]	Affymetrix U133A	>22,000	10 SCC (I)	5 SCC (stage I)	246 DE genes between high-aggressive vs. low-aggressive tumors. 27 top genes could predict group
	[418]	cDNA	11,168	50 NSCLC (30AC, 16 SCC, 4 LCC)	6 NSCLC (3 SCC, 3 non-SCC)	98-gene NSCLC signature (refined to 25-gene signature), 19-gene SCC signature and 12-gene non-SCC signature.
	[419]	Affymetrix U95Av2	20,951	25AC (5 BAC, 10 invasive, 10 BAC + invasive)	–	319 DE genes between 3 invasive subclasses. 30 DE genes between BAC and BAC + invasive subclasses
	[420]	cDNA	>40,000	48 SCC, 9AC, 30 NL	–	432 DE genes separated SCCs into two subgroups with significant survival differences
	[421]	Affymetrix U95Av2 and HuGeneFL	5377	86AC (69 pN0, 17 pN+) [395]	69AC (52 pN0, 17 pN+) [388]	318-gene set to predict pN status, predicted pN+ patients had worse DFS and OS
	[422]	Affymetrix U95Av2 and HuGeneFL		86AC (I, III) [395]	84AC(I–III) [388]	37-gene signature for survival
	[405]	Affymetrix U95Av2 and HuGeneFL, cDNA	2848	231AC 31 [387], 72 [395], 128 [388]		Bronchoid, squamoid and magnoid subtypes within AC correlated with OS
	[423]	Meta-analysis of 7 datasets, in-house and published (5 as training set, 2 as test set)	4905	197 NSCLC (156AC, 37 SCC, 4 other)	(1) 63AC (stage I) (unpublished data sets), (2) 64AC and SCC (stage I)	Correlating expression with survival identified a 64-gene signature for stage I NSCLC
	[424]	cDNA	1185	17AC, 11 SCC (I–III)		12 Genes correlated with survival
	[425]	Affymetrix U133Plus2	>54,000	45AC, 44 SCC (I–III)	(1) 11AC, 14 SCC; (2) 84AC; (3) 15 SCC	Lung metagene model predicted likelihood of recurrence
	[426]	Affymetrix U133A	>22,000	129 SCC (I–III)	36 SCC (25 stage I, 9 stage II, 1 stage III, 1 stage IV)	50-gene signature correlated with survival
	[427]	Affymetrix U95Av2 and HuGeneFL	>4000	41AC (I) [395] and 60AC (I) [388] (Patients selected if alive/dead at 30 months)	34AC (I) [378]	80-gene model (comprising a 49-gene meta-analysis model, 28-gene cancer-biased model [428], and 3 genes from literature) to predict prognosis. Refined to 10-gene model
	[429]	Oligo	22,575	10 pN+ AC and matched lymph node metastases, 11 pN- AC	–	Unsupervised HC clustered 8/10 primary/metastases pairs together. 75-gene signature of metastasis (comparing pN+ with pN-ACs)
	[430]	Oligo	22,323	48AC (I–II)	55AC [388], 40AC [378]	54-gene signature to predict recurrence
	[431]	Oligo	22,323	51 SCC (I–III)	58 SCC (I–III) [426]	111-gene signature correlated with recurrence

(continued)

Table 28.4 (continued)

Analysis type	Reference	Platform[a]	No. of probes/ elements	Samples in training set (TNM stage)[b]	Samples in test set (TNM stage)[b]	Analysis and findings[c]
	[432]	cDNA	11,168	2 LCC cell lines (1 highly metastatic, 1 parent)	50 NSCLC [418], and 62AC, 78 breast AC, 60 medulloblastomas [415]	45-gene metastasis signature could predict prognosis in lung and breast data sets
	[433]	Oligo	672	60AC, 52 SCC, 13 other	24AC, 31 SCC, 5 other	16-gene signature to predict OS, 5-gene signature confirmed with qRT-PCR
	[434]	Affymetrix U133Plus2	>54,000	138 NSCLC (I–III) (75 SCC, 63AC)	56 NSCLC (I–III) (32 SCC, 25AC)	6-gene clinicopathologic signature to predict DFS
	[435]	Affymetrix HU6800	4966AC; 12,990 SCC	86AC (I,III) [395], 129 SCC (I–III) [426]	84AC [388], 45AC and 46 SCC [378]	Developed two 50-gene signatures in AC and SCC datasets to predict OS
	[436]	Affymetrix U133A	>22,000	256AC (I–III)	186AC (I–III)	Multi-site blinded study developed several classifiers to predict OS where prediction accuracy was improved with inclusion of clinical data
	[437]	Oligo	44,000	103 NSCLC (57 SCC, 33AC, 13 other) (I–II)	69 NSCLC (35 SCC, 23AC, 11 other) (I–II)	72-gene signature to predict RFS and OS
	[438]	qRT-PCR	158	147 [439]	91AC and SCC [425], 130 SCC [426], 59 SCC [430], 48AC [440]	6-gene signature to predict OS
	[441]	Affymetrix U133A, U133Plus2	>22,000	129 SCC [426]	85 NSCLC (44 SCC, 41AC) [425], 138 NSCLC (76 SCC, 62AC) [434], 327AC [436]	12-gene signature for OS
Treatment						
	[384]	cDNA	23,040	22AC, 11 SCC	–	Correlation of sensitivity to 6 anticancer drugs
	[383]	cDNA	1185	29 NSCLC (II–IV), 18 SCLC	–	3 Genes correlated with survival
	[381]	cDNA	27,648	17AC (III–IV)	10AC, 1 SCC (III–IV)	12-gene signature to predict gefitinib sensitivity
	[382]	cDNA	1185	[383]	–	3 Genes correlated with outcome of chemotherapy
	[442]	Affymetrix U133Plus2	>54,000	42 NSCLC cell lines	–	Identified signature correlating with erlotinib sensitivity
	[377]	Affymetrix U133A	>22,000	29 LC cell lines	19AC, 40AC [378]	180-gene signature for EGFR TKI sensitivity
	[378]	Affymetrix U133Plus2	>54,000	74 NSCLC	–	Gene expression signature that reflects activation status of oncogenic pathways which in turn, can predict sensitivity to components that target pathway
	[380]	Affymetrix U133	45,000	11 NSCLC cell lines (3AC, 3 SCC, 2 LCC, 2 BsAC, 1 AdSq)	12 NSCLC cell lines (7AC, 3 LCC, 2 SCC)	Gene signature associated with EGFR-TKI sensitivity
	[443]	Affymetrix U133A	>22,000	10 NSCLC cell lines	19 NSCLC cell lines	Identified signatures of sensitivity to docetaxel, paclitaxel, gemcitabine, vinorelbine, 5-FU, SN38, cisplatin, and carboplatin

(continued)

Table 28.4 (continued)

Analysis type	Reference	Platform[a]	No. of probes/ elements	Samples in training set (TNM stage)[b]	Samples in test set (TNM stage)[b]	Analysis and findings[c]
	[390]	cDNA	32,256	15 SCLC, 37 NSCLC [384], 14AC [381]	–	Identified DE in SCLC and advanced AC with history of extensive chemotherapy
	[444]	Affymetrix U133A	>22,000	16 NSCLC cell lines	9 NSCLC cell lines	Nine-gene classifier to predict sensitivity to HDAC inhibitors
	[445]	Affymetrix U133A	>22,000	62 NSCLC (I–II)	96 NSCLC [436], 48 NSCLC [425], 79 SCC [426], 133 NSCLC [437]	15-gene signature to predict sensitivity to cisplatin/vinorelbine
Smoking						
	[385]	cDNA	18,432	19AC (I–II)	–	45 DE genes between smokers and nonsmokers
	[386]	Affymetrix U133A	>22,000	18 SCC, 9AC, 8 NL	–	23-gene signature that differentiated between bronchial epithelium of nonsmokers, smokers, and cancers
	[446]	Affymetrix U133A and B		66 NSCLC (54AC, 6 SCC, 5 LCC, 1 AdSq), 13 NSCLC cell lines (12AC, 1 NSCLC), 7 NL, 6 HBEC lines	LOOCV (Train 2/3, Test 1/3)	65-gene signature of NSCLC nonsmoking *nAChR* α6β3 phenotype
	[447]	Affymetrix U133A		58AC, 49 NL (20 never, 26 former, 28 current smokers)	19 NSCLC, 21 NL	Signature of cigarette smoking

Unless otherwise specified, all samples were primary lung tumor samples
[a] *Affymetrix* Affymetrix GeneChip®, *cDNA* cDNA microarray or membrane, *Oligo* Oligo microarray
[b] *AC* adenocarcinoma, *SCC* squamous cell carcinoma, *LCC* large cell carcinoma, *LCNEC* large cell neuroendocrine carcinoma, *SCLC* small cell lung carcinoma, *NL* normal lung
[c] *OS* overall survival, *DFS* disease-free survival, *DE* differentially expressed, *HC* hierarchical clustering, *TRU* terminal respiratory unit, *nAChR* nicotine acetylcholine receptor

28.6.2 DNA Methylation

The spatial arrangement and three-dimensional structure of DNA in the nucleus is controlled through the interdigitation of DNA binding proteins such as histones and their modifiers, the Polycomb-Trithorax proteins, and the DNA methyltransferase enzymes [451]. DNA methylation is involved in the epigenetic gene regulation in mammals. Cellular transformation coincides with multiple changes in chromatin architecture, thereby affecting genome integrity and gene expression. The process of DNA methylation involves the covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides which tend to be clustered in islands at the 5′ ends of many genes, and can be considered an epigenetic modification of DNA. In general, methylated genes are inactive whereas unmethylated genes tend to be active. DNA methylation appears to be associated with genetic repression and is necessary for proper embryonic development. The methylated CpG residues could either interfere directly with the binding of specific transcription factors to DNA, bind to specific repression factors or convert chromatin to an inactive form by altering its structure. The mammalian DNA methylation process requires two components, the DNA methyl-transferases (DNMTs) and the methyl CpG binding proteins (MBDs) [452, 453]. DNMTs establish and maintain methylation patterns whereas MBDs are thought to be involved in reading methylation marks. Potential mechanisms include spreading of DNA methylation from repetitive sequences into promoter-associated CpG islands secondary to loss of transcriptional activators, gain of methylation secondary to hyperexpression of transcriptional repressors, primary hypermethylation due to hyperexpression of methyltransferases, and inter-allelic transfer of methylation via gene pairing [454]. A proposed model of DNA methylation and cancer proposes that global hypomethylation in at-risk cells contributes to genomic instability through increased mitotic recombination events, whereas CpG island methylation in cancer cells leads to transcriptional silencing of growth regulatory genes [452]. One of the major reasons for the explosion in knowledge regarding tumor methylation is thanks to the rapid technological advances in this area. Frommer et al. demonstrated that bisulfite deamination of cytosine and 5-methylcytosine differentially to yield uracil and thymine respectively coupled

with PCR techniques allowed simplified analysis of DNA methylation [455]. More recently, Weber et al. has developed an antibody based immunocapture method termed methyl-DNA immunoprecipitation (MeDIP) involving purification of methylated DNA using a monoclonal antibody raised against 5-methylcytidine [456]. When combined with high-resolution genomic microarrays, this approach allows correlations between DNA methylation and histone modifications to be made, demonstrating its usefulness in epigenetic research [457]. These days, the very sensitive bisulfite based methylation specific PCR (MSP) is perhaps the most popular tool for examining DNA methylation patterns, although whole-genome microarray-based and next-generation sequencing based approaches are gaining popularity due to their ability to profile several thousands of genes in a single experiment. The principles of MSP and other methylation detection techniques, such as restriction digestion by methylation sensitive enzymes, COBRA, PyroMeth, SnaPmeth, bisulfite SSCP, and bisulfite sequencing have been reviewed [458]. Some techniques such as restriction landmark genomic scanning (RLGS) and arbitrarily primed PCR (AP-PCR) are genome wide techniques, often methylation sensitive restriction enzyme based, for analyzing the DNA methylation status of CpG islands [459]. Other high throughput techniques including array based tests, MethyLight, MALDI-TOF mass spectrometry and next-generation sequencing approaches based on bisulfite conversion are increasingly being utilized and will result in better understanding of lung cancer epigenomics [460–462]. Nonetheless, the ease of detection means that there is great interest in developing potential clinical useful lung cancer biomarkers for diagnosis and prognosis [463, 464].

28.6.3 DNA Hypomethylation

The link between cancer and abnormal methylation has been known since 1983, with the demonstration that cancer genomes are relatively hypomethylated compared to normal counterparts [465]. Hypomethylation in cancer cells is thought to be primarily due to loss of methylation from repetitive regions of the genome, with resulting genomic instability [452]. Apart from global genomic demethylation which in many cancers is an early event, gene specific hypomethylation also occurs which can result in functional changes in gene expression. Studies of oncogene transformed normal human bronchial epithelial (NHBE) cells and clinical lung cancer samples have demonstrated alterations in methylation [466, 467] during lung carcinogenesis. The global demethylation is known to results in the derepression of parasitic DNA, loss of imprinting, and upregulation of the expression of a number of usually silent genes. Examples of genes altered in these ways include loss of imprinting of the H19, IGF2, and MEST genes. Imprinting is the epigenetic modification in a specific parental chromosome that leads to differential expression of the two alleles in the offspring, thus loss of imprinting is loss of the normal allele specific gene expression, which may result in deregulated cell growth. Other genes upregulated as a result of global hypomethylation include those encoding cancer testes antigens (CTAs) proteins including the MAGE family [453]. Surrogate markers of global hypomethylation including repetitive elements such as long interspersed nuclear element 1 (LINE-1) and Alu elements which constitute approximately 30 % of the genome [468] have been implicated in several cancer types [469]. Recent investigations of LINE-1, have demonstrated its potential utility as a prognositic marker for stage IA lung cancer showing poorer survivals in subjects with LINE-1 hypomethylation [470]. LINE-1 hypomethylation also appears to be strongly associated with genomic instability [468].

28.6.4 DNA Hypermethylation

In contrast, site-specific hypermethylation of CpG islands in gene promoters is now the most well characterized epigenetic modification in cancer. It is found in nearly every human cancer type and is associated with transcriptional silencing of gene expression. The genes silenced by promoter hypermethylation tend to be tumor or growth suppressor genes, and is an alternative to the classic loss of one TSG and mutation of the other to satisfy Knudson's two hit hypothesis. Indeed there are regions of chromosomes where allele loss and hypermethylation may be the predominant method on TSG inactivation, e.g., RASSFI1 at chromosome 3 CDKN1A and HIC1 are 17p13.3. It is known that exposure to cigarette smoke induces lung cancer in mice via both genetic and epigenetic pathways [471]. Indeed some of the DNA methylation changes involve the same genes that are altered in human lung cancers [472]. There are now many reports of somatically acquired DNA methylation in the genes involved in lung cancer. We have reported on methylation specific PCR to examine gene promoter methylation in lung cancer. DNA was examined from 107 resected NSCLC and corresponding normal lung tissue [473–475]. Methylation in the tumor samples was detected in 40 % for RARB, 26 % for TIMP-3, 25 % for CDKN2A, 21 % for MGMT, 19 % for DAPK, 18 % for ECAD, 8 % for p14 ARF, and 7 % for GSTP1, whereas it was not seen in the vast majority of the corresponding non-malignant tissues. A total of 82 % of the NSCLCs had methylation of at least one of these genes; 37 % had one gene methylated, 22 % had two genes methylated, 13 % had three genes methylated, 8 % had four genes methylated, and 2 % had five genes methylated. Aberrant CDKN2A and FHIT methylation corresponded with downregulation [473, 476] of gene expression. The advent of whole-genome methylation

profiling has provided new means of identifying novel methylation biomarkers. Rauch and colleagues utilized this technology to characterize DNA methylation changes in the genome of lung SCC [477] identifying 57 CpG islands consistently hypermethylated in all samples. Most of these mapped to homeobox genes which are increasingly being implicated in lung carcinogenesis [478]. Twelve of the 57 CpG islands were independently validated using combined bisulfite restriction analysis (COBRA) with methylation frequencies ranging from 70 to 100 % [477]. Whole genome methylation profiling has also been used to investigate the role of methylation in radiosensitivity of cancer cells [479]. Kim and colleagues identified 1091 genes differentially methylated between cell lines treated with ionizing radiation and normal lung cell lines concluding that examination of key differentially methylated genes in radiosensitive patients is necessary considering the use of DNA methylating agents currently in clinical use [479]. The Cancer Genome Atlas (TCGA) project has also provided extensive insights into epigenomic changes occurring in lung adenocarcinomas and squamous cell carcinomas [367, 480]. Assessment of 230 lung adenocarcinomas using Illumina Beadarray technology identified a distinct CIMP positive phenotype for lung adenocarcinomas while interrogation of 178 lung squamous cell carcinomas, showed that DNA hypermethylation was accompanied by distinct changes in mRNA expression [367, 480]. Similarly, methylation differences have been associated with cisplatin resistance, and prognostic methylation signatures identified for early stage tumors [481, 482]. In addition, several groups have identified differences in the methylation profiles of smoking and never smoking lung adenocarcinomas, with Tan et. al. identifying a potential diagnostic signature that could distinguish smoking related lung adenocarcinomas with a sensitivity of 88.9 % and specificity of 83.2 % [483, 484]. Several studies have also used integrative approaches to identify methylated candidates accompanied by concordant changes in mRNA expression [481, 485, 486]. The explosion in methylation based studies, highlights the potential for DNA methylation changes to be used as diagnostic and prognostic biomarkers in NSCLC. Thus, we and others have documented examples of epigenetic gene silencing by de novo methylation of TSGs in lung cancers, e.g., RB1, VHL, CDKN2A, DAPK, GSTP1, and MGMT [473–475]. Conversely in SCLC, regional hypermethylation has been found at chromosome 3p, but the precise gene target is uncertain [487]. Many other genes are now shown to be methylated to varying degrees in the different subtypes of lung cancer, from primary lung cancers and also lung cancer cell lines. These include genes such as TSLC1 [488]; CDH13[489], hSRBC [490], SPARC [491], DBC1 [28], TCF21 [492], LHX6 [493], and KEAP1 [494] among others. The genes altered by DNA methylation include those involved in cell cycle regulation (e.g., CDKN2A), DNA repair (e.g., MGMT), apoptosis (e.g., DAPK, caspase 8, FAS, TRAILR1), RAS signaling (RASSF1A, NORE1A), and invasion (e.g., cadherins, TIMP3, laminin family) with more detailed lists available from reviews [464, 495]. Some of these pathways affected by epigenetic change are those described as being hallmarks of cancer [5].

28.6.5 Histone/Chromatin Modifications

Chromatin remodeling and histone modifications including methylation, acetylation, and phosphorylation contribute to gene silencing and activation. Mutations, amplifications, and other genomic alterations can disrupt the balance between histone deacetylases (HDAC) and histone acetylases (HATs) resulting in repression or activation of genes [496]. Hypoacetylation may result in repression of tumor suppressor gene function through activation of HDACs and suppression of HATs while hyperacetylation can activate genes that would normally be silenced. Suberoylanilide hydroxamic acid (SAHA), a novel HDAC inhibitor, has been shown to inhibit tumor cell growth by decreasing the number of cells in S-phase [497] and decreasing tumor development in mice treated with the tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [498]. Other HDAC inhibitors including CI-994, trichostatin A, and OSU-HDAC-44 have also been shown to significantly decrease tumor cell growth in NSCLC [499–501]. Interestingly, the novel HDAC inhibitor YCW1 has been shown to have synergistic effects with cisplatin, inhibiting tumor growth and significantly suppressing lung cancer metastases via inhibition of the focal adhesion complex [502]. Therefore, HDAC inhibitors may be useful in increasing histone acetylation and gene transcription through inhibition of lung cancer cell growth.

28.6.6 microRNAs

miRNAs are a recently discovered family of small, RNA molecules encoded in the genomes of plants and animals. These highly conserved ~21-mer RNAs regulate gene expression by binding to the 3′-untranslated regions (3′-UTR) of specific target mRNAs. Acting as a crucial member of the RNA Induced Silencing Complex (RISC), miRNAs control gene expression at the posttranscriptional level by degrading or repressing target mRNAs [503–506]. The biogenesis and control of miRNAs involves complex regulatory networks that control cropping and dicing [507]. miRNAs are expressed in animal cells and are dynamically regulated in response to physiological and developmental cues. It is thought that there are cell-type specific miRNA milieus with unique sets of miRNAs controlling gene expression.

Furthermore, each miRNA may, in computational predictions, have as many as hundreds of target genes—if correct, this means that up to a third of protein-encoding genes are regulated by miRNAs in humans [508]. There is mounting evidence of the importance of miRNA mediated gene regulation in human cancers. Firstly, miRNAs participate in the regulation of a wide range of cellular pathways including development, proliferation, differentiation, apoptosis, angiogenesis, methylation, metastasis, and invasion [509–518]. Second, miRNA expression profiling shows differential expression of nearly all miRNAs across cancer types, expression profiles parallel the developmental origins of tumors, expression patterns reflect the mechanisms of transformation, and extensive dysregulation of miRNA expression in tumors compared with normal tissues [519]. Third, several genes implicated in human cancers have been experimentally validated as miRNA targets including PTEN TSG, SMAD-1 transcriptional regulator, polycomb gene EZH2, and MYCN basic HLH transcription factor proto-oncogene [520, 521]. Furthermore, miRNA genes are located nonrandomly in the genome. Although only representing 2–3 % of all human genes, more than half of them are clustered at fragile sites, minimal regions of LOH, and breakpoint sites which are implicated in cancer [522, 523]. In addition to this association with genomic regions involved in cancers, aberrant methylation, SNPs, mutations, miRNA regulation by cancer-associated transcription factors, and dysfunctional miRNA biogenesis, have all been implicated in their altered expression in human malignancy [524–529].

The miR-17-92 cluster of miRNAs is highly expressed in many lymphomas that contain amplified 13q31-31 chromosomal sequences [530]. When co-expressed with MYC in hematopoietic stem cells in mice, miR-17-92 appears to augment the oncogenic effect of MYC. This miRNA cluster, when upregulated by MYC, actually translationally downregulates a MYC transcriptional target, E2F1 [528]. These interesting findings suggest that this cluster may act as either an oncogene or a TSG, depending on circumstances, functioning within a regulatory network with c-Myc and E2F1 to balance cell death and proliferation [528, 531]. Nonetheless overexpression of an oncogenic miRNA may therefore posttranscriptionally downregulate target TSGs [532]. In keeping, miR-17-92 is similarly implicated in lung cancer, with miR-17-92 cluster overexpression and occasional gene amplification, especially in small-cell lung cancer [511].

Conversely oncogenes may also be regulated by miRNAs and miRNAs are frequently downregulated in cancers, with loss of a tumor suppressing (TSG) miRNA potentially causing posttranscriptional overexpression of target oncogenes—the archetype being RAS and let-7. Several studies have demonstrated that let-7 expression is reduced in lung cancers and that let-7 causes growth inhibition and regulates the RAS oncogene in vitro [533–535]. These findings, together with the demonstration of RAS overexpression in cancers with low let-7, strongly implicate let-7 as a tumor suppressor in lung, whereby downregulation of let-7 causes increased expression of the RAS oncogene, and thus contributes to carcinogenesis [527, 535]. Moreover, some let-7 family members are located in genomic regions commonly deleted in lung cancer and reduced let-7 expression correlated with a worse prognosis in resected lung cancers [522, 535].

Microarrays can also be used to detect miRNA deregulation in cancers [536]. Comparing primary lung cancers and corresponding lung tissues, differentially expressed miRNAs appear to be associated with FRA fragile sites [537]. Others were located in regions deleted or amplified in various types of cancers. For example mir-21 at 17q23.2 was amplified and upregulated in neuroblastoma and lung cancer [527], and mir-126 at 9q34.3, was associated with FRA17B and downregulated in NSCLC and hepatocellular carcinoma [537]. Of clinical importance is the finding that high mir-155 and low let-7a-2 expression correlated with reduced survival in adenocarcinomas [537]. Numerous studies have now published potential miRNA gene signatures for prediction of outcome and survival in lung cancer patients [538–542], however little consistency has been observed between these signatures. This may be due to differences in methodology (microarrays compared to real time PCR), sample type (tumor tissue compared to serum) or cohorts (ethnicity). Differences in miRNA expression profiles between histological subtypes of lung cancer have sparked interest in identifying single miRNAs or miRNA signatures for histological differentiation. Indeed, miR-205, has been reported as a lung squamous cell carcinoma (SCC) specific miRNA, able to identify lung SCCs with 96 % sensitivity and 90 % specificity [543].

Recent advances in our understanding of the involvement of miRNAs in lung carcinogenesis have demonstrated that miRNA expression is altered from the early stages of bronchial carcinogenesis [544]. Furthermore, dysregulated miRNA expression has been shown to be associated with exposure to established or potential lung carcinogens, including cigarette smoke and diesel exhaust particles [545–547]. Schembri et al. observed downregulation of miR-218 in human bronchial epithelial cells exposed to cigarette smoke extract [545]. Subsequently, our work demonstrated significant downregulation of miR-218 in the tumors of smokers compared to never smokers [546] and Wu et al. reported downregulation of miR-218 in combination with upregulation of its target gene Paxillin as an independent predictor of survival in NSCLC [548]. Specific miRNAs have also been implicated in EGFR and p53 signaling pathways involved in response to chemotherapeutic agents such as EGFR tyrosine kinase inhibitors, Carboplatin and Cisplatin [549–554]. Further exploration of the role these miRNAs play may lead to the identification of novel approaches to maximize chemosensitivity and reduce development of chemoresistance.

Finally, recent reports have demonstrated that these single stranded miRNAs are able to be detected in multiple clinical specimens including whole blood, serum, plasma, sputum and malignant effusions [555–560]. This important discovery enhances the potential for development of clinical miRNA biomarkers. Despite these significant advances in our understanding of miRNA biology in lung cancer, further investigation is required to fully demonstrate the extent of their involvement in lung carcinogenesis and potential use as diagnostic or prognostic biomarkers and novel therapeutic agents in lung and other human malignancies.

28.7 Tobacco Smoke Carcinogens

It is thought that tobacco smoke is responsible for 85–90 % of all cases of lung cancer [561]. Tobacco smoke contains thousands of substances including carcinogens, co-carcinogens, and tumor promoters. The polycyclic hydrocarbons (such as benzo(a)pyrene), nitrosamines, and aromatic amines are the three major classes of carcinogens in tobacco smoke. There has been much study of the nitrosamines which are derived from nicotine during the burning of tobacco, especially 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Indeed, NNK and its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), are potent carcinogens that are specific for the lung in rodents. The carcinogenic effects of tobacco smoke in the lung involve the induction of carcinogen-activating and inactivating enzymes, as well as covalent DNA adduct formation which may result in DNA misreplication and mutation. DNA adducts have been identified in the bronchial tissue of lung cancer patients and their levels correlate with the amount of tobacco smoke exposure. Furthermore, benzo(a)pyrene, a major cigarette smoke carcinogen, was shown to form adducts selectively along the TP53 gene of bronchial epithelial cells, at the nucleotide positions known to be the major mutational hot spots in lung cancer [562]. Thus, targeted adduct formation rather than phenotypic selection may determine the pattern of TP53 mutations in lung cancer, and the data provided a direct etiological link between a defined chemical carcinogen and lung cancer.

28.8 Lung Cancer Susceptibility: Candidate and Genome Wide Association Studies (GWAS)

Only a proportion of smokers develop lung cancer despite the fact that tobacco smoke is implicated in most cases of lung cancer. Clearly, there are other important factors operating apart from the simple inhalation of tobacco smoke. It has, for instance, been postulated that individuals may exhibit genetic polymorphisms in carcinogen metabolizing pathways that would lead to inherited differences in lung cancer risks associated with tobacco smoking. The relationships of human lung cancer to polymorphisms of phase I pro-carcinogen-activating and phase II-deactivating enzymes and intermediate biomarkers of DNA mutation, such as DNA adducts, oncogene and tumor suppressor gene mutation, and polymorphisms have been compiled [563].

The important genetic variations in lung cancer include polymorphisms at the cytochrome P450 gene (CYP) loci and the glutathione S-transferases (GST) M1 gene cluster. An important enzymatic group relevant to lung cancer includes the phase II GST enzymes, which are encoded by a multigene family whose proteins serve to detoxify mutagenic electrophiles [564]. The gene products are suggested to play a part in detoxifying epoxides of certain carcinogenic polycyclic aromatic hydrocarbons (PAH). These enzymes conjugate glutathione to a substrate generally resulting in its inactivation and excretion, although in certain cases this can lead to activation of a pro-carcinogen to a mutagenic product. Four gene families are known (A, M, P, and T) and large inter-individual variations in enzyme activity are known to exist for several GSTs [565]. The GSTM1 locus is homozygously null or deficient in approximately 40–50 % of the population. Several case–control studies have linked the null phenotype to an increased risk of lung cancer [566, 567], but there have also been conflicting studies [568, 569]. A meta-analysis of 12 comparable studies suggested that GSTM1 null is overrepresented in lung cancer cases with an overall odds ratio (OR) of 1.41 (95%CI of 1.23–1.61), which did vary depending on ethnic origin, being lower in Caucasians (OR 1.17) [570]. Although the increased risk was modest, it potentially translates to a large number at the population level, and the authors suggested that ~17 % of new lung cancer cases could be attributable to GSTM1 deficiency. As GSTM1 is only weakly expressed in the lung, interest has also focused on the protein product of GSTP1, which in fact, is the major GST protein in the lung [565, 571]. This enzyme has activity for many epoxides of PAH and could theoretically compensate for loss of GSTM1 activity. Increased expression of the GSTP1 gene has been reported in lung tissue [565]. In the only study of lung cancer, the combination of GSTM1 null and the G allele at a GSTP1 codon 104 polymorphism (A to G substitution replacing isoleucine with valine) was shown to be associated with more hydrophobic-DNA adducts and lung cancer risk in Norwegian patients [572]. Importantly, this polymorphism is known to functionally change enzyme kinetics [573].

The structural gene for the phase I enzyme, cytochrome CYP1A1 which catalyzes a range of human carcinogens, has a MspI restriction polymorphism (m2) associated with an amino acid substitution in the enzymatically active site that results in a protein of high activity [574]. In the Japanese

population, this polymorphism has been shown to be associated with an increased lung cancer risk [575]. On the other hand, the data in Caucasians is controversial as this association was not demonstrated in some older studies of Caucasians where the polymorphism is much rarer [576–578]. On the other hand, a study of 490 Caucasians found an estimated OR of 2.08 for the CYP1A1 m2 allele (heterozygotes and homozygotes) and lung cancer development [579]. Finally, NSCLC patients with at least one susceptible allele of the MspI polymorphism were associated with a shortened survival [580]. Another polymorphism at residue 462 (Ile/Val) of the CYP1A1 heme-binding region, has also been associated with lung cancer risk in Japan [581]. The Val protein demonstrates twice as much activity as the isoleucine protein [575] but there appear little or no in vitro difference in their kinetic properties [582, 583]. In any case, the results are mixed as CYP1A1 Val/Val was twice as prevalent in Brazilian lung cancer patients [584], borderline in a German cohort [585] and not increased in a Finnish cohort [576]. In our case–control study of predominantly Caucasian Australian NSCLCs, we reported that carriers of the valine allele (Ile/Val or Val/Val genotypes) were significantly overrepresented even when adjusted for potential confounders, particularly in women [586]. In the complex process of multistep carcinogenesis, it is unlikely that a single factor can completely account for an individual's susceptibility to lung cancer. For any given exposure to an environmental carcinogen, it is more likely that it is the interaction between several genetic susceptibility factors that determines the risk of lung cancer. Consequently, the possibility that it is the combination of the bioactivating phase I (P450) and the inactivating phase II (e.g., GSTs) carcinogen metabolizing enzyme polymorphisms that is crucial to lung cancer risk is biologically plausible and attractive. The CYP1A1 MspI polymorphism in conjunction with the GSTM1 null genotype has been reported to carry a ninefold increased risk of lung cancer [567]. This was confirmed in a second cohort of Japanese subjects where the OR of a combined CYP1A1 m2/m2 and GSTM1 null genotype versus nonsmoker controls was 21.9 (95 % CI 4.7–112.7), albeit lowered when smoking controls were compared. In the only non-Japanese cohort to be tested, a Swedish study found too few appropriate subjects for analysis [577]. Thus, whilst the data suggests a link for combination genotypes and lung cancer risk in Japanese populations, the relatively lower prevalence of the m2 and Val allele in non-Japanese populations (estimated 12 % and 3–16 % respectively) [563] has meant that it may not have been adequately studied in Caucasian populations. We have studied CYP1A1 haplotypes in a case–control study of 1040 non-small-cell lung cancer (NSCLC) cases and 784 controls to investigate three CYP1A1 variants, CYP1A1*2A (rs4646903; thymidine to cytosine substitution at nucleotide 3801 (3801T>C)), CYP1A1*2C (rs1048943; 2455A>G; substitution of isoleucine 462 with valine (exon 7)) and CYP1A1*4 (rs1799814; 2453C>A; substitution of threonine 461 with asparagine (exon 7)) [587]. CYP1A1 haplotypes (in allele order CYP1A1*4, CYP1A1*2C, CYP1A1*2A) CGC and CGT were associated with an increased risk of lung cancer, whereas AAT was associated with decreased lung cancer risk in this population. Thus, risk haplotypes were identified for CYP1A1 in NSCLC, and CYP1A1 polymorphisms were identified as a minor risk factor for NSCLC. In the Australian setting, we reported that another pair of polymorphisms in carcinogen metabolizing enzymes (CYP1A1 Ile462Val and MPO G-463A) interacted to increase risk of adenocarcinoma [588] supporting the concept that multiple genes of modest effect can interact to confer genomic-based susceptibility to lung cancer. In the context of the explosion of epidemiologic studies on the prevalence of gene variants and their associations with human lung cancer that will result from completion of the human genome sequence a proper assessment of genome based risk will require collaborative efforts such as the Human Genome Epidemiology (HuGE) Network and careful meta-analyses of many primary studies [589].

CYP2D6 (debrisoquine 4-hydroxylase), another P450 metabolizing enzyme, bioactivates nicotine as well as NNK. The relationship between lung cancer risk and the CYP2D6 polymorphism has been the subject of numerous studies and is still controversial [590, 591]. While some studies have suggested a reduction in the risk of lung cancer with the poor metabolizer phenotype and/or genotype [592], others have indicated little or no role for the CYP2D6 polymorphism in lung cancer [591, 593, 594].

In addition to linkage studies and candidate gene approaches, the genome-wide association study (GWAS) methodology uses an association testing approach on a genome-wide scale, testing large numbers of SNPs using standard microarray platforms [6]. The key advantage of this approach is that it enables testing of SNPs across the entire genome without needing a prior hypothesis about the identity of causal genes [6] as traditionally done through genetic association studies. Disadvantages include lack of coverage of rare variants, or ability to analyze gene-gene interaction, gene-environment interaction, epigenomics, or other principles that can explain variance in heritability.

A number of genome-wide association studies have now been published for lung cancer (Table 28.5). The most frequent genetic association for lung cancer in smokers was observed with SNPs in chromosomal region 15q25 containing the genes for the neuronal nicotinic acetylcholine receptor (nAChR) subunits (cholinergic receptor, nicotinic, alpha 3 and 5: *CHRNA3* and *CHRNA5*). This chromosomal region has also been associated with smoking behavior and intensity, e.g., cigarettes per day, in a number of GWAS. Whilst the association of smoking behavior with nicotine receptor SNPs could partly explain the relationship with lung cancer, many of the GWAS and subsequent validation studies [595]

Table 28.5 Genome-wide association studies of lung cancer

Study	Lung cancer cases (discovery set)[a]	Controls (discovery set)	Arrays (nos. of SNPs)	Chromosomal regions and main associated genes
Spinola [599]	335 Smokers	338 Smokers	Affymetrix (116, 204)	10p *KLF6*
Amos [600]	1154 Smokers	1137 Smokers	Illumina (317, 498)	15q *CHRNA3*
Hung [601]	1989 Smokers	2625 Smokers	Illumina (139, 317)	15q *CHRNA3, CHRNA5*
Liu [602]	194 with familial lung cancer	219 Smokers and nonsmokers	Affymetrix (500, 568, 906, 703)	15q various genes
Thorgeirsson [603]	1024 Smokers	32,244 Controls	Illumina (207, 306)	15q *CHRNA3*
McKay [604]	3259 Smokers	4159 Smokers	Illumina (194, 315)	5p *TERT-CLPTM1L,* 15q *CHRNA3*
Wang [605]	1952 Smokers	1438 smokers	Illumina (511, 919)	5p *CLPTM1L*, 6p *BAT3-MSH5,* 15q *CHRNA3*
Broderick [606]	1978 Smokers, and meta-analysis	1438 Smokers, and meta-analysis	Meta-analysis	5p *TERT-CLPTM1L*, 6p *BAT3-MSH5*, *TNXB,* 15q *CHRNA3*
Landi [607]	5739 Smokers	5848 Smokers	Illumina (515, 922)	5p *TERT-CLPTM1L*, 15q *CHRNA3*
Hsiung [608]	584 Cases (never smoking females with lung adenocarcinoma)	585 Controls (never smoking females)	Illumina (610, 901)	5p15 *TERT-CLPTM1L*
Li [596]	377 Never smokers	377 Never smokers	Illumina (373, 397, 532, 592)	13q31.3 *GPC5*
Miki [609]	1004 with lung adenocarcinoma	1900 Controls	Illumina (610, 901)	3q28 *TP63*, 5p15 *TERT*
Yoon [610]	621 Cases (smokers and never smokers)	1541 Controls (smokers and never smokers)	Affymetrix (500, 568)	3q29 *C3orf21*, 5p *TERT-CLPTM1L*
Hu [611]	2331 Cases (smokers and never smokers)	3077 Controls (smokers and never smokers)	Affymetrix (703, 906)	3q28 *TP63*, 5p15 *TERT-CLPTM1L*, 13q12 *MIPEP-TNFRSF19*, 22q12 *MTMR3-HORMAD2-LIF*
Ahn [612]	446 Never smokers	497 Healthy controls	Affymetrix (906, 703)	18p11 *FAM38B*
Dong [613]	833 Cases with SCC	3094 Controls	Affymetrix (906, 703)	12q23 *SLC17A8-NR1H4*
Lan [614]	5510 Never-smoking female lung cancer cases	4544 Controls	Various	3q28 *TP63*, 5p15, 6p21 *HLA*, 6q22 *ROS1, DCBLD1*, 10q25 *VTI1A*, 17q24 *BPTF*
Shiraishi [615]	1722 Cases (smokers and never smokers)	5846 Controls (smokers and never smokers)	Illumina (709, 857)	3q28 *TP63*, 5p15 *TERT*, 6p21 *BTNL2*, 17q24 *BPTF*
Timofeeva [616]	Meta-analysis: 14,900 cases (smokers and never smokers)	29,485 Controls (smokers and never smokers)	Various	5p15, 6p21, 15q25 for NSCLC; 9p21 for SCC
Kim [617]	285 Female never smokers with lung cancer	1455 Controls	Affymetrix (440, 794)	2p16 *NRXN1*

[a]In Table 28.5, replication study samples sizes have not been included. For details of these, see http://www.genome.gov/gwastudies

have adjusted for measures of smoking intensity. With this adjustment, the association with nicotine receptor SNPs has remained positive in many studies. The mechanism postulated is that variation in the nicotine receptor pathway, due to genetic variation, is contributing directly to lung cancer susceptibility, in addition to or independent of smoking intensity. Another chromosomal region, at 13q, has been associated with lung cancer in never smokers [596].

28.9 Familial Lung Cancer

Hereditary predisposition to lung cancer is rare. However, analysis of the genomic aberrations provide insight into lung cancer susceptibility and biology. Gazdar et al. reported a 29-year-old female proband with a minimal smoking history and a 4.4 cm lung adenocarcinoma [597]. *EGFR* analysis of

the tumor revealed an L858R mutation, as well as a T790M mutation which was also detected in her germ line DNA in peripheral blood monocytes. 14 of the tested family members over a number of generations were also carriers, and of these, three, who were never smokers, also developed lung cancer (in addition to the proband). Yamamoto et al. reported a family of Japanese descent with multiple members with lung adenocarcinoma, inherited in an autosomal dominant pattern [598]. The proband was a 53-year-old lady who was a very light former smoker with multiple lung adenocarcinomas. The use of whole-exome sequencing found a novel germ line mutation (G660D) in the transmembrane domain of the human epidermal growth factor receptor 2 (*HER2*) gene, which, on functional testing, activated akt and p38. Overall, these reports of germ line mutations in familial lung cancer are very uncommon and their applicability to sporadic lung adenocarcinoma remains to be determined.

28.10 Differentiation Factors

Some genetic abnormalities of oncogenes and tumor suppressor genes are common to all lung cancers but others appear more specific for certain histological subtypes. RAS mutation is specific to NSCLC, RB1 mutation relatively predominant in SCLC and CDKN2A/CDKN2A mutation mostly occurs in NSCLC. Aberrant expression of CD44 is considered to be a marker for NSCLC [4, 619]. It is possible that such specificities may be important for the phenotypical development of that histological subtype, but the possibility that it is a secondary phenomenon remains. In support of the former paradigm, the in vitro introduction of oncogenes and tumor suppressor genes into lung cancer cell lines can induce phenotype transition among subtypes of lung cancer. For instance, the v-Ha-RAS gene was shown to change SCLC phenotype of lung cancer cells into a NSCLC phenotype [619].

A few normal bronchial epithelial cells and a substantial proportion of lung cancers, particularly exemplified by SCLC, exhibit a neuroendocrine phenotype. The genetic factors responsible for neuroendocrine differentiation in either normal lung or lung cancer are not well elucidated. Candidate molecules include the achaete-scute family of basic helix-loop-helix transcription factors which play a critical developmental role in neuronal commitment and differentiation of both Drosophila and vertebrates. The human achaete-scute homologue-1 (hASH1) gene is selectively expressed in normal fetal pulmonary neuroendocrine cells, as well as in lung cancers with neuroendocrine features [620]. Additionally, pulmonary neuroendocrine cells appear absent in newborn mice with ASH1 disruptions and antisense mediated ASH1 depletion from lung cancer cells results in a significant decrease in the expression of neuroendocrine markers [620].

28.11 Transgenic Animal Models

Transgenic and knockout mice are being increasingly used to understand lung cancer pathogenesis. Different transgenic animals have been generated which show dramatic developmental abnormalities including lung structure, suggesting crucial roles of the genes in normal lung development [97]. Early animal models included mice overexpressing mutant alleles of TP53, lacking E2F1, or which express a truncated nuclear retinoic acid receptor β (RARβ) [210, 240, 621].

Since then an increasing variety of transgenic animals have been developed for human lung cancer [622]. Expectedly perhaps, many of molecular aberrations identified in human tumors or cell lines are also found in the mouse models. There are mouse models for spontaneous and chemically induced lung tumors. These make use of the inherent differences in susceptibility to and incidence of spontaneous lung tumors between different inbred strains such as A/J or SWR mice [622]. Indeed three pulmonary adenoma susceptibility (PAS) loci were mapped from recombinant inbred strain crosses derived from susceptible A/J and resistant C57BL/6 strains [623]. Transgenic murine lung cancer models are based on the introduction of human lung cancer genetic aberrations into the mouse germ line or pulmonary tissue. The resulting murine lung tumors resemble in many aspects their human lung tumors counterparts. Various transgenic models have also been developed where oncogene expression is targeted to a specific subset of lung epithelial cells, thus allowing us to examine the role of these oncogenes in lung tumor initiation and progression. The models include transgenic (CC10, Sp-C, TGFβ, RARB, HRas), conditional transgenic (KRas), regulatable by mifepristone or doxycycline (Sp-C, FGF), spontaneous knockin (KRas and Kras/Trp53−/−), and conditional knockouts (Trp53, RB1) [622]. Interestingly, most models are for adenocarcinoma, with no currently reported somatic models for SCCs, and only a single model for SCLC (several others for neuroendocrine lung cancers).

Transgenic animal models are likely to contribute in the future to the currently limited knowledge of the functional relationship between the genes required in normal lung development and those mutated in lung cancer. These will be useful for many purposes, including testing agents for chemoprevention, e.g., with EGFR inhibitors [624] and for better understanding of the biology of oncogene stimulation (e.g., the requirement for continuous stimulation in a KRas adenocarcinoma model [625], experimentally supporting the oncogene addiction paradigm). Another important goal is improved understanding of the metastatic cascade through the generation of compound, inducible mouse models with relevant conditional dominant or recessive oncogenes that lead to a metastatic phenotype.

28.12 Telomerase Activation

Vertebrates have special structures at the ends of their chromosomes (telomeres) which are composed of 5–15 kb pairs of a guanine-rich hexameric repeat (TTAGGG)n. In normal somatic cells there is a progressive degradation of telomeres with aging. The loss of these telomeric repeats during normal cell division is thought to represent an intrinsic cellular clock by gradually shortening the telomere. The progressive telomere shortening is believed to lead to senescence and thus govern normal cellular mortality. Germ cells and some stem cells compensate for the telomere shortening as they possess a telomerase activity which replaces the hexameric repeats at the chromosomal ends. Normal somatic cells however, do not express telomerase, presumably because they do not need to replicate or only replicate to a finite degree. Thus, immortal cells may be able to proliferate indefinitely because they express telomerase activity whereas the vast majority of normal adult cells do not. The length of terminal telomeric restriction fragments is altered in various types of tumors, including lung cancer. Telomere shortening was detected in 14 of 60 primary lung cancers, while 2 cases showed telomere elongation [626].

Telomerase is upregulated or reactivated in almost 90 % of all human cancers. Approximately all SCLC and 80–85 % of NSCLC were demonstrated to express high levels of telomerase activity as measured by a highly sensitive telomere replication amplification protocol (TRAP) [626–628]. A high level of telomerase activity was associated with increased cell proliferation rates and advanced pathologic stage in primary NSCLC [628]. In addition, telomerase activity and/or expression of the RNA component of human telomerase were frequently dysregulated in carcinoma in situ lesions, implicating its early involvement in the multistage development of lung cancer [629]. Catalytic subunits of human telomerase have been cloned and appear expressed in human cancers [630]. Their expression and regulation need to be studied in lung cancer. It also remains to be seen whether a novel mechanism for lengthening their telomeres, named ALT (Alternative Lengthening of Telomeres) found in some immortalized cell lines with no detectable telomerase activity, is relevant to those lung cancers without high levels of telomerase expression [631, 632].

28.13 Lung Cancer Stem Cells

Somatic stem cells can be regarded as cells with a relative undifferentiated phenotype but with multipotent differentiation capacity. Other features include a low rate of self-renewal, an extended life span, and existence in a specific microenvironment, termed stem cell niches [633]. The cancer stem cell hypothesis suggests that tumors are derived from a single cell which is transformed into a cancer-initiating cell (cancer stem cell) that has the capacity to give rise to another stem cell and also a progenitor cancer cell that proliferates and form tumors [634]. This concept is important to cancer medicine since one explanation for the observation of cancer recurrence despite curative intent chemotherapy or radiotherapy has been postulated to result from survival of cancer stem cells because of their low proliferation rate and potential drug resistance.

Distal airway epithelial cells have the capacity to self-renew after naphthalene inhalation, implying the presence of stem cells in the pool of (neuro) epithelial cells along the bronchial lining [635]. Stem cells can produce daughter cells or transient-amplifying (TA) cells, which often have a limited proliferation capacity and a distinct marker profile before they enter a terminal differentiation state. In the lung, Clara and alveolar type II cells seemingly fit this TA cell phenotype since both do proliferate but finally differentiate into ciliated and alveolar type I cells, respectively.

Direct proof of lung cancer stem cells is so far lacking. However, a population of bronchoalveolar duct junction cells called bronchoalveolar stem cells (BASCs) have been isolated—these were resistant to bronchiolar and alveolar damage, proliferated during epithelial cell renewal in vivo, exhibited self-renewal and were multipotent in clonal assays [636]. Moreover, BASCs expanded in response to oncogenic K-ras in culture and in precursors of lung tumors in vivo, leading to the hypothesis that BASCs are a stem cell population that maintains the bronchiolar Clara cells and alveolar cells of the distal lung and that their transformed counterparts give rise to adenocarcinoma. Recently, a small sample of lung cancers were shown to displayed a small side population subset of cells where the side population phenotype is linked to their ability to efflux Hoechst 33342 dye, a characteristic found in stem cells [637].

Stem cells do not generally exhibit DNA hypermethylation at tumor suppressor genes [638]. Transcription of these genes is governed by patterns of promoter chromatin histone methylation under the control of polycomb proteins [639, 640]. Malignant stem cells have additional repressive histone marks of the type associated with aberrant DNA hypermethylation in adult cancers. In colorectal cancers these same marks occur more frequently in promoters of genes specifically methylated in tumors than in genes with a constitutive methylation state [641]. In lung cancer a high proportion of hypermethylated loci have at least one polycomb repressive complex 2 mark [642], which is further evidence of the presence of a stem cell phenotype within lung cancers. Biological properties specific to lung cancer stem cells present opportunities to target this cell population with the aim of preventing tumor reconstitution after treatment.

28.14 Evading Host Immunity

The major role of the immune system is to distinguish between self and nonself. The concept of immune surveillance postulates that tumor cells express various novel tumor-specific epitopes which represent potential immune non-self-targets. Thus, established cancers are thought to have effectively avoided immune recognition and elimination. Several different genetic mechanisms for escaping this immune surveillance have been elucidated in lung cancer patients. It has been shown that the class I major histocompatibility complex (MHC) antigen expression is downregulated in human cancers including lung cancer [643, 644]. These class I MHC molecules mediate presentation of endogenous antigenic peptides to cytotoxic T-lymphocytes. In addition, rare mutations of the β2-microglobulin gene whose protein product comprises a component of the class I molecules, have also been reported in lung cancer cells [645]. Indeed, reintroduction of the β2-microglobulin gene into the mutated cells restored the MHC class I expression [645]. For the stable assembly of MHC class I complex and subsequent recognition by cytotoxic T-lymphocytes, transporters associated with antigen presentation (TAP1 and TAP22), are necessary for conveying intracellular peptides into the endoplasmic reticulum for complex formation with class I MHC. The TAP1 transporter has been shown to be downregulated in lung cancer [644, 646]. Although the TAP1 gene does not appear somatically mutated in lung cancer, one SCLC cell line was found to be transcriptionally silent for its wild-type allele and only expressed a genetically defective TAP allele, leading to an acquired loss of antigen presenting ability [647]. The Fas (CD95) system is an important mediator of T-cell cytotoxicity. The FasL ligand can induce activated T-cells to undergo apoptosis, and this mechanism is thought to contribute to development of immune privilege sites. It is notable that lung cancer cells (both lines and primary tumors) express FasL and coculture of lung cancer cell lines with a Fas-sensitive human T-cell line induced apoptosis in the T-cells [648]. Thus, the expression of FasL by neoplastic lung cells can be speculated to be a potential mechanism for evading host immunity by the peripheral deletion of tumor-reactive T-cell clones [649, 650].

28.15 Tumor Angiogenesis

For either primary or metastatic tumors to grow, it is essential that they receive an adequate blood supply which is provided by recruiting new blood vessels from the surrounding host vasculature. The development of new vessels toward and within the tumor is required for adequate nutrient delivery and necessary at the beginning and end of metastatic dissemination. Without angiogenesis, tumors are unable to grow larger than 2–4 mm in diameter. During angiogenesis, normally quiescent endothelial cells become invasive, breaching their own basement membrane to invade the stroma during the development of new capillary buds. The number of microvessels in the tumor area, representing an index of angiogenesis, has been shown to correlate with an increased risk of metastatic disease and a worse overall survival for several types of human cancer including lung cancer. The higher the microvessel density, the poorer is the clinical outcome [651, 652]. The process of tumor angiogenesis is complex and controlled by a diverse family of inducers and inhibitors governing angiogenesis and regulating endothelial cell proliferation and migration [653]. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are amongst the most important tumor angiogenesis inducers. In lung cancer, VEGF expression was significantly lower in TP53-immunohistochemically negative (wild-type) and lowly vascularized NSCLCs [654, 655]. VEGF expression was higher in lung cancers with nodal metastasis than those without [656]. In squamous cell lung carcinomas, VEGF expression was inversely correlated with overall survival of patients and Flt-1, the receptor of VEGF, was also shown to be frequently expressed [657]. Both the basic fibroblast growth factor (bFGF) and its receptor (FGFR-1, Flg) are variably expressed in NSCLCs. Whether there is an association between bFGF expression and prognosis is currently controversial [658, 659]. In either case, expression of another angiogenic/endothelial cell chemotactic factor, platelet-derived endothelial cell growth factor (PD-ECGF, also called gliostatin), correlated with tumor angiogenesis and adverse prognosis in a study of N0 NSCLC patients [660]. Many angiogenesis inhibitors are stored as cryptic parts of larger molecules. These inhibitors include angiostatin, a fragment of plasminogen, and endostatin, a fragment of collagen XVIII. Endostatin administered to Lewis lung carcinoma-bearing mice was shown to regress tumors without inducing drug resistance during six treatment cycles [661]. Another inhibitor, thrombospondin-1 (TSP-1) is regulated by the wild-type TP53, such that loss of TP53 function (a common feature of lung cancers) was associated with a reduction in the levels of this angiogenesis inhibitor [662]. Restoration of TP53 function upregulates TSP-1 and impairs tumor cells angiogenesis. Another angiostatic factor, interferon-γ-inducible protein 10 (CXCL10) was shown to inhibit human NSCLC tumorigenesis and spontaneous metastases [663].

Thus, it is the balance between angiogenesis inducers and inhibitors expressed from tumor cells which appears to affect the ability of the normally quiescent vasculature to form new capillaries [653]. Another example comes from the finding that an imbalance in the expression of the angiogenic or angiostatic CXC chemokine family favors angiogenesis in NSCLC [664]. The isolation and synthesis of molecular

regulators has enhanced our understanding of angiogenesis and, angiogenesis inhibitors as well as inducers need to be carefully studied in lung cancer. Such studies have led to the development of targeted therapies against pro-angiogenic molecules particularly against one of the key mediators, VEGF. Bevacizumab, a recombinant humanized monoclonal anti-VEGF antibody, is the most clinically advanced antiangiogenic agent in NSCLC [665]. In a phase III study, bevacizumab showed significantly improved overall and progression-free survival when used in combination with standard first-line chemotherapy in patients with advanced NSCLC but some tumor-related bleeding adverse events occurred, predominantly those with squamous cell histology or centrally located tumors.

28.16 Conclusions

Our understanding of the role of the molecular genetic changes that occur during lung cancer pathogenesis, although in its early stages, is advancing rapidly with several specific genes and pathways being identified. The gene products are being classified into several important growth and cell cycle regulatory pathways. Some of these markers appear to be important for carcinogenesis in general, whereas others show more frequent involvement in lung cancers.

There has been much emphasis on the molecular diagnosis of early disease or pre-invasive changes. Most cases of lung tumors which are detected by imaging, have grown to at least 1 cm in diameter, with a mass of a gram or more, and contain at least 10^8–10^9 cells, estimated to be about 30 doubling times from single malignant cell. This only leaves a narrow window of opportunity for intervention before the tumor burden reaches the lethal range, about 10^{12} cells (about a kilogram or another 10 doubling times) or metastatic disease develops. In other words, about three quarters of the natural history of the cancer has occurred by the usual time of clinical detection by traditional methods. The considerable number of genetic lesions identified in overt lung cancer justifies attempts to detect corresponding lesions in preneoplastic lung tissue. If there are a few critical and consistent early lung cancer clonal markers, one could envisage an opportunity to detect preneoplastic or early lesions by testing a panel of selected markers. An example frequently cited is the finding of TP53 mutations in a retrospective analysis of sputum samples obtained from patients undergoing screening procedures who subsequently developed lung cancer [666]. The hope is that intervention with chemoprevention or other means can block progression to multiple other genetic lesions in these same cells and the development of overt malignancy. Otherwise early lung cancer biomarkers including genetic susceptibility markers are also likely to impact on clinical efforts to identify people most at risk of lung cancers for most effective and cost-effective screening and early detection techniques such as low dose spiral CT scans.

The challenge is to continue translating understanding of the disrupted cellular physiology into clinical benefit for the patient by expanding the diagnostic and therapeutic armamentarium. Effective strategies may include: early molecular diagnosis; identification of high risk individuals for developing lung cancer; early treatment studies; rational development of novel therapies such as tumor vaccines; genetic inhibition of growth factor loops by regulation of agonists and antagonists and by other mechanisms such as monoclonal antibodies; epigenetic alteration of gene expression such as by alteration of methylation; manipulating intrinsic tumor chemo- or radiosensitivity; blocking the expression of activated oncogenes; and replacing defective tumor suppressor genes. Knowledge from molecular studies has already spawned new agents at the bedside, particularly the targeted therapies of EGFR tyrosine kinase inhibitors, angiogenesis inhibitors, and agents targeted to the EML-ALK and ROS1 fusion abnormalities. Dual specificity or combination targeted drugs are in clinical trials. Individualizing treatment based on molecular tumor characteristics will increasingly become practical, with the ultimate aim of maximizing therapeutic success and minimizing toxicity aided by post-genomic tools such as arrays, proteomics, and animal models. Stem cell directed strategies also appear highly promising, as do emerging immunomodulatory medicines, including monoclonal antibodies that target key immune molecules, anti-CTLA4, anti-PD1/PDL1. So far, these treatments appear to have potential efficacy in metastatic lung cancer in early clinical trials.

Thus, the classic paradigm proposed by Boveri and Little that cancer is essentially a genetic disease at the cellular level, and now confirmed by modern genetic/genomic, epigenetic/epigenomic techniques, and coupled with the emergence of proteomics will afford us new approaches to the prevention, early detection, and treatment of lung cancer. There is now a rational hope that new therapies directed against one or more likely several of these targets that are drivers of the carcinogenic process may be successful in more effectively treating advanced lung cancer.

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Molecular Basis of Skin Carcinogenesis

29

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29.1 Introduction

The incidence of skin cancer exceeds the incidence of all other human cancers combined [1]. Epidemiologic, clinical, and biologic studies indicate that solar ultraviolet (UV) radiation is the major etiologic agent in skin cancer development. Wavelengths in the UVB range of the solar spectrum (290–320 nm) are absorbed by the skin producing erythema, burns, immunosuppression, DNA mutations, and nonmelanoma skin cancers (NMSC) [2].

Multiple genetic alterations have been shown to have a direct correlation with cancer development. The majority of these mutations can be found within three categories of genes: proto-oncogenes, tumor suppressor genes, or DNA repair genes. A mutation in one of these groups or any combination can cooperate to induce a neoplastic condition. The proto-oncogenes act as crucial growth regulators in normal cell division, while the tumor suppressor genes act as negative growth regulators [3].

The *p53* tumor suppressor gene is involved in the cell cycle arrest and activation of programmed cell death. Mutations in the *p53* gene have been detected in 50 % of all human cancers and in almost all skin carcinomas [4]. The majority of carcinomas have missense mutations that produce a full-length protein with altered function. Often the other allele is lost resulting in loss of heterozygosity (LOH) which is particularly high (40–80 %) in carcinomas of the colon, lung, and bladder [5]. Several studies have shown that the p53 tumor suppressor gene is susceptible to UV-induced mutations and plays a critical role in the induction of NMSC.

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In squamous (SCC) and basal (BCC) cell carcinomas of the skin the frequency of LOH is much lower with a higher proportion of both p53 alleles being independently mutated [6]. Mouse skin models have shown that standard chemical initiation/promotion protocols results in LOH, whereas repeated carcinogen experiments (like UV exposure) results in independent mutations on both p53 alleles [7]. Upon repeated exposure to UV radiation, DNA lesions in the *p53* gene are transformed into mutations, mainly C→T or CC→TT transitions at dipyrimidine sites (UV signature mutations), thereby initiating the molecular process of skin carcinogenesis [8]. Thousands of p53 mutant cell clones are found in sun-exposed skin that appears quite normal. The frequency of UV signature mutations in the *p53* gene is high in precancerous lesions and reaches 50–90 % in human BCCs and SCCs and 100 % in murine UV induced skin tumors [9, 10].

In addition to the disrupted p53-dependent mechanism, development of NMSC also may involve dysregulation of the Rb-controlled pathway of the cell cycle progression including inactivation of the *CDKN2A* tumor suppressor gene in the 9p21 region [11].

29.2 Acute Effects of UV Radiation

29.2.1 UV-Induced DNA Damage

Irradiation of DNA or cells with ultraviolet light induces the formation of several types of mutagenic DNA lesions. The most frequent lesions induced by UVB radiation are the cis-syn cyclobutane pyrimidine dimers (CPDs) and the pyrimidine pyrimidone photoproducts (PPs) [12, 13]. Several minor photoproducts such as purine dimers and pyrimidine mono-adducts are also formed [14].

CPDs are formed between the 5,6 bonds of any two adjacent pyrimidine bases. PPs are characterized by a stable bond between position 6 and 4 of the neighboring pyrimidines and appear to form preferentially at 5′-TC and 5′-CC

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_29

sequences. They are formed at levels considerably lower than those of CPDs [15, 16]. These unique lesions in the DNA give rise to unique mutations in the *p53* tumor suppressor gene. Consequently UVB induces mostly C→T and CC→TT transitions at the dipyrimidine sequence which are therefore called UV signature mutations [17].

The genotoxicity of UVA has most commonly been linked to endogenous photosynthesizers causing reactive oxygen species mediated induction of DNA damage [18, 19]. More recently it has been reported that UVA can induce CPDs in rodent cells. The poor absorbance of UVA by DNA itself favors the idea that type I (direct reaction) or type II (indirect through reaction of the excited photosensitizer molecule with oxygen) photosensitization reactions occurring within cells are primarily responsible for UVA induced DNA damage [20]. In addition, UVA may produce H_2O_2 through activation of flavin containing oxidases or through release of free iron in the irradiated cells from the iron storage protein ferritin [21, 22]. UVA has been shown to induce 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a typical oxidative DNA lesion, as well as generate DNA strand breaks in a variety of experimental systems [23, 24].

29.2.2 Induction of p53 Tumor Suppressor Protein

p53 codes for a 53 kDa phosphoprotein involved in gene transcription and control of the cell cycle by coordinating transcriptional control of regulatory genes. Human *p53* is a highly conserved 11 exon gene that is located on the short arm of chromosome 17 that is about 20 kb in size. p53 protein forms tetramers through interactions between recognize specific binding sites on target genes and stimulate their activation. Mutant forms of *p53* rarely exhibit mutations in the DNA binding domain [25, 26].

Normally there is little p53 protein in the cell, but numerous studies have shown that in response to UV damage, high levels of p53 protein are induced. This has been demonstrated in cell culture, mouse epidermis, and human tissue [27]. With high levels of p53 protein, there is a G1 arrest, allowing the cellular repair pathway to remove DNA lesions before DNA synthesis and mitosis and an increase in apoptosis. Therefore *p53* aids in the DNA repair or the elimination of cells that have excessive DNA damage [3, 25]. *p53* is stabilized as early as 6 h after UV exposure reaching a peak at 12 h. Nelson and Katzan found the DNA lesions induced by UV radiation were pyrimidine dimers and that *p53* was induced when these lesions were accompanied by excision repair-associated DNA strand breaks [28].

UV radiation induces the activating phosphorylation of the p53 protein at multiple serine residues, including Ser15, Ser20, Ser33, Ser37, Ser46, and Ser392. It has been also shown the involvement of the ATM, ATR, p38, ERK1/2, and JNK-1 mitogen activated protein (MAP) kinases in the phosphorylation of various p53 serine residues in response to UV radiation [29–31].

29.2.3 Cell Cycle Arrest and DNA Repair

Accumulation of activated p53 protein induces a cell cycle arrest at G1 phase which allows the repair of DNA damage before is replication in the S phase.

The p21Waf1/CIP1 gene encodes an inhibitor of cyclin dependent kinase (CDK) whose induction is associated with the expression of wild-type p53. p21 inactivates the CDK cyclin complex by forming a complex of CDK2, cyclin A, PCNA, and p21 leading the cell into G1 arrest. Formation of these complexes leads to the accumulation of hypophosphorylated retinoblastoma protein (Rb) causing the release of the E2F transcription factor which is necessary for DNA synthesis [32, 33].

The tumor suppressor activity of p53 is in turn inhibited by the Mdm2 protein, which targets p53 for rapid degradation. The gene encoding Mdm2 is itself activated by p53 thus providing a negative autoregulatory loop. If normal DNA repair is not achieved, cells proliferate abnormally which can lead to carcinogenesis [34].

The induction of DNA damage appears to play a central role in photocarcinogenesis and efficient removal of DNA lesions by cellular repair processes appears to be a critical step in the prevention of tumor formation. Interaction of UV with DNA can give rise to different forms of DNA damage which is normally removed by nucleotide excision repair (NER). The most relevant DNA damage generated after UV irradiation is the formation of CPDs. These lesions are primarily repaired by NER which removes bulky DNA damage in two distinct sub-pathways [35]. Damage existing in actively transcribed genes is removed by a quick mechanism called transcription-coupled repair (TCR) [36]. Damage prevailing in other parts of the genome is removed with a slower mechanism by the global genome repair (GGR) [37]. Defects in both sub-pathways of NER can lead to three distinct diseases: Xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD). These diseases provide important model systems for investigating aspects of DNA repair since they are all deficient in NER but in different sub-pathways.

p53 directly affects NER by inducing the transcriptional activation of several downstream genes. Cells lacking functional p53 are defective in removing DNA photoproducts which are normally repaired by NER complexes [38].

In addiction to that, there is the base excision repair pathway (BER) which removes lesions caused by oxidative damage. It has been postulated that p53 affects BER by

regulating DNA polymerase β which is directly involved in DNA repair/synthesis [39]. Cells lacking p53 are deficient in BER [40].

29.2.4 Apoptosis

Keraratinocytes respond to UV-induced DNA damages by growth arrest or induction of the programmed cell death—apoptosis. *p53* plays a major role in the control of both of these processes. In fact these two *p53*-dependent mechanisms are believed to maintain the integrity of the genome and to protect cells from the accumulation of mutations. Consequently, induction of apoptosis of keratinocytes by UV radiation is a protective phenomenon relevant in limiting the survival of cells with irreparable DNA damage [41].

p53-induced apoptosis involves several mechanisms and among them the bcl-2 gene family seems to play a crucial role. The bcl-2 gene family is composed of the pro-apoptotic gene group (*Bax, Bad, Bak, Bcl-xs, Bik, Bim,* and *Mtd/Bok*) and the anti-apoptotic gene group (*Bcl-2, Bcl-w, Bcl-xL,* and *Mcl-1*). Bax and Bcl-2 proteins are homologous but they have opposing effects on apoptosis and they may form heterodimers in cells [42]. Thus their interaction is critical to the ability of Bcl-2 to block cell death. p53 induces apoptosis by disrupting the balance between these proteins, upregulating the *Bax* gene, and downregulating the *Bcl-2* gene [43].

Another *p53* induced apoptotic mechanism is through the expression of Fas (APO-1/CD95) and its ligand. Fas receptor is a target for transcriptional activation by *p53* and a target for *p53*-mediated cytoplasmic redistribution to the cell surface [44]. The Fas-Fas ligand interaction results in the cleavage of pro-caspase 8 and release of cytochrome c from mitochondria, followed by activation of the Apaf-1-caspase 9-caspase 3 pathway which results in apoptosis [45]. Hill et al. in 1999 demonstrated that Fas-Fas ligand interactions are essential for the induction of sunburn cells in UV-irradiated mouse skin and the elimination of aberrant cells following skin exposure to UV radiation. UVB irradiation induced the rapid overexpression of Fas and Fas ligand in murine skin. The formation of sunburn cells was found to depend on Fas ligand expression since their formation was significantly reduced in Fas ligand-deficient mice [46].

29.3 Chronic Effects of UV Radiation

29.3.1 Mutations in p53 Tumor Suppressor Gene

Following chronic UV exposure, mistakes associated with DNA repair and replication can result into mutations in the *p53* gene, especially C → T and CC → TT transitions at dipyrimidine sites, considered as UV molecular signature. The p53 mutation in keratinocytes is probably an initiating event in UV skin carcinogenesis [8].

As UV signature mutations in the *p53* gene are already present in benign precursor lesions of squamous cell carcinomas (AK), they appear to be an early step in the UV carcinogenesis. In the experiments with hairless mice, microscopic clusters of epidermal cells overexpressing mutant *p53* occur long before skin carcinomas become visible [47]. Such clusters are also found in sun-exposed human skin [48]. Most of human NMSC found to bear mutations in the *p53* gene. Brash et al. in 1991 showed *p53* mutations in 58 % of human SCCs analyzed (3/24 showed CC → TT transition and 5/24 had C → T base change) [49]. In a recent study, Bolshakov et al. analyzed 342 human NMSC and found *p53* mutations in 28/80 aggressive SCCs and in 28/56 non aggressive SCCs. About 71 % of the detected *p53* mutations were UV signature mutations [50].

Experiments to determine the timing of p53 mutation in relation to skin cancer development have been performed in the mouse model of photocarcinogenesis because this model is easily referable to UV irradiation and sample collection at various time points during the carcinogenesis protocol. Ananthaswamy et al. have shown that p53 mutations in mouse skin arise very early during UV carcinogenesis by using a very sensitive mutation-specific PCR technique for the early detection of *p53* mutations in UVB irradiated C3H mice. They were able to detect p53 mutations at 4th week of UV irradiation and the frequency of p53 mutations increased progressively and reached 50 % at 12th week of chronic UV exposure. The real *p53* mutations frequency observed in this study is probably higher than the one described because only tandem CC → TT mutations were analyzed [51]. In addition to C3H mice, Ouhtit et al. used SKH-hr1 mice to determine the timing of p53 mutations during UV carcinogenesis. Interestingly, *p53* mutations were detected as early as 1st week of chronic UV irradiation and the mutation frequency reached 80–90 % by 4–8 weeks [52]. Both the early appearance and the high frequency of *p53* mutations in UV irradiated SKH-hr1 mice skin can be explained with the fact that either C → T or CC → TT mutations were analyzed in this study.

29.3.2 Fate of p53 Mutations After Discontinuation of UV Exposure in Mice

It has been widely demonstrated in animal models that UV acts as a tumor promoter and induces cell proliferation by stimulating the production of various growth factors and cytokines as well as activation of their receptors [53]. Repeat UV exposure of the skin results in clonal expansion of initiated p53-mutant cells. Two mechanisms are believed to con-

tribute to the selective expansion of p53-mutant cells: (1) their resistance to UV-induced apoptosis, and (2) their proliferative advantage over normal keratinocytes in response to stimulation with UV radiation [54, 55].

Zhang et al. have shown that every successive UVB exposure allows p53-mutant keratinocytes to colonize adjacent epidermal stem cell compartments without incurring any additional mutation [56]. Chronic UV irradiation of the skin quickly induces apoptosis resistance and stimulates hyperproliferation throughout the epidermis as an adaptive response [52]. Nevertheless, discontinuation of UV irradiation has been shown to result in the fast spontaneous regression of some mutant p53 clones in mouse skin [57]. Melnikova et al. investigated whether discontinuation of UV exposure before the onset of skin tumors results in the disappearance of *p53* mutations in the skin of SKH-hr1 mice. Their results indicated that despite discontinuation after 8 weeks, UV irradiation results in 100 % skin tumor incidence, although the kinetics of tumor occurrence is greatly delayed. In terms of human relevance these results suggest that early life exposure to UV may introduce *p53* gene mutations in epidermal keratinocytes as well as keratinocytes progenitors. While some *p53* mutated keratinocytes may be eliminated via differentiation and epidermal desquamation, others, may still persist and eventually give rise to skin tumors even in the absence of further UV exposure. Thus, cancer development can be delayed but not definitively abrogated upon further avoidance of exposure to UV radiation [58].

29.4 Oncogene Activation and Tumor Suppressor Inactivation in Skin Carcinogenesis

29.4.1 Ras Oncogene Activation and Skin Cancer

The *ras* family of proto-oncogenes encodes small GTP binding proteins involved in intracellular signal transduction of mitogenic signals arising from activation of growth factor receptors. Mammalian *ras* genes are important constituents of these mitogenic signalling pathways and when activated, they contribute to dysregulated cellular growth [59].

In mammals, keratinocytes respond to UVB irradiation with a marked downregulation of the ras extracellular signal regulated kinase (ERK) signalling cascade. The downregulation precedes a decrease in the steady state levels of cyclin D1 that mediates the action of ERK. The increased activity of *ras* and phosphorylation of ERK in these cells are maintained by the production of molecules that stimulate the epidermal growth factor receptor (EGFR). Irradiation of keratinocytes increases the phosphorylation of EGFR above the basal levels. This leads to the increased recruitment of the adaptor proteins to the UVB activated EGFR. At the same time, UVB causes the inactivation of *ras* and a subsequent dephosphorylation of ERK [60].

Normal skin has some *ras* mutations in the form of T:A → C:G base change at codon 27. Exposure of the skin to UVR induces photoproducts along several exons of the *ras* proto-oncogenes (*H-ras, K-ras,* and *N-ras*). These photoproducts included both cyclobutane pyrimidine dimers and pyrimidine pyrimidone photoproducts [61].

In NMSC activated *ras* genes were frequently found in BCCs, AK, and SCCs of the sun-exposed areas. This *ras* gene activation is the result of an aberrant repair of UV induced pyrimidine dimers. Those activating mutations were found mostly at codon 12 of the *K-ras* gene and codons 12, 13, and 61 of the *H-ras* gene [62].

29.4.2 PATCH Tumor Suppressor Gene Inactivation

PATCH gene is located on chromosome 9q22.3 regions and spans about 34 kb in genomic DNA. It is formed of 24 exons with two alternate exon1. The encoded protein is the receptor for sonic hedgehog, a secreted molecule implicated in tumorigenesis. The normal function of PATCH protein is to convey extracellular growth regulatory signals to the nucleus [63].

PATCH gene is the candidate tumor suppressor gene for familial and sporadic BCCs. Its mutations are frequent in BCCs and they are predominantly UV specific C–T transitions. This mutation are significantly more frequent in BCCs associated with xeroderma pigmentosum than in sporadic BCCs and they may be associated with allelic loss at the chromosomal region 9q22.3 harboring PATCH gene [64, 65]. Alternatively, occasional nonsense mutations were found in SCCs isolated from individuals with histories of multiple BCCs [66].

29.4.3 CDKN2A Inactivation

The CDKN2A locus at 9p21, is frequently inactivated in human cancers and it consists of two overlapping genes that encode two unrelated proteins, $p16^{INK4a}$ and $p14^{ARF}$, functioning as cell cycle inhibitors. $p16^{INK4a}$ and $p14^{ARF}$ share the same exon 2 but have a distinct exon 1, exon 1α and exon 1β, respectively. Because exon 1β splices into common exons 2 and 3 in a different reading frame, the resulting $p14^{ARF}$ bears no similarity to $p16^{INK4a}$. It is well established that $p14^{ARF}$ plays a role in cell cycle control linking the $p16^{INK4a}$/Rb pathway and the p53/Rb pathway. Upon phosphorylation of Rb, E2F is activated and promotes induction of $p14^{ARF}$, which in turn sequesters MDM2 and thereby prevents degradation and nuclear export of p53. Both $p16^{INK4a}$ and $p14^{ARF}$ transcripts

by their interactions with pRb and p53, are important in regulating the proliferation of normal and tumorigenic squamous epithelial cells [66, 67].

Inactivation of the tumor suppressor gene *CDKN2A* can occur in a variety of genetic mechanisms including mutations and deletions. In addition, hypermethylation of the CpG islands of gene promoters is an effective means of gene silencing in a variety of cancers. Inactivation of *CDKN2A* by deletion, mutation or promoter hypermethylation in a wide range of malignancies has been documented [68].

It has been shown by Soufir et al. that SCCs from xeroderma pigmentosum patients contain mutations in *CDKN2A* gene in 13/28 SCCs and 54 % of mutations detected at CDKN2A locus were UV signature mutations [69].

In order to determine the involvement of *CDKN2A* gene in sporadic SCCs, Saridaki et al. performed the allelic imbalance analysis and the mutational analysis on 22 SCCs and on 5 Bowen's disease specimens. Their results indicated that 52 % of specimens exhibited loss of heterozygosity (LOH) in at least one microsatellite marker whereas only 2/27 samples exhibited microsatellite instability. Mutational analysis revealed the presence of a base substitution in exon 1α of 1 tumor and the presence of a C → T transition in exon 2 in a second tumor [70].

Brown et al. analyzed 30 cutaneous SCCs from immunosuppressed 29 patients and 10 immunocompetent patients and have shown that the total frequency of 9p21 alterations was 76 %, with abnormalities of $p16^{INK4a}$ detected in 53 % of tumor analyzed and of $p14^{ARF}$ in 64 % of the tumors. Promoter methylation was the predominant mechanism of inactivation for both genes. Biallelic events were common [71].

Murao et al. examined the epigenetic abnormalities of a wide range of cancer related genes (*CDH1*, *p16*, *p15*, *RB1*, *p14*, *DAPK1*, *MGMT*, *RASSF1*, *PTEN*, *PRDM2*, and *p53*) in 20 sporadic SCCs from 20 immunocompetent patients. Their results showed that although the frequency of methylation of $p16^{INK4a}$, Rb1 and $p14^{ARF}$ was not high, methylation of these genes in combination with mutation analysis of *CDKN2A* and *p53* revealed that 70 % of cases had abnormalities of the RB1/p16 and/or p53 pathway through either genetic or epigenetic mechanisms, except for epigenetic abnormalities of *p53* itself [72].

All these findings emphasize the importance of *CDKN2A* tumor suppressor gene in the pathogenesis of NMSC.

29.5 Conclusions

UV radiation present in the sunlight is a potent carcinogen. Recent advances in cellular and molecular biology have clarified some of the mechanisms of photocarcinogenesis including the formation of DNA photoproducts, DNA repair, mutation of proto-oncogenes and tumor suppressor genes. It is well established that UV radiation induces mutations in the *p53* gene and that these mutations arise very early during photocarcinogenesis. Accumulation of several mutations in key genes due to chronic exposure to sunlight may lead to the development of skin cancer. Skin cancers do not arise immediately after exposure to UV light, mutated p53 or other tumor suppressor genes must remain latent for a long period of time. In addition to mutations in the *p53* tumor suppressor gene, genetic alterations in *CDKN2A* gene leading to loss of expression of $p16^{Ink4a}$ and $p14^{ARF}$ proteins may also play an important role in the development of human NMSC. Several studies have shown that human NMSC harbor unique mutations in the *p53* gene as well as inactivation of the *CDKN2A* gene. While mutations in the *p53* gene are induced by UV radiation and represent tumor initiating events, the majority of the alterations detected in the *CDKN2A* gene do not appear to be UV dependent. Probably these genetic alterations arise spontaneously, probably during tumor progression.

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Molecular Biology of Leukemias

30

L. Jeffrey Medeiros and Sergej N. Konoplev

30.1 Introduction

Normal hematopoiesis is a meticulously controlled process of cell division and differentiation arising from hematopoietic stem cells with self-renewal capacity. This process is regulated through several signaling pathways that appear to be, to some degree, redundant. In many neoplastic processes, alteration of one or several signaling pathways has been observed, implicating these pathways in pathogenesis.

Conventional cytogenetics analysis, which began in the 1970s, was a great boon to our understanding of acute and chronic leukemias. Approximately 50 % of de novo acute leukemias have distinctive molecular abnormalities, most frequently chromosomal translocations [1] (Table 30.1). These translocations typically affect genes that are involved in transcription and differentiation. In many translocations, two genes are disrupted and recombined to form a novel fusion gene from which chimeric mRNA is transcribed and protein is translated. Other translocations involve the juxtaposition of intact genes adjacent to genes with strong promoters or enhancers that upregulate gene expression. Inversions and interstitial deletions can also occur that juxtapose genes and have the same effects as translocations [1].

The advent of additional molecular methods and their application to leukemias furthered our understanding of the molecular pathogenesis of these neoplasms. Methods that contributed greatly included Southern, Northern and Western blot analysis, fluorescence in situ hybridization, various polymerase chain reaction (PCR)-based methods, and Sanger (first generation) sequencing. In general, molecular methods were used initially to assess single genes, or small numbers of genes. More recently, high-throughput methods such as array-based comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) arrays, and next (or second)-generation sequencing have resulted in an explosion of new data. It is now clear from the results of studies that have used whole genome, whole exome, transcriptome (mRNA), and targeted sequencing studies that acute and chronic leukemias are commonly associated with a number of somatic gene alterations and that involve key cellular pathways. Examples of involved pathways include transcriptional regulation of hematopoietic cell development, cell signaling related to tyrosine kinases or cytokine receptors, and chromatin modulation. These studies also have uncovered abnormalities in genes previously unknown to be involved in oncogenesis.

Although next-generation sequencing has shown many gene mutations in leukemias, other molecular mechanisms also have been implicated in leukemogenesis. Gene amplification can result in gene overexpression. Numerical gains or losses of chromosomes, such as trisomies or monosomies due to nondisjunction, are detected in a large subset of acute and chronic leukemias. Gene deletions, like those arising from partial chromosomal deletions or unbalanced translocations, can result in tumor suppressor gene inactivation or loss. Hypermethylation (i.e., epigenetic changes) is another mechanism of gene inactivation. Often more than one mechanism is involved, leading to the accumulation of genetic lesions culminating in leukemogenesis, or subsequently contributing to disease progression.

Traditionally, genes implicated in cancer pathogenesis have been divided into oncogenes and tumor suppressor genes. Oncogenes are considered to play an important role in pathogenesis having acquired constitutive activation through translocation or sporadic mutation, whereas tumor suppressor genes are involved in pathogenesis through their inactivation of gene/protein function. Point mutation, causing missense or nonsense mutations resulting in abnormal or truncated protein products, is a common mechanism of tumor suppressor gene inactivation. Although some genes involved in leukemogenesis follow this paradigm (for example, typical oncogenes *HOX* and EVI-1 and typical tumor-suppressor genes *PU.1*

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_30

Table 30.1 Cytogenetic and molecular abnormalities in acute myeloid leukemia

Chromosomal translocations	Genes	Comments
Involving RARAα		
t(15;17)(q21;q21)	*PML*	DNA binding, cell proliferation
	RARα	Transcription factor
t(11;17)(q23;q21)	*ZBTB16 (PLZF)*	Zn-finger transcription factor
	RARα	Transcription factor
t(5;17)(q32;q21)	*NPM1*	RNA transport/processing
	RARα	Transcription factor
t(11;17)(q23;q21)	*NUMA1*	Nuclear mitotic apparatus
	RARα	Transcription factor
del(17)(q21)	*PRKAR1A*	cAMP-dependent protein kinase type I alpha regulatory subunit
	RARα	Transcription factor
t(4;17)(q12;q21)	*FIP1L1*	FIP1-like 1
	RARα	Transcription factor
t(X;17)(p11;q12)	*BCOR*	BCL6 corepressor
	RARα	Transcription factor
t(2;17)(q32;q21)	*NABP1 (OBFC2A)*	Oligonucleotide/oligosaccharide-binding fold containing 2A
	RARα	Transcription factor
Involving core-binding factor		
t(8;21)(q22;q22.3)	*RUNX1T1*	Zn-finger transcription factor
	RUNX1	*Runt*-like transcription factor
t(3;21)(q26;q22)	*MECOM EAP*	Contains Zn-finger motif
	RUNX1	Runt-like transcription factor
t(12;21)(p13;q22)	*ETV6 (TEL)*	*ETS*-like transcription factor
	RUNX1	*Runt*-like transcription factor
t(1;21)(p36;q22)	Unknown	Rare in AML and MDS
t(5;21)(q13;q22)	*RUNX1*	*Runt*-like transcription factor
t(17;21)(q11;q22)		
inv(16)(p13;q22)	*CBFβ*	Stabilizes CBF binding to DNA
t(16;16)(p13;q22)	*MYH11*	Smooth-muscle myosin gene
del(16q)		
Involving KMT2A (MLL)		
t(11;v)(q23;v)	*KMT2A (MLL)*	*Drosophila trithorax* homology
	Variable	Many partner genes, *AF-9* is commonest
Involving nucleoporin genes		
t(6;9)(p23;q34)	*DEK*	Putative transcription factor
	NUP214 (CAN)	Nucleoporin
Cryptic	*SET*	Unknown function
	NUP214 (CAN)	Nucleoporin
t(7;11)(p15;p15)	*HOXA9*	Homeobox gene
	NUP98	Nuclear-pore complex gene
inv(11)(p15;q22)	*DDX10*	DEAD-box putative RNA
	NUP98	Helicase
		Nuclear-pore complex gene
Involving genes of the ets family		
(t16;21)(p11;q22)	*FUS (TLS)*	EWS-like RNA-binding protein
	ERG	*ETS*-like transcription factor
t(12;22)(p13;q11)	*ETV6 (TEL)*	*ETS*-like transcription factor
	MN1	Cloned from meningioma
Other translocations		
t(8;16)(p11;p13)	*KAT6B (MOZ)*	Monocytic leukemia Zn finger
	CREBBP (CBP)	Transcriptional activation
t(9;22)(q34;q11)	*ABL1*	Tyrosine kinase
	BCR	Unknown function

and *RARα*), some genes demonstrate overlapping features of both oncogenes and tumor-suppressor genes (such as *CBF*, *CEBPA*, *GATA-1*, and *NPM1*), suggesting that this traditional dichotomy is an oversimplification [2–7].

A new concept of gatekeeper genes has been recently introduced to our understanding of hematologic malignancies. This concept was first established in solid tumors (e.g., colon cancer) where inactivation of a particular pathway is thought to be the first genetic alteration in pathogenesis [8]. Several studies suggest that the genetic alterations affecting hematopoietic transcription factors may be the initiating event resulting in the establishment of a malignant clone, but then secondary events are required for disease manifestation. For example, a myelodysplastic syndrome (MDS) known to precede acute myelogenous leukemia (AML) is often associated with *RUNX1* mutations, which might be an initiating event in AML [9]. Additionally, cytogenetic and genetic abnormalities identified in therapy-related MDS and AML might be initiating genetic events in leukemogenesis [10]. Concordant AML in identical twins, which share the initiating clone carrying the same translocation affecting the *MLL* gene, supports the idea of gatekeepers in leukemias, as does the observation of *RUNX1* or *CEBPA* mutations in hereditary AML syndromes [11].

Some authors have proposed the concept that leukemogenesis requires the collaboration of at least two classes of mutations: class I, which activate signal-transduction pathways and confer a proliferation advantage to hematopoietic cells; and class II, which affect transcription factors and serve primarily to impair hematopoietic differentiation [12]. While some hematologic malignancies appear to follow this scheme, for example, acute promyelocytic leukemia (APL) carries *PML-RARα* impairing differentiation and many cases also have *FLT3* mutations leading to increased proliferation, there are too many exceptions to believe that this concept can be universally applied for all hematologic malignancies.

30.2 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is an umbrella term for a group of neoplasms composed of immature cells demonstrating differentiation into one of the myeloid lineages. The approach to classification of AML has changed dramatically over the years, from a purely morphologic system used in the French–American–British (FAB) classification [13] to a system that incorporates morphologic, immunophenotypic, genetic, and clinical features as proposed by the World Health Organization (WHO) classification [14]. The WHO classification divides AML in four major categories: (1) AML with recurrent genetic abnormalities, (2) AML with myelodysplasia-related changes, (3) Therapy-related myeloid neoplasms, and (4) AML, not otherwise specified (NOS).

From the point of view of molecular mechanisms, cases of AML with recurrent genetic abnormalities represent biologically unique entities. Cases of therapy-related AML and AML with myelodysplasia-related changes have overlapping biologic and cytogenetic features. The fourth category, which basically recapitulates the older approach of FAB classification, represents a heterogeneous group driven by a large variety of molecular alterations.

30.2.1 AML with Recurrent Genetic Abnormalities

30.2.1.1 AML Associated with t(8;21)(q22;q22.3): *RUNX1–RUNX1T1* (*AML1/ETO* or *RUNX1/MTG8*)

The t(8;21)(q22;q22.3) has been identified in 8–12 % of de novo AML [14]. Less commonly, t(8;21) has been identified in therapy-related myeloid neoplasms [15]. In adults, de novo AMLs with t(8;21) respond well to AraC-containing chemotherapy with a high rate of complete remission and relatively long survival. However, the presence of t(8;21) in pediatric AML cases may be less predictive of good prognosis [16]. Morphologically, most cases are classified as M2 using the FAB classification although only a subset of M2 cases carry t(8;21). The myeloblasts in these leukemias typically have distinctive cytological features, characterized by abundant evidence of maturation, including single slender Auer rods with tapered ends and salmon-colored cytoplasmic granules (Fig. 30.1) [17].

The t(8;21) is a reciprocal chromosomal translocation involving the *RUNX1T1* (*ETO)* gene on chromosome 8 and the *RUNX1* (*AML1*) gene on chromosome 21. As a result of

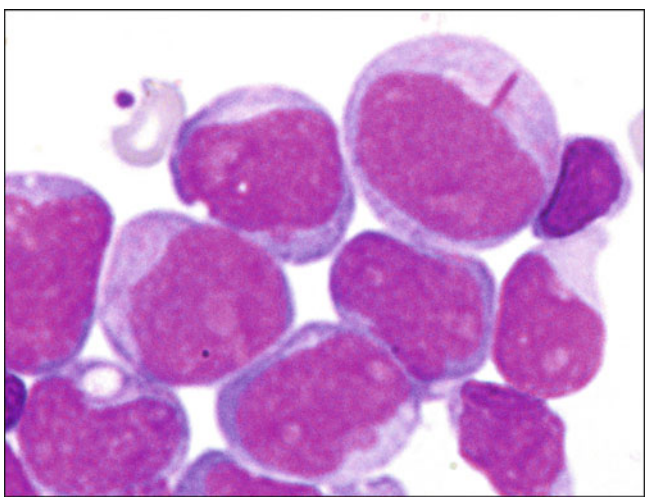

Fig. 30.1 Typical morphology of acute myeloid leukemia with t(8;21). Blasts show single long slender Auer rods with tapered ends. Wright-Giemsa stain, 1000×.

this translocation, the *RUNX1T1* and *RUNX1* genes are disrupted and fused forming an *RUNX1-RUNX1T1* fusion gene, located on the derivative chromosome 8. The *RUNX1* and *RUNX1T1* genes are fused in a $5' \rightarrow 3'$ orientation. The fusion protein includes the promoter and *runt*-like domains of the *AML1* protein; the translocation domain of normal *RUNX1* is replaced by sequences derived from the *RUNX1T1* gene. A reciprocal *RUNX1T1- RUNX1* fusion gene located on the derivative chromosome 21 has not been identified. One proposed mechanism for the role of *RUNX1-RUNX1T1* in leukemogenesis is competitive inhibition of normal RUNX1 protein. The breakpoints in the *RUNX1* gene are consistently detected in intron 5. The breakpoints in the *RUNX1T1* gene are also relatively uniform, in the 5′-end of the gene.

The *RUNX1* gene encodes for one of many members of a family of heterodimeric transcriptional regulatory proteins. The *RUNX1* gene is highly homologous with the *Drosophila runt* gene, and encodes core binding factor (CBF) α, the human counterpart of the murine nuclear polyoma enhancer binding protein (PEBP2). The binding of RUNX1 protein to DNA occurs via the *runt*-like central domain and also requires heterodimerization with CBF. The latter protein does not bind to DNA but improves the binding affinity of RUNX1. The *RUNX1* gene is normally expressed by myeloid and T-cells and is thought to play an important role in hematopoietic differentiation. The *RUNX1T1* gene (also referred to in some studies as *CDR* or *MTG8)* encodes a transcription-factor protein that has two zinc-finger-motifs at its C-terminus. The *RUNX1T1* gene is expressed in brain, lung, and gonads, but is not normally expressed in hematopoietic cells.

A variety of methods are available to detect t(8;21). This translocation is not difficult to recognize using conventional cytogenetics, which detects t(8;21) in over 95% of cases (Fig. 30.2). However, rare cases have been reported in which the translocation was not detected by cytogenetics, but was detected by other molecular methods [18]. FISH effectively identifies the t(8;21) (Fig. 30.3). RT-PCR methods detect

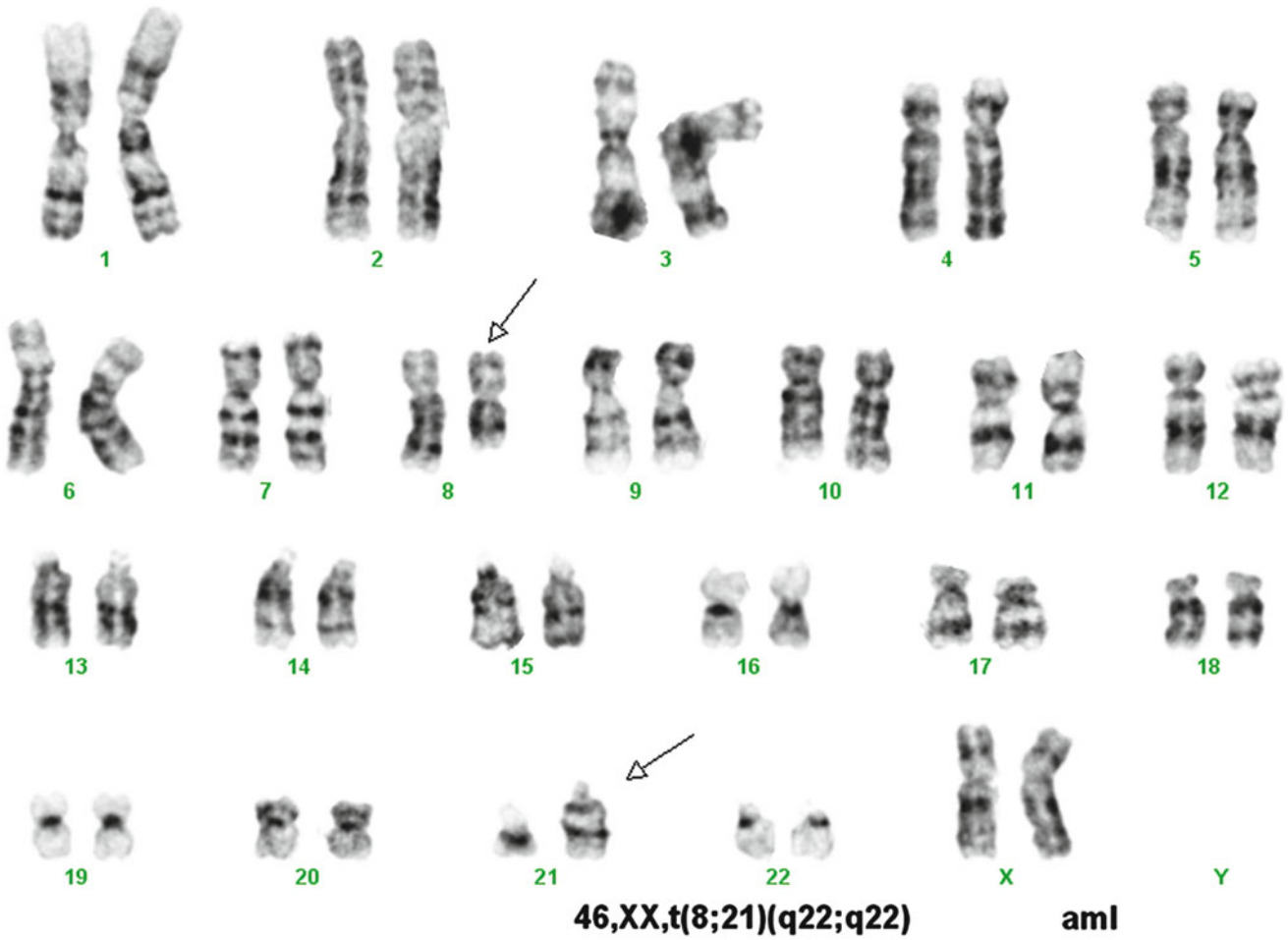

Fig. 30.2 Karyotype of a case of acute myeloid leukemia with t(8;21). Conventional cytogenetic analysis demonstrates t(8;21) as a sole cytogenetic abnormality. GTG banding technique.

almost all cases with the t(8;2l). RT-PCR methods, because of their extraordinary sensitivity, also have been used to monitor residual disease. However, studies have shown the persistence of low level *RUNX1-RUNX1T1* transcripts in patients in complete clinical remission after chemotherapy or stem cell transplantation, and who have remained in complete remission with prolonged clinical follow-up [19, 20]. Thus, RT-PCR results do not appear to effectively predict risk of relapse after therapy in patients in clinical remission [19, 20].

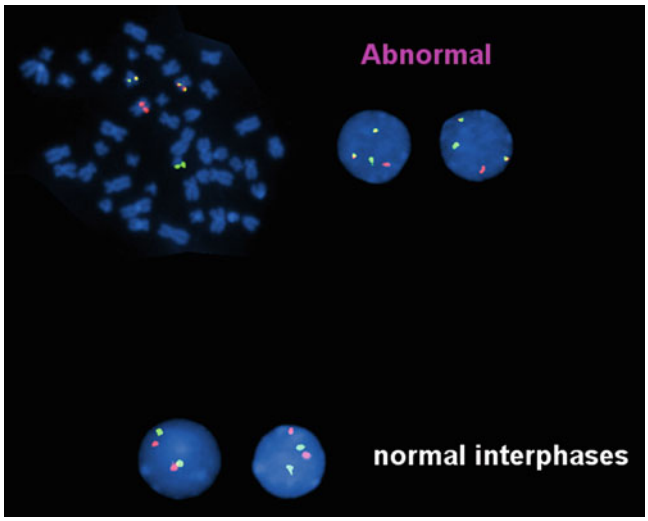

Fig. 30.3 Fluorescence in situ hybridization (FISH) analysis of a case of acute myeloid leukemia with t(8;21). Dual color, dual fusion probe (Vysis) in which an *RUNX1T1* probe is *red* and an *AML1* (*RUNX1*) probe is *green*. In the event of t(8;21) (*upper panel*) the involved chromosomes show juxtaposition of the *red* and *green* probes producing a *yellow* fusion signal. The uninvolved chromosomes 8 and 21 show *red* and *green* signals, respectively. This technique allows analysis of both metaphase (*left*) and interphase (*right*) cells. In the absence of t(8;21), cells show two *red* and two *green* signal (*lower panel*).

Cases with *RUNX1-RUNX1T1* transcripts appear to follow the class I and class II mutation rule as *RUNX1-RUNX1T1* fusions can be detected in long term survivors who are presumably cured of their disease. Knockin mice expressing an *RUNX1-RUNX1T1* fusion gene do not develop leukemia until they are exposed to mutagens presumably causing mutations that complement *RUNX1-RUNX1T1*. Taken together, these findings support the hypothesis that a single genetic event is not sufficient to cause acute leukemia.

30.2.1.2 AML with inv(16)(p13q22) or t(16;16)(p13;q22), (*CBFβ/MYH11*)

The inv(16)(p13q22) and, more rarely, the t(16;16)(p13;q22) have been identified in 5–12 % of de novo AMLs (Fig. 30.4) [21]. In most cases, the blasts have monocytic cytological features and are associated with immature eosinophils containing coarse basophilic cytoplasmic granules (Fig. 30.5) [21]. The inv(16) also has been identified in rare cases of CML in blast crisis [22].

The detection of inv(16) in de novo AMLs is a favorable prognostic finding. Patients with AML with inv(16) usually have a higher rate of complete response to chemotherapy, longer duration of remission, and prolonged survival as compared with patients with other forms of AML. The inv(16)(p13q22) involves the *CBF* (core binding factor or *PEBP2*) β subunit gene situated on chromosome 16q22 and the *MYH11* (smooth muscle myosin heavy chain) gene located at chromosome 16p13. The inversion results in a *CBPβ-MYH11*

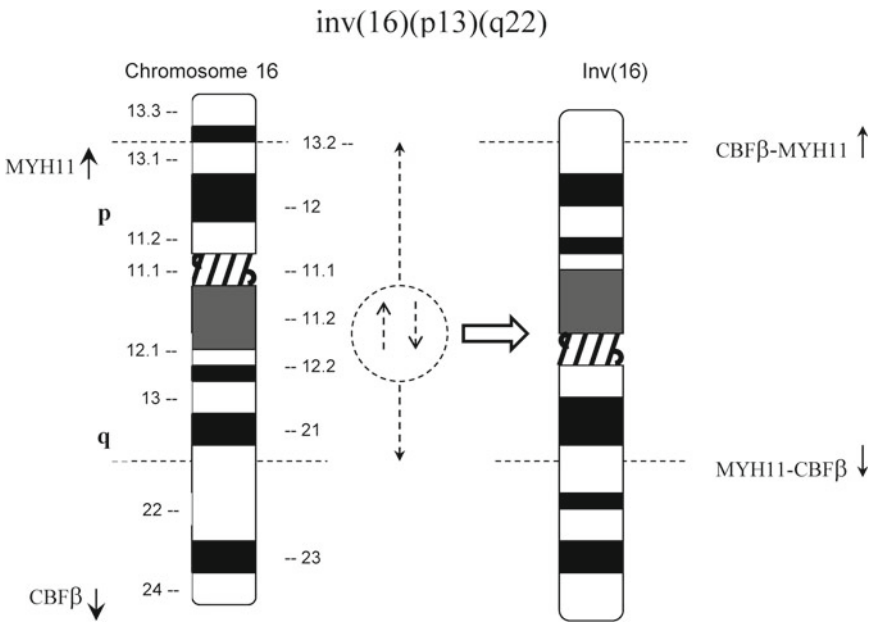

Fig. 30.4 Schematic representations of inv(l6)(p13q22) and t(16;16)(p13;q22). *Top panel*, In inv(l6), most of the *CBFβ* gene at 16q22 is joined upstream of the 3′ end of *MYH11* at 16p13 forming a chimeric *CBFβ-MYH11* fusion gene. *Bottom panel*, The t(16;16) results in a similar chimeric gene on 16p+.

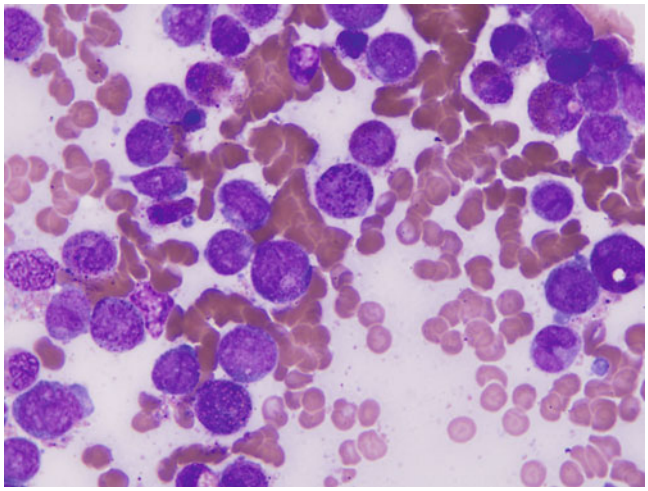

Fig. 30.5 Typical morphology of acute myeloid leukemia with inv(16)(p13q22) or t(16;16)(p13;q22). Immature eosinophilic precursors demonstrate coarse, irregularly sized basophilic granules. Wright-Giemsa stain, 1000×.

fusion gene from which chimeric mRNA and a novel protein are generated [23]. Both *CBFβ* and *MYH11* are oriented 5′→3′ and are transcribed in the centromeric to telomeric direction. The t(16;16) also involves the *CBFβ* and *MYH11* genes, and results in an identical *CBFβ-MYH11* fusion gene. A reciprocal *MYH11-CBFβ* mRNA or protein has not been identified.

The CBF protein has two components, α and β, of which there are three α subunits and one β subunit. The α subunits share a *runt* domain sequence that allows binding of protein to DNA and the β subunit. The β subunit binds to an α subunit and stabilizes CBF binding to DNA. The normal *CBFβ* gene spans 50 kb with 6 exons. The breakpoints in the *CBFβ* gene are relatively constant. In most cases, the breakpoint occurs in intron 5, at nucleotide 495 (corresponding to amino acid 165). However, a small subset of cases with a more proximal breakpoint at nucleotide 399 (amino acid 133) has been reported. The normal *MYH11* gene encodes for the smooth-muscle form of the myosin heavy chain, and the gene is a member of the myosin II family [23]. Although the function of the CBFβ-MYH11 protein is not completely known, it is thought to bind to the enhancer or promoters of a number of genes involved in hematopoietic cell differentiation [21]. Gene expression profiling studies of cases of AML with inv(16)(p13q22) have shown upregulation of the NF-κB pathway and genes associated with high cell proliferation [24].

The breakpoints in the *MYH11* gene are more variable than *CBFβ*; a number of different breakpoint sites have been reported, although most occur in a small 370 bp intron [21, 24]. This common breakpoint corresponds to nucleotide 1921 in the *MYH11* gene. Despite the variability in the different fusion genes generated by the inv(16) and t(16;16), one form of the fusion gene is created in approximately 85 % of cases, involving nucleotide 495 of the *CBFβ* and 1921 of *MYH11* genes. This fusion gene results in the generation of CBFβ-MYH11 protein that includes the first 165 amino acids of the normal CBFβ protein and a relatively small tail portion of the normal MYH11 protein.

Conventional cytogenetic methods, Southern blot analysis, and RT-PCR, have been used to detect the inv(16)(pl3q22) and t(16;16). One major advantage of conventional cytogenetics is that this method will also identify additional chromosomal abnormalities that are present in up to 50 % of cases. However, one potential disadvantage of karyotyping is that the inv(16) can be difficult to recognize or it may be misinterpreted as a del(16). RT-PCR analysis identifies *CBFβ-MYH11* transcripts in most cases of AML, with either the inv(16) or the t(16;16), demonstrating that the genetic consequences of the inversion and translocation are identical [22]. Immunohistochemical analysis using an antibody specific for the C terminus of CBFβ-MYH11 also can be used. Cases with inv(16) show nuclear expression, unlike other types of AML [23].

30.2.1.3 Acute Promyelocytic Leukemia (AML with t(15;17)(q24.1;q21.2), *PML/RARα* and Variants)

The t(15;17)(q24.1;q21.2) occurs exclusively in acute promyelocytic leukemia (APL), classified in the FAB system as M3. APLs represent 5–13 % of all de novo AMLs [26]. There is variation in the genetic predisposition to the development of APL with a higher incidence of APL in adult Latinos compared with non-Latinos in the USA, and a higher incidence in children from Central and South America and Italy [27]. The presence of t(15;17) consistently predicts responsiveness to a specific treatment, all-*trans* retinoic acid (ATRA). Retinoic acid is a ligand for the retinoic acid receptor alpha (*RARα*) that is involved in the t(15;17). ATRA is thought to overcome the block in cell maturation, allowing the neoplastic cells to mature and be eliminated. Additional genetic abnormalities are rare in APL suggesting that the t(15;17), by itself, is sufficient for neoplastic transformation. This hypothesis was confirmed by an animal model in which transgenic mice expressing the PML-RARα protein in myeloid cells developed APL and responded to ATRA therapy [28]. Of interest, ubiquitous and unrestricted expression of *PML-RARα* in embryos is lethal.

Two morphologic variants of APL have been described, typical and microgranular; both variants carry the t(15;17). In the typical or hypergranular variant (Fig. 30.6) promyelocytes have numerous azurophilic cytoplasmic granules that often obscure the border between the cell nucleus and cytoplasm. Cells with numerous Auer rods in bundles (the so-called faggot cells) are common. In contrast, in the microgranular variant (Fig. 30.6) promyelocytes contain

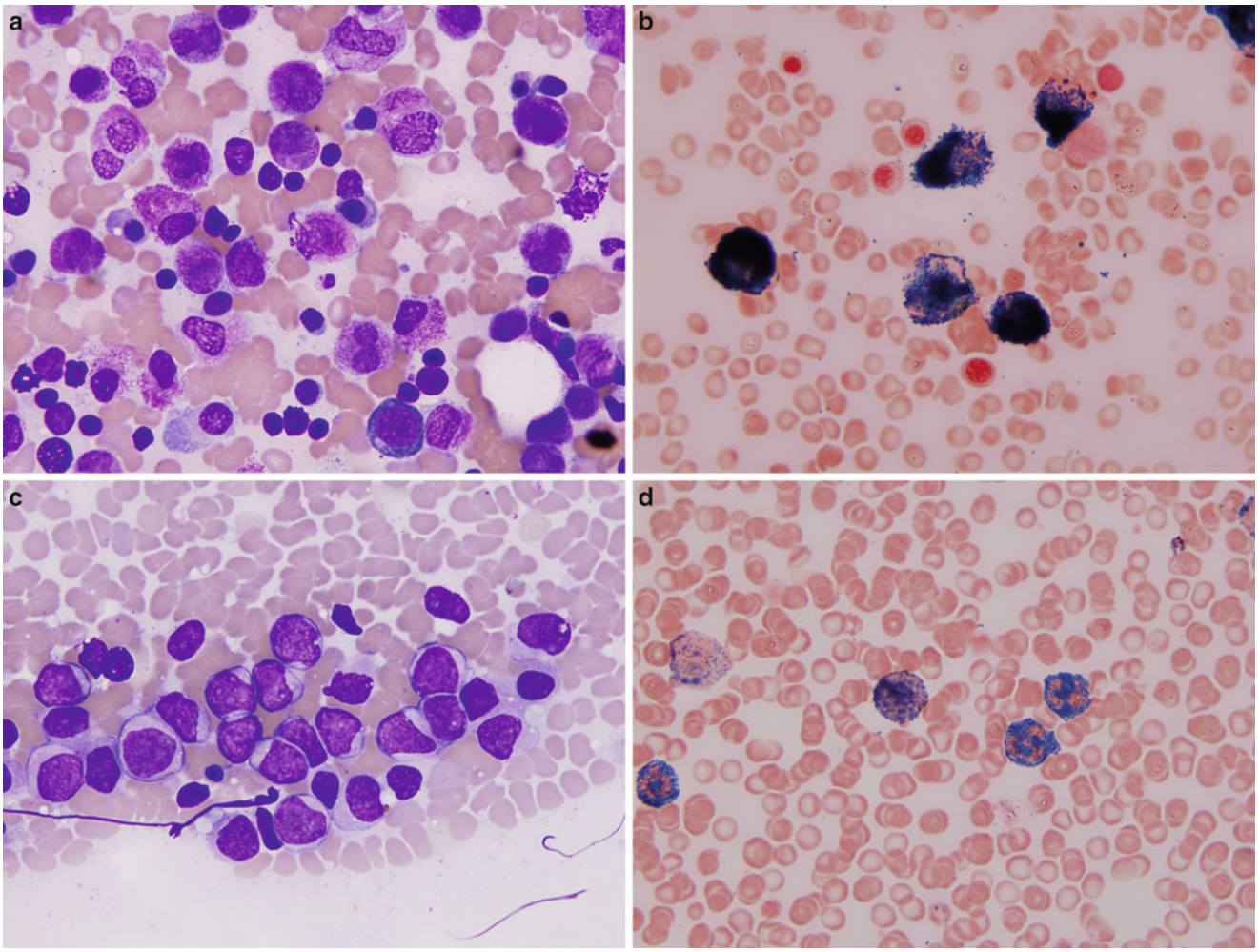

Fig. 30.6 Morphologic variants of acute promyelocytic leukemia. (**a**, **b**) *Classical variant.* Wright-Giemsa stain (**a**) shows abundant progranulocytes with numerous granules and occasional bundles of Auer rods (faggot cells). Myeloperoxidase cytochemistry (**b**) shows numerous positive granules in all neoplastic cells covering entire cell surface. (**c**–**d**) *Microgranular variant.* Wright-Giemsa stain (**c**) shows immature cells with irregular shaped, convoluted nuclei, and abundant cytoplasm containing small granules that are difficult to appreciate by routine microscopy. However, myeloperoxidase cytochemistry (**d**) highlights numerous granules similar to the classical variant. Original magnification 1000×.

numerous small cytoplasmic granules that are difficult to discern with the light microscope on routinely stained smears, but which are highlighted by myeloperoxidase cytochemical staining and are easily detected by electron microscopy.

The t(15;17) is a balanced and reciprocal translocation in which the *PML* (*promyelocytic leukemia*) gene on chromosome 15 and the *RARα* gene on chromosome 17 are disrupted and fused to form a hybrid gene (Fig. 30.7) [29]. The *PML-RARα* fusion gene, located on derivative chromosome 15, encodes a chimeric mRNA and protein. The *PML* and *RARα* genes are oriented in a head-to-tail orientation. The function of the normal *PML* gene is poorly understood. The gene is ubiquitously expressed and encodes a protein characterized by an N-terminal region with two zinc-finger-like motifs, known as a ring and a B-box, and thought to be involved in DNA binding [29]. A dimerization domain is also present. The normal PML protein appears to have an essential role in cell proliferation. The *RARα* gene encodes a transcription factor that binds to DNA sequences in *cis*-acting retinoic acid-responsive elements. High-affinity DNA binding also requires heterodimerization with another family of proteins, the retinoic acid X receptors. The *RARα* protein, from N-terminal to C-terminal, has transactivation, DNA binding, heterodimerization, and ligand-binding domains. The normal *RARα* protein plays an important role in myeloid differentiation.

There are three major forms of the *PML-RARα* fusion gene, corresponding to different breakpoints in the PML gene. The breakpoint in the *RARα* gene occurs in the same general vicinity in all cases, within intron 2. Approximately 40–50 % of cases have a *PML* breakpoint in exon 6 (the so-called long form, termed *BCR1*), 40–50 % of cases have the

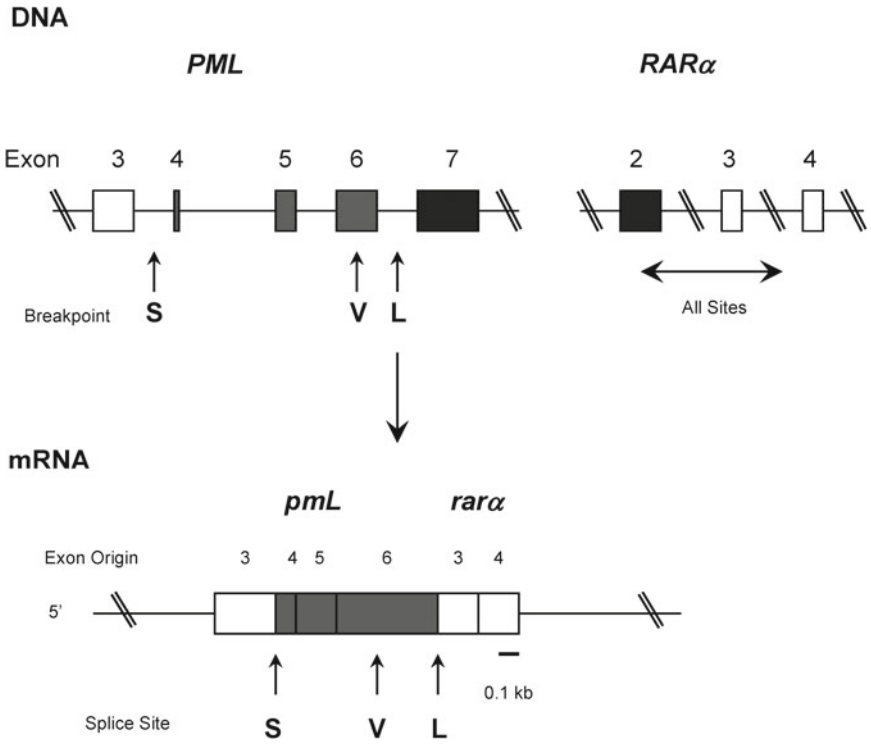

Fig. 30.7 Structure of the PML and RARα genes. Schematic representation of the germ line *PML* and *RARα* genes (*top*), the *PML-RARα* fusion mRNA transcript (*middle*), and the long (L) form of the fusion protein (*bottom*). The lines represent introns, *arrows* indicate three breakpoints, *open boxes* represent the exons present in each type of *PML-RARα, shaded boxes* represent exons excluded from the short (S) form but included in the long (L) or variable (V) forms, and *solid boxes* indicate exons excluded from all types.

PML breakpoint in exon 3 (the so-called short form, termed *BCR3*), and 5–10 % of cases have a breakpoint in *PML* exon 6 that is variable (the so-called variable form, termed *BCR2*). In each form of the translocation, the PML-RARα fusion protein retains the 5′-DNA binding and dimerization domains of PML and the 3′-DNA binding, heterodimerization, and ligand (retinoic acid) binding domains of *RARα*. Recent studies indicate that the different forms of *PML-RARα* fusion mRNA correlate with clinical presentation or prognosis. In particular, the BCR3 type of *PML-RARα* correlates with higher leukocyte counts at time of presentation and is more commonly present in the microgranular variant. Higher leukcocyte count and microgranular variant morphology are adverse prognostic findings in APL patients. PML-RARα transcript type does not correlate with survival [30].

In addition to the PML-RARα protein, the t(15;17) results in two other abnormal proteins. An aberrant PML protein is expressed in virtually all cells with the t(l5;17), as a result of alternative mRNA splicing. This PML protein retains its DNA binding capacity and could possibly play a role in neoplastic transformation. A *RARα-PML* fusion gene is also formed, located on the derivative chromosome 17, and RARα-PML protein is expressed in approximately 75 % of cases of APL. Because this protein lacks the DNA-binding regions of both normal PML and RARα, it is not thought to play a role in leukemogenesis. Furthermore, RARα-PML expression does not correlate with response to ATRA in vitro or with clinical outcome in vivo.

A number of methods can be used to detect the t(15;17). Conventional cytogenetic methods detect the t(l5;l7) in 80–90 % of APL cases at time of initial diagnosis (Fig. 30.8). Suboptimal clinical specimens and poor-quality metaphases explain a large subset of cases with negative results. Fluorescence in situ hybridization (FISH) (Fig. 30.9) is another useful method for detecting the t(15;17) in APL. Different methods employ probes specific for either chromosome 15 or chromosome I7 (or both), and commercial kits are available. In the past, Southern blot hybridization was used to detect gene rearrangements that result from the t(15;17) but this approach is cumbersome. RT-PCR is another method, convenient and reliable, and detects the various *PML-RARα* fusion transcripts.

Polyclonal and monoclonal antibodies reactive with the PML and RARα proteins have been generated and immunohistochemical studies assessing the pattern of staining are useful for diagnosis [29, 31, 32]. In normal cells, PML protein is localized in 5–20 spherical structures per nucleus, known as PML oncogenic domains (POD) that are highlighted in a punctate pattern of staining by the anti-PML antibody (Fig. 30.10). RARα protein is not present in these structures. In contrast, in APL cells immunostaining for either PML or RARα reveals a microgranular pattern. Thus, the PML-RARα fusion protein is present in the microgranules. The fusion protein may prevent PML from forming normal PODs because treatment with ATRA allows PML reorganization into these domains.

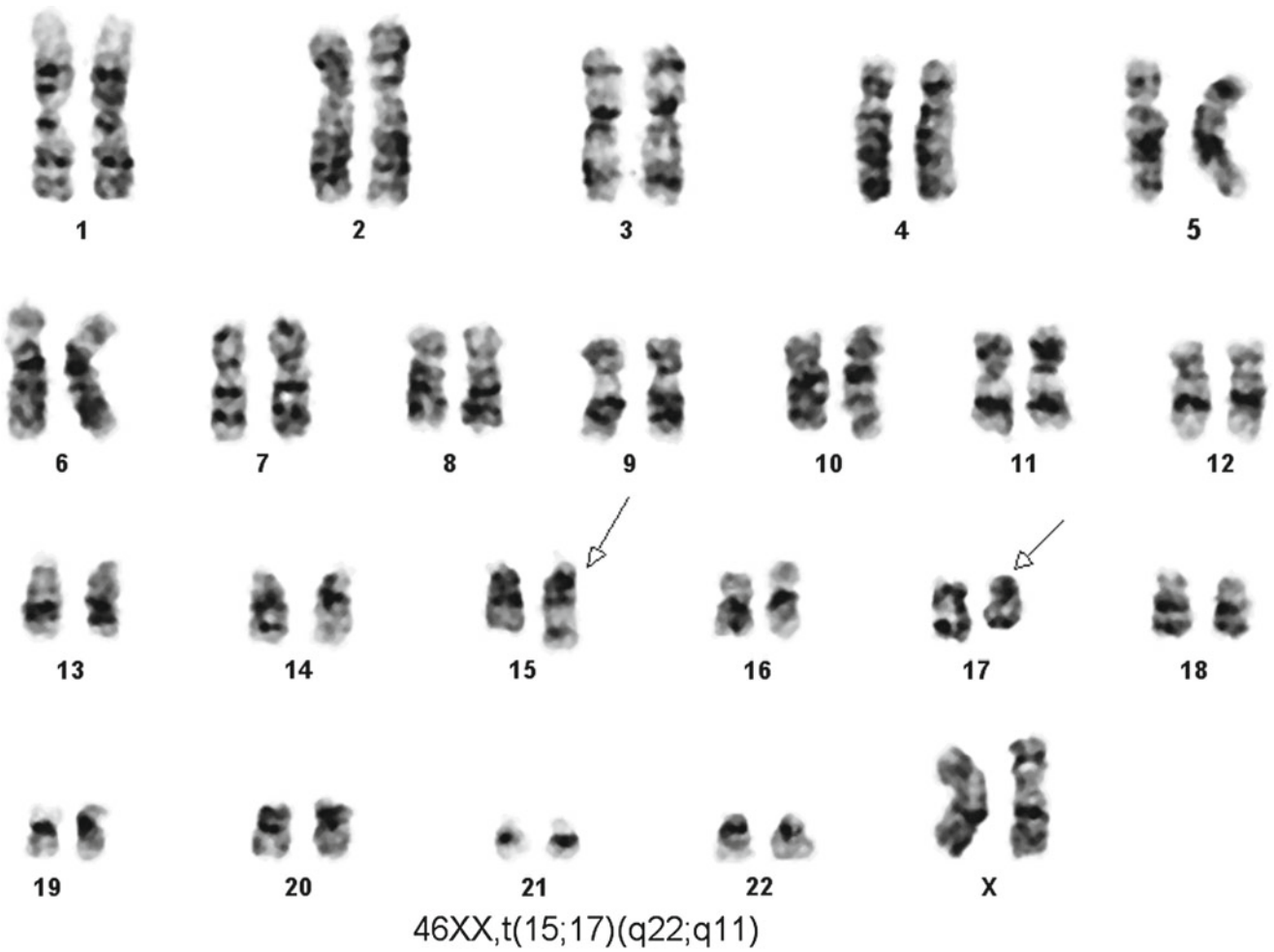

Fig. 30.8 Karyotype of a case of acute promyelocytic leukemia. Conventional cytogenetic analysis using G banding by using trypsin and Giemsa stain (GTG banding technique) demonstrates t(15;17) as a sole cytogenetic abnormality.

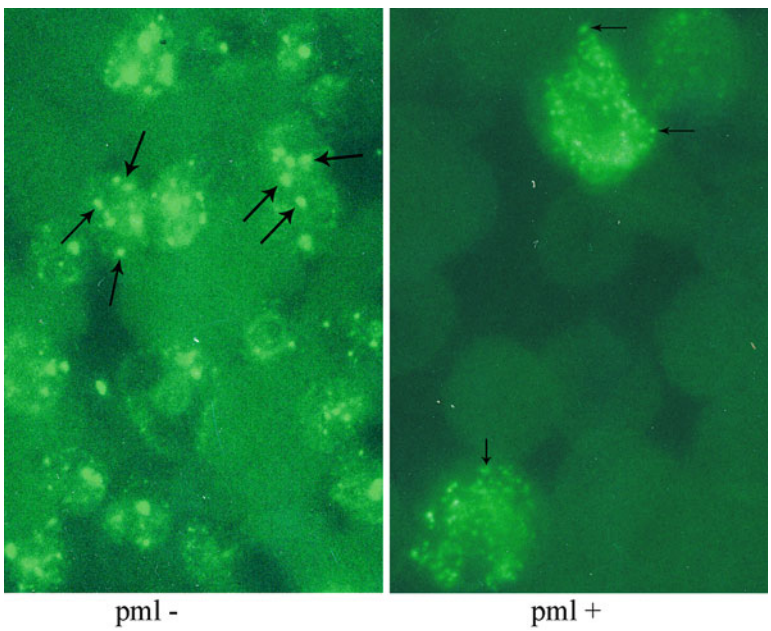

Fig. 30.9 Immunofluorescence study using an antibody specific for PML is used to highlight cellular PML oncogenic domains (POD). In the absence of t(15;17), a chunky granular or punctate pattern is observed (left). In the presence of t(15;17), a microgranular pattern is observed (right).

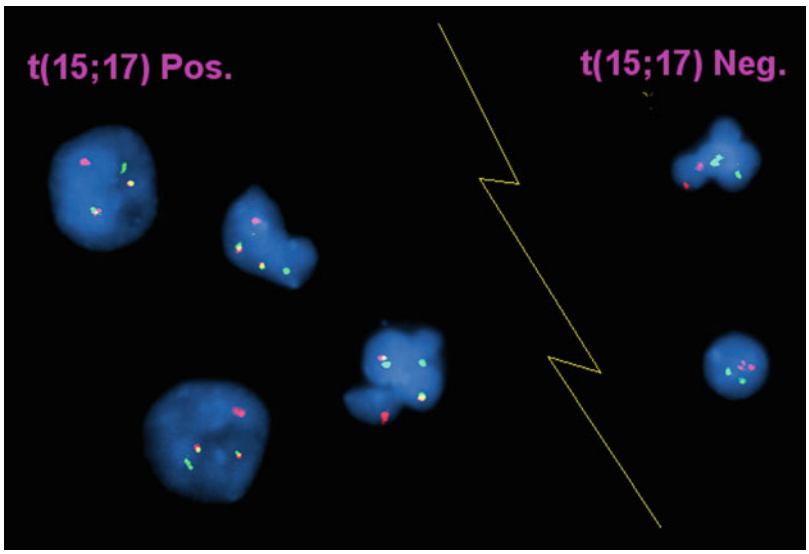

Fig. 30.10 Interphase FISH of acute promyelocytic leukemia. Dual color, dual fusion probe (Vysis) in which the *PML* probe is red and the *RARα* probe is green. In the event of t(15;17) (4 cells to the left of the image) the involved chromosomes show juxtaposition of the red and green probes producing a yellow fusion signal. Uninvolved chromosomes show red and green signals, respectively. In the absence of t(15;17), cells show two red and two green signals (2 cells to right of the image).

For the diagnosis of residual disease or early relapse after therapy, conventional cytogenetic studies, Southern blot analysis, and immunohistochemical methods are limited by low sensitivity. In contrast, RT-PCR and FISH methods are very useful. The sensitivity of RT-PCR, which can detect one cell with the *PML-RARα* gene in 1×10^5 benign cells, makes this method most useful for monitoring residual disease after therapy. For the first few months after therapy, RT-PCR results may be positive with no correlation with relapse rate. However, longer than 3 months after therapy a positive RT-PCR result significantly correlates with an increased rate of relapse [30]. FISH methods offer an advantage over RT-PCR as FISH studies may detect variant forms of the t(15;17) that are negative by RT-PCR. However, several *bona fide PML/RARα* APL cases with negative FISH results and positive PCR results have been reported in the literature [32]. These cases stress the role of morphological assessment in the establishing the diagnosis of APL and also illustrate that a negative FISH result does not eliminate the diagnosis of APL [32].

t(11;17)(q23;q21)

The t(11;17)(q23;q21) has been rarely identified in AML, in <1 % of all cases. Many patients with t(11;17)-positive AML present with a bleeding diathesis. The blast cells in these cases have cytological features intermediate between the blasts of M2 and the promyelocytes of M3 (FAB). Others have noted that many of these cases have distinctive cup-like nuclei [33]. Unlike APL, patients with t(11;17)-positive AML respond poorly to ATRA.

The t(11;17) is a reciprocal and balanced translocation involving the *PLZF* (*promyelocytic leukemia zinc finger*) gene on chromosome 11 and the *RARα* gene on chromosome 17 [34]. There is some variability in the breakpoints within the *PLZF* gene. The breakpoints in the *RARα* gene occur in the second intron, similar to those that occur in the t(15;17). The translocation results in the creation of two fusion genes, *PLZF-RARα* and *RARα-PLZF*. Both fusion genes may play a role in leukemogenesis as the structure of both chimeric proteins suggests that either can bind to DNA and influence transcription. The normal *PLZF* gene is expressed in a tissue specific manner, particularly in cells of myeloid lineage. *PLZF* is a member of a large family of zinc-finger transcription factors and is probably involved in myeloid differentiation. The poor ability of ATRA to induce differentiation in t(11;17)-positive AML suggests that disruption of the normal *PLZF* gene, rather than the *RARα* gene (as is the case in APL), is the primary leukemogenic event [35]. RARα-PLZF binds to normal PLZF which represses CRABP1 (cellular retinoic acid binding protein 1), thereby resulting in CRABP1 upregulation and thought to be involved in retinoic acid resistance. PLZF-RARα induces MYC expression and may repress normal PLZF in a dominant negative fashion.

Other Rare Variants

Rare partner genes fused with *RARα* have been identified, greatly aided by the advent of next-generation sequencing methods of cases in which conventional cytogenetics, FISH, and RT-PCR were negative, but the tumors were morphologically typical and had strong myeloperoxidase reactivity. One of the first partner genes was *NPM1* (*nucleophosmin*) in t(5;17)(q32;q21.2). The t(5;17) is a balanced and reciprocal translocation involving *NPM1* on chromosome 5 and the *RARα* gene on chromosome 17. The NPM and *RARα* genes are fused in a $5' \rightarrow 3'$ direction. The breakpoint region in the *RARα* gene is identical to the breakpoint in t(15;17). Other rare gene partners of *RARα* include: *NUMA1* (nuclear mitotic apparatus) [36], *STAT5B* (signal transducer and activator of transcription 5b) [37], *PRKAR1A* (cAMP-dependent protein kinase type I alpha regulatory subunit) [38], *FIP1L1* (FIP1-like 1) [39], *BCOR* (BCL6 corepressor) [40], *OBFC2A*

(oligonucleotide/oligosaccharide-binding fold containing 2A) [41], and *IRF2BP2* (interferon regulatory factor 2 binding protein 2) (C. Cameron Yin, personal communication).

30.2.1.4 AML with t(9;11)(p22;q23), *MLLT3–MLL*

The t(9;11)(p22;q23) represents the most common translocation involving chromosome 11q23 in patients with AML and accounts for approximately 2 % of all adult and 9–12 % pediatric AML cases [42, 43]. AMLs with the t(9;11) may be either de novo or therapy-related. De novo neoplasms occur in both children and adults. Patients with de novo AML with the t(9;11) tend to have a more favorable outcome than adults with AML associated with other 11q23 abnormalities. In addition, therapy-related cases of AML have been reported in patients treated previously with chemotherapeutic agents that target topoisomerase II. The t(9;11) is a balanced and reciprocal translocation that disrupts the *MLL* gene on chromosome 11q23 and the *MLLT3* gene (also known as *AF9* gene or *LTG9* for the leukemia translocation gene) on chromosome 9p22. The translocation results in the formation of an *MLL–MLLT3* fusion gene located on the derivative chromosome 11.

The *MLL* gene (myeloid–lymphoid leukemia or mixed lineage leukemia) gene, also known as *ALL1, HRX*, or *HRTX*, is a relatively large gene that is homologous to the *Drosophila trithorax* gene. *MLL* is promiscuous and translocations can involve many partners, including *MLLT3*. *MLL* consists of 36 exons distributed over 100 kb, and produces a 12 mRNA that encodes a 3968 amino acid protein with an estimated molecular weight of 430 kDa [44]. Recent experiments have indicated that MLL is normally processed via a cytoplasmic cleavage event into a 320 kDa amino terminus (MLL-N), and a 180 kDa carboxy terminus fragment [44]. A number of protein motifs/domains have been identified in the primary structure of MLL, including AT hooks, a DNA methyltransferase domain, PHD domains, a transactivation domain, and a SET domain. An important function of MLL is maintaining HOX gene expression during embryonic development. It has been shown that loss of MLL function in flies and mice results in embryonic lethality and homeotic transformation [44]. The ability of MLL fusion proteins to regulate the expression of HOX genes might be partially responsible for immortalization of myeloid progenitor cells. MLL also influences HOX gene expression via direct binding to promoter sequences [45] and HOXA9 is highly expressed in MLL leukemias [46]. MLL-ENL fusion protein was shown to immortalize immature myelomonocytic cells in vitro. The transplantation of these cells into mice resulted in myeloid leukemia [47]. By fusing a truncated MLL protein to an inducible dimerization domain, Martin et al. have shown that MLL fusion proteins must dimerize to immortalize hematopoietic cells and inhibit their differentiation [48]. Biochemical analysis of MLL suggests that it normally functions as a transcriptional regulator, and expression of MLL fusion proteins has been shown to be leukemogenic in mice.

Despite the large size of the *MLL* gene, most (but not all) *MLL* translocations associated with hematologic malignancy can be mapped to an 8.3 kb breakpoint cluster region (bcr). This region encompasses *MLL* exons originally designated exons 5–11. Subsequent studies revealed that *MLL* exon 4 actually consists of three discrete exons (now designated exons 4A, 4B, and 4C). There is a correlation between the locations of the breakpoints in de novo versus therapy-related AML. Using the *XbaI* restriction enzyme, the 8.3 kb fragment of DNA can be divided into a 5′ 4.6 kb region I and a 3′ 3.9 kb region II [49]. De novo AMLs more commonly have breakpoints in region I. In contrast, most cases of therapy-related AML have region II breakpoints [49]. The presence of scaffold-attachment regions and possible topoisomerase II consensus binding sites in region II may explain the increased likelihood of region II breakpoints in therapy-related AML [49].

Conventional cytogenetics is an excellent method to detect 11q23 abnormalities. This technique allows the detection of translocations involving 11q23 and also identifies all possible partner chromosomes. However, a subset of AMLs with 11q23 abnormalities may have a normal karyotype [50]. FISH techniques are also useful, and may detect a small subset of cases not recognized by conventional cytogenetics. The clustering of breakpoints within the *MLL* gene is well-suited to their detection by Southern blot analysis [51]. Using *BamHI* digested DNA, a single *MLL* cDNA probe spanning an 8.3 kb genomic fragment detects most of the common and uncommon 11q23 translocations as gene rearrangements. The introns between exons 5 through 11 are very large and thus standard PCR methods cannot be used for detecting 11q23 translocations. However, the cDNA corresponding to exons 5 through 11 is <700 bp, allowing RT-PCR analysis. A panel of primers needs to be used to detect the l lq23 translocations because of the number of possible partner chromosomes. Multiplex RT-PCR approaches are most convenient.

One of the more unusual features of leukemias associated with *MLL* fusion is that there is a very brief latency period. Leukemias with *MLL* fusions have been detected in newborns [52] and even in aborted fetuses [53]. The concordance rate of leukemia in monozygotic twins who have *MLL* fusions is almost 100 % [54]. It has been hypothesized that additional mutations occur very rapidly in cells that acquire *MLL* fusions [55]. One possible mechanism is that *MLL* fusion leads to a mutator phenotype [55]. *MLL* fusions have been shown to impair DNA double strand break recognition [56] and cell cycle checkpoints [57], both of which can lead to increased accumulation of mutations. It is also possible that the insult caused by *MLL* translocation can cause additional mutations, especially if the individual had an inherited deficiency in DNA repair or toxin metabolizing enzymes.

This possibility is supported by studies that show that certain isoforms of NQO1 or CYP3A4 confer an increased risk of infant leukemia [58] or therapy-related leukemia [59].

30.2.1.5 AML with t(6;9)(p23;q34); *DEK-NUP214*

The t(6;9)(p23;q34) (Fig. 30.11) occurs in 1–2 % of cases of AML [60]. Patients with AML and the t(6;9) are commonly of young age (<40 years old), and have a poor prognosis [60]. Approximately 50 % of patients achieve clinical remission with chemotherapy, but relapse is common. A subset of patients presents with aggressive MDS rather than AML; others present with overt AML with multilineage dysplasia [60].

The t(6;9) disrupts the *DEK* gene at chromosome 6p23 and the *CAN* (*NUP214*) gene at chromosome 9q34, resulting in a *DEK-CAN* fusion gene on the derivative chromosome 6 [61]. The breakpoints in t(6;9) are clustered. The breakpoints in the *DEK* gene occur in one intron, known as *icb*-6 (for intron containing breakpoint on chromosome 6). The breakpoints in the *CAN* gene also cluster in one intron, known as *icb*-9. The *DEK* gene is approximately 40 kb in size and encodes a 43 kDa protein, located in the cell cytoplasm, and thought to be a transcription factor. The *CAN* gene is relatively larger (over 140 kb) and encodes a 214 kDa protein. The normal CAN protein is a component of the nuclear-pore complex, involved in transport of mRNA and proteins between the cytoplasm and the nucleus [61]. The DEK-CAN fusion protein, of predicted size of 165 kDa, has a nuclear distribution, suggesting it is a part of the transcription factor system [61]. It has been suggested that DEK-CAN initiates AML within a subpopulation of hematopoietic stem cells [62].

The t(6;9) is effectively detected by conventional cytogenetics, which also allows the detection of additional abnormalities that may occur with disease progression; trisomies of 8 and 13 are the most common additional abnormalities [60]. The clustering of breakpoints in *icb*-6 of *DEK* and *icb*-9 of *CAN* also allows convenient detection by using RT-PCR methods. *FLT3* gene mutations are common in t(6;9)-positive AML [60, 63].

30.2.1.6 AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*

AML associated with inv(3) or t(3;3) is uncommon, representing 1–3 % of all cases of AML. This disease occurs in adults and men and women are equally affected [64]. De novo

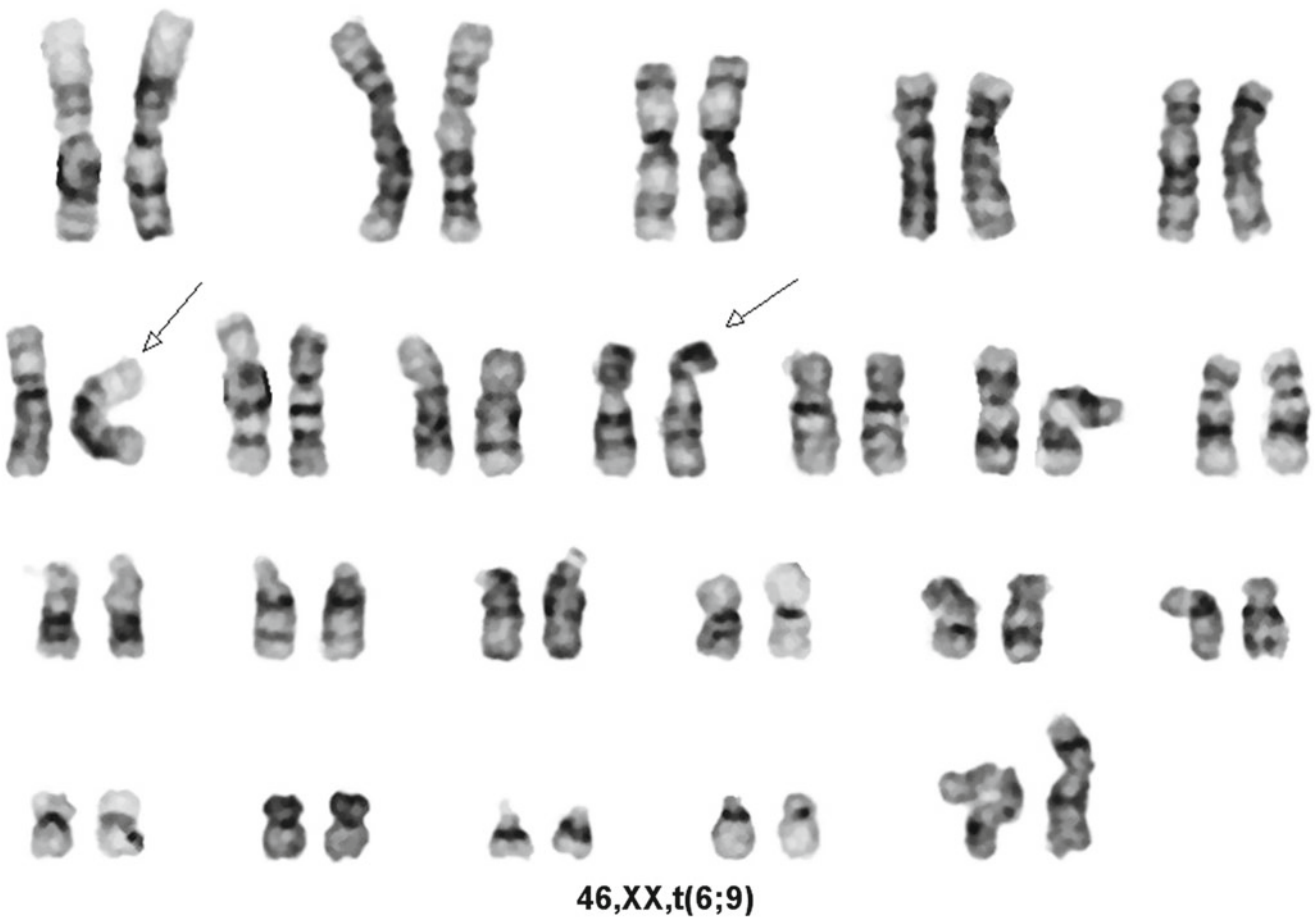

Fig. 30.11 Karyotype of a case of acute myeloid leukemia with t(6;9)(p23;q34). GTG banding technique.

AML and cases arising from MDS have been described [65]. Inv(3) or t(3;3) is usually associated with other cytogenetic abnormalities and is rarely a sole abnormality.

Patients with AML with inv(3) or t(3;3) can present with prominent hepatosplenomegaly [65]. Platelet counts in the peripheral blood at presentation may be increased, normal or decreased, and demonstrate a wide range of values, from 20 to 1731×10^9/L [64, 65]. AML with inv(3) or t(3;3) is associated with increased number of micromegakaryocytes and monolobated and bilobated megakaryocytes in the bone marrow (BM). Many cases also show dysplastic changes in the erythroid and/or myeloid lineages [65, 66].

It has been shown that the chromosomal breakpoints in 3q26 are widely scattered in the 5′ or the 3′ regions of the *EVI1(Ecotropic viral integration site 1)* gene currently known as *MECOM* (*MDS1 and EVI1 complex locus*) gene [67], whereas the chromosomal breakpoints in the 3q21 region are restricted to a relatively narrow area. Two different clusters have been identified downstream of the *RPN1* (*ribophorin I*) gene [68]. Alternative splicing of *EVI1* results in the creation of *MDS1/EVI1* transcripts. Suzukawa et al. suggested that the housekeeping gene *RPN1* acts as an enhancer of *EVI1* expression, resulting in leukemogenesis [68]. It has been demonstrated that ectopic expression of *EVI1* in immature hematopoietic cells interferes with erythroid and granulocytic development [69]. While there is a single report in the literature describing good response of two patients with inv(3) to thalidomide and arsenic trioxide [70], in general, the prognosis of patients with inv(3) or t(3;3) is poor with short survival [66, 71].

30.2.1.7 Acute Megakaryoblastic Leukemia with t(1;22)(p13;q13); *RBM15-MKL1*

Most cases of AML with t(1;22) have been reported in infants [72]. The largest series published to date describes 39 cases with a median age of 4 months (range, 1 day to 36 months) [72]. The t(1;22) is a sole cytogenetic abnormality in more than half of the cases and is a sole cytogenetic abnormality much more often in infants younger than 6 months of age (19 of 23, 83 % of cases), than in older infants (3 of 16, 19 % of cases) [72]. There is a slight female predominance; all patients reported had not had evidence of Down syndrome [72]. In rare cases, *RBM15–MKL1* transcripts result from 3-way translocations involving 1p13 and 22q13, such as t(1;14;22) or t(1;22;4) [73]. Almost all patients present with organomegaly [72]; a subset of patients was initially diagnosed with a solid tumor [72]. Moderate leukocytosis with circulating blasts, marked anemia, and variable thrombocytopenia are usually observed at presentation [72, 73]. Most (~90 %) patients in the largest series reported [72] fulfilled the diagnostic criteria for AML M7. Patients usually have a hypercellular or normocellular BM; however, some patients have hypocellular BM [73]. Blasts may exhibit typical morphologic features of megakaryocytic differentiation (i.e., cytoplasmic basophilia, blast clumping, cytoplasmic blebs) or have an undifferentiated appearance [72]. The blasts usually express immunologic markers of megakaryocytic differentiation (i.e., CD42b, CD41a, Factor VIII) [72]. A subset of blasts also expresses CD34 and HLA-DR; some blasts may express the myelomonocytic marker, CD33 [72].

The t(1;22)(p13;q13) results in a fusion of *RNA-binding motif protein-15*(*RBM15*) and *megakaryocyte leukemia -1* (*MKL1*) genes [74]. t(1;22)-positive blasts express both reciprocal fusion transcripts, *RBM15–MKL1* and *MKL1–RBM15*, as detected by RT-PCR [74]. However, the predicted *RBM15–MKL1* chimeric protein encompasses all putative functional motifs encoded by each gene, which makes it the candidate oncoprotein of t(1;22). Ma et al. suggested in cases with *RBM15–MKL1* the *MKL1 SAP* domain is expected to aberrantly relocalize the *RRM* and *SPOC* motifs of *RBM15* to sites of transcriptionally active chromatin, deregulating RNA processing and/or Hox and Ras/MAP kinase signaling and altering the normal proliferation or differentiation of megakaryoblasts [74]. The prognosis of patients with AML with t(1;22) is intermediate and appears to have improved dramatically with modern therapy. The largest series published in 1999 reported a 53 % complete remission rate and median survival of 8 months (range, 1 day to 104 months) [72]. A more recent report of 11 pediatric patients published in 2003 described 6 of 11 patients being alive after more than 2 years follow-up [73].

30.2.1.8 AML with Mutated *NPM1*

The *NPM1* (nucleophosmin) gene, mapping to chromosome 5q35, contains 12 exons [74]. It encodes for three alternatively spliced nucleophosmin isoforms: B23.1, B23.2, and B23.3. Nucleophosmin is a highly conserved phosphoprotein that is ubiquitously expressed in tissues [74]. The bulk of NPM resides in the granular region of the nucleolus, but NPM shuttles continuously between nucleus and cytoplasm [74]. NPM, through its involvement in ribosome biogenesis, plays a central role in cell growth and proliferation. *NPM1* mutations result in cytoplasmic accumulation of NPM that can be detected by immunohistochemical analysis.

NPM1 functions both as an oncogene and a tumor-suppressor gene depending on gene dosage, expression levels, interacting partners, and compartmentalization [2]. *NPM1* appears implicated in promoting cell growth, as its expression increases in response to mitogenic stimuli and above normal amounts are detected in highly proliferating and malignant cells. On the other hand, *NPM1* contributes to growth suppressing pathways through its interaction with *ARF*.

NPM1 mutations are relatively specific for AML [75]. Although rare cases of *NPM1* mutations have been described in patients with chronic myelomonocytic leukemia and MDS, many of these patients rapidly progress to overt AML [76].

NPM1 mutations are relatively common in de novo AML, but rare in AML secondary to myeloproliferative/myelodysplastic disorders and therapy-related AML. *NPM1* mutations are characteristically heterozygous and retain a wild-type allele. In children with AML, the frequency of *NPM1* mutation ranges from 2.1 % in Taiwan to 6.5 % in Western countries accounting for 9–26.9 % of all childhood AML with normal karyotype. In adult AML patients, the frequency of *NPM1* mutations ranges between 25 and 35 % accounting for 46–64 % of adult AML with normal karyotype [74, 77–79]. Except for two cases involving the splicing donor site of *NPM1* in exon 9 or exon 11 [80], *NPM1* mutations are restricted to exon 12. Loss of *NPM1* mutations at relapse is rare and may be due either to emergence of a different leukemic clone or inability to detect mutations because few leukemic blasts are infiltrating the BM. AML patients with a wild type *NPM1* gene at diagnosis rarely acquire *NPM1* mutations during the course of disease, suggesting that mutations are unlikely to play a role in disease progression.

NPM1 mutations are associated with a normal karyotype and do not occur in core binding factor leukemias and acute promyelocytic leukemia [75]. Chromosomal abnormalities observed in 14 % of *NPM1* mutated AML cases usually arise in subclones and are probably secondary events associated with clonal evolution. Several studies have shown a strong correlation between *NPM1* mutations and *FLT3-ITD* [77, 78].

NPM1-mutated AML cases show a wide morphologic spectrum, but *NPM1* mutations are more frequent in the FAB M4 and M5 categories [78] and in AML with prominent nuclear invaginations (cuplike nuclei) [33]. More than 95 % of *NPM1*-mutated AML cases are CD34+ [81]. NPM-mutated AML cases with a normal karyotype often have high blast counts, high *FLT3-ITD* and LDH serum levels, extramedullary involvement (mainly gingiva and lymphadenopathy) and higher platelet counts. Bone marrow in *NPM1*-mutated AML cases frequently shows an increased number of megakaryocytes exhibiting dysplastic features [82]. After induction therapy, AML with a normal karyotype carrying mutated NPM shows a higher complete remission rate than AML with normal karyotype without *NPM1* mutations [83]. *NPM1* mutation status, however, is not an independent predictor for responsiveness to chemotherapy [81].

Conventional screening of *NPM1* mutations is usually performed by PCR using a fluorescence conjugated primer prior to fragment analysis by capillary electrophoresis. Immunohistochemical analysis to detect abnormal cytoplasmic localization of mutated NPM1 protein is another common screening method [81, 84]. Alternative methods for detecting *NPM1* mutation include denaturing high performance liquid chromatography and fluorescence resonance energy transfer technique, and direct sequencing.

30.2.1.9 AML with Mutated *CEBPA*

The *CCAAT/enhancer-binding protein a* (*CEBPA*) gene encodes a protein member of the basic region leucine zipper (bZIP) transcription factor family, playing a crucial role in granulopoiesis [85]. There are two main categories of *CEBPA* mutations: (1) C-terminal mutations that occur in the bZIP domain, which are usually in-frame and predict mutant proteins lacking DNA binding and/or homodimerization activities, and (2) N-terminal nonsense mutations that prevent expression of the full-length protein and result in truncated isoforms with dominant-negative activity. Some patients present with biallelic mutations at the C-terminus, whereas others are heterozygous for separate mutations or have a C-terminal mutation coexisting with a mutation in the N-terminus [86, 87].

CEBPA mutations usually occur in AML with a normal (diploid) karyotype and represent 15–19 % of cases in patients with normal cytogenetics. *CEBPA* mutations are associated with a favorable prognosis [79, 86, 88], and significantly better event-free survival [87, 89], disease-free survival [89], and overall survival [87, 89]. At diagnosis, patients with *CEBPA* mutations often have higher percentages of peripheral blood blasts, lower platelet counts, less lymphadenopathy and extramedullary involvement, less frequent *FLT3/ITD* and *FLT3/TKD* mutations, and rare *MLL/PTD* mutations compared with AML patients without *CEBPA* mutations [86]. Complete remission rates do not differ between patients with and without *CEBPA* mutations [79, 86, 88], but complete remission duration [86], disease-free survival [88], event-free survival [79], and overall survival [88, 96] are better for patients with *CEBPA* mutations. On multivariate analysis, *CEBPA* mutational status adds prognostic information to that provided by *MLL/PTD* and *FLT3/ITD* status, age, and resistant disease after the first course of induction therapy with regard to complete remission duration and by *FLT3/ITD* status, age, and WBC for overall survival [86]. In another study, *CEBPA* mutations, *BAALC* expression, and *FLT3/ITD* status were prognostic for disease-free survival, and *CEBPA* mutations, age, *BAALC* expression, and *FLT3/ITD* status were prognostic for overall survival [88]. Fröhling et al. [86] analyzed clinical outcomes in patients with N-terminal nonsense *CEBPA* mutations, patients with other *CEBPA* mutation types, and patients with wild-type *CEBPA* and found that the complete remission duration was the longest in patients with N-terminal mutations, followed by the complete remission duration in patients with other mutations and in those without *CEBPA* mutations. However, in pairwise comparisons, there were no significant differences in the complete remission duration between patients with N-terminal mutations and those with other mutations or between patients with other mutations and patients with wild-type *CEBPA* [86].

30.2.2 AML Associated with Translocations Not Included in the AML with Recurrent Genetic Abnormalities Category of the Current WHO Classification

30.2.2.1 AML with 11q23 (*MLL*) Abnormalities Other Than t(9;11)

Translocations involving the 11q23 locus are detected in 3–10 % of all patients with de novo AML [90]. In infants (age <1 year), AML with 11q23 translocations typically presents with hyperleukocytosis and has a poor prognosis [91]. Translocations involving chromosome 11q23 are found in more than 70 % of leukemia patients younger than 1 year of age whether the immunophenotype is designated AML or ALL. In adults, AMLs with 1lq23 translocations most commonly exhibit monocytic maturation and are classified as FAB M4 or M5. These AMLs do not have specific clinical features and may have a poorer prognosis than AMLs without 11q23 translocations. In addition, 80–90 % of therapy-related AMLs that occur in patients previously treated with topoisomerase II inhibitors are associated with 11q23 translocations. Translocations involving the 11q23 locus are not restricted to AML and are associated with different hematologic malignancies including precursor T or B lymphoblastic leukemia, MDS, and Burkitt lymphoma [51, 90]. In addition, *MLL* has been labeled a promiscuous oncogene since translocations involving over 60 partner genes or regions have been identified [90].

AML/MDS with t(11;16)(q23;p13.3). The t(11;16) is a rare recurrent translocation that has been identified in therapy-related AML and MDS, occurring almost exclusively in patients treated previously with agents that inhibit topoisomerase II [92]. The t(11;16) is a reciprocal translocation that disrupts the *MLL* gene at 11q23 and the CBP cAMP response element or CREB-binding protein) gene at 16p13.3, resulting in the formation of an *MLL-CBP* fusion gene located on the derivative chromosome 11 [92]. A *CBP-MLL* fusion gene is also produced, but not in all cases, and therefore is not thought to be leukemogenic. The normal CBP protein is a transcriptional adaptor/coactivator protein.

Other 11q23 translocations in AML. Other 11q23 translocations less commonly identified in AMLs include the t(6;11)(q27;q23), t(11;19)(q23;p13.3), t(11;19)(q23;p13.1), t(1;11)(p32;q23), t(l;11)(q21;q23), t(11;17)(q23;q21), and t(10;11)(p11;q23). In each of these translocations, the *MLL* gene is disrupted and its 5′ end is fused with the 3′ end of the partner gene: *AF6* (*MLLT4*) in the t(6;11), *ENL* (*MLLT1*) in the t(11;19)(q23;19p13.3), *ELL* in the t(11;19)(11q23;19p13.1), *AFlp* in the t(1;11)(p32;q23), *AFlq* in the t(l;11)(q21;q23), *AF17* (*MLLT6*) in the t(11;17), and *AF10* (*MLLT10*) in the t(10;11) [93]. The formation of these fusion genes, all located on the derivative chromosome 11, results in the generation of a novel chimeric protein.

30.2.2.2 11q23 Rearrangements Identified in AMLs with Normal Cytogenetics and Trisomy 11

MLL gene rearrangements have been shown in ~10 % of AML with a normal karyotype. All were shown to have partial tandem duplications (PTD) of the 5′ part of the *MLL* gene [50]. These mutations occur in 3–10 % of adult AML cases, but are uncommon in pediatric AMLs. MLL-PTDs are extremely frequent in conjunction with trisomy 11 [50, 94, 95] and are associated with an unfavorable outcome [50, 96]. Another type of intrachromosomal rearrangement of the *MLL*, intrachromosomal amplification, also occurs. This type most likely leads to increased *MLL* expression in AML and MDS [97].

30.2.2.3 AML with inv(11)(p15q22)

The inv(11)(p15q22) has been identified in a very small number of de novo and therapy-related cases of AML [98]. This inversion disrupts the *DDX10* gene at chromosome 11q22 and the *NUP98* gene at 11p15, creating the *NUP98-DDX10* fusion gene. A reciprocal *DDX10-NUP98* fusion gene is also created and may be expressed, but is not thought to be involved in neoplastic transformation [98]. The normal *DDX10* gene is large, spanning 200 kb, and is composed of at least 12 exons. This gene is a DEAD-box putative RNA helicase gene that encodes a protein that may be involved in ribosomal assembly [99]. The *DDXl0* gene is ubiquitously expressed in normal tissues. The normal *NUP98* gene encodes a nuclear-pore complex protein that is also ubiquitously expressed.

30.2.2.4 AML with t(16;21)(p11;q22)

Most neoplasms with the t(16;21) have been AMLs, but rare cases of CML in blast crisis and MDS with the t(16;21) have been described [100]. Patients with AML associated with the t(16;21) are generally young and have a poor prognosis. The t(16;21) is a reciprocal and balanced translocation that disrupts the *TLS/FUS* gene at chromosome 16p11 and the *ERG* (*ETS*-related gene) gene on chromosome 21q22 [101]. As a result two fusion genes are created, *TLS/FUS-ERG* on the derivative chromosome 21 and *ERG-TLS/FUS* on the derivative chromosome 16. Although both fusion genes may be expressed, only the *TLS/FUS-ERG* fusion gene is consistently expressed in all cases, suggesting that this chimeric protein is involved in leukemogenesis [101]. Both the *TLS/FUS* and *ERG* genes are oriented 5′ → 3′. The normal *TLS/FUS* gene encodes an RNA-binding protein that is highly homologous to the *EWS* gene involved in Ewing sarcoma. The TLS/FUS protein plays a role in activating transcription. The normal *ERG* gene, a member of the *ETS* proto-oncogene superfamily, also encodes an RNA-binding protein and is a potent transcriptional activator [101]. At least four different transcripts of the *TLS/FUS-ERG* have been identified. These

transcripts result from variability in the breakpoints and alternative RNA splicing. In most cases, the breakpoint in the *ERG* gene is tightly clustered in one intron. The breakpoints in the *TLS/FUS* gene are more variable [100].

30.2.2.5 AML with t(12;22)(p13;q11)

The t(12;22)(p13;q11) is found in AML and rare cases of MDS and CML. The t(12;22) is a reciprocal translocation involving the *ETS variant 6* (*ETV6*) gene (*known previously as TEL oncogene)* at chromosome 12p13 and the *MNI* (meningioma) gene at chromosome 22q11 [102]. The translocation disrupts the *ETV6* and *MNI* genes, resulting in the formation of *MNI-ETV6* and *ETV6-MNI* fusion genes. The *MNI-ETV6* fusion gene is likely to encode the leukemogenic protein, based on the predicted structure of the fusion protein, which is consistent with an altered transcription factor. The *MNI-ETV6* fusion gene also has been constantly expressed in the small number of neoplasms analyzed, unlike the *ETV6-MNI* fusion gene [102]. The normal *ETV6* gene is very large, exceeding 150 kb, and is a member of the *ETS* gene family of transcription factors. *ETV6* is ubiquitously expressed in tissues. The normal *MNI* gene is less well-known. The gene was originally cloned from a t(4;22)(p16;q11) identified in a case of sporadic meningioma [103]. The gene spans 70 kb, has at least two exons separated by a large intron, and encodes a protein predicted to have 1319 amino acids. The breakpoints in the *MNI* gene appear to be clustered in the 5′-region of the intron [103].

30.2.2.6 AML with t(8;16)(p11;p13)

The t(8;16)(p11;13) is identified in <1 % of all cases of AML. Most AMLs with the t(8;16) exhibit monocytic differentiation and are morphologically classified using the FAB as M4 or M5 [104]. The blasts often exhibit evidence of erythrophagocytosis. Both de novo and therapy-related AMLs with the t(8;16) have been reported. De novo cases commonly occur in children and adolescents (<18 years of age) [104]. The t(8;16) is a reciprocal translocation, involving the *MOZ* (monocytic leukemia zinc-finger protein) located at chromosome 8p11 and *CREB binding protein* (*CREBBP*) gene located at 16p13 [105]. The translocation disrupts these genes, resulting in the formation of a *MOZ-CBP* fusion gene on the derivative chromosome 8. A *CREBBP-MOZ* fusion gene is also created, but is thought to be nonfunctional. The *MOZ* gene encodes a 225 kDa protein that is widely expressed in tissues. The protein has both zinc-finger and acetyltransferase domains and may mediate leukemogenesis by aberrant acetylation of chromatin [105]. The *CREBBP* gene spans 190 kb and encodes a protein involved in transcriptional activation. Mutations in the *CREBBP* gene have been identified in a rare genetic disease, the Rubinstein–Taybi syndrome, characterized by mental retardation, dysmorphic cranial features, and digital abnormalities [106].

30.2.2.7 AML with t(3;21)(q26;q22)

The t(3;21)(q26;q2.2) has been reported in cases of chronic myelogenous leukemia (CML) in blast crisis, in MDS, and in AML following treatment with topoisomerase II inhibitors such as etoposide [107]. The breakpoint in the *AML1* gene on chromosome 21 may be identical to that in the t(8;21), but more often occurs approx. 60 kb downstream. The chromosome 3q26 locus is the site of three different genes that are involved in the t(3;21): from telomere to centromere, *EAP, MDS1*, and *EVI1*. These genes are located within 200 kb of each other. In each of these translocations, the 5′-end of the *AML1* (*RUNX1*) gene, including sequences that encode the *runt*-like domain, is fused to the 3′-end of one of these partner genes [107].

In the *AML1-EAP* fusion gene, the 3′-*EAP* sequence is very small and it is not fused to *AML1* in reading frame. Thus, the AML1-EAP chimeric protein lacks transactivation activity and may exert its effect by inhibiting normal AML1 protein. In contrast, the *AML1*-MDS1 and *AML1-EVI1* fusion genes contain 3′-sequences of the *MDS1* and *EVI1* genes, suggesting that their proteins possibly have unique functions, in addition to inhibiting normal AML1 protein. One potential explanation for the occurrence of these different translocations, not proven, is that the entire 3q26 region is transcribed as a result of the t(3;21), and different fusion genes result from alternative splicing of mRNA [108]. The normal EAP, MDS1, and EVI1 proteins are not normally expressed in hematopoietic cells, but are expressed as a result of the t(3;21).

30.2.2.8 AML with t(9;22)(q34;q11)

The existence of AML associated with t(9;22)(q34;q11) has been controversial in the past, in large part, because distinguishing AML from an initial presentation of CML in blast crisis (phase) can be difficult. The presence of Philadelphia chromosome and *BCR/ABL* fusion gene transcripts are not reliable criteria, as some cases of AML with these features have been reported [109], including cases in which *BCR/ABL* fusion transcripts were acquired in MDS. To complicate the issue, the karyotype in CML blast crisis is often very complex. Some authors believe that the only reliable way to distinguish these entities is by assessing therapeutic response or by testing maturing hematopoietic elements for the *BCR/ABL*, present in CML only. The t(9;22)(q34;q11) is rarely identified in apparently de novo cases of AML, in 1–2 % of cases [109, 110]. The t(9;22) involves the *ABL1* gene at chromosome 9q34 and the *BCR* gene at chromosome 22q11. The translocation results in a *BCR-ABL* fusion transcript. Two different forms of the t(9;22) have been detected in AML that result in either a 190 kDa or 210 kDa fusion protein [110]. In both translocations, the breakpoint in *ABL1* occurs in the same general area, in the proximal portion of the gene. In the $p190^{bcr\text{-}abl}$ form, the breakpoint in *BCR* occurs in the first

exon. In contrast, in the $p210^{bcr\text{-}abl}$ form the breakpoint occurs in the 5.8 kb major breakpoint region of *BCR*. The detection of the $p190^{bcr\text{-}abl}$form of the t(9;22) in a case of AML is evidence to support the diagnosis of de novo AML. *NPM1* mutations have been identified in a subset of de novo AML associated with t(9;22) [109], but no *ABL1* mutations, known to occur in a subset of untreated CML patients, have been reported [109].

30.2.2.9 AML with t(7;11)(p15;p15)

The t(7;11)(p15;p15) is a rare translocation in AML. Morphologically, most AMLs with the t(7;11) exhibit granulocytic maturation and are classified as FAB-M2; coexistent myelodysplastic features are common [111]. Most patients with t(7;11)-positive AML have been Japanese adults, suggesting a genetic predisposition for this type of AML [111].

The t(7;11) disrupts the *HOXA9* gene at chromosome 7p15 and the *NUP98* gene at 11p15, resulting in formation of the *NUP98-HOXA9* and *HOXA9-NUP98* fusion genes [105, 111]. The *NUP98-HOXA9* fusion gene, located on the derivative chromosome 11, is thought to be involved in leukemogenesis, based on the predicted structure of the chimeric protein [105, 111]. The reciprocal *HOXA9-NUP98* fusion gene is probably nonfunctional, and was not amplifiable by RT-PCR in a subset of cases tested [105]. The normal HOXA9 gene is a member of the homeobox gene family, and is involved in development and differentiation. The HOXA9 gene encodes a class I homeodomain protein that is expressed in hematopoietic cells and the kidney [105, 111]. The normal *NUP98* gene is relatively small, with five exons, and encodes a component of the nuclear-pore complex that is 98 kDa.

30.2.2.10 Gene Mutations Associated with AML

FLT3 Gene

FMS-like receptor tyrosine kinase 3 (*FLT3*), also known as *fetal liver kinase 2* (*FLK-2*) and *stem cell tyrosine kinase 1* (*STK-1*), belongs to a class III receptor tyrosine kinase family that also includes *KIT*, *FMS*, and *platelet-derived growth factor receptor* (*PDGFR*) [112]. The human *FLT3* gene is located on chromosome 13q12 and contains 24 exons [112]. *FLT3* has been demonstrated to exist in two forms: a membrane-bound protein of 158–160 kDa that is glycosylated at N-linked glycosylation sites in the extracellular domain and an unglycosylated protein of 130–143 kDa that is not membrane bound [113]. FLT3 is preferentially expressed by hematopoietic stem cells as well as in the brain, placenta, and liver [114]. The ligand of FLT3, which is expressed as a membrane-bound or soluble form by bone marrow stromal cells, stimulates the stem cells by itself or in cooperation with other cytokines [113, 115–117]. FLT3 is expressed on the surface of a high proportion of AML and stimulation of FLT3 enhances proliferation and reduces apoptosis [118].

Two unique forms of *FLT3* gene mutation have been described. Initially, an internal tandem duplication (ITD) in the juxtamembrane domain–coding sequence of *FLT3* was described (Fig. 30.12) [119]. Subsequently, a missense point mutation at the D835 residue and point mutations, deletions, and insertions in the codons surrounding D835 within a *FLT3 kinase domain* (KDM) were found [120, 121]. *FLT3/ITD* and *FLT3/KDM* occurs in 15–35 and 5–10 % of adults with AML, respectively, and are associated with a poor prognosis [122, 123]. In addition, extremely high levels of *FLT3* transcripts have been demonstrated in a proportion of AML patients without *FLT3* mutations, and are also associated with a poor prognosis [124]. Rarely, both types of mutations occur simultaneously in AML; this may be related to clonal progression [125].

FLT3 mutations lead to constitutive activation resulting in increased receptor signaling, which in turn is invovled in tumorigenesis. *FLT3/ITD* is constitutively phosphorylated on tyrosine residues and forms homodimers and, if the ITD-containing *FLT3* is cotransfected with wild-type *FLT3*, a heterodimer with wild type *FLT3* [122]. *FLT3/ITD* or *FLT3/KDM* result in autonomous proliferation of murine interleukin 3-dependent cell lines, such as 32D, and induce consti-

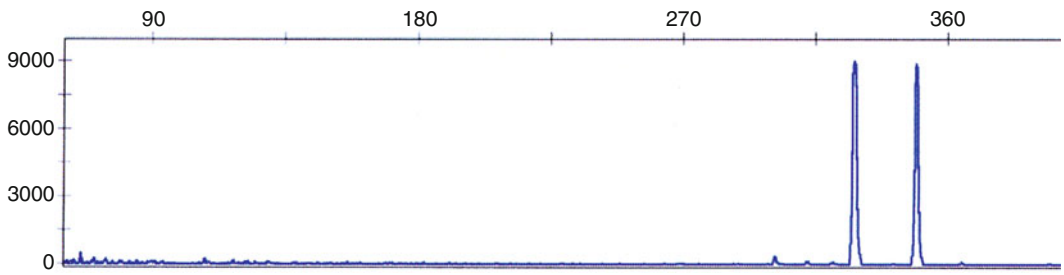

Fig. 30.12 Multiplex fluorescence-based PCR method used to detect internal tandem duplication (ITD) of FLT3. DNA is assessed by amplification of the juxtamembrane domain using primers from exons 11 and 12. The presence of second, larger peak signifies that *FLT3/ITD* is present.

tutive activation of downstream signaling molecules, such as signal transducer and activation of transcription 5 (STAT5), MAP kinase, SHC, AKT, and BAD [122, 126, 127]. 32D cells are known to differentiate into mature neutrophils in response to granulocyte colony-stimulating factor (G-CSF). However, both *FLT3/ITD*-expressing and *FLT3/KDM*-expressing 32D cells do not differentiate into mature neutrophils when they are treated with G-CSF [128]. These results indicate that *FLT3* mutations are involved in cascades for autonomous cell proliferation and differentiation block. In addition, mutant *FLT3* has antiapoptotic effects on leukemia cells [127].

It has been shown that *FLT3/ITD* induces an oligoclonal myeloproliferative disorder in mice, but not AML, suggesting that additional mutations impairing hematopoietic differentiation and/or proliferation may be necessary for AML development [129]. Transplantation of *FLT3/ITD*-transfected bone marrow cells into *PML/RARα* transgenic mice clearly shortens the latency period and increases penetrance for developing acute promyelocytic leukemia-like disease [130].

FLT3 mutations are mainly found in de novo AML, and are less frequent in AMLs that have developed from MDS or therapy-related AML [131, 132]. In low grade MDS, *FLT3/ITD* or *FLT3/KDM* occurs in approximately 3 % of cases. However, the frequency increases in advanced-stage MDS, and in up to 15 % in patients with AML that has developed from MDS [131, 132]. The frequency of *FLT3* mutations in AML is associated with patient age. *FLT3/ITD* has been found in about 25 % of adult AML patients, but is higher (~30 %) in patients older than 55 years [133]. In contrast, *FLT3/ITD* has been found in approximately 10 % of pediatric AML patients [134, 135] and is rare in infant AML. *FLT3* mutations are frequently found in AML with normal or intermediate-risk cytogenetic characteristics and with t(15;17), but are infrequent in patients with core binding factor translocations, such as t(8;21) and inv(16). Recently, it has been demonstrated that the *NPM1* gene is mutated in more than 60 % of adult AML patients harboring *FLT3* mutations [77–79].

FLT3/ITD is strongly associated with leukocytosis and an increased percentage of blast cells in the peripheral blood and BM of AML patients [131, 132]. The association of *FLT3/KDM* with leukocytosis is controversial. Only one study has demonstrated significant leukocytosis in adults who have AML with *FLT3/KDM* [121]. Several large-scale studies have shown the impact of *FLT3* mutations on patient outcome. *FLT3/ITD* is a strong adverse predictive factor for overall survival, disease-free survival, and event-free survival within the intermediate-risk cytogenetic category. The clinical impact of *FLT3/KDM* on long-term outcome is controversial. Recent meta-analysis of four published studies including 1160 adult AML patients suggests an adverse effect of *FLT3/KDM*s on long term outcome [136].

RAS Gene

RAS is a GTP-dependent, 21 kDa protein that is localized at the inner side of the cell membrane. RAS is encoded by the *RAS* gene family, including *KRAS* located at 12p12.1, *HRAS* located at 11p15.5, and *NRAS* located at 1p13.2. RAS binds guanine nucleotides, displays intrinsic GTPase activity, and transduces signals from growth factor receptors to downstream effectors. The GTPase-mediated cycling between *RAS* bound to GTP (active conformation) versus GDP (inactive conformation) serves as a regulatory switch for signal transduction.

In AML, activating somatic mutations in the first two exons of *RAS* have been described, including codons 12, 13, and 61 of *N-RAS*, and sporadically in *K-RAS* [137]. These point mutations disrupt *RAS* function by diminishing GTPase activity. The reported frequency of *N-RAS* mutations in AML ranges from 10 to 27 % [137]. *K-RAS* mutations at the same codons can be found in approximately 5–10 % of pediatric and adult AML patients [138]. *H-RAS* mutations are rare in hematologic tumors [137]. In two studies, the presence of *RAS* mutations correlated with low blast counts in the BM and a better survival of patients in an age-adjusted analysis [139]. However, in other reports patients with *RAS* mutations showed a trend to a worse outcome compared to patients without tyrosine kinase or *RAS* mutations [138].

Patients with MDS and *N-RAS* mutations have a very high risk of progression to AML suggesting that these mutations might represent an important progression factor in MDS. On the other hand, analyses of *RAS* mutation status in patients at first diagnosis and at relapse revealed a marked instability of these mutations, with loss of mutations in some cases or new mutations in different codons. This may indicate that RAS mutation is not a leukemia-initiating event [140]. *RAS* has been shown to be an important mediator of G-CSF-induced and thrombopoietin-induced differentiation and to enhance the function of CEBPA in promoting myeloid differentiation [141].

KIT Gene

Activating mutations of *KIT*, which are well-known in mast cell disorders and in gastrointestinal stromal tumors, also occur in AML. These mutations usually occur in one of two regions: the juxtamembrane region that functions as a negative regulatory region [142], and residues in the activation loop. *KIT* mutations are infrequent in AML in general, but are more common in patients with core binding factor AMLs, ranging from 2 to 20 % [143].

G-CSF Receptor

Approximately 20 % of patients with severe congenital neutropenia have *G-CSF receptor* point mutations. These mutations result in a truncation of the C-terminal region which is crucial for G-CSF signaling [144]. The mutated receptor

interrupts signals required for normal myeloid cells. Patients with these mutations have an increased risk of progression to AML [144]. In 32D cells, the expression of a truncated G-CSF receptor resulted in increased proliferation without neutrophilic differentiation [145]. Transgenic mice expressing mutant G-CSF receptor (truncated at amino acid 715) develop a form of neutropenia in which myeloid progenitors react to G-CSF with hyperproliferation and neutrophilia [146]. Transgenic mice overexpressing a form of the receptor truncated at amino acid 718 or 731 have low neutrophil counts compared with wild-type controls and increased numbers of immature myeloid cells in the bone marrow [146, 147]. These effects are attributed to ligand-induced internalization of the receptor [148], reduced ability of the mutant receptor to activate STAT3, and reduced STAT5 inhibition by SOCS3 [149]. These features might be responsible for increased risk of AML in patients with severe congenital neutropenia.

IDH1 and *IDH2*

The isocitrate dehydrogenase (IDH) family of enzymes has three members: IDH1, IDH2, and IDH3. These enzymes catalyze the oxidative decarboxylation of isocitrate to produce CO2 and α-ketoglutarate (αKG). IDH1 and IDH2 are NADP+-dependent while IDH3 is NAD+-dependent. IDH1 contains a C-terminal peroxisomal localization sequence and is localized in both peroxisomes and cytosol; IDH2 and IDH3 are located in mitochondria [150]. A mutation in the *IDH1* gene was first discovered in colorectal cancer and subsequently shown in brain gliomas. Later, mutations in *IDH1* and *IDH2* were identified in AML as well as MDS [151, 152]. In addition, *IDH2* mutations have been identified in T-cell lymphomas, but to date, there have been no reports of cancer-associated mutations in *IDH3*.

In the initial report by Mardis and colleagues, *IDH1 R132* mutation was detected in approximately 8 % of AML cases analyzed and was associated with normal cytogenetics [153]. Subsequently *IDH2* mutations were detected in patients with AML. The initial study cited the frequency of IDH1 and IDH2 mutations in patients with AML as 7.7 and 15.4 %, respectively [153]. In subsequent studies, focused on a relatively homogenous group of adult patients with de novo AML with diploid cytogenetics, mutations in *IDH1* and *IDH2* were found in 14 and 19 % of cases, respectively [151, 152]. *IDH1* and *IDH2* mutations are not mutually exclusive as several patients harboring both an *IDH1* and *IDH2* mutation have been detected [152, 155]. Unbiased sequencing also has revealed the presence of 12 nonsynonymous recurring mutations (including *IDH1*) in more than one AML genome. The data implicate these alterations as important for leukemogenesis [153].

Recent studies across large patient cohorts have shown that *IDH1 and IDH2* mutations are strongly associated with the presence of *NPM1* mutation [153–156]. An analysis of cytogenetically diploid AML patients has shown that IDH mutations adversely affect prognosis [152]. Retrospective clinical trial data also have shown that IDH1 or *IDH2* mutations are associated with a worse prognosis in younger AML patients with diploid cytogenetics and mutated *NPM1* without *FLT3-ITD* [152, 155].

TP53 Gene

The *TP53* tumor-suppressor gene, located at chromosome 17p13, is mutated in a wide variety of human cancers including AMLs. *TP53* encodes for a nuclear phosphoprotein that binds to DNA and influences the expression of a variety of genes. p53 protein appears to be involved in cell proliferation, apoptosis, and DNA repair. Over 200 cases of AML have been assessed for *TP53* abnormalities. Point mutations were identified in about 6 % of cases. *TP53* gene mutations have been identified in all morphologic types of AML except acute promyelocytic leukemia. Presence of *TP53* mutations correlates with older patient age, presence of myelodysplasia, and poorer prognosis [157].

30.2.2.11 Other Abnormalities in AML

STAT Activation

Consitutive activation (phosphorylation) of signal transducer and activator of transcription (STAT) 3 and STAT5 has been shown in cases of AML [126]. For full transcriptional activity, STAT proteins must be phosphorylated on serine residues, often by MAP kinases, and this also has been observed in AML blasts. Aberrant regulation of gene expression contributes to constitutive activation of STAT proteins. STAT3 and STAT5 can be activated by mutationally activated isoforms of KIT and FLT3 in AML blasts [126]. SOCS proteins are potent repressors of STAT activation, and their ability to repress transcription occurs through aberrant DNA-methylation of regulatory regions upstream of their transcriptional initiation site.

Constitutive activation of STAT3 and STAT5 appears to work as a signal integration site for several pathways on the way to leukemic transformation. The transforming ability of constitutively activated STAT5 protein on mouse bone marrow was demonstrated using retroviral transduction/transplantation studies. STAT3 or STAT5 are necessary for tyrosine kinase-mediated transformation of myeloid cells.

CXCR4 Expression

CXC chemokine receptor 4 (CXCR4) is one of a number of chemokine receptors defined by their ability to induce cell migration towards a chemotactic cytokine gradient (chemotaxis). CXCR4 has received much attention in the literature because it is the receptor for stromal derived factor (SDF-1α), also known as CXCL12, and the CXCR4-CXCL12 axis is

essential for the migration of normal cells to the bone marrow microenvironment [158]. CXCR4-CXCL12 also appears to play a role in metastases of both hematopoietic and solid tumors [158, 159]. CXCR4 is expressed and functional in a subset of AML cases and expression correlates with poorer prognosis [159, 160]. In addition, CXCR4 has been reported to be expressed more frequently and at a higher level in AML cases associated with *FLT3/ITD* mutations [161]. Based on this last observation, a link between CXCR4 expression and *FLT3/ITD* mutation has been proposed. One group has demonstrated that CXCR4 expression is associated with a poorer prognosis in AML patients with normal cytogenetics and unmutated *FLT3* [160].

Antigen-Receptor Gene Rearrangements in AML

Immunoglobulin heavy chain (IgH) and /or T-cell receptor (TCR) β-chain and γ-chain gene rearrangements have been detected in less than 5 % of cases of AML, using Southern blot analysis [162]. However, a study of AMLs using PCR-based methods found a substantially higher number of cases with monoclonal IgH gene rearrangements. *T-cell receptor* rearrangements are more common in AML cases with stem cell immunophenotypic features. Many AML cases with antigen-receptor gene rearrangements are undifferentiated, often TdT-positive, and express one or more lymphoid-associated antigens, suggesting that these leukemias are arising from an early BM precursor cell with multilineage potential [162]. Patients with AML associated with antigen receptor gene rearrangements are reported to have a poorer prognosis than patients with AML without these gene rearrangements.

30.2.2.12 Therapy-Related AML

Patients with therapy-related acute myeloid leukemia (t-AML) present either early, within 3 years of cessation of all chemotherapy, or late, 7 or more years after completion of therapy. Early t-AMLs often display balanced translocations, especially involving the *MLL* gene, whereas late t-AMLs often shown unbalanced rearrangements, cytogenetic abnormalities of chromosomes 5 and 7, and are often preceded by MDS [163]. Clinicopathologic studies with multivariate analysis have implicated topoisomerase II inhibitors, particularly the epipodophyllotoxins etoposide and teniposide, in the generation of balanced *MLL* translocations in the patients with early t-AML [164]. Data from the initial series of cases with t-AML related to topoisomerase II inhibitors suggested that the epipodophyllotoxins were more highly associated with t-AML than other topo II agents, and that dose and schedule of therapy were important.

Cytotoxic chemotherapeutic agents are associated with cases of late t-AML. Gene mutation studies using next-generation sequencing methods have shown that mutation profiles in t-AML differ greatly from de novo AML cases. In particular, *TP53* mutations are much more common in t-AML.

30.2.2.13 AML in Patients with Down Syndrome

Children with Down syndrome (DS) have a 10-fold to 20-fold increased risk of developing acute megakaryoblastic leukemia when compared with their non-DS counterparts [165]. Recently, an important role for the hematopoietic transcription factor *GATA1* has been demonstrated in DS patients with either transient myeloproliferative disorder (also known as transient abnormal myelopoiesis) or acute megakaryoblastic leukemia [166]. Expression of *GATA1* is essential for normal maturation of erythroid cells and megakaryocytes [167]. In two studies of 41 DS patients with acute megakaryoblastic leukemia or transient myeloproliferative disorder, missense mutations in *GATA1* were identified in over 90 % of cases; the mutations were not identified in subsequent remission samples and were determined to be acquired [168]. In contrast, *GATA1* mutations were not seen in four cases of acute lymphoblastic leukemia in DS patients [168].

Genomic profiling studies have shown that cases of transient myeloproliferative disorder often have only trisomy 21 and *GATA1* mutations. In contrast, in patients that progress to acute megaryoblastic leukemia a number of other genes are mutated including cohesin genes, genes that regulate epigenetics (e.g., *EZH2*), *CTCF*, *JAK* family genes, *MPL*, and *RAS* pathway genes [169].

30.2.2.14 Hypoxia and Hypoxia-Related Changes

Recently, hypoxia and hypoxia-related changes in the BM have attracted the attention of investigators analyzing AML and other malignancies. In normal BM, hypoxia is critical for development of hematopoietic stem cells [170]. A critical role in mediating the effect of hypoxia on hematopoietic stem cells is played by hypoxia-inducible factor 1 alpha (HIF1α) [171]. Decrease in oxygen tension in BM can lead to the selective outgrowth of AML clones with a survival advantage in the severely hypoxic microenvironment [172]. Data obtained from animal models indicate that AML progression within BM is associated with further decreases in overall oxygenation and expansion of hypoxic areas [172]. Hypoxia also affects the exposure of AML cells to systemic chemotherapy and the antitumor immune response [173]. The overexpression of HIF1α in primary AML and acute lymphoblastic leukemia BM samples correlates with poor chemotherapy outcomes [174, 175]. For these reasons, hypoxia-activated agents, novel cytotoxic drugs preferentially activated under low oxygenation conditions, may have an active role in the treatment of AML and other BM malignancies. Recent studies have demonstrated that HIF1α plays a role in cancer progression by activating transcriptional programs for maintaining the self-renewal and multipotent capacities of cancer stem cells in a hypoxic environment [176–178]. HIF1α was also shown to be required for stem cell functions in mouse lymphoma and human AML [179].

One of the new approaches in AML therapy is hypoxia-activated prodrugs such as TH-302. TH-302 is a 2-nitroimidazole hypoxia-activated prodrug of the cytotoxin bromo-isophosphoramide mustard. In vitro, TH-302 demonstrates activity against solid tumors, multiple myeloma cells, and AML cells under low oxygen tension [173, 180, 181]. In clinical trials, TH-302 alone and in combination with chemotherapy was very effective in solid tumors and hematologic malignancies including AML and multiple myeloma [182, 183].

30.3 Blastic Plasmacytoid Dendritic Cell Neoplasm

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare tumor that arises from precursors of plasmacytoid dendritic cells (also known as plasmacytoid monocytes or professional type 1 interferon producing cells). The age range of patients with BPDCN is quite broad, from young children to the elderly and males are more often affected. Patients with BPDCN usually present with skin disease and involvement of bone marrow. Most patients also present with low-level peripheral blood involvement and lymphadenopathy occurs in about 50 % of patients. Overall survival is poor, in the range of 1–2 years despite therapy [184, 185].

Conventional cytogenetic analysis commonly shows a complex karyotype but there are no consistent recurrent abnormalities. Deletions of a number of chromosomal loci have been reported with del(5q) most common. Rare cases reported have had T-cell receptor gene rearrangements, but in most cases of BPDCN the *IgH* and *TCR* genes are germ line.

Gene expression profiling of BPDCN has pointed to activation of the NF-kB pathway as a potential therapeutic target [186]. A number of gene mutations have been identified using whole exome or more targeted sequencing approaches including *TET*, *IKZF3*, *HOXB9*, *UBE2G2*, and *ZEB2*. A single case of a child with BPDCN associated with a *CLTC-ALK* fusion gene has been reported [187].

TET mutations are most common in BPDCN, in up to 40 % of cases [184]. *TET* is also mutated in cases of MDS and AML. TET normally converts 5-methyl-cytosine to 5-hydroxymethylcytosine and is expressed in early hematopoietic precursors. TET is involved in epigenetic regulation and hematopoiesis. *TET* mutations are usually heterozygous and result in loss of function, presumably allowing uncontrolled expansion of hematopoietic cells [187].

30.4 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is an umbrella term for a group of neoplasms composed of immature lymphoid cells that are derived from the bone marrow. Previously, the FAB system classified these neoplasms on the basis of their morphological features as L1, L2, or L3. It is now recognized that the distinction between L1 and L2 has little biological and clinical meaning, and that L3 cases represent Burkitt lymphoma/leukemia [188].

Far more important in ALL are their immunophenotypic and molecular features. Cases of ALL can be divided into immature B-cell and T-cell neoplasms and each of these categories has a number of molecular subgroups. Approximately 80–90 % of ALL cases are of immature B-cell lineage, and include neoplasms once designated as non-B/non-T, null, and common ALL. The remaining 10–20 % of ALL cases are of T-cell lineage. Heritable syndromes predisposing to ALL and *germ line* mutations in known tumor-suppressor genes are uncommon, implying that most cases probably arise from somatic mutations in immature lymphoid cells.

Immature B-cell and T-cell neoplasms can present as either ALL or lymphoblastic lymphoma. At the molecular level leukemic and lymphomatous B-cell or T-cell neoplasms appear to be similar. For this reason, the current WHO classification designates these tumors as B or T lymphoblastic leukemia/lymphoma. However, as most studies of these tumors have focused on cases that presented as leukemia we use the terms B-ALL or T-ALL or lymphoblastic lymphoma (LBL).

30.4.1 B-Cell ALL

Based on antigen expression, B-ALLs can be further subclassified into as many as five subgroups: (1) pre-pre-B-ALL, (2) early pre-B-ALL, (3) pre-B-ALL, (4) transitional B-ALL, or (5) mature B-ALL, although subdivision into fewer subgroups based on the expression of CD10, cytoplasmic IgM, and surface immunoglobulin is the usual practice. Nearly all B-ALLs carry monoclonal *IgH* gene rearrangements. In addition, 40–50 % of ALLs also contain *Igk* gene rearrangements, and 20–25 % contain *Igλ* gene rearrangements. The results suggest a hierarchy of *Ig* gene rearrangements in developing B lymphocytes: IgH rearrangement first followed by *Igk* and then *Igλ*.

Lineage infidelity is common in B ALLs and the TCR genes are frequently rearranged [189]. TCR-δ is rearranged or deleted (after rearrangement) in as many as 80 % of cases. The TCR-γ and TCR-β genes are rearranged in 50–60 %, and 20–30 % of cases, respectively. As a result, either the *TCR* or *Ig* genes can he assessed to determine clonality, but evaluation of individual antigen receptor genes is less useful for lineage determination.

Standard cytogenetic studies have shown the presence of multiple nonrandom chromosomal translocations in B-ALL. The molecular and cytogenetic findings are summarized in Table 30.2. A number of the most frequent and best-characterized molecular abnormalities are discussed more specifically here.

Table 30.2 Cytogenetic and molecular abnormalities in acute lymphoblastic leukemia

Chromosomal translocations	Genes	Comments
B-cell lineage		
t(9;22)(q34;q11)	*ABL1*	Tyrosine kinase
	BCR	Unknown function
Involving CRLF2		
t(X; 14)(p22.3;q32)	*CRLF2*	JAK-associated signaling pathway
t(Y; 14)(p11.3;q32)		Activator
	IgH	Ig heavy-chain enhancer
Involving TCF3 (E2A)		
t(1;19)(q23;q13)	*PBX1*	Homebox gene
	TCF3 (E2A)	bHLH transcription factor
(17;19)(q22;p13)	*HLF*	bZIP transcription factor
	TCF3 (E2A)	bHLH transcription factor
Involving KMT2A (MLL)		
t(11;v)(q23;v)	*KMT2A (MLL)*	*Drosophila trithorax* homology
	Variable	Many partner genes
		Infancy, high risk
Involving ETV6 (TEL) and CDKN1B (KIP1)		
t(12;21)(p12;q22)	*ETV6 (TEL)*	*ETS*-like transcription factor
	RUNX1	Runt-like transcription factor
t(5;14)(q31;q32)	*IL-3*	Cytokine gene
	IgH	Ig heavy-chain enhancer
Involving *MYC*		
t(8;14)(q24;q32)	*MYC*	bHLH transcription factor
	IgH	Ig heavy-chain enhancer
t(2;8)(p12;q24)	*Igk*	Igk chain enhancer
	MYC	bHLH transcriptor factor
	Igλ	Igλ chain enhancer
Gene amplification		
Hyperdiploid karyotype		Favorable prognosis
DHFR		Associated with p53 mutations
T-cell lineage		
Involving TAL1, TAL2, and LYL1		
Interstitial deletions	*TAL1*	bHLH transcription factor
	STIL (SIL)	Gene-expressed T cells
		Up to 30 % T-ALL
t(1;14)(p32;q11)	*TAL1*	bHLH transcription factor
	TCR-δ	TCR enhancer
t(1;7)(p33;q35)	*TAL1*	bHLH transcription factor
	TCR-β	TCR enhancer
t(7;9)(q34;q32)	*TCR-β*	TCR enhancer
	TAL2	bHLH transcriptor factor
t(7;19)(q34;q13)	*TCR-β*	TCR enhancer
	LYL1	bHLH transcription factor
del(9)(p21-22)	*P16INK4A*	Cell-cycle inhibitor
	P16INK4B	Cell-cycle inhibitor
Involving TLX1 (HOX11)		
t(10;14)(q24;q11)	*TLX1 (HOX11)*	Homeobox gene
	TCR-qδ	TCR enhancer
t(7;10)(q34;q24)	*TCR-β*	TCR enhancer
	TLX1 (HOX11)	Homeobox gene

(continued)

Table 30.2 (continued)

Chromosomal translocations	Genes	Comments
Miscellaneous		
t(8;13)(p11;q11)	*FGFR1*	Growth-factor receptor
	ZNF198	Novel gene, Zn finger
t(8;14)(q24;q11)	*MYC*	bHLH transcription factor
	TCR-δ	TCR enhancer
Involving LMO1 (TTG1) and LMO2 (TTG2)		
t(11;14)(p15;q11)	*LMO1 (TTG1)*	LIM protein
	TCR-δ	TCR Enhancer
t(11;14)(p13;q11)	*LMO2 (TTG2)*	LIM protein
	TCR-δ	TCR Enhancer
Involving KMT2A (MLL)		
t(11;v)(q23;v)	*KMT2A (MLL)*	*Drosophila trithorax* homology
	Variable	Many partner genes
Involving NOTCH1 (TAN1)		
t(7;9)(q34;q34.3)	*TCR-β*	TCR Enhancer
	NOTCH1 (TAN1)	*Drosophila notch*-like
Involving TCL1A (TCL1)		
t(14;14)(q11;q32.1),	*TCR-δ*	TCR Enhancer
inv(14)(q11;q32.1)	*TCL1A (TCL1)*	Homology to *MTCP1* of T-CLL
t(7;14)(q35;q32.1)	*TCR-β*	TCR Enhancer
	TCL1A (TCL1)	Homology to *MTCP1* of T-CLL

30.4.1.1 B-ALL with t(9:22)(q34;q11.2); BCR-ABL

The Philadelphia chromosome (Ph) or t(9;22)(q34;q11), is among the most prevalent recurrent chromosomal translocations in B-ALL, detected in 20–33 % of adults and 5 % of children with this disease [190]. This translocation disrupts and fuses a portion of the *ABL1* gene at chromosome 9q34 with a portion of *BCR* locus at 22q11 forming *BCR-ABL1* (Figs. 30.5, 30.13, and 30.14). Two forms of the t(9;22) occur. In 25–50 % of adult cases of B-ALL, the translocation appears identical to that found in CML; a fusion protein with a molecular weight of 210 kDa is formed. In the remaining 50–75 % of adult cases and most childhood cases of B-ALL, the breakpoint on chromosome 22 is 100 kb upstream of the BCR region, resulting in a smaller fusion protein with a molecular weight of 190 kDa. This p190 protein has enhanced tyrosine kinase activity and transforming capability compared to p210 protein [191]. The t(9;22) is usually detected by conventional cytogenetics, FISH, and RT-PCR.

BCR-ABL1-Like B-ALL

This entity was initially identified by gene expression profiling which showed that these tumors have an expression profile very similar to B-ALL with t(9;22)(q34;q11) even though these tumors lack the translocation [192–195]. This entity is usually mutually exclusive with B-ALL with recurrent cytogenetic abnormalities such as *ETV6-RUNX1*, *E2A-PBX1*, *ERG* rearrangement, *MLL* rearrangement, or hyperdiploidy [192, 195, 196]. The reported incidence of BCR-ABL1-like B-ALL varies from 10 % in children to 30 % in young adults, and these tumors are present at higher frequency in patients with Down syndrome [192, 195, 196], but the true incidence might be higher taking into consideration the novelty of the entity and lack of reliable tools to diagnose it in the community. Patients with BCR-ABL1-like B-ALL have a poorer outcome, but these tumors have the potential to respond to tyrosine kinase inhibitors [193–195].

From the molecular perspective, *BCR-ABL1-like* B-ALL can be divided in two groups based on the underlying activation of either the JAK-associated signaling pathway or the ABL-associated signaling pathway [195, 197]. This distinction is critical for proper patient management. Most commonly, the JAK-associated signaling pathway is activated by *CRLF2 protein encoded by CRLF2* gene (chromosome Xp22.3/Yp11.3) overexpression, which can be a result of either *IGH-CRLF2* translocations or deletions that create *P2RY8-CRLF2* fusions. *CRLF2* overexpression is often (about 50 %) associated with additional mutations in *CRLF2*, *IL7R*, *JAK1*, or *JAK2* [196]. Less commonly, *CRLF2* overexpression can result from rare translocations such as *BCR-JAK2*, *STRN3-JAK2*, or *PAX5-JAK2* [195, 197]. The ABL-associated signaling pathway is usually activated via a large number of gene fusions that involve *ABL1;* partners include *NUP214*, *ETV6*, *RANB2*, and *RCSD1*. Fusion genes that involve *JAK2* and rarely *PDGFRβ* are also reported with partners that include *PAX5*, *EBF1*, and *STRN3* [195, 197, 198].

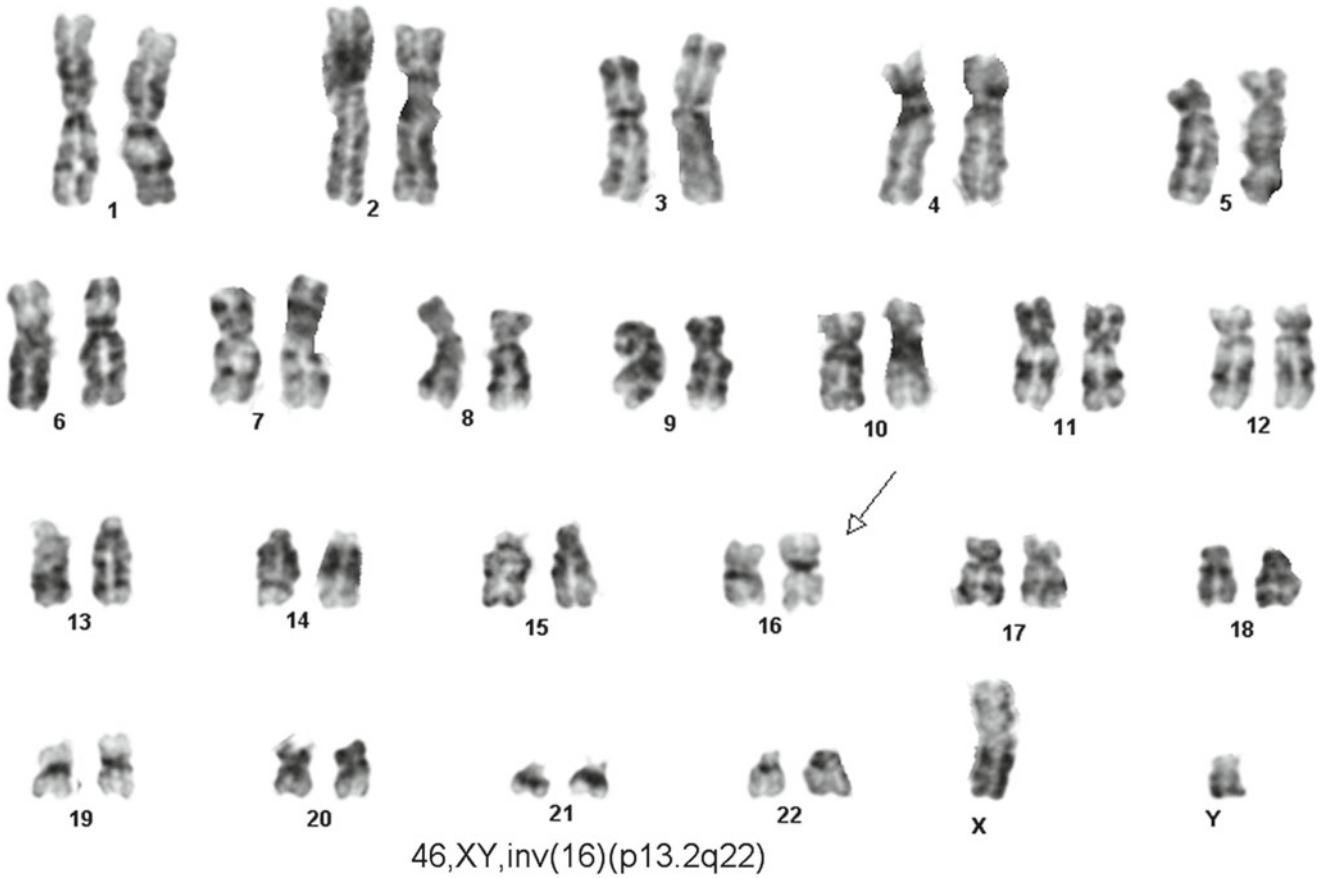

Fig. 30.13 Karyotype of a case of acute myeloid leukemia with inv(16)(p13q22). Conventional cytogenetic analysis demonstrates inv(16) as a sole cytogenetic abnormality. GTG banding technique.

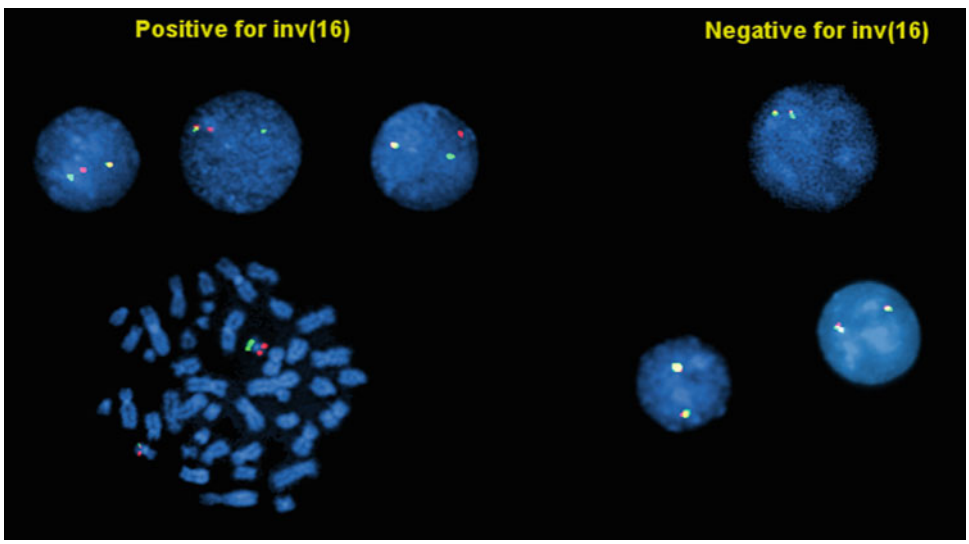

Fig. 30.14 Fluorescence in situ hybridization (FISH) analysis of a case of acute myeloid leukemia with inv(16)(p13q22). Dual color, breakapart probe (Vysis) in which the 5′ CBFβ probe positioned centromeric to the inv(16) breakpoint region is *red*, and the 3′ CBFβ probe positioned telomeric to the inv(16) breakpoint is *green*. In the event of inv(16) or t(16;16), *red* and *green* signals become separated, and a cell has one *red*, one *green*, and one fused (*yellow*) signal (*left panel*). In the absence of inv16 or t(16;16), a cell contains two fused (*yellow*) signals (*right panel*). This technique allows analysis of both metaphase and interphase cells (*right*).

30.4.1.2 B-ALL with t(v;11q23); *MLL* Rearranged

A large number of partners on different chromosomes have been reported in translocations involving *MLL* at the 11q23 locus [1]. In B-ALLs, 11q23 abnormalities are detected as translocations involving the following partner chromosome loci: 9p11, 9p21-p22, 4q21, 19p13, 1q21, 1p32, 6q27, 12p13, 17q21, 17q25, 20q13, and Xq13; of these, t(4;11), t(9;11), and t(11;19) are most prevalent. The t(4;11) (q2l;q23), which occurs in about 2 % of childhood B-ALLs and 5–6 % of adult B-ALLs, confers a poor prognosis [1]. The gene located at 4q21 is named *AF4* or *FEL*. Although most ALLs with 11q23 abnormalities are of B-cell lineage, a small subset of cases possess an immature T-cell immunophenotype and *MLL* fusions are found in 4–8 % of T-ALL [46, 199].

In infants, 11q23 abnormalities are found in more than 70 % of leukemias in patients <1 year of age whether the immunophenotype is designated AML or ALL [200]. Some infant leukemias express antigens characteristic of both lymphoblasts and monoblasts, and are sometimes designated acute biphenotyic leukemias. Infants diagnosed with ALL harboring an 11q23 rearrangement have a particularly poor prognosis. Recently it has been found that the receptor tyrosine kinase *FLT3* is highly expressed in *MLL*-rearranged ALL as compared with other acute leukemias [46].

30.4.1.3 B-ALL with t(12;21)(p13;q22); *ETV6-RUNX1* (*TEL-AML1*)

ETV6 (TEL) is an *ETS*-like gene on 12p12 that is a transcription factor. The t(12;21) results in a chimeric gene comprised of the *ETV6* gene and the *RUNX1 (AML1)* gene at 21q22. The chimeric protein contains the bHLH domain of *ETV6* fused to the DNA-binding subunit of the RUNX1/CBFP transcription-factor complex [201]. Although this translocation is detectable by routine cytogenetic studies in less than 5 % of pediatric B-ALL cases, cryptic *ETV6-RUNX1*fusions have been found using RT-PCR methods in 22–27 % of pediatric patients, representing the most frequent genetic lesion in childhood B-ALL [201]. *ETV6-RUNX11* is associated with an excellent prognosis, with event-free survival rates approaching 90 % [201–203]. The favorable prognostic impact of *ETV6-RUNX1* is independent of age and leukocyte count [202].

Epidemiologic studies have identified the *ETV6-RUNX1* translocation in neonatal blood spots indicating that the translocation is present in blood cells at birth, up to 5–10 years before the development of leukemia [11]. These data suggest that the *ETV6-RUNX1* translocation is an initiating event in this leukemia. Most, but not all, 12p cytogenetic abnormalities in childhood B ALL involve *ETV6* [204]. These cases are associated with a non-hyperdiploid DNA content and a favorable prognosis [204].

30.4.1.4 B-ALL with t(5;14)(q31;q32); *IL3-IG*

A small subset of B-ALLs is associated with eosinophilia and carries the t(5;14)(q31;q32) [205]. The chromosome 5 breakpoints are tightly clustered within the promoter region of the *interleukin-3(IL-3)* gene, whereas the chromosome 14 breakpoints occur within *IgH* [205]. The translocation results in the head-to-head juxtaposition of *IL-3* with the *IgH* gene-joining region. In all likelihood, the translocation results in overexpression of IL-3, inducing eosinophilia and stimulation of the leukemic clone, presumably via an autocrine loop.

30.4.1.5 B-ALL with t(1;19)(q23;p13.3); *TCF3-PBX1* (*E2A-PBX1*)

The t(1;19) is found in approximately 30 % of B-ALLs [206]. It is most prevalent in leukemias with a pre-B cell (cytoplasmic Ig μ positive) immunophenotype. This translocation juxtaposes the *PBX1* gene on chromosome lq23 with the *TCF3* gene on chromosome 19p13 [206]. The *PBX1* gene is a member of the homeobox gene family. The *TCF3* gene encodes an enhancer-binding transcription factor [207]. *TCF3* contains the basic helix-loop-helix (bHLH) domain responsible for sequence-specific DNA binding and dimerization, and plays a critical role in lymphocyte development [208]. Breakpoints are clustered allowing for PCR-based methods to be used for detection [207].

30.4.1.6 B-ALL t(17;19)(q22;p13); *HLF/ TCF3*

In the t(17;19) the *HLF* (hepatic leukemia factor) gene located on chromosome 17 is juxtaposed with the *TCF3* gene on chromosome 19p13 [209]. The translocation creates a fusion gene that encodes for a chimeric protein containing the basic region/leucine zipper domain of the HLF protein fused to the transactivation domain of the TCF3 protein.

30.4.1.7 Hyperdiploid B-ALL

Childhood precursor B-ALL patients with a hyperdiploid karyotype (defined as >50 chromosomes or a DNA index ≥1.16) have a favorable prognosis, presumably because their disease is more responsive to chemotherapy [210]. These tumors show non-random gains of chromosomes X, 4, 6, 10, 14, 17, 18, and 21. Studies have shown that trisomy of both chromosomes 4 and 10 identify a subgroup of pediatric patients with an extremely favorable 4-year event-free survival and are likely to be cured with antimetabolite-based chemotherapy [211]. Increased gene copy numbers on these chromosomes may contribute to leukemogenesis and/or enhance responsiveness to chemotherapy.

Gene expression studies have shown that hyperdiploid cases represent a separate genetically defined subset of B-ALL [212]. Activating mutations of *FLT3* have been identified in approximately 20 % of cases of hyperdiploid B-ALL [213, 214]. *CREB-binding protein* (*CREBBP*) mutations, mostly in the HAT domain, have been identified in about 50 % of cases [215].

30.4.1.8 Hypodiploid B-ALL

About 2–3 % of B ALL cases exhibit hypodiploidy, and these patients have a poor prognosis. Hypodiploidy is defined as less than 44 chromosomes and can be further broken out into two subgroups: near haploid with 24–31 chromosomes and low-hypodiploid with 32–39 chromosomes with each subgroup distinct at the molecular level [216].

Near haploid B-ALL cases commonly have mutations in *NF1*, *IKZF3*, and other genes that activate the RAS pathway. Low-haploid B-ALL cases are often associated with *TP53* mutations. These mutations may be sporadic or *germ line* with the latter group a part of Li–Fraumeni syndrome [216].

30.4.2 T-Cell ALL

T-cell ALLs can be subclassified based on the presence of cytoplasmic or membrane expression of CD3 [217] as follows: TI—immature subgroup or pro-T-ALL defined by the expression of only CD7; TII—pre-T-ALL expressing CD2 and/or CD5 and/or CD8; TIII—or cortical T-ALL expressing CD1a; and TIV—mature T-ALL expressing surface CD3 and lacking CD1a. Depending on the mutually exclusive membrane expression of αβ or γδ TCR, two subgroups, group A and group B, are distinguished.

In a subset of early-thymic T-ALLs, only rearrangements of *TCR-δ* or both *TCR-δ* and *TCR-γ* are identified with the *TCR-β* in the germ line configuration [189]. In the remaining early-thymic cases, and all of the mid-thymic and late-thymic ALLs, each of the *TCR* are rearranged. *TCR-δ* is often deleted after rearrangement. Although *TCR-α* is difficult to analyze due to its large size, *TCR-α* gene transcripts have been detected in late-thymic T-ALLs using Northern-blot hybridization. These findings suggest a hierarchy of gene rearrangements in normal developing T lymphocytes: *TCR-δ* rearrangement occurs first, followed by rearrangement of *TCR-γ* and *TCR-β*, followed by rearrangement of *TCR-α*. Lineage infidelity occurs frequently in T-ALLs; *IgH* rearrangements occur in about 20 % of cases. However, *Ig light chain* rearrangements are rare.

A number of nonrandom chromosomal translocations involving candidate proto-oncogenes have been described in T-ALLs. Tumor-suppressor genes also may be involved. Some of the more common abnormalities are discussed here.

30.4.3 Early T-Cell Precursor T-ALL

Early T-cell precursor (ETP) T-ALL represents a clinically aggressive subset of tumors with the following immunophenotype: cytoplasmic CD3+, CD5 dim+ or negative, myeloid antigens+, and both CD1a and CD8 negative. Early T-cell precursor T-ALL accounts for about 12 % of all pediatric T-ALL cases [218]. Cytogenetic studies found that these patients often show 5q, 13q, and 11q chromosomal deletions, and in contrast to the other T-ALL subtypes, usually do not have 9p deletions involving the *CDKN2A/B* tumor suppressor genes. Gene expression profiling studies have shown that ETP T-ALL exhibits prominent overexpression of *LYL1* and *LMO2* [219].

At the molecular level, ETP T-ALL appears to be quite heterogeneous [220]. Multiple chromosomes show structural alterations and a number of gene or pathway abnormalities have been identified in subsets of ETP T-ALL [221, 222]. Major groups of alterations in ETP T-ALL include: loss of genes that regulate hematopoietic development, such as *ETV6*, *GATA3*, *IKZF1*, and *RUNX1*; overexpression of stem cell genes, such as *BAALC*, *WT1*; gene mutations in *NRAS* or *KRAS* that activate the RAS pathway; mutations involving the cytokine receptors *JAK1*, *JAK3,* and *IL7R*), and inactivating mutations of genes involved in epigenetic regulation (e.g., *EZH2*, *MLL, DNMT3A*, *CREBBP*). *FLT3* mutations are also frequent and *NOTCH1* mutations are rare in ETP T-ALL, unlike other types of T-ALL [221, 222].

The profile of gene mutations in ETP T-ALL is closer to acute myeloid leukemia that it is to other types of T-ALL and the gene expression profile of ETP T-ALL supports this idea, being more similar to hematopoietic stem cells or early myeloid precursors than to T-lymphoblasts [221, 222].

30.4.3.1 T-ALL with *TAL-1* Rearrangements

The *TAL-1* gene is rearranged in 25–30 % of T-ALLs and is much more common in childhood than in adult T-ALL cases [223, 224]. *TAL-1* is located at chromosome 1p32. It encodes a protein with a bHLH domain that is a member of a family of transcription factors involved in growth regulation and differentiation. The *TAL-1* gene is not normally expressed in T-cells [224].

In most cases with *TAL-1* rearrangements, small interstitial deletions in the *TAL-1* locus result in the joining of the *TAL-1* gene with a gene known as *SIL* that is normally expressed in T cells. This joining is thought to be due to illegitimate recombination mediated by the VDJ recombinase mechanism using heptamer-nonamer-like sequences flanking each gene [225]. In these cases, no abnormalities are detectable by standard cytogenetic analysis. In a small subset of cases the t(1;14)(p32;11) is present. This translocation places the *TAL-1* gene in continuity with the *TCR-δ enhancer*. A similar translocation, the t(1;7)(p33;q35), places the *TAL-1* gene in continuity with the *TCR-β enhancer*. Gene rearrangements involving *TAL-1*, both deletions and translocations, are identifiable by Southern blot or PCR analyses. This group of T-ALLs also has a distinct gene expression profile.

30.4.3.2 T-ALL with del(9)(p21-22) *CDKN2B/CDKN2A*

Deletions of chromosome 9p21-p22 occur in a subset of T ALLs with a frequency of up to 50 % in childhood cases [226]. Recent studies have identified several closely related candidate tumor-suppressor genes at this locus: two cell-cycle inhibitors, *CDKN2A* (p16) at 9p21 and *CDKN2B* (p15) located 12 kb centromeric to the p16*INK4A* [226]. The *CDKN2A* and *CDKN2B* genes encode 16 and 15 kDa proteins, respectively, that inhibit CDK4 and CDK6. CDK4 and CDK6 are involved in regulating cell-cycle progression from the G_1 to S phase, and their inhibition provides a cell with a growth advantage [226].

Using FISH, homozygous (and less frequently heterozygous) *CDKN2A* locus deletions have been identified in T-ALLs [226, 227]. *CDKN2A* deletions have been identified in most cases with 9p21-p22 deletion as shown by conventional cytogenetics as well as in cases in which conventional cytogenetics are normal. The *CDKN2B* locus has a similar frequency of homozygous deletions in T-ALLs, although this locus has been analyzed less commonly. *CDKN2A* and *CDKN2B* deletions correlate with a poorer survival [210, 228].

The breakpoints in *CDKN2A* have been found to occur in two clusters (bcrα and bcrβ) that are located near heptamers whose sequences resemble those involved in VDJ recombination [228]. In one study, all T ALL cases also contained short deletions, GC-rich tandem nucleotide additions, or clone-specific junctional sequences in *CDKN2A*. These combined findings suggest tumor-suppressor gene inactivation by an illegitimate VDJ recombinational mechanism.

30.4.3.3 T-ALL with t(10;14)(q24;q11) or t(7;10)(q34;q24)

Approximately 5–10 % of T-ALLs carry the t(10;14) involving the *T-cell leukemia homeobox 1 (TLX1) previously known as homeobox 11(HOX11)* gene on chromosome 10q24. T-ALLs with the t(7;10)(q34;q24) also involve the *TLX1* gene. *TCR-α/δ* and *TCR-β* are located at 14q11 and 7q34, respectively. In adults, HOX11 was expressed by 33 % of T-ALLs, compared with only 3 % of neoplasms in children [229]. The *HOX11* gene is a homeobox gene involved in transcriptional regulation [230]. It is not normally expressed in T-cells but is expressed in the adult liver and fetal spleen of animals [230]. Overexpression of this gene in the thymus of transgenic mice appears to correlate with the development of T-cell neoplasms. In most cases, the breakpoint on chromosome 10 is upstream of *HOX11*, placing it under the influence of a TCR enhancer, thereby resulting in deregulation and increased expression of the normal HOX11 protein [230]. HOX11 expression in T-ALL is associated with a favorable prognosis in both children and adults treated with modern intensive therapy [219, 231]. Forced expression of HOX11 in murine BM gives rise to T-ALL-like malignancies after long latency intervals suggesting that additional mutations are required.

The *TLX3*/*HOX11L2* gene, located at chromosome 5q35, can be activated by translocation near the *BCL11B* locus as a result of t(5;14)(q35;q32), or by fusion to the *TCR-δ* locus as a result of t(5;14)(q35;q11). Although t(5;14)(q35;q32) and t(5;14)(q35;q11) are commonly not recognized by conventional cytogenetic techniques, almost 20 % of childhood and 13 % of adult T-ALL patients demonstrated a *HOX11L2* gene translocation by FISH [232]. Rare variants have been reported: t(5;14) that involves *NKX2-5*, another homeobox gene instead of *TLX3* [233]; t(5;7)(q35;q21) involving *TLX3* and *CDK6* [234] and t(5;14)(q35;q11) juxtaposing *TLX3* and *TCR α/δ* genes [235]. *TLX3* is very similar to *TLX1* and microarray studies indicate that *TLX1*+ and *TLX3*+ T-ALLs cluster together, suggesting common mechanisms of action [219, 236]. Patients with *TLX3* expressing T-ALL may not have the favorable outcome reported for patients with *TLX1*+ cases [219, 237].

30.4.3.4 T-ALL with t(10;11)(p13;q14)

The t(10;11)(p13;q14) is a recurrent translocation in T ALL, but it is also found in a wide spectrum of hematologic malignancies [238, 239]. The t(10;11) fuses *CALM* (clathrin assembly protein-like lymphoid-myeloid leukemia gene, also called *PICALM*) to *AF10* (also called *MLLT10*). *CALM-AF10* fusion is detected in 9–10 % of T-ALL and is restricted to cases expressing γδ TCR [240]. The translocation is often cryptic and requires molecular detection by either FISH or RT-PCR. Several different fusion transcripts have been described. AF10 breakpoints correlate with stage of maturation arrest: the more 5′ breakpoints are associated with mature γδ T-ALLs and the more 3′ breakpoints are associated with immature γδ T-ALLs [240]. These findings suggest a possible role for AF10 protein in γδ TCR lineage commitment at the early stages of hematopoietic differentiation. Prognosis is globally poor, particularly for the most immature cases [240].

Gene expression studies have disclosed upregulation of the *HOXA5*, *HOXA9*, and *HOXA10* genes and their cofactor *MEIS1* [236, 241]. This finding strongly indicates common oncogenic pathways for T-ALL with either *CALM-AF10* or *MLL* translocations. Unique to *CALM-AF10* is the overexpression of *BMI1*, a gene located at 10p12.3 in close vicinity to *AF10*. *BMI1* is essential for self-renewal of normal and leukemic stem cells [242]. *BMI1* controls cell proliferation by inhibiting the *CDKN2A* locus and is thus an alternative to deletion of the *CDKN2A* locus observed in 70 % of T-ALL cases, but is not present in *CALM-AF10* T-ALLs.

30.4.3.5 NOTCH-1 Mutations in T-ALL

The *NOTCH-1* gene was initially discovered by its involvement and activation in the rare t(7;9) in T-ALL [243] and activated *NOTCH-1* was shown to induce T-ALL in mouse models [244, 245]. *NOTCH1* is an important player in T-cell commitment decisions of the common lymphoid precursor [246] and in the assembly and signaling of the TCR in immature thymocytes [247]. *NOTCH1* may also play a role in differentiation by controlling TCF3/E2A protein turnover [248]. *NOTCH1 activating* mutations have been found in the heterodimerization domain (HD) domain and the PEST domain in ~60 % of T-ALL of all molecular subtypes [248]. Mutations in the HD result in ligand independent intracellular NOTCH1 (ICN) production; mutations in PEST extend the half-life of ICN transcription activator complex. Combined HD and PEST mutations have been found in ~15 % of cases and shown to have a synergistic effect on NOTCH1 activation [248].

The finding of *NOTCH1* mutations in all molecular subtypes of T-ALL suggests that these mutations occur in immature progenitors. When several T-cell leukemia cell lines were tested with a drug known to inhibit gamma-secretase, cell-cycle arrest was detected, which was subsequently proven to be *NOTCH-1* specific [248]. The mechanism of *NOTCH1* transforming activity is still unclear and probably results from the deregulation of its normal functions during T-cell development, and its role in maintenance of self-renewal capacity of stem cells [249].

30.4.3.6 T-ALL Associated with Translocations Involving TCR Loci

Other translocations have been identified in T-ALLs by conventional cytogenetics, often involving the TCR loci: TCR-α/δ at 14q11, TCR-β at 7q34, and TCR-γ at 7p15. These translocations, present in small subsets of T-ALL, involve known or candidate proto-oncogenes brought into continuity with an antigen-receptor locus, resulting in inappropriate oncogene overexpression. Rearrangements joining the candidate oncogene *TCL1* on chromosome 14q32 to the TCR locus at l4q11 have been described in acute as well as in chronic T-cell leukemias arising in ataxia telangiectasia [250].

The t(7;9)(q34;q32) activates the *TAL-2* gene, a transcription factor with a bHLH motif bearing great sequence homology to *TAL-1*. The *TAL-2* gene is located on chromosome 9q32, and t(7;9)(q34;q32) juxtaposes *TAL-2* next to sequences from the *TCR-β* locus on chromosome 7q34. The t(7;19)(q34;p13) is another translocation in T-ALL that involves the *LYL-1* gene at chromosome 19p13. The *LYL-1* gene encodes another member of this subgroup of bHLH transcription factors that is inappropriately altered. The t(7;19)(q34;p13) results in its head-to-head juxtaposition of *LYL-1* with the *TCR-β* locus and truncation of its mRNA.

30.4.3.7 T-ALL with t(11;14)(p15;q11) or t(11;14)(p13;q11)

The genes encoding the LIM domain only proteins *LMO1* (*RTBN1*, *TTG-1*) and *LMO2* (*RTBN2*, *TTG-2*) located on chromosome 11p15 and 11p13, respectively, are rearranged in T-ALL [251]. Most common are the t(11;14)(p15;q11) and t(11;14)(p13;q11) juxtaposing *LMO1/LMO2* to the *TCR α/δ* locus due to erroneous VDJ joining [252]. The frequency of translocations in T-ALL with *LMO1* is less than 1 %, whereas it may be nearly 7 % for *LMO2* [251]. These genes are not normally expressed in T-cells. The LMO1 and LMO2 proteins are members of a subset of nuclear-localizing zinc-finger proteins containing cysteine-rich LIM domains and likely function as regulators of transcription [252]. *LMO2* has been demonstrated to be essential in erythropoiesis using homologous recombination in mice [253]. Dysregulation may occur either by putting the gene under the control of the TCR-δ enhancer or, in those cases lacking this enhancer on the translocated allele, by disturbing the negative regulatory region of the TCR-δ gene promoter [254]. The incidence of T-cell malignancies in transgenic mice is tied to the level of transgene expression.

Translocations involving *TCR-β* and *LMO1* or *LMO2* loci also have been reported [255]. Abnormal expression of LMO1/2 has been found in 45 % of T ALL cases, even in the absence of typical chromosomal changes [256], but often in association with deregulation of LYL1 (LMO2) or TAL1 (LMO1 and 2). Aberrant activation of LMO2 via retroviral integration has been reported recently in two T-cell lymphoproliferative disorders in children participating in a gene therapy trial of X-linked severe combined immunodeficiency [256].

30.4.3.8 T-ALL with 11q23/MLL Rearrangements

MLL/KMT2A rearrangements are found in 4–8 % of T-ALL [257]. The preferential partner is *ENL* (*MLLT1*). Translocation (11;19)(q23;p13.3) encoding *MLL-ENL* is often found in young adolescents and carries a better prognosis than other *MLL* translocations [257]. Other partners (*AF10*, *AF6*, *AF4*, *AFX1*) are occasionally seen. Gene expression profiling analysis has shown increased expression of a subset of *HOX* genes (*HOXA9*, *HOXA10*, *HOXC6*) and of *MEIS1*, the *HOX* gene co-regulator. This appears to be a central mechanism of leukemic transformation by MLL-oncoprotein in T-ALL [199]. These neoplasms represent a distinct molecular subtype with a specific expression profile characterized by differentiation arrest at an early stage of thymocyte development and commitment to γδ lineage [199, 236].

30.4.3.9 T-ALL with t(7;9)(q34;q34.3)

The t(7;9)(q34;q34.3) occurs in up to 5 % of T-ALL. This translocation juxtaposes sequences from the *TAN1* gene on 9q with the TCR-β locus on 7q34, leading to inappropriate

expression of a truncated gene product [243]. The *TAN1* gene is a mammalian homolog of the Drosophila *NOTCH* gene that encodes a transmembrane protein [243, 258]. Normally, TAN1 is a membrane protein involved in signal transduction during cellular differentiation. The abnormal *TAN1* protein resulting from the translocation aberrantly localizes to the nucleus and induces T-cell neoplasms when introduced into the BM of mice [243].

30.4.3.10 T-ALL with inv(14)(q11q32.3)

The inv(14)(q11q32.3), commonly found in ataxia-telangiectasia, also has been reported in T-ALL. This inversion arises from interlocus VDJ recombination and may signify a propensity towards abnormal recombinational activity rather than being oncogenic in-and-of itself.

30.4.3.11 *ABL1* Gene Rearrangement in T-ALL

The Philadelphia chromosome is detected in ≤1 % cases of T-ALL [259]. *ABL1* is a ubiquitously expressed cytoplasmic tyrosine kinase that has been shown to play a role in TCR signaling [260]. The *NUP214-ABL1* fusion is specific to T-ALL and found in up to 6 % of cases [261]. The fusion gene is found on amplified episomes, which are not visible cytogenetically, or only exceptionally in homogeneously staining regions [294]. FISH or RT-PCR studies are required to detect the *NUP214-ABL1*. These leukemias are characterized by ectopic expression of *TLX1* (*HOX11*) or *TLX3* (*HOX11L2*) and deletion of CDKN2A locus [261, 263]. Other *ABL* fusion variants have rarely been reported. Like *BCR-ABL*, *ETV6-ABL* resulting from t(9;12)(q34;p13) is found in CML, B-ALL and exceptionally in T-ALL. The t(9;14)(q34;q32) encoding a *EML1-ABL* fusion protein has been described in a single T-ALL case [264].

30.4.4 Other Abnormalities in T-ALL

In T-ALL with a relatively mature immunophenotype (CD2-, CD4+), *HOXA* genes (especially *HOXA10* and *A11*) can be upregulated as a consequence of cryptic inv(7) or t(7;7), which juxtaposes the *TCR-β* enhancer within the *HOXA* locus [265]. FISH analysis disclosed these rearrangements in up to 5 % of cases. Expression-array analysis of *HOXA*+ T-ALL precursor indicates arrest after lineage choice but before β selection [236]. Another recurrent aberration in T-ALL is del(6q). The common deleted region is of variable size but is focused around 6q16. The target gene(s) have not yet been formally identified [266].

To date, T-ALL has received less attention than B-ALL in studies using whole genome sequencing, RNAseq, and other high-throughput methods. T-ALL cases usually have multiple gene mutations, in the range of 5–10 in children and ~20 in adult cases. In addition to genes already mentioned, mutations have been reported in *PHF6* (~40 %), *FBXW7* (~20 %), *DNMT3A* (~18 %), *RUNX1* (~15 %), *CNOT3* (~10 %), *RPL5* (~10 %), *RPL10* (~10 %), and *PTEN* (~10 %). *FLT3* mutations are rare in T-ALL, but more common in neoplasms with stem cell features [267, 268]. Activating mutations of *N-RAS* have been detected in 10 % of a series of pediatric T-ALL [269]. Other studies indicate that *RAS* is highly activated in 50 % of T-ALL [270] suggesting a key role for *RAS* activation in T-ALL pathogenesis.

30.5 Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) primarily affects adults and is distinguished by neutrophilic leukocytosis, basophilia, eosinophilia, anemia, and splenomegaly, among other findings [271]. The bone marrow at diagnosis is usually hypercellular with granulocytic hyperplasia. The disease course is an continuum from more indolent to aggressive disease that frequently entails three phases: (1) an initial chronic phase that lasts 3–4 years; (2) a transitional accelerated phase that lasts approximately 6 months in which there is a progressive left shift in myeloid maturation, often associated with new cytogenetic abnormalities; and (3) blast phase or crisis characterized by a proliferation of myeloid, lymphoid, or less commonly megakaryoblastic or erythroid blasts.

Chronic myelogenous leukemia is a monoclonal neoplasm in which 90–95 % of cases show cytogenetic evidence of a distinct chromosomal abnormality, a small derivative chromosome 22, designated the Philadelphia chromosome (Ph), and subsequently shown to be the t(9;22)(q34;q11) (Fig. 30.15). The t(9;22)(q34;q11) disrupts the *ABL1* and *BCR* genes and forms a novel fusion gene, *BCR-ABL1*. This disease is now defined by the presence of *BCR-ABL1* and, as a result, the definition of CML has been extended to include rare morphologic variants, once not recognized as CML, as well as cases that lack cytogenetic evidence of the Ph, as long as *BCR-ABL1* is present (shown by other methods).

30.5.1 *ABL1*

The *ABL1* gene is a cellular homolog of the transforming sequence of *Abelson murine leukemia virus* (v-*ABL*), located on chromosome 9q34. *ABL1* spans over 230 kb. By convention the exons of *ABL1* are numbered lb, la, and a2-a11 from 5′→3′ (Fig. 30.14). The 5′-exons (1b and 1a) of *ABL1* undergo alternative splicing to exon a2. This normally yields 6 kb and 7 kb mRNA transcripts that encode two alternative proteins, type la and 1b $p145^{abl}$ that differ in their N-terminal amino acid sequence. The normal *ABL1* resides, at least in part, in the nucleus and contains the regulatory *src*-homology domains SH2 and SH3, a tyrosine kinase domain, a DNA-

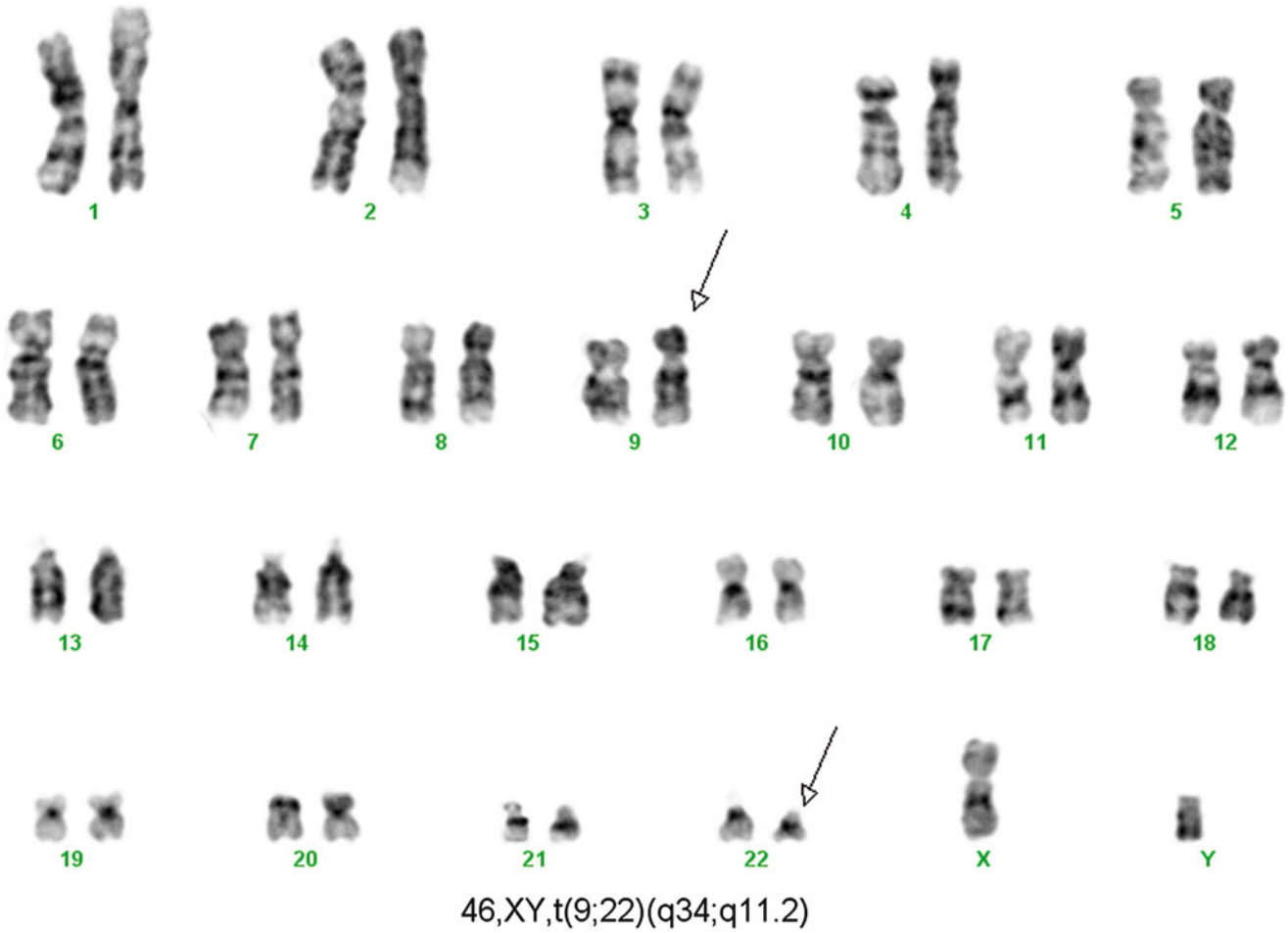

Fig. 30.15 Karyotype of a case of chronic myelogenous leukemia in chronic phase. Conventional cytogenetic analysis demonstrates the t(9;22). GTG banding technique.

binding domain, and actin-binding domains. In CML, the breakpoint on chromosome 9 can occur over a 300 kb segment of the *ABL1* gene, upstream of exon 1b, between exons lb and la, or between exons 1b and a2.

30.5.2 *BCR*

The *BCR* locus is located on chromosome 22q11 (Fig. 30.16). The breakpoints on chromosome 22 are localized to a 5.8 kb region, originally called the breakpoint-cluster region or *BCR*. It is now referred to as the major breakpoint-cluster region (*M-BCR*) because two additional breakpoint-cluster regions have since been described. The *M-BCR* is part of a larger gene on chromosome 22, known as *BCR*. It encompasses exons e12-e16 of *BCR* that historically are referred to as M-*BCR* exons bl-b5. In its entirety, the *BCR* gene consists of 21 exons that stretch over a region of 130 kb. It encodes a protein ($p160^{bcr}$) with dimerization, phosphoserine/threonine-rich SH2 binding, *DBL*-like (a guanine-nucleotide-releasing factor for the GTP-binding protein CDC24M), and GTPase activating protein for $p21^{RAC}$ (GAP^{RAC}) domains. The subcellular localization of BCR protein shifts from being predominantly cytoplasmic in interphase cells to being largely perichromosomal during mitosis. The overall features suggest that BCR protein may be involved in signal transduction pathways and cytoskeletal/chromatin organization. The *BCR* breakpoints in CML most frequently arise between M-*BCR* exons b2 (e13) and b3 (e14), or between M-*BCR* exons b3 (e14) and b4 (e15). A breakpoint either 5′ or 3′ of M-*BCR* is uncommon, occurring in less than 5 % of cases.

30.5.3 *BCR-ABL1* and $p210^{BCR-ABL1}$

The aforementioned translocations result in an aberrant fusion genes, *BCR-ABL1* located on the derivative chromosome 22 (Ph), and *ABL1-BCR* on the derivative chromosome 9, respectively. Despite the absolute position of the *ABL1*

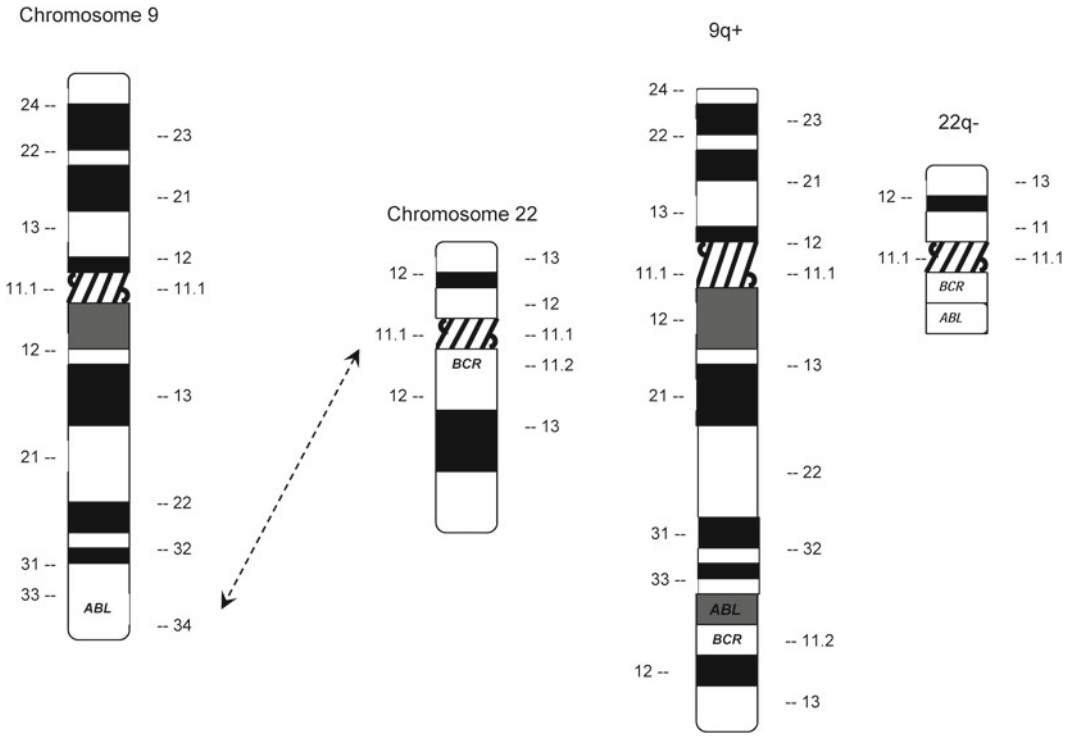

Fig. 30.16 Schematic representation of the t(9;22)(q34;q11) in chronic myelogenous leukemia. Compare the normal chromosomes (*left set*) to the derivative chromosomes (*right set*). The t(9;22) is a balanced and reciprocal translocation involving the *ABL1* gene at 9q34 and the *BCR* gene at 22q11. The derivative 22q- is known as the Philadelphia chromosome.

breakpoint, processing of primary *BCR-ABL1* transcripts generally produces a fusion mRNA with a e13-a2 junction, a e14-a2 junction, or occasionally both types due to alternative splicing [110]. Translation yields two similar $p210^{BCR\text{-}ABL1}$ fusion protein variants that differ only slightly in amino acid sequence without affecting clinical outcome. Regardless of which molecular species is produced, $p210^{BCR\text{-}ABL1}$ is uniformly expressed in CML, aberrantly localizes to the cytoplasm, and possesses enhanced tyrosine kinase activity and transforming capabilities [110]. Moreover, a CML-like syndrome can be induced in irradiated mice reconstituted with BM progenitor cells transfected with a retrovirus containing *BCR-ABL1* constructs [272].

BCR-ABL1 plays a crucial role in the initiation of CML. The *BCR-ABL1* oncogene encodes $p210^{BCR\text{-}ABL1}$ composed of N terminal *BCR* and C-terminal *ABL1* sequences. The *BCR* first exon is a critical component of the transforming capability exerted by *BCL-ABL1*. The serine/threonine kinase domain of BCR protein is able to bind to the SH2 regulatory domain of *ABL1* and other putative signal transduction proteins such as the SH2 domain of the GRB2 (for growth factor receptor binding protein 2) adaptor protein. Its coiled-coil oligomerization domain brings about tetramerization of *BCR-ABL1*. The net effect is to initiate constitutive tyrosine kinase activity and activate the F-actin-binding domain contributed by ABL1. The RAS-related signal-transduction proteins are likely substrates of $p210^{BCR\text{-}ABL1}$, resulting in disruption of these important signaling pathways [273]. For example, $p210^{BCR\text{-}ABL1}$ binds GRB2 and CRKL (CT10 regulator of kinase-like adapter protein), each of which can bind SOS (son of sevenless; a guanine-nucleotide releasing factor) and then activate the RAS signaling pathway [274]. Activation of F-actin binding probably leads to aberrant subcellular localization by promoting attachment to the cytoskeleton, thereby interfering with translocation to the nucleus. MYC protein also has been implicated in BCR-ABL1-induced transformation [275]. Other aspects concerning the pathogenesis of CML are beyond the scope of this chapter and are reviewed elsewhere [274].

30.5.4 Variant *BCR-ABL1* Transcripts

A minor breakpoint cluster region (m-BCR) that occurs upstream of M-*BCR* between exons el and e2 produces an ela2 transcript that encodes a smaller $p190^{BCR\text{-}ABL1}$ fusion protein. The m-BCR is involved in nearly two-thirds of Ph+ ALLs, but only rarely in CML or AML. The rare cases of CML associated with $p190^{BCR\text{-}ABL1}$ share certain characteristics with chronic myelomonocytic leukemia such as an absolute monocytosis and a decreased neutrophil to monocyte ratio.

A micro breakpoint-cluster region (μ-bcr) also has been found at the 3′ end of the *BCR* gene, between exons e19 and e20, that encodes a e1a2 transcript and a larger ($p230^{BCR\text{-}ABL1}$) fusion protein [276]. Rare cases associated with $p230^{BCR\text{-}ABL}$ show a greater degree of myeloid maturation and had been incorrectly characterized as chronic neutrophilic leukemia in the past [276]. It is now clear that $p230^{BCR\text{-}ABL}$ cases are a variant of CML and chronic neutrophilic leukemia is a clinicopathologic distinct entity.

30.5.5 *ABL1-BCR*

In contrast to *BCR-ABL1*, the reciprocal chimeric *ABL1-BCR* on derivative chromosome 9 encodes a product that has no known pathogenic consequence. Although the fusion transcript bas been detected in about 40 % cases of CML by RT-PCR, the ABL1-BCR protein has not been identified. It has been suggested that *ABL1-BCR* expression might account for some differences in presentation or clinical outcome

30.5.6 Accelerated Phase and Blast Crisis of CML

Additional cytogenetic changes often accompany progression of chronic phase CML into accelerated phase and are almost always present in blast crisis [271]. The most frequently observed cytogenetic abnormalities include trisomy 8, isochromosome 17, an additional Ph, or isochromosome 19. The t(3;21)(q26;q22), monosomy 7, and loss of chromosome Y occur less commonly [277]. At the molecular level, a variety of low-frequency acquired events have been reported in association with transformation, suggesting that maturation arrest may occur as a result of the interruption of several pathways. Gene amplification of *MYC* also has been implicated in progression of CML. Alterations of the *TP53* gene have been found in the myeloid and megakaryocytic variants of blast crisis, accounting for progression in approximately 25 % of CML patients. Rarely, translocations associated with specific types of AML, such as t(15;17)(q22;q21), t(8;21)(q22;q22), and inv(16)(p13q22), can occur at time of blast crisis.

Theories regarding the potential role of a second Ph, such as increased gene expression or a new *BCR-ABL1* rearrangement, are intriguing but the evidence is inconclusive. Homozygous deletion of the $p16^{INK4A}$ tumor-suppressor gene has been reported in up to 50 % of CML patients with lymphoid blast crisis [278]. Structural abnormalities of the *RB1* gene with loss of protein expression have been reported with an incidence of 11–29 % in lymphoid blast crisis of CML [279]. The apparent emergence of $p190^{BCR\text{-}ABL1}$ in $p210^{BCR\text{-}ABL1}$-positive CML patients also has been put forth as a putative mechanism of transformation. However, some reports indicate that low-level co-expression of $p190^{BCR\text{-}ABL1}$ with $p210^{BCR\text{-}ABL1}$ can occur due to alternative splicing and both transcripts are detectable by RT-PCR in most cases of CML with M-bcr breakpoints [280, 281].

The clinical course of CML patients was dramatically changed with the introduction of imatinib into clinical practice. Imatinib inhibits the BCR-ABL1 tyrosine kinase by competitive binding at the ATP-binding site [282]. In patients with newly diagnosed chronic-phase CML, treatment with imatinib results in a high complete cytogenetic remission rate and a high molecular remission rate with low or undetectable amounts of *BCR-ABL1* transcripts [283]. The high rates of complete cytogenetic response with imatinib necessitate molecular monitoring by quantitative RT-PCR to measure residual disease [284]. It was demonstrated that the reduction of BCR-ABL1 transcripts was associated with better prognosis.

With the success of imatinib therapy, which has dramatically extended the life of many CML patients, the problem of imatinib resistance has come to the forefront. This can be manifested as primary refractoriness to therapy or relapses after the patients has received therapy for a prolonged interval of time. Mutations in *ABL1*, most often involving the kinase domain, have been identified that explain, in part, imatinib resistance. Second and third generation tyrosine kinase inhibitors have been developed that are valuable in the treatment of CML patients at time of relapse, and upfront for some CML patients with high-risk disease. Dasatinib, nilotinib, and ponatinib are three examples of more recently developed drugs that have a role in managing CML patients. Other causes of imatinib resistance involve genomic amplification of *BCR-ABL1* or may be mediated through mechanisms independent of *BCR-ABL1* such as activation of the mTOR/Akt pathway, loss of functional *TP53*, or *SRC* activation [285, 286]. The utility of detecting mutations before imatinib therapy is not well established [287]. This is an active area of research and the reader referred to reviews discussing imatinib resistance in CML [288].

Sanger sequencing can detect *BCR-ABL1* mutations in approximately 50 % of CML patients resistant to imatinib, but not all mutations have the same effects. Certain mutations in the kinase domain, such as Y253F/H, E255K/V, T315I, or H396P/R, are highly imatinib resistant and usually indicate the need for alternative therapy, whereas patients with other mutations, such as M244V, M351T, or F359V, can benefit from an increased imatinib dose [289]. Various ABL1 mutations are more or less sensitive to either dasatinib or nilotinib; the T315I mutations is resistant to both drugs but sensitive to ponatinib [290].

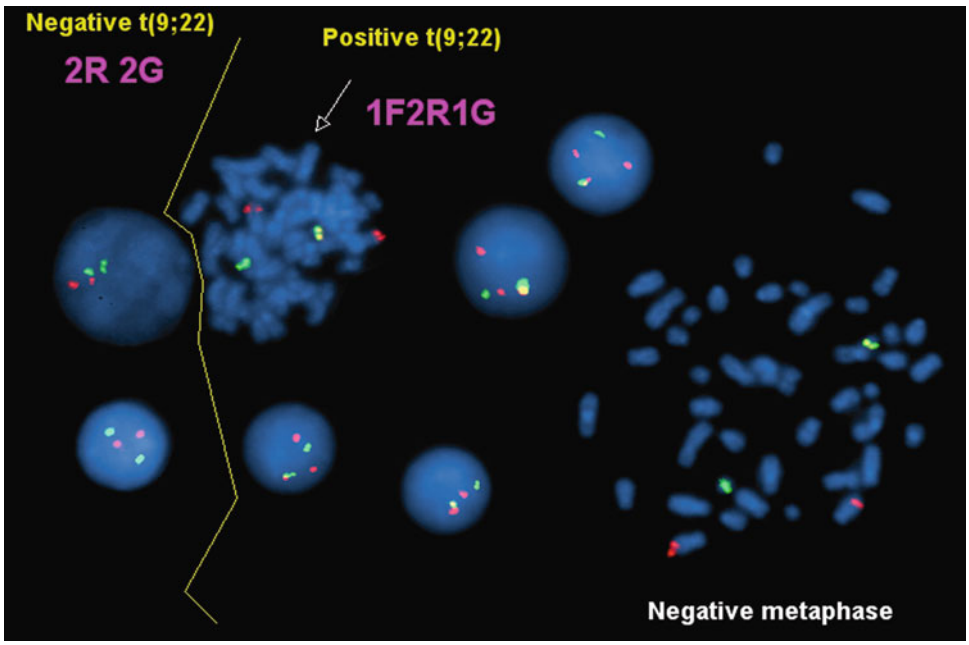

Fig. 30.17 Fluorescence in situ hybridization (FISH) analysis of a case of chronic myelogenous leukemia in chronic phase. *BCR/ABL1 ES* dual color probe (Vysis) in which the BCR probe is *green*. This probe spans approximately 300 kb beginning between *BCR* exons 13 and 14 (M-bcr exons 2 and 3). The *ABL1* probe is *red* and spans an area centromeric of the *ASS* gene to well telomeric of the last *ABL1* exon. In the absence of t(9;22), a cell has two *red* and two *green* signals. In the presence of t(9;22) and preserved *ASS* gene, a cell shows one *green* (native *BCR*), one large *red* (native *ABL1*), one smaller *red* (*ASS*), and one fused (*yellow*) signal pattern (*middle panel*). In a case of *ASS* deletion, an abnormal nucleus shows one *green*, one large *red*, and one *yellow* (fused) signal (not shown).

30.5.7 Molecular Methods for Diagnosis and Detection of Minimal Residual Disease in CML

The diagnosis of CML is most often made by morphologic analysis with conventional cytogenetic analysis showing the presence of a small derivative chromosome 22 (Ph) that is created as a result of t(9;22)(q34.q11). Approximately 95 % of all cases of CML have evidence of the Ph as detected by conventional cytogenetic analysis. Routinely 20 metaphases are examined and therefore the analytic sensitivity is about 5 %. Despite the availability of more sensitive methods, conventional karyotypic results have prognostic as well as diagnostic significance. Karyotypic results after therapy are divided into prognostic groups: complete response (no Ph+ metaphases); major response (1–34 % Ph+ metaphases), and partial response (35–90 % Ph+ metaphases). A complete response correlates with lower levels of BCR-ABL1 by quantitative RT-PCR and longer survival.

More sensitive techniques such as FISH (Fig. 30.17) or RT-PCR (Fig. 30.18) are also very popular because they permit assessment of minimal residual disease following therapy [291]. FISH routinely assesses 200 nuclei and unlike conventional cytogenetics does not require dividing cells. This method allows a semiquantitative determination of the percentage of nuclei positive for *BCR-ABL1* and has an analytical sensitivity of approximately 0.5 %. FISH studies have shown that not all *BCR-ABL1* translocations are identical in that variably sized deletions of chromosome 9 occur and have prognostic significance [292]. However, FISH is highly focused on detecting *BCR-ABL1* and cannot provide information about other chromosomal abnormalities that may be present.

Quantitative RT-PCR is the most sensitive method for detecting *BCR-ABL1* with an analytical sensitivity of at least 0.001 % or 1 positive cell in 100,000 negative cells. Most laboratories also measure a control gene that is present in near constant amounts in the cell and tumor burden is expressed as a *BCR-ABL1*–control ratio. Quantitative RT-PCR for *BCR-ABL1* transcript levels is the best approach to assess for minimal residual disease and assessment of serial specimens over time is an excellent means of assessing risk of relapse, permitting stratification of patients into risk groups.

Great efforts have been made to standardize qRT-PCR techniques by definiting an internal standard for *BCR-ABL1* quantification and an international scale for results [284]. The monitoring of *BCR-ABL1* transcript levels is important for early detection of imatinib resistance at the molecular level. Imatinib resistance is defined as lack or loss of complete molecular response [293]. Complete molecular response is defined as undetectable *BCR-ABL1* transcripts by

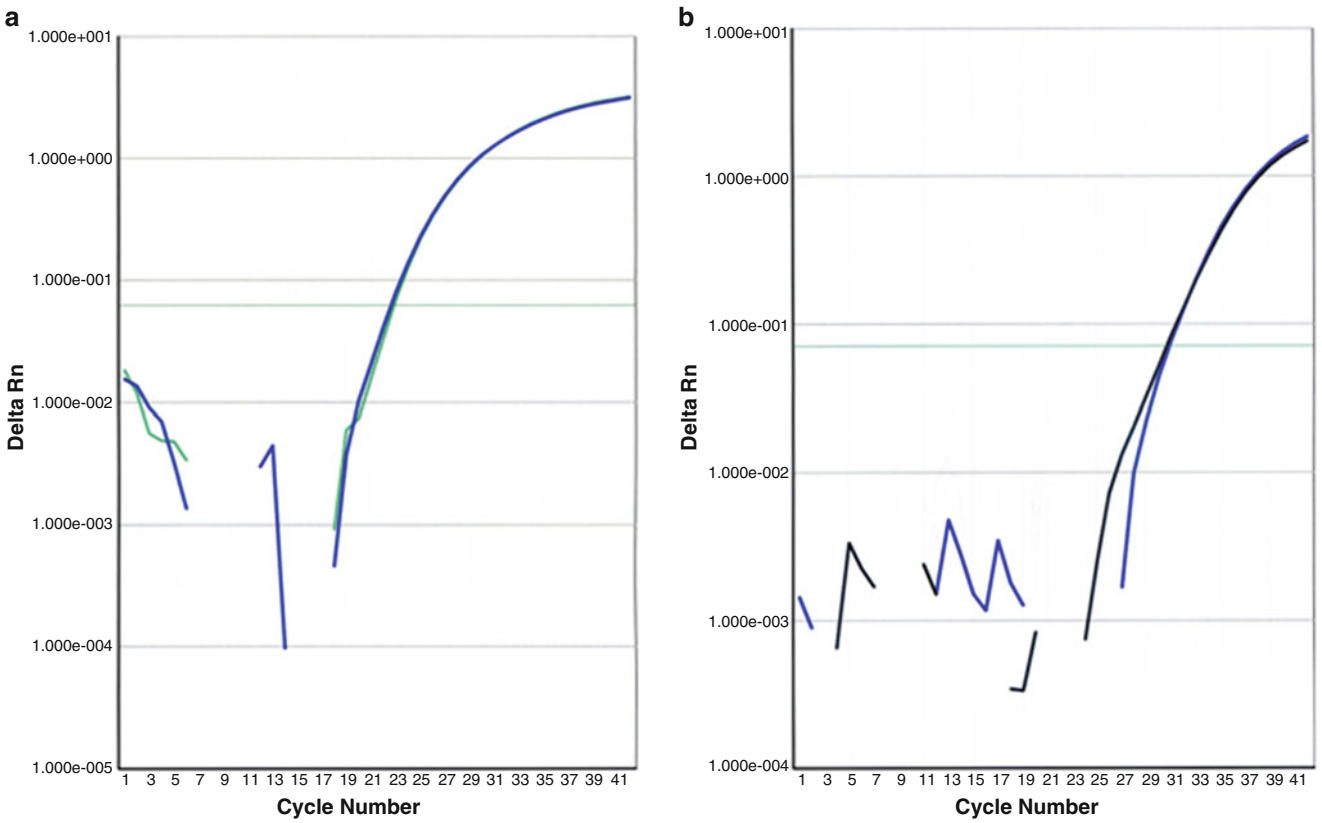

Fig. 30.18 Real-time PCR for BCR/ABL1. In addition to establishing diagnosis, this technique is widely used for monitoring the therapy response. A,B RT-PCR for *BCR/ABL1* in the same patient with CML before (**a**) and 3 months after (**b**) therapy with imatinib. Lower level of *BCR/ABL1* transcript is reflected in large number of cycles (31 versus 23) required to reach the positive threshold (*green line*). Note the different scale of the *y*-axis in two cases.

RT-PCR. Major molecular response is defined quantitatively as a >3 log reduction of *BCR-ABL1* transcripts or a *BCR-ABL1/ABL1* ratio of <0.1 % [294]. Molecular relapse, in complete cytogenetic responders, is considered to be an increase in *BCR-ABL1* transcript levels by a log of 0.5–1 [293]. The current recommendation is to judge the possibility of relapse after therapy based on at least two consecutive samples after more than one negative result [293].

30.6 Other Myeloproliferative Neoplasms and Myelodysplastic/ Myeloproliferative Neoplasms

In addition to CML, the current WHO classification lists a group of myeloproliferative neoplasms (MPN) including polycythemia vera, essential thrombocythemia, chronic idiopathic myelofibrosis, chronic neutrophilic leukemia, and chronic eosinophilic leukemia/hypereosinophilic syndrome. The MDS/MPN category includes chronic myelomonocytic leukemia, atypical chronic myeloid leukemia (*BCR-ABL1* negative), and juvenile myelomonocytic leukemia. These entities are defined based on a constellation of clinicopathologic and laboratory findings, and, unlike CML, some of these diseases lack unifying molecular abnormalities. However, a few important molecular abnormalities in these diseases have been identified.

30.6.1 *JAK2* Mutations

The Janus family of tyrosine kinases (*JAK1, JAK2, JAK3, TYK2*) are cytoplasmic tyrosine kinases that mediate signaling downstream of cytokine receptors [295]. Activation of the JAK-cytokine receptor complex results in JAK-mediated phosphorylation of substrate molecules, including STAT proteins that have critical roles in cellular proliferation, cell survival, and immune responses [296]. *JAK2V617F* is the most common mutation in cases of MPN. This mutation is found in virtually all patients with polycythemia vera (PV) and approximately half of those with essential thrombocythemia (ET) or chronic idiopathic myelofibrosis (CIMF) [297].

V617 resides within the pseudokinase, JH2 domain, a residue that is highly conserved between species [298]. Molecular modeling of JAK2 kinase suggests that the JH2 domain mutation lies adjacent to the site that stabilizes the activation loop of the kinase in its inactive conformation. A mutation at this point conceivably destabilizes this conformation, allowing the loop to flip into active conformation leading to constitutive activity of JAK2-cytokine receptor complex [299]. In addition to point mutations, *JAK2* can be altered by chromosome translocations, including *ETV6-JAK2* via t(9;12)(p24;p13), seen in T-ALL or B-ALL as well as MDS/MPN; *PCM1-JAK2* via t(8;9)(p22;p24) seen in eosinophilia-associated MDS/MPN, ALL, AML, and T-cell lymphoma; and *BCR-JAK2* via t(9;22)(p24;q11.2) seen in MDS/MPN [300, 301].

JAK2V617F is a G to T somatic mutation of *JAK2*, at nucleotide 1849 in exon 14, resulting in substitution of phenylalanine for valine at codon 617 [297]. Initially reported in PV, ET, and CIMF [297, 303–305], *JAK2V617F* mutation also has been described in MDS/MPN and MDS, although at a lower frequency [306, 307]. Some researchers express doubt that *JAK2V617F* is the initiating or even a mandatory event in MPN and MDS/MPN [308, 309]. Those concerns are supported by observations of *JAK2V617F*-negative leukemia clones arising from *JAK2V617F*-positive MPN [310].

Most recently, other *JAK2* mutations have been described in *JAK2V617F*-negative patients with PV and in idiopathic erythrocytosis; most of these cases demonstrate one of four exon 12 *JAK2* mutant alleles and induce cytokine-independent/hypersensitive proliferation in erythropoietin receptor-expressing cell lines and a PV-like phenotype in mice [311]. The four newly described exon 12 mutations, which include both in-frame deletions and tandem point mutations, appear to be specific to either PV or idiopathic erythrocytosis.

30.6.2 *CALR* Mutations

The *CALR* (*calreticulin*) gene, located at chromosome 19p13.3-p13.2, is a multifunctional protein located in the endoplasmic reticulum, cytosol, and cell surface of the cell. Calreticulin is important in calcium homeostasis, has a transport chaperone function, and is involved in cell adhesion. *CALR* mutations have been identified in about 25 % of ET and CIMF cases, in over 50 % of cases negative for *JAK2* mutation [312]. In most but not all studies, *CALR* and *JAK2* mutations have been mutually exclusive. The exact role of *CALR* mutations is not well established, but is likely involved in JAK-STAT signaling. In patients with ET, *CALR* mutations have been associated with a higher platelet count, lower hemoglobin level and leukocyte count, and a lower risk of thrombosis.

30.6.3 *MPL* Mutations

The *MPL* gene, located at chromosome 1p34, encodes a receptor for thrombopoietin, which is essential for megakaryopoiesis. *MPL* mutations have been described in familial thrombocytosis [313] and congenital amegakaryocytic thrombocytopenia [314]. Recently, somatic gain of function mutations involving codon 515 of the *MPL* gene (*MPLW515L* and *MPLW515K*) have been identified in approximately 5 % of CIMF patients and 1 % of ET patients [308]. *MPL* and *JAK2* mutations are not mutually exclusive [308].

30.6.4 *ASXL1* Mutations

The *ASXL1* (*additional sex combs-like transcriptional regulator 1*) gene, located at chromosome 20q11, is involved in regulation of the polycomb repressor complex and other functions. Mutations of *ASXL1* have been reported in about 6 % of cases of essential thrombocythemia (in addition to cases of MDS and AML) [315].

30.6.5 *CSF3R* Mutations

The *colony-stimulating factor 3 receptor (CSF3R)* gene, located on chromosome 1p35-p34.3, is mutated in about 60 % of cases of chronic neutrophilic leukemia and atypical CML. These mutations tend to cluster in distinct regions of the gene and result in downstream JAK activation [316].

30.6.6 *PDGFR* Mutations

Rearrangement of the *platelet-derived growth factor receptor α* (*PDGFRA*) gene, located on chromosome 4q12, most often occurs as a consequence of del(4)(q12) resulting in *FIP1L1-PDGFRA* fusion gene. *FIP1L1-PDGFRA* occurs in a very small subset of patients who present with the phenotypic features of both chronic eosinophilic leukemia (CEL) and systemic mastocytosis and predicts complete hematologic and molecular response to imatinib therapy [317, 318]. *PDGFRA* can also be activated through other mechanisms such as t(4;10)(q12;p11) resulting in an in-frame fusion gene *KIF5B-PDGFRA;* t(4;22)(q12;q11) forming a *BCR-PDGFRA* fusion gene, and ins(9;4)(q33;q12q25) forming *CDK5RAP2-PDGFRA* fusion gene [319, 320]. Clinically, patients with MPN associated with t(4:10)(q12;p11), t(4;22)(q12;q11), or ins(9;4)(q33;q12q25) present as CEL.

Rearrangements of *platelet-derived growth factor receptor α* (*PDGFRα*) gene, located on chromosome 5q31-32, are found in several cases of MPN, most of which are associated with eosinophilia. The examples include t(5;12)(q33;p13)

resulting in *ETV6-PDGFRα* fusion gene; t(5;17)(q33;p13) resulting in *RABAPTIN5-PDGFRβ*; t(5;17)(q33;p11.2) resulting in *HCMOGT-1-PDGFRα*; t(5;14)(q33;q24) resulting in *NIN-PDGFRα*; t(5;14)(q33;q32) resulting in *KIAA1509-PDGFRα*; t(5;15)(q33;q22) resulting in *TP53BP1-PDGFRα*; t(1;5)(q23;q33) resulting in *PDE4DIP-PDGFRα*; t(5;7)(q33;q11.2) resulting in *HIP1-PDGFR α;* t(5;10)(q33;q22) resulting in *H4-PDGFR α* [321–323].

30.6.7 Mutations Involving RAS Signaling Pathway

Mutations involving the RAS signal transduction pathway are most prevalent in juvenile myelomonocytic leukemia (JMML) [324]. JMML is often associated with monosomy 7 [325, 326]. Known hypersensitivity of myeloid progenitors in JMML to granulocyte-macrophage colony-stimulating factor (GM-CSF) [327] has recently been attributed to altered RAS signaling as a result of mutations affecting one of the pathway regulatory molecules such as *RAS*, *PTPN11*, and *NF1* [326, 328]. *PTPN11* mutations are found in approximately 30 % of patients with JMML, whereas NF1293 and RAS29 mutations have lower incidence of approximately 15–20 %; all three mutations appear to be mutually exclusive [326, 328].

RAS mutations are also found in other MPN or MDS/MPN including chronic myelomonocytic leukemia (CMML). *RAS* mutations in JMML often represent single nucleotide substitutions involving the P loop domain of either *KRAS* (approximately 15 %) [326, 329] or *NRAS* (approximately 30 %) [330] and have been shown to cause the disease phenotype in mice [331]. BRAF mutations have been reported in about 40 % of cases of CMML [332].

Neurofibromatosis type 1 (*NF1*) located on chromosome 17q11.2 is a tumor suppressor gene that encodes for a 327-kDa neurofibromin protein that inactivates RAS through acceleration of RAS-associated GTP hydrolysis. *Germ line NF1* mutations cause von Recklinghausen disease. Children with type 1 neurofibromatosis are predisposed to JMML [333] and demonstrate loss of heterozygosity of *NF1* in myeloid progenitor cells [334]. Primary leukemia cells from such patients accumulate RAS in the active GTP-bound state [335]. It was shown that *NF1* knockout mice developed an MPD-like disease with GM-CSF hypersensitivity [335, 336].

PTPN11 (*Protein-Tyrosine Phosphatase, Nonreceptor-type, 11*), located on chromosome 12q24.1, encodes for the nonreceptor protein tyrosine phosphatase, SHP-2, that transmits signals from growth factor receptors to RAS. *Germ line PTPN11* mutations are found in approximately 45 % of patients with Noonan syndrome, and these patients are at risk of developing JMML [336]. On the other hand, more than 30 % of patients with JMML demonstrate *PTPN11* mutations [328, 337]. In mice, retroviral transduction of *PTPN11* mutant alleles in bone marrow cells induces GM-CSF hypersensitivity and an MPD-like disease [338], which suggests an important role of *RAS* mutations in pathogenesis of JMML.

30.7 8p11 Myeloproliferative Syndrome

The 8p11 myeloproliferative syndrome (EMS) is also known as stem cell leukemia/lymphoma syndrome, or as myeloid and lymphoid neoplasms associated with FGFR1 abnormalities in the current WHO classification (Fig. 30.19). Patients with this entity most often present with a MPN or MDS/

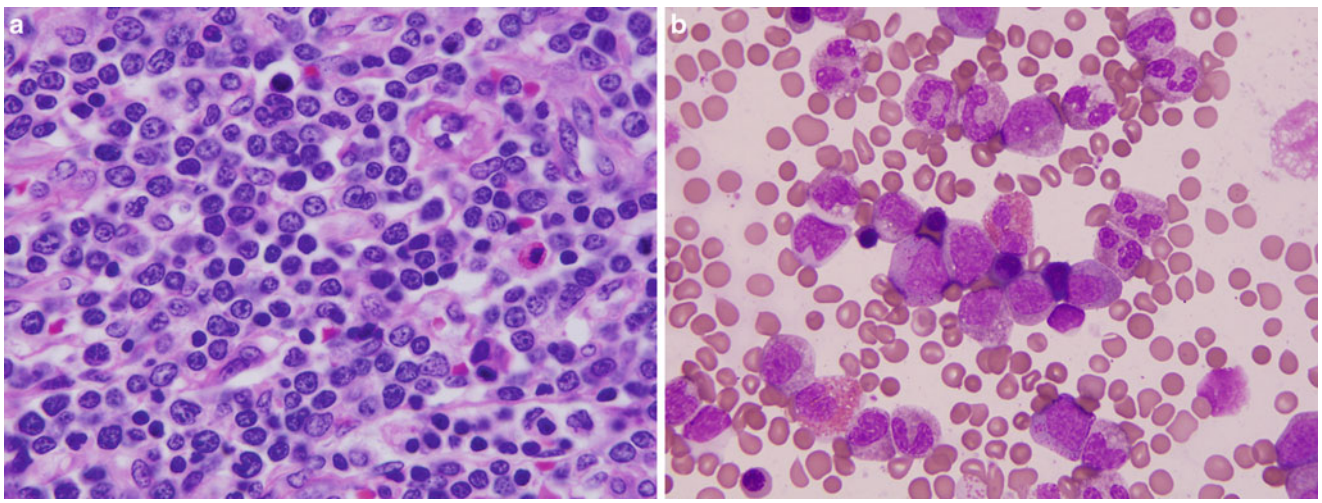

Fig. 30.19 A case of 8p11 myeloproliferative syndrome with t(8;13). (**a**) The lymph node demonstrates involvement by blastic neoplasm that most closely resembles precursor T-cell lymphoblastic lymphoma with minor myeloid foci. (**b**) The bone marrow is hypercellular and is consistent with a myeloproliferative syndrome (**a**, hematoxylin–eosin stains, 1000×; **b**, Wright-Giemsa, 1000×).

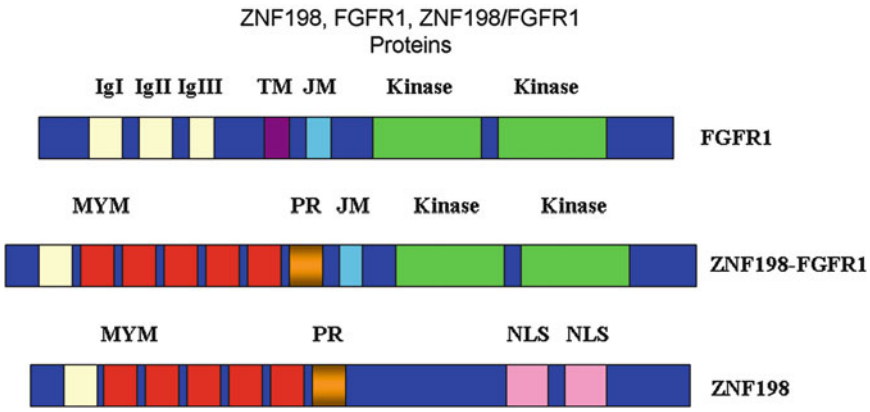

Fig. 30.20 Schematic representation of ZNF198 (ZMYM2), FGFR1, and ZNF198 (ZMYM2)/FGFR1 fusion proteins. The fusion is the result of t(8;13)(p11;q12), the most common translocation in 8p11 myeloproliferative syndrome.

MPN associated with eosinophilia [339]. Many patients develop an immature lymphoid neoplasm, most often classified as T ALL/LBL, although in some of these neoplasms there is evidence of mixed lineage [339]. Patients with EMS have a high risk of subsequent acute myeloid leukemia.

The fibroblast growth factor receptor 1 (*FGFR1*) at chromosome 8p11 is always involved. The most common partner is *ZMYM2* (previously known as *ZNF198)* at 13p11. The t(8;13) results in the joining of *FGFR1* with the novel *ZMYM2* gene on chromosome 13q11 [339]. The *ZMYM2-FGFR1* fusion transcript encodes a 76 kDa protein localized predominantly to the cytoplasm. Conventional cytogenetics is most commonly used to detect the t(8;13), but FISH and RT-PCR methods also can be used.

A number of other partner genes can be involved including *FOR* in the t(6;8)(p17;p11), *FGFR1OP2* in the ins(12;8)(p11;p11p22), *BCR* in the t(8;22)(p12;q11); *TPR* in the t(1;8)(q25;p11.2); *TIF1* in the t(7;8)(q34;p11), *MYO18A* in the t(8;17)(p11;q23), *HERV-K* in the t(8;19)(p11;q13.3), and *CEP110* in the t(8;9)(p12;q33) (Fig. 30.20) [339]. In all translocations, *FGFR1* and the partner gene are disrupted and recombined to form novel fusion genes that constitutively activate *FGFR1*. The different translocations show some clinical correlations. Patients who develop T-ALL/LBL or mixed T-cell/myeloid lymphomas often carry *ZMYM2-FGFR1*. Patients with *BCR-FGFR1* often present with a CML-like disorder. The *ZMYM2-FGFR1* and *BCR-FGFR1* have been shown to induce CMPD in mice [340].

30.8 Chronic Lymphoid Leukemias

30.8.1 Chronic Lymphocytic Leukemia

Virtually all cases of chronic lymphocytic leukemia (CLL) express surface Ig, usually at a low level, and have *IgH* and *Ig light chain* gene rearrangements (k>λ). Surface Ig expression requires a functional Ig protein and two other critical proteins, B29 (Igβ and CD79b) and mb-1 (Igα and CD79a), that are essential components of the B-cell antigen-receptor complex. Mutations in the B29 gene and/or abnormalities of B29 expression (at the mRNA level) are common in CLL [341]. The diminished surface Ig on CLL cells may result in these cells being unresponsive to antigen binding, perhaps preventing initiation of growth signals or apoptosis cascades.

A milestone in understanding of CLL was the observation that somatic mutations of the *IgV* (*immunoglobulin variable region*) genes occur in a subset of CLL cases, and that the absence or presence of these mutations correlates with clinical course [342]. Mutations occur in both the *IgH* and *Ig light chain* genes, but *IgHV* analysis is easier and adequate for prognostic purposes. An arbitrary cutoff of 2 % or more mutations divides CLL into two prognostic groups. Patients with CLL in which the *IgHV* show <2 % somatic mutations (unmutated CLL) have a poorer clinical outcome. It appears that CLL cells in this subset receive continuous anti-apoptotic and/or proliferating microenvironmental stimuli via the BCR leading to more aggressive disease. In contrast, CLL patients in which the *IgHV* show ≥2 % mutations (mutated CLL) have a clinically indolent course [342]. A difference in outcome was also demonstrated in CLL patients receiving an autologous stem-cell transplant (ASCT); all patients with unmutated *IGHV* genes relapsed and progressed after a 4-year follow-up, whereas most patients with mutated *IGHV* genes remained in molecular remission [343].

Initially it was thought that CLL cases with unmutated *IgHV* (U-CLL) were derived from naive B-cells, and that CLL cases with mutated *IgHV* CLL (M-CLL) derived from antigen-experienced B-cells. It is now known that all CLL cells bear the surface membrane immunophenotype of being antigen experienced (CD27) and activated (expression of CD23, CD25, CD69, and CD71). Gene expression profiling has shown that CLL cells have a profile similar to memory

B-cells irrespective of *IgHV* mutational status [344]. These findings suggest that all cases of CLL have a common cellular origin and/or common mechanism of transformation and a continued requirement for antigen after the transformational event [345]. However, comparative analysis of U-CLL and M-CLL suggests that the neoplastic cells, by differing in their *IgHV*, have different antigen encounter histories [345]. It also has been suggested that an antigen-driven process might be critical for determining clinical features and for modulating disease outcome, irrespective of mutation status in B-CLL [346].

Biased use of *Ig* gene variable (V) segments has been described in CLL cases. The *VH1* family of the *IgH* gene and the *VIIIb* family of the *Igk* gene are used in the formation of *Ig* gene rearrangements more often than can be explained by chance alone. DNA sequence analysis of the variable V, diversity (D) and joining (J) segments involved in *IgH* and *Igk* gene rearrangement has shown that a subset of CLL have somatic mutations [346].

The frequency of chromosomal abnormalities detected in CLL depends on the method used. Conventional cytogenetic analysis detects abnormalities in a substantially lower proportion of cases as compared with FISH. The significant false-negative rate of conventional cytogenetic analysis is attributed to the low proliferation rate and therefore poor growth of CLL cells in culture [348]. Using conventional cytogenetic analysis, chromosomal abnormalities have been identified in up to 50 % of cases of CLL, with trisomy 12 being the most common chromosomal abnormality (15–20 % of cases), followed by structural abnormalities of chromosomes 11, 13, and 14. The most common chromosomal abnormality in CLL detected by FISH is deletion of 13q14, followed by deletion of 11q22.3-q23.1 and trisomy 12 (Fig. 30.21) [348].

There is a correlation between genomic aberrations and the *IgHV* mutation status. Unfavorable aberrations, such as del(11q) and del(17p), occur more frequently in *IgHV* unmutated, and del(13q14), a favorable finding, occurs more often in the *IgHV* mutated subgroup [348, 349]. Approximately two thirds of the *IgHV* unmutated CLL cases show no unfavorable genomic aberrations, indicating a differential influence of these factors [349].

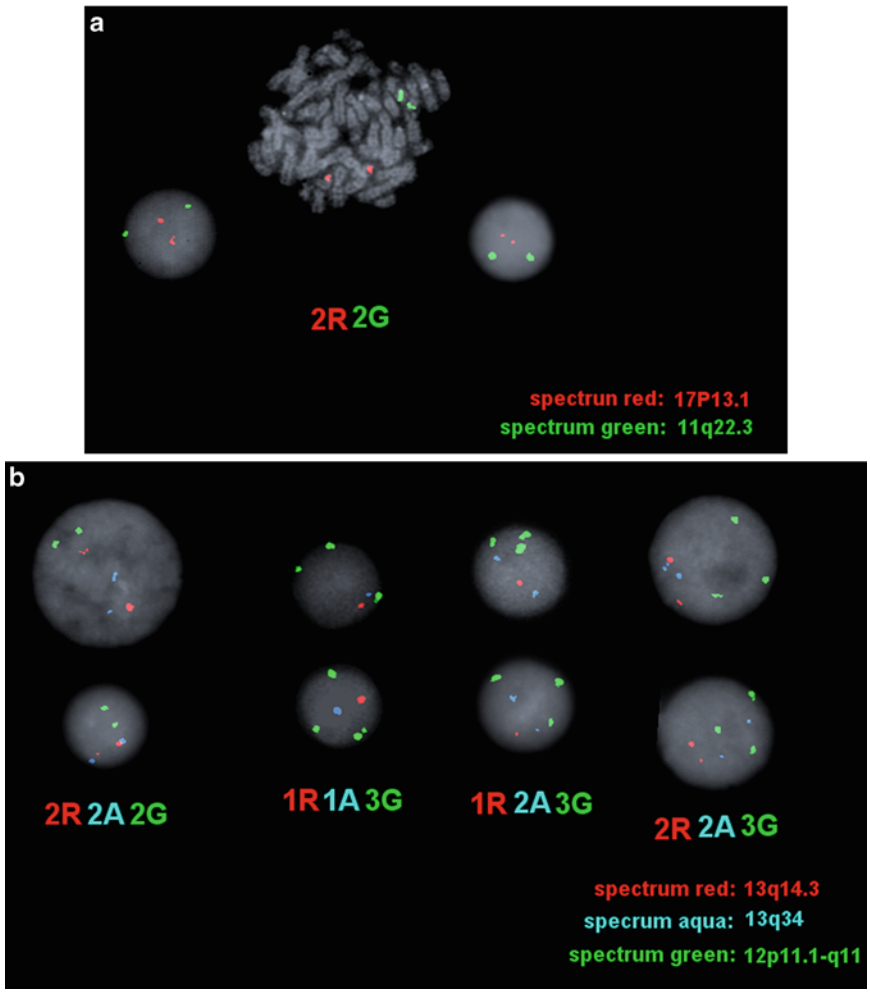

Fig. 30.21 Fluorescence in situ hybridization (FISH) analysis of chronic lymphocytic leukemia. The probes are part of a commercially available panel (Vysis). (**a**) Probe mixture 1 assesses 17p13.1 (p53) and 11q22.3 (ATM). In a normal cell with two intact copies of chromosomes 17 and 11, two *orange*, and two *green* signals will be observed. (**b**) Probe mixture 2 assesses 13q14.3 (D13S319), 13q34, and 12p11.1-q11. In a normal cell with two intact copies of chromosome 13 and chromosome 12, two *orange*, two *aqua*, and two *green* signals are observed. Any deviation of the normal pattern indicates deletion or gain a corresponding chromosomal region.

30.8.2 CLL with 13q14 deletion

13q14 deletions occur in 40–50 % of CLL cases using FISH. Studies using FISH and CGH have frequently shown *RB1* deletion, located at 13q14, as well as 13q deletions. Monoallelic loss of the *RB1* gene was detected in 30 % of CLL patients, but disruption of both alleles is rare. Additional loci D13S25 and D13S319 located distal to band 13q14, which are more frequently deleted in CLL, were identified suggesting a novel tumor suppressor gene located in these regions [350]. Deletion or downregulation of two microRNA genes, *miR15* and *miR16*, has been recently implicated in the pathogenesis of CLL [351].

30.8.3 CLL with Structural Aberrations of Chromosome 11

Structural aberrations of chromosome 11 are reported in 12–25 % of CLL [348, 352]. The 11q22-q23 locus is frequently deleted in CLL and is rich in tumor suppressor and other genes including *FDX*, ataxia telangiectasia mutated (*ATM*), *KMT2A*, *PZLF*, *Mre11*, and *RDX* [352]. The del(11q22.3) group of patients often has a poor prognosis, and acquisition of this abnormality is associated with clonal evolution and disease progression [353]. Tumor cells carrying del(11q22.3) often show upregulation of genes that control cell cycle progression and signaling pathways [354, 355]. The *ATM* gene frequently shows *germ line* as well as somatic mutations in CLL patients. More than two thirds of CLL cases have monoallelic loss of *ATM*. The other nondeleted allele often bears point mutations, most frequently in the PI-3 kinase domain [356]. Recently, three new candidate genes in the 11q22-23 region, *NPAT* (cell-cycle regulation), *CUL5* (ubiquitin-dependent apoptosis regulation), and *PPP2R1B* (component of the cell-cycle and apoptosis regulating *PP2A*) have been shown to be significantly downregulated in CLL cases with 11q deletion [357].

30.8.4 CLL with 17p13 Aberration

The *TP53* gene, located on 17p13, plays a crucial role in apoptosis induction or cell cycle arrest after DNA damage. *TP53* mutations occur in 10–15 % of patients with CLL [358]. Acquisition of *TP53* mutations is a late event in CLL, detected in about 10 % of early CLL cases but in more than 30 % of CLL patients who subsequently develop large B-cell lymphoma [359]. *TP53* abnormalities in CLL are associated with advanced stage at presentation, resistance to purine nucleoside analogues, high incidence of transformation and disease progression in 1–2 years, and poorer clinical outcome [359]. *TP53* deletions are detected by FISH in 10–20 % cases and independently predict poor survival and resistance to chemotherapy in CLL patients [359].

Wild-type *p53* protein is a target of *MDM-2*-mediated ubiquitination and subsequent degradation and, thus, has a short half-life in early-stage tumor cells in CLL [360]. The protein product of mutated *TP53* has a prolonged survival in cells, and it is thus possible to detect *p53* expression by immunocytochemistry [361]. *p53* expression serves as an independent prognostic variable in CLL and is associated with *TP53* gene mutations, advanced disease, resistance to therapy, and poor survival [361].

30.8.5 CLL with Trisomy 12

Trisomy 12 is reported in 10–20 % of CLL patients as assessed by conventional cytogenetic analysis and in 20–40 % of CLL patients as assessed by interphase FISH [362]. The occurrence of trisomy 12 is more common in CLL with atypical morphological and immunophenotypic features [362]. CLL cases with trisomy 12 often have unmutated *IgVH*. The acquisition of trisomy 12 is associated with advanced-stage disease, shorter time to progression, and significantly poorer median survival rates [362]. Detailed structural analyses of chromosome 12 identified a minimally gained region limited to bands 12q13-q15. A number of genes including *CDK2*, *CDK4*, *STAT6*, *APAF-1*, and *MDM-2* are located in this region and play critical roles in the regulation of oncogenesis, cell cycle control, and apoptosis; however, the role of these genes in the pathogenesis of CLL has not been well characterized.

30.8.6 CLL with 6q deletion

Deletions of the long arm of chromosome 6 are detected in 4–6 % of CLL cases by conventional cytogenetics and up to 9 % cases by FISH [363]. Deletions of 6q are often associated with high leukocyte counts, extensive lymphadenopathy, and splenomegaly. Cases with del(6q) often show atypical morphologic features, including large lymphocytes, immunoblast-like cells, prolymphocytoid cells, and cleaved lymphocytes. Usually these neoplasms have a classic immunophenotype with CD38 positivity, and an intermediate frequency of *IGHV* somatic hypermutation.

30.8.7 Chromosomal Translocations in CLL

A small subset of cases of CLL, less than 5 %, are associated with chromosomal translocations that involve the immunoglobulin gene loci. These cases differ somewhat from other CLL cases in that patients may be younger or the tumors show atypical morphologic or immunophenotypic features.

t(14;18)(q32;q21). Conventional cytogenetic studies have rarely identified the t(14;18)(q32;q21) in CLL [348]. In addition, the *BCL2* gene at 18q21 can be involved in t(2;18)(p11;q21) or t(18;22)(q21;q11), involving the *Igk* (chromosome 2p11) or *Igλ* (chromosome 22q11) gene loci [365]. The breakpoint on chromosome 18 usually occurs within the variant cluster region, 5′ to the *BCL2* gene, but may also occur 3′ to the *BCL2* gene, at a location intermediate between the major and minor breakpoint cluster regions [365]. Errors in the VDJ recombination process may be involved in the generation of the t(2;18) and t(18;22), as a heptamer-like recognition sequence is present upstream of the *BCL2* gene [365]. Despite the relative infrequency of chromosomal translocations involving *BCL2* in CLLs, virtually all CLL cases express BCL2 protein. The BCL2 protein is known to inhibit apoptosis (programmed cell death). Because chemotherapeutic drugs act by inducing apoptosis, BCL2 expression in CLL appears to be a major mechanism for drug resistance.

t(14;19)(q32;q13). The t(14;19)(q32;q13) involves the *BCL3* gene at chromosome 19q13. This translocation is rare in CLL. The *BCL3* gene encodes a protein of the IkB family and is involved in regulating the NF-kB family of transcription factor proteins. Cases with the t(14;19) are associated with atypical lymphocyte morphology manifested by a mixture of small and larger lymphocytes, increased prolymphocytes (usually <10 %), and cleaved/indented nuclei. Trisomy 12 is common. In addition, such patients present at a younger age and with rapid disease progression [366].

30.8.7.1 CLL with 8q24/*MYC* Rearrangements

A small subset of CLL cases are associated with translocations involving chromosome locus 8q24 with either the *IgH* (14q32) or the *Ig kappa* (2p12) or *Ig lambda* (22q11) loci. FISH analysis has confirmed *MYC* gene rearrangements in these cases. Clinically, patients with 8q24/*MYC* rearrangements tend to show morphologic evidence suggestive of disease progression including increased (>10 %) prolymphocytes or full blown prolymphocytic transformation (>55 % prolymphocytes) [367].

30.8.7.2 CLL with t(2;14)(p16;q32)

Rare cases of CLL have been associated with t(2;14)(p16;q32) involving the *BCL11A* gene at chromosome 2p16. These tumors are associated with atypical morphologic features and often exhibit plasmacytic differentiation. The *IgHV* are often unmutated in these tumors [368].

30.8.7.3 CLL with t(11;14)(q13;q32)

In older literature, t(11;14)(q13;q32) was reported in a small subset of cases of CLL, up to 5 %. In retrospect, these cases were most likely indolent variants of mantle cell lymphoma in leukemic phase. The translocation involves the *CCND1* (also known as BCL1 and cyclin D1) at chromosome 11q13 and the *IGH* gene at chromosome 14q32. *CCND1* encodes cyclin Dl protein, which is involved in progression of the cell cycle from G_1 to S phase. The t(11;14) is common in mantle cell lymphoma, being present in virtually all cases (Figs. 30.22 and 30.23) [369].

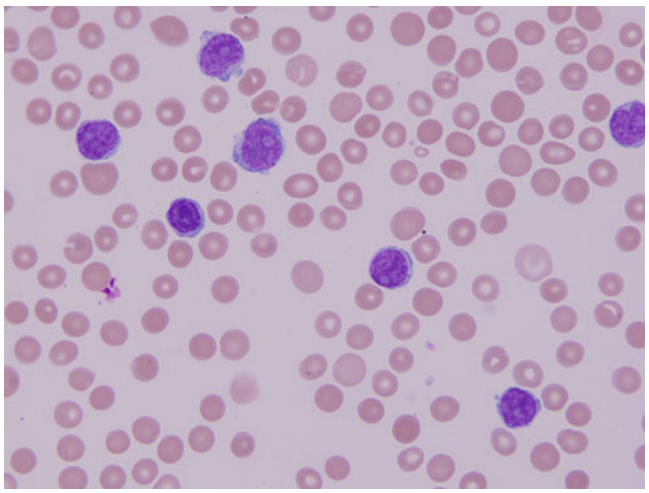

Fig. 30.22 Mantle cell lymphoma with unusual prolymphocyte-like morphology. Wright-Giemsa, 1000×. This case had the t(11;14)(q13;q32).

30.8.8 Gene Mutations in CLL

Using next-generation sequencing approaches, CLL has been shown to be highly heterogeneous. Overall, a number of genes are mutated in CLL, but no specific gene is mutated in greater than ~10 % of cases, with most genes mutated in <5 % of cases. In addition, a subset of CLL cases has very few gene mutations.

The genes most commonly mutated in CLL include *NOTCH1, SF3B1*, *TP53*, *MYD88*, and *BIRC3 (API2). NOTCH1* mutations have been reported in approximately 10 % of CLL cases and have been correlated with unmutated *IGV* and a poorer prognosis. Other genes are mutated in <5 % of cases and involve many different cellular functions. *SF3B1* is involved in mRNA splicing, *TP53* in DNA repair, *MYD88* is an adaptor protein involved in Toll-like receptor and interleukin-1 receptor signaling, and *BIRC3* in apoptosis regulation. *MYD88* mutations are more common in the mutated type of CLL.

30.8.9 Richter Syndrome

A small subset of CLL/SLL patients can subsequently develop diffuse large B-cell lymphoma (DLBCL), so-called

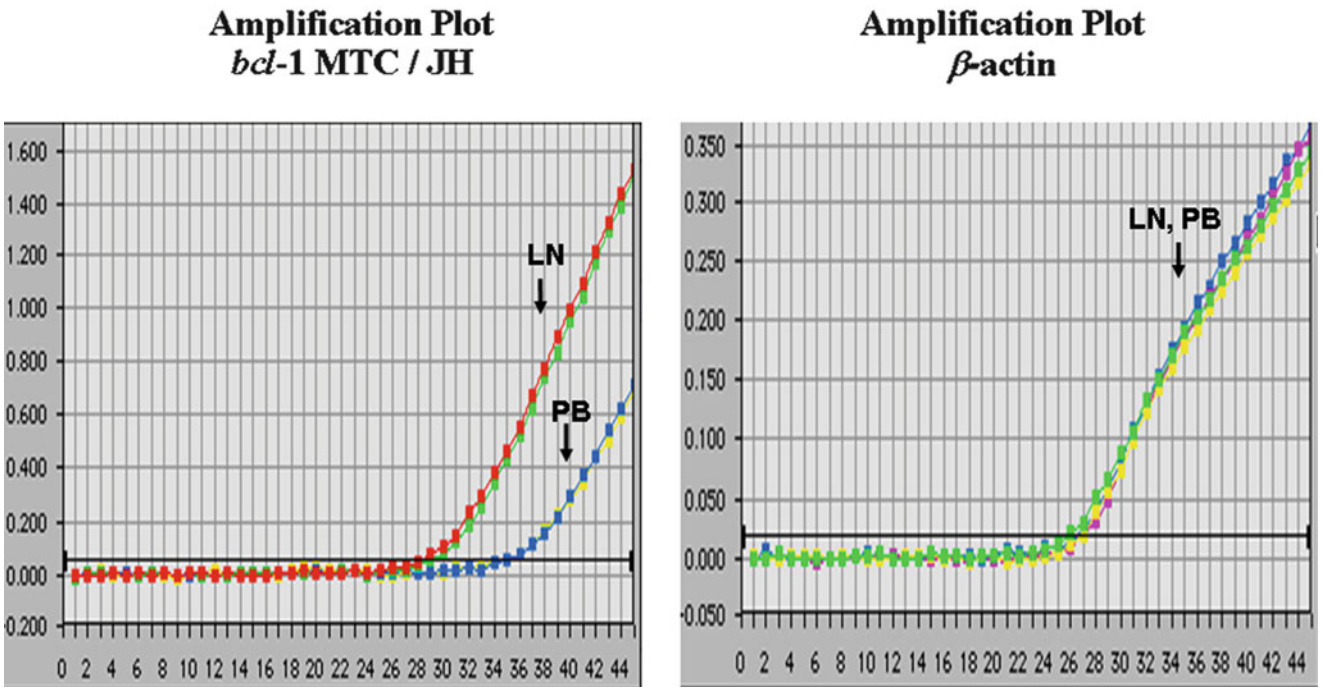

Fig. 30.23 Real-time PCR for BCL1/IgH. *Left* histogram shows DNA level in peripheral blood and lymph node of a patient with mantle cell lymphoma. *Right* histogram shows similar levels of β-actin DNA in both peripheral blood and lymph node used as a control.

Richter syndrome [370]. The overall frequency of this occurrence ranges from 2 to 10 % of patients in various reports, or about 0.5–1 % per year. In 70–80 % of patients, the CLL/SLL and DLBCL are clonally related and therefore the DLBCL is a form of histologic transformation. The risk of Richter transformation is increased in patients with high tumor stage, high-risk cytogenetic abnormalities, unmutated *IGHV* region genes, and expression of ZAP70 and/or CD38. At a genetic level, DLBCL arising as part of Richter syndrome is genetically distinct from de novo DLBCL [371].

30.8.10 Epigenetic Changes

Gene methylation is another mechanism of gene inactivation and with the advent of new methods for assessing DNA methylation, the importance of epigenetic changes in human cancers is well recognized [372].

Genome-wide patterns of DNA methylation in CLL are rather specific for CLL cells and can be reproduced in different patients [373, 374] suggesting that DNA methylation plays a specific role in CLL pathogenesis. Aberrant methylation has been described for specific genes, for example, hypomethylation of BCL2 and TCL1 [375]. Unbiased genome-wide screens of CLL have shown specific methylation of the promoter region of *TWIST2* [376] and of *DAPK1* [377]. When DNA methylation analyses were performed on the promoter region of *ZAP-70*, a specific CpG was methylated in 51 of 53 ZAP70-negative CLL cases. By contrast, no methylation was found in 30 of 32 ZAP70+ CLL cases [378].

Apart from their role in pathogenesis and their potential utility in diagnosis, epigenetic modifications are also an interesting target for therapy as epigenetic modifications are reversible (in contrast with genetic aberrations). Several compounds have been tested on cells in vitro and in clinical studies and shown to be biologically and clinically active. An inhibitor of histone deacetylases (depsipeptide FR901228) was shown to selectively target CLL cells in vitro as compared with normal blood B-cells and BM-derived progenitors [379].

30.8.11 B-Cell Prolymphocytic Leukemia

B-cell prolymphocytic leukemia (B-PLL) is a rare and ill-defined disease of mature B-cells with prolymphocytic morphology. In many studies, a cutoff of 55 % has been suggested for the number of prolymphocytes required for the diagnosis of B-PLL. In retrospect, it seems clear that cases designated as B-PLL in the past included cases of CLL in prolymphocytoid transformation as well as cases of prolymphocytoid variant of mantle cell lymphoma associated with t(11;14)(q13;q32) and cyclin D1 overexpression. In addition, a subset of patients with splenic marginal zone lymphoma can progress into a leukemic phase with >55 % prolymphocytes [380].

Most cases of B-PLL carry monoclonal *IgH* and *Ig light chain* gene rearrangements. The IgHV demonstrate somatic mutations consistent with antigen selection at a higher frequency than occurs in CLL [381]. Cytogenetic data obtained from cases of B-PLL are sparse and conflicting due to rareness of the disease and its poorly defined nature. In a series of 18 B-PLL patients using FISH, del(13q14) was detected in 55 % and monoallelic del (11q23) in 39 % of cases, respectively [382]. Other abnormalities reported include t(6;12), del (6q) structural aberrations of chromosome 1, and trisomy 12 [383]. Other known B-cell oncogenes (such as *BCL2*, *BCL3*, *BCL6*, and *MYC*) are not rearranged. The *TP*53 gene is frequently mutated in B-cell PLL, in approximately 50 % of cases [384].

A recent study of 13 B-PLL cases used gene expression profiling and showed that B-PLL cases cluster with either CLL or mantle cell lymphoma. Not surprisingly, cases with t(11;14)(13;q32) clustered with mantle cell lymphoma. However, a subset of cases without t(11;14) also clustered with cases of mantle cell lymphoma [385].

30.8.12 Hairy Cell Leukemia

Hairy cell leukemia is a rare type of B-cell lymphoma in which patients present with splenomegaly, and bone marrow involvement with abdominal lymphadenopathy in a subset of patients. Patients commonly present with pancytopenia and monocytopenia, and distinctive neoplastic lymphocytes with villous cytoplasmic projects (hairy cells) can be detected in the blood. Hairy cells have a mature B-cell immunophenotype and often express CD11c, CD22, (bright), CD103, and annexin A1.

Conventional cytogenetic studies have not shown recurrent abnormalities. Cases of HCL carry monoclonal *IgH* and *Ig light chain* gene rearrangements. The *IgHV* often show somatic mutations. In 2011, Tiacci and colleagues showed *BRAF* V600E mutation, in which valine (V) is replaced by glutamic acid (E), in virtually all cases of HCL [386]. The mutation occurs within the activation loop of *BRAF* and likely explains activation of the *RAF-MEK-ERK* pathway that is characteristic of HCL [387]. A small subset of HCL cases have *MAP2K1* mutation in the same pathway.

30.8.13 Splenic Marginal Zone Lymphoma/Leukemia

Splenic marginal zone lymphoma (SMZL) is an uncommon mature B-cell neoplasm, representing 1–2 % of all non-Hodgkin lymphomas. Patients tend to be elderly and they present predominantly with subdiaphragmatic disease characterized by massive splenomegaly, BM involvement, intra-abdominal lymphadenopathy, and hepatomegaly. Most patients have circulating lymphoma cells and patients can present an elevated leukocyte count that can be very high. Conventional cytogenetic and array CGH studies have shown del(7q22-36) in up to 50 % of patients and trisomy 3q in about 25 % of cases.

Recent sequencing studies have shown a number of gene mutations. *NOTCH2* mutations are common, in about 25 % or more of cases [388, 389]. Martinez and colleagues have shown a large number of other genes that are mutated in SMZL implicating activation of NF-kB, B-cell receptor signaling, cytoskeletal genes, and chromatin remodeling [390]. Parry et al. have suggested three genes in the minimally deleted region of 7q possibly being involved in pathogenesis: CUL1, EZH2, and FLNC [391].

30.8.14 T-Cell Prolymphocytic Leukemia

T-cell prolymphocytic leukemia (T-PLL) is a disease of prolymphocytes with a mature T-cell immunophenotype. Typical clinical features of T-PLL are splenomegaly, generalized lymphadenopathy, skin infiltration, and serous effusions. The disease usually affects elderly patients with a median age at presentation 63 years (range: 33–91 years).

A unique feature of T-PLL is dysregulation of the T-cell leukemia 1 (*TCL1*) oncogene [392]. TCL1 deregulation is detected in 70–80 % of T-PLL but not in other T-cell neoplasms [393]. In most cases, *TCL1* is insertionally activated by the T-cell receptor α/δ enhancer via inv(14)(q11q32.1) or t(14;14)(q11;q32.1) [394]. Rarely, it can also rearrange with the TCR-β chain locus on chromosome 7 in translocation t(7;14)(q35;q32.1) [392, 395]. In a small subset of T-PLL cases, the TCL1 homolog MTCP1 is dysregulated as a result of t(X;14) [396].

The 14q32.1 breakpoint region has three additional genes, *TCL1b, TNG1* (*TCL1 neighboring gene 1*), and *TNG2*, that have an expression pattern similar to *TCL1* in that they are not expressed in normal T-cells, but are expressed in T-PLL cell lines and cells from T-PLL patients [397]. Activation of *TCL1* through hypomethylation of its promoter also has been described [375].

In non-lymphoid cell line models, dysregulated TCL1 has been shown to bind to the pleckstrin homology (PH) domain of AKT and to regulate its activity, suggesting that TCL1 homodimers may act by promoting AKT dimerization, thus facilitating AKT transphosphorylation [398, 399]. In vitro TCR engagement results in rapid recruitment of TCL1 and AKT to transient membrane activation complexes that include TCR-associated tyrosine kinases [400]. Pharmacologic inhibition of AKT activation alters the localization, stability, and levels of these transient TCL1-AKT complexes and reduces tumor cell growth [400]. Experimental introduction and knockdown of *TCL1* influence the kinetics and strength of TCR-mediated AKT activation [400]. These

data indicate that TCL1 represents a highly regulated modulator of TCR-mediated AKT growth signaling in T-PLL.

Mutations involving *JAK1*, *JAK3*, *STAT5B*, and *IL2RG* are common in T-PLL, in up to 75 % of all cases. Constitutive activation of *STAT5B* is one result of these mutations and offers a potential target for therapy [401].

30.8.15 Adult T-Cell Leukemia/Lymphoma

Approximately 15 million persons in the world carry human T-cell lymphotropic virus 1 (HTLV-I) infection. The virus is common in endemic regions such as Africa, Middle East, the Caribbean basin, coastlines of South America, southwestern Japan, and some southern Pacific Islands. These locations mirror the transport of slaves from Africa in past centuries. The seroprevalence of HTLV-I infection in the USA is very low; 0.01 % of normal blood donors show serologic evidence of infection.

Adult T-cell leukemia/lymphoma is caused by HTLV-I. About 1–5 % of individuals seropositive for the virus eventually develop adult T-cell leukemia/lymphoma (ATLL), usually after many years. The are at least four clinical variants of ATLL: acute, lymphoma-like, chronic, and smoldering. Most patients with leukemic involvement by ATLL have the acute form. These patients commonly exhibit leukemia, lymphadenopathy, hepatosplenomegaly, skin lesions, osteolytic lesions, and hypercalcemia. Adult T-cell leukemia/lymphoma is a neoplasm of mature T-cell lineage, often with a T regulatory cell immunophenotype; CD4+ CD25+, and FOXP3+, and negative for CD1a, CD8, and TdT.

The HTLV-I provirus genome includes *gag*, *pol*, and *env* genes and other genes encoding Tax, Hbz, Rex, p12, p30, and p21 [402]. Transcriptional activation by Tax occurs through its interaction with cellular transcription factors such as NF-kB and the members of the cAMP responsive element binding proteins (CREB)/activating transcription factor (ATF) family [403]. Heterologous promoters of *IL-2 (interleukin-2), IL-13, IL-15, TGF-β (tumor growth factor-β), GM-CSF (granulocyte/macrophage colony-stimulating factor)*, *MYC, TNF-β (tumor necrosis factor-β)*, and others contain NF-kB binding sites and are transactivated through an interaction between Tax protein and NF-kB/Rel proteins [404, 405]. Other genes such as those for *IL-1, IL-6, major histocompatibility complex class I, and PTHrP (parathyroid hormone-related protein)* are activated by Tax through unknown mechanisms [406]. Transcriptional repression of some genes, such as *DNA polymerase-β*, *p18*, and *TP53*, by Tax protein also has been described [407]. Other effects of Tax include interaction with *CDKN2A* resulting in activation of CDK4, interaction with and inhibition of checkpoint kinase-1 (Chk1), and promotion of nuclear translocation of NF-kB through binding its cytoplasmic inhibitor, IKK [408]. The dysregulated expression of these genes with important roles in cell-cycle regulation, apoptosis, and proliferation leads to the development of HTLV-I-related diseases and contributes to their clinical features [409, 410].

In addition to the important role of Tax in pathogenesis, there are many other abnormalities. Conventional cytogenetics often shows complex karyotypes, pointing to numerous gene alterations. Comparative genomic hybridization has shown many gains and losses of chromosomal loci. *TP53* is mutated in 20–40 %. *CDKN2A* can be inactivated by methylation. A number of pathways have been shown to be activated in ATLL including NF-kB, JAK-STAT, mTOR-Akt, NOTCH, and RhoA [411].

30.8.16 Hepatosplenic T-Cell Lymphoma/Leukemia

This disease tends to involve younger adults, more often men, who present with splenomegaly, hepatomegaly, and BM involvement by an aggressive T-cell lymphoma/leukemia. Patients have short survival, often less than 2–3 years. Pathologic examination of tissues involved by HSTCL shows a sinusoidal pattern of involvement in the spleen, liver, and bone marrow. Patients can present with cytopenias and low-level involvement of the blood occurs in a patient subset; however, the total leukocyte count is often in the normal range. The neoplastic cells have an immature cytotoxic immunophenotype (TIA-1+, granzyme B-) and usually express the T-cell receptor (TCR) γ/δ, although ~20 % of cases express TCR α/β.

Conventional cytogenetic analysis has shown isochromosome 7q in 50–60 % of all cases. A variant ring-7 chromosome also has been reported less commonly. Isochromosome 7q results in loss of one copy of 7p and gain of one copy of 7q. Using array CGH, Ferreiro reported that the minimally deleted region as 7p22.1p14.1 and the minimally gained region as 7q22.11q31.1 [412]. Losses involving 7p are thought to be critical driver genetic alterations whereas gains at 7q are thought to provide growth advantages and may be involved in chemoresistance. Genes suggested as important at these loci include *CHN2* (which encodes β-chimerin) at 7p and *ABCB1*, *RUNDC3B*, and *PPPAR9A* at 7q. The *JAK-STAT* pathway also appears to be important in HSTCL; *STAT5B* and *STAT3* mutations have been reported in approximately 40 and 10 % of HSTCL cases, respectively [413].

30.8.17 T/NK Large Granular Lymphocyte Leukemia

Large granular lymphocyte (LGL) leukemias are proliferations of neoplastic LGLs that are further characterized, based on surface CD3 expression and TCR gene rearrangement, as

being of either T or NK cell lineage [414]. The median age of patients at diagnosis of LGL leukemias is 55–60 years, but these tumors has been reported in all age groups [415]. LGL leukemia is characterized by a persistent increase in the number of peripheral blood LGLs and is commonly associated with neutropenia and anemia [415]. Previously, an increase in the peripheral blood LGLs greater than 2.0×10^9/L lasting for more than 6 months was used as the necessary criterion for the diagnosis. However, some authors advocate establishing a diagnosis of T-LGL leukemia at lower level of LGL lymphocytosis, provided a monoclonal *TCR* rearrangement is detected.

The cytopenias characteristic of LGL leukemias are thought to be the result of leukemic cells exerting an inhibitory effect on normal hematopoiesis, rather than as a result of BM infiltration or hypersplenism [415]. Abnormalities in the FAS/FASL pathway resulting in dysregulated apoptosis are thought to play an important role in the pathogenesis of LGL leukemias [416]. Activated cytotoxic lymphocytes are normally eliminated through FAS-mediated apoptosis. However, LGL leukemic cells are resistant to FAS-mediated cell death [416]. Furthermore, leukemic LGLs express high levels of Fas and FasL and high levels of soluble Fas has been reported in the sera of patients with LGL leukemia [416]. These high levels of soluble Fas may block Fas-mediated apoptosis of LGL leukemic cells and contribute to the neoplastic process [417]. Reports have shown a correlation between serum levels of Fas ligand and disease activity and normalization of these levels with successful therapy [416, 418].

Neutropenia in LGL patients may be secondary to neutrophil destruction resulting from FAS-mediated apoptosis by direct contact of LGL cells expressing FAS ligand (FASL) or through paracrine effects of soluble FASL [415, 416].

Recent studies have shown a high frequency of *STAT3* mutations in cases of LGL leukemias. Koskela and colleagues identified *STAT3* mutations in approximately 40 % of cases [419]. The mutations occurred exclusively in exon 21, encoding the Src homology 2 domain, and a number of hotspots were also reported. In <5 % of LGL leukemias *STAT5B* mutations also have been reported and appear to be mutually exclusive with *STAT3* mutations [420].

30.9 Summary

Characterization of the molecular abnormalities in leukemias is important for a number of reasons. From the scientific viewpoint, these studies help to identify the genes and mechanisms involved in normal hematopoiesis and in the pathogenesis of leukemia. Further progress may help to identify targets for rational drug design or gene therapy. From a more immediate clinical perspective, information gleaned from these studies has improved the accuracy of diagnosis, aided in the design of assays to detect minimal residual disease, helped to predict response to therapy, and has provided criteria for selecting high-risk patient groups who may benefit from intensive but highly toxic chemotherapy protocols or stem cell transplantation.

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Molecular Mechanisms in Non-Hodgkin Lymphoma

31

John Hunt, Yuri Fedoriw, and William N. Rezuke

31.1 Introduction

Hematologic malignancies are a heterogeneous group of disorders that include neoplasms derived from cells of myeloid and lymphoid lineages. These malignancies can be categorized in many ways, but the most commonly used classification today is the World Health Organization's Classification of Tumours of Haematopoietic and Lymphoid Tissues (WHO classification) [1]. The WHO classification is largely based on the Revised European American (REAL) classification, which divided this diverse group of disorders into what were felt to be distinct or real clinicopathologic entities on the basis of combined morphologic, immunophenotypic, clinical, and genetic features [2]. The lymphoid neoplasms include immature B-cell and T-cell neoplasms (precursor lymphoblastic leukemia/lymphoma), mature B-cell and T-cell neoplasms (including the group of disorders collectively known as non-Hodgkin lymphomas (NHL) and plasma cell myeloma) and Hodgkin lymphoma. A review of Surveillance, Epidemiology, and End Results (SEER) data stated that in 2005 the United States would have approximately 93,420 incident cases of lymphoid neoplasms with approximately 38,000 deaths. Over the 10-year course of SEER data that were reviewed, the B-cell lymphomas showed a modest decrease in incidence (approximately 1 % per year), while T-cell neoplasms increased and Hodgkin lymphoma incidence remained stable [3]. Because lymphomas comprise a significant number of new cancer diagnoses, it is important to understand the pathogenesis of these disorders and how this information can be used for diagnosis, prognosis, and guidance of therapy.

The diagnosis of hematologic malignancies often requires a multi-parameter approach, correlating morphologic evaluation of traditional hematoxylin and eosin-stained tissue sections or Romanowsky-stained smears along with a variety of ancillary studies, including cytochemical and histochemical stains, immunohistochemical studies, flow cytometric analysis, cytogenetic analysis, and molecular genetic techniques. Of these, immunohistochemistry is favored among many pathologists because of the ability to directly correlate with the tissue morphology and convenience in working with readily available paraffin-embedded tissue (PET) (Fig. 31.1). Other methods may be more sensitive and quantitative than immunohistochemistry, but each method has advantages and disadvantages. For instance, flow cytometric analysis can detect small populations of aberrant B-cells and T-cells in a background of normal lymphocytes, a feat that is often not possible with routine immunohistochemical analysis. However, this technique requires viable cells in suspension for analysis and allows only limited correlation with morphologic findings. Molecular studies by polymerase chain reaction (PCR) or Southern blotting are very sensitive in fresh or frozen tissue, but sensitivity may be decreased in some assays using the more commonly available PET. Any of these ancillary studies can produce false positive and false negative results, and the interpretation of results must be made in the context of the traditional morphologic findings and clinical findings.

This chapter will focus on the underlying pathogenesis of lymphoproliferative disorders with some reference on how that information can be used for diagnostic and prognostic purposes. First, there will be an overview of normal B- and T-cell development, noting that the various stages of lymphocyte development can be roughly correlated with different types of lymphoma. A description of immunoglobulin

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_31

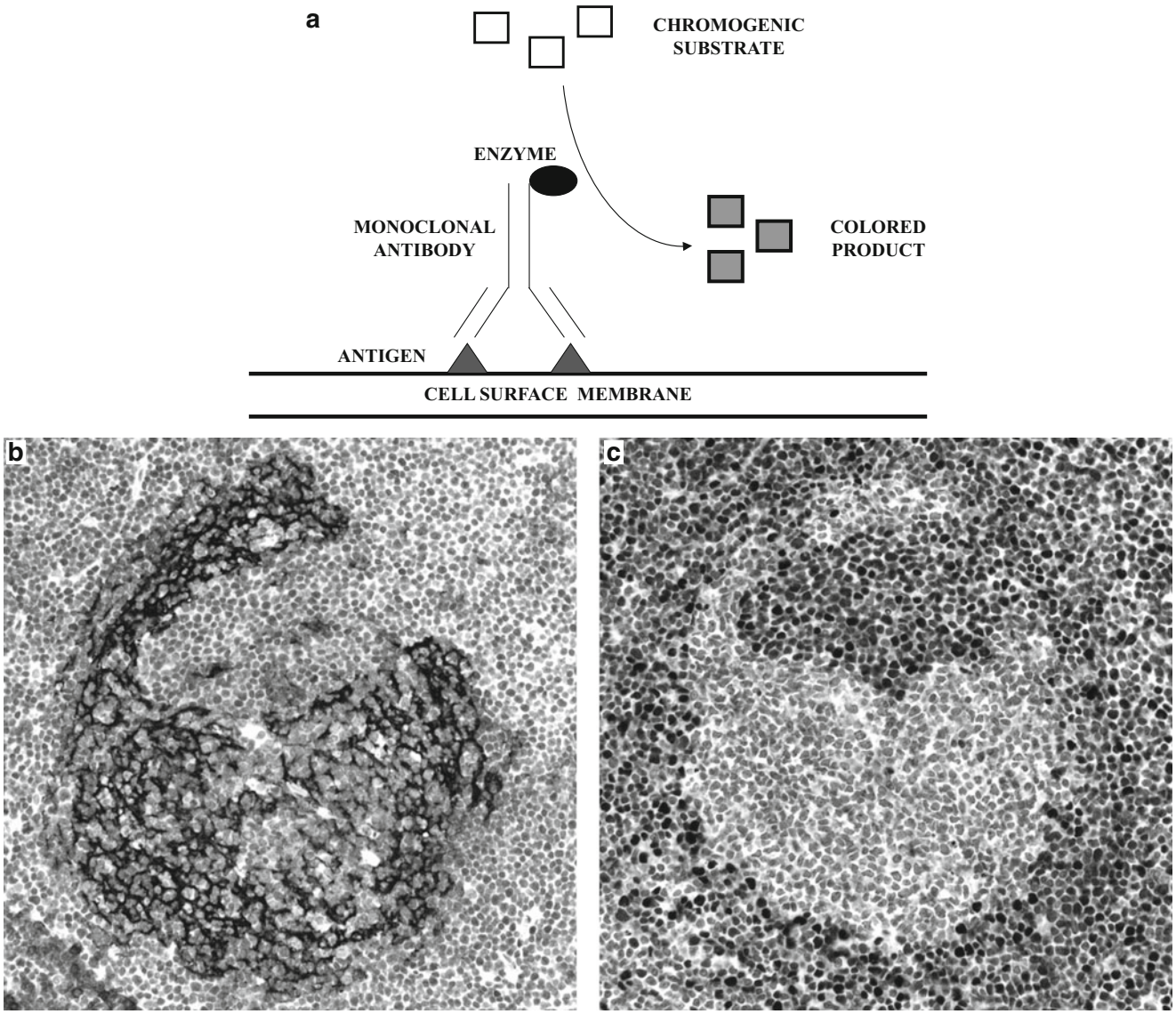

Fig. 31.1 Immunopathological detection of cell surface antigens. (**a**) Diagram illustrating the basic principle of immunopathological methods. An enzyme is linked to a monoclonal antibody, which recognizes a specific cell surface membrane antigen. A chromogenic substrate is added and is enzymatically converted to a colored product, which is visualized microscopically. (**b**) Lymph node biopsy with partial disruption of the follicular dendritic cell meshwork (highlighted by antibody to CD23) in a case of mantle cell lymphoma. The mantle cell lymphoma cells are not immunoreactive for CD23. (**c**) The diagnosis of mantle cell lymphoma is supported by nuclear immunoreactivity for cyclin D1.

gene rearrangement and T-cell receptor gene rearrangement is imperative since this process provides the basis for the detection of clonality of B- and T-cells and because it highlights some of the differences in subtypes of lymphomas. Because an in depth discussion of the pathogenesis of lymphoproliferative disorders is enough to fill several textbooks, a select group of non-Hodgkin lymphomas will be reviewed with particular attention to how the pathogenetic mechanisms can be used to classify, diagnose, and in some cases serve as a potential therapeutic targets. Finally, there will be a brief discussion of molecular profiling and microRNAs, two areas in which our understanding of lymphomagenesis stands to grow a great deal in the coming years.

31.2 Physiology of B-Cell and T-Cell Development

31.2.1 Normal B-Cell Development

The B lymphocytes arise from pluripotent stem cells in the bone marrow, which subsequently migrate to secondary lymphoid organs including lymph nodes, spleen, and various mucosal sites (e.g., Waldeyer's ring, Peyer's patches). There are two phases of B-cell development: the antigen-independent phase of B-cell differentiation that occurs in the bone marrow, independent of antigen exposure and giving

rise to naïve B-cells, and the antigen-dependent phase of differentiation that primarily occurs in the extramedullary compartment and secondary lymphoid organs after exposure to antigen [4, 5]. Definable intermediate stages of the antigen-dependent phase have been characterized, ultimately related to the germinal center reaction in secondary lymphoid organs and proliferation of memory B-cells and immunoglobulin-secreting plasma cells.

An orderly progression of morphologic, molecular, and immunophenotypic changes allows recognition of distinct stages of B-cell development from the progenitor B-cells to the terminally differentiated plasma cells. The stages of B-cell development are illustrated in Fig. 31.2. At the molecular level, the genes coding for the immunoglobulin heavy and light chain proteins undergo sequential rearrangements early in B-cell development (Fig. 31.2). Initially, the immunoglobulin mu heavy chain located on chromosome 14q32 undergoes rearrangement, followed by kappa light chain rearrangement on chromosome 2p12 and lambda light chain rearrangement on chromosome 22q11 [6]. Subsequent transcription and translation of the mu heavy chain gene results in the appearance of cytoplasmic mu heavy chain protein, which defines the pre-B-cell stage of development. Antigen-independent differentiation leads to mature, but naïve B-cells that produce intact surface immunoglobulin molecules (surface IgM and IgD), including two heavy and two light chains (Fig. 31.3a). In the lymph node, the primary antigen response leads to development of B-immunoblasts, which are capable of progressing to IgM-secreting mature B-cells. Several days after antigen exposure, the T-cell-dependent germinal center reaction occurs. This complex reaction involves rapid proliferation of surface Ig-negative centroblasts that have switched off the gene encoding the protein bcl-2, rendering them susceptible to death via apoptosis. In addition, expression of bcl-6 protein and somatic mutation of the bcl-6 and immunoglobulin variable region genes (discussed below) occur. As centroblasts mature to centrocytes, mutations in the immunoglobulin variable region (IgV) occur and expression of surface Ig resumes. Centrocytes whose IgV mutations result in immunoglobulins that recognize self-antigens or show decreased antigen affinity undergo programmed cell death. By contrast, centrocytes whose Ig mutations yield immunoglobulins with increased antigen affinity are saved from apoptosis and resume expression of bcl-2. These veterans of the germinal center reaction gives rise to antigen-specific memory B-cells which can subsequently differentiate into plasma cells.

As illustrated in Fig. 31.2, a variety of cellular antigens can be detected at different stages in normal B-cell development. The CD (cluster designation) numbers have been applied to many of these antigens to standardize the nomenclature and allow easier comparison among studies using various antibody clones that recognize the same surface marker. The earliest antigens identified on precursor cells of B lineage are terminal deoxynucleotidyl transferase (TdT), the progenitor cell antigen CD34, the common acute lymphoblastic antigen (CALLA, CD10) and HLA-Dr. However, none of these antigens are B-lineage specific. Specific B-lineage associated antigens, CD19, CD79a, cytoplasmic

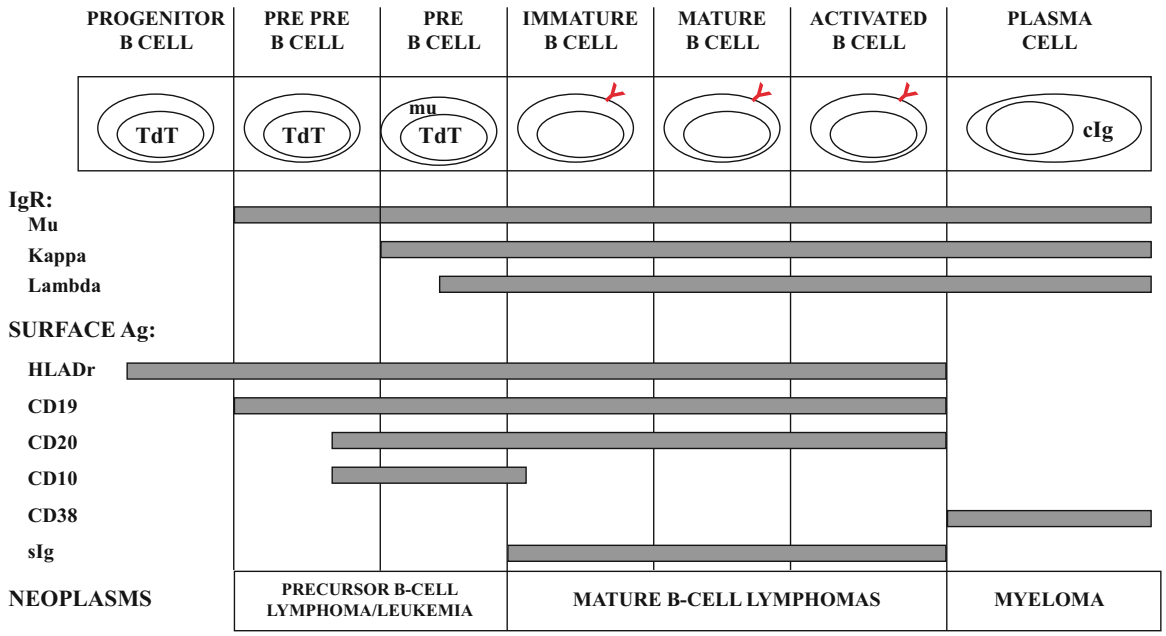

Fig. 31.2 Normal stages of B-cell development with immunological and molecular genetic changes at different stages. See text for discussion. *TdT* terminal deoxynucleotidyl transferase, *mu* cytoplasmic mu heavy chain, *cIg* cytoplasmic immunoglobulin, *IgR* immunoglobulin rearrangement, *sIg* surface immunoglobulin.

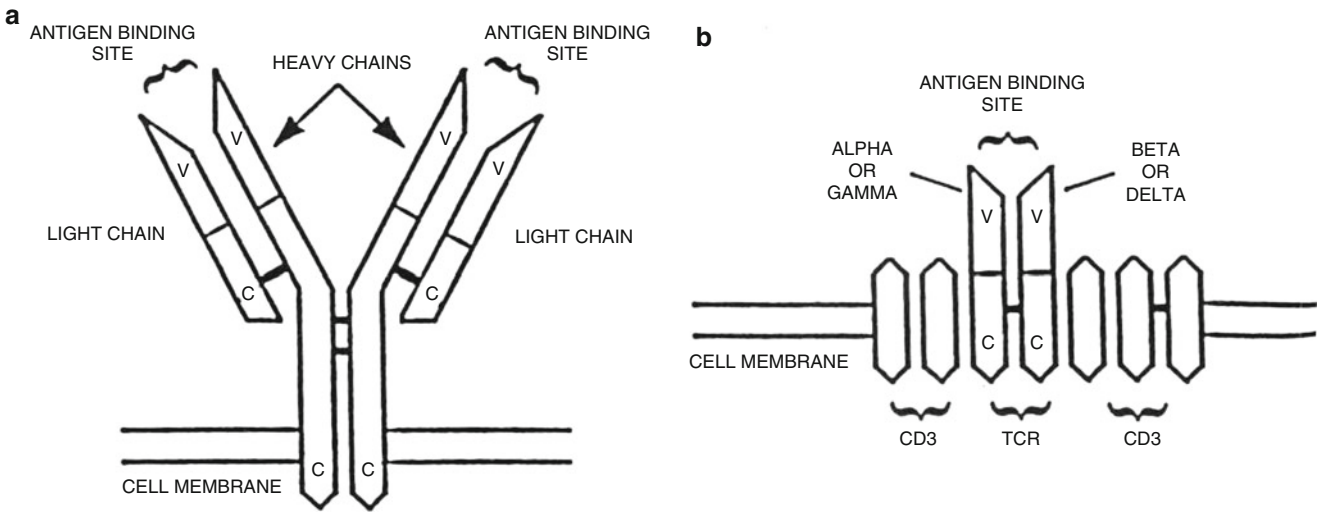

Fig. 31.3 Schematic diagram of immunoglobulin and T-cell receptors. (**a**) The immunoglobulin protein is a heterodimer composed of two heavy and two light chains, each of which has variable (V) and constant (C) regions. (**b**) The T-cell receptor (TCR) is also a heterodimer composed of either one α (alpha) and one β (beta) chain or one γ (gamma) and one δ (delta) chain. Each of the TCR proteins has variable (V) and constant (C) regions. CD3 is a complex of five proteins associated with the TCR.

CD22 and mu heavy chain, and the nuclear transcription factor, B-cell specific activator protein (BSAP, pax-5) are expressed in the pre-B stage of B-cell development [1, 7]. As B-cells mature, there is loss of CD10 and acquisition of the B-cell antigen, CD20 as well as surface immunoglobulin. CD10 is noteworthy in that its expression resumes for the germinal center stage of development, and at the same time, the anti-apoptosis protein, bcl-2 is lost but returns after the germinal center stage. As a B-cell matures to a terminally differentiated plasma cell, the majority of B-cell surface antigens are no longer expressed and CD138 (syndecan-1) appears [8]. The plasma cell is also characterized by presence of abundant cytoplasmic immunoglobulin.

One fundamental theory of lymphoid neoplasia is that disorders of lymphoid cells represent neoplastic transformation of cells at various stages of normal development [9]. This notion has been used in the classification of lymphoid malignancies [1]. For example, precursor B-cell acute lymphoblastic lymphoma/leukemia immunophenotypically mimics normal precursor B-cells by showing expression for TdT, HLA-Dr, CD10, CD19, variable CD20, and (cytoplasmic) mu heavy chain (Fig. 31.2), although the pattern of antigen expression in neoplastic populations differs from that seen in normal precursor B-cells [10]. The mature B-cell neoplasms represent different stages of B-cell development, including mantle cell lymphoma, which represents a neoplasm of CD5-positive naïve B-cells that have not undergone the germinal center reaction [11] and follicular lymphoma, which has an immunophenotype and molecular profile analogous to B-cells at the germinal center stage of development with expression of CD10, bcl-6, and somatic hypermutation of IgH and bcl-6 genes [12]. Plasma cell myeloma is the neoplastic counterpart of the normal plasma cell.

31.2.2 Normal T-Cell Development

T-lymphocytes also arise from pluripotent stem cells in the bone marrow. However, in contrast to B-cell development in which the earliest stages of maturation occur in the bone marrow, progenitor T-cells migrate from the bone marrow to the thymus where the early stages of T-cell development occur [13, 14]. Subsequently, mature T-cells circulate in the peripheral blood and seed peripheral lymphoid tissues, which include paracortical areas of lymph nodes and periarteriolar lymphoid sheaths of the spleen.

Figure 31.4 shows the normal stages of T-cell development in the thymus, which are analogous to B-cell development and occur in an orderly fashion. T-lymphocytes possess a surface membrane protein complex referred to as the T-cell receptor, which is structurally similar to the B-cell surface immunoglobulin receptor (Fig. 31.3b). The T-cell receptor is a heterodimer protein comprised of either one α (alpha) and one β (beta) polypeptide chain, which combine to form the αβ T-cell receptor, or one γ (gamma) and one δ (delta) polypeptide chain, which combine to form the γδ T-cell receptor [15–18]. Approximately 1–5 % of circulating T-cells expresses the γδ T-cell receptor while the remainder expresses the αβ T-cell receptor [18, 19]. T-cells bearing the γδ receptor comprise a greater percentage of T-lymphocytes at mucosal sites and in the spleen. The genes that code for each of the polypeptide chains that make up the T-cell receptor undergo sequential rearrangements early in T-cell development. The first T-cell receptor gene to rearrange is δ, which is followed sequentially by γ, β, and α genes. The genes coding for the α and δ polypeptide chains are located on chromosome 14q11, the β chain gene on chromosome 7q34 and the γ chain gene on chromosome 7p15 [6].

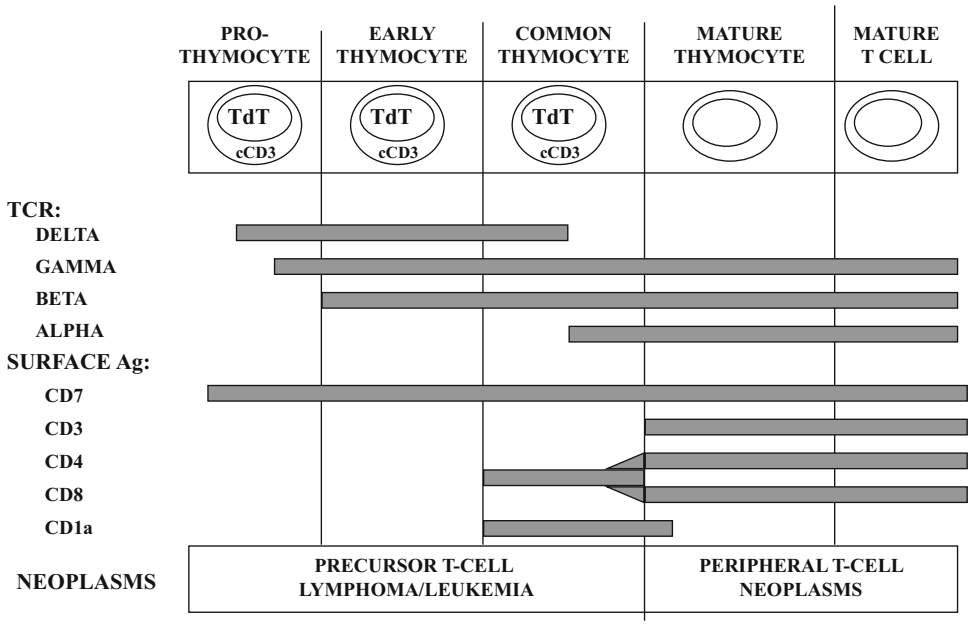

Fig. 31.4 Normal stages of T-cell development with immunological and molecular genetic changes at different stages. See text for discussion. *TdT* terminal deoxynucleotidyl transferase, *cCD3* cytoplasmic CD3, *TCR* T-cell receptor rearrangements, *Ag* antigen.

Analogous to developing B-cells, a variety of cellular antigens can be detected at different stages of normal T-cell development (Fig. 31.4). The earliest antigens expressed are TdT and the T-lineage antigen, CD7. The CD3 antigen, which is a protein complex associated with the T-cell receptor (Fig. 31.3b), is primarily a cytoplasmic antigen early in T-cell development and only appears on the cell surface later in development at the same time as the T-cell receptor β chain. The earliest thymocytes lack expression of CD3, CD4, and CD8, but as development progresses, the developing T-cells acquire CD1a, CD2, and CD5. The common thymocyte stage of T-cell development is characterized by coexpression of the CD4 (helper/inducer) and CD8 (cytotoxic/suppressor) T-cell subset antigens. Phenotypically mature T-cells (also referred to as peripheral T-cells) lack TdT, lack CD1a and express surface CD3 with either CD4 or CD8, but not both. After leaving the thymus, naïve T-cells home to the paracortex of lymph nodes and periarteriolar lymphoid sheaths of the spleen. T-cells undergo antigen-dependent differentiation similar to B-cells with evolution through a T-immunoblast stage, giving rise to antigen-specific T-helper cells (CD4 positive), T-suppressor cells (CD8 positive) which show coexpression of either αβ or γδ T-cell receptor [16].

Similar to B-cell non-Hodgkin lymphoma, the T-cell neoplasms represent neoplastic transformation of cells at various stages of normal T-cell development [9] although the correlation is not quite as well characterized as it is for the mature B-cell neoplasms. For example, precursor T-cell lymphoblastic lymphoma/leukemia may exhibit an immunophenotype which resembles the normal common thymocyte, showing expression for TdT, CD1a, cytoplasmic CD3 and CD7, as well as coexpression for CD4 and CD8 (Fig. 31.4). Examples of T-cell non-Hodgkin lymphoma, which are neoplastic counterparts to relatively mature or peripheral T-lymphocytes, include anaplastic large cell lymphoma, cutaneous T-cell lymphoma (mycosis fungoides), and peripheral T-cell lymphoma [1].

31.2.3 B-Cell Surface Immunoglobulin and T-Cell Receptor Gene Rearrangement

The B-cell surface immunoglobulin receptor and T-cell receptor are involved in the process of antigen recognition by normal B-cells and T-cells, respectively. These receptors are structurally similar being heterodimer proteins linked by disulfide bonds and are composed of both variable (V) and constant (C) regions [6] (Fig. 31.3). The variable regions of these proteins are similarly involved in antigen recognition. The constant region of the immunoglobulin heavy chain protein defines the different immunoglobulin subclasses (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM, IgD, and IgE) [5]. The genes that code for the B-cell and T-cell receptors are also structurally similar and consist of a large number of exons or coding sequences referred to as a *supergene family*. The exons undergo a process of DNA recombination or rearrangement leading eventually to transcription and translation and the production of functional receptor proteins [5, 6, 15, 18, 20].

A general scheme illustrating the process of B-cell surface immunoglobulin and T-cell receptor gene rearrangement is shown in Fig. 31.5. The *germline configuration* refers to non-rearranged DNA. The exons which code for the variable regions of the immunoglobulin and T-cell receptors are referred to as variable (V) segments, diversity (D) segments, and junctional (J) segments, and those which code for the constant regions are referred to as (C) segments. The process of gene rearrangement first involves the selective apposition

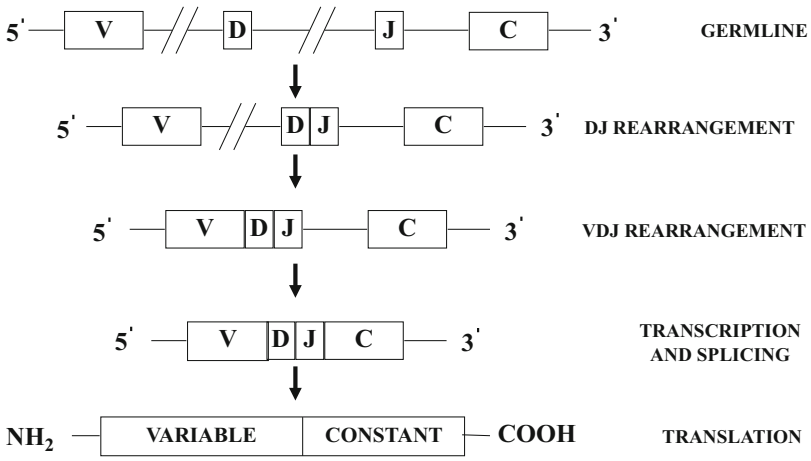

Fig. 31.5 Schematic diagram illustrating the sequential steps involved in immunoglobulin and T-cell receptor gene rearrangements. See text for discussion. *V* variable segments, *D* diversity segments, *J* junctional segments, *C* constant segments.

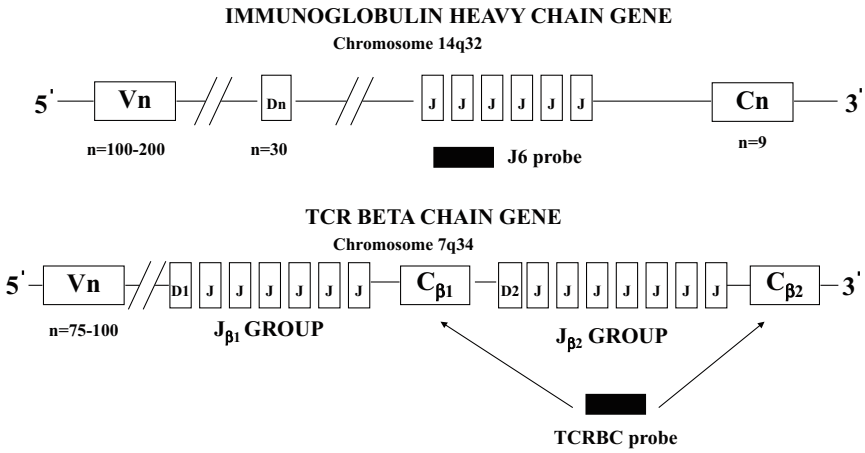

Fig. 31.6 Schematic diagram of the immunoglobulin heavy chain and the T-cell receptor beta chain supergene families. The specific sites of recognition for probes used for Southern blotting are typically associated with consensus sequences in the joining (J_6 probe) and constant regions (TCRBC probe).

of one D segment with one J segment by deletion of the intervening coding and noncoding DNA sequences resulting in a DJ rearrangement. A similar process of rearrangement opposes a V segment, located in the 5′ direction to D and J to form a VDJ rearrangement. Transcription to messenger RNA (mRNA) then occurs even though the rearranged VDJ segments are not yet directly opposed to C segments, which are remotely located in the 3′ direction. Subsequent splicing of the mRNA with deletion of noncoding sequences results in apposition of VDJ with C to form a VDJC mRNA, which can then be translated into a surface immunoglobulin or T-cell receptor protein.

The genes coding for the immunoglobulin heavy chain proteins and T-cell receptor beta and delta chain proteins include V, D, J, and C segments. The genes coding for the kappa and lambda light chain proteins and the T-cell receptor alpha and gamma proteins include only V, J, and C segments without D segments [5, 6, 20]. A schematic diagram of the immunoglobulin heavy chain and the T-cell receptor beta chain supergene families is shown in Fig. 31.6. The immunoglobulin heavy chain locus contains 123 V_H segments, 27 D segments, 6 J_H segments [21]. The V_H segments, which include 79 pseudogenes and 44 segments that have an open reading frame, can be further divided into 7 families based on the presence of conserved or homologous DNA sequences, which are common to each member of a family. The V_H families are designated V_H1 to V_H7. The T-cell receptor beta chain gene includes 75–100 V_β segments, which can be further divided into several families, and two tandem DJC complexes referred to as D1J1C1 and D2J2C2. Each DJC complex contains one D segment and one C segment. The first DJC complex contains six J_β segments ($J_{\beta1}$ group) and the second DJC complex contains seven J_β segments ($J_{\beta2}$ group) [6, 15, 20].

The humoral (B-cell) and cell mediated (T-cell) systems must be able to recognize a wide variety of environmental antigens even though only a finite quantity of DNA is present in each cell. The complex process of DNA recombination or rearrangement involving the surface immunoglobulin and T-cell receptor allows for tremendous diversity of both the humoral and cell mediated systems, and hence, the ability of these systems to detect a wide variety of antigens [5, 6, 15, 18, 20]. The large number of V, D, J, and C segments results in many combinations, which can be transcribed and translated to millions of different antigen receptors.

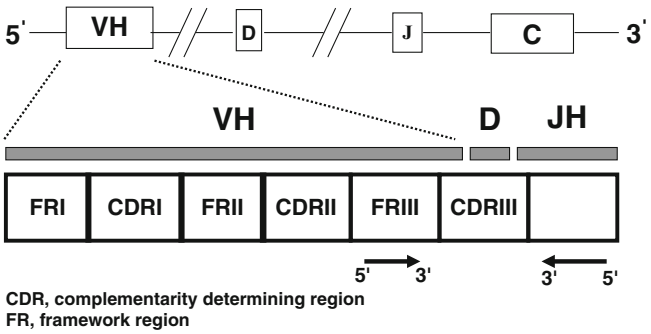

Fig. 31.7 Schematic diagram illustrating the rearranged immunoglobulin heavy chain region. Variable primer sets targeting the complementarity-determining regions or framework regions are used for polymerase chain reaction and southern blot studies to determine B-cell clonality. *Arrowheads* identify restriction enzyme cleavage sites.

Further diversification of the immunoglobulin receptor genes is achieved through a process of *somatic hypermutation*, which occurs normally within germinal center B-cells. In addition to conserved or homologous DNA sequences within V segments referred to as *framework regions* (FR), individual immunoglobulin heavy and light chain V segments contain *complementarity-determining regions* (CDR), which contain nucleotide sequences that encode for the antigen-binding site (Fig. 31.7). The CDRs contain hypervariable nucleotide sequences, which tend to undergo somatic hypermutation. This process results in a series of DNA point mutations with consequent amino acid substitutions within the antigen-binding site. The end product of somatic hypermutation involving the CDRs is enhanced antibody diversity, affinity, and specificity for target antigens [5, 6, 15, 18, 20].

31.3 Molecular Mechanisms in Non-Hodgkin Lymphoma

31.3.1 Background

Non-Hodgkin lymphoma comprises a heterogeneous group of lymphoid neoplasms, which occur because of neoplastic transformation of lymphocytes at different stages of B-cell and T-cell development [4, 13]. The wide variety of B-cell and T-cell non-Hodgkin lymphomas reflects the varying stages of lymphocyte development and the complexity of the immune system. The clinical and pathological characteristics of the non-Hodgkin lymphomas are summarized in a comprehensive manner in the World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues (WHO Classification) [1].

Centrally important to establishing a diagnosis of non-Hodgkin lymphoma is the identification of an abnormal clonal lymphoid population since B-cell and T-cell non-Hodgkin lymphomas represent monoclonal proliferations of B-cells and T-cells, respectively. A major application of molecular genetic methods (e.g., PCR, Southern blot) in the evaluation of non-Hodgkin lymphoma involves the determination of B-cell and T-cell clonality. These methods are considered to be the gold standard for determining clonality but are utilized primarily when a definitive diagnosis (malignant or benign) cannot be determined by morphologic or immunopathologic methods. For B-cell neoplasms, clonality can often be determined immunopathologically by demonstrating the presence of monoclonal surface immunoglobulin [22]. In contrast, for T-cell malignancies, there is no immunopathological equivalent to monoclonal surface immunoglobulin, although qualitative or quantitative loss of normal T-cell antigen expression is considered to be presumptive evidence of T-cell neoplasia [22]. Thus, molecular genetic approaches for the determination of clonality in T-cell non-Hodgkin lymphoma are especially important.

Other uses for molecular genetic techniques in the assessment of lymphoid malignancies include determination of B-cell or T-cell lineage, detection of chromosomal translocations, and detection of minimal residual disease. The latter application has become increasingly useful in evaluating patients before and after bone marrow transplantation particularly in acute lymphoblastic leukemias [23]. The clinical significance of minimal residual disease detection in chronic lymphoproliferative disorders remains a matter of debate and warrants more study [24, 25]. The detection of a specific chromosomal translocation may be very useful in the subclassification of non-Hodgkin lymphoma. For example, in a lymph node with suspected follicular lymphoma, the detection of a translocation, t(14;18), involving the *BCL2* proto-oncogene, would confirm this diagnosis. Similarly, in the appropriate context, the detection of a translocation, t(11;14), involving the *CCND1* proto-oncogene, would confirm a diagnosis of mantle cell lymphoma or a t(8;14) involving the c-*MYC* oncogene would support a diagnosis of Burkitt lymphoma [26].

There are many potential mechanisms of carcinogenesis and lymphomagenesis. In some instances, the details of the pathways involved in the transformation of a benign cell to a malignant one are generally well understood, but in most the pathogenesis is a matter of ongoing study. Some diseases can almost be defined by a single, recurrent cytogenetic or molecular alteration that leads to dysregulation of the cell cycle and proliferation of abnormal cells. For example, in mantle cell lymphoma and Burkitt lymphoma, the alteration involves placement of a normally carefully regulated gene that promotes cell growth and division (*CCND1* and *MYC*, respectively) under the regulatory control of the constitutively active immunoglobulin heavy chain gene. In a similar fashion, the common molecular rearrangement in follicular lymphoma puts the normally tightly controlled expression of

the *BCL2* gene under the control of the immunoglobulin heavy chain gene. However, in this case, the product of the upregulated *BCL2* gene does not lead to cell proliferation but rather a blockade of apoptosis, again culminating in an uncontrolled overgrowth of cells. Marginal zone B-cell lymphoma is associated with multiple different translocations that all lead to dysregulation of the NF-kB pathway which plays many roles in cell proliferation and differentiation. The mechanisms involved in the production of functional immunoglobulins (e.g., VDJ recombination, somatic hypermutation, immunoglobulin class switching) provide the molecular milieu for development of many of the familiar translocations in B-cell lymphomas [27].

In contrast, relatively few of the T-cell lymphoproliferative disorders have recognizable recurrent genetic abnormalities. One that does is T-cell prolymphocytic leukemia, which has a mechanism similar to the B-cell lymphoproliferative disorders listed above, placing an oncogene under control of the promoters of the T-cell receptor α/β locus. In the case of anaplastic large cell lymphoma (ALCL), the creation of a novel fusion protein (derived from a translocation involving nucleophosmin (*NPM*) and anaplastic lymphoma kinase (*ALK*) genes) with a constitutively active tyrosine kinase domain drives cell proliferation. Another T-cell lymphoma, adult T-cell leukemia/lymphoma (ATLL) has a different mechanism of oncogenesis, being caused by a human retrovirus, HTLV-1.

One thing that has become clear with our understanding of these molecular hallmarks of disease (e.g., *IGH/BCL2* rearrangement in follicular lymphoma, *NPM/ALK* rearrangement in ALCL, HTLV-1 infection in ATLL) is that the presence of the t(14;18), t(2;5), or HTLV-1 infection should be regarded as "required but not sufficient" for malignant transformation. This conclusion must be reached, as many normal subjects can be shown to have molecular evidence of these rearrangements while never manifesting signs of the disease. The discussion of these lymphoproliferative disorders will examine how these molecular alterations can lead to cell cycle disruption and some possible additional hits that may help produce a clinical lymphoma, as well as touch on how the use of molecular diagnostic tests can be used to diagnose and follow these disorders.

31.3.2 Mantle Cell Lymphoma (t(11;14)(q13;q32), *GH-CCND1*)

Mantle cell lymphoma (MCL) is a pathologically distinct B-cell lymphoproliferative disorder [28] that is characterized by upregulation of cyclin D1, most often due to the presence of a translocation involving the *BCL1* (B-cell leukemia/lymphoma-1) region on chromosome 11q13 and the *IGH* gene on chromosome 14q32. MCL makes up between 5 and 10 % of all B-cell NHL and shows a male predominance. MCL occurs over a wide age range, but is more prominent in older patients with a median age of approximately 60 years. Compared to other B-cell non-Hodgkin lymphomas composed of small lymphocytes, MCL has a poor prognosis with a median survival time of 3–5 years even with aggressive therapy.

Classic cases show effacement of lymph node architecture by a population of small to medium-sized lymphocytes with irregular nuclear contours, evenly dispersed chromatin and scant cytoplasm. A population of nucleolated cells is distinctly absent in typical cases. Scattered mitotic figures and solitary epithelioid macrophages are identified (Fig. 31.8a, b). The neoplastic cells can be distributed in a variety of patterns, including nodular pattern, diffuse pattern, and a mantle zone pattern. Several studies have reported an association between the growth pattern and prognosis [29–31]. Occasional cases of otherwise typical mantle cell lymphoma show moderate to abundant cytoplasm, resembling marginal zone B-cells. Plasma cell differentiation is not typical of mantle cell lymphoma, but has been reported. A more aggressive blastoid variant is characterized by larger cells with more open chromatin, scant cytoplasm, and variably prominent nucleoli (Fig. 31.8c). Leukemic presentations of mantle cell lymphomas also occur, and may present with a marked peripheral blood leukocytosis, raising a differential diagnosis with prolymphocytic leukemia.

Flow cytometric immunophenotyping shows that the neoplastic cells in MCL express the B-cell associated antigens CD19, CD20, CD22, and FMC-7. Because this is a lymphoma of mature B-cells, the neoplastic cells express surface immunoglobulins, often IgM and IgD heavy chains and moderate to bright kappa or lambda light chains. One of the phenotypic hallmarks of MCL is coexpression of the T-cell associated antigen, CD5 without coexpression of CD23, CD10, or bcl-6. This immunophenotypic profile supports the notion that mantle cell lymphomas are derived from a population of CD5-positive naïve pre-germinal center B-cells.

Recognition of t(11;14)(q13;q32) as the genetic hallmark of MCL has greatly improved reproducibility in diagnosis and allowed greater study of this disorder [32]. This translocation involves the *BCL1* locus on chromosome 11 and the immunoglobulin heavy chain locus on chromosome 14, with the result that the *CCND1* gene (formerly known as *PRAD1* because of its association with parathyroid adenoma), which encodes for the protein cyclin D1, comes under regulatory control of the immunoglobulin heavy chain enhancer. This translocation leads to overexpression of the cyclin D1 protein, which is not typically expressed at high levels in B lymphocytes [33]. The cyclin D1 mRNA produced as a result of this translocation has two main forms, each of which contains the complete coding region for a 36 kDa form of cyclin D1: a 4.5 kb form with a long 3′ untranslated region and a 1.5 kb form that lacks the long 3′ untranslated region. Cyclin D1 promotes the transition of cells from the G1 phase into the S phase of the cell cycle by binding to cyclin-dependent kinases 4 and 6 (CDK4 and

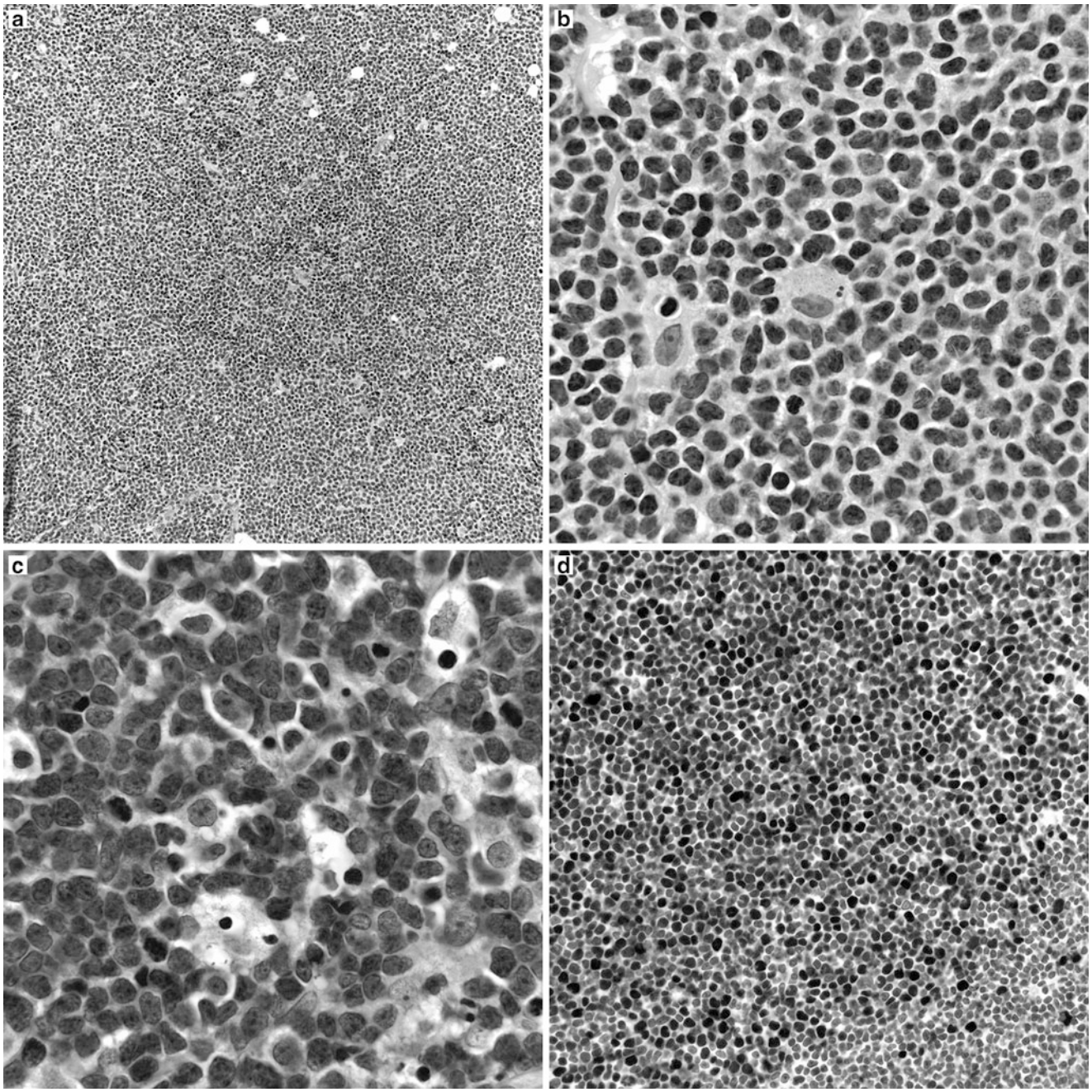

Fig. 31.8 Morpohologic features of mantle cell lymphoma. (**a**) Low magnification image showing a vaguely nodular pattern of growth. (**b**) Classical mantle cell lymphoma cytology with small to medium cells showing slightly irregular nuclear contours, inconspicuous nucleoli, and scant cytoplasm. Scattered epithelioid macrophages are present. (**c**) Blastoid variant of mantle cell lymphoma with larger, nucleolated nuclei and more open chromatin. (**d**) Cyclin D1 immunohistochemistry highlighting nuclear immunoreactivity in the neoplastic cells.

CDK6). These complexes of cyclin D1 and CDK4 or CDK6 lead to phosphorylation of retinoblastoma 1 (Rb1), eliminating its suppressive effects on the cell cycle by leading to the release of the transcription factor E2F and progression into the S phase. Other molecular pathways are affected including downregulation of the *TP53* tumor suppressor gene [34]. Because the t(11;14) with cyclin D1 overexpression is found in virtually all cases, it is felt to be one of the early events in the development of mantle cell lymphoma [35]. However, since it can be seen in normal individuals without MCL and since mouse models with the classic t(11;14) do not develop mantle cell lymphoma without other genes also being affected, it is also felt that this is not sufficient for full transformation. Additional genetic hits are common in MCL, and cytogenetic studies have demonstrated that MCL has one of the highest levels of genetic instability among lymphoproliferative disorders. Many of the additional genetic abnormalities seen in mantle cell lymphoma seem to target either cell cycle regulation (e.g., through deletions of

chromosome 9p21 resulting in the loss of two genes, the CDK inhibitor $p16^{INK4a}$ (interferes with Rb1 function) and $p14^{ARF}$ (downregulates the p53 pathway)) or the DNA damage response (mutations of the *ATM* (ataxia-telangiectasia mutated) gene, which has a role in the cellular response to DNA damage) [33, 34] (Fig. 31.9). Better understanding of the molecular mechanisms leading to mantle cell lymphoma may lead to therapeutic agents that directly target these various pathways [36].

Some groups have asserted that cyclin D1 positivity should be a part of the definition of mantle cell lymphoma [37]. However, by gene expression profiling, a small subset of cyclin D1-negative mantle cell lymphomas has been identified, which have the usual morphologic features and unique gene expression signature of mantle cell lymphoma [38]. This report suggests that other cyclin D proteins may substitute for cyclin D1 in lymphomagenesis in such cases. Like cyclin D1, other members of the cyclin D family of proteins are involved in cell cycle control, particularly as regulators of progression from the G1 to S phase of the cell cycle.

The diagnosis of MCL is based on a combination of morphologic immunophenotypic, cytogenetic, and clinical data. Identification of cyclin D1 overexpression by immunohistochemical analysis (Fig. 31.8d) has been shown to be a specific and sensitive marker for MCL [39]. Immunohistochemical markers for proliferation (especially Ki-67) have been shown to be of prognostic value [29, 40], a finding shown to be associated with a distinct genetic signature [41]. Classical cytogenetic studies allow identification of the t(11;14) (Fig. 31.10a). Since the specific breakpoints in the *BCL1* region are spread over a wide area (Fig. 31.10b), PCR analysis for a specific breakpoint is not as sensitive as other techniques in detecting the rearrangements. FISH analysis has been shown to be a very sensitive marker for detection of the t(11;14) in MCL [42]. Again, it should be borne in mind that while detection of cyclin D1 abnormalities is quite sensitive for mantle cell lymphomas, there seems to be a subset of MCL cases that lack a t(11;14) and cyclin D1 overexpression [38]. It should also be noted that these findings are not entirely specific to MCL). For example, cyclin D1 overexpression by immunohistochemical analysis is seen in hairy cell leukemia, and some cases of plasma cell myeloma not only show overexpression of cyclin D1 by immunohistochemical analysis but even exhibit an identical t(11;14).

31.3.3 Follicular Lymphoma (t(14;18)(q32;q21), *IGH-BCL2*)

Follicular lymphoma (FL) is the second most common NHL seen in the United States, after diffuse large B-cell lymphoma, accounting for greater than 20 % of NHL [43]. Similar to MCL, there is a hallmark cytogenetic abnormality that involves placing a gene whose expression is normally tightly regulated under the control of the immunoglobulin heavy chain promoters. This translocation involves the *BCL2* region located at chromosome 18q23. Unlike cyclin D1 in MCL, this gene does not regulate cell proliferation, but rather serves as an inhibitor of programmed cell death (apoptosis). Follicular lymphoma is a disease predominantly of adults, with a median age in the sixth decade and uncommonly occurring in patients under the age of 20. Morphologic grading of follicular lymphoma allows separation

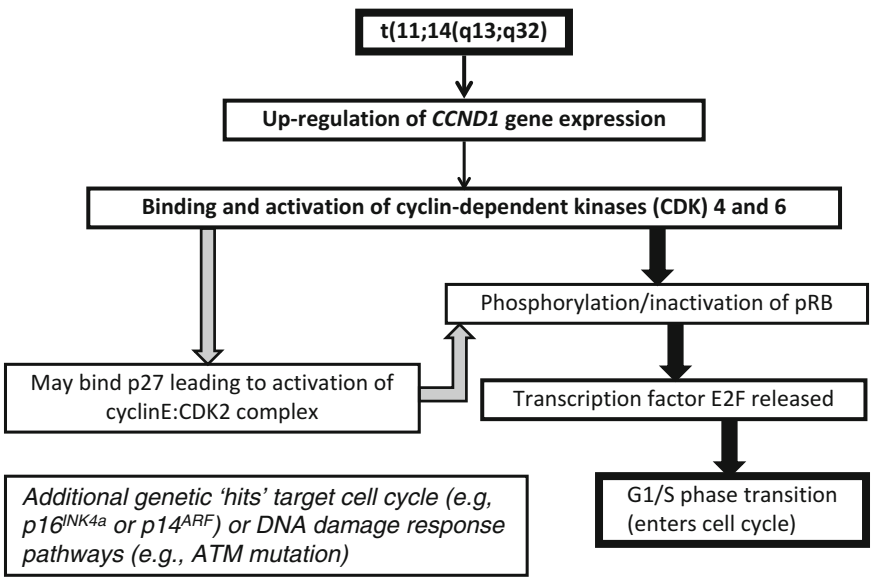

Fig. 31.9 Molecular mechanism in mantle cell lymphoma. The t(11;14)(q13;q32) leads to overexpression of cyclin D1 (*CCND1*), which binds to cyclin-dependent kinases (CDK) 4 and 6. This leads to over-phosphorylation of pRB, releasing the transcription of E2F and progression from the G1 to S phases of the cell cycle. Deregulation of pRB via other cyclins is also possible. Subsequent genetic abnormalities target the cell cycle and DNA repair pathways.

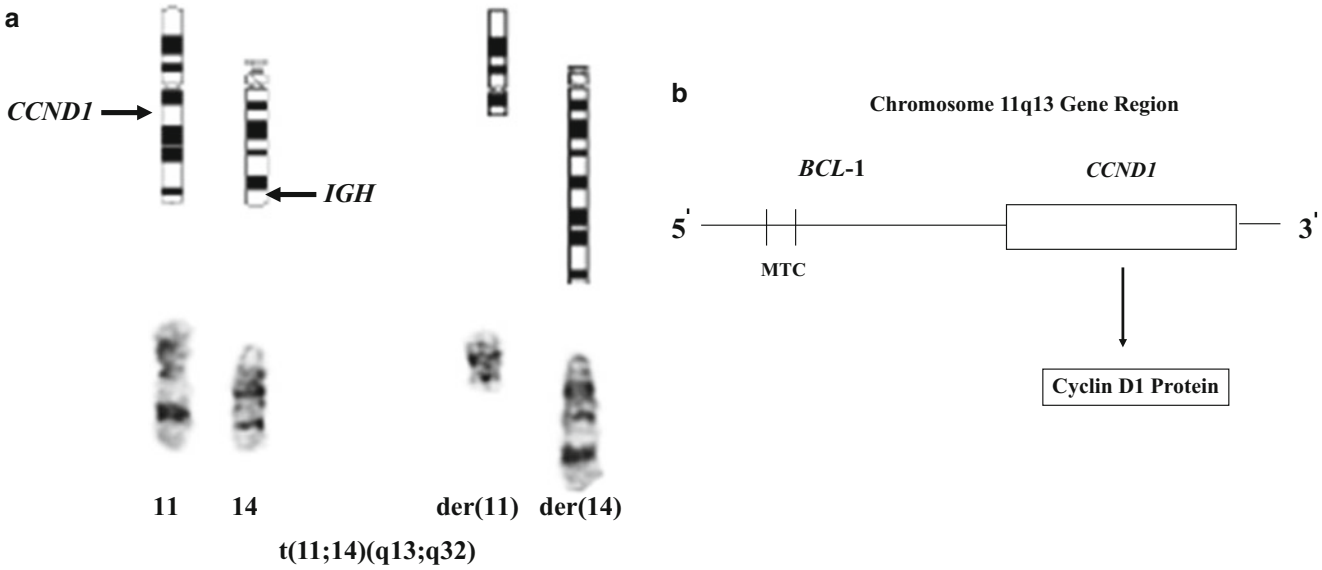

Fig. 31.10 Cytogenetics of mantle cell lymphoma. (**a**) Derivative chromosomes produced by the t(11;14) translocation (Giemsa-stained chromosomes and ideogram). (**b**) Schematic diagram of the chromosome 11q13 gene region, which includes the *BCL1* locus and the *CCND1* gene. The breaks in the 11q13 gene region are widely scattered within the *BCL1* locus; however, approximately 30–40 % of breaks occur within the MTC (major translocation cluster) region of the *BCL1* locus. The *CCND1* gene encodes for the cyclin D1 protein.

into more indolent forms (grades 1 and 2), which have a median survival of up to 10 years and more aggressive forms (grades 3A and 3B) which have a median survival of less than 5 years.

Follicular lymphoma commonly presents as lymphadenopathy and excised lymph nodes show effacement of normal lymph node architecture by closely packed follicles with attenuated or absent mantle zones. At low magnification, neoplastic follicles lack the normal light zone/dark zone polarization seen in reactive germinal centers (Fig. 31.11a). The cytologic features in follicular lymphomas vary considerably, but the most common finding is a predominant population of small to medium-sized lymphocytes with angulated or cleaved nuclei having clumped chromatin and inconspicuous nucleoli. These small cleaved cells are called centrocytes and have scant cytoplasm, although they occasionally have monocytoid differentiation with more abundant cytoplasm or exceptionally exhibit a signet ring appearance (Fig. 31.11b). Also present in follicular lymphomas are scattered large cells with vesicular chromatin, one or multiple nucleoli often adjacent to the coarse, thickened nuclear envelope, and moderate amounts of amphophilic cytoplasm. It is important to recognize these large centroblasts because the number of centroblasts per high power field forms the basis for morphologic grading. Cases with fewer than 15 centroblasts per high power field are considered grade 1–2 (Fig. 31.11c) and those with greater than 15 centroblasts per high power field is classified as grade 3 [44] (Fig. 31.11d). Grade 3 follicular lymphomas are further subdivided into grade 3A, which shows admixed small centrocytes, and Grade 3B which is composed of sheets of centroblasts without admixed centrocytes. Although the predominant pattern of growth is often follicular, some cases have areas with a diffuse pattern of growth. Even in otherwise typical cases of FL, areas with large sheets of centroblasts present in a diffuse pattern should be diagnosed as diffuse large B-cell lymphoma. Admixed follicular dendritic cells and T-cells are present in varying numbers, a finding that has received a lot of recent attention since molecular studies show that benign components of lymphoma may be associated with outcome [45].

Morphologically and immunophenotypically, the cells of follicular lymphoma resemble those of the normal follicle center. These are mature B-cells that show expression of CD19, CD20, and CD22 along with coexpression of CD10. Also like their normal follicle center cell counterparts, they show expression of bcl-6. Follicle center cells do express surface immunoglobulin, although this may be downregulated to very low levels. Mitotic activity in low-grade follicular lymphomas is usually decreased relative to that in normal reactive follicles.

Follicular lymphomas are characterized by a recurrent translocation involving the *BCL2* gene located at chromosome 18q21 and the immunoglobulin heavy chain locus at chromosome 14q32 (Fig. 31.12). This places production of the anti-apoptotic gene, *BCL2* under the regulation of *IGH* enhancers, resulting in overproduction of a structurally intact and functional bcl-2 protein. Physiologically, bcl-2 expression is present in naïve B-cells but is downregulated as these cells enter the germinal center. If somatic immunoglobulin hypermutation in the germinal center results in antibodies with poor antigen affinity or avidity or antibodies that recognize self-antigens, the lack of bcl-2 permits the cell to undergo apoptosis. However, if the process leads to increased

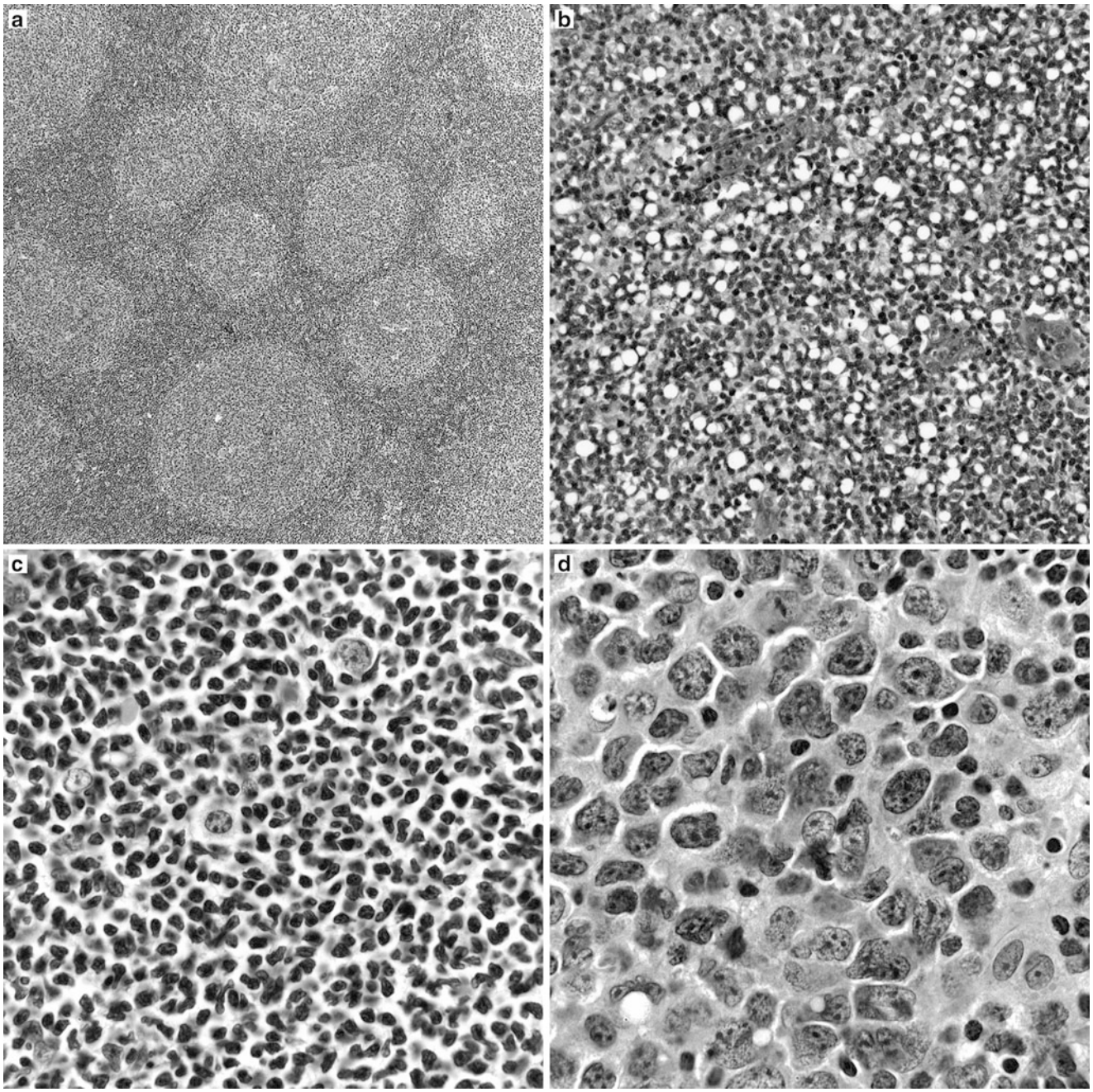

Fig. 31.11 Follicular lymphoma. (**a**) Low-magnification image showing closely packed neoplastic follicles. (**b**) Signet-ring cell forms containing cytoplasmic immunoglobulin are seen in an unusual morphologic variant of follicular lymphoma. (**c**) Cytologic findings including numerous cleaved cells within the neoplastic follicles and rare large cells with multiple peripherally arrayed nucleoli (centroblasts) (grade 1). (**d**) Cytologic features in grade 3 follicular lymphoma with numerous centroblasts.

antibody affinity for non-self-antigens, the cell will reactivate bcl-2 and escape programmed cell death.

The constitutive overexpression of bcl-2 protein in abnormal follicle center cells due to the t(14;18) represents an early and important step in the development of follicular lymphoma. The translocation occurs during V(D)J rearrangement at the pre-B-cell stage of development [46], but the cells ultimately mature and expand in germinal centers [47]. However, t(14;18) is not the sole abnormality required for transformation. This assertion is supported by the fact that a high percentage of normal individuals who never develop follicular lymphoma can be shown to harbor this translocation in low numbers of cells [48]. In addition, experimental mouse models with this same translocation first develop follicular hyperplasia but only later, presumably after additional genetic changes, progress to follicular lymphoma [49].

These t(14;18) positive cells in normal individuals do have a survival advantage over other follicle center cells and

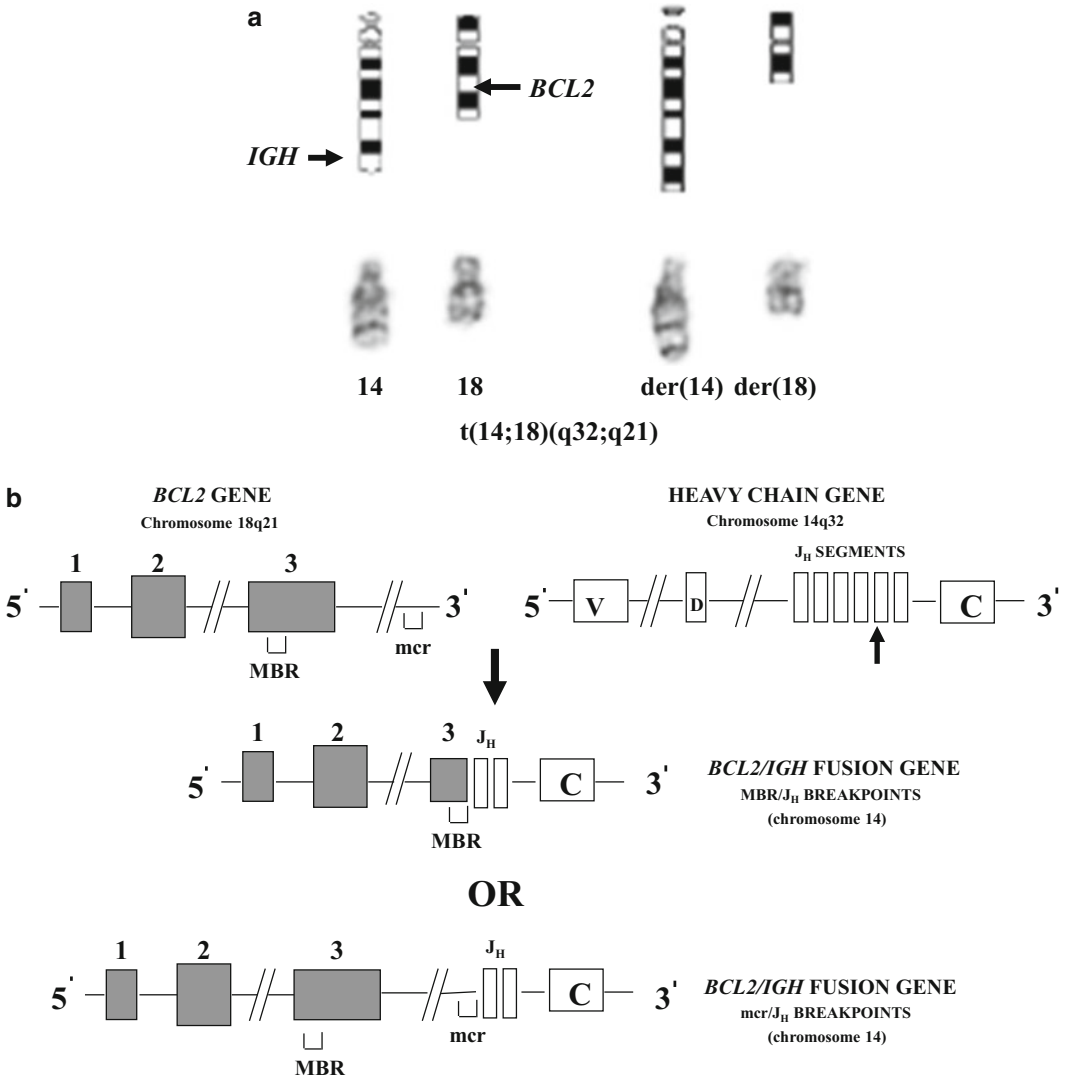

Fig. 31.12 Cytogenetics of follicular lymphoma. (**a**) Derivative chromosomes produced by the t(14;18) translocation (Giemsa-stained chromosomes and ideogram). (**b**) Schematic diagram showing the translocation of *BCL2* from chromosome 18 (*shaded boxes*) to the heavy chain gene (IgH) on chromosome 14 (*open boxes*) resulting in a *BCL2*/*IGH* fusion gene. For *BCL2*, most breaks occur in either the MBR or mcr regions. Breakpoints in the heavy chain gene involve J_H segments (*arrowhead*). The translocation may involve MBR and J_H breakpoints (*middle panel*) or mcr and J_H breakpoints (*lower panel*).

can undergo multiple rounds of rearrangement and somatic mutation in the germinal center. It is tempting to speculate that the additional time in the follicle center predisposes those cells to accumulation of additional genetic damage. Although these further genetic steps are thought to be required to complete transformation to follicular lymphoma, there are few secondary genetic alterations that occur in a significant proportion of follicular lymphomas, and this stepwise progression is currently ill-defined [50]. Several chromosome abnormalities were seen in FL in greater than 20 % of cases in one series (loss of 1p32-36, loss of 6q11-27, +7, +12, and + X) [51], but the specific genes involved have not been elucidated. It is noteworthy that a small number of follicular lymphomas (especially among those with grade 3B morphology) do not show a t(14;18) but rather have alterations in *BCL6* on chromosome 3q27. Histological and clinical progression of FL can be associated with a variety of molecular alterations including some that are also seen in mantle cell lymphoma via *CDKN2A* ($p16^{INK4a}$-ARF), *MYC* rearrangements, or mutations of *BCL6* or *TP53* [50].

Recent studies investigating the role of the microenvironment in the development and progression of FL have shown that the neoplastic cells have close interactions with T-cells, follicular dendritic cells (FDCs), macrophages, and other stromal cells. The stromal cells in some FL have a phenotype typical of FDCs in normal germinal centers, while in others, there is an absence of most FDC markers. This phenotype did not correlate with nodularity or architectural pattern, but it did correlate with the density of T-cells within neoplastic follicles. Those cases in which FDCs expressed normal germinal

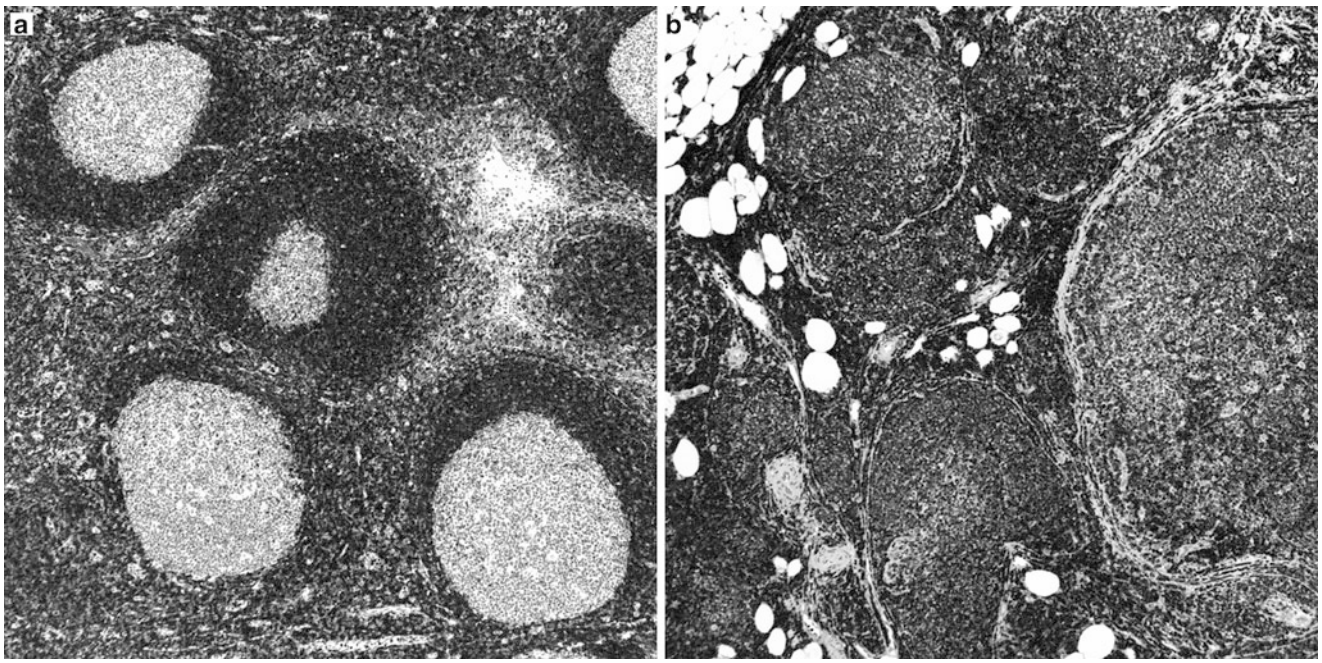

Fig. 31.13 BCL-2 expression in normal and neoplastic follicles. (**a**) Bcl-2 immunohistochemical study on a reactive lymph node showing absence of BCL-2 in reactive follicle centers. (**b**) Bcl-2 immunohistochemical study shows that virtually all cells, including those in follicle centers, are immunoreactive in this case of follicular lymphoma.

center type FDC markers had higher numbers of intrafollicular T-cells. It is speculated that follicular lymphomas with extranodal spread, which have few FDCs, may have lost their requirement for mature FDC or germinal center T-cell signals for growth. The loss of these features in a patient with follicular lymphoma may herald early disease progression [52]. In addition, molecular profiling studies of follicular lymphoma patients showed that survival in follicular lymphoma patients was correlated with molecular features at diagnosis and had little to do with subsequent acquisition of oncogenic abnormalities. Instead, the gene expression profile that best predicted survival was derived from non-malignant cells in the tumors [45]. Other studies have suggested that increased numbers of lymphoma associated macrophages were associated with a worse prognosis [53] although the predictive value of this parameter may not hold true in the setting of rituximab (monoclonal anti-CD20) therapy [54].

The diagnosis of FL can be made solely on morphologic grounds, although correlation with immunophenotypic and immunohistochemical findings is often employed. The expression of bcl-2 in follicle centers helps distinguish benign and malignant follicles (Fig. 31.13a, b). Expression of bcl-2 protein is not specific for follicular lymphoma, since bcl-2 is also expressed in normal T-cells, in non-germinal center B-cells and in other types of lymphoproliferative disorders. This marker is expressed only in about 90 % of FL cases and in the appropriate morphologic or immunophenotypic setting, lack of bcl-2 expression does not exclude a diagnosis of FL. FISH analysis for a *BCL2* rearrangement can be useful and is more sensitive than PCR in detecting the rearrangement [55, 56]. PCR analysis for immunoglobulin gene rearrangements can yield false negative results in FL, depending on the primer sets used. This is in part due to the high degree of somatic hypermutation present in cases of FL. Using more comprehensive BIOMED-2 primer sets and protocols yielded improved sensitivity by assessing IgH as well as Igk and Igλ probes. Of note, analysis for T-cell receptor gene rearrangements also highlighted a clonal T-cell gene rearrangement in 13 % of 109 cases tested [57]!

31.3.4 Extranodal Marginal Zone B-Cell Lymphoma, MALT type (t(11;18)(q21;q21), *API2-MALT1*; t(14;18)(q32;q21), *IGH-MALT1*; t(1;14)(p22;q32), *IGH-BCL10*; t(3;14)(p14.1;q32), *IGH-FOXP1*)

Marginal zone B-cell lymphomas (MZBCL) comprise a heterogeneous group of typically indolent B-cell lymphomas that make up approximately 7–8 % of all cases of NHL. Although there is morphologic overlap between the various forms of MZBCL, there are significant differences in clinical presentation, cytogenetic findings, and to a degree in immunophenotype that warrant their separation in the WHO classification [1]. The most commonly encountered forms of marginal zone B-cell lymphomas are those associated with mucosa-associated lymphoid tissue (MALT). Splenic

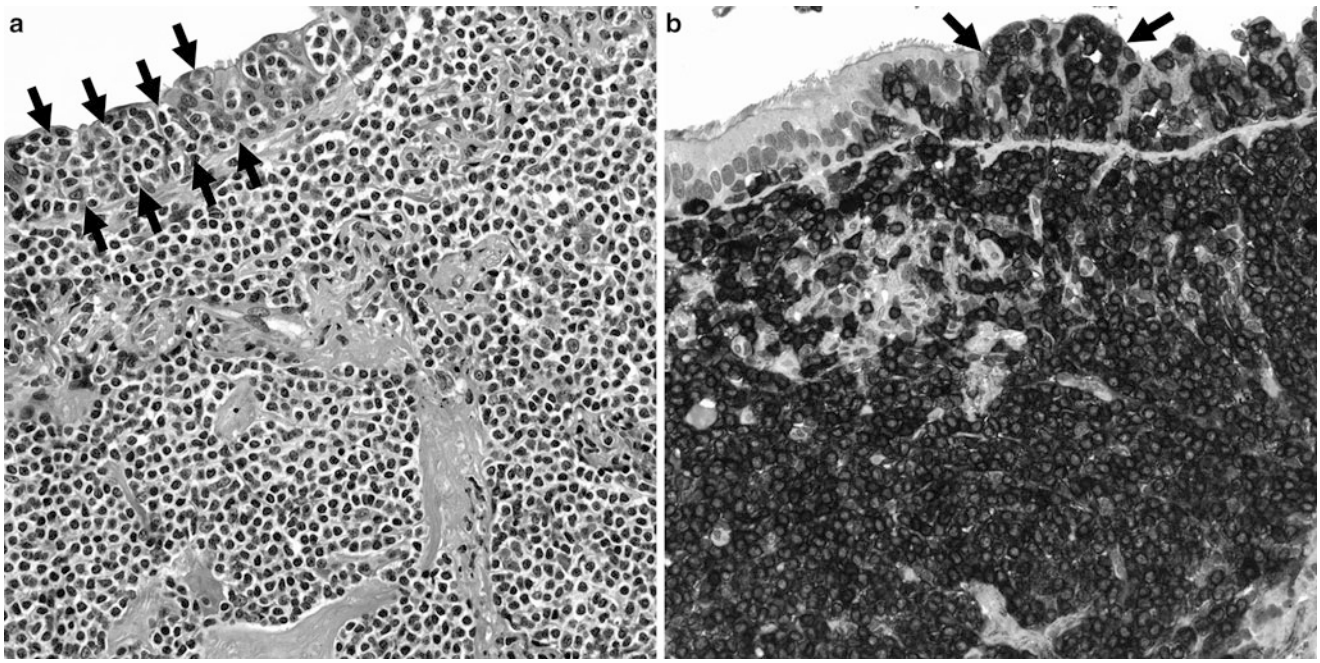

Fig. 31.14 Histology of extranodal marginal zone B-cell lymphoma, MALT type. (**a**) Lung biopsy with infiltrate of small lymphocytes with abundant cytoplasm with infiltration of bronchial epithelium (*arrows*). (**b**) Immunohistochemical study for CD79a shows predominance of B-cells and highlights the lymphoepithelial lesion (*arrows*).

marginal zone B-cell lymphoma with or without circulating villous lymphocytes and nodal marginal zone lymphoma are much less common.

The marginal zone is seen as a layer of small lymphocytes with moderate amounts of pale cytoplasm that surround the mantle zone and germinal center. Marginal zones are commonly found in Peyer's patches and other MALT sites, mesenteric lymph nodes, the spleen and occasionally in peripheral lymph nodes [58]. In extranodal MZBCL of MALT tissues (MALT lymphomas), the neoplastic cells frequently surround reactive follicle centers mimicking the normal marginal zone [59]. Similar morphologic findings are seen in splenic and primary nodal MZBCL. The marginal zones are expanded and sometimes fuse to form large sheets of the pale-staining cells. In MALT lymphoma, there is often infiltration of the epithelium by small groups of neoplastic cells, forming lymphoepithelial lesions (Fig. 31.14a). The neoplastic cells of MZBCL are usually small to medium-sized lymphocytes with abundant cytoplasm (classically described as monocytoid), although cytologic variability is common, even within a given case. Plasma cell differentiation is common [60].

By immunohistochemical analysis the neoplastic cells are shown to be mature B-cells with expression of CD19, CD20, CD22, and CD79a (Fig. 31.14b). Although exceptions occur, MZBCL is typically regarded as negative for CD5 and CD10. A subset of cases will show coexpression of the T-cell/macrophage associated marker, CD43. These lymphomas do not express cyclin D1 and usually lack expression of bcl-2 [61]. Molecular studies show that MZBCL are derived from post-germinal center B-cells with evidence of ongoing somatic mutation.

MZBCL are usually associated with chronic immune stimulation, either from exogenous (typically infectious) or endogenous (auto-antigen) exposure. Examples of this connection include the association that of *H. pylori* (Fig. 31.15) infection and gastric MALT lymphomas, hepatitis C and splenic marginal zone B-cell lymphoma, and MALT lymphomas involving the salivary gland and thyroid in patients with Sjögren syndrome and Hashimoto thyroiditis, respectively. Evidence of the association between infection and the gastric MALT lymphoma is the most clearly defined, and includes the fact that up to 90 % of patients with gastric MALT have the infection, the fact that eradication of the infection leads to regression of the lymphoproliferative disorder in a significant number of patients, and the fact that infection with *H. pylori* leads to similar lymphoid proliferations in animal models. There is increasing evidence of an association between *C. jejuni* and immunoproliferative small intestinal disease (IPSID), *B. burgdorferi* and cutaneous MZBCL, and *C. psittaci* and ocular adnexal MALT lymphoma [62]. In patients with hepatitis C (HCV), there is a question of whether the chronic infection leads to an increase in lymphoproliferative disorders in general or to an increase in specific subtypes such as splenic MZBCL [63]. Supportive evidence of a causative relationship between HCV and some cases of NHL (particularly MZBCL) includes series of patients who show a response to antiviral therapy with interferon and ribavirin [64, 65]. It has been suggested that the chronic immunologic response in patients with these infections or autoimmune disease leads to DNA damage. This damage is likely related in part to the effects of associated acute inflammation (particularly

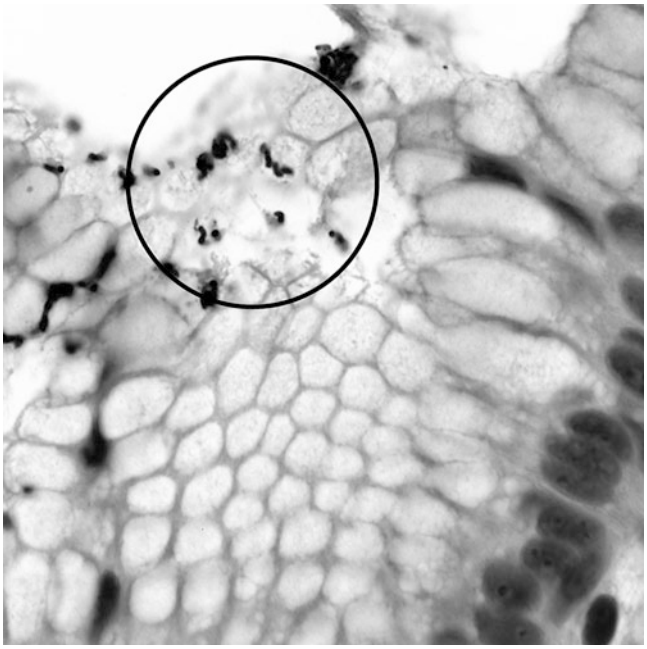

Fig. 31.15 Infection with *H. pylori*. Gastric biopsy with encircled area showing *H. pylori*, which has been shown to be the major causative agent in the development of gastric MALT lymphomas.

the release of reactive oxygen species) and in part to the process of proliferation and differentiation of B-cells associated with chronic inflammation [66].

Several recurrent balanced translocations have been described in association with MALT lymphomas, the most common of which are t(11;18)(q21;q21) (Fig. 31.16a) involving the *API2* and *MALT1* genes, t(14;18)(q32;q21) involving *IGH* and *MALT1* genes, and t(1;14)(p22;q32) (Fig. 31.16b) involving the *BCL10* and *IGH* genes. The translocations involving chromosome 14 share a common theme with MCL and FL with upregulation of a gene by placing it under regulatory control of the IgH promoters. The t(11;18) is different in that it involves the formation of a novel protein, which fuses parts of the *API2* gene product and *MALT1* gene product with the result that the MALT1 activity is constitutively expressed. Despite the various genes and mechanisms involved in these translocations, they ultimately result in activation of the necrosis factor kappa B (NF-κB) pathway. Other genetic alterations are known to occur with MALT lymphoma, including the recently described t(3;14)(p13;q32), involving the *FOXP1* gene on chromosome 3 and the *IGH* gene [67]. This translocation was shown to be present in 10 % of 91 MALT lymphomas evaluated, with a predilection for the thyroid, ocular adnexae, and skin. There appears to be potential for a geographic influence on distribution of these translocations related to sites of involvement of lymphoma as a large study from the US showed discrepancies with what was found in European and Japanese studies of distribution [68]. The t(11;18) and t(1;14) are most commonly associated with GI tract and pulmonary MALT lymphomas, while the t(14;18) is typically seen in other sites [66]. The reasons for the site predilections are not well-understood, but may in part be related to the types of antigenic stimulation that drives the lymphoid proliferation.

The t(11;18), t(14;18), and t(1;14) share a common theme in cases of MZBCL. Each ultimately leads to constitutive upregulation of the NFkB (Fig. 31.17). In the resting state, NFkB is sequestered in the cytoplasm because of the inhibitory effects of the IkB protein. In stimulated cells, IkB is phosphorylated and targeted for elimination by ubiquitin. In the absence of the inhibitory protein, NFkB can enter the nucleus, where it activates transcription of genes involved in inflammation, immunity, apoptosis, and cell survival. The gene for *BCL10* is located on chromosome 1p22, and expressed in many normal tissues. This protein binds to MALT1 to activate NFkB in the normal state. The *MALT1* gene at chromosome 18q21 encodes a paracaspase that does not activate NFkB without interacting with other proteins. BCL10 and MALT1 can bind together, leading to oligomerization of the caspase-like domain of MALT1 and physiologic activation of the NFkB pathway. The *API2* gene on chromosome 11q21 (also called cIAP2) is an inhibitor of apoptosis protein that inhibits active caspases that promote apoptosis. In each of the three translocations, there is upregulation of this system. In two translocations, there is upregulation of one of the partners in this activation pathway (BCL10 is overexpressed in t(1;14) and MALT1 is overexpressed in t(14;18)), leading to constitutive upregulation of the NFkB pathway. In t(11;18), there is a novel fusion protein, which allows for self-oligomerization of MALT1 and the upregulation of NFkB pathway without the participation of BCL10. In each case, the changes lead to activation of NFkB essential modulator (NEMO or IKKγ) resulting in phosphorylation and degradation of inhibitory kB proteins (IkB). The end result is that movement of NFkB to the nucleus is uncontrolled and leads to enhanced cell survival and proliferation [62, 66]. It is interesting to note that in MALT lymphoma cases, the t(1;14) and t(14;18) are often present with additional chromosomal abnormalities, while the t(11;18) is often the sole abnormality. It is also noteworthy that cases with the t(11;18) translocation rarely undergo transformation [69].

Because of the sites of involvement and the mechanisms of sampling those sites, the diagnosis of marginal zone B-cell lymphoma of MALT type is frequently made on small fragments of tissue. Morphologic and immunohistochemical findings are important; however, isolation of a B-cell clone by immunohistochemistry alone can be challenging. The lack of a specific aberrant immunophenotype in most cases can also render diagnosis difficult. In many cases, establishing clonality of the associated plasma cell population can serve as a surrogate for clonality in the lymphoid population [70]. In some instances, PCR analysis is required to establish clonality. FISH studies can also be performed looking for specific translocations, as listed above. The finding of a

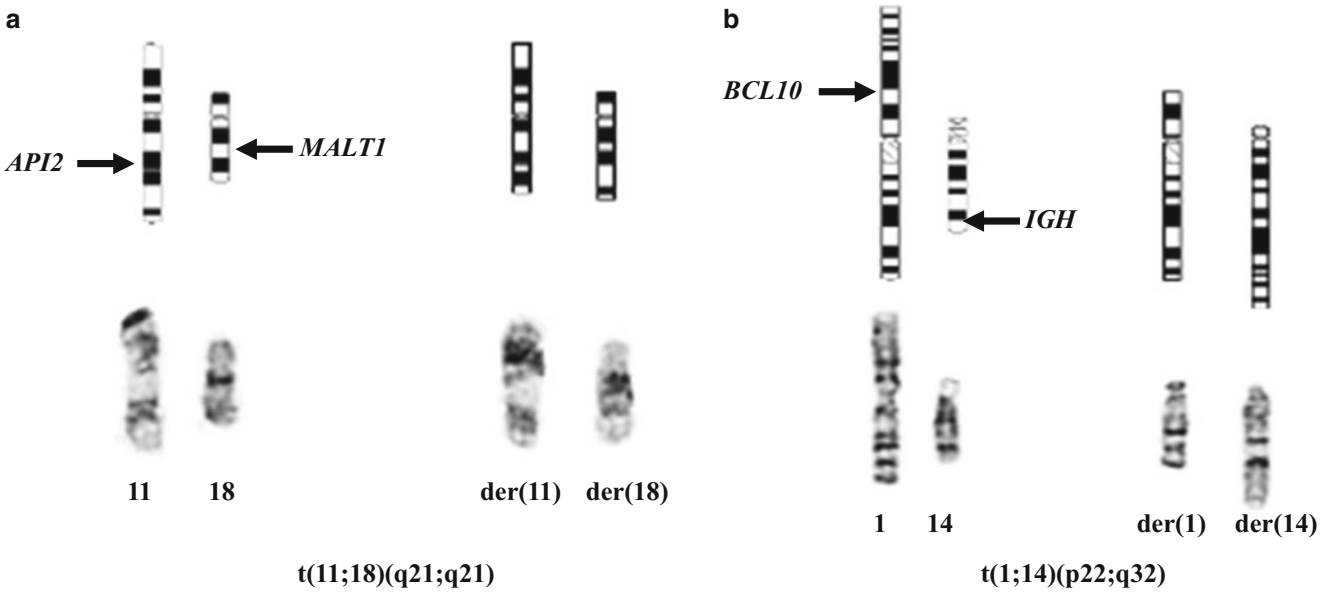

Fig. 31.16 Translocations in MALT lymphoma. (**a**) Translocation involving *API2* on chromosome 11 and *MALT1* gene on chromosome 18 that produces a novel API2-MALT1 fusion product that is capable of self-oligomerization and constitutive activation. (**b**) Translocation involving the *BCL10* gene on chromosome 1 and the *IGH* gene on chromosome 14 leads to overexpression of *BCL10*.

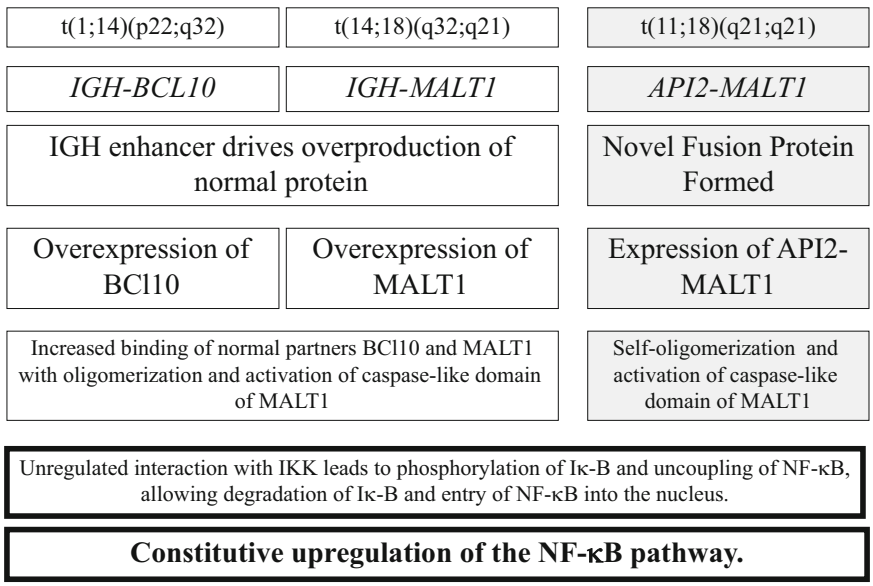

Fig. 31.17 Mechanisms by which NF-kB is upregulated in marginal zone B-cell lymphoma. The three common translocations, t(11;18), t(1;14), and t(14;18) in MALT lymphomas lead to increased activity of MALT1 and BCL10, which upregulates function of NF-kB pathway and leads to cell proliferation.

t(11;18) in a gastric MALT lymphoma by FISH can be of value clinically, since patients with this translocation are unlikely to respond to antibiotic therapy for *H. pylori* [71].

31.3.5 Anaplastic Large Cell Lymphoma (t(2;5)(p23;q35), *NPM-ALK*

The term anaplastic large cell lymphoma (ALCL) was first used in 1985 to describe a group of lymphoproliferative disorders that showed immunoreactivity for the Ki-1 antibody raised against Hodgkin lymphoma but that had distinctive morphologic features [72]. This lymphoma accounts for approximately 2 % of NHL [73], with a predilection for young patients and shows a striking male predominance [1]. The disease comprises a heterogeneous group that can be largely divided by the presence or absence of expression of anaplastic lymphoma kinase (ALK) which is caused by certain translocations. Patients with ALK overexpression have a remarkably good prognosis, while those that lack it have a poor prognosis, similar to patients with peripheral T-cell lymphoma, unclassified. This difference is so striking that it has led to the suggestions that the ALK-positive (ALK+) and ALK-negative (ALK−) forms be separated into two distinct entities.

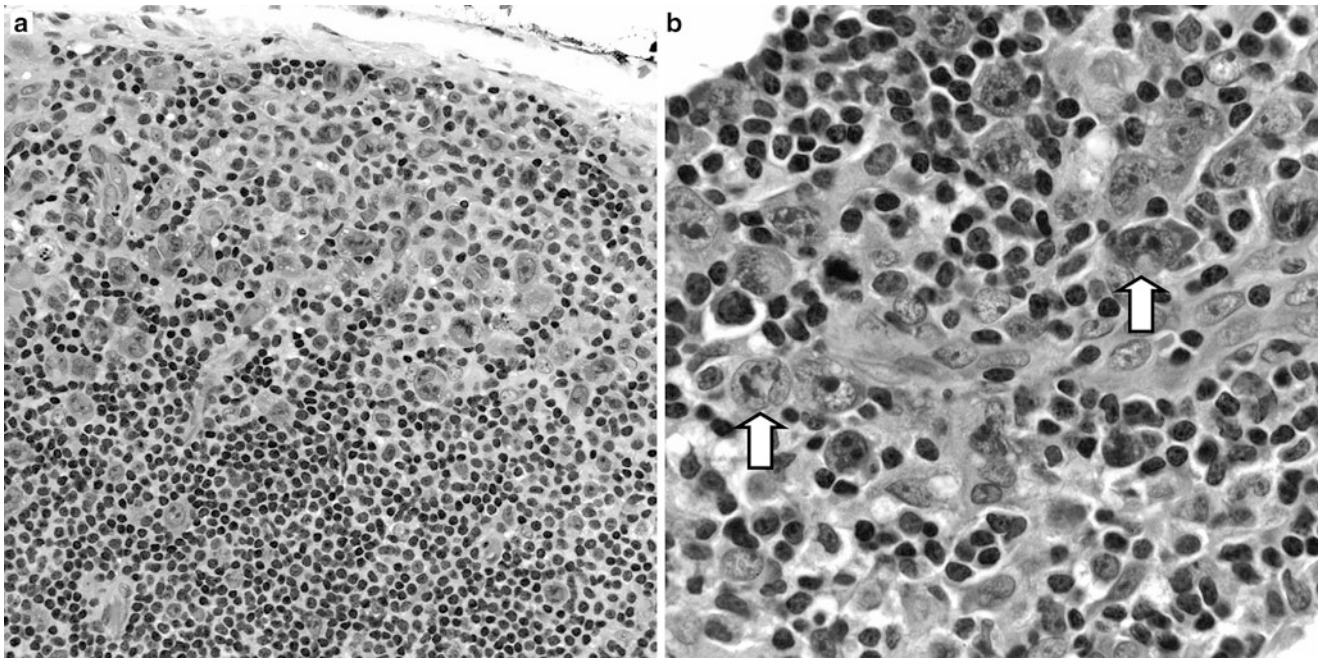

Fig. 31.18 Morphology of anaplastic large cell lymphoma. (**a**) Lymph node with predominantly sinusoidal and perisinusoidal infiltration by atypical lymphoid cells. (**b**) "Hallmark" cells of ALCL (*arrows*) with large, reniform/embryoid nuclei and prominent paranuclear clearing.

Lymph nodes involved by ALCL often show preservation of lymph node architecture with the population of abnormal cells showing a peculiar sinusoidal pattern of distribution (Fig. 31.18a). In other cases, there may be diffuse effacement of the lymph node architecture. The so-called hallmark cell is a cell classically described as having a reniform, embryoid or horseshoe-shaped nucleus, and moderate to abundant cytoplasm often with a distinct perinuclear eosinophilic region which may represent a prominent Golgi apparatus (Fig. 31.18b). There are several morphologic variants of ALCL, including the common variant, small cell variant, lymphohistiocytic variant, and a sarcomatoid variant. Despite the variation in the overall morphology, the hallmark cells remain a constant finding, which can be very useful in establishing the diagnosis [74]. Morphologically, the ALK+ and ALK− variants do not show substantial differences, although it has been suggested that the t(2;5), which is the most common translocation leading to ALK positivity is more frequent in the monomorphic and small cell variants [75]. A group of Ki-1 (CD30) positive cutaneous lymphoid proliferations with similar cytologic features is also recognized. The clinical features of the CD30-positive cutaneous lymphoproliferative disorders are quite variable, and they rarely express ALK by immunohistochemistry.

Aside from the uniform expression of CD30, the immunologic features in ALCL are variable. Most cases show immunophenotypic evidence of T-cell differentiation with expression of one or more T-cell markers including CD2, CD3, CD5, and CD7. ALCL is somewhat more frequently immunoreactive for CD4 than for CD8. The null cell type expresses no lineage-specific antigens. Whether or not the neoplastic cells express T-cell markers, the majority show evidence of clonal T-cell receptor gene rearrangement [73]. Curiously, the neoplastic cells express the epithelial membrane antigen, which along with the tendency for the neoplastic cells to cluster and involve lymph node sinuses can cause confusion with carcinoma at the time of diagnosis. Most cases express one or more cytotoxic molecules such as granzyme B, perforin, TIA-1. In contrast to some cases of Hodgkin lymphoma, there is no expression of EBV and no expression of the B-cell specific activator protein (BSAP, *PAX5*) [1]. Rare cases show expression of CD15 [74].

Anaplastic large cell lymphoma has been shown to be associated with a variety of translocations. The first described was the t(2;5)(p23;q35) [76, 77] which was later shown to involve the nucleophosmin gene (*NPM*) located at chromosome 5q35 and a previously uncharacterized receptor tyrosine kinase dubbed anaplastic lymphoma kinase (ALK) located at chromosome 2p23 [78] (Fig. 31.19). NPM is a ubiquitously expressed nuclear shuttling protein that carries proteins to the nucleolus. Its function depends on an N-terminal oligomerization motif and nuclear localizing domains at the C-terminus. ALK is a transmembrane receptor tyrosine kinase most closely related to leukocyte tyrosine kinase and is a member of the insulin receptor superfamily. It carries an intracytoplasmic tyrosine kinase catalytic domain that is activated by forming homodimers after ligand binding [79]. The ALK protein shows relatively limited expression in

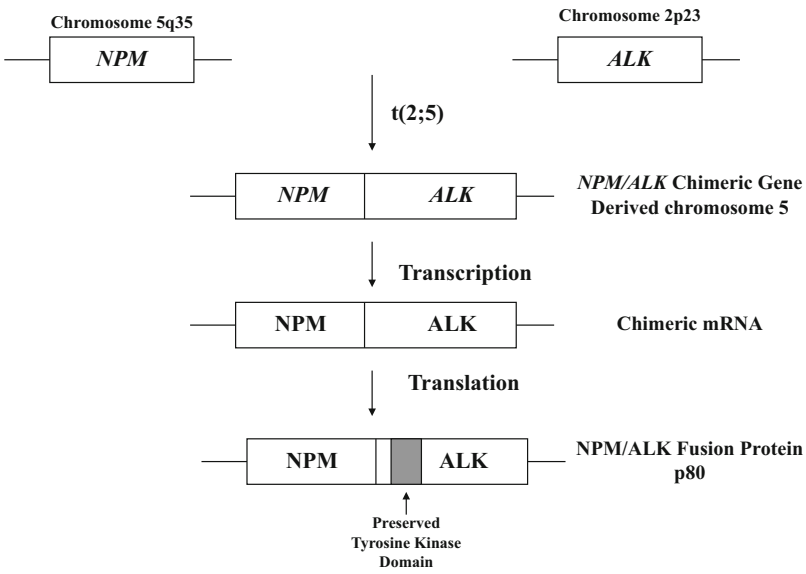

Fig. 31.19 Schematic diagram of the t(2;5)(p23;q35). This translocation juxtaposes the *NPM* and *ALK* genes on the derived chromosome 5 resulting in a *NPM/ALK* chimeric gene, which codes for an 80-kd, NPM/ALK fusion protein (designated p80). The NPM/ALK fusion protein includes a preserved tyrosine kinase domain (*shaded area*).

cells of neural origin and is not expressed in normal hematopoietic cells. The t(2;5) leads to the production of a novel chimeric protein that carries the 5′ oligomerization motif of NPM that allows homodimerization of the fusion protein leading to constitutive activation of the 3′ tyrosine kinase domain derived from ALK. Numerous genes can be the 5′ partner for *ALK* in ALCL (including clathrin heavy chain-like 1 on chromosome 17, tropomyosin 3 on chromosome 1, *TRK*-fused gene on chromosome 3, and others) and most supply domains that lead to spontaneous oligomerization allowing the ALK fusion kinases to auto-phosphorylate and activate downstream pathways [80]. ALK overexpression has been associated with other tumors including inflammatory myofibroblastic tumors, neuroblastoma, and some solid tumors. The oncogenic potential of NPM-ALK fusion has been demonstrated in mouse models, with development various tumor types including B-cell lineage lymphomas [81] and/or T-cell lineage lymphomas [82]. It is also important to note that similar to with the t(11;14) of MCL and t(14;18) of FL, normal patients can be demonstrated to carry NPM-ALK fusion transcripts can be demonstrated in non-neoplastic cells, suggesting that this is an important but not sufficient step in the development of ALCL [83].

Constitutive activation of the ALK tyrosine kinase interacts with numerous cellular pathways involved in cell-cycle progression, survival, and proliferation. For instance, constitutive activation of signal transducers and activators of transcription (STATs) by tyrosine kinases leads to their translocation to the nucleus where they regulate transcription of genes involved in apoptosis, the cell cycle, and cell growth. Constitutive activation of the STAT3 pathway is consistently present in ALK-positive ALCL. Other proteins, including JAK2 and STAT5 can also play a role in ALCL pathogenesis [84]. Two other relevant and relatively well-characterized pathways affected by ALK fusions include the Ras-extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol 3-kinase (PI3-K)-Akt pathway. The Ras-ERK, JAK3/STAT3, and PI3-K-Akt pathways interact at multiple points in mediating the effects of ALK activity, with the first playing a major role in cell proliferation and the latter two involved with anti-apoptotic effects and cell survival [80]. The role of CD30 in the pathogenesis is unclear, but its consistent expression in these tumors suggests it may have a role.

Although the presence of ALK overexpression in ALCL defines a relatively discrete population, they do not seem to have much in common with ALK-negative ALCL cases. Indeed, there is some debate about considering ALK+ ALCL a discrete entity and classifying the ALK− lymphomas otherwise typical of ALCL with the group of peripheral T-cell lymphoma, unspecified because of differences in clinical presentation and outcome. Gene profiling studies show that ALK+ ALCL and ALK− ALCL hold some mechanisms in common (expression of anti-apoptotic molecules and certain kinases) but differ in other ways (such as level of expression of signal transduction molecules (greater in ALK+ cases) and transcription factors (less in ALK+ cases)) [85]. Another gene expression profile study confirmed the discrepancy between ALK+ and ALK− subgroups and showed subgroups even among the ALK+ ALCL cases. The common type clustered together with increased expression of genes associated with inflammatory/immune response and stress responses, while the morphologic variants were in a different group showing increased expression of genes involved in cell adhesion and cell migration. The authors postulated that this clustering among ALK+ cases may reflect expression of different genes during the course of the disease [86].

Diagnosis of ALCL hinges on recognition of morphologically abnormal cells showing CD30 expression (Fig. 31.20a).

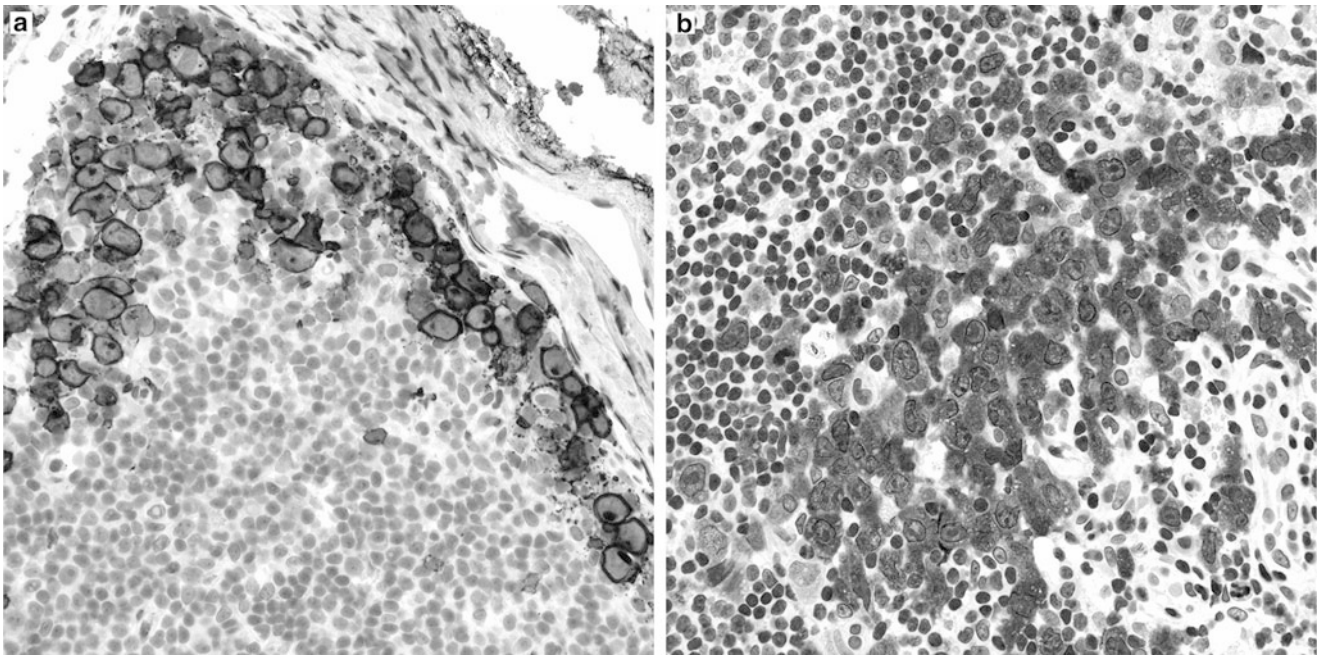

Fig. 31.20 Immunohistochemistry in the diagnosis of ALCL. (**a**) CD30 highlights the sinusoidal infiltration of the lymph node. (**b**) Immunohistochemical study for antibody to anaplastic lymphoma kinase (ALK) showing only cytoplasmic immunoreactivity, suggesting an *ALK* translocation involving a partner gene other than nucleophosmin (*NPM*), which would show nuclear and cytoplasmic immunoreactivity.

Discrimination from cases of Hodgkin lymphoma is aided by the expression of other markers such as *PAX5*, ALK, and T-cell markers in many cases. The presence of ALK expression is an important prognostic variable that can be demonstrated by immunohistochemical analysis and the pattern of immunoreactivity to ALK can provide some insight into the translocation partners involved [79] (Fig. 31.20b). Cytogenetics and FISH studies can be useful in demonstrating the characteristic translocations and additional genetic abnormalities. Finally, although T-cell markers are not expressed all cases, the large majority will exhibit clonal T-cell receptor gene rearrangements, including those that are of null cell phenotype.

31.3.6 Adult T-Cell Leukemia/lymphoma (Integrated, Clonal HTLV-1)

Unlike the chronic inflammation and infection-associated MALT lymphomas, another group of lymphomas can be directly caused by viruses. The main transforming viruses implicated in lymphomagenesis include Epstein-Barr Virus (EBV), Human Herpesvirus-8 (HHV8), and Human T Lymphotropic Virus-1 (HTLV-1). EBV is consistently linked with the endemic form of Burkitt lymphoma and the consistent presence of EBV DNA and proteins in the cells in such cases is a strong argument for an etiologic role of the virus. Other evidence includes serologic evidence of infection preceding the development of lymphoma and the fact that the EBV itself is monoclonal in the lymphoma cells. This virus is also found in the vast majority of extranodal NK/T-cell lymphomas (nasal type), most post-transplant lymphoproliferative disorders, and a subset of Hodgkin lymphoma. Another virus more recently associated with non-Hodgkin lymphoma, particularly in the setting of infection with the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) is HHV8 (also known as Kaposi sarcoma-associated herpesvirus (KSHV)). This virus is closely associated with the primary effusion lymphomas, which are clinically aggressive lymphomas. The exact mechanism of oncogenesis in such cases is under investigation, but pathways involving p53, the retinoblastoma gene product, cyclin-dependent kinase 6, and NFkB have been implicated [87]. A viral etiology has been established for greater than 20 years in adult T-cell leukemia/lymphoma (ATLL), which is caused by infection with a retrovirus known as HTLV-1 [88].

ATLL is a disease of mature CD4+ T-cells and shows distinctive geographical clustering in areas with high seroprevalence for HTLV-1. The clinical manifestations of ATLL vary with acute, lymphomatous, chronic, and smoldering forms recognized by the WHO [1]. Most patients with ATLL present with lymphadenopathy and many have organomegaly. The presence of hypercalcemia and lytic bone lesions is common, which is likely caused by increased numbers of osteoclasts and accelerated bone resorption [89]. The peripheral blood of involved patients shows varying numbers of

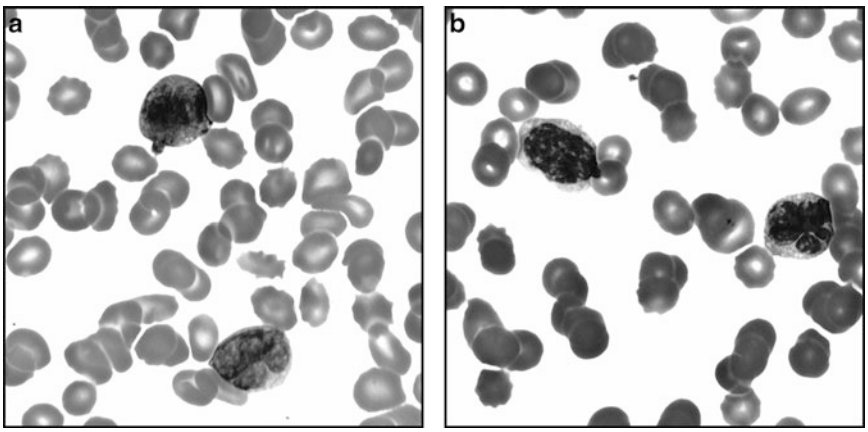

Fig. 31.21 Adult T-cell leukemia/lymphoma. (**a**, **b**) Flower cells in the peripheral blood of a patient with adult T-lymphocytic leukemia/lymphoma.

abnormal lymphocytes including those with markedly convoluted nuclear contours (so-called flower cells) (Fig. 31.21). The neoplastic cells have a mature T-cell phenotype and show expression of T-cell antigens including CD2, CD3, CD5, and CD4 and typically lack expression of CD7 and CD8. The malignant cells also show expression of CD25. There is not a specific karyotypic abnormality associated with development of ATLL, but multiple chromosomal abnormalities may be seen [90], including loss of heterozygosity on chromosome 6q, which has been reported in up to 40 % of cases [91]. The genetic hallmark of ATLL is the integration of HTLV-1 at a random site in the genome. The viral genome and the site of integration are clonal within a given patient's neoplastic population.

HTLV-1 is a type C human retrovirus with a positive single-stranded RNA genome. The encapsulated virion contains the viral RNA, a protease, reverse transcriptase, and integrase. Upon entering the cell, possibly via a ubiquitous glucose transporter [92], the virus is converted into double-stranded DNA and is integrated randomly into the DNA of the host cell as a provirus. The infection is lifelong, and preferentially infects CD4-positive T-cells. The virus optimizes its RNA by using RNA ribosomal frameshifts, transcript splicing patterns, and even makes use of the negative strand for encoding functional proteins [93]. Given that the virus replicates with the cells it infects (as an integrated provirus) using cellular polymerases, the genome of the virus is quite stable and viral loads are low. Based on the low level of genetic variability, and low incidence (2–6 %) of ATLL among infected populations, it is presumed that development of leukemia is related to host factors [90]. The variation in host factors may also help explain the various clinical manifestations of infection with HTLV-1, including HTLV-1-associated myelopathy/tropical spastic paraparesis and the associations with infective dermatitis, Sjögren syndrome, etc.

The molecular pathways involved in development of lymphoma revolve around the viral Tax protein and a recently discovered gene encoded on the minus strand of the viral RNA that encodes for HTLV-1 bZIP factor, also called HBZ [94]. Tax increases expression of viral genes and stimulates expression of cellular genes via cell signaling pathways of NFkB, cyclic AMP response element-binding protein (CREB) among others. The Tax protein affects these pathways by interacting with cellular transcription factors or other modulators of cell function. It has also been shown to inactivate the tumor suppressors p53, $p16^{INK4a}$, and others [89]. Because of its activities in promoting transcription of its own genome, cytokines, and receptors, anti-apoptosis genes and its inhibition proteins involved in tumor suppression and DNA repair, Tax seems a likely candidate for the transforming factor in ATLL. However, it also serves as a target for the host cytotoxic T-cell response and its overexpression leads to destruction of infected cells. The ATLL cells can lose expression of Tax protein by several mechanisms, including deletion or hypermethylation of the promoter for production of viral Tax, which is a 5′LTR, or various missense or nonsense mutations leading to loss of production of functional Tax. The loss of Tax via these mechanisms allows the cells to evade the host immune system [95]. Another way of downregulating the expression of Tax was found in the form of *HBZ*. This protein was expressed in all ATLL cells and no cases with abortive mutations were found, suggesting that this gene is important in oncogenesis. The *HBZ* gene seems to function in perpetuating the infected cell line in the protein form by suppressing Tax expression and evasion of the immune response, and in the mRNA form by increasing expression of E2F1, a gene pivotal in control of the G1 to S phase transition in the cell cycle [96]. Ultimately, these mechanisms of immune system surveillance and proliferation are important in driving the process of ATLL, but more complex interactions with host immunologic and genetic factors must play a role in the development of neoplasia since the large majority of patients infected with HTLV-1 do not develop this fatal complication.

Diagnosis of ATLL is made by correlating the clinical, morphologic, immunophenotypic, and genetic findings. Patient demographic data may be a key feature in leading one to consider the diagnosis. The presence of atypical circulating lymphocytes (flower cells) confirmed by immunophenotyping, and serologic data to confirm the presence of HTLV-1 infection are also invaluable. PCR analysis, directed at detecting the presence of integrated proviral sequences is a sensitive technique for identifying ATLL and T-cell receptor studies show clonal rearrangements, although molecular analysis for ATLL is not usually necessary for diagnosis [97]. The presence of hypercalcemia and lytic bone lesions is a helpful but not specific finding as these are also characteristic of plasma cell myeloma.

31.4 Future Directions in Understanding the Molecular Basis of Lymphoma

31.4.1 Molecular Profiling

Morphologic and immunohistochemical methods of analysis have improved our classification of lymphomas in the past 20 years. However, there are still large groups of NHL that are not further classifiable with routinely used methodologies. Two of the major groups include diffuse large B-cell lymphoma and peripheral T-cell lymphoma, unspecified. There are morphologic, immunohistochemical, genetic, and clinical differences among these groups of lymphomas, but their subclassification with routine methods is poorly reproducible. A recent trend in tumor analysis is gene expression profiling using DNA or RNA libraries and applying these to tissue microarrays composed of samples from a host of different cases. This process allows one to examine genes that are upregulated or downregulated on a case by case basis and then group cases based on similarities and differences in how those genes are expressed [98].

The seminal article in gene expression profiling details a study that examined thousands of genes expressed in normal cells as well as chronic lymphocytic leukemia/small lymphocytic lymphoma, follicular lymphoma, and diffuse large B-cell lymphoma [99]. The expression of genes expressed in subsets of normal cells was compared to that in neoplastic cases. Hierarchical clustering was used to compare samples and to group cases with similar profiles. It was found that among the heterogeneous category of diffuse large B-cell lymphomas, two major groups were present—a group with a an pattern of gene expression that resembled germinal center B-cells (germinal center B-cell-like) and a second group with a profile more akin to activated peripheral blood B-cells (activated B-cell-like). These groups were shown to have significantly different outcomes when treated with similar therapeutic regimens. It was further noted that although these two groups were distinct, there was heterogeneity within each group. The possibility that the differences in molecular signatures and clinical outcomes were sufficient to consider the ABC type and GCB type as two distinct entities was raised. Subsequent gene profiling studies of DLBCL have largely substantiated this initial work, although differences exist, including the presence of a third subgroup by some authors [100, 101]. In addition to substantiating the findings of the original study, other groups have applied various technologies including self-organizing maps to simplify and organize the vast amounts of data generated [102]. Another group applied fuzzy neural networks to aid in sorting data and found that the number of genes evaluated can be reduced from several thousand to less than 10 with reasonable predictive value [103]. Because gene expression profiling is not yet readily available for such distinction, several groups have proposed immunohistochemical algorithms for distinguishing the ABC from GCB types of large B-cell lymphoma [104, 105] (Fig. 31.22).

Along with value in subclassification and prognostication, the use of gene expression profiling can be helpful in further defining existing disease entities. For instance, it is a well-known issue that distinction of Burkitt lymphoma from other forms of B-cell lymphoma, which is extremely important for optimal therapy, can be difficult or impossible on a pure histologic basis [106] (Fig. 31.23). With the judicious use of immunohistochemical analysis, cytogenetics and FISH, the classification of these cases can frequently be made, but there is a subset of cases that defies precise classification [107]. Molecular studies of cases of Burkitt lymphoma, Burkitt-like lymphoma, and diffuse large B-cell lymphoma show distinctly different gene expression profiles, emphasizing the fact that the current tools used for classification of these lymphomas is not ideal. One group identified a molecular signature for Burkitt lymphoma using eight cases that met all WHO criteria for that diagnosis. Using that signature in an analysis of numerous cases of mature, aggressive B-cell lymphomas, the authors found that among cases with the molecular signature of Burkitt lymphoma, 25 % had morphologic features that were more typical of diffuse large B-cell lymphoma and only 65 % had classical or atypical Burkitt morphology [108]. Another study published at the same time analyzed over 300 cases of aggressive lymphoma and found that all 25 cases of pathologically confirmed Burkitt lymphoma were correctly identified and distinguished from DLBCL. However, some cases originally diagnosed as DLBCL had the molecular signature of Burkitt lymphoma and vice versa. The findings underscore the difficulties in diagnosis of BL using currently accepted criteria. Because of significant differences in therapeutic regimens and clinical outcome between cases of DLBCL and Burkitt lymphoma, molecular profiling studies may be the best way to ensure correct therapy, although such testing methods are not currently available in clinical laboratories [109].

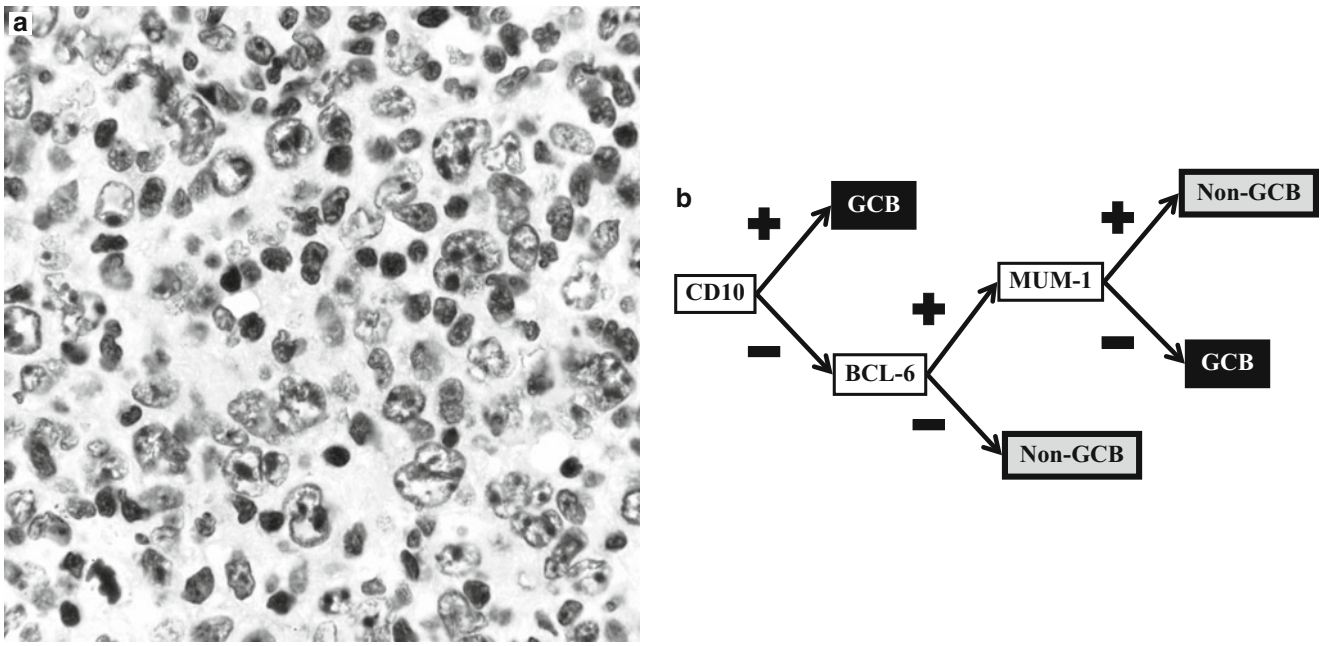

Fig. 31.22 Diffuse large B-cell lymphoma. (**a**) Diffuse large B-cell lymphoma with large atypical cells showing multiple nuclei, vesicular chromatin, and thickened nuclear contours (centroblastic morphology). (**b**) A suggested algorithm is the Hans algorithm, which uses a small number of immunohistochemical studies as a surrogate for gene profiling studies in the separation of non-germinal center B-cell type (non-GCB) from the germinal center B-cell (GCB) types of DLBCL.

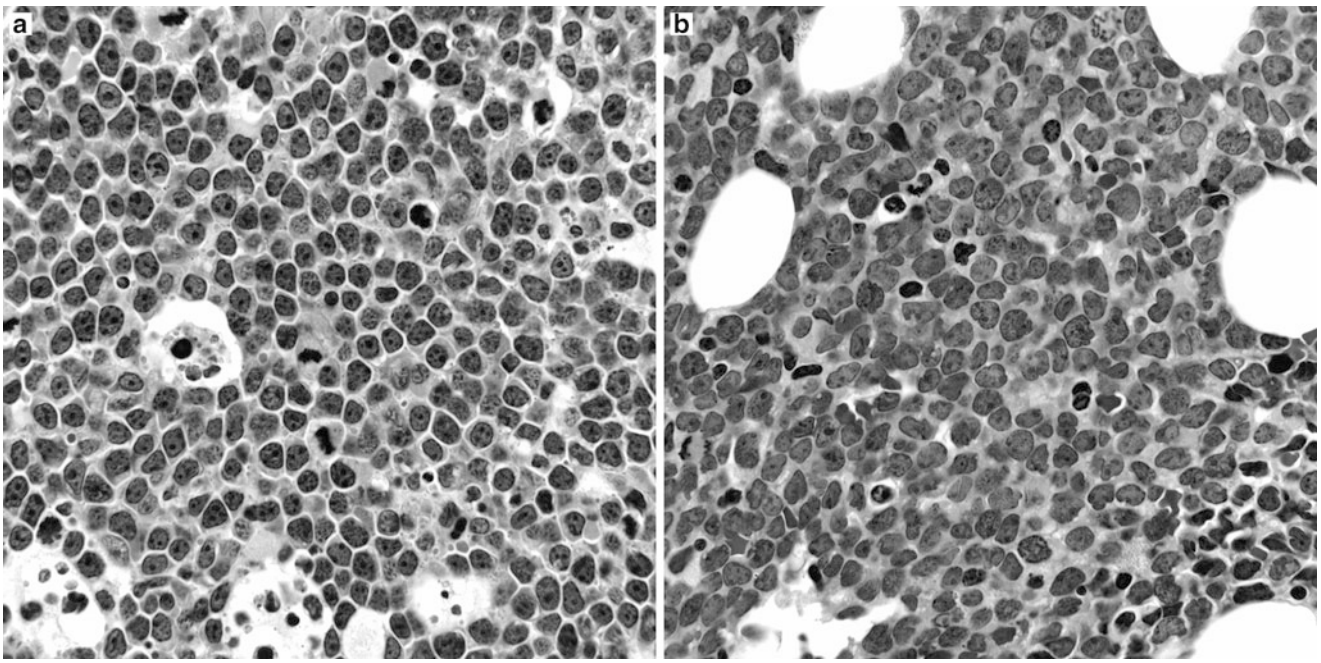

Fig. 31.23 Aggressive B-cell lymphomas. (**a**) Aggressive B-cell lymphoma immunophenotypic findings and morphologic findings that would be compatible with double hit lymphoma. Despite a *MYC* rearrangement in the form of a t(8;14), the presence of an additional t(14;18) argues against classification as Burkitt lymphoma. (**b**) Burkitt lymphoma with classic morphology and isolated t(8;14). There is often morphologic and immunophenotypic overlap among aggressive B-cell lymphomas; however gene expression profiling has been shown to be a reproducible method of discriminating Burkitt lymphoma, which requires more aggressive therapy from others that might be better treated with more conventional DLBCL therapy.

These and other findings and others are exciting and may lead to further subclassification of DLBCL, and may help lead to more precise classification of existing entities. However, this use of this data is a work in evolution. It is noted, for instance, that as therapies have evolved and with the addition of anti-CD20-directed rituximab, the differences in outcome related to various prognostic factors, including those noted at the time of Alizadeh's original study may be somewhat lessened [110]. Utilization of molecular profiling could be of great value in ensuring that patients with aggressive disease receive appropriate therapy. In addition, the use of these techniques to further delineate the pathogenetic mechanisms of lymphomas may lead to novel therapies more precisely directed at disrupted molecular pathways, such as specific inhibitors of cyclin-dependent kinases in mantle cell lymphoma or inhibitors of the NFkB pathway in various lymphomas with deregulation of this pathway.

31.4.2 MicroRNAs

A relatively new player in the understanding of molecular mechanisms of disease is the microRNA. These small molecules, first characterized in *C. elegans* in 1993 [111] were not well-appreciated for their widespread function in physiologic and pathologic processes until years later [112]. The term microRNA was coined by Lee and Ambros in 2001 [113]. Study of microRNA structure and function has been extensive, and several hundred microRNAs have been described and cloned in humans [112]. MicroRNAs are conserved across various organisms, and play important roles in many cellular and developmental processes. A single microRNA can influence large numbers of genes, and can serve as tumor suppressors or as oncogenes [114]. Their expression has been shown to be dysregulated across the spectrum of epithelial, mesenchymal, and hematopoietic malignancies.

The molecular profiling studies involving DNA libraries and proteomics have been shown to be effective in distinguishing various tumor types. Similarly, it has been shown that there are unique microRNA fingerprints that distinguish normal cells from tumor cells and tumors from one another. Among lymphoid neoplasms, the use of a microRNA-microchip to analyze chronic lymphocytic leukemia found that microRNA profiles were associated with expression of ZAP-70, with the mutational status of the immunoglobulin heavy chain variable region and with the length of time from diagnosis treatment [115]. Although many microRNAs were found to be dysregulated, there were two microRNAs that were abnormal in cases of CLL, *miR-15a* and *miR-16-1* that were felt to behave as tumor suppressors. The ability to distinguish accurately between patients with CLL into good prognosis and bad prognosis groups is only one of the many useful pieces of information that can be gleaned from the study of microRNAs. Another study showed that microRNA expression profiles were able to distinguish accurately between acute lymphoblastic leukemia and acute myeloid leukemia. It was found that using expression patterns of just two to four microRNAs, one could accurately distinguish ALL from AML in greater than 97 % of case analyzed [116].

Other recent studies have shown interactions between microRNAs and other oncogenes, supporting their role in cancer development. For instance, it has been shown that Myc activation in tumor cells results in widespread changes in the expression of microRNAs, predominantly in the form of downregulation, a finding that suggests that microRNA expression plays a role in Myc-mediated tumorigenesis [117].

Like the gene expression profiling that relies on DNA, microRNA gene expression profiling has identified patterns that can be used in the classification and prognosis of various tumors. They can be upregulated or downregulated and can serve as oncogenes or tumor suppressors [118]. The study of these small molecules has some advantages over the study of mRNA, particularly that they are stable in routinely available tissue (including paraffin-embedded tissue), and that it seems that a limited number of microRNAs may be sufficient for classification of tumors. It is hoped that these small molecules will have a big impact on understanding the biology of cancer and in either serving as a target for novel therapies or at least helping illuminate other mechanisms of tumorigenesis that can be targeted.

31.5 Conclusion

Our understanding of the molecular basis of tumors continues to grow, but there is much yet to learn. It has been known for many years that cancer is not a single disease but many, and that neoplasia is a multi-step process. As we have seen, many of the molecular hallmarks of common diseases are simply markers, but do not cause the disease *per se*. For this reason, in the realm of clinical diagnostics it is important to not equate clone with malignant. The detection of low levels of aberrant transcripts by highly sensitive methods similarly does not translate to the presence of early malignancy. The principles of classification espoused in the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues [1] are highly applicable. There needs to be correlation of morphologic, immunophenotypic, clinical, and molecular data. It is through this integration that we are able to advance our understanding of these disorders and further study them. At this point, the use of tools like cytogenetics, FISH, and PCR can help us to diagnose, classify, and prognosticate lymphoproliferative disorders. It is hoped that the use of powerful molecular techniques like molecular profiling to study disease and the discovery of novel ways in which gene expression is con-

trolled will lead to better characterization and understanding of disease processes. This understanding should lead to development of novel therapies, and ideally improve clinical outcome in patients suffering from these disorders.

Acknowledgement Special thanks to Ms. Jaye Smith, CLSp CG for assistance in preparing the cytogenetic images.

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The Molecular Pathology of Head and Neck Squamous Cell Carcinoma

32

Laura J. Tafe

32.1 Introduction

In the United States, there were an estimated 49,000 new cases of head and neck cancer diagnosed in 2010, the majority of which were squamous cell carcinomas [1]. Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer worldwide by incidence [2]. HNSCC arises in the oral cavity, oropharynx, larynx, or hypopharynx. The overall 5-year survival for all stages and races is approximately 63 %, but this varies from 30 to 80 % for patients with distant metastases versus localized disease, respectively [1].

The pathogenesis of HNSCC has been associated with environmental risk factors such as alcohol, tobacco, and regional/ethnical substance chewing which may act synergistically. During the past decade there has been a decreasing incidence of HNSCC in several sites which has been attributed to a decrease in tobacco use [3]. However, there has been an increase in oral tongue and especially oropharyngeal tumors which is speculated to be because of increasing high-risk Human Papilloma Virus (HPV) infections [4]. In addition, patients with certain inherited disorders, such as Fanconi anemia and Li-Fraumeni syndrome, and genetic susceptibility have a predisposition for HNSCC. There is growing evidence that HNSCC associated with HPV infection is a unique clinical and biologic subset of HNSCC. Currently, there are two models of pathogenesis in HNSCC. One, in so-called conventional HNSCC, is associated with environmental substance use and the second driven by oncogenic HPV infection.

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32.2 Etiology and Biology of Conventional HNSCC Associated with Environmental Substance Use

Tobacco, alcohol, and regional/ethnical substance, such as the betel quid (a combination of betel leaf, areca nut, and slaked lime; when combined with tobacco this is called gutka), use are classical risk factors for development of HNSCC. Exposure to these carcinogenic substances over time leads to multiple opportunities for DNA damage and often a stepwise accumulation of genetic events leads to carcinogenesis. In a simplified approach, carcinogenesis develops from overexpression of oncogenes and or suppression of tumor suppressor genes. A number of genetic alterations have been described in HNSCC and the interplay among all of these changes that lead to carcinogenesis are not yet completely understood.

Conventional HNSCC is thought to evolve through a multistep process of genetic, epigenetic, and metabolic changes secondary to exposures to the above carcinogens. Clinically, this is reflected through the progression from normal mucosa to precancerous and dysplastic lesions to eventual carcinoma. However, not all precancerous lesions develop into carcinoma. Oral leukoplakias have an approximate 1–2 % rate of transformation into cancer [4]. Histologically, conventional HNSCC usually have some degree of keratinization and are classified as well to poorly differentiate based on the amount of keratin produced (Fig. 32.1a). It appears that an accumulation of changes are necessary for progression. Alterations in chromosomal region 9p21 are seen in 70–80 % of oral dysplastic lesions. This region, known as the *CDKN2A* locus encodes the p16 and $p14^{arf}$ tumor suppressors, which are frequently inactivated by promoter hypermethylation [5]. Loss of 3p is another common alteration in early carcinogenesis; this region includes the *FHIT* (fragile histidine triad) and *RSSFIA* tumor suppressor genes which are commonly inactivated by promoter methylation or exonic deletion. Loss of heterozygosity (LOH) at chromosomal regions 4q, 8p, 11q, 13q, 14q, and 17p are common in dysplastic lesions,

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_32

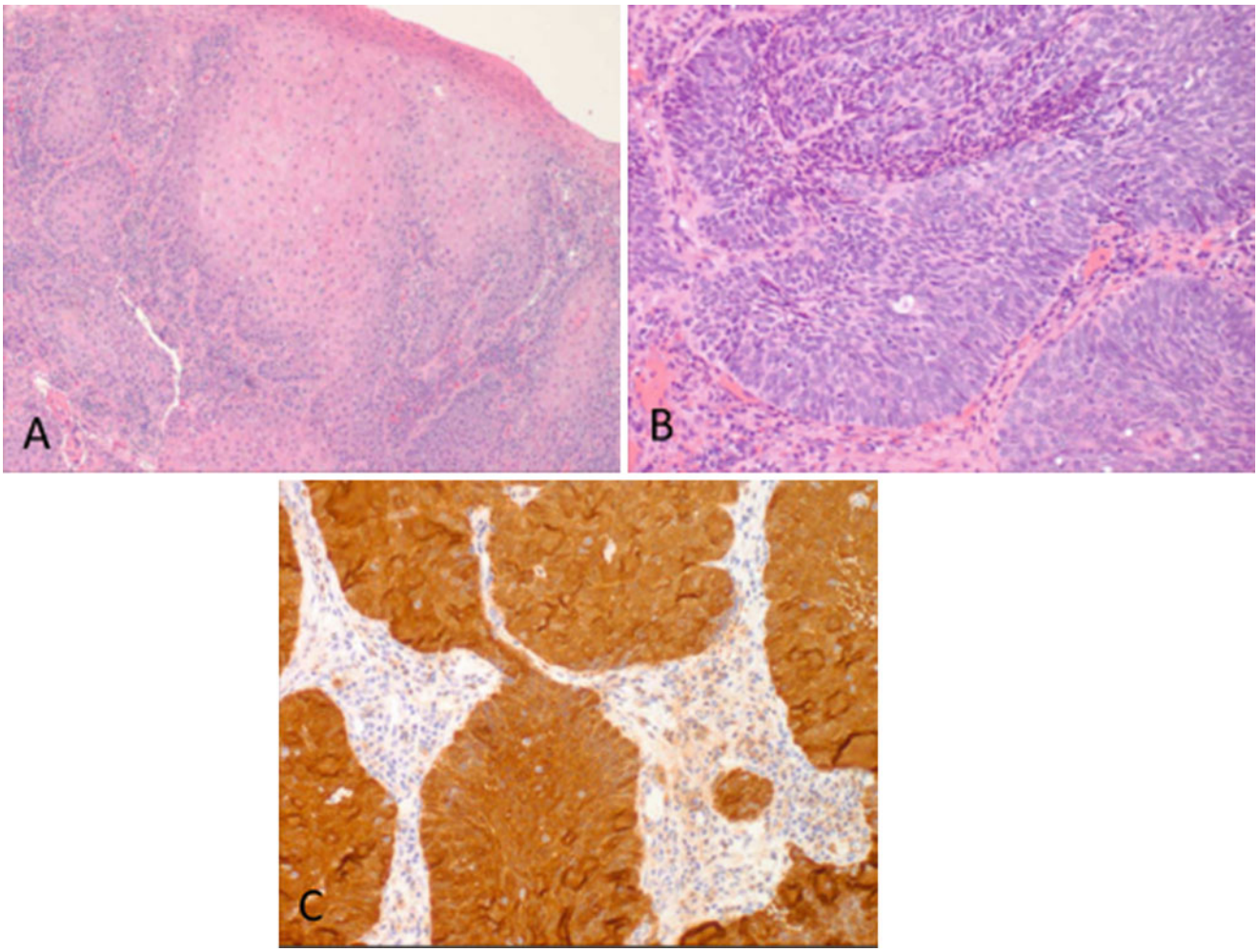

Fig. 32.1 The histology of conventional and HPV-associated head and neck squamous cell carcinomas (HNSCC). (**a**) A well differentiated conventional type HNSCC with keratinization (hematoxylin-eosin, 100×). (**b**) HPV-associated HNSCC which lacks keratin and has a poorly differentiated basaloid histology (hematoxylin-eosin, 200×). (**c**) The same tumor in part **b** with diffuse and strong nuclear and cytoplasmic p16 staining (p16 immunohistochemistry, 200×).

p53 mutations or deletions and LOH at 13p and 18q are associated with increased genomic instability and aneuploidy are commonly seen in carcinomas [5]. Additional events important to the events in carcinogenesis include the activation of telomerase, and overexpression of epidermal growth factor receptor (EGFR), cyclooxygenase-2 (COX-2), and cyclin D1 (11q13) [6]. Loss of heterozygosity (LOH), promoter methylation, and somatic mutations are all mechanisms of genetic alterations that play a role in the multistep progression model of HNSCC carcinogenesis. Below is further discussion on several of the molecular alterations that seem to play an important role in tumorigenesis.

32.2.1 Field Cancerization/Precursor Lesions

The study of pre-malignant lesions and the concept of field cancerization have been best studied and described in oral squamous cell carcinomas (OSCCs) in part because of the accessibility of these lesions for clinical observation and biopsy. The term field cancerization was proposed in 1953 by Slaughter et al. to explain the high incidence of local recurrences of HNSCC after treatment and the high likelihood of multiple independent tumors arising in the oral mucosa [4, 7]. Oral leukoplakias are precursor lesions that are visible to the naked eye. However, evidence suggests that there are precursor lesions that cannot be seen on a macroscopic level. Initially histologic studies and now molecular studies have shown that dysplasia and genetic changes are frequently seen in the grossly normal appearing tissues surrounding carcinomas [4]. This is significant because of the real potential that if these areas of genetically abnormal epithelium are not resected at the time of surgical excision of the tumor they are likely an important source of local recurrences and secondary primary tumors. Approximately 10–30 % of resected cancers recur despite negative surgical margins [4, 8]. Studies have shown that the carcinoma and their adjacent fields are often clonally related, supporting the

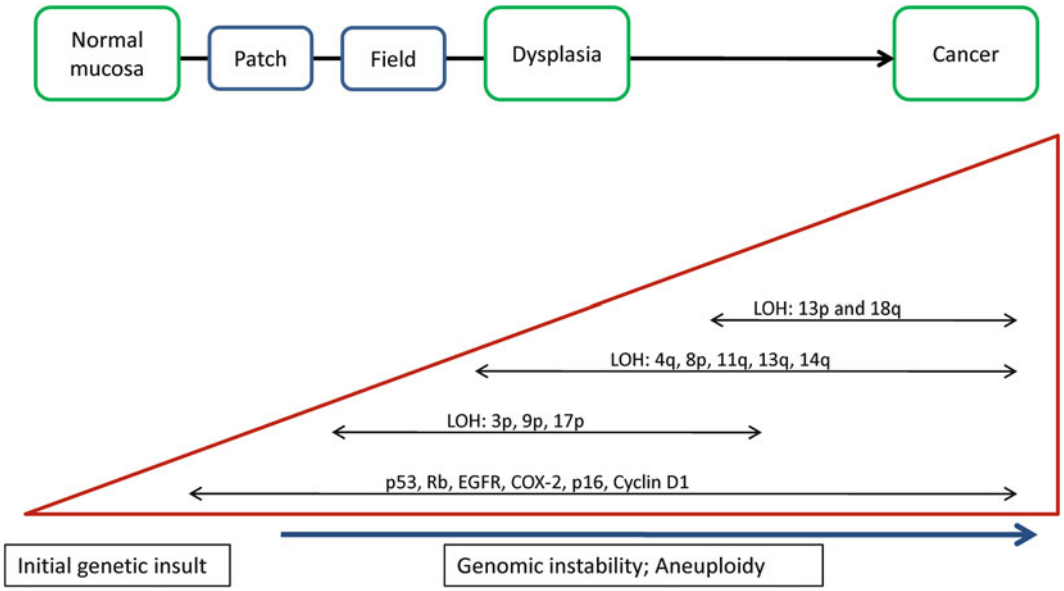

Fig. 32.2 The multistep genetic progression model of conventional head and neck squamous cell carcinoma (HNSCC) carcinogenesis [4–6, 71]. An initial genetic insult occurs (e.g. exposure to mutagenic tobacco products) which leads to a series of genetic events along the pathway to carcinogenesis. Histologically, this manifests as normal mucosa transitioning to dysplasia and eventually frank carcinoma. Events associated with dysplasia and early phases of carcinogenesis include loss of heterozygosity (LOH) at 3p, 9p, and 17p. Accumulated genetic changes lead to genetic instability and aneuploidy characteristic of carcinoma. This also includes mutations and epigenetic silencing of genes as well as upregulation and overexpression of genes all leading to deregulation of the cell cycle and uncontrolled proliferation.

hypothesis that the field precedes the invasive carcinoma [9]. It is hypothesized that a stem cell acquires the genetic alteration and passes it onto it progeny eventually evolving into a field that develops a cancer. In addition, van Houten et al. have shown that the mucosa of patients with carcinoma may harbor focal patches with *TP53* mutations that are not identical to the tumor (i.e. not clonally related), these *TP53* mutated patches or clonal units are considered to represent the first oncogenic changes in the mucosa and these findings suggest that exposure to carcinogens, such as tobacco and alcohol, leads to multiple unrelated clonal patches [8]. These findings are the basis of the patch-field-tumor-metastasis progression model for the development of HNSCC (Fig. 32.2) [4, 9].

32.2.2 Alterations in Signaling Pathways

32.2.2.1 p53 and Rb Pathways

One of the first genetic changes in HNSCC is disruption of the p53 pathway. *TP53* is a tumor suppressor gene which induces apoptosis and is an important regulator of the cell cycle. Disruption of p53, through *TP53* mutations, LOH at 17p or the interaction with viral proteins, is one of the most common genetic alterations seen in HNSCC. Somatic *TP53* mutations have been identified in 60–80 % of HNSCC [4]. In a multicenter study of 560 patients with HNSCC, Poeta et al. screened the tumors for *TP53* mutations using the Affymextix GeneChip p53 assay for high-throughput detection of mutations in exons 2-11 of *TP53* with sequencing for confirmation. They defined two categories of mutations based on the location of the mutation and the predicted amino acid alterations. Disruptive mutations were defined as stop codons in any region or non-conservative mutations located inside the key DNA-binding domain, and non-disruptive mutations are non-conservative or conservative mutations outside the DNA-binding domain. The results of their study showed that the presence of a disruptive *TP53* mutation was strongly associated with a decreased overall survival ($P<0.001$) [10].

Studies have shown that p53 immunohistochemistry (IHC), looking specifically for protein overexpression, does not correlate with *TP53* mutation status for several reasons: (1) *TP53* mutations at splice sites, frame shifts, or nonsense mutations would produce a truncated p53 protein that may not be detectable by IHC; (2) other causes of overexpression such as retention and secondary stabilization of wild-type p53; and (3) poor tissue processing (i.e. over fixation, delayed fixation, or inadequate tissue processing) which would result in p53 degradation [11].

P53, when activated by DNA damage, is an important intracellular regulator of apoptosis by affecting regulation of expression of apoptosis-regulatory proteins. These regulatory molecules include the Bcl-2 family of proteins which have either anti-apoptotic (Bcl-2, Bcl-XL) or pro-apoptotic (Bax, Bak) effects. Thus, alterations in p53 expression can change the ratio of anti-apoptotic to pro-apoptotic regulatory proteins which can promote tumor cell survival and resistance to chemo and radiation therapies [5]. For example, Nichols et al. reported that in a subset of patients with oropharyngeal squamous cell

carcinoma (OPSCC), pretreatment Bcl-2 expression was an independent risk factor (independent from HPV status) for treatment failure and distant metastasis after therapy [12].

The retinoblastoma (Rb) pathway (p16-cyclin D-CDK4/6-Rb axis) can be deregulated at a number of points. Inactivation of the *CDKN2A* gene (region 9p21), which encodes for p16, a key regulatory molecule, by mutation, deletion, LOH or a combination of these is another frequent occurrence in HNSCC [4]. Humans have three cyclin D proteins referred to as cyclin D1-3 which are present in most proliferating cells. *CCND1*, which encodes cyclin D1 (region 11q13), a key activator of the Rb pathway, shows gain or amplification in more than 80 % of HPV-negative HNSCCs [13]. The Rb protein itself can be inactivated by HPV oncoproteins, as discussed below. Taken together, disruption of the p53 and Rb pathways lead to disordered cell cycle regulation and immortalization of the neoplastic cells.

32.2.2.2 Tyrosine Kinase Receptors: The EGFR and MET Pathways

Epidermal growth factor receptor (EGFR) is a member of the Erb family of four tyrosine kinase receptors that form homo- or heterodimers upon ligand binding and initiate signaling cascades through the Ras-MAPK, PI3K-PTEN-AKT, and phospholipase C pathways. Studies have shown that EGFR is overexpressed in many HNSCCs. However, these were based on immunohistochemistry studies with variations in antibodies and techniques; therefore, the reported prevalence rates of overexpression are widely variable. Overexpression may be a result of receptor cross phosphorylation (autocrine activation) or gene amplification; rates of EGFR amplification are reported to be 10–30 % [4]. EGFR copy number alterations have been associated with a poor prognosis and this observation did lead to a clinical trial that showed improved locoregional control and progression-free survival with radiotherapy in combination with the EGFR-specific antibody cetuximab (Erbitux) versus radiotherapy alone [14, 15].

EGFR has multiple tyrosine phosphorylation sites that interact with different partner proteins with different intracellular signaling, thus the real role of EGFR in HNSCC is not clear and maybe different for different tumors. In addition, Epidermal Growth Factor (EGF)-bound EGFR is able to translocate to the nucleus and function as a transcription factor or coactivator of genes involved in cell proliferation. *CCND1*, the gene for Cyclin D1, appears to be one of the targets induced by intranuclear EGFR, linking the mitogenic stimulation of EGF-EGFR to cell cycle promotion [16].

Few activating *EGFR* mutations have been found in HNSCC, which is not entirely surprising when taken in the context of the lung carcinoma data which have shown that activating *EGFR* mutations are essentially exclusive to adenocarcinoma histology [17]. The most common EGFR alteration, seen in up to 42 % of the HNSCCs analyzed, is the *EGFR* variant III (EGFRvIII) truncation mutation, which has also been observed in gliomas and non-small-cell lung carcinomas [5, 16]. EGFRvIII leads to constitutive activation of the receptor in the absence of ligand or receptor overexpression. These mutants also seem to show resistance to chemotherapy and targeted EGFR monoclonal antibody treatments, but on univariate analysis was associated with better disease control [18, 19].

MET (the receptor for hepatocyte growth factor (HGF); also known as scatter factor) is another receptor tyrosine kinase that is also able to activate the AKT and Raf-MAPK pathways. MET and its ligand HGF play a role in invasion and metastasis through up regulation of the expression of matrix metalloproteinases MMP-1 and MMP-9 [5]. *MET* (region 7q31) mutations and gene amplification have been identified in HNSCC, but biomarkers to predict response to anti-MET treatment have not yet been identified [4].

32.2.2.3 NOTCH1 Pathway

One unexpected finding from whole exome sequencing of a set of HNSCC was the identification of *NOTCH1* mutations. While *NOTCH1* has been implicated in the tumorigenesis of several lymphomas and, *NOTCH1* mutations have also been identified in lung SCC, they are not frequent in other solid tumors [20, 21]. *NOTCH1* inactivating mutations are seen in approximately 10–19 % of HNSCC making it second only to TP53 as most frequently mutated gene in HNSCC [20, 22, 23]. NOTCH1 is a transmembrane receptor with extracellular and intracellular domains. The intracellular domain is cleaved and translocation to the nucleus is necessary to turn on target genes which are necessary for cell differentiation (including keratinocytes) and embryonic development [22, 24]. In the setting of HNSCC *NOTCH1* appears to act as a tumor suppressor gene where *NOTCH1* mutations lead to silencing of the pathway. However, in a study by Sun et al. in about 30 % of tumors, there was an increase in gene copy number and expression of the NOTCH1 receptor or ligands and subsequent downstream pathway activation. These findings suggest a bimodal pattern of alterations in the NOTCH pathway in HNSCC and this may lend itself to the use of NOTCH pathway targeted therapeutics [22].

32.2.2.4 PI3K-PTEN-AKT Pathway

The PIK3-PTEN-AKT pathway is also important in HNSCC. The class Ia PIK3s, form heterodimers with a receptor tyrosine kinases, such as EGFR, or adaptor molecules, are activated by phosphorylation and lead to downstream activation of AKT, a serine/threonine kinase. Activated AKT subsequently activates a number of downstream proteins including apoptosis inhibitors, cell cycle inhibitors, and transcription factors which promote cell proliferation and survival. PTEN is a tumor suppressor that counteracts the activation of AKT. This pathway can be deregulated at a number of points. Homozygous deletions or inactivating mutations in *PTEN* (region 10q23) have been

reported in 10 % of HNSCC; in these tumors, the AKT pathway cannot be turned off so the cells proliferate uncontrollably [25]. One of the PIK3 subunits, the p110α catalytic peptide, is encoded for by *PIK3CA*, located on the 3q26 locus which is frequently gained in HNSCC. *PIK3CA* somatic mutations have also been reported in 10–20 % of HNSCC [4]. In one study, *PIK3CA* mutations were more frequently seen in HPV-positive tumors versus HPV-negative tumors, and were associated with activation of mTOR suggesting a possible role for PI3K/mTOR inhibitors in *PIK3CA* mutant HNSCC [26].

32.2.3 Immortalization

Human telomeres, repetitive tandem DNA repeats at the ends of the chromosomes, naturally shorten after each cell division which purposefully limits the life span of cells. In tumor cells, this process likely needs to be overcome to maintain proliferation. The enzyme telomerase elongates telomeric DNA by reverse transcription via its catalytic subunit TERT (telomerase reverse transcriptase). Telomerase or TERT activity is detected in up to 80 % of HNSCC, allowing for the potential of limitless replication of the tumor cells [4, 5]. Recurrent mutations in the *TERT* promoter have been described in several tumor types; in laryngeal squamous cell carcinomas, univariate survival analysis showed that *TERT* promoter mutations were significantly associated with poor survival with a hazard ratio (HR) of 1.52 (95 % CI, 1.05–2.20; *p* 0.03) [27].

32.2.4 Angiogenesis

Angiogenesis is very important in the development of cancer; tumors require a new blood supply in order to invade and metastasize. This is achieved through a shift in the balance between pro-angiogenic and anti-angiogenic factors. Solid tumors induce neo-angiogenesis by producing angiogenic factors, such as vascular endothelial growth factor (VEGF) [5]. In one meta-analysis, VEGF expression seemed to be associated with worse overall survival in HNSCC patients [28]. More studies are needed to further elucidate the role of VEGF and its potential as a therapeutic target in HNSCC.

32.2.5 Invasion and Metastases

HNSCCs classically invade locally and metastasize primarily to cervical lymph nodes. Steps involved in invasion and metastasis involve a complex process of: (1) alterations in cellular adhesion and the cytoskeleton, (2) cellular migration and dissolution of the basement membrane and extracellular matrix, (3) movement into and survival in the blood stream, (4) extravagation at the metastatic site with (5) subsequent growth and neovascularization [5]. Epithelial-to-mesenchymal transition (EMT) is a process associated with invasion and metastases where cells shift from an epithelial to a mesenchymal phenotype such that their shape changes and they become more motile. Alterations in e-cadherin, a cell surface molecule, are associated with EMT. Integrin that mediate cell-cell and cell-matrix interactions play a role in the motility and invasion of tumor cells. Enzymes, secreted by the tumor cells such as matrix metalloproteinases (MMP) are likely involved with the remodeling of the extracellular matrix around invasive and metastatic tumor cells [5].

32.2.6 Gene Expression Profiles

Messenger RNA (mRNA) expression analysis has been used to categorize HNSCC into subtypes based on expression profiles. Unsupervised hierarchical clustering is a powerful discovery tool that is often used for this purpose and heatmaps of mRNA expression values of a large number of classifier genes are used to visualize the clustering into subtypes. Walter et al. mRNA expression microarrays on 138 tumor samples using an 840 gene classifier and The Cancer Genome Atlas (TCGA's) preliminary data on HNSCC have resulted in consistent classification of HNSCC into four molecular subtypes: basal (BA), mesenchymal (MS), atypical (AT), and classical (CL) based on the characteristics of the genes that are highly expressed in each subtype [29, 30]. These studies also confirm the finding of four gene expression subtypes reported by Chung et al. in 2004 [31]. When comparing the HNSCC subtypes to those reported in lung SCC by Wilkerson et al and the TCGA, there is a striking correlation with the HNSCC BA, MS, and CL subtypes with lung SCC basal, secretory, and classical subtypes, respectively [21, 32].

The BA signature is similar to that seen in basal cells of human airway epithelium; MS tumors had elevated expression of epithelial-to-mesenchymal transition (EMT) associated genes. The AT tumors have a strong HPV-positive signature (although only 14 HPV-positive cases where included in the study) and lacked either *EGFR* amplification or deletion of 9p; and, the CL subtype showed high expression of genes associated with cigarette smoke exposure as well as deletion of 3p and 9p, amplification of 3q, *EGFR*, and *CCND1*. Each subtype is associated with distinct expression patterns and chromosomal gains and losses. For example, gain of 3q, a common alteration in SCC, contains *TP63*, *PIK3CA*, and *SOX2* was associated with variable expression of these genes in the different subtypes. The CL and AT subtypes show higher SOX2 expression relative to the MS and BA subtypes, whereas, BA subtype showed the highest level of p63 expression of all. Interestingly, while MS had 3q copy number gains, the target genes above did not show expression levels any higher than normal tonsil [29]. These findings

suggest that there is likely differential usage of transcription factors (i.e. *SOX2* and *TP63*) and oncogenes (i.e. *PIK3CA*) that may contribute to the heterogeneity seen in HNSCC and help define the expression subtypes [29].

32.2.7 miRNA

MicroRNAs (miRNAs) are a class of noncoding regulator RNA molecules that are expressed in a tissue-specific manner and are believed to control the expression of up to 30 % of genes via a negative regulation on their target messenger RNA (mRNA) by either targeting the mRNA for degradation or blocking translation. Several excellent reviews on the role of miRNAs in human cancers have recently been published and the discovery of miRNAs is changing how we understand the molecular pathways)associated with carcinogenesis [33, 34].

Within HNSCC, researchers have confirmed deregulation of a number of miRNAs in tumor cells versus corresponding normal tissues but, with considerable variation across studies. However, there does appear to be at least a core set of miRNAs comprising miR-21, miR-31, miR-115, miR-155, and miR-205 which are consistently up-regulated and let-7b, miR-26b, miR-107, miR-133a/b, miR-138, and miR-139 which are consistently down regulated in HNSCC [35, 36]. Because a single miRNA may have hundreds or more potential gene targets, the causal role played by any individual miRNA in the carcinogenesis transformation is difficult to define and the relationship of miRNAs to different etiologies of HNSCC (e.g. HPV-positive cases versus conventional) is not entirely clear. HPV infection, by disrupting the p53 and pRb pathways, may lead to a signature increase of miR-16, miR-25, miR-92a, and miR-378 and the decrease of miR-22, miR-27a, miR-29a, and miR-100 [37]. Recent studies have reported progression of leukoplakia to squamous cell carcinoma associated with miRNA-345, miRNA-21, and miRNA181b; higher rates of locoregional occurrences and sorter survival associated with low expression levels of miRNA-205 and Let-7d; and, miRNA-451 expression a strong predictor for relapse [38–40]. We can anticipate many future studies evaluating the role of miRNAs in HNSCC for their potential as markers of prediction, prognosis, monitoring of disease, and targeted therapeutics.

32.3 Etiology and Biology of HPV in HPV-Associated HNSCCs

HPV-positive HNSCC tumors are different from conventional HNSCC in their clinicopathologic features and molecular pathogenesis (Table 32.1). While the incidence of HPV-unrelated HNSCC is decreasing, the incidence of HPV-related HNSCC has risen by about 11 % in the last two decades [3]. HPV type-16 (HPV-16) was discovered in the 1970s and since then, its role as an oncogenic virus has been well established and particularly well characterized in cervical cancer [41, 42].

Low-risk HPV has long been known to be associated with respiratory papillomas. The role of high-risk HPV in HNSCC has been studied since the 1980s and it was found that the same *E6* and *E7* viral oncogenes that were carcinogenic in cervical cancer were also involved in development of squamous cell carcinoma in the upper aerodigestive tract, especially in the oropharynx [43]. The majority (~90 %) of these cancers are associated with HPV-16 [44]. A case-control study in 1998 showed that the presence of HPV in the oral cavity was associated with an increased risk of oropharyngeal cancer independent of tobacco or alcohol exposure [45]. In 2000, Gillison et al. showed for the first time that HPV-positive HNSCC is most commonly associated with HPV-16, oropharyngeal sites, high grade non-keratinizing or minimally keratinizing morphology (Fig. 32.1b) and a more favorable clinical outcome than HPV-negative tumors. Overall, their patients with HPV-positive tumor had a 59 % reduction in risk of death from cancer in comparison to patients with HPV-negative tumors [46].

Table 32.1 Differences in the clinical and biologic features between HPV-negative and HPV-positive HNSCC

Characteristic	HPV-negative HNSCC (approximately 80 %)	HPV-positive HNSCC (approximately 20 %)	References
Incidence	Decreasing	Increasing	[3]
Etiology	Smoking, alcohol abuse	Multiple sexual partners	[66, 78]
Patient age	Greater than 60	Less than 60	[79]
Site of origin predilection	None	Oropharynx	[46]
Field cancerization	Yes	Unknown	[8]
Precursor lesions	Step-wise progression of dysplasia	None identified	[59]
Histology	Keratinizing	Non-keratinizing or minimally keratinizing	[59, 66]
TP53 mutations	Frequent	Infrequent	[51]
P16 protein overexpression	Infrequent	Frequent—detectable by IHC	[64]
Chromosomal instability (CIN)	Frequent (65 %)	Infrequent	[4, 51]
Prognosis	Poor	Favorable	[46, 69]

Adapted in part from Leemans et al. [4]

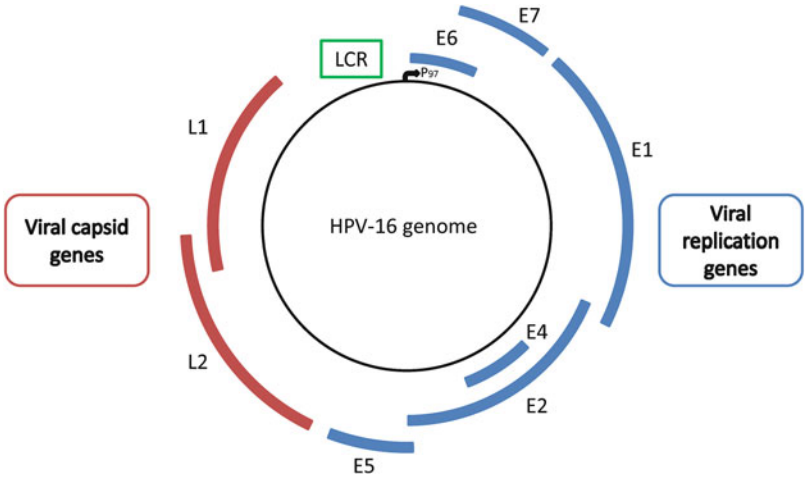

Fig. 32.3 HPV 16 genome. HPV 16, the major type of high risk HPV seen in HNSCC, has a 7904 base pair, double-stranded circular DNA genome which encodes a set of early (E1, E2, E4, E5, E6, and E7) and late genes (L1, L2) on one of the DNA strands. The viral Long Control Region (LCR) contains elements that regulate transcription and replication. P_{97} is the transcriptional promoter. The early genes are involved in viral replication while the late genes encode for the major and minor capsid proteins. E6 and E7 play a role in carcinogenesis by binding to p53 and retinoblastoma protein (pRb), respectively. Adapted from Lin et al. [48].

HPV is a circular, non-enveloped double-stranded DNA virus that is strictly epitheliotropic (preferentially infecting squamous epithelial cells). There are greater than 100 known viral subtypes and the ones known to be associated with cervical carcinogenesis have been designated as high-risk HPVs (most notably HPV-16 and HPV-18) [47]. The HPV genome is around 8000 base pairs and is organized into three regions: early region, late region, and the Long Control Region (LCR). The genome encodes at least six early genes (*E1*, *E2*, *E4*, *E5*, *E6*, and *E7*) and two late genes (*L1* and *L2*) on one strand. The early genes are responsible for regulation of viral DNA replication and the late genes encode the viral capsid [48]. The LCR does not code for viral proteins but contains replication and transcriptional control sequences that interact with viral (E1 and E2) and host proteins (Fig. 32.3) [49].

HPV infection is presumed to be via microtrauma and infection of the basal epithelial cells and in the majority of instances, infection is transient and self-limiting by the host's immune response [48]. The immature epithelial basal cells allow for expression of the early HPV genes whereas in more mature and terminally differentiated cells viral transcription shifts to the late genes which allows for release of newly formed virions away from the host's immune surveillance in the submucosa. Most often HPV remains episomal but, in some persistent infections, high risk HPVs may integrate into the host genome. With integration there is often deletion of *E2*, *E4*, *E5*, *L1*, and *L2* genes. The loss of E2, a transcriptional repressor of E6 and E7, allows upregulation of the viral oncogenes *E6* and *E7*, and their expression causes inactivation of p53 and retinoblastoma (Rb) pathways, respectively [48]. The E6 oncoprotein targets the p53 protein for ubiquitination and degradation thus disrupting the p53 pathway, which plays a role in the host's response to DNA damage (Fig. 32.4). E6 also activates telomerase which allows cells to regenerate chromosomal telomers after cell division [50]. The E7 oncoprotein inactivates pRb, an important cell cycle regulatory protein, through direct interaction (Fig. 32.5). Integration leads to disordered cell cycle regulation (genomic instability and repression of apoptosis) in the infected cells which is considered to be the mechanism of carcinogenesis [42].

Data support that HPV-positive HNSCCs form a distinct molecular entity within HNSCC based on different genetic profiles. In a case- control study of the genetic patterns found in tumors positive for HPV DNA and E6 and E7 mRNA versus tumors negative for HPV DNA and E6 and E7 mRNA, Braakhuis et al. found that the HPV DNA—E6/E7 mRNA positive tumors had less LOH than the HPV-negative tumors (14 % versus 81 %) which was statistically significant for the markers tested specifically on 3p, 9p, and 17p ($P < 0.05$). In addition, none of the HPV-positive tumors harbored mutations in *TP53* but, mutations were found in 75 % of the HPV-negative tumors ($P < 0.001$). Interestingly, cases that were HPV DNA positive but negative for E6 or E7 mRNA had similar LOH pattern and presence of *TP53* mutations as the HPV-negative group. This suggests that the E6 and E7 transcriptional activity is driving these tumors [51]. This group has shown that 77 % of dysplastic lesions (early in the carcinogenesis pathway of conventional HNSCC) exhibited LOH at 3p, 9p, and/or 17p, therefore the lack of LOH in the HPV DNA and E6 and E7 mRNA positive cases is a significant finding which supports the concept that HPV infection with E6 and E7 integration is an early and likely initiating oncogenic event [51]. In addition, the disruption of these oncoproteins

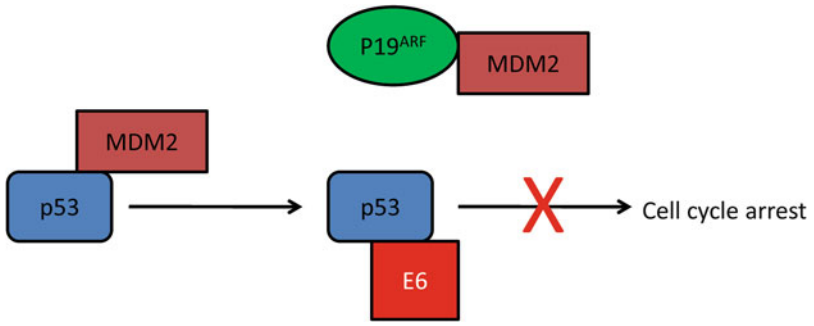

Fig. 32.4 Simplified p53 pathway [76]. p53 is a tumor suppressor which acts as a transcription factor. When bound to Mdm2, p53 in inactive and unstable. P19ARF binds Mdm2, decreasing its affinity for p53. Unbound p53 is stable and active and induces cell cycle arrest for DNA repair and if necessary, apoptosis. The E2F transcription factor in the pRb pathway binds to the promoter region of p19ARF and is a potent inducer of apoptosis through the p53 pathway. Therefore, inactivation of pRb leads to activation of p53 in the cells. The HVP oncoprotein E6 directly binds p53 and targets it for ubiquination and degradation. Thus the E7 and E6 proteins inactivate pRb and p53 pathways leading to genomic instability and repression of apoptosis.

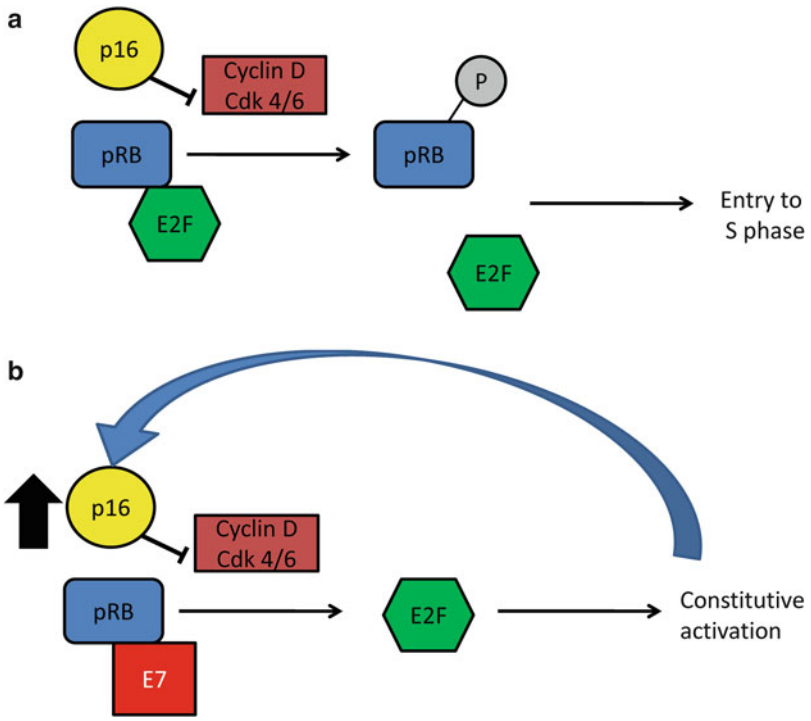

Fig. 32.5 p16-pRB pathway. (**a**) In a normal cell, p16 acts as a tumor suppressor to block the activity of the CDK4/6 Cyclin D kinase complex. This complex is responsible for increasing the phosphorylation of pRB. In the hypophosphorylated form (early G1) pRB binds the E2F transcription factor and inactivates it. In the phosphorylated state, pRb releases E2F which leads to transcription and progression into the S phase of the cell cycle. (**b**) In an HPV infected cell that has undergone viral DNA integration into the host genome, E7 binds to the E2F site on pRb, thus blocking the regulatory control of pRb leading to constitutive activation of E2F transcription. The expression of p16 is greatly increased in a futile attempt to provide negative feedback to the pathway. Adapted from Munger et al. [77].

in squamous cell carcinoma cell lines via short hairpin RNAs have resulted in increased levels of p53 and pRb and induction of apoptosis, supporting that these pathways remain intact within HPV infected tumor cells [52]. The absence of *TP53* mutations in HPV-positive tumors has also been seen by others [46, 53]. A number of other studies have demonstrated differences in gene expression between HPV-positive and HPV-negative tumors with the use of microarray comparative genomic hybridization (CGH) [13, 54, 55].

In recent years the spectrum of HPV-associated HNSCC has expanded beyond the oropharynx to include sinonasal squamous cell carcinoma and a fraction of EBV negative nasopharyngeal carcinomas. As in OPSCC, the HPV-associated sinonasal carcinomas have a favorable prognosis;

Table 32.2 Common HPV detection methods

Testing method	Targeted molecule	Tissue type	Advantages	Disadvantages	Detects biologically relevant infection	Detection method
P16 IHC	Cellular protein	FFPE	High sensitivity (100 %) Easy to assess	Low specificity (79 %)	Yes—a surrogate marker for E7 integration	SA
HPV-16 in situ hybridization	DNA	FFPE	Visualization of nuclear localization of HPV DNA Easy to assess	Lower sensitivity than TA methods	Not directly but, a punctuate signal is suggestive of viral integration	SA
RT-PCR E6 or E7	mRNA	Fresh or frozen	High sensitivity and specificity	Poor performance in FFPE Not practical for most labs	Yes—considered the "gold standard" for identifying biologically relevant infection	TA
Consensus- or type-specific PCR and genotyping	DNA	Fresh, frozen, FFPE	High sensitivity	Inter assay variation	No—cannot determine biologically relevant from transient infection or contamination	TA
Real-time PCR	DNA	Fresh, frozen, FFPE	High sensitivity and specificity	Requires tissue microdissection	No but gives an estimate of viral load. Copy number >1 viral genome per cell is highly specific for biologically relevant HPV infection	TA

Adapted from Robinson et al. [62]
IHC immunohistochemistry, *FFPE* formalin-fixed paraffin-embedded tissue, *SA* signal amplification, *TA* target amplification, *PCR* polymerase chain reaction, *RT-PCR* reverse transcriptase PCR

the clinical significance of HPV in nasopharyngeal carcinomas is not yet known [56–59].

The 2005 World Health Organization (WHO) classification of HNSCC is principally based on histologic pattern and degree of differentiation [60]. Lewis and Chernock make the compelling argument that because much has changed in our understanding of HNSCC this should be reflected in the anticipated revised WHO classification. In particular, HPV-associated OPSCC have a different etiology and prognosis than conventional HNSCC, and deserve to be considered separately [59].

32.3.1 Detection of HPV in HNSCC

A number of methods have been used to detect HPV in HNSCCs (Table 32.2) and studies reporting on the prevalence of HPV-positive tumors ranges from 14 to 72 %, most likely because of differences in the sites of origin and in HPV detection methods used in various studies [61]. More likely, approximately 20 % of HNSCCs contain transcriptionally active (i.e. integrated) HPV [4]. The choice of detection method is dependent on the type of specimen available (fresh/frozen, formalin-fixed, paraffin-embedded (FFPE), fine needle aspiration (FNA), or cytology), the preservation of the target molecule (protein, DNA, RNA), the desire to detect a broad group of DNA types or target a specific genotype. The detection methods are based on either target amplification techniques (PCR based) or signal amplification using chemical or colorimetric indicators [62]. For an HPV test to be useful in routine clinical diagnostic practice it needs to be reliable at classifying HPV-driven tumors (clinically sensitive and specific), reproducible, subject to standardization and quality assurance and be economically feasible for the laboratory [62].

Polymerase chain reaction (PCR) is a technique that is able to amplify specific rare DNA sequences in a background of diverse DNA molecules. It is a qualitative technique and therefore provides no quantitative information about the target DNA. Because of its high analytical sensitivity it may provide false-positive results. In the case of HPV testing in HNSCC, a positive result does not distinguish among DNA integrated into the host genome (i.e. biologically significant), a transient infection or a contaminated specimen. Consensus PCR using GP5+/6+ primer sets, directed to the L1 gene which encodes the major capsid protein, is commonly used [63]. Consensus PCR products can then be genotyped by a variety of methods including a linear array assay and reverse line blotting [64, 65].

Real-time PCR can amplify HPV genotype-specific sequences, compare them to the amplification of an endogenous control gene such as β-globulin and provide quantitative measurement of the viral copy number per cell number analyzed. Smeets et al. have found that a copy number of >1 viral genome per cell is highly specific for biologically relevant infection [64].

In situ hybridization (ISH) for high risk HPV is performed on glass slides with bright-field microscopy and provides a visual determination of the signal localization to the infected cell nucleus. A punctuate nuclear staining pattern is highly suggestive of viral integration into the host genome as opposed to a more diffuse pattern seen with episomal (non-integrated) virus [66]. Two ISH testing strategies are available using either genotype-specific probes (HPV-16 or HPV16/18) or a high-risk HPV probe cocktail [63].

It has been proposed that p16 overexpression, which is detectable by IHC, can be used as a surrogate marker for HPV

DNA integration into the host genome (Fig. 32.1c) [67]. In one study, this method had 100 % sensitivity in screening for transcriptionally active infection. However, there are HPV independent pathways that can drive oncogenesis and lead to increased p16 expression so the specificity was only 79 % [64]. A significant benefit of the p16 IHC is that, unlike some in situ assays, it is not specific to HPV type.

The National Comprehensive Cancer Network (NCCN) and the College of American Pathologists (CAP) recommend routine HPV testing for all oropharyngeal squamous cell carcinomas (OPSCCs). At this time there are no guidelines for the best testing strategy for determination of HPV status in HNSCCs. However, the College of American Pathologists has developed a work group with this focus. Two diagnostic algorithms have recently been proposed. The first utilizes p16 IHC followed by HPV DNA (using the GP5+/6+ primers) detection with a polymerase chain reaction (PCR) amplification technique. This method was validated for formalin-fixed paraffin-embedded (FFPE) biopsy samples by comparing with HPV E6/E7 mRNA in matched frozen tissue samples [64]. HPV E6/E7 mRNA is considered the reference test of choice or the gold standard for transcriptionally active and biologically significant HPV infection. Although more reliable than standard PCR for HPV DNA (which can have false-positives), reverse transcriptase (RT)-PCR assays for E6 and E7 transcripts do not work well in FFPE tissue specimens.

The second algorithm utilizes p16 IHC followed by in situ hybridization (ISH) for HPV DNA [68]. This algorithm has been clinically validated as an independent prognostic marker through its use in clinical trials and epidemiological studies [63, 69].

Most pathologists and oncologists agree on the importance of testing OPSCCs for high-risk oncogenic HPV because this provides prognostic information and in the future may be used in treatment decision making. In addition, in patients with metastatic HNSCC of unknown origin, a positive HPV status could direct the search for the primary tumor to the oropharynx [62].

32.4 Standard Treatment and Targeted Therapeutics

A majority of patients with HNSCC present with locally advances stage III or IV cancer that is usually treated with a combination of chemotherapy, radiation, or surgery. In general, two approaches are taken: either (1) induction chemotherapy (cisplatin is the first choice) followed by radiation therapy or (2) concurrent chemo-radiotherapy [70]. Patients who present with earlier stage I or II disease are most often treated with radiation or surgery and have an excellent prognosis although they are still at risk for local recurrence or second primary tumors [71].

Anti-EGFR monoclonal antibodies (mABs), which specifically recognize the extracellular ligand-binding domain of EGFR, are clinically being used in patients with HNSCC. But, there are currently no validated testing methods for selecting patients likely to respond to EGFR targeted therapies. Bonner et al. demonstrated in a phase III study, significant survival benefit (from 29.3 to 49.0 months) and local-regional control (from 14.9 to 24.4 months) with the addition of cetuximab (Erbitux) to radiation therapy in patients with stage III and IV HNSCC [14]. Based on these result, additional monoclonal antibodies (panitumumab and matuzumab) are being used in clinical trials. In 2006, the U.S. Food and Drug Administration (FDA) approved cetuximab for use in combination with radiation therapy (RT) for the treatment of locally or regionally advanced HNSCC or as a single agent in patients with recurrent or metastatic HNSCC who had failed platinum-based therapies.

The small-molecule EGFR tyrosine kinase inhibitor (TKI) erlotinib (Tarceva) bind to the receptor's intracellular ATP-binding site, thus inhibiting autophosphorylation. In lung cancer, TKIs have been shown to have higher affinity for the TK domains in mutated EGFR, thus in HNSCCs, which rarely have EGFR activating mutations, and TKIs may be less effective than mABs [72]. However, TKIs are actively being studied as targeted therapies in HNSCC and phase I and II trials have demonstrated a modest increase in progression-free and overall survival [51, 52]. A phase III trial of gefitinib compared to methotrexate for recurrent HNSCC did not show improved survival compared to methotrexate [72]. Additional clinical trials are needed to further elucidate the therapeutic prospects of TKIs in HNSCC.

The EGFRvIII variant is another possible target for therapy. EGFRvIII is the result of the deletion of amino acids 6-273 in the EGFR extracellular domain which leads to a reduced affinity to mABs. Targeted immunotherapy for the EGFRvIII is currently in development for glioblastomas. If immunotherapy is shown to be applicable to HNSCC, this would be a promising therapeutic target for cetuximab-refractory tumors that express EGFRvIII [72].

The anti-VEFF mAb, bevacizumab is another agent being evaluated in clinical trials that has shown some efficacy in HNSCC. Phase I and II trials of bevacizumab in combination with anti-EGFR agents, pemetrexed and chemoradiation have shown some promising results and phase III trials are underway [73, 74]. Additional promising targeted therapeutic agents include lapatinib (a selective dual inhibitor of both EGFR and HER2 TKs), sunitinib (a TKI that targets RET, VEGFR, PDGFR, and KIT), cediranib (selective inhibitor of VEGF signaling), bortezomib (the first proteasome inhibitor selected for development as an anti-cancer agent), figitumumab, and cixutumumab (insulin growth factor receptor (IGF-1R) mAbs) [73].

TP53 gene therapy is another fascinating treatment modality that is being investigated in HNSCC. Advexin, which is an adenovirus that enters the cell via a cytomegalovirus promoter, delivers a functional wild-type *TP53* gene into the tumor cells, thus triggering growth arrest and/or apoptosis leading to tumor inhibition and necrosis. The therapy is delivered via injection to the tumor site and is well tolerated with flu-like symptoms being the most common side effect. Although this form of gene therapy is associated with tumor response, it has not yet been shown to improve patient survival [73]. Studies of the use of such agents combined with other targeted therapies and chemoradiation therapy are ongoing.

Currently, despite the prognostic benefit of HPV-positive tumors, there are not different treatment regimens being utilized for HPV-positive versus HPV-negative tumors. Ang et al. in a retrospective study of patients with stage III or IV oropharyngeal squamous cell carcinoma (SCC) treated with radiation and combined cisplatin, were able to classify these patients into low, intermediate, or high risk of death based on tobacco use, stage, and HPV status. HPV status was an independent prognostic factor for survival in these patients [69]. Several current de-escalation clinical trials are underway trying to evaluate if current treatment paradigms need to be altered based on HPV status to reduce the acute and chronic treatment related morbidity. Several approaches are currently being evaluated including replacing cisplatin with cetuximab to reduce toxicity. RTOG 1016 (NCT01302834) is one such phase III trial involving p16 positive OPSCC patients randomized to weekly cetuximab or cisplatin with concurrent accelerated intensity-modulated radiation therapy. The trial enrolment has been completed but provisional data will not be available for several years. Another approach is induction chemotherapy followed by decreased chemo-radiotherapy dose in good responders. ECOG-1308 (NCT01084083) is a phase II trial that includes induction chemotherapy (cisplatin, paclitaxel, and 90 cetuximab) followed by low dose (54 Gy) or standard dose (70 Gy) radiation therapy with cetuximab, dependent on response to induction chemotherapy. Preliminary results for this trial show that a favorable response to induction chemotherapy may identify a group of HPV+ patients with a low 1-year failure rate with the lower radiation dose. The third approach to de-escalation therapy includes surgical resection with or without adjuvant chemo-radiotherapy randomized based on histopathology features (such as extra nodal extension). The excellent article by Masterson et al. provides a more extensive review of current de-escalation protocols for HPV-associated OPSCC [75].

In the next several years, we can expect that clinical trials, epidemiologic observations about tumor HPV status and smoking history and ongoing genomic research will lead to a more molecular-based classification of HNSCC and a better understanding of targeted therapies and biomarkers that will drive more individualized therapies for HNSCC patients.

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Ovarian Cancer

33

Amy D. Brockmeyer and Louis Dubeau

33.1 Introduction

Ovarian carcinomas account for more deaths annually than any other gynecological cancer in the US. More than twice as many women die of ovarian cancer than endometrial cancer in spite of the fact that cancer of the endometrium is more frequent than that of the ovary. The 5 years survival of patients affected by these tumors has improved only modestly, from 45.4 to 48.6 % during the last two decades [1]. The high mortality associated with ovarian carcinomas is due largely to the hidden anatomical location of the ovaries, which complicates the detection of abnormalities on physical examination, and the fact that these tumors typically remain asymptomatic until they spread outside the ovary, at which time they are difficult to eradicate. Hopefully, advances in our understanding of the histogenesis of ovarian epithelial tumors as well as of their underlying molecular mechanisms will lead to more effective strategies aimed at their early detection and prevention. This, in turn, should have a major impact on the morbidity and mortality associated with these tumors.

The majority of ovarian tumors are epithelial (carcinomas). Tumors of this organ that are derived from other cell types, such as germ cell tumors, stromal tumors, and others, share little in common with those of epithelial origin and will not be addressed in this chapter.

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33.2 Classification of Ovarian Epithelial Tumors

Ovarian epithelial tumors are a heterogeneous group that comprises several histological subtypes. Basic knowledge of the most common subtypes is essential to understand issues related to their cell of origin, risk factors, and other biological characteristics.

33.2.1 Histological Classification

The four most frequent subtypes of ovarian carcinomas as well as their distinctive morphological features and are shown in Table 33.1. The question of whether or not these different lesions share a common cell of origin has been the subject of debate among pathologists for several decades. Differences in the risk factors, clinicopathological characteristics, and molecular genetic features of individual subtypes suggest that they are distinct disease entities. However, the fact that different subtypes occasionally co-exist, resulting in mixed lesions that show features of two or more subtypes adjacent to each other within the same tumor mass, provides a strong argument that they are related.

An intriguing characteristic shared by the major subtypes of ovarian epithelial tumors is their resemblance to tumors that originate in other organs of the reproductive tract (Table 33.1). Serous ovarian carcinomas are morphologically similar to tumors arising in the fallopian tubes. This resemblance is so striking that pathologists have agreed several decades ago not to attempt to determine the exact origin of large serous tumors involving both the ovary and the fallopian tubes. It is by pure convention that these tumors are labeled as ovarian, except in rare cases where an origin from the tubes can unequivocally be demonstrated. Endometrioid ovarian carcinomas are morphologically identical to carcinomas arising in the endometrium. Here again, the striking resemblance has led to diagnostic dilemmas related to the fact that when an endometrioid ovarian tumor coexists with

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_33

Table 33.1 Most common subtypes of ovarian carcinomas

Name	Frequency (%)	Morphological features of well differentiated lesions	Segment of the reproductive tract with similar features
Serous	50	Columnar cells with a prominent ciliated border that often form finger-like projections around a fibro-vascular core (papillae) within the inner lining of cysts filled with serous fluid	Fallopian tube and other extra-uterine Müllerian tissues
Endometrioid	25–30	Glandular structures lined by low columnar cells that may be filled with bloody material	Endometrium
Mucinous	10–15	Columnar cells filled with clear mucus pushing cell nucleus toward the basal pole; typically forming complex cystic structures	Endocervix
Clear cell	5	Low columnar to cuboidal cells with clear cytoplasm and hobmail appearance; often forms small glandular structure or solid nests	NA
Others	<5	Heterogeneous group that includes tumors so poorly differentiated that their exact lineage cannot be determined as well as rare subtypes such as tumors showing features of transitional epithelium (malignant Brenner tumor) and others	NA

an endometrial tumor in the same patient, it is not possible to determine whether the ovarian tumor represents a primary lesion or a metastasis from the endometrial lesion. Mucinous ovarian carcinomas are identical to endocervical carcinoma. Clear cell carcinomas are identical to the clear cell variant of endometrial carcinomas. The observation that ovarian carcinomas are similar to tumors arising in these other sites of the reproductive tract is not based solely on morphological arguments, as recent studies have shown that the profile of homeobox genes expressed in serous, endometrioid, and mucinous ovarian carcinomas is similar to that expressed in tumors of the fallopian tubes, endometrium, and endocervix, respectively [2]. This phenomenon has implications regarding the site of origin of ovarian epithelial tumors.

33.2.2 Toward a Molecular Classification of Ovarian Carcinoma Subtypes

There are several examples of molecular genetic alterations in ovarian epithelial tumors that are strongly associated with specific tumor histological subtypes. For example, genetic factors associated with familial ovarian carcinoma predisposition are subtype-specific. Indeed, mutations in BRCA1 or BRCA2 are associated with strong predisposition to serous ovarian carcinomas but not with any of the other tumor subtypes. Germline mutations in DNA repair enzymes leading to microsatellite instability are associated with endometrioid ovarian carcinomas. In addition, specific somatic mutations are more common in certain subtypes of ovarian carcinomas than others. The association of PTEN mutations with endometrioid tumors [3, 4] and the more frequent (although not exclusive) presence of K-RAS or B-RAF mutations in mucinous and serous tumors [5–9] are additional examples. Mutations in ARID1A, a gene encoding a key component of the SWI–SNF chromatin remodeling complex called BAF250a, are associated with endometrioid and clear cell ovarian carcinomas but not with serous ovarian carcinomas [10]. These differences underscore the importance of not lumping different ovarian carcinoma subtypes as if they represented a single entity in research studies.

The development of analytical tools to examine global expression profiles over the last decade has allowed investigators to compare the spectrum of gene expression in different subtypes of ovarian epithelial tumors. Not surprisingly, the current data suggests fundamental differences in the expression profile of each major ovarian epithelial tumor subtype [11–14]. As more data accumulates, it might be possible to identify panels of markers specific for individual subtypes that might assist pathologists in the diagnosis of poorly differentiated ovarian tumors. This could be valuable in ruling out, for example, tumor subtypes associated with a less favorable prognosis such as clear cell carcinomas. In addition, profiling studies comparing tumors from patients with rapid clinical course to those from patients with more favorable outcomes suggest that panels of markers could be developed and used as predictors of clinical aggressiveness or therapeutic response independent of classical predictors such as tumor stage or grade [15, 16]. Finally, these studies could provide important clues about cell lineage derivation and histogenesis. For example, the expression profile of clear cell carcinoma of either the endometrium or the ovary was reported to be remarkably similar to that of clear cell carcinomas of the kidney [14]. This is interesting in light of the fact that the embryological origin of much of the reproductive tract is related to renal development.

33.3 Risk Factors for Ovarian Epithelial Tumors

Knowledge of genetic and environmental factors associated with predisposition to a specific cancer type can provide insight into the mechanisms underlying its development. This is particularly true of ovarian epithelial tumors, where strong predisposing factors have been well established.

33.3.1 Reproductive Factors

Most ovarian cancers occur sporadically, without evidence of genetic predisposition. Ovulation is the most well established risk factor for the sporadic form of these cancers [17, 18]. Interruption of ovulatory activity protects against the development of this disease independently of whether such interruption is achieved through pregnancy or oral contraceptives, although there is evidence of late pregnancies being more protective than earlier ones. For example, use of oral contraceptives for 5 years results in an approximately 40 % decrease in lifetime ovarian cancer risk, which is similar to the protective effect of five pregnancies after the first [19].

An initial explanation for the association between ovulation and ovarian cancer predisposition was based on the notion that these tumors originate in the coelomic epithelium lining the ovarian surface. Fatallah [20] reasoned over four decades ago that the chronic breakage and repair of the ovarian surface epithelium that results from monthly releases of the egg might lead to predisposition to malignant transformation of this epithelium (the incessant ovulation hypothesis). This hypothesis seemed attractive given the known association between cancer predisposition and cellular proliferation, one of the consequences of chronic repair. However, it fails to provide a comprehensive explanation for the current epidemiological data. For example, the disproportionately increased protective effect of late pregnancies compared to early pregnancies, as well as the progressive rise in ovarian cancer incidence after menopause, cannot be readily accounted for by the incessant ovulation hypothesis [17, 18]. Although the incessant ovulation theory is still widely quoted, a currently more favored hypothesis stipulates that it is the hormonal changes associated with the normal menstrual cycle that may have a lasting effect on predisposition of the cell of origin of ovarian epithelial tumors to neoplastic transformation. Estradiol, which is unopposed during the first half (follicular phase) of the menstrual cycle, stimulates growth of benign and malignant ovarian epithelial tumor cells in vitro, while progesterone, which is elevated during the second half (luteal phase) of the cycle, inhibits the growth of the same cells [21]. The fact that pituitary gonadotropins, which have high circulating levels around menopause, stimulate the growth of ovarian epithelial tumors in vitro suggests that hormonal changes associated with menopause may also play a role [21]

It is possible that each follicular phase of the menstrual cycle, characterized by unopposed estrogen stimulation and by elevated levels of follicular stimulating hormone, favors growth stimulation. Such stimulation may be accentuated at the end of the follicular phase due to the rapid surge in levels of luteinizing hormone that triggers ovulation. Each luteal phase, in contrast, is characterized by growth inhibition due to increased levels of progestins. This scenario of growth stimulation followed by growth inhibition might contribute to the increased risk of tumor development in women with uninterrupted menstrual cycles. The protective role of either pregnancy or oral contraceptive could, in turn, be partly due to the interruption of such a scenario. Alternatively, a study examining the long-term effects of oral contraceptives in macaques suggested that the direct action of progestins is primarily responsible for the protective effects of oral contraceptives [22].

Another explanation for the association between ovulation and ovarian cancer risk is known as the stromal hyperactivity hypothesis, which stipulates that although most ovarian follicular cells undergo apoptosis following release of the egg and the ensuing luteinization period, some may persist and retain their hormone-producing ability [23]. This would result in accumulation of steroid producing cells proportionate to the number of lifetime ovulations. Indeed, the basal levels of circulating estradiol were higher in premenopausal women with a greater lifetime number of ovulatory cycles in a longitudinal study [23].

In a recent population-based case-control study involving 477 patients with ovarian epithelial tumors and 660 controls, there was a 51 % reduction in risk of developing ovarian cancer in women who had given birth after the age of 35 compared to nulliparous women. Although prior births further reduced the risk, the magnitude of the protective effect of an early pregnancy was less than that of a pregnancy occurring after age 35 [17]. These observations underscore the complexity of the link between ovulatory activity and risk of sporadic ovarian cancer, which may in fact be the net result of several factors. In that regard, a role for androstenedione, which is the major ovarian hormone after the menopause and is suppressed by oral contraceptives, has also been suggested [24]. A role for this hormone is further supported by the fact that its circulating levels were found to be higher in the serum of patients with ovarian cancer compared to that of matched controls [25].

33.3.2 Inflammatory Factors

Although reproductive factors associated with the menstrual cycle are by far the strongest risk determinants of ovarian cancer, a role for inflammation has also been suggested [26]. Application of talc on the perineal area has consistently been associated with increased risk of ovarian cancer. Inflammatory conditions such as pelvic inflammatory disease have also been associated with such increase [27–32]. The association between endometriosis and endometrioid ovarian carcinoma [26, 33–38] is often regarded as further support for a role of inflammation in ovarian cancer predisposition, but this association can also be explained by the hypothesis that ovarian epithelial tumors arise in components of the secondary

Müllerian system. However, the apparent association between pelvic inflammatory diseases not involving endometriosis and ovarian cancer risk [39, 40], as well as the evidence for a protective effect of anti-inflammatory drugs [41–44] provide further support for the notion that inflammation can influence the risk of ovarian cancer.

33.3.3 Smoking

Multiple studies have linked cigarette smoking with risk of mucinous ovarian cancer, but not of other ovarian cancer subtypes [45–47]. This parallels the reported effect of smoking on histologically similar cancers of both the gastrointestinal tract and cervix. The proposed mechanism of carcinogenesis is a combination of direct DNA damage by carcinogens in cigarette smoke and the ability of these carcinogens to accumulate in mucin-secreting cells. Interestingly, not only is smoking not shown to increase serous or endometrioid cancer rates, but also it has been shown to decrease the relative risk of clear cell ovarian cancer. This suggests that the mechanism of carcinogenesis may be different for mucinous cancer than for other ovarian epithelial tumor types and may be more related to environmental carcinogens than to hormonal influences, as smoking is known to lower circulating estrogen levels.

33.3.4 Diet

The influence of diet has also been studied as it pertains to ovarian cancer. Data regarding the role of dietary saturated fat is controversial. One retrospective study showed an increased risk of mucinous tumors in women with diets high in saturated fats [48] while another large study found only a weakly positive, non-linear association between ovarian cancers of all subtypes and no difference for the mucinous subtype [49]. Although milk consumption and, more precisely, consumption of galactose, which is high in countries with elevated risks of ovarian carcinoma, has been proposed as a risk factor for this disease, recent data could not confirm this association, including in individuals with a functional polymorphism in an enzyme involved in galactose metabolism [50–52].

33.3.5 Genetic Factors

Approximately 15 % of all ovarian carcinomas are familial [53]. Almost all of these cases are due to germline mutations in the BRCA1 or BRCA2 genes, which are also associated with hereditary breast cancer. Approximately 40 % of women carrying a germline BRCA1 mutation will develop ovarian cancer in their lifetime while the risk for BRCA2 mutation carriers is about 20 % [54–60]. Given that the risk of ovarian cancer in the general population is only 1.7 %, cancer-causing mutations in either one of these two genes are highly penetrant. The only major subtype of ovarian epithelial tumors that has a well-defined familial component other than serous tumors is endometrioid. These tumors, which are often associated with microsatellite instability due to replication error repair deficiencies, are the fourth most common cancer type associated with the HNPCC syndrome [61].

Although the isolation of the BRCA1 gene more than a decade ago [62] raised hopes that elucidation of its biological function would shed light on the mechanisms underlying ovarian (as well as breast) cancer development, little progress has been made to date in spite of extensive data on the cellular function(s) of this gene. Part of the difficulty comes from the fact that although BRCA1 influences a large number of cellular functions potentially important in controlling cancer development, there is little insight into which function is most closely associated with familial cancer. The fact that the *BRCA1* locus is associated with several splice variants, with at least one, IRIS, possibly showing effects that are opposite to those of the full-length BRCA1 protein, complicates this issue further [63].

Individuals with germline BRCA1 mutations are predisposed almost exclusively to cancers of the breast and ovaries in spite of the fact that this gene product is expressed ubiquitously in most cell types. Cellular processes associated with the full-length BRCA1 nuclear protein that are often invoked as potentially underlying the alleged tumor suppressor function of this protein include cell cycle regulation, regulation of apoptosis, DNA repair, chromatin remodeling, transcriptional regulation, X chromosome inactivation, and post-translational modification [64–68]. These are global processes important in most cells. Thus, if any of these processes were primarily responsible for cancer predisposition in mutation carriers, the resulting cancers would be expected to affect a large number of cell types. Thus, current knowledge of the normal function of BRCA1 is difficult to reconcile with the site specificity of the tumors that develop in mutation carriers. This, plus the fact that BRCA1 mutations are rare in the sporadic form of ovarian cancer, suggests that this gene may act indirectly, perhaps by controlling cells that are not direct precursors, but that nevertheless influence the cells of origin of ovarian tumors.

It is with this idea in mind that Chodankar et al. [69] hypothesized that loss of BRCA1 function could influence ovarian tumorigenesis cell non-autonomously, by disrupting interactions between cells that control the menstrual cycle, the most important risk factor for sporadic ovarian carcinoma, and cells from which ovarian epithelial tumors originate. Given the central role of granulosa cells in regulating progression through the normal menstrual cycle, plus the fact that these cells secrete a variety of hormones such as estradiol,

Müllerian inhibiting substance, and others that are known to influence ovarian cancer cell growth in vitro, these authors used the *cre-lox* system to inactivate the *Brca1* gene in mouse granulosa cells specifically. The mice indeed developed benign tumors that were clearly of epithelial origin (as opposed to an origin from granulosa cells) in strong support of a cell non-autonomous mechanism. Although it is not clear whether a similar mechanism is also applicable to humans, these results strongly suggest that ovarian cancer predisposition in BRCA1 mutation carriers is due, at least in part, to decreased BRCA1 expression in ovarian granulosa cells, thereby disrupting control mechanisms that these cells exert on the cells from which ovarian epithelial tumors originate. The finding by Hu et al. [70] that down-regulation of BRCA1 in primary cultures of human granulosa cells results in increased expression of aromatase, the rate-limiting enzyme in estradiol biosynthesis, is well in line with this hypothesis. It is not clear whether the same mechanism is also responsible for breast cancer predisposition in BRCA1 mutation carriers. The fact that ovulatory activity, which is largely controlled by ovarian granulosa cells, has a strong influence on sporadic breast cancer predisposition in addition to ovarian cancers suggests that the mechanisms of predisposition to breast cancer in mutation carriers could indeed be similar. This idea is further strengthened by the demonstration that oophorectomy can protect against breast cancer in BRCA1 mutation carriers [71].

33.3.6 Potential Link Between Genetic and Reproductive Risk Factors

Another intriguing aspect of the genetic risk factors for ovarian carcinoma is that BRCA1 and BRCA2 are rarely abnormal in the sporadic form of this disease. A possible explanation that would also account for the site specificity of the tumors that develop in BRCA1 and BRCA2 mutation carriers is that inactivation of either one of these two genes might amplify the effects of risk factors for sporadic ovarian carcinoma. It is possible, for example, that such mutations could result in slight alterations in the dynamics of the menstrual cycle by increasing the length of the follicular phase resulting in increased estrogen stimulation unopposed by progesterone. The net result would be an amplification of the consequences of the menstrual cycle on ovarian cancer risk. The fact that pregnancy or oral contraceptive use, both of which have a strong protective effect against sporadic ovarian cancer, are also protective in BRCA1-2 mutation carriers [72, 73] is supportive of this idea. Hong et al. [74] tested this hypothesis by measuring the relative lengths of the different phases of the estrus cycle in mice harboring a Brca1 mutation in their ovarian granulosa cells and showed that indeed, the average length of the proestrus phase, which corresponds to the follicular phase of the human menstrual cycle, was longer than in wild type littermates. In addition, circulating levels of estradiol were higher in mutant mice than in wild type following inoculation of gonadotropins. They concluded that mice carrying a Brca1 mutation had both increased and prolonged estrogen stimulation unopposed by estrogen, raising the possibility that similar changes are also present in human BRCA1 mutation carriers [74].

33.4 Origin of Ovarian Epithelial Tumors

A fascinating aspect of ovarian cancer research is the persisting debate among scientists as to where and from which cell type these tumors actually originate. An answer to this fundamental question is essential to the understanding of the biology of the normal counterpart of these tumors, of the risk factors for this disease, and to the development of effective protective measures. This is also important for the identification and characterization of ovarian carcinoma precursor lesions and for the development of strategies aimed at their early detection.

33.4.1 Origin of the Theory That Ovarian Epithelial Tumors Arise in the Coelomic Epithelium

It has been widely accepted for the most part of the last century that ovarian epithelial tumors arise from the single mesothelial cell layer that lines the ovarian surface, which is also called ovarian surface epithelium [75]. This cell layer is also called coelomic epithelium because it is continuous with and identical to the mesothelial cell layer that lines all pelvic and abdominal surfaces. It was once believed, in the early part of the last century, that various cell types present in the normal mature ovary, including follicular and germ cells, were embryologically derived from the portion of the coelomic epithelium that lines the ovarian surface. It is for this reason that this cell layer was named germinal epithelium, a name that continues to be used today. The idea that ovarian epithelial tumors arose from this cell layer was attractive given such an alleged role in ovarian development. It is now well established that germ cells do not form from the coelomic epithelium and although the exact origin of ovarian follicular cells continues to be debated, there are strong morphological, functional, and molecular arguments that they are of mesonephric origin [76]. It is intriguing that although the original embryological arguments that led to the development of the theory that ovarian carcinomas originated in the overlying coelomic epithelium are no longer valid, this theory persisted, probably due to delays in the formulation of an alternative hypothesis.

33.4.2 Issues Relevant to the Identification of the Cell of Origin of Ovarian Epithelial Tumors

33.4.2.1 Morphological Arguments

Several histological observations cannot be readily accounted for by the idea that ovarian epithelial tumors are derived from the coelomic epithelium. First, this cell layer rarely, if ever, shows pre-neoplastic changes. Although a handful of microscopic cancers have been described in the coelomic epithelium lining the ovarian surface, these are extremely rare and an origin from the fallopian tubes or other components of the Müllerian tract is difficult to rule out. Second, and even more compelling, ovarian epithelial tumors do not resemble mesotheliomas, which is what would be expected if they originated from the coelomic epithelium, but are similar to epithelial tumors arising from other organs of the female reproductive tract as already pointed out (Fig. 33.1, Table 33.1). The fact that the most common ovarian epithelial tumor subtypes resemble tumors originating in either fallopian tubes, endometrium, or endocervix is intriguing because not only do these other components of the reproductive tract share a common embryological origin that is different than that of the ovary, but there are also no normal cells resembling either fallopian tubes, endometrium, or endocervix within normal ovaries. If ovarian carcinomas indeed arose from the ovarian surface, they would be the only example of somatic tumors that are better differentiated than their cell of origin.

33.4.2.2 Embryological Arguments

The fallopian tubes, endometrium, and endocervix all share a common embryological origin that is distinct from that of the ovaries. They are derived from two ducts, called Müllerian ducts, which are completely separate from each other initially as they develop adjacent to the ureters of the mesonephros, which is the functioning kidney of the embryo. It is because of the close association with this renal system that the Müllerian ducts are also called paramesonephric. The distal portions of the two Müllerian ducts converge and eventually fuse in the midline during fetal development. It is this fused segment that develops into the upper third of the vagina,

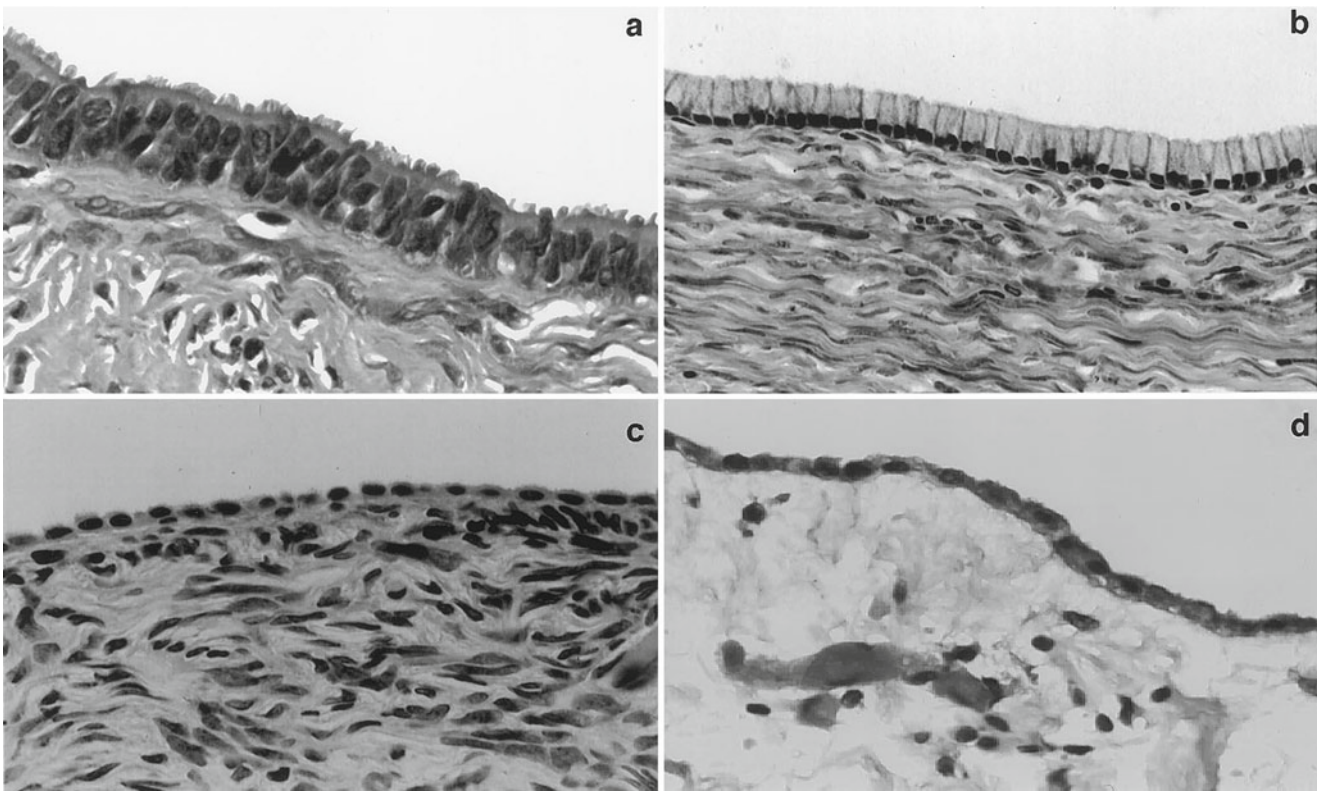

Fig. 33.1 Morphological comparison between the ovarian surface epithelium, serous or mucinous cystadenomas and peritoneal mesothelium. Photographs of benign tumors are shown because they better illustrate the morphological features of interest due to better differentiation. (**a**) Serous cystadenoma characterized by tall columnar cells with prominent cilia. Such cells are reminiscent of the epithelial cells lining normal fallopian tubes. The mucin-secreting cells lining the mucinous cystadenoma shown in (**b**) are reminiscent of cells lining normal endocervix. The differences between the epithelial lining of these two cystadenoma subtypes and that of the ovarian surface (**c**) are readily apparent. The morphological characteristics of cells lining normal ovarian surface epithelium, which are flat to low cuboidal, are much closer to those of the cells lining the abdominal peritoneum shown in (**d**). (Reproduced from Gynecol Oncol vol 72, p. 438, 1999 with permission).

the exocervix and endocervix, and the body of the uterus. The proximal portions of the Müllerian ducts remains unfused, giving rise to the fallopian tubes. In the adult, the epithelial lining of the exocervix and upper vagina is replaced by squamous epithelium derived from the lower vagina. The epithelial linings of the endocervix, endometrium, and fallopian tubes form a continuum, with gradual transitions but no sharp boundaries between those different organs. It is puzzling how the ovary, which is not derived from the Müllerian ducts, could give rise to tumors identical to tumors of Müllerian origin.

33.4.2.3 Molecular Biological Arguments

The notion that ovarian epithelial tumors resemble tumors derived from the Müllerian tract is not only supported by morphological arguments. Cheng et al. [2] studied the expression status of genes involved in body segmentation and morphogenesis in different components of the female reproductive tract. Expression of individual members of this gene family, called HOX genes, is highly specific for different body segments. These authors showed that ovarian surface mesothelium, fallopian tube epithelium, endometrium, and endocervix each expressed a different set of HOX genes. When they examined the expression status of these genes in different subtypes of ovarian epithelial tumors, they found that serous ovarian carcinomas expressed the same set of HOX genes expressed in normal fallopian tube epithelium. Likewise, endometrioid ovarian carcinomas expressed the same set of HOX genes as normal endometrium and mucinous ovarian carcinomas had a HOX gene expression profile similar to that of the endocervix. These results are highly supportive of the idea that these different ovarian tumor subtypes originate in Müllerian epithelium as opposed to the coelomic epithelium.

33.4.2.4 Primary Peritoneal Tumors

Hypotheses about the origin of ovarian epithelial tumors must take into account the fact that tumors that are histologically and clinically indistinguishable from ovarian carcinomas can arise outside the ovary. Such tumors, which are often referred to as primary peritoneal carcinomas, are confined to women and may be seen in individuals in whom the ovaries were removed several years ago for reasons other than cancer.

33.4.3 The Coelomic Epithelium Hypothesis

The idea that ovarian epithelial tumors arise from the portion of the coelomic epithelium that lines the ovarian surface is still favored by many in spite of the arguments discussed. Proponents of this theory account for the fact that these tumors have morphological and molecular features characteristic of Müllerian tumors by stipulating that the coelomic epithelium is not the direct precursor of ovarian tumors, but must first change into Müllerian-like epithelium through a process known as metaplasia. According to this theory, it is the rich hormonal environment of the ovary that triggers such changes. It is further hypothesized that this is most likely to happen in portions of the coelomic epithelium that have invaginated within the ovarian parenchyma, resulting in the formation of small cystic structures referred to as inclusion cysts. This readily accounts for the fact that benign ovarian epithelial tumors as well as most carcinomas are cystic in nature. This theory also accounts for the observation that small cysts within the ovary are often lined by cells with features suggestive of Müllerian differentiation while such features are extremely rare on the ovarian surface itself. Finally, proponents of this theory account for the presence of primary peritoneal tumors by stipulating that the hormonal environment in fertile women can trigger Müllerian metaplasia in coelomic epithelial cells away from the ovary in addition to cells on or within the ovary.

33.4.4 The Müllerian Hypothesis

There is little evidence that hormonal stimuli can trigger metaplastic changes within the coelomic epithelium although such changes are central to the notion that this epithelium is the site of origin of these tumors. Because of the various arguments raised so far, it was proposed by the author nearly two decades ago that this theory, in spite of its wide acceptance, should be revisited and that the notion that ovarian epithelial tumors arise directly from Müllerian elements should be given due consideration [77]. An obvious site in the Müllerian tract that might contribute to tumors likely to be diagnosed as ovarian carcinomas is the fallopian tubes. Indeed, pathologists have acknowledged for several decades that many lesions diagnosed as primary serous ovarian tumors are in fact of fallopian tube origin because these two organs are so close to each other and the morphology of the tumors is so similar that it is usually impossible to tell them apart. It is by pure convention that serous tumors from this area are categorized as ovarian unless morphological features are present that clearly reveal an origin from fallopian tubes. Strong support for this notion comes from reports from several groups that the fimbriated end of the fallopian tubes is a frequent site of pre-neoplastic changes such as dysplasia in surgical specimens from women undergoing prophylactic procedures due to familial predisposition to ovarian cancer [78–81]. These dysplastic lesions also showed differences in expression of regulators of cell cycle progression and of apoptosis such as p53, p21, and p27 [80].

It is clear that the fallopian tubes are not the only site of origin of serous carcinomas arising in the tubo-ovarian region because some tumors do not involve the tubes and

because benign serous cysts that are lined by the same cell type present in ovarian carcinomas are frequently seen within the ovary as well as in the para-tubal region with no connection to the tubes. In addition, a tubal origin is unlikely for endometrioid and mucinous ovarian carcinomas. It was proposed that these lesions could originate in other derivatives of the Müllerian ducts, which are common in the tubo-ovarian region and often impinge on the ovary [77]. Such derivatives are often referred to as the secondary Müllerian system [82] and include structures such as endosalpingiosis, which are lined by cells similar to those lining the fallopian tubes, endometriosis, which are lined by cells similar to endometrial glands, and endocervicosis, which are lined by cells similar to those lining the endocervix. In fact, small cysts lined by serous or mucinous epithelium and morphologically indistinguishable from ovarian serous or mucinous cystadenomas are frequent outside the ovaries (para-ovarian and para-tubal cystadenomas). The frequency of such extra-ovarian cysts is so high that pathologists often do not mention them in surgical pathology reports unless they are large enough to be clinically relevant. Such extra-ovarian cysts, when they increase in size, usually engulf the ovary within their wall because of their close proximity to this organ, at which point they would be categorized as ovarian cystadenomas.

Further support for the notion that endometrioid carcinomas arise in endometriosis is available from epidemiological [26, 33, 35, 38], histopathological [34, 36], as well as molecular biological evidence [37]. Additional evidence that primary peritoneal tumors arise in Müllerian tissues comes from a statistical argument made by Quddis et al. [83]. These authors reviewed all cases of endosalpingiosis and endometriosis of the omentum seen at their institution over a 12-year period. They reported that the endosalpingiosis to endometriosis ratio in this cohort was similar to the ratio of primary peritoneal serous to endometrioid carcinomas, [84] supporting the view that these two malignant tumor types arise from these two benign lesions, respectively.

Dubeau used these arguments to suggest that ovarian epithelial tumors develop exclusively in derivatives of the Müllerian ducts [77, 85]. Many serous ovarian carcinomas originate in fallopian tubes, a notion that has been accepted by pathologists for several decades. Serous tumors that do not originate in the tubes arise in endosalpingiosis, which is defined as tubal epithelium outside the tubes. Most serous carcinomas from the tubo-ovarian area, even if they originate outside the ovary, have reached a large enough size to involve the ovary by the time they come to medical attention and are thus categorized as ovarian. Those that arise in foci of endosalpingiosis that are far enough from the ovaries or tubes to spare both of these organs are categorized as primary peritoneal. Thus, the 3 serous tumor types currently categorized to as ovarian, tubal, or primary peritoneal all originate in serous Müllerian epithelium according to this hypothesis and are regarded as a single disease entity. With regard to the other histological subtypes of ovarian epithelial tumors, it is proposed that mucinous tumors arise in endocervicosis (defined as endocervical tissue outside the cervix) while endometrioid tumors arise in endometriosis (defined as endometrial tissues outside the uterus). Endosalpingiosis, endometriosis, and endocervicosis, which are the most important components of what is referred to as the secondary Müllerian system, can also give rise to intra- and extra-ovarian cystadenomas, which are the benign counterparts of ovarian carcinomas. This theory provides a straightforward explanation for the otherwise unaccounted finding that either tubal ligation or hysterectomy, which undoubtedly result in the destruction of components of the secondary Müllerian system, is protective against ovarian cancer based on numerous epidemiological studies [86–95].

The main differences between the classical theory invoking the ovarian surface epithelium as the site of origin of ovarian epithelial tumors and the Müllerian hypothesis are illustrated diagrammatically in Fig. 33.2. The Müllerian hypothesis implies that the term ovarian in ovarian carcinomas is somewhat of a misnomer given that most of these tumors arise outside the ovary. Dubeau suggested the term extra-uterine Müllerian carcinomas, further subdivided into serous, endometrioid, mucinous, and clear cell, as being more appropriate [85].

33.5 Animal Models of Ovarian Cancer

33.5.1 Spontaneous Ovarian Tumor Development in Animals

Until recently, the development of a suitable animal model for spontaneous ovarian carcinoma has been complicated by the fact that these tumors are rare in most animals including lower mammals. Knowledge of the reasons for the relatively low incidence of spontaneous ovarian epithelial tumors in lower mammals compared to humans could provide important clues about the origin and risk factors of the human tumors. Tumors resembling human ovarian carcinomas are frequently present in the domestic hen [84]. The high frequency of ovarian tumors in those animals has been linked to the activity of incessant egg production, similar to the relationship between incessant ovulation and ovarian cancer risk in humans. Wild hens or other wild birds, in which continuous egg production is not artificially induced, do not develop ovarian tumors. These observations raise the possibility that ovarian carcinomas result from an artifact of civilization, that of incessant ovulation, as chronic menstrual cycling was unlikely in early humans due to more frequent pregnancies and longer lactation periods.

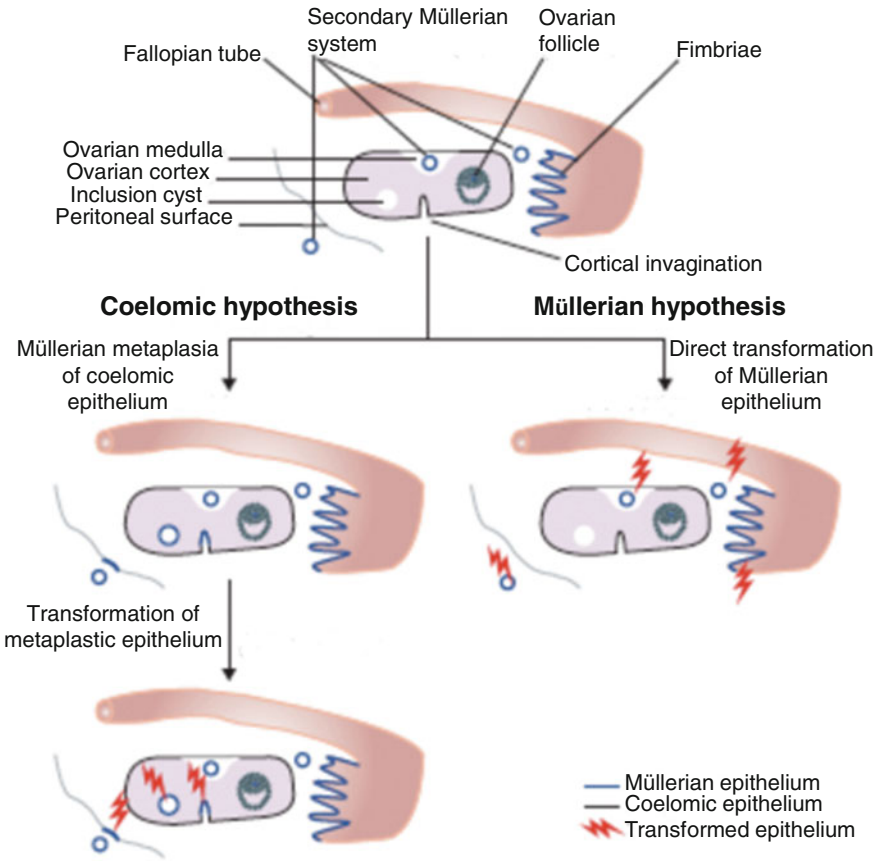

Fig. 33.2 Coelomic versus Müllerian hypotheses for the origin of ovarian, tubal, and primary peritoneal carcinomas. According to the coelomic hypothesis, cortical invaginations and cortical inclusion cysts, which are initially lined by coelomic epithelium (*thin black line*), undergo metaplasia, and change to Müllerian-like epithelium (*thicker blue lines*) before undergoing malignant transformation (lightning signs). The coelomic epithelium covering peritoneal surfaces outside the ovary can give rise to primary peritoneal tumors only after undergoing metaplasia to acquire characteristics of Müllerian epithelium. No intermediary metaplastic step is necessary with the Müllerian hypothesis, which stipulates that Müllerian-like tumors arise directly and exclusively from Müllerian epithelium that is already present, either in the fimbriae or in components of the secondary Müllerian system. (Reproduced with minor modifications from Lancet Oncol vol 9, p. 1193, 2008 with permission).

33.5.2 Models Based on Targeted or Conditional Manipulations of the Mouse Genome

The isolation of BRCA1 and BRCA2, the main genetic determinants of familial ovarian carcinoma, first raised the hopes that inactivating the genes encoding the analogous proteins in mice would lead to the creation of animal models for ovarian cancer based on genetic manipulations relevant to the human disease. These approaches initially failed because mice lacking a functional Brca1 die during early embryological development. Also disappointing was the fact that none of the mice carrying heterozygous inactivation of any of these two genes were prone to cancer development. Although mutants encoding the Brca1-delta11 splice variant of Brca1, which lacks the nuclear localization signal of the full-length protein, are viable, most of the tumors that developed in these animals were lymphomas or sarcomas [96]. Models of Brca2 knockout compatible with survival were likewise associated with predisposition to lymphomas predominantly [97–99].

Models based on conditional Brca1 and Brca2 alleles have mostly been targeted to mammary epithelium. A model where Brca1 inactivation was targeted to the ovary was developed by Chodankar et al. [69], who used a truncated form of the Fshr promoter, which is expressed in granulosa cells specifically, to create a conditional Brca1 knockout. The embryos were viable and fertile. A majority of Brca1 knockout mice had grossly visible cystic tumors either attached to the ovary, to the uterine horns, or with no demonstrable attachment to either of these organs. All tumors except one resembled human serous cystadenomas, the benign counterparts of ovarian serous carcinomas. Strikingly, these tumors carried only the non-recombined (wild type) form of the floxed Brca1 allele; the recombined (mutant) form was present only in granulosa cells. These findings provide strong support to the notion that tumor predisposition in BRCA1 mutation carriers is mediated, at least in part, via a cell non-autonomous

mechanism where granulosa cells control from a distance, in a BRCA1-dependent manner, neoplastic transformation in the tissue from which ovarian epithelial tumors originate. Elucidation of the mediator(s) of such transformation in this mouse model could lead to the development of better strategies for the prevention of these tumors in human populations at risk. The fact that the tumors that developed in this model were not confined to the ovary, but were seen along the entire Müllerian tract including in tissues from the para-ovarian and para-uterine areas, is supportive of the hypothesis that ovarian epithelial tumors are of Müllerian origin.

Several authors have succeeded in inducing tumors in mouse ovarian surface epithelium using conditional manipulations of the mouse genome targeted to ovarian surface epithelial cells. Orsulic et al. [100] demonstrated that the superimposition of a p53 mutation on any two of the oncogenes c-Myc, k-Ras, or Akt in targeted ovarian surface epithelial cells was sufficient to induce tumors in those cells. Flesken-Nikitin et al. [101] performed intrabursal administration of a vector expressing Cre recombinase in mice carrying floxed alleles of both p53 and Rb1, resulting in malignant transformation of the ovarian surface epithelium. Dinulescu et al. [102] similarly used intrabursal inoculations of a vector expressing Cre recombinase to induce expression of a conditional oncogenic allele of K-ras in the ovarian surface epithelium. The authors argued that the epithelial cells expressing this allele resembled the endometrial lining, providing us with an experimental model for endometriosis. Endometrial stroma, an important diagnostic feature of human endometriosis, was absent in these lesions. When inactivation of the Pten tumor suppressor was superimposed on the oncogenic K-ras allele, invasive tumors were obtained that were morphologically similar to the endometrioid subtype of ovarian carcinoma. Wu et al. [103] targeted the ovarian surface epithelium for dysregulation of the PI3K/Pten and Wnt/beta-catenin pathways, both of which are constitutively active in human endometrioid carcinomas, by conditional inactivation of Pten and Apc. Mice carrying those mutations developed carcinomas morphologically similar to human endometrioid carcinomas. These models are all based on the assumption that the targeted tissue, the ovarian coelomic epithelium, is the site of origin of ovarian epithelial tumors, a hypothesis that was favored by a majority of scientists.

Conditional inactivation of Brca1 in mouse ovarian surface epithelium resulted in hyperplasia, epithelial invaginations, and inclusion cysts [104]. This model could be valuable in understanding the potential relationship between such changes and ovarian carcinoma development. Epithelial ovarian inclusion cysts were also observed in Cd1 and Smad2 dominant negative mice after chronic superovulation from inoculations of gonadotropin hormones [105].

A transgenic model for ovarian cancer was developed by Connolly et al. [106], who used the mouse homolog of the human mullerian inhibitory substance type two receptor to drive expression of SV40 large T antigen. This receptor, which has a highly restricted tissue distribution, is expressed in a large majority of human ovarian epithelial tumors. The resulting transgenic mice developed highly invasive and metastatic tumors at a young age in the tubo-ovarian areas. A specific area where this model can be particularly attractive is for investigating the merit of targeting the Müllerian inhibitory substance type two receptor for the treatment of ovarian cancer [107, 108].

33.6 Ovarian Epithelial Tumors as Models of Cancer Progression

33.6.1 Classification of Ovarian Epithelial Tumors Based on Their Malignant Potential

Serous, mucinous, and endometrioid ovarian epithelial tumors can be further subdivided based on their malignant potential (Fig. 33.3). At one end of the spectrum are benign tumors lacking the ability to infiltrate into adjacent tissues and lacking metastatic ability. These tumors are often fluid-filled cysts (hence their designation as cystadenomas) lined by a single layer of epithelial cells resembling the lining of either fallopian tubes (serous cystadenomas) or endocervix (mucinous cystadenomas). Benign tumors of the endometrioid subtype (endometriomas) are usually filled with bloody material because they respond to the cyclic hormonal events of the normal menstrual cycle resulting in bleeding at the time of menses. At the opposite end of the spectrum are fully malignant lesions, which can be further subdivided based on histological grade. Increasing the attractiveness of ovarian epithelial tumors as a model for cancer progression is the existence of an additional category, called tumors of low malignant potential (LMP) or tumors of borderline malignancy, which are regarded as intermediate between the clearly benign and fully malignant lesions. This concept of semi-malignant tumors, which is not a feature of most other cancer models, was first advanced by Howard Taylor in 1929 [109]. It took an additional 40 years before such ovarian tumors, which are associated with a more favorable prognosis than their frankly malignant counterparts regardless of stage of presentation, became accepted as a clinical entity [110, 111]. Both the International Federation of Gynecology and Obstetrics (FIGO) and the World Health Organization (WHO) have classified ovarian epithelial tumors as benign, malignant, and low malignant potential [112].

Ovarian tumors of low malignant potential are characterized by absent or minimal invasive potential, although they can spread outside the ovary and implant onto peritoneal surfaces. Since they are distinguished from carcinomas

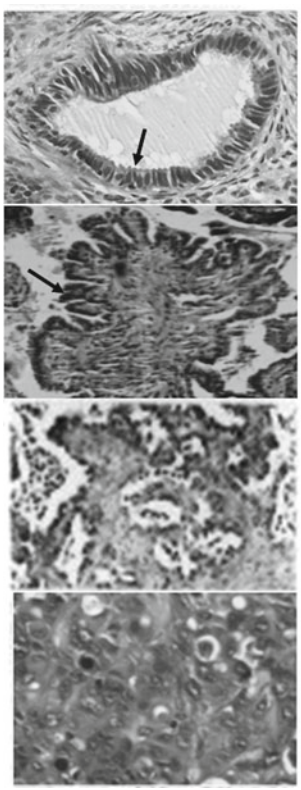

Fig. 33.3 Classification of ovarian epithelial tumors based on their malignant potential.

primarily based on differences in their invasive ability, progress in understanding their molecular determinants and in elucidating the basic molecular differences between these tumors and ovarian carcinomas could shed light on the mechanisms underlying this hallmark of the malignant phenotype. Unfortunately, the data so far have not been telling in that regard.

A fundamental molecular genetic difference between tumors of low malignant potential and carcinomas seems to be that mitotic errors leading to somatic losses of heterozygosity, a hallmark of malignancy, are rare in LMP tumors [113]. Although such losses can be demonstrated in these tumors, it is clear that these events are not frequent enough to play an important role in their development. On the other hand, some of the molecular features associated with the malignant phenotype are present in LMP tumors. For example, these tumors usually express telomerase [114], a feature of the malignant phenotype, and global DNA methylation levels or levels of DNA methylation status in centromeric and juxtacentromeric sequences in these tumors are closer to those seen in carcinomas than in cystadenomas [115, 116]. Although these results strengthen the notion that LMP tumors are intermediate between benign and frankly malignant ovarian epithelial tumors, they shed little light on their underlying mechanisms. Studies at the individual gene level have likewise been unrevealing. Although mutations in specific protein kinases have been associated with LMP tumors and may be more frequent in these tumors than in carcinomas [6, 117–119], such mutations are nevertheless seen in a large number of cancers of various types as well as in some ovarian cystadenomas and their presence in LMP tumors sheds little light on the distinguishing molecular features responsible for the phenotypic differences between these tumors and either ovarian cystadenomas or carcinomas. Along the same lines, expression profiling studies have suggested that although a panel of genes or specific pathways may be more frequently associated with LMP tumors [11, 16, 120], there are so far no clues as to the molecular determinants of the fundamental difference between these tumors and carcinomas, the ability of the latter to infiltrate into adjacent tissues.

33.6.2 Insights from Work with In Vitro Systems

Scientists have attempted to obtain further insight into the biology and molecular mechanisms of ovarian cystadenomas, tumors of low malignant potential, and carcinomas by studying the behavior of these cells in tissue culture. Several authors succeeded in culturing the mesothelial cell layer lining the ovarian surface [121–124]. Cultures of epithelial cells derived from rete ovarii, which could be of Müllerian origin and play a role in ovarian tumorigenesis although this structure is usually regarded as of mesonephric origin [76, 77], were also reported [125]. Godwin et al. [126] reported a high transformation rate in cultured ovarian surface mesothelial

cells, suggesting that they may indeed be prone to malignant development.

One of the difficulties in investigating the molecular mechanisms underlying the development of ovarian tumors of low malignant potential has been the inability to culture these tumors beyond primary explants [127]. The same problem also applies to benign tumors (cystadenomas). It is possible to extend the in vitro life span of either cystadenomas or LMP tumors by introducing viral oncoproteins such as SV40 large T antigen [128], but studies based on such models are complicated by the consequences of such oncoproteins on the malignant phenotype. Nevertheless, these approaches have led to a number of observations with potentially important implications. Although these tumors clearly show continuous growth in vivo and, like carcinomas, almost always express telomerase, expression of this enzyme is not detected in cultures of LMP tumors transfected with SV40 large T antigen. Also, these cells are not immortal in culture despite the fact that this antigen prolongs their in vitro life span to about 50 population doublings [129, 130]. It is therefore possible that only a small fraction of tumor cells, perhaps with stem cell features, are primarily responsible for sustained proliferation in vivo.

Work with in vitro cultures of cystadenomas and tumors of low malignant potential transfected with SV40 large T antigen also led to the observation that although ovarian cystadenomas typically undergo severe numerical chromosomal alterations resulting in aneuploidy when they reach the phenomenon of in vitro crisis toward the end of their in vitro lifespan, cultures of tumors of low malignant potential remain remarkably stable through the crisis period [129, 130]. It is tempting to relate such chromosome stability in culture to the fact that these tumors are typically diploid and genetically stable in vivo [131, 132]. In fact, aneuploid LMP tumors are associated with a more aggressive clinical course and their response to chemotherapeutic agents may be more typical of ovarian carcinomas, raising the possibility that at least some of those tumors are carcinomas incorrectly diagnosed as LMP tumors [132–135]. Indeed, the possibility of using ploidy status as a diagnostic tool to help distinguish ovarian LMP tumors from carcinomas has been suggested [131]. Further understanding of the mechanisms underlying the apparent protection against chromosomal instability that appear to be present in LMP tumors should further increase our understanding of the development of aneuploidy, one of the hallmarks of cancer.

33.6.3 Molecular Genetic Model for Ovarian Carcinoma Development

Table 33.2 lists a number of abnormalities that have been associated with the development of these tumors, none of which are specific for ovarian cancer. A diagram illustrating global as well as selected specific abnormalities distinguishing ovarian cystadenomas, LMP tumors, and carcinomas is shown in Fig. 33.4. This diagram is primarily applicable to the serous subtype of ovarian tumors, as K-RAS, H-RAS, and B-RAF mutations are not common in all subtypes and PTEN mutations, which are frequent in endometrioid carcinomas, are not featured in this illustration. Mutations in BAF250a [10], which are frequent only in the endometrioid and clear cell subtypes (Table 33.2), are likewise not mentioned in the Fig. 33.4. The complexity of molecular genetic changes present in ovarian carcinomas clearly increases with

Table 33.2 Molecular genetic alterations associated with sporadic (non-familial) ovarian carcinomas

Category	Gene or locus	Comment
Oncogenes	*Her-2/neu*	Receptor tyrosine kinase; over-expression is associated with poor prognosis and therapeutic response
	K-RAS, H-RAS, B-RAF	Mutations are frequent in serous and mucinous low grade carcinomas and tumors of low malignant potential
	AKT-2	Member of a subfamily of protein-serine/threonine kinases
	Cyclin E	Over-expression in ovarian carcinomas has been associated with poor clinical outcome
Tumor suppressor genes	*P53*	Regulator of cell cycle progression and apoptosis
	PTEN *BAF250a*	Phosphatase that results, in part, in inhibition of cell death; it is mutated in a significant proportion of endometrioid ovarian carcinomas Component of the SWI–SNF chromatin remodeling complex
	NOEY2	Induces p21 and down-regulates cyclin D
	SPARC2	Encodes a calcium binding matrix protein that contributes to cell adhesion
	DOC-2	Binds GRB-2 upstream of RAS
Chromosomes with frequent losses of heterozygosity	*3p, 6p, 6q, 7q, 9p, 11p, 11q, 13q, 17p, 17q, 19q, 22q, Xp, Xq*	Multiple candidate tumor suppressor genes have been reported on these chromosomes, but their role in ovarian tumorigenesis remains unclear
Altered signal transduction pathways	*PI3K/PTEN*	
	Wnt/beta-catenin	
	EGF-R	

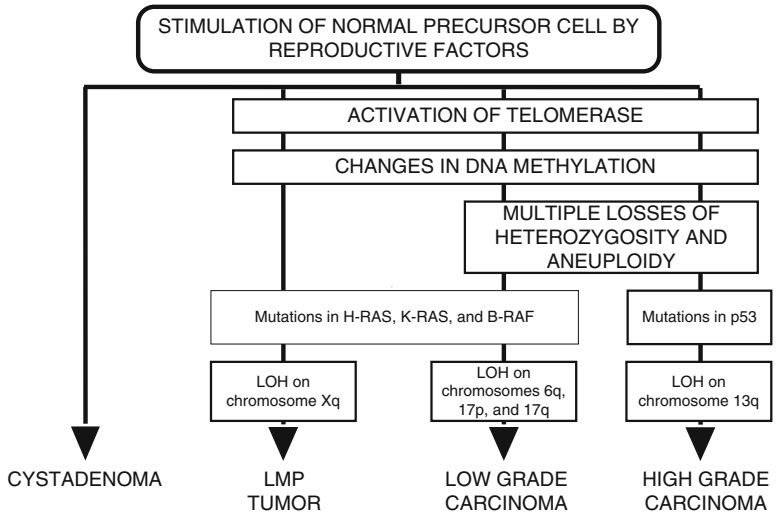

Fig. 33.4 A genetic model for ovarian epithelial tumor development. This diagram is not meant to feature all molecular genetic changes that have been associated with the development of ovarian epithelial tumors. It emphasizes the global genetic mechanisms as well as the most important locus-specific differences that distinguish ovarian cystadenomas, tumors of low malignant potential, and carcinomas. It is mostly applicable to serous tumors.

increasing tumor histological grades, which can be regarded as a measure of a tumor's biological aggressiveness [113, 136–138]. However, as is apparent from Fig. 33.4, the grade of ovarian carcinomas is not only a function of the mere number of molecular genetic abnormalities present in a given tumor genome, as specific molecular abnormalities appear strongly associated with high histological grades [113, 136–139]. For example, losses of heterozygosity in certain chromosomal regions, such as 6q, 17p, and 17q, appear frequent in ovarian tumors of all histological grades [113] while losses in chromosome 13 are frequent only in those of high histological grades [139, 140]. It may be that the gene(s) targeted by losses of heterozygosity in chromosome 13 control(s) cellular pathways associated perhaps not with cell cycle regulation, but with differentiation or other determinants of tumor grade. Another point illustrated in Fig. 33.4 is that although loss of heterozygosity, which is an important mechanism of inactivation of tumor suppressor genes in most human cancers, is frequent in ovarian carcinomas, this abnormality is rare in the biologically less aggressive ovarian epithelial tumors. Perhaps tumor suppressor gene inactivation, which is an important consequence of such losses, is not a feature of cystadenoma or low malignant potential tumor development. Mutations in the p53 gene, which are among the most frequent tumor suppressor gene alterations in all cancers, are present in nearly 100 % of high grade serous ovarian carcinomas but are very rare in low grade carcinomas as well as in ovarian low malignant potential tumors and cystadenomas [141]. Alterations in DNA methylation are associated with tumors of low malignant potential as well as carcinomas but not with cystadenomas, suggesting fundamental differences in the mechanisms underlying the development of these benign ovarian tumors [115, 116]. This conclusion is further strengthened by the fact that telomerase is usually not detected in cystadenomas while it is expressed in most tumors of low malignant potential and carcinomas [114]. Given that this enzyme is regarded as necessary for continuous cell growth, its absence in most cystadenomas suggests that these tumors may have a limited life span in vivo, an idea consistent with the observation that benign ovarian cysts frequently regress or remain unchanged in post-menopausal women [142].

The only exception to the rarity of losses of heterozygosity in LMP tumors is losses affecting the X chromosome, which are present in about 50 % of the cases [113]. However, these losses appear to arise through a different mechanism than that responsible for most losses occurring in carcinomas because they are small interstitial chromosomal deletions as opposed to losses involving large segments such as entire chromosomes or chromosomal arms, which usually result from mitotic errors. The gene(s) targeted by the interstitial allelic losses in LMP tumors is/are still not known. The fact that the reduced allele invariably affects the inactive copy of the chromosome suggests that the targeted gene(s) escape(s) X chromosome inactivation. This suggestion is attractive because individuals born with a single X chromosome (Turner syndrome) show abnormal ovarian development (gonadal dysgenesis). Thus, the presence of the inactive X chromosome is necessary for normal ovarian development. It is conceivable that abnormalities in the same gene during adult life may lead to tumorigenesis. In that regard, it is intriguing that BRCA1, a protein involved in the control of

familial ovarian carcinoma, is thought to interact with the X chromosome and has been suggested to play a role in X chromosome inactivation [143–146].

33.6.4 Relationship Between Ovarian Cystadenomas, Tumors of Low Malignant Potential, and Carcinomas

The fact that ovarian epithelial tumors are subdivided into benign, low malignant potential, and malignant lesions raises the question of whether these represent distinct disease processes or are part of a single disease continuum where tumors first develop as cystadenomas and later progress to more aggressive lesions. The answer is not only important for our understanding of ovarian tumor development, but is also relevant to the clinical management of cystadenomas and tumors of low malignant potential, which often occur in women of reproductive ages. Arguments in favor of a continuum come from morphological observations that areas histologically indistinguishable from typical ovarian cystadenomas are sometimes found contiguous to carcinomas. The most straightforward interpretation for these lesions, which are sometimes called cystadenocarcinomas, is that the histologically malignant areas arose from the pre-existing morphologically benign areas. This interpretation implies that any molecular genetic change associated with carcinomas, but normally not present in solitary cystadenomas, should be confined to the histologically malignant portions of cystadenocarcinomas. However, losses of heterozygosity and p53 mutations, which are both frequent in carcinomas and absent or at least very rare in solitary cystadenomas, are usually concordant in all portions of ovarian cystadenocarcinomas including the morphologically benign areas [147, 148]. Concordance for aneuploidy was likewise shown in different regions of cystadenocarcinomas using interphase cytogenetic approaches [149]. It seems clear, based on these observations, that the histologically benign portions of cystadenocarcinomas are genetically different from typical (solitary) cystadenomas. This conclusion supports the idea that cystadenomas do not generally progress to malignancy unless they carry a genetic predisposition to such progression such as, for example, a mutation in the p53 gene.

Another argument against the notion of a disease continuum is the presence of specific genetic abnormalities that are more frequent in tumors of low malignant potential than in carcinomas. Interstitial deletions of a small region of the X chromosome are a feature of LMP tumors but not of carcinomas [113]. In addition, mutations in the K-RAS and B-RAF genes appear to be more frequent in LMP tumors. Since mutations in these genes are also seen in low grade carcinomas [5–9], Shih and Kurman [150] suggested a dual mechanism for carcinoma development where high grade tumors develop de novo while low grade lesions arise in pre-existing LMP tumors. The fact that LMP tumors appear to be intrinsically more stable than either cystadenomas or carcinomas also argues against the notion that they are precursors of high grade ovarian carcinomas, which are typically highly aneuploid [130].

33.7 Strategies for Early Detection of Ovarian Carcinoma

33.7.1 Screening Strategies for Early Detection in Populations at Risk

The ability to detect ovarian carcinoma precursor lesions before they develop into fully mature cancers would undoubtedly have a profound effect on morbidity and mortality. The poor prognosis currently associated with these lesions is largely due to the fact that they are most often detected after they spread outside the ovary, at which time they are difficult to eradicate. All cancer screening tests that have had a significant impact on disease morbidity and mortality allow detection of pre-cancerous or pre-invasive lesions in addition to localized cancers. This is true of the PAP test used for the detection of pre-invasive cervical cancers (cervical dysplasia), of mammography for the detection of pre-invasive breast cancer (detection of microcalcifications associated with atypical ductal hyperplasia and ductal carcinoma in situ), of prostate biopsy for the detection of prostatic epithelial neoplasia in individuals with elevated PSA, etc. Although this has not been fully established, it is perhaps not the ability to detect localized cancers, but the ability to detect pre-malignant lesions that accounts for the bulk of the impact that these various screening methods have had on disease mortality. Although it is clear that localized cancers (stage I) generally have a better prognosis than disseminated cancers, these cancers could be inherently less aggressive biologically, implying that they are less likely to metastasize. Their prognosis could therefore remain more favorable even if they are not detected until they become clinically manifest. This view is supported in the ovarian model by expression profiling studies comparing localized versus metastatic ovarian cancers, which suggest that they indeed could be regarded as distinct disease entities [151].

One of the problems with developing a sensitive screening protocol for precursors of ovarian carcinomas is that not only the nature of the precursor lesion itself is unclear, but also there is still debate as to where these tumors actually originate. There is therefore a great deal of effort focused on the development of alternate approaches with enough sensitivity and specificity for ovarian carcinomas to allow detection of early disease in populations at risk. Given that transabdominal and transvaginal ultrasound is commonly

used in the evaluation of pelvic masses, the potential of this technique as a screening tool was extensively investigated. However, current data suggest that this approach alone not only lacks specificity, but may be of little value to diagnose ovarian carcinomas before they metastasize [152]. Measurement of serum CA125, a glycoprotein encoded by the *MUC16* gene that has been used extensively as a marker of disease recurrence following adjuvant chemotherapy for ovarian carcinoma, is not specific for this disease and is elevated in only 50–60 % of patients with stage I ovarian carcinomas. Although measurements of rate of change in serial CA-125 measurements can increase the sensitivity of this marker based on the fact that it tends to gradually increase in women with cancer while it remains stable in those with benign conditions, the sensitivity of this approach falls short of meeting the needs of a practical screening tool [153]. Combining serial measurements of CA-125 with transvaginal ultrasound was evaluated as a means of further increasing both sensitivity and specificity. However, there was no difference in overall incidence of ovarian cancer or stage at diagnosis in women screened by this method compared to matched controls in a study of 21,935 women [154]. We are still awaiting data from an ongoing trial based on utilizing rate of rise of CA-125 as an adjunct to ultrasound as a mode of increasing positive predictive value [155].

More recently, investigators have used gene expression profiling technologies and proteomic tools in an effort to identify novel markers associated with ovarian cancer. Lu et al. [156] were able to distinguish normal from malignant ovarian epithelial cells based on expression levels of 5 markers identified from gene expression profiling analyses. In another study, proteomic approaches based on a panel of three markers, combined with CA-125 measurements, distinguished patients with stage I/II ovarian cancer from healthy controls with a specificity of 94 % [157]. Similarly, Gorelik et al. [158] used multianalyte profiling to compare the amounts of multiple cytokines in women with stage I/II ovarian carcinoma and healthy controls. These authors showed strong correlation between marker levels and early stage disease. It is hoped that further progress with these approaches will lead to the development of a panel of markers which, when used alone or in combination with CA-125 measurements or transvaginal sonography, will increase our ability to detect early ovarian carcinomas in populations at risk.

33.7.2 Early Detection of Residual or Recurrent Disease

Patients diagnosed with advanced ovarian carcinoma are usually first treated with surgical debulking of all visible disease greater than 1 cm, followed by adjuvant chemotherapy. Although most patients show good initial therapeutic responses, a large proportion with no evidence of residual disease after completion of the initial chemotherapeutic regimen undergo later recurrences. The development of sensitive methods for the detection of minimal residual disease in treated patients should therefore enhance our ability to identify those at higher risk of recurrence. In addition, it is possible that further therapeutic interventions may be most effective for small, subclinical tumors. Of all current surveillance modalities, second-look procedures provide the most accurate assessment of response to chemotherapy in patients with advanced epithelial ovarian cancer [159–161]. These procedures refer to laparotomies or laparoscopies, performed usually 6 weeks after completion of chemotherapy, in patients who display no clinical evidence of residual disease. These procedures were widely used to aid physicians in deciding whether to stop, change, or continue chemotherapy in patients undergoing treatment until recently. However, the fact that up to 50 % of patients in whom no residual carcinoma was detected during such procedures subsequently developed disease recurrence [160] has prompted most centers to abandon these procedures except when mandated by research protocols. Currently, most patients are followed up with serial measurements of CA-125 and CT scan. Although rising CA-125 levels constitute a good indicator of disease recurrence, this approach is not sensitive enough to allow detection of residual disease immediately after adjuvant chemotherapy.

Recent progress in our understanding of the molecular genetic changes associated with cancer development may provide us with novel sensitive approaches to better evaluate the presence or absence of residual disease in patients treated for advanced ovarian carcinoma. In that regard, the presence of detectable telomerase activity in abdominal washings may be of some value. Duggan et al. [162] showed that the presence of such activity is a more sensitive indicator of the presence of disease than cytological examination. Half of patients with negative second-look procedures tested positive for telomerase in a subsequent study [163]. A follow up study of these patients is not yet completed, but preliminary results show that patients with negative second-look procedures have a shorter survival if they test positive for telomerase (unpublished results from the author's laboratory), raising the possibility that this marker could identify a subset of patients for whom further chemotherapy could be beneficial.

33.8 Concluding Remarks

Although progress in decreasing the incidence and improving mortality rates associated with ovarian carcinoma has lagged behind progress made with other gynecological cancers, it is hoped that current efforts will have a significant impact in the foreseeable future. A better understanding of the precursor lesion for these tumors, combined with efforts

aimed at the identification of specific serum markers expressed early in disease development, should lead to better screening strategies applicable to the general population. In addition, further progress in understanding the biology of these tumors as well as of their underlying genetic mechanisms should lead to more effective therapeutic protocols based on specific molecular profiles.

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Molecular Pathology of Bone and Soft Tissue Tumors

34

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34.1 Introduction

Bone and soft tissue sarcomas are a heterogeneous and infrequent group of tumors. They make up roughly less than 5 % of neoplasms in adult patients and 10 % of childhood tumors. Despite this difference in the proportion, they are, in absolute numbers, more frequent in the adult age [1]. Some of these tumors, such as synovial sarcoma, ES, and osteosarcoma, are more usual in children, adolescents, and young adults, while some neoplasms such as leiomyosarcoma or liposarcoma are more frequent in patients over 55 years of age.

They are classified according to its specific type of cellular differentiation. Many of them exhibit similar features to those from healthy cells of the mesenchymal tissue, such as cartilage, adipose tissue, or muscle, in chondrosarcoma, liposarcoma, and rhabdomyosarcoma (RMS), respectively; others, though, lack a differentiation program reminiscent of any given normal tissue, for example synovial sarcoma or ES. It is unknown which is the cell type that originates sarcomas, especially considering that there are hardly ever any precursory lesions, quite the opposite than in the case of carcinoma. It is believed that some of the molecular alterations which are described later on affect the mesenchymal stem cell (or cells) to induce them to a neoplastic differentiation program (phenotype). That is why the recent WHO classification on sarcomas has dropped the histogenesis concepts to focus on a combination of parameters that include morphology, phenotype, and genotype. We know very little of the origin of sarcomas. However, some studies point to mesenchymal stem cells (MSC) as the possible origin for some types of sarcomas, such as ES [2–4]. We know some environmental risk factors are associated to some specific types of sarcoma, such as vinyl chloride and hepatic angiosarcoma; or ionizing radiation, which is associated to various types of sarcomas. There are four syndromes of family cancers that are associated to sarcomas, but pathogenesis is based in most of them on the presence of acquired mutations.

Sarcoma diagnosis did not need to be particularly precise 40 years ago, when we lacked highly effective treatments for sarcomas, they were lethal tumors. Current development of therapies is consistent with that of pathology; a timely diagnosis should integrate histopathology, immunophenotype, and molecular techniques.

34.2 Which Cells Give Rise to Sarcomas?

The genesis of most types of sarcomas is unknown, and the elucidation of the cell of origin is a key for discovering the early molecular mechanisms involved in the genesis of the different types of sarcomas as well as the identification of reliable molecular markers and possible therapeutic targets. The cellular context contributes to the phenotype; for example, the transfection of *EWS-ets* fusions into different cellular models resulted in diverse outcomes ranging from the induction of cell cycle arrest or apoptosis to dedifferentiation [5–8].

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_34

Recently, Pan Q et al. showed that MSC derived from bone marrow and liver suffers spontaneous transformation. Interestingly, these transformed cells implanted into imunodeficient mice induced sarcoma-like tumors [9]. Moreover, the tissue source of MSC does not seem to be relevant with regard to development of a particular type of sarcoma [10, 11]. However, the MSC differentiation state seems to play a main role in order to define the sarcoma phenotype [11].

34.2.1 Chondrosarcoma

Several theories about the histogenesis of dedifferentiated chondrosarcoma have been proposed: (1) Originally, it was thought that a well-differentiated cartilaginous tumor cell dedifferentiates into a primitive undifferentiated tumor cell [12]. (2) The two components (anaplastic and cartilaginous) are derived from two separate clones of primitive spindle cells (collision tumor), one of which differentiates into a low-grade chondrosarcoma, while the other fails to differentiate and displays features of high-grade sarcoma [13–15]. (3) Both components of dedifferentiated chondrosarcoma display numerical aberrations of chromosome 7 [16], suggesting that both components are derived from a single abnormal clone or cell (common primitive mesenchymal progenitor). (4) Bovée et al. [17] analyzed a case of a dedifferentiated chondrosarcoma and showed compelling evidence for a monoclonal origin (common primitive mesenchymal progenitor), since both components have specific genetic alterations in common. Combination of comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) results revealed that both components share an identical p53 mutation (somatic 6 bp deletion in exon 7) and deletion of the same copies of chromosome 13. However, the presence of many additional different genetic alterations suggests that the separation of the two clones was a relatively early event. The p53 alteration being an early event in chondrosarcomas is in contrast with the literature, since p53 overexpression and mutation were mainly found in high-grade chondrosarcomas [18–20].

34.2.2 Ewing Sarcoma (ES)

ES is a group of pediatric or young adult sarcomas characterized by EWS-ETS gene fusions, mainly EWS-FLI-1 [2, 3]. The expression of neural markers in ES points to a potential mesenchymal or neuroectodermal origin. Recently, Neural Crest Stem Cells (NCSC) have been shown permissive to EWS fusion. However, MSC remains as the most acceptable candidate to be the origin cell in this neoplasia [10].

Bone marrow-derived human mesenchymal stem cells (hMSCs), which are classic mesodermal derivatives, express an extensive assortment of neural genes, and therefore, are predisposed to differentiate to neural and glial lineages [21]. Considering that most ES occur in bone and soft tissue, hMSCs serve as one possible source of cells prone to EWS-FLI1 transformation. Simulation of the expression of EWS-ETS in hMSCs from healthy donors would likely recapitulate the early signaling events underlying in oncogenic transformation. In fact, the development of ES from primary BM-derived mesenchymal cells (MPCs) has been already described in mice [22] and the profiles of different *EWS-FLI1*-silenced Ewing cell lines converge toward that of mesenchymal stem cells [23]. Riggi et al. found out that the EWS-FLI1 fusion protein, can transform MPCs to form ES-like tumors in mice [22]. Moreover, EWS-FLI1 expression in the absence of other pro-oncogenic events is sufficient to induce MPC transformation, suggesting that, in the appropriate cellular microenvironment, it may constitute the initiating event in ES pathogenesis. MPCs display a high degree of plasticity and can differentiate into osteocytes, adipocytes, neurons, and chondrocytes [24]. Despite their bone marrow origin, MPCs can migrate to a broad range of tissues, including soft tissues, where most sarcomas develop [25]. Based on these observations and the hypothesis that sarcomas arise from pluripotent mesenchymal cells, the possibility is that MPC whose developmental program is deranged and blocked in differentiation at an early stage, might provide insights on the origin of other translocation-related sarcomas. However, EWS-FLI1 does not transform hMSC, suggesting the involvement of secondary events to the fusion [26]. This highlights the need of research on MSC or even earlier precursor cells. Recent studies show that some MSC could originate from the neuroepithelium, neural crest stem cells, and from hematopoietic stem cells. Based on this and other data, the Stamenkovic group suggested an hMSC of hematopoietic or neuroectodermal origin as the possible origin cell for ES [27]. Additionally, the some authors also found that pediatric hMSCs transduced with EWS-FLI1 showed ES features of cancer stem cells (CSCs) in vitro but the ability of the CSCs to generate tumors in immunosuppressed mice was not shown [28].

More recently, our group also characterized MSCS obtained from sarcoma patients (hMSC-P). These cells are more similar to hMSC derived from healthy donors than to ES cells. hMSC-P lacked EWS gene rearrangements but showed a few immunophenotypical features also, present in ES cells, such as CD99 expression. Interestingly, ES were sensitive to anti-CD99 therapy while MSCs were not [10].

34.2.3 Leiomyosarcoma

Sanerkin postulated that primary leiomyosarcoma of the bone does not necessarily arise from the media of blood vessels; it might also conceivably develop through advanced myogenic metaplasia of a sarcoma originating from fibroblastic tissue [29]. Yamashina described a case of a primary

leiomyosarcoma in the breast and from the analysis of ultrastructural features of this case led to suggest a tumor of pluripotent mesenchymal cell nature rather than of vascular smooth muscle cell origin [30]. Leiomyosarcoma can also take part into a carcinosarcoma [31] and the establishment of a leiomyosarcoma cell line (HTMMT) derived from human uterine carcinosarcoma was described by Ishiwata et al. [32]. The findings of a monoclonal origin of carcinosarcomas support the hypothesis of a single totipotential stem cell divergence [33]. The divergence hypothesis proposes the differentiation of a single totipotential stem cell into separate epithelial and mesenchymal directions. Jin et al. described that most uterine and ovarian carcinosarcomas are monoclonal [34]. Hernando et al. generated a Tagln-cre/Pten$^{loxP/loxP}$ mice and sarcomas developed responded to rapamycin (an mTOR inhibitor used as an immunosuppressor), showing that *Pten* genetic inactivation in the mouse smooth muscle lineage predisposed smooth muscle cells to transformation and led to mTOR constitutive activation, which was strongly associated with sarcomagenesis in their model [35]. Although required, *Pten* inactivation seemed insufficient to completely promote leiomyosarcoma formation. In humans, *PTEN* loss may not be the only molecular alteration in this pathway responsible for leiomyosarcoma genesis. Interestingly, Rubio et al. reported that adipose-derived MSCs and also Bone marrow MSCS both lacking p53 alone or in combination with Rb induces leiomyosarcoma-like tumors when implanted into immunodeficient mice [11, 36].

34.2.4 Liposarcoma

Regarding the origin cell of liposarcoma, it is known that these tumors can arise in any location, and have an unusual pattern of metastases to the soft tissues, bone, visceral surfaces, and lung. Myxoid liposarcomas (M-LPS) are associated with specific chromosomal translocations that give rise to fusion genes. Embryonic fibroblasts from double-transgenic FUS-CHOP animals showed a similar phenotype to that of human liposarcomas, including the presence of lipoblasts with round nuclei, accumulation of intracellular lipid, induction of adipocyte-specific genes, and a concordant block in the differentiation program [37]. Riggi et al. suggested that expression of FUS-CHOP may be the initiating event in myxoid liposarcoma pathogenesis, and that MPCs may constitute one cell type from which these tumors originate [38]. Domoto et al. identified the *TLS/FUS-CHOP* target gene *DOL54* of the M-LPS and round cell liposarcoma oncogene (RC-LPS). The *DOL54* gene product is closely associated with adipogenic differentiation [39]. They examined *TLS-CHOP* and *DOL54* expressions in M-LPS/RC-LPS, well-differentiated liposarcoma, observing *DOL54* expression in 50 % of M-LPS/RC-LPS cases (in which *TLS-CHOP* was also expressed). Rodriguez et al. reported recently that FUS-CHOP expression in mouse but not in human adipose-derived mesenchymal stem/stromal cells lacking p53, induced liposarcoma [40].

34.2.5 Malignant Fibrous Histiocytoma (MFH)

The so-called malignant fibrous histiocytoma (MFH), now termed high-grade undifferentiated pleomorphic sarcoma, has been postulated that represents a transformed adult hMSC [41]. A stem cell-specific gene expression pattern revealed that the profile of hMSCs was significantly associated with that of MFH. Wnt2 mediates commitment to differentiation while Wnt5a mediates a viability checkpoint in hMSCs. They showed that the tumor suppressor function for specific Wnt2 signaling in hMSCs may mediate differentiation of β-catenin in conjunction with Wnt5a/JNK. Nevertheless, other authors have reported that Wnt3a signaling is inhibitory to hMSC differentiation [42]. The differences in these results suggest that interactions between specific Wnts and Wnt receptors (i.e., frizzled family) may result in potential activation of different downstream pathways [43].

34.2.6 Osteosarcoma

The genesis of the different types of osteosarcoma remains speculative. Several research groups are working on characterizing cancer stem cells, and functional studies are being carried out to assess the tumorigenic potential of these cells. In primary osteosarcoma of the breast, an origin from totipotent mesenchymal cells of the breast stroma or the transformation from a preexisting fibroadenoma or phyllodes tumor has been suggested [44, 45]. The origin of osteosarcoma in testis from undifferentiated mesenchymal cells or from a malignant transformation of preexisting teratomatous elements is still unclear [46].

Recently, Rubio et al. reported osteogenic progenitors derived from bone marrow-MSC defective in Rb and p53 are the cell of origin for osteosarcoma [11]. The same authors showed that intra-bone or periosteal inoculation of MSCs derived from bone marrow or adipose tissue both of them deficient in p53(−/−) or p53 (−/−) and Rb (−/−), induced metastatic osteoblastic osteosarcoma (OS) in immunosuppressed mice. They also showed the main role played by bone microenvironment in osteosarcoma development [47].

34.2.7 Rhabdomyosarcoma

RMS is a common pediatric soft tissue sarcoma that resembles developing fetal skeletal muscle. Keller et al. generated a Cre-mediated conditional knock-in of *PAX3-FKHR* into

the mouse *Pax3* locus, suggesting that terminally differentiating skeletal muscle myofibers have the capacity to give rise to alveolar RMS, which can express early myogenic markers (e.g., myogenin, MyoD, and Pax7), known *PAX3-FKHR* targets (e.g., c-Met, Bcl-XL), and markers of proliferation (e.g., C-Myc and Cyclin D1) [48, 49]. *Pax3-Fkhr* activation was restricted to a target pool of terminally differentiating Myf6-expressing skeletal muscle. *PAX3-FKHR* homozygosity and *Ink4a/ARF* or *Trp53* pathway loss of function significantly accelerates alveolar RMS formation, raising the possibility that some or most alveolar RMS arise from postnatal, terminally differentiating, Myf6-expressing myofibers. Odelberg et al. demonstrated that fully differentiated mature skeletal muscle is able to dedifferentiate into earlier precursor myogenic forms or even into different lineages [50]. These findings reconsider the widely held assumption that only muscle stem cells give rise to RMS. The embryonal subtype of RMS is not associated with these fusion genes. Tiffin et al. suggested that myogenic satellite cells are the origin of these tumors [51]. The pattern of *PAX7* and *MET* (*c*-met) expression (a marker for the myogenic satellite cell lineage), corroborated this hypothesis.

34.2.8 Synovial Sarcoma

Synovial sarcomas are divided into biphasic, monophasic, and poorly differentiated sarcomas on the basis of histopathology. Biphasic synovial sarcoma expresses generally *SYT-SSX1* gene fusion which it is associated with a worse prognosis, and the monophasic subtype shows predominantly, but not exclusively, the expression of *SYT-SSX2* fusion genes [52]. The relationship between fusion gene and the histological subtype suggests that *SYT-SSX1* facilitates the acquisition of epithelial features by undifferentiated tumor cells [53]. De Torres et al. suggested that *SYT-SSX1* fusion transcript collaborates in the epithelial differentiation of mesenchymal tumors [54]. The differences in the degree of transcriptional derepression of E-cadherin by SYT-SSX1 and SYT-SSX2 result in different propensities for epithelial differentiation [55]. These findings strengthen the concept that the protein interactions with Snail or Slug are important in the modulation of mesenchymal to epithelial transition and vice versa in mesenchymal differentiation and neoplasia. The ultrastructural features together with the common epithelial (cytokeratin) and mesenchymal (vimentin) immunophenotype suggest that both epithelioid and synovial sarcoma probably share a similar histogenetic background. Araki et al. suggested a lineage relationship between synovial sarcoma cells and smooth muscle-like mesenchymal cells in a subset of synovial sarcomas expressing the basic calponin gene [56]. Capecchi and colleagues reported a mouse model of synovial sarcoma based on conditional expression of the human SYT-SSX2. Tumors were generated within Myf5 lineage, pointing out the cell of origin to myoblasts [57]. Since they suspected tumor origin in their model to be postnatal, the best cellular candidate was Myf5-expressing myoblasts arising from activated satellite cells (muscle stem cells). However, quiescent satellite cells themselves, postnatally, could also be a potential source of synovial sarcoma. Recently, some authors also suggested a non-myoblast origin for synovial sarcoma [58].

34.2.9 Technologies Applied to Classification and Discovery of Early Events in the Genesis of Sarcomas

Genome-wide expression profiling leads to an improved classification of bone and soft tissue tumors [59, 60], and its combination to light/electron microscopy and immunohistochemical techniques contributes to a better understanding of the genesis of sarcomas. It could also allow a better understanding of poorly described normal connective-tissue counterparts of these tumors, such as fibroblasts, myofibroblasts, and pericytes, because some of the highly expressed genes are implicated in the pathogenesis of these type of tumors.

Once the possible origin cell of a given sarcoma type would be discovered, a major challenge would be to gain insights into the origin of the genetic alterations taking place in that particular cell type. Emerging technologies allow us to analyze posttranslational histone modifications and their roles in regulating chromatin structure and function. Epigenetic regulation of gene expression is mediated in part by posttranslational modifications of histone proteins, which in turn modulate chromatin structure [61, 62]. The core histones H2A, H2B, H3, and H4 are subject to dozens of different modifications, including acetylation, methylation, and phosphorylation.

It is known that embryonic stem (ES) cell differentiation is accompanied by changes in chromatin accessibility to several key developmental genes, including a large-scale opening of the HoxB locus [63, 64]. Furthermore, Polycomb-group proteins play an essential role in maintaining the pluripotent state of ES cells and show markedly reduced expression upon differentiation [65–67]. However, little is known about the overall structure of ES cell chromatin, how it is established, or how it contributes to the maintenance of pluripotency [68].

Exciting findings can be obtained from genome-wide maps of chromatin state. Because the cellular state may be closely related to chromatin state, ChIP-Seq studies by using Solexa® Technology could reveal the mechanisms triggering genetics instabilities. ChIP-Seq is a sequencing-based alternative to ChIP-chip assays. For ChIP-chip studies, DNA fragments that interact with a protein of interest are identified by

hybridization of DNA to the array. Mikkelsen et al. reported the development of ChIP-Seq method for mapping ChIP (chromatin immunoprecipitation) enrichment by sequencing, and described its application to create chromatin state maps for pluripotent and lineage-committed mouse cells [69]. They generated genome-wide chromatin state maps of mouse embryonic stem cells, neural progenitor cells, and embryonic fibroblasts and the resulting data defined three broad categories of promoters based on their chromatin state. Cancer cells are the most obvious targets, as they are frequently associated with epigenetic defects and many appear to have acquired characteristics of earlier developmental stages. Kwon et al. described a sensitive ChIP-DSL technology to detecting in vivo DNA-protein interactions by coupling ChIP with a DNA selection and ligation strategy, permitting analysis of many fewer cells than required by the conventional ChIP-on-chip method [70]. Recently, Riggi et al. reported that EWS-FLI1 acts directly on reprogramming genes by both activating new enhancers and inactivating conserved enhancers. The chimeric fusion acts on the chromatin remodeling which, in turn, induces repression of tumor suppressors and activation of oncogenes [71].

34.3 Molecular Alterations

34.3.1 Acquired Somatic Mutations

Five major types of acquired mutations are detected in sarcomas. *Deletion*: Any loss of genetic material smaller than a chromosome. It may affect a chromosome's arm, a gene, or a small number of base pairs. *Amplification:* The presence of various copies of a single gene whose structure is otherwise normal. *Translocation:* The interchange of genetic material between two non-homologous chromosomes. Frequently, there is no material loss but it simply moves to another place; in this case, we refer to balanced translocations. *Inversion:* This is the result of two ruptures in a chromosome that are followed by reinsertion of the original fragment in reverse order. *Point mutation:* In this type of mutation, one base is substituted by another.

34.3.2 Family Syndromes

Although most cases of mutations turn up sporadically, there are, however, four well-characterized syndromes of family cancer associated to sarcoma. Firstly, patients with RB mutations in the germ-line have a frequency of developing osteosarcomas higher than the general population [72]. Patients with Li-Fraumeni syndrome, showing p53 gene germ-line mutations [73], have a higher incidence for a wide group of sarcomas, and typically earlier than 40 years of age. Another type of sarcoma, malignant peripheral nerves sheath tumor, is frequently observed as a consequence of a neurofibromatosis type 1 (NF-1) associated to the loss of NF-1 gene in the germ-line [74].

34.3.3 Types of Sarcomas Regarding the Complexity of Molecular Alterations

There are two types of sarcomas with regard to the complexity of molecular alterations. On the one hand, there is a group of sarcomas that is more frequent in children and adolescents, their cytogenetic alterations are relatively simple, balanced translocation in general [75]. From the molecular point of view, they are characterized by translocations or punctual mutations which suppose the beginning of the process of carcinogenesis.

A second group of sarcomas, such as the osteosarcoma or malignant fibrous histiocytoma [76], are characterized by a very complex karyotype and lack of gene fusions. In fact, they show a clear chromosome and genomic instability.

The mutations of tumor-suppressing genes p53, INK4A, or RB are found on both subtypes of tumors and are related with tumor progression. Therefore, it is worth mentioning that they have no diagnostic interest, but may determine the prognosis of some sarcomas [77].

34.3.4 Mutations in Sarcomas

There are two types of mutations of clinical value in sarcomas: (1) translocations and (2) point mutations.

34.3.4.1 Translocation

Various types of sarcomas show characteristic translocations [2] In fact, the deepest advances in the knowledge of its pathogenesis have been carried out in these types of tumors [75]. Gene fusions generated from these translocations are the initiating events of many sarcomas and are probably essential in some subtypes of these tumors. These translocations break up certain genes, recombine them, and create gene fusions with new structures and functions by combining functional domains that are usually found in separate molecules. Most chimeric proteins are transcription factors, namely, proteins that have the capacity for union to regions regulating the transcription of some genes. These genes are usually involved in certain key functions of the cell, such as cell proliferation or survival. As a result of translocations, gene fusions represent almost always aberrant transcription factors. Two noteworthy exceptions are: COL1A1-PDGFB in dermatofibrosarcoma protuberans, a growth factor; and ETV6-NTRK3 in congenital fibrosarcomas, a protein with

tyrosine kinase activity [75]. As gene fusions and their products are specific to each tumor type, and they are practically seen in all cases of a large group of sarcomas, their characterization is not only important from the pathogenesis point of view, but it offers great diagnostic and therapeutic opportunities.

34.3.4.2 Point Mutations

Mutations are another type of specific findings in sarcomas, usually beyond the bone-soft tissue location for example, c-*kit* activating mutations in GIST, or inactivating mutations on hSNF5/INI1 in rhabdoid tumors.

34.4 More Relevant Translocations and Their Tumor Type

EWS-FLI1 fusions are detected in roughly 90–95 of Ewing sarcomas [3]; EWS-ERG are present in 5–10 % of cases, while 1 % stand for some other type of fusion of EWS with a member of the ETS family of the transcription factor group [3]. It is specific to this neoplasia, as PCR studies of other tumors that could be included in differential diagnosis, such as central primitive neuroectodermal tumors, neuroblastomas, RMS, adamantinoma and giant cell tumors, have repeatedly yielded negative results [78].

In addition to the usual prognostic factors of this neoplasia (stage, localization/volume of the primary tumor, age, and response to treatment), recent studies have assessed the contribution of molecular heterogeneity (Table 34.1) toward prognosis in ES. There are at least 18 structural chances of gene fusions in this neoplasia. There are two sources of variability. On the one hand, *EWS* fusion companion (*FLI1*, *ERG*, *ETV1*, *E1A,* or *FEV*), and on the other hand, the localization of the translocation rupture point within each gene involved. It has been reported that patients with localized ES expressing the most common chimeric transcript (EWS exon 7 united to FLI1 exon 6; 63 %) have a better prognosis than those with some other type of fusion [79, 80]. However, prognostic differences based on this have not been found in the EuroEwing99 prospective multicentric trial [81].

In the case of the *desmoplastic small round cell tumor* (SRCDT), the *EWS* gene is bound to the *WT1* gene. At first, *WT1* was known to be an altered tumor suppressor gene in Wilms tumor (nephroblastoma); in fact, *EWS-WT1* is the first example of a constant rearrangement of a tumor suppressor gene. The chimeric transcript EWS-WT1 has been found in 97 % of cases that were studied. This is very helpful for diagnosis [82]; it also suggests the chimeric protein is important for the tumor development. It is, as in many other sarcomas, an aberrant transcription factor that regulates the expression of genes that partially coincide with usual WT1 targets. One of them is PDGFA, a fibroblastic growth factor, which is probably involved in the characteristic fibrosis of this neoplasia [83]. BAIAP3 is another factor that regulates the exocytosis process, and thus the secretion of growing factors [84].

EWS binds to *ATF1* in the *clear cell sarcoma* (soft tissue malignant melanoma) [85] and in the *angiomatoid fibrous histiocytoma* [86]. EWS binds to a DNA-binding domain of a transcription factor, as in the Ewing tumor. Contrary to ATF1, EWS-ATF1 fusion works as a transcriptional activator, probably altering the regulation of genes usually controlled by ATF1. The chimeric RNA is detected both in fragments of frozen tumor and formalin-fixed paraffin-embedded material [87].

EWS-CHN fusion, generated from a t(9;22), is observed in the extraskeletal myxoid chondrosarcoma, curiously, not from myxoid chondrosarcoma of osseous origin [88]. CHN codifies a nuclear receptor having a binding domain to DNA. The fusion protein has an EWS amino-terminal domain bound to the whole CHN reading frame; this yields a nuclear receptor more active than the native one. This receptor is involved in the control of cellular proliferation by modulating the response to diverse growth factors. There are some infrequent variants of this fusion. Genomics studies have shown that this tumor has a high expression on PPARG. There are some specific inhibitors against this molecule, which could become a therapeutic target [89].

In addition, a gene analogous to *EWS*, *FUS*, is involved with *CHOP (DDIT3)* in gene fusion in 90 % of cases comprising *round cell/myxoid liposarcoma* (*FUS-CHOP*) [90]. CHOP (DDIT3) is a transcription factor. In TLS-CHOP (FUS-DDIT3), TLS binding domain to RNA is replaced by CHOP binding domain to DNA. The relation between myxoid liposarcoma and round cells is confirmed by detecting FUS-CHOP fusions in tumors made up, as a whole or in part, of round cells [90]. Approximately 5 % of cases show EWS-CHOP fusions, wherein EWS has an analogous role to FUS. Thus, proteins having the ability to bind to RNA, FUS, and EWS seem to be functionally similar, while the component providing the binding domain to DNA, CHOP, is specific in this neoplasia.

34.4.1 Alveolar Rhabdomyosarcoma

Alveolar rhabdomyosarcoma is associated to a characteristic translocation, t(2;13), and another less common one, t(1;13). This is the result of *PAX3* and *PAX7* gene fusion, respectively, with forkhead in RMS (*FKHR*) localized in 13q14 [91]. *PAX* genes are transcription factors regulated during the embryo development; they are essential for the genesis of some organs. PAX3 and PAX7, in particular, are expressed in the neural tube and are fundamental for its correct formation, and for the migration of myoblasts to upper and lower

Table 34.1 Chromosomic translocations and the molecular findings described in sarcomas

Tumor type	Cytogenetic finding	Molecular trace	Reference
Ewing sarcoma	t(11;22)(q24;q12)	EWSR1-FLI1	Delattre et al. [100]
	t(21;22)(q22;q12)	EWSR1-ERG	Sorensen et al. [240]
	t(19;der(ins.inv(21;22)))	EWSR1-ERG	Maire et al. [241]
	t(16;21) (p11;q22)	FUS-ERG[a]	Shing et al. [242]
	t(7;22;)(p22;q12)	EWSR1-ETV1	Jeon et al. [243]
	t(17;22)(q12;q12)	EWSR1-ETV4	Kaneko et al. [244]
	t(2;22)(q33;q12)	EWSR1-FEV	Peter et al. [245]
	t(6;22)(p21;q12)	EWSR1-POU5F1	Yamaguchi et al. [246]
	t(1;22) (q36.1;q12)	EWSR1-PATZ1	Mastrangelo et al. [247]
	t(2;22)(q31;q12)	EWSR1-SP3	Wang et al. [248]
	t(20;22)(q13;q12)	EWSR1-NFATc2	Szukhai et al. [249]
	t(2;16)(q35;p11)	FUS-FEV	Ng et al. [250]
	t(15;19)(q14;p13.1)	BRD4-NUT[a]	Mertens et al. [251]
	t(4;19)(q35;q13)	CIC-DUX4	Kawamura-Saito et al. [252]
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	EWSR1-WT1	Ladanyi et al. [253]
Round cell, myxoid liposarcoma	t(12;16)(q13;p11)	FUS-DDIT3	Crozat et al. [254]
	t(12;22)(q13;q12)	EWSR1-DDIT3	Panagopoulus et al. [255]
Epithelioid pleomorphic liposarcoma	t(12;16)(q13;p11)	FUS-DDIT3 (CHOP)	Cecco et al. [256]
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	EWSR1-CHN	Clark et al. [257]
	t(9;17)(q22;q11)	hTAFII68-CHN	Attwooll et al. [258]
	t(9;15) (q22;q21)	TCF12-CHN	Sjogren et al. [259]
	t(3;9)(q12;q22)	TFG-CHN	Hisaoka et al. [260]
Clear cell sarcoma	t(12;22)(q13;q12) t(2;22)(q33;q12)	EWS-ATF1 EWS-CREB1	Zucman et al. [85] Antonescu et al. [261]
Angiomatoid fibrous histiocytoma	t(12;22)(q13;q12) t(2;22)(q33;q12)	EWS-ATF1 EWS-CREB1	Hallor et al. [86] Rossi et al. [262]
Synovial sarcoma	t(X;18)(p11.23;q11)	SS18-SSX1	Crew et al. [263]
	t(X;18)(p11.21;q11)	SS18-SSX2	Clark et al. [264]
	t(X;18)(p11;q11)	SS18-SSX4	Skytting et al. [265]
	t(X;20)(p11;q13.3)	SS18L1-SSX1	Storlazzi et al. [266]
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	PAX3-FOXO1	Galili et al. [267]
	t(1;13)(p36;q14)	PAX7-FOXO1	Davis et al. [268]
	t(2;2)(q35;p23)	PAX3-NCOA1	Wachtel et al. [194]
	t(2;8)(q35;q13)	PAX3-NCOA2	Sumegi et al. [269]
Embryonal rhabdomyosarcoma	t(2;8) (q35; q13)	PAX3-NCOA2	Sumegi et al. [269]; Hosoi et al. [270]; Yoshida et al. [271]
	t(6;8) (p21;q11.2) t(8;11)	SRF-NCOA2 TEAD1-NCOA2	Mosquera et al. [272]
Dermatofibrosarcoma protuberans	t(17;22) (q22;q13)	COL1A1-PDGFB	Simon et al. [273]
Infantile fibrosarcoma/cellular mesoblastic nephroma	t(12;15)(p13;q25)	ETV6-NTRK3[a]	Knezevich et al. [96]
Alveolar soft part sarcoma	t(X;17)(p11;q25)	TFE3-ASPL [a]	Ladanyi et al. [274]
Low grade endometrial stromal sarcoma[b]	t(7;17)(p15;q21)	JAZF1-SUZ12	Koontz et al. [275]
Inflammatory myofibroblastic tumor	t(1;2)(q25;p23)	TPM3-ALK [a]	Lawrence et al. [276]
	t(2;19)(p23;p13)	TMP4-ALK	Lawrence et al. [276]
	t(2;17)(p23;q23)	CLTC2-ALK	Bridge et al. [277]
	t(2;2)(p23;q13)	RANBP2-ALK	Ma et al. [278]
Low-grade fibromyxoid sarcoma	t(7;16)(q33p11) t(11;16)(p11;p11)	FUS-CREB3L2 FUS-CREB3L1	Storlazzi et al. [279] Mertens et al. [280]

(**EWSR1** is also named as EWS); (**FLI-1** is also named as EWSR2, SIC-1) (**ETV4** is also known as E1AF, E1A-F, PEA3,PEAS3); (**DDIT3** is also named as CHOP; CEBPZ, CHOP10; GADD153; MGC4154); (**NR4A3** is also named as CHN, TEC, CSMF, MINOR, NOR1); (**FOXO1** is also named as FKHR, FKH1 y FOXO1A); (**SS18** is also named as SYT, SSXT, MGC116875). (SUZ12 is also name as JJAZF1)

[a]These fusions are also present in other tumors (carcinomas, leukemias or lymphomas) (reviewed in Ordonez et al. [3])

[b] More information can be obtained from Lee et al. [281]

extremities. PAX3 could suppress myoblast differentiation; this could lead to the undifferentiated phenotype of this tumor. Some amplification has been detected concerning gene fusions in some tumors with *PAX7-FKHR* fusions; this suggests that translocation and amplification may be sequential mechanisms in the oncogenesis of this neoplasia. As for PAX3-FKHR fusion, an overexpression of transcriptional origin was detected on PAX3 non-associated to gene amplification [92]. These differences concerning PAX3 and PAX7 overexpression mechanisms are analogous to those observed at clinic level. PAX7-FKHR tumors tend to turn up in younger patients and are associated to a lower rate of metastasis and a better survival than those with PAX3-FKHR, even though they have a similar morphology [93].

34.4.2 Synovial Sarcoma

Synovial sarcoma is characterized due to t(X;18); it generates a fusion between *SS18* (*SYT*) gene on chromosome 18 with one *SSX* gene: *SSX1* and *SSX2*. They are located in two subregions of chromosome Xp11 (23 and 21, respectively); there are, though, rarer fusions. This fusion codifies an abnormal nuclear transcription factor which alters chromatin remodeling; this may induce some changes in the gene expression patterns. Transcripts are detected in almost all synovial sarcomas through RT-CPR. The synovial sarcoma is a clear example of correlation tumor phenotype and transcript type. Biphasic synovial sarcoma is associated with SYT-SSX1 fusions, which are present both in epithelial and fusocellular elements, while monophasic synovial sarcoma is usually present with SYT-SSX2. In addition, patients at clinic level with SYT-SSX2 show a relatively low risk of relapses, while tumors with SYT-SSX1 variant show a high proliferative rate and a worse prognosis [52, 94]. However, the prognostic value of these fusions is still under discussion [95].

Translocation t(17;22) in *dermatofibrosarcoma protuberans* and giant cell fibroblastoma generates a fusion involving *COL1A1*, a collagen gene and *PDGFB*, a protein-modifying gene with growing factor function. This fusion sets *PDGFB* under the control of *COL1A1* promoter, thus removing all elements repressing *PDGFB* transcription. In the case of congenital fibrosarcoma, t(12;15) translocation unites *ETV6 (TEL)* gene with neurotrophin 3 receptor (*NTRK3*) [96]. Curiously, this fusion is also specifically seen in mesoblastic nephroma, acute myeloblastic leukemia, and secretory carcinoma of the breast, a rare variant of the infiltrating ductal carcinoma of the breast [97]. This phenomenon suggests that the same gene fusion may cause different neoplasms, and depends on the characteristics of the cell where this occurs. These two examples represent the exception confirming the general rule that gene fusions generate new ETV6-NTRK3 transcription factors in a chimeric kinase tyrosine, which may contribute to oncogenesis by deregulation of transduction pathways of signals generated by NTRK3, while PDGFB-COL1A1 probably acts as an autocrine growing factor.

34.5 Chimeric Proteins Resulting from Translocations: Structure and Function

34.5.1 Chimeric Protein Structure: ES Oncoprotein as an Example

Sarcomas are paradoxical examples of how a disease with a relatively low prevalence can bear prominent biological significance because of their molecular mechanisms of generation and progression. Fusion genes resulting from chromosomal translocations can be transcribed and translated into chimerical proteins. Let us first take Ewing sarcoma oncoproteins as an example [3, 98].

The ES family of tumors (ES) comprises morphologically heterogeneous tumors that are characterized by nonrandom chromosomal translocations involving the EWS gene and one of the several members of the ETS family of transcription factors [99]. The translocation t(11;22)(q24;q12) is the most common one, associated to 90 % of cases, and this high specificity suggests that the product of this rearrangement is involved in the formation of these malignancies. This translocation leads to in-frame fusion of EWS at 22q12 to FLI1 at 11q24 and the formation of EWS-FLI1 on der[22] comprising the 5' end of EWS and the 3' end of FLI1 [100]. The fusion gene encodes an oncoprotein consisting of the N-terminal domain of EWS and the C-terminal DNA-binding domain of transcription factors such as ETS family, activating transcription factor-1, Wilms' tumor 1, and nuclear orphan receptors [101].

EWS protein is predicted to be an RNA-binding protein containing the transcriptional activation domain(s) in the N-terminal domain and the RNA recognition motif and three arginine-glycine-glycine repeats (RGG boxes 1–3) in its C-terminal domain [102, 103]. The transcriptional potency of the N-terminal domain of EWS observed in its various tumorigenic fusion proteins suggests that EWS may function as a transcription factor. In the EWS-FLI1 fusion protein, the RNA-binding motif containing the C-terminal half of EWS is replaced by the DNA-binding domain (DBD) of the FLI1 protein. EWS belongs to a subgroup of RNA-binding proteins called the TET family, which also includes liposarcoma/fusion protein (TLS/FUS) and human TATA-binding protein-associated factor (hTAFII68) [90, 104]. They all contain a distinctive conserved 87-amino-acid RNA recognition motif/ribonucleoprotein consensus sequence (RRM/RNP-CS) motif commonly found in most RNA-binding proteins and possess a number of R-G-G (arginine-glycine-glycine)

repeats that also seem to facilitate binding to RNA. A zinc finger domain with four cysteines is present in their C-terminal domain. In addition, mammalian TET family members share glutamine-rich N-terminal domains consisting of a series of degenerate repeats. In Fig. 34.1, we present a schematic view of EWS protein domains.

Three key features indicate that the EWS protein is encoded by a housekeeping gene; it is expressed ubiquitously, its expression is stable throughout the cell cycle, and its mRNA has a long half-life [90]. Although more remains to be known about the role of native EWS, it is a nuclear protein that appears to be recruited to promoter regions where it associates with other factors to act as a promoter-specific transactivator [105, 106]. It also functions as a transcriptional coactivator in a cell type-specific and promoter-specific manner. It requires CBP/p300 for hepatocyte nuclear factor 4 (HNF4)-mediated transcriptional activation [107, 108]. Although EWS fusion proteins function as sequence-specific transcription factors, the role of native EWS protein and the regulatory mechanism controlling the coactivator function of EWS are largely unknown.

FLI1 is a member of the ETS family of transcription factors which activate specific target genes by binding to their cognate DNA sequences through their DNA-binding regions, usually located at their carboxyl termini [109, 110]. The replacement of the native transcription activation domain(s)

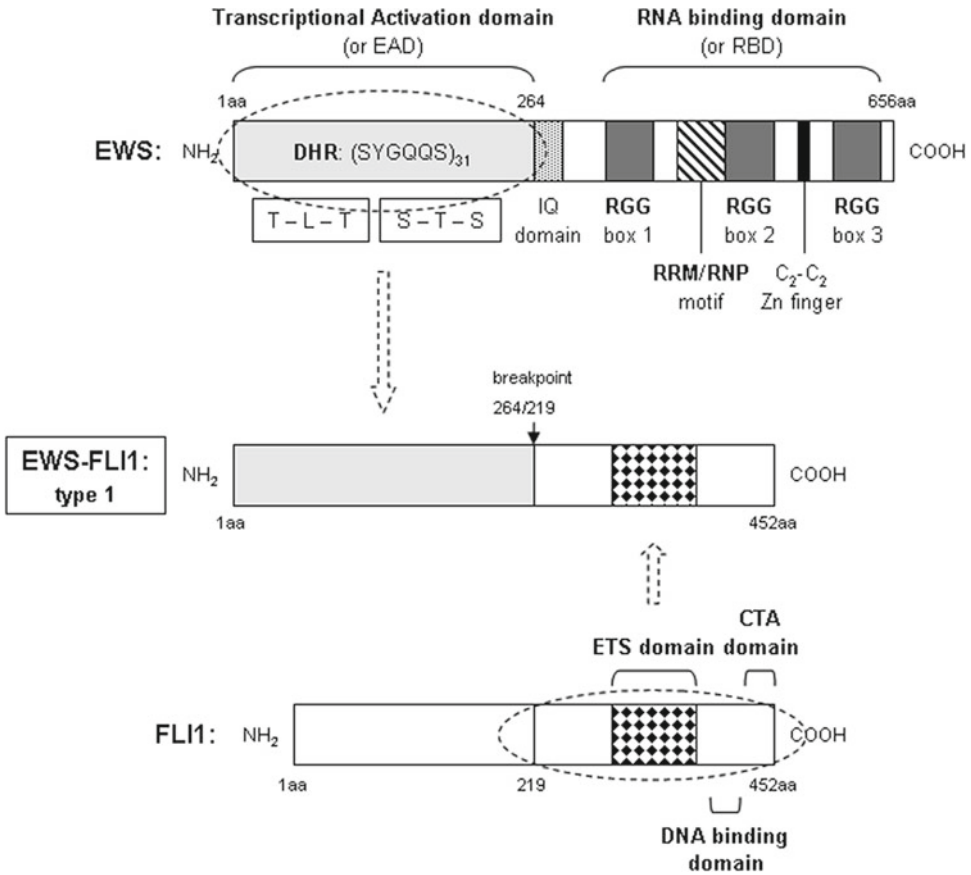

Fig. 34.1 Schematic view of the structural features of a prototypic sarcoma chimeric protein, and the native proteins it arises from. EWS-FLI1 type 1 protein is associated with ES. Primary and secondary structures, as well as functional regions of native EWS, FLI1, their breakpoints, and the resulting fusion protein are depicted. EWS N-terminal domain contain amino acid residues 1–264, referred to as the EWS-activation domain (EAD). It contains 31 degenerate hexapeptide repeats (DHR) with the consensus sequence SYGQQS and 7 other Tyr residues. The C-terminal domain begins with the IQ domain, a region susceptible of binding calmodulin and being phosphorylated. Next, it has a distinctive conserved 87-amino-acid RNA recognition motif/ ribonucleoprotein consensus sequence motif (RRM/RNP domain) commonly found in most RNA-binding proteins, and possess a number of RGG (arginine-glycine-glycine) repeats that also seem to facilitate binding to RNA. A zinc finger domain with four cysteines (C_2-C_2 Zn finger-like) is also present close to the C-terminal end. FLI1 is a transcription factor, with a naturally occurring transactivating region in the N-terminal end. It encloses a highly conserved, sequence-specific, ETS-type DNA-binding domain. FLI1 also has contains a C-terminal end transactivating domain (CTA). The chromosomal t(11;22)(q24;q22) translocation in ES results in the fusion of the N-terminal domain of EWS and the DNA-binding domain of FLI1, generating the oncoprotein EWS-FLI1. The transactivating domain of EWS is retained, while the RNA-binding domain is lost, replaced by the sequence-specific DNA-binding domain of the FLI1 protein. The type 1 chimeric protein is represented entirely schematically. Types 2 or 3 can be deduced from type 1 by the insertion of 84 or 22 additional amino acids from EWS or FLI1, respectively.

of FLI1 by the N-terminal region of EWS converts the non-transforming activator, FLI1, into a transforming protein with new transcriptional activation potential. The ETS family of transcription factors is defined by a conserved ETS domain that recognizes a core DNA motif of GGAA/T [111]. This family of approximately 30 genes including FLI1, ERG, ETV1, E1AF, FEV, and ZSG controls a variety of cellular functions in cooperation with other transcription factors and cofactors. Target genes include oncogenes, tumor suppressor genes, and genes related to apoptosis, differentiation, angiogenesis, and invasion [111, 112]. Recently, Riggi et al. reported that EWS-FLI1 acts on chromatin remodeling playing a main role in Ewing sarcoma biology [71].

34.5.2 Protein–Protein Interactions Studies Provide Structural and Functional Insights into the Mechanisms of Action of Native Proteins in Sarcomas

We can next take a look inside the archetypical case of the ES chimera protein. In the EWS-FLI1 fusion protein, both the N-terminal domain of EWS and the DBD of FLI1 are necessary for the transforming activity of Ewing cells [101, 102]. To assess the contribution of the N-terminal domain of the EWS protein to the formation of human solid tumors, it is important to understand the normal function(s) of EWS. Although much is known about the oncogenic functions of chimeric EWS fusion proteins that result from chromosomal translocations, the cellular role of the normal EWS protein is not well characterized.

Bertolotti et al. investigated the putative role of EWS in RNA polymerase II (Pol II) transcription by comparing its activity with that of its structural homolog, hTAFII68 [105]. The structural homology between EWS and the transcription factor hTAFII68 (70 % similarity among the full-length proteins) strongly suggested that there may be a functional homology between these proteins. The authors demonstrated that a portion of EWS is able to associate with the basal transcription factor TFIID, which is composed of the TATA-binding protein (TBP) and TBP-associated factors (TAFIIs). In vitro binding studies revealed that both EWS and hTAFII68 interact with the same TFIID subunits, suggesting that the presence of EWS and that of hTAFII68 in the same TFIID complex may be mutually exclusive. Moreover, EWS is not exclusively associated with TFIID but, similarly to hTAFII68, is also associated with the Pol II complex. The subunits of Pol II that interact with EWS and hTAFII68 were identified, confirming the association with the polymerase. On the other hand, using Ewing cell nuclear extracts, they studied the association of EWS and the oncogenic fusion protein, EWS-FLI1, with different multiprotein complexes. These experiments suggest that EWS and EWS-FLI1 behave differently since EWS-FLI1 cannot stably associate with either TFIID or Pol II in Ewing cell nuclear extracts. These observations suggested that EWS and EWS-FLI1 may play different roles in Pol II transcription.

Employing yeast two-hybrid cloning to study protein–protein interactions, Petermann et al. isolated the seventh largest subunit of human RNA polymerase II (hsRPB7) as a protein that specifically interacts with the amino terminus of EWS [113]. This association was confirmed in nuclear extracts, where hsRPB7 was found to co-purify with EWS-FLI1 but not with FLI1. Overexpression of recombinant hsRPB7 specifically increased gene activation by EWS-chimeric transcription factors, whereas replacement of the EWS portion by hsRPB7 in the oncogenic fusion protein restored the transactivating potential of the chimera. Those results suggested that fusion to FLI1 causes as structural change of the amino-terminal EWS domain, making it accessible for interaction with hsRPB7. Presumably, the physical interaction of the amino terminus of EWS with hsRPB7 contributes to the transactivation function of EWS-FLI1 and, since hsRPB7 has characteristics of a regulatory subunit of Pol II, may influence promoter selectivity.

Most chimeric proteins in many sarcoma types function as aberrant transcription factors. One step further in the characterization of the RNA-binding capabilities of such proteins was the revelation by proteomics techniques of the association of EWS protein to the IRES of central proteins in some pathogenic processes. This was the case in hepatitis C virus-infected cells [114]. The authors used shotgun peptide sequencing to identify proteins in quadruplicate protein affinity extracts of lysed cells, obtained using a biotinylated IRES. EWS protein contains an RRM RNA recognition motif and a zinc finger RNA-binding region, through which interaction occurs. From a mechanistic point of view, protein binding to the IRES is an indication of the importance of that protein, both for its functional value as well as a potential drug target. In particular, EWS may shuttle from the nucleus to the cell surface [115] and function as a transcriptional cofactor with CBP/p300 [106]. This interpretation is particularly interesting in view of the proposed role of the nucleolus as a gateway for viral infection and site of replication for many viruses. Although to our knowledge this point has not been studied in sarcomas, the evidences gathered to date indicate that it might be interesting to investigate the potential role of EWS as an IRES-binding protein.

Despite the numerous reports aiming at the function of TET proteins as a partner of fusion proteins in different sarcoma translocation types, little evidence has been collected about the cellular function of the normal proteins, let alone from proteomic approaches. Anumanthan et al. reported the interaction between a WD domain-containing protein, serine-threonine kinase receptor-associated protein (STRAP) and EWS, using matrix-assisted laser desorption/ionization,

time-of-flight and tandem mass spectrometry (MALDI-ToF MS/MS) [116]. STRAP interacts with EWS in the nucleus and induces inhibition of EWS transactivation function. Their research found that normal EWS protein is upregulated in human cancers, suggesting a cooperative role of these two proteins in tumorigenesis. Results suggested that STRAP inhibits the transactivation function of EWS by displacing p300 from the functional transcriptional complex. In consequence, this study provided a novel TGF-ß-independent function of STRAP and described a mechanism by which STRAP regulates the physiologic function of oncogenic EWS protein in the nucleus.

Gaining insight into the role of TET proteins in RNA metabolism, Guipaud et al. characterized one of the precise cellular functions of these multifunctional proteins, DNA pairing [117]. Conservation across species suggests that TET proteins have important physiological roles. TET proteins are thought to be involved in RNA transcription and/or processing [118, 119]. Besides these functions, several lines of evidence suggested a new role for TLS/FUS in DNA transaction and in DNA double-stranded break rejoining [120, 121]. Particularly, they addressed the question whether EWS displays pairing on membrane (POM) activities. Since EWS share similar features with TLS/FUS (structure, partners, and affinities), they raised the question whether it also exhibits POM activities. Accordingly, they applied the assay to 2-DE coupled to MS analysis for a global screening of catalysis of homologous DNA POM activities. This test allowed them to identify EWS, in addition to hTAF(II)68, TLS/FUS, but no other proteins, indicating a feature specific to a protein family whose members share extensive structural similarities.

This common activity suggests a role for TET proteins in genome plasticity control. The results suggest for TET proteins a role in DNA metabolism in addition to their role in pre-mRNA splicing and transcription regulation. Proteins that function at the interface between transcription and RNA metabolism affect genetic stability by regulating transcription-associated recombination. Significantly, the homologous DNA pairing activity of TLS/FUS is supported by the phenotypes of TLS/FUS-deficient mice [122, 123]. Based on these findings and on their DNA pairing activities, it is thus tempting to speculate on a role for POM proteins in genome plasticity control. This common enzymatic feature could also be of importance in understanding tumor development involving these proteins.

34.5.3 The Role of Chimeric Proteins in Sarcoma Molecular Mechanisms

Much of the understanding about the function of sarcoma genes is derived from studies of oncogenic fusions that arise from gene rearrangement after chromosomal translocation. And, obviously, even more research has been devoted to understand the mechanisms of action of the chimera proteins themselves, as necessary (but not sufficient?) effector of the tumorigenic cascade of events that ultimately cause the onset of tumoral alterations. However, although extensive work has been performed on cellular, molecular, and genomic lines of attack, little knowledge has been reported so far related to proteomics approaches (Fig. 34.2).

An alternative proteomic approach has been recently developed in our laboratory, aiming at identifying differences in protein expression and posttranslational modifications attributable to the loss of EWS-ETS protein activity: an interference shRNAi model for EWS-FLI1 was constructed in the TC71 ES cell line [124]. Protein expression levels were analyzed in cell extracts of the shRNAi clone model to gain insights into the differential changes associated with the interference of the EWS-FLI1 transcript [125]. Two-dimensional PAGE analyses were performed to visualize differences in a plethora of different conditions. Most of the changes associated with the shRNAi clone were presented as an overexpression of their protein levels (Fig. 34.2). In contrast, about one third of the proteins showed a clear reduction of their expression amounts. Further examination of those results by a MALDI-ToF MS analysis enabled the identification of significant protein changes. Interestingly, many of the identified proteins with the highest scores were molecules directly involved in RNA and DNA processing and functioning. Given the fact that the protein chimera EWS-FLI1 acts as an aberrant transcriptional factor, this evidences suggest that the interference model is altering several protein regulations, in pathways directly and indirectly related to EWS-FLI1, which in consequence brings about changes in expression patterns for molecules involved in nucleic acid activities.

Proteomic expression pattern showed differences for nuclear proteins. For instance, a component of the RNA polymerase II holoenzyme complex that is involved in DNA recombination, DNA repair, and protein folding, in agreement with previously commented reports about the involvement of TET-ETS fusion proteins in direct regulation of RNA polymerase II. Similarly, we have observed alterations in a structural constituent of the ribosome, which binds RNA during translation. An aminoacyl-tRNA synthetase was also selected; through the regulation of Akt, and therefore, modulation of the HSP90 stress-responsive activity, the biological function of this tRNA synthetase may play a role in the response of *ES* cell machinery to stress conditions [126]. Another factor engaged in protein translation is a component of the polyribosome, whose molecular function is the binding of mRNA. This protein has been reported to participate in Wnt signaling, which, in turn, has been proven to play an important role in the development of some types of sarcoma [41, 127]. The list includes more proteins involved in DNA/RNA metabolisms, as well as some others implicated in RNA translation and protein synthesis.

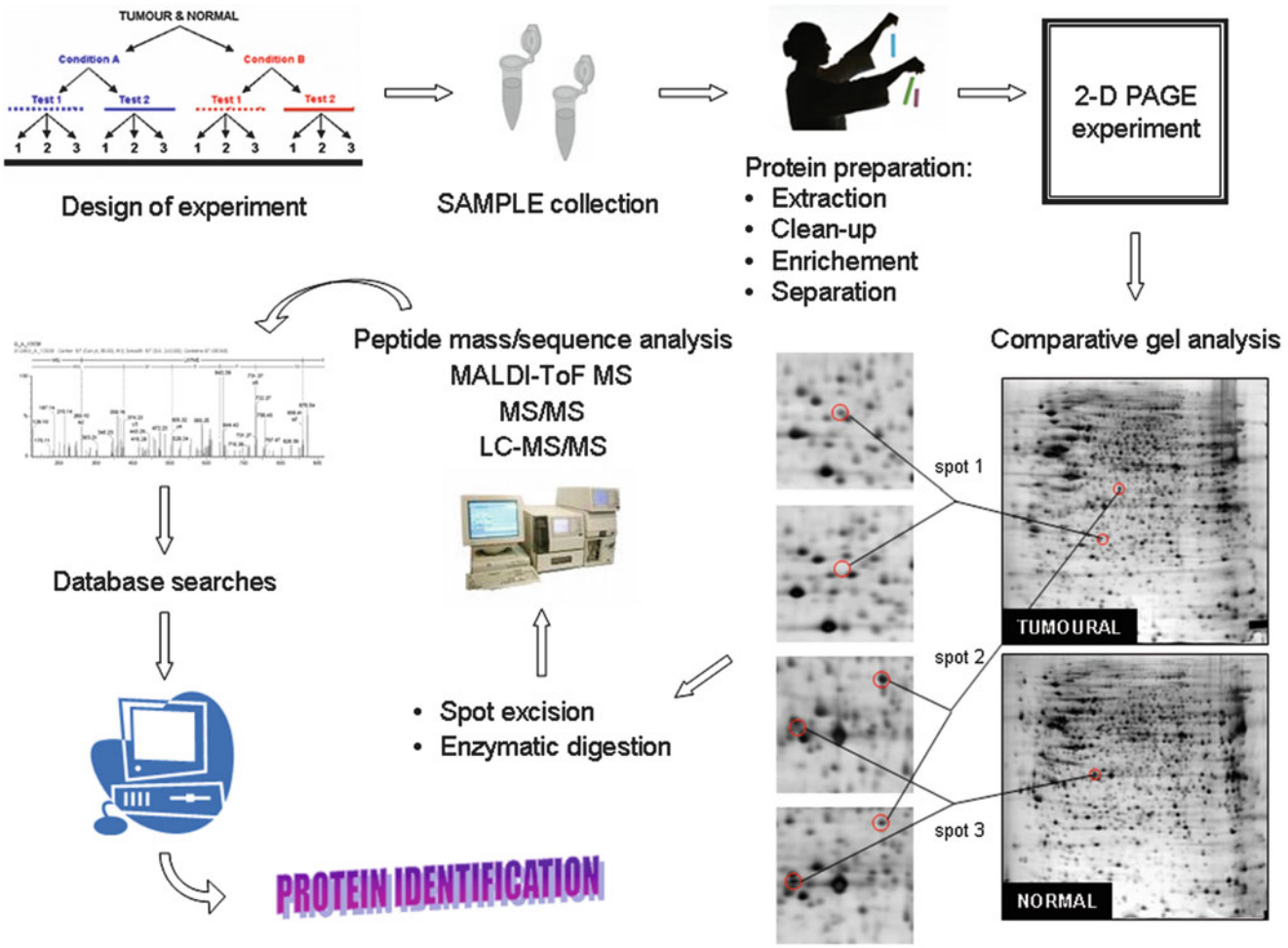

Fig. 34.2 Flow-chart diagram illustrating the experimental steps taken in a proteomics approach to characterize and identify the protein(s) present in a clinical sample. (1) Samples are collected following the recommendations from the donor tumor bank, (2) protein extracts are separated from the cellular debris, and (3) prepared for the proteomic analysis through two-dimensional electrophoresis (2-D PAGE). After isoelectric focusing in a pH gradient the electrophoretic separation is completed in a second polyacrylamide gel dimension. Proteins can be then visualized after staining (chemically or radioactively). A close-up 2-D PAGE image of an assay showing protein expression differences in an experimental ES cell line TC71 versus a control illustrates the proceeding of the comparison, that eventually renders information that can be linked with the pathogenesis of the studied disease; (4) proteins are digested by proteolytic enzymes to smaller peptides and their precise identity is achieved using mass spectrometry (generally performed in two ways); (5) either, precise molecular weights are measured using MALDI-ToF instruments, and the results compared with protein sequences from available databases. Or, (6) tandem mass spectrometry gives information about protein)sequence that is subjected to the search in databases; (7) finally, the identification of the protein(s) in the initial sample can be solved with almost full certainty in 90 % of the cases.

Besides that aspect of chimeric protein regulation, there is another group of proteins altered in sarcoma cells, whose functions are associated to cell cycle and cell division activities. Another protein involved in cell cycle is a serine/threonine phosphatase, essential for cell division. From previous analysis (immunohistochemistry, flow cytometry), it has been suggested that this phosphatase is involved in the accelerated growth of malignant tumor cells in sarcoma.

Finally, we identified in the shRNAi clone a group of targets related to cellular protein metabolic processes. In particular, several of them are molecules engaged in protein binding, folding, or rearranging of polypeptide bonds and structures in specific protein species. It seems that the chimera protein inhibits, in some way, several effectors that have to do with the assembling of tertiary structures of certain proteins. The overexpression of these or similar proteins in the RNA-interfered model may provide some clues about the molecular changes accompanying the redifferentiation program of cells after inhibition of the fusion gene induction. Alternatively, other candidates in this group are connected with proteolytic degradation processes. This is the case of a proteasome subunit. It has been recently pointed that EWS-FLI1 plays an important role in the prevention of senescence, leading to the unlimited growth and oncogenesis of ES cells through a decrease in the stability of p27 protein, due to increased action of Skp2-mediated 26S proteasomal degradation [128].

These results partly correlate with data from a gene expression microarray experiment, where transcript levels from the shRNAi clone and controls were registered by qRT-PCR. Further and deeper studies are currently being accomplished in our group to characterize the effect of another variables, such as the stability of the RNA inhibition with time progression, posttranslational modifications (e.g., phosphorylation, acetylation, etc.), and to quantitate the magnitude of these changes, both at the protein level, and at the translational level.

34.5.4 Functional Studies of Proteins Determining the Tumorigenicity of Sarcomas: An Overview of Protein Targets Identified

The depiction of cellular functionality that explains and corroborates the mechanisms by which cancer originates and eventually propagates has traditionally been entrusted to genomic and post-genomic strategies. For example, microarray analyses have identified differentially expressed genes from many classes that might contribute to the different steps of cancer generation and progression [129, 130], but these expression changes only suggest functional importance. RNA-mediated interference (RNAi) screens in mammalian cells can functionally assess the genome in high throughput, and these screens for cancer phenotypes have identified new gene targets [131]. However, such screens are limited because RNAi knockdown occurs over many hours, and because proteins that turn over slowly cannot be efficiently depleted. The alternative would be to develop a high-throughput approach that directly addresses protein function and is temporally restricted. Such an approach could identify new and different targets for tumor invasion or other disease-relevant processes.

Jay and colleagues exploited a previously developed technology for functional proteomics to identify and validate proteins required for cancer invasion in fibrosarcoma cells [132]. This technique, known as fluorophore-assisted light inactivation (FALI), affords the possibility of targeting a particular surface protein for destruction using the specificity of an antibody [133]. The authors revealed that the molecular chaperone heat shock protein 90 (HSP90) plays a crucial role in the invasion of fibrosarcoma cells. HSP90 was shown to promote maturation of the extracellular metalloprotease MMP2, and therefore, was identified as an important extracellular mediator of invasion.

Making also use of the FALI technology and libraries of monoclonal antibodies and phage-expressed single-chain antibodies, the same group identified CD155/PVR as a key protein in cell motility during cell invasion and migration in fibrosarcoma [134]. CD155 was found to be highly expressed in multiple cancer cell lines and primary tumors including glioblastoma. They showed that CD155 is recruited to the leading edge of migrating cells where it co-localizes with actin and alpha v-integrin, known mediators of motility and adhesion.

34.6 Cellular Signaling Pathway Categories

The cell signaling pathways (Fig. 34.3), cell surface adhesion molecules, receptor tyrosine kinases, growth factors, transcription factors, and early developmental genes expressed in these tumors identify potential candidates for therapeutic intervention and diagnostic development.

A tight clustering has been observed analyzing the gene expression data from 181 tumor types, representing 16 classes of human bone and soft tissue sarcomas on a 12,601-feature cDNA microarray [59]. On one hand, the dendrogram is populated primarily by the more poorly differentiated or dedifferentiated tumors and the typically adult sarcomas with the major branches being formed by the malignant fibrous histiocytoma tumors, leiomyosarcoma, dermatofibrosarcoma, hemangiopericytoma, and liposarcoma. On the other hand, the tightly clustered groups are assigned to the pediatric sarcomas, Ewing sarcoma, synovial sarcoma, osteosarcoma, and RMS. This could denote the existence of common signaling pathways for each branch.

34.6.1 Tyrosine Kinases

ES proliferation and survival is also determined by autocrine and paracrine activation of growth factor receptors and their ligands as insulin-like growth factor 1 [135]. EWS-FLI1 shRNA interference affects IGF-1/IGF-1R survival pathway and its downstream targets (Fig. 34.4) [136]. shRNAi clone showed a marked decrease in IGF-1 transcript and protein levels, both early and late stages (Fig. 34.4a, b), but there were no significant changes in IGF-1R protein level (Fig. 34.4b). shRNAi clone was more sensitive to the action of IGF-1R signaling pathway inhibitors (Fig. 34.4c). A higher apoptotic index was detected, as analyzed by FACS for apoptosis using annexin V (Fig. 34.4d) as well as the reduction of proteins involved in the activation of IGF-1R signaling pathway after treatment with AEW571 combined with inhibitors LY294002 and PD98059 (Fig. 34.4e).

Benini et al. analyzed the contribution of the 2 major pathways of the intracellular IGF-1R signaling cascade to the overall effects elicited by IGF-1 in Ewing sarcoma [137] Both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K) signaling pathways appeared to be constitutively activated in Ewing sarcoma, likely due to the presence of the IGF-1R-mediated autocrine

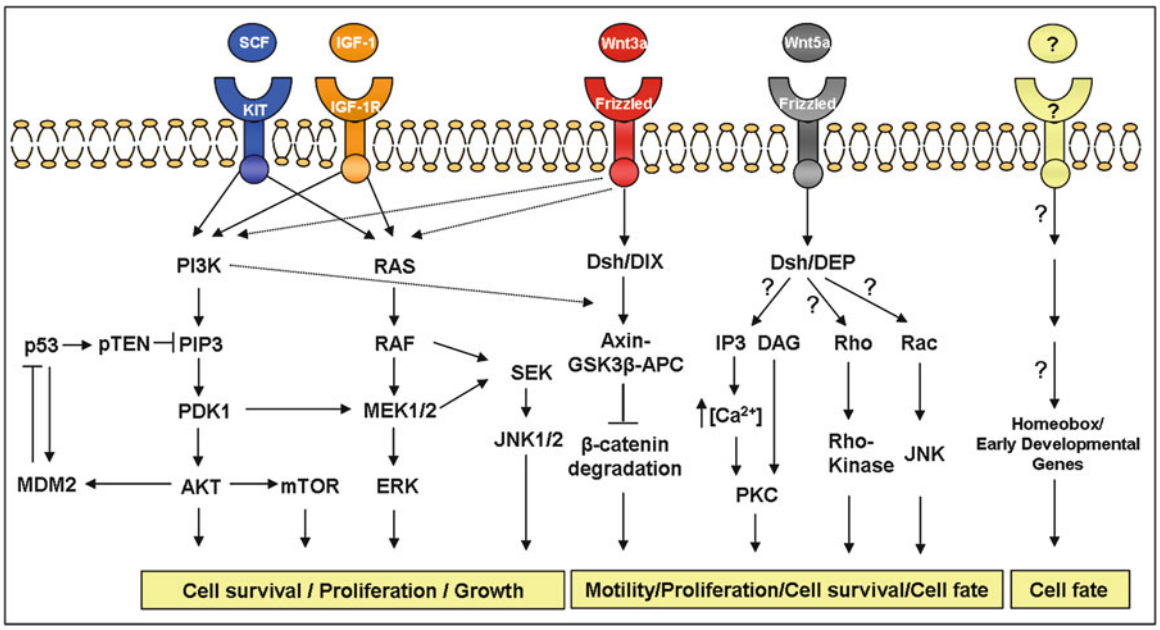

Fig. 34.3 Main cell signaling pathways described in sarcomas. This figure shows the importance of certain specific pathways in sarcomas. The Wnt-frizzled signaling pathway has been found in both, ES and synovial sarcoma. The MEK/MAPK and PI3-K signaling pathways contribute to the malignant behaviour of ES cells and gastrointestinal stromal tumor. The AKT-mTOR pathway plays a critical role in the development of leiomyosarcomas. In dashed lines are shown possible pathways interactions.

loop. Exogenous IGF-1 completely reverted LY294002-induced growth inhibition by abrogating anti-proliferative and pro-apoptotic effects of the PI3-K inhibitor. By contrast, IGF-1 could not rescue cells from growth inhibition induced by PD98059. MEK/MAPK blockade also significantly reduced the migratory ability of ES cells, both in basal and IGF-1-induced conditions, and increased chemosensitivity to doxorubicin, a leader drug in the treatment of ES patients.

Hernando et al. analyzed the PI3K-AKT signaling cascade in a cohort of sarcomas and found a new and critical role for the AKT-mTOR pathway in smooth muscle transformation and leiomyosarcoma genesis [35]. PTEN is a dual–function lipid and protein phosphatase originally identified as a protein encoded by a tumor suppressor gene, found to be frequently mutated in sporadic cancers and hereditary disorders. The main substrate of PTEN is phosphatidylinositol-3,4,5-triphosphate (PIP3), a second-messenger molecule generated by the action of PI3Ks. PIP3 activates the serine-threonine kinase AKT, involved in cell survival, proliferation, and growth. Other alterations, such as IGF-1 or IGF-1R overexpression, also frequently observed in leiomyosarcoma [138], could ultimately account for the observed upregulation of this pathway.

The putative aberrant signaling provided by c-*kit* overexpression may be dispensable for Ewing sarcoma development and is unlikely to constitute a critical therapeutic target [139, 140] Baird et al. found highly expressed tyrosine kinases or receptor tyrosine kinases associating with half of the tumor groups [59] Along with known highly expressed kinases, such as *KIT* in gastrointestinal stromal tumor and *PDGFRB* in dermatofibrosarcoma protuberans, they additionally found *JAK1* in Ewing sarcoma, *FLT1* in hemangiopericytoma, *EGFR* and *PDGFRA* in synovial sarcoma, and several *FGFR* in osteosarcoma.

34.6.2 WNT Signaling Pathway

The *WNT* signaling pathway (Fig. 34.3), involved in the development of brain and the peripheral nervous system, has been found to play a critical role in the formation of several cancers [141], including synovial sarcoma [142–144], Ewing sarcoma [145], and malignant fibrous histiocytoma [41]. Wnts activate a canonical pathway that is characterized by accumulation of β-catenin in the cytoplasm. In the absence of Wnt signaling, cytoplasmic b-catenin forms a complex with Axin, adenomatous polyposis coli (APC) gene product, glycogen synthase kinase-3b (GSK-3b), and casein kinase I-a (CKIa). Phosphorylation of β-catenin by CKIa and GSK-3b results in its ubiquitination and rapid degradation [146–148]. Wnt binding to Fz and LRP5 or LRP6 disrupts this degradation machinery by a process that involves Disheveled (Dvl)-dependent recruitment of Axin to the plasma membrane [147]. As a result, unphosphorylated

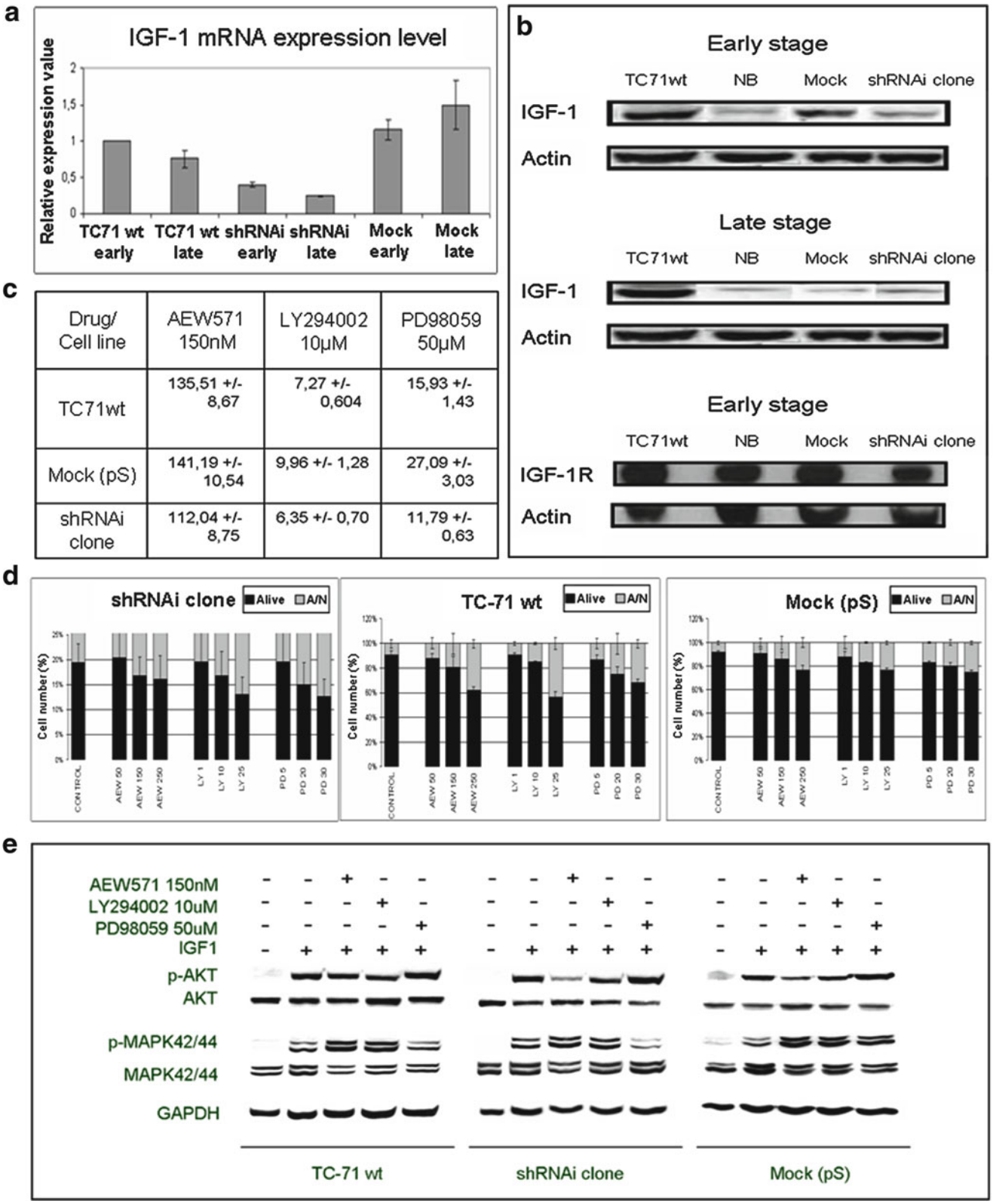

Fig. 34.4 EWS-FLI1 shRNA interference affects IGF1/IGF1R survival pathway and its downstream targets. (**a**) IGF1 mRNA expression level in TC71wt, shRNAi and mock (early and late stages) determined by qRT-PCR. (**b**) IGF1 and IGF1R protein levels as assessed by Western Blot. (**c**) IC50 of proliferation after AEW571, LY294002 or PD98059 treatment at 72 h of incubation measured by MTT assay. shRNAi clone is more sensible to the action of drug and inhibitors. (**d**) Apoptotic index after AEW571, LY294002 or PD98059 treatment at 72 h of incubation cells were seeded on 24 well plates, 72 h after cells were analyzed by FACS for apoptosis using an annexin V detection kit assay. The means ± standard deviations (error bars) of 4 independent experiments are shown. (**e**) Effects of AEW571 combined with inhibitors LY294002 and PD98059 on the activation of IGF1R signaling pathway. All conditions were treated with AEW571 during 15 min and with the inhibitors for 2 h, before IGF1 stimulation (50 ng/ml) during 15 min (serum-free conditions). Data obtained from Herrero et al. 2010. Permission applied [136].

β-catenin accumulates in the cytoplasm and nucleus, forming a complex with members of the TCF/LEF-1 family of transcription factors that activates transcription of target genes such as c-myc and cyclin-D1 [149–151]. Mutations of Axin, APC, and β-catenin have been characterized in numerous human malignancies including colon cancer, malignant melanoma, hepatocellular carcinoma, endometrial carcinoma, ovarian carcinoma, and prostate cancer [152, 153]. A common outcome of these mutations is the accumulation of free β-catenin, mimicking constitutive Wnt activation. Saito et al. showed that APC mutations also occur in sarcomas, especially in synovial sarcoma, and the possible inactivation of the APC gene by missense mutations was thought to contribute to the accumulation of b-catenin in synovial sarcoma [154]. Baird et al. also found evidence implicating this pathway in synovial sarcoma [59]. *WNT5A* expression was pronounced, as was the expression of the *WNT* signaling targets *FZD1, CDH4, EN2, TLE4*, and *TLE1*. *TLE1, WNT5A, FZD1*, and *TLE4* were ranked among the top 50 discriminating genes for the synovial sarcoma, and both *WNT5A* and *FZD1* showed heavy staining on the tissue microarray as well. Uren et al. showed evidence that Wnt/Frizzled signaling is functional in Ewing sarcoma cell lines [145]. They observed a marked stimulation of the β-catenin/canonical Wnt pathway in ES cells treated with Wnt-3a. Wnt-3a induced morphologic changes characterized by the formation of long cytoplasmic extensions in ES cells. β-catenin/canonical Wnt signaling enhanced ES motility, contributing to metastasis, and it could occur through either autocrine or paracrine modes of Wnts since they are expressed in bone, muscle, and soft tissues. They also observed chemotaxis of ES cells in response to Wnt-3a. Both canonical and non-canonical Wnt pathways have been shown to modulate cell motility and tumor metastasis, but non-canonical Wnt pathway is pending of demonstration in ES.

34.6.3 Homeobox and Early Developmental Genes

The homeobox genes are involved in early embryonic development and in the determination of cell fate. There is interest in defining the relationship between deviant expression of these early developmental genes and their role in cancer. Specifically, aberrant homeobox gene expression has been linked to the development of leukemia, testicular cancer, breast carcinoma, as well as several other tumors [155]. Several homeobox genes have been identified in the gene expression profiles of various sarcomas: *MEOX2* in synovial sarcoma [144], *HOXA5* in liposarcoma [60], and *MEOX1* in dermatofibrosarcoma protuberans [156].

34.6.4 Study of Cell Regulation by Proteomics: Proteomic Analysis of Responses to Gene Regulation, Chemicals, Viral Infections, or Therapeutic Treatment in Sarcoma

Alternative proteomics approaches can also combine the disciplines of protein chemistry, molecular biology, and histopathology to paint a portrait of the protein circuitry in diseased cells. It allows the identification of the molecular circuitry of various proteins in a tumor by noting their state of activation (translocation and phosphorylation) and correlative expressions. So far, this approach has been only utilized to depict the mTOR pathway in mesenchymal chondrosarcoma [157].

The combination of 2-DE, MALDI-ToF MS, and bioinformatics offers to the biomedical researcher an opportunity to study the profile of changes in protein levels. Taking advantage of such combination of proteomic tools, and to better understand the mechanisms by which ascochlorin (ASC) regulates physiological or pathological events and induces responses in the pharmacological treatment of cancer, Chang and collaborators performed differential analysis of the proteome of the human osteosarcoma cells U2-OS in response to ASC [158]. In addition, the authors established the first two-dimensional map of the U2-OS proteome. They identified 117 proteins whose expression showed consistent differences in their expression patterns with ASC treatment. Most of the proteins downregulated in U2-OS cells treated with ASC are associated with tumor growth, suggesting that ASC may be useful as a potent clinical suppressor of tumor invasion, a topic of considerable interest in the biological chemistry of chemotherapeutic agents.

In many cases, as seen in the previous examples, sarcoma cell lines are employed as model systems wherein mechanistic molecular schemes are studied. Control of cell-cycle progression, mediation of invasion and migration, evasion of apoptosis, etc. constitute checkpoints that are usually investigated in cell line models of different types of tumors in response to different agents. E2F1, for example, is an essential transcription factor that regulates cell-cycle progression and apoptosis. Overexpression of E2F1 sensitizes neoplastic cells to apoptosis and leads to tumor growth suppression, making it an interesting target for anticancer therapy. Li et al. performed a differential proteome analysis to identify proteins associated with E2F1 activity in inducible p53-deficient Saos-2ERE2F1 osteosarcoma cells [159]. Thirty-three proteins were reproducibly identified, and most of them were the products of genes known to be cancer related. Proteome analysis provided, in this case, new information that may be considered when using E2F1 as a drug.

Using different models of sarcoma xenografts (SK-N-MC and IMR32 (neuroblastoma), RH1 and RH30 (RMS), and KHOS/NP (osteosarcoma), Izbicka et al. evaluated and compared the effects of docetaxel and paclitaxel [160]. The approach used immunoblotting and surface-enhanced laser desorption/ionization (SELDI) MS to assess the drug effects on the expression of the beta-tubulin isotypes and apoptotic markers (Bcl-2, Bax, Bcl-XL). However, the results of this anticancer activity showed no apparent correlation with drug effects on those proteins. The drugs had significantly different, yet highly heterogeneous effects on the tumor levels of the proteins. In contrast, six protein species identified by proteomic profiling were consistently and differentially regulated by docetaxel and paclitaxel in all xenografts.

The mechanisms underlying the clinical outcome of interferon alpha (IFNalpha) treatment of pleomorphic sarcoma (PS) were also investigated using proteomic approaches. A proteomic analysis of 120 signaling components in growth arrested, apoptotic PS cells showed that the relative endogenous expression levels of the IFNAR2 (the IFNalpha/beta receptor) isoforms influence the cytostatic and pro-apoptotic effect of IFNalpha on PS cells [161]. The levels of the receptors may dictate the signaling pathways triggered by the ligand, such as to cause exclusively cell cycle arrest or induce programmed cell death.

The study of ES has provided, as well, a few examples of how proteomics may deepen our understanding about the mechanisms regulating the response to different treatments. The disease expresses several deregulated autocrine loops mediating cell survival and proliferation that contribute to its pathogenesis. Insulin-like growth factor I receptor (IGF1R) and KIT are transmembrane receptors that mediate two of these loops [139, 162], and are therefore directly involved in the growth and survival properties of ES [163–165]. Their blockade is a promising therapeutic approach for this neoplasm. Martins et al. reported the in vitro impact of IGF1R/KIT pathway blockade on ES cell lines [166] and afterwards they extended their observations to the level of proteomic changes induced by this treatment, to find and validate new possible therapeutic targets. Two-dimensional PAGE analysis and MALDI-ToF studies of ES cell lines treated with ADW742 and/or Imatinib (specific IGF1R/KIT inhibitors) revealed a large panel of differentially expressed proteins, some of them related to stress-induced response. Among them, the changes in protein expression between cell lines sensitive and resistant to IGF1R/KIT inhibitors were particularly significant in the case of HSP90 [167].

Response to stress is a key mechanism conditioning drug sensitivity. Stress-protective HSP are often overexpressed in neoplastic tissues and cancer cell lines [168–170], and therefore HSP inhibition might become a new therapeutic strategy to inhibit multiple receptor pathways. The authors illustrated that inhibition of HSP90 with 17-AAG (a specific HSP90 inhibitor) and siRNA reduced ES cell line growth and survival, especially in cell lines that previously showed resistance to IGF1R/KIT pathway blockade. 17-AAG treatment induced HSP90 client protein degradation, including AKT, c-KIT or IGF1R, by inhibiting their physical interaction with HSP90. Animal models confirmed that HSP90 inhibition, alone or combined with IGF1R inhibition, significantly reduced tumor growth and expression of client proteins. The authors postulate, for the first time in ES, that in addition to the levels of expression and basal activation of IGF1R/KIT, the development level of the stress response mechanism is another important determinant of sensitivity to ADW/IMA in ES cell lines. Importantly, targeting HSP90 function might be of therapeutic value in ES, especially in cases of previous resistance to IGF1R/KIT pathway blockade.

In the same sense, another study has recently confirmed the susceptibility of targeting Heat Shock Proteins (HSP) as a potential anticancer therapy in ES and neuroblastoma (NB) [171]. In the study, to perform a comprehensive evaluation of the HSP response, the authors firstly identified the HSP expressed in ES and NB cell lines by a sensitive proteomic profiling. Cell lysates were analyzed by a combination of 1-D PAGE and automated nano high-performance-liquid-chromatography coupled with electrospray quadrupole time-of-flight (nano HPLC-ESI-Q-TOF). Then they studied the response to doxorubicin (an antibiotic used as an anticancer drug) mediated by HSP on the cell lines. As previously described, quercetin was shown to inhibit HSP at transcriptional level [172, 173]. The effect of quercetin caused a potent doxorubicin sensitization in NB and, to a lesser extent, in ES cells. In the experiments, quercetin exerted an intense pro-apoptotic effect and increased significantly the pro-apoptotic effect of doxorubicin. These results were mediated by HSP in all cases, and even to an extent in which, interestingly, quercetin sensitizing effect was higher in cells with higher HSP levels. The sensitization to doxorubicin found in NB and ES cell lines might be of potential clinical interest because maximal quercetin effect was observed at quercetin concentrations that could be reached in plasma following intravenous administration without serious side effects.

34.7 Applications: Molecular Diagnosis of Translocation

34.7.1 Techniques for Detecting Translocations

Quantitative RT-PCR is a common technique in our clinical setting, especially when we have frozen samples in stock. cDNA is used as template and primers exon sequences flank

the rupture points of translocations. Amplified products are resolved in an agarose gel. Optionally, DNA product in agarose gel may be transferred onto a nylon membrane and incubated with DNA complementary probes. Some authors suggest the use of quantitative RT-PCR for diagnosis [174].

Rupture points in translocations usually take place in certain introns. However, the site within the intron where the rupture takes place is very variable. As a result the fusion structure at genomic DNA level is less predictable than that at RNA level, where it has a more constant number of exons from each gene involved in the fusion. This is the reason why RNA is the material of choice for detecting translocations in sarcomas using RT-PCR. We therefore insist on the creation and maintenance of tumor banks, as frozen material is the best source of good quality RNA.

Other diagnostic methods include *conventional cytogenetics* and fluorescent *in situ* hybridization (FISH). Specific probes flanking rupture points in translocations *(fusion signal probes)* are marked with fluorescent molecules. At least two probes are necessary for detecting sequences, one for each gene involved. Each probe is marked with a different fluorochrome. We recently developed a FISH probe to detect EWS-FLI translocations which allow us to show that MSC-P does not show the chimeric rearrangement [10]. Other times, probes are designed for detecting any gene rearranging in the fusion *(split signal probe)*. The advantage of the FISH method is that it can yield trustful results even when the amount of available tissue is scarce or when there is only paraffin-embedded material left, and it can be used for tissue microarrays [175], or it can even be used in tumor tissue samples. The rub is that a fluorescent microscope is necessary, and this makes the FISH method a technique difficult to be integrated in a small pathology lab. The CISH technique, wherein immunofluorescence is substituted by a chromogen (similar to those employed in immunohistochemistry), is employed in diagnostic routine for detection of gene amplifications (e.g., HER2 in breast carcinoma) but at least with current technology has a poor use for detecting translocations [175]. In the future, this technique could be used for detecting translocations in sarcomas if it were suitably improved.

Very differently to what takes place with lymphomas, a few types of proteins can be detected with *immunohistochemistry*. The alveolar sarcoma in the soft tissues is a good example of it; it shows a t(X;17)(p11;q25) translocation and a TFE3-ASPL fusion. An effect derived from fusion is the TFE3 overexpression. This can be detected by over-the-counter monoclonal antibodies, and represents a practically specific finding which is shared by some kidney carcinomas in children [176]. Another example is seen in the desmoplastic tumor of small round cell tumor, wherein a fusion is detected; it includes the EWS amino-terminal end and WT1 carboxy-terminal. Immunohistochemistry can be accomplished by antibodies directed to WT1 amino domain and carboxy-terminal [177]. The fusion is detected thanks to the immunoreactivity of the latter and the absence of it within the first domain. Similarly, NPM-ALK fusion can be detected in 60 % of inflammatory myofibroblastic tumors; the overexpression of ALK1 carboxyl domain is detected thanks to a specific antibody [178], while in other similar morphological lesions (nodular fasciitis, fibromatosis) no immunoreactivity is found for ALK1. Last example is the atypical lipomatous tumor/well-differentiated liposarcoma (ALT-WDLPS) and dedifferentiated liposarcoma (DDLPS) which may be difficult to distinguish from benign adipose tumors and from poorly differentiated sarcomas, respectively. Genetically, they are characterized by amplification of *MDM2* and *CDK4* genes on chromosome 12q13-15. MDM2 and CDK4 immunostainings, which correlate with gene amplification, are helpful adjuncts to differentiate ALT-WDLPS from benign adipose tumors and to separate DDLPS from poorly differentiated sarcomas [179]. Although in practice it is hard to have specific antibodies for each epitope of interest, this system could be employed in some other translocations that are present in sarcomas.

34.7.2 Detection of Translocation in Clinical Material

Despite the fact that sometimes we have to rely on a small or morphologically distorted biopsy, a great advantage of molecular pathology techniques is the fact they provide material fixed in formaldehyde and embedded in paraffin [180]. RT-PCR can detect them in roughly 50 % of cases, depending on the author's experience. RNA is extracted with a longer proteolytic treatment than usual and employing primers that delimit a very short PCR product. For instance, the French Sarcoma Group [181] proved, through a study that includes the detection of SYT-SSX fusions, characteristic of the synovial sarcoma in 250 tumors fixed with several usual fixatives and embedded in paraffin, that the technique's sensitivity is over 95 % (as long as tissues are not fixed with Bouin) and specificity reaches 100 %. FISH output is, at least, similar to that of RT-PCR with regard to paraffin-embedded material [180].

As for cytologic material, not only extensions but also material obtained through fine needle aspiration cytology is a good source of high-quality RNA for cytogenetic and molecular studies [79, 80, 182]. Routinely, once the diagnosis is certain, we carry out an additional sample for molecular studies. RT-PCR and FISH are especially easy applicable techniques for cytological samples from mesenchymal tumor origin.

34.7.3 Should Molecular Techniques to Detect Translocations Always Be Carried Out?

It is obvious that anatomopathological diagnosis, in most sarcomas, is carried out with many difficulties when it is represented under its usual clinical-pathological context (age, localization, etc). To answer this question, we would like to make a distinction between essential and convenient indications. The following are essential ones:

- The appearance of an unusual morphological variant (e.g., a synovial sarcoma, poorly differentiated, which appears in the usual place and age for a 27-year-old male, close to knee joint; a tumor similar in morphology to an adamantinoma on the frontal tibia side, but having areas with round cells, may suggest the differential diagnosis with a ES).
- The appearance of a sarcoma with a usual morphological aspect, but at an unusual age. It is applicable to round cell or spindle-shaped sarcomas, having translocations that are characteristic in patients over 40 years (Fig. 34.5).
- The appearance of a sarcoma with a usual morphologic aspect, but in an anomalous localization (e.g., a cutaneous ES, vesical or renal; they may be mistaken for small cell/neuroendocrine carcinomas).
- Differentiation between a sarcoma and some other tumor which may simulate it, the latter not being a sarcoma. The most usual example in my experience is that regarding a fusocellular tumor in the costal ribs which suggests a differential diagnosis between synovial sarcoma in pleura and pleural mesothelioma or a malignant solitary fibrous tumor; or the differential diagnosis between a melanoma and a clear cell sarcoma of soft tissues.
- Detection of point mutations in genes is key for cellular signaling. The best example at present is detecting the presence and exonic localization of c-kit mutations for the differential diagnosis of GIST and diagnosis of their secondary resistances to imatinib/sunitinib.

When in previous situations it was *necessary* to resort to molecular techniques to be able to confirm a diagnosis, perhaps it may be convenient, from a dimension more bound to research, to carry out molecular assessments in as many cases as possible. This must be specially taken into account with regard to the design of clinical trials, wherein molecular data may become inclusion criteria for patients or in the case of tumor banks whose value is proportional to the quantity and quality of clinical and molecular data attached to each sample.

34.8 Minimal Residual Disease Detection

RT-PCR is extremely specific and sensitive; it permits the detection of very low levels of tumor cells, even if they are mixed with a high number of healthy cells, as in the case of bone marrow samples, or peripheral blood. Therefore, it is a robust method for carrying out a molecular staging, or monitoring the response to treatment. Determining the presence of cells with chimeric transcripts by RT-PCR has an unquestionable potential in clinical applications, as tumor staging is the variable having more prognostic value in sarcomas. Presumably some patients with localized disease may have an unfavorable prognosis due to a minimal metastatic disease presence, undetectable by conventional methods. There are, for instance, several groups who report that the detection

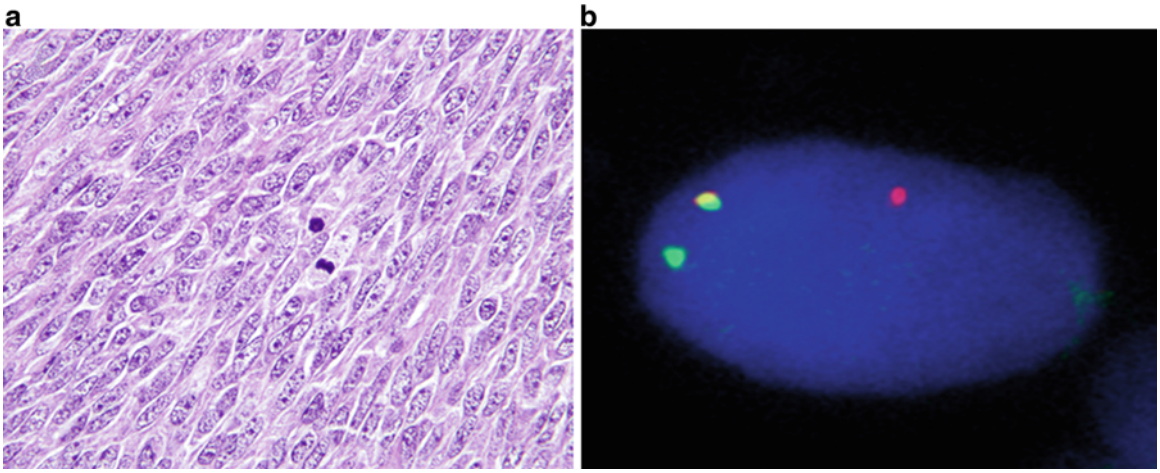

Fig. 34.5 Molecular pathology applied to diagnosis. This case corresponds to a 72 year-old woman with a solitary nodule in the lung, with a wide involvement of the pleura. Tumor morphology shows (**a**) a spindle cell malignant tumor; differential diagnosis in this location included a synovial sarcoma and a sarcomatoid mesothelioma. Demonstration of SYT rearrangement by FISH (**b**), characteristic of synovial sarcoma, seen as a splitting of one pair of green-red signals, was useful to confirm the diagnosis of synovial sarcoma.

of cells having EWS-FLI1 or EWS-ERG fusions in peripheral blood or bone marrow in patients with localized disease is associated to tumor progression or systemic relapses of Ewing tumor [79, 80, 183]. Similar results have been observed by detecting PAX3-FKHR fusions, which are characteristic of alveolar RMS [184]. However, there are still a large number of methodological and clinical questions to resolve in order to know what sort of prognostic and therapeutic implications entail the detection of any circulating disease through RT-PCR.

34.9 Translocations as Therapeutic Targets

From now on, determining the molecular mechanisms involved in the genesis of the different sarcomas may have very important consequences at the time of improving treatments for patients who suffer from these neoplasms. The point is that new drugs can be designed for acting specifically on some of these gene alterations. As we have expressed previously, some chimeric proteins have kinase tyrosine activity; some of them may respond to imatinib (Gleevec). For example, some clinical studies on the treatment of dermatofibrosarcoma protuberans with imatinib have been published and they show an excellent response rate [185]. Let us not forget this tumor overexpresses a tyrosine kinase activity, and imatinib shows an intense action against it [186]. Some other chimeric proteins will probably benefit from new drugs, as shown in Table 34.2. Lastly, in the case of chimeric proteins (mostly in sarcomas) as transcription factors, the RNA interference phenomenon has been put into practice experimentally on, for instance, some cellular lines in Ewing tumor with EWS-FLI1 fusion; a diminution in both, the transcription and translation of the fusion, as well as a marked modification of the tumorigenic capacity of tumor cells, both in cellular and animal models, was observed (Fig. 34.4) [124, 187]. Interestingly, some recent reports have shown some drugs with certain specificity towards EWS-FLI inducing tumor cell death [188]. These results are very stimulating as they open the door to new therapeutic possibilities, although it will not be easy to transfer this model to clinical trials. Besides, it is believed this experimental technique, together with opposite experiments, namely, studies on inducible expression of fusions [189] will be useful for the research of target genes in chimeric proteins; these genes, in turn, could become therapeutic targets.

Table 34.2 Gene fusions that trigger tyrosine kinase activity

Tumor	Protein	Imatinib treatable?
Dermatofibrosarcoma protuberans	COL1A1-PDGFB[a]	Yes
Infantile fibrosarcoma	ETV6-NTRK3	No
Inflammatory myofibroblastic tumor	TMP3/TMP4/ CLTC2-ALK	No

[a]Through PDGFR

34.10 Sarcoma Classification

34.10.1 Contribution of Translocations

(a) At present, we accept that both Ewing sarcoma and peripheral primitive neuroectodermal tumor of bone and soft tissues (pPNT) are the same entity, having a variable degree of neural differentiation, due fundamentally to the fact that they share an equal function (for instance, EWS-FLI1, to mention the most frequent one) [190].
(b) TLS-CHOP fusions are present both in round cell liposarcomas and myxoid liposarcoma; this confirms it is the same entity, something we suspected, as tumors having areas with both patterns had been previously reported.
(c) Most extraskeletal myxoid chondrosarcomas have EWS-CHN fusion, which is absent in bone myxoid chondrosarcomas. This fact, together with some clinical–pathological differences, suggests they are diverse entities.
(d) Some undifferentiated RMS, having a solid growing pattern, and liable to be diagnosed as embryonary, turn up in localizations and age groups typical for alveolar RMS. They also have PAX3-FKHR fusion, very typical of the latter, and represent the solid variant of alveolar RMS.
(e) DSRCT is not only peculiar for its clinical presentation in the form of peritoneal nodules in male adolescents, and for its divergent immunophenotype, but also for its characteristic gene fusion EWS-WT1, which was actually reported simultaneously to the tumor entity.

34.10.2 Genomics Contribution: Application of Multiple Expression Techniques

Even though genomic and proteomic mass analysis techniques may be employed for sifting mutations or detecting polymorphisms, the study of gene expression profiles is the most straightforward application in the field of gene expression. Although all cells have the same genes, only 5 % of them are expressed in a particular cell at a particular moment. Therefore, the study of gene expression through multiple expression techniques yields a privileged piece of information on the function and state of a healthy tumor cell. Whatever the technology employed in the multiple expression studies (DNA or oligonucleotides arrays), it is advisable to mention the result that has been obtained consists in the relative level of expression of a problem sample with regard to that of control. That is to say, it represents quantitative

changes in the gene of the problem sample between two different samples. These differences may reach several hundred-fold. The great amount of data obtained calls for the application of a bioinformatics technique in an attempt to group all studied problem samples with regard to their similarity in expression patterns. There are two systems of grouping, called supervised or non-supervised. In supervised grouping systems some previous knowledge of the analyzed objects is taken into account (e.g., anatomopathological diagnosis) for carrying out a comparison between two tumor groups differing in a specific characteristic (e.g., liposarcoma versus leiomyosarcoma, good response versus bad response). On the other hand, non-supervised analysis is carried out without paying attention to any previous knowledge in order to avoid any bias in the classification [191].

There are few studies employing this technology in sarcomas. Nielsen and col., for instance, employed various types of cDNA microarrays to analyze 46 samples from all types of sarcomas [60]. First carrying out a non-supervised study they found five large groups of genes that express coordinately. Three of these groups correspond to GIST, synovial sarcomas, and schwannomas, the fourth group represented leiomyosarcomas, and the fifth one represented a group of tumors that included all malignant fibrous histiocytomas, pleomorphic liposarcomas, and a second group of leiomyosarcomas. It is comforting, and not surprising, that a non-supervised analysis comprising tens of thousands of genes reproduced quite well the classification of sarcomas pathologists have been using for the last 100 years; nevertheless, this analysis represents certain refinement in the classification of sarcomas. As an example, malignant fibrous histiocytoma is not an entity itself; it represents a group of high-grade sarcomas including liposarcomas and pleomorphic leiomyosarcomas. Secondly, Nielsen et al. carried out a supervised analysis (that is to say having the diagnosis in mind) to see what genes make one type of tumor different from another. In this study, they saw some genes codifying proteins already known and employed for differential diagnosis in sarcomas through histochemistry (e.g., Kit for GIST, or S-100 for schwannomas), but also tens of genes with differential expression, unknown until now, that could be employed from now on in the differential diagnosis [60].

Some studies are rather focused on specific groups of tumors, as in the case of a study that supports the classification of clear cell sarcomas in soft tissues as a subtype of melanoma thanks to its expression profiles [192]. However, some other studies yield contradictory results, for instance, with regard to differentiation between synovial sarcoma and malignant peripheral nerve sheath tumor (malignant schwannoma). While a study by Nielsen and col., previously mentioned, sets them apart, an article appeared almost simultaneously showing them to be associated in the same group. A number of methodological aspects can be in the origin of this situation.

A new group of studies seems to be more useful and practical. For instance, a new study carried out by a British group [193] is capable of finding a few genes whose expression levels arrange, at the diagnostic instant, leiomyosarcoma patients into two groups, showing a clearly different probability of producing metastasis during the course of the disease. A study including 29 RMS (alveolar and embryonary), well characterized from the cytogenetic point of view, finds a group of genes that differentiate both subtypes, and, best of all, finds a new type of translocation when a patient showing no known translocations and included in the alveolar RMS group was re-studied [194]. A profound study on the genomic profile of extraskeletal myxoid chondrosarcomas points out this tumor has a high PPARG expression; there are specific inhibitors against it and it could become a therapeutic target [89]. A complete expression study was published on 181 sarcomas, with the help of cDNA arrays, wherein some important molecular targets are identified in some tumor subtypes [59]. The authors have made a public database available on the Internet (http://Watson.nhgri.gov/sarcoma).

Multiple expression techniques must be regarded as a potentially useful complement to the usual diagnostic techniques [195]. The aim of microarray technology is not based on classifying tumors that can be classified by simpler, more economical, more conventional methods, but to get, on a genomic level, markers yielding new information on a prognostic or therapeutic basis [196]. It does not seem probable, in the short or medium term, that conventional techniques will be substituted by new molecular pathology techniques.

Contradictions shown in the genomic studies, previously mentioned, reflect the limitations these techniques show at present. They are very expensive, available in few centers and reproducibility is affected by the type of *arrays* or the sampling carried out in the tumor samples. An interesting study published recently [197] makes us reflect on the effect sampling has on the reproducibility of microarray studies. It is worth noting that the pathologist's role is to portray sarcomas (and other types of tumors) as heterogeneous entities with regard to their morphology and molecular characteristics, and therefore it is crucial, for the quality of the experiment, to carefully select the tumor areas to be studied. On the other hand, it is worth bearing in mind that results of multiple expression studies are not the objective themselves, and must be validated by RT-PCR, or immunohistochemistry on tissue section.

Bearing that in mind, it is expected that high output techniques, both genomic and proteomic, which allow a massive study of human tumor samples, will have a profound impact on the classification of sarcomas, and they will provide new diagnostic and response markers over drugs. However, we must have in stock a series of easy-to-implement tools for the clinical sphere; that is to say, its information must be easily integrated with that generated by platforms already existing.

Current platforms, for instance, are not directly applicable to the daily diagnostic routine of a National Health System such as ours. The introduction of proteomics and genomics in the clinical field will probably be carried out in the short run through the design and validation of markers that may employ the existing technological platforms in this medium, such as immunochemistry, FISH or flow cytometry, and only in the medium run through the design and validation of new genomic and or proteomic tools. In other words, it is predictable, for instance, that most markers that are derived from studies of microarrays will be adapted through immunohistochemistry to the clinical-pathological context by the development of new antibodies that may be used in paraffin-embedded material. A good example of this is the finding and subsequent validation of two new markers that are very useful in the clinical management of GIST. This was thanks to genomics and immunohistochemistry studies carried out in large series of patients. These markers are DOG1 [198] and protein kinase C theta (PKC) [199]; they are not only markers, and specific to a greater extent, but also probable therapeutic targets.

34.10.3 Proteomic Profiling and Classification of Sarcomas

Proteomics is the large-scale study of proteins, particularly their expression levels, structures, and functions. It can be defined as the study of the proteome, the complete set of proteins produced by a species, using the technologies of large-scale protein separation and identification. The term proteomics was coined in 1994 by Marc Wilkins, who defined it as "…*the study of proteins, how they're modified, when and where they're expressed, how they're involved in metabolic pathways and how they interact with one another*…" [200]. In oncological research, proteomics is an emerging technology that can evaluate normal and abnormal protein expression in tissues. To date, tissue profiling has been successfully utilized to detect proteins specific to almost any type of cancer.

For instance, Suehara et al. performed a global protein expression study on soft-tissue sarcoma (STS) in order to develop novel diagnostic biomarkers and allow molecular classification [201]. Two-dimensional electrophoresis was used to generate the global protein expression profiles of 80 soft-tissue sarcoma samples with seven different histological backgrounds. They found that 67 protein spots distinguished the subtypes of soft-tissue sarcoma. Among them, the expression pattern of a reduced group of proteins could discriminate certain types of sarcomas into low-risk and high-risk groups, which differed significantly with respect to survival. These results open the road in proteomics to develop novel biomarkers for diagnosis and molecular classification of soft-tissue sarcomas. Identification of proteins associated with survival in specific cases of sarcomas will allow delineation of a high-risk group that may benefit from adjuvant therapy leading to likely clinical benefit.

In a similar approach, Holt et al. described tissue profiling by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) as a tool for orthopedic research [202]. The usefulness of proteomic over histopathological analyses to predict STS behavior is reported to be based in its ability to objectively predict tumor grade and behavior. They detected differentially expressed proteins in high-grade and low-grade STS.

Recently, a differential approach was used to highlight proteomic changes between human osteosarcoma cells and mature osteoblasts [203]. Changes in expression profiles for 17 proteins were ascertained in human mature osteoblasts compared to pre-osteoblasts (differentiation markers). Results also showed a relative over-expression of 8 proteins (proliferation and tumor indicators), as well as under-expression of proteins also found downregulated in pre-osteoblasts (specific markers of osteoblast differentiation). In particular, three markers reported in the article exhibited features potentially related to the osteosarcoma metastatic potential. These findings also confirmed the differences between cell lines and primary human cell cultures.

34.11 Mouse Models of Sarcoma

Roughly, mouse animal models of sarcomas can be divided into two: the xenograft models and genetically engineered mouse models (GEM). Xenograft models are developed by implantation of tissue of human (xenogeneic or xenograft model) or mice origin (syngeneic model) mainly into immunocompetent mice. These models have been used mainly to test the effects of new drugs [160, 204, 205]. Such is the case of the Pediatric Preclinical Testing Program supported by the National Cancer Institute (NCI), aimed to identify new drugs active against childhood cancers, which makes use of a panel of 61 childhood tumor xenografts [205]. Xenograft models have been used also to gain knowledge about the pathogenesis and histogenesis of ES and to generate a biobank of sarcoma samples [2]. However, despite of the great amount of information that these models provide, they have some limitations. For instance, normal architecture present in the patient tumor specimens is altered in xenografts and the genetic heterogeneity is diminished as a result of the selective pressure of cell culture or tissue explantation. Moreover, in these models is not possible to assess accurately the metastasis formation, which is a key issue in the survival of patients. Therefore, the data obtained by using these models should be taken into account carefully, especially when a drug is checked [206].

Development of GEM has allowed gaining insight into the origin cell of sarcomas (Table 34.3). Several strategies

Table 34.3 GEM of fusion genes of sarcomas[a]

Model	Fusion	Promoters	Expression place	Phenotype	Reference
– Conditional transgenic (Cre-loxP) – Deletion of p53	EWS-FLI1	Prx1 (Cre mouse)	Primitive mesenchymal tissues of the embryonic limb bud	Poorly differentiated sarcomas	Lin et al. [214]
Conditional knock-in (Cre-loxP)	SYT-SSX2	– Rosa26 (loxP mice) – Myf5 (Cre mouse) – Myf6 (Cre mouse) – PAX-7; PAX3 (Cre mouse)	Myoblast Myocyte, myofiber Progenitors cells	Sinovial Myopathy Lethality	Haldar et al. [57]
Conditional knock-in (Cre-loxP)	EWS-FLI1	Rosa26 (loxP mice) MX1-cre	Bone marrow Liver, spleen, and hematopoietic tissues	Myeloid/erythroid leukemia	Torchia et al. [207]
Invertor	EWS-ERG	EWS endogenous (loxP mice) Rag1 (Cre mouse)	Lymphocyte	Leukemia	Forster et al. [210]
Conditional knock-in (Cre-loxP)	PAX3-FKHR	Pax7 (Cre mouse)	Progenitor cells (satellite)	No tumor	Keller et al. [49]
– Conditional knock-in (Cre-loxP) – Knock-out Trp53 or Ink4/ARF	PAX3-FKHR	Myf6 (Cre mouse)	Myofiber	No tumor Rhabdomyosarcoma	Keller et al. [48]
Transgenic	FUS-CHOP	EF1-α	All tissues	Liposarcoma	Perez losada et al. [208, 282]

[a]Table obtained from Ordonez et al. [2]

have been used to develop genetic engineered models of tumors showing translocations as sarcomas and hematological malignancies. These models can be generated by injection of cDNA into fertilized oocytes (transgenic mouse) or through gene targeting in embryonic stem cells. The embryo lethality as a consequence of the expression of fusion genes is the main problem to overcome [57, 207].

Some transgenic mice have been engineered to induce the expression of fusion genes in a nonphysiological manner under the control of exogenous promoters or enhancer elements [206, 208]. In its simpler version transgenic mouse are developed by pronuclear injection of gene-fusion cDNA constructs under the control of exogenous both ubiquitous or tissue specific promoters.

Another big group of models expresses the fusion genes from their native promoters through knock-in technology. Some of them make use of specific recombinases as Cre allowing a spatiotemporal control of the chimeric gene expression. These models include the knock-in model, the conditional knock-in model, the translocator model, and the inverter model [209–212]. These models use homologous recombination in embryonic stem cells (ESC) to induce gene fusions and transfer them into the mouse germ line. The knock-in strategy approach of creating fusion genes by homologous recombination in ESC was initially tested in the development of hematological malignancies [209]. However, the knock-in model has two important limitations as the embryonic lethality usually conferred by the fusion genes and the difficulty of obtain cell-specific effects. These problems can be solved by generating conditional mouse models using site specific recombinases that allow a spatiotemporal control of the chimeric fusion gene. The P1 bacteriophage Cre-Lox system is the most widely use. The Cre-recombinase mediates the recombination between loxP sites, a pair of inverted repeat DNA elements. Depending on the orientation and location of loxP sites, recombination mediates by Cre induces deletion, inversion, or translocation of the sequence of interest. To generate a Cre-lox mice expressing the fusion gene is necessary to engineered two transgenic mouse lines, a cre mouse, and a loxP mouse. The cre mouse harbors the Cre-recombinase gene under the control of a tissue specific promoter. The loxP mouse can be engineered to harbor the chimeric fusion gene and a stop cassette upstream of the fusion place or gene flanking by loxP sites in the same orientation. After breeding the two mouse lines, expression of Cre in the Cre-lox mice induces the excision of the stop cassette and subsequently expression of the fusion gene.

34.11.1 Ewing Sarcoma

Until 2001 only a few studies with models of ES, mainly xenograft had been developed [213]. At present, there have been also reported several GEM models harboring ES gene fusions but they did not show the expected ES phenotype [207, 214].

Xenograft models of ES were developed in mice implanted with murine primary bone-derived cells and mesenchymal

progenitor cells expressing EWS-FLI1 to gain knowledge about the origin of ES [22, 215]. Several ES metastatic models were also developed by injecting ES tumor cells through the tail vein of immunocompetent mice. These models resemble human ES metastatic pattern, including skeletal and extraskeletal locations [187, 216]. Recently, a different strategy to induce metastasis formation was reported. Gonzalez et al. injected cells transduced with the EWS-FLI fusion types 1, 2, and 3 into the left cardiac ventricle of athymic mice. The aim of this approach was to overcome the limitation impose by the fact that lung metastasis formation after injection of cells via tail vein could not reflect necessarily metastatic activity as lungs bear the first capillary bed that cells face after tail vein injection [217]. This model is useful to test the effects of drugs and new systems in inhibiting the tumor growth.

At present, a GEM of ES has not been developed and even there was no report indicating any attempt to achieve that model until recently [207]. The embryo lethality as a result of the expression of the fusion genes characteristic for ES is probably the main explanation to the no existence of a transgenic model to date [207, 210]. *Torchia* et al. bypassed the embryo lethality induced by EWS-FLI1 and published the first GEM of EWS-FLI1 [207]. They generated a GEM of EWS-FLI1 by using a knock-in conditional approach, using a loxP-flanked (floxed) transcription stop (loxP-STOP-loxP). The cre mouse was engineered to produce a Cre-inducible recombinase under the promoter Mx1 after administration of α/β interferon (pIpC). However, EWS-FLI expression after Cre induction after a single administration of pIpC did not generate ES tumors but instead myeloid/erythroid leukemia and provoked the death of the animals about 15 days after administration. Moreover, a group of animals that were not pIpC administered developed a similar pathology as pIpC-treated mice indicating a basal expression of Cre and consequently of EWS-FLI1 [207]. More recently, a conditional transgenic GEM was created by inducing type-1 EWS-FLI1 expression in the primitive MSC of the embryonic limb buds, making use of specific recombinases (Cre-lox system), which allow for a spatial-temporal control of the chimeric gene. However, it did not induce tumor formation, but did elicit several limb defects. Animals developed poorly differentiated sarcomas (but not ES) only in the setting of p53 deletion [214].

A different strategy was used by the Professor Rabbitts´ group, who generated T-cell lymphomas by conditional expression in haematopoietic cells of the EWS-ERG, a characteristic ES fusion gene. This goal was achieved by making use of the inverter model, (a new version of *knock-in* strategy using loxP sites) in which the target gene is orientated in the reverse direction for transcription [210]. Recently, Torres et al. using a different approached named as, RNA-guided CRISPR-Cas9 system, generated de novo EWS-FLI translocations in vitro [218].

34.11.2 Osteosarcoma

Various syngeneic and xenogeneic models of osteosarcoma have been developed. A syngeneic orthotopic model of primary osteosarcoma and pulmonary metastases was developed by implantation of tumor pieces of mouse osteosarcoma into the tibia of nude mice. These tumor pieces derived from two mouse osteosarcoma cell lines (K7M2 and K12) which showed different aggressive behavior regarding the metastatic potential [219]. The histology of these tumors was very close to the human counterpart. Another xenogeneic model of primary osteosarcoma and pulmonary metastatic lesions resembling the human lessons was developed by orthotopic implantation of a rat osteosarcoma cell line name UMR 106 into the proximal tibia of athymic mice [220]. This model has been used to study the role of urokinase plasminogen activator system (uPA) and its receptor (uPAR) showing that downregulation of uPAR expression provokes reduction in tumor growth and metastases [220, 221]. Another metastatic murine model established by using LM8 murine osteosarcoma cells which metastasizes to lung after subcutaneous implantation, has been used recently to test the efficacy of new anti-osteosarcoma treatments as gemcitabine an interleukin-18 [222, 223]. Xenograft models of osteosarcoma by using human cell lines (U2OS, SaOS-2, HOS, KRIB, and 143b) have also been reported. U2OS model of primary and metastatic disease was developed by subcutaneous and intravascular injection of cells, respectively. In this study it was assessed the anti-angiogenic effect of transfecting the alkaline phosphatase gene [224]. However, at present it has not been reported a study of testing drugs using this model. Tail vein injection of SaOS-2 cells and derived cells such as SAOS-LM6, SAOS-LM7 in athymic mice has allowed establishing other metastatic model of human sarcoma. This model was used to study the effects of new anti-metastatic drugs such as adriamycin, interleukin 12, gemcitabine [225–227]. More recently an orthotopic murine model of primary and metastatic osteosarcoma has been induced by implanting the non-transformed SaOS-2 cell line into the tibia, probing be useful in testing the anti-tumor effects of pigment epithelium-derived factor (PEDF) [228–230]. Other models have been established by using derivatives of the human osteosarcoma cell line HOS, such as KRIB, MNNG/HOS, and 143B, created by using several transforming agents as v-Ki-Ras, N-methyl-N´-nitro-N-nitroguanidine, and Ki-Ras, respectively [231]. Orthotopic intratibial inoculation of KIRB cells induced primary and secondary lesions in lungs in 100 % of mice. As review by Dass et al. other models derived from the HOS cell line derivatives has been used to test the effects of new treatments [231]. A study published recently showed a different tumorigenicity potential of HOS, MNNG/HOS, and 143B cell lines after orthotopic intratibial administration in mice. HOS mice did not develop tumors

showing the lowest grade of tumorigenicity while 143B mice develop primary a metastatic lesions showing the higher grade. The use of the three cell lines was proposed as a model to study the osteosarcoma biology [232]. Nevertheless, these models have the great inconvenient that after transformation several signaling pathways are altered and therefore do not longer mimic the human disease.

34.11.3 Liposarcoma

Some xenografts models of liposarcoma have been developed to study the biology of tumor, the efficacy of new drugs and the histogenesis [38, 233]. Perez Losada et al. generated a transgenic GEM of liposarcoma by pronuclear injection into fertilized eggs of a cDNA construct including the human FUS-CHOP gene under the control of the elongation factor 1α promoter (EF-1α). Despite the use of a ubiquitous promoter, animals developed specifically liposarcomas and the phenotype of these tumors was very close to the human phenotype, mimicking the chromosomal translocation effects [208]. Moreover, induction of FUS-CHOP seemed not to be lethal for the mice as compare with other sarcoma fusion genes such as EWS-FLI1, EWS-ERG, SYT-SSX, Pax3-Fkhr [57, 207, 208, 210].

34.11.4 Synovial Sarcoma

Haldar and col. developed a model of synovial sarcoma by inducing the expression of SYT-SSX2 fusion gene in myoblast. When the expression of the chimeric gene occurred in other cells of skeletal muscle lineage animals not developed synovial sarcoma phenotype but myopathy or embryonic lethality. They used a conditional *knock-in* strategy in which two transgenic mouse lines (loxP mouse and Cre mouse) were generated. The loxP mouse was engineered to express SYT-SSX2 from the endogenous Rosa26 promoter on chromosome 6. Between the promoter and the SYT-SSX2 cDNA there was a transcriptional termination signal (Neo-PA) flanked by loxP sites. In absence of Cre, SYT-SSX2 is not transcribed but in the presence of Cre, Neo-PA is excised and the transcription starts. The Cre mouse was engineered to induce Cre in those myoblasts expressing Myf5. This is a myogenic regulatory factor playing a crucial role in the specification of skeletal muscle lineage and whose expression starts early in embryogenesis. They also investigated the effects of expression of SYT-SSX2 in other skeletal muscle cells, which were less or more undifferentiated than Myf5 expressing myoblasts. When the chimeric protein was induced in more undifferentiated cells such as myocytes and myofibers expressing the Myf6 myogenic regulatory factor, mice developed myopathy. When SYT-SSX2 was induced in less differentiated cells such as Progenitors expressing Pax3 or Pax7 mice suffer embryonic lethality [57]. All these findings indicate the relevance of correct selection of the target cell line in which the fusion gene should be expressed. Recently, the same investigators, using a tamoxifen-inducible CreER strategy, suggested a possible non-myoblast origin for synovial sarcoma as well [58].

34.11.5 Alveolar Rhabdomyosarcoma

A conditional knock-in strategy was used to generate an alveolar RMS model, by inducing the expression of PAX3-FKHR gene in muscle skeletal cells by using a Myf6-Cre mouse line [48]. However, the only expression of PAX3-FKHR did not produce an alveolar RMS phenotype and additional inactivation of the Ink4a/Arf and Trp53 pathways was necessary [48]. Interestingly, expression of PAX3-FKHR early in development in muscle satellite cells by using a Pax-7-Cre did not trigger RMS [49, 234].

On the other hand, another GEM of RMS has been published. PTC heterozygous knock-out mice develop spontaneous RMS in a model of basal cell carcinoma [235]. Other models of RMS were developed in *knock-out* mice of p16-INK4a (−/−) transgenic for the hepatocyte growth factor/scatter factor [236]. Double *knock-out* mice Trp53−/−; Fos−/− developed invasive RMS [237]. On the other hand, an HER-2/neu transgenic mouse coupled with p53 inactivation can induce RMS [238]. Tsumura et al. developed a pleiomorphic RMS mouse model by conditional activation of RAS and P53 loss [239].

34.12 Conclusions

Sarcoma research is expected to be extremely fruitful in the next years. Both US and European opinion leaders agree in that major needs for translational research in sarcomas include some of the following ones: (1) focus research on key areas of sarcoma biology, including, i.e., cell of origin, targets of chimeric proteins, secondary genetic alterations indicating possible new tumor suppressor genes; (2) new targeted therapies in sarcomas, using key signal transduction molecules or the translocations themselves; (3) development of sarcoma-specific animal models, allowing availability of good experimental models for target discovery; (4) availability of high-throughput technologies to screen cases at the genomic DNA, RNA, or protein levels; (5) development of sarcoma-specific sample repositories (biobanks) including frozen tumor samples, cells, and plasma from sarcoma patients; and (6) development of collaborative networks of sarcoma research, allowing for interchange of materials, researchers, and sarcoma centers.

Among all of them, we believe priority should be given to the development of large research networks that avoid current fragmentation in sarcoma research, allowing for a more efficient use of research funds, and also to the development of feasible animal models carrying the genetic background of human sarcomas, especially in the case of translocation-bearing sarcomas.

Acknowledgements The authors would like to thank the members of the Diagnostic Molecular Pathology and Molecular Pathology of Sarcoma Laboratory at CIC-Salamanca, and at Hospital Virgen del Rocío- IBiS, Sevilla, as well as pathologists and oncologists of the Grupo Español de Investigación de Sarcomas (GEIS) for their collaboration and commentaries. They would also like to acknowledge: AECC (Asociación Española contra el Cáncer); European Commission (NoE EuroBoNet); the European Project EuroSARC (FP7-HEALTH-2011-two-stage, Project ID 278742 EUROSARC); Ministry of Health of Spain-FEDER (PI081828, RD06/0020/0059 RD12/0036/0017, PT13/0010/0056, PI110018), Instituto de Salud Carlos III (Contrato postdoctoral Sara Borrell CD06/00001), Regional Government, Castilla y León, Asociación Española Contra el Cáncer, Fundación Ramón Areces, Fundación Marcelino Botín, and Fundación Solórzano for their financial support. JLO was sponsored by the CSIC and the European Social Fund (postdoctoral grant JAE DOC) and by the AECC.

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Molecular Biology of Human Brain Tumors

35

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35.1 Introduction

The Central Brain Tumor Registry of the USA estimates the annual incidence of primary brain and central nervous system (CNS) tumors at 7.3/100,000 for malignant and 13.3/100,000 for nonmalignant tumors [1]. A true comparison of incidence numbers across different time periods and countries is difficult given the inconsistency of data collection methods and the diversity in diagnostic criteria. Available diagnosis rates for malignant gliomas spanning the last three decades do not allow the determination of an increase or decline of age adapted incidence [2, 3], despite remarkable changes of environment and lifestyle in industrial nations.

The etiology of most brain tumors is largely unknown. In gliomas, familial clustering is found in about 5 % and in 1 % an autosomal dominant inheritance is assumed [4]. Besides family history, demographic risk factors listed are age and gender. Most brain tumors such as gliomas occur in older adults with a slight predominance in males [1]. However, this might be different depending on origin and location of the tumor. For example, brainstem gliomas, medulloblastomas or intracranial ependymomas are usually found in children and adolescents [1]. Even though children constitute only 7 % of all CNS tumors, these tumors comprise 25 % of all cancers in infants and are the most frequent solid tumor and the second most common neoplasm in their age group after leukemia [1, 5]. The only established environmental risk factor is exposure to elevated ionizing radiation [2, 3]. Other environmental carcinogens possibly related to increased brain tumor formation have been examined, such as n-nitroso compounds and polycyclic aromatic hydrocarbons, but no correlation was found [2, 3]. Nonionizing radiation such as cellular phones or smoking have also been extensively evaluated, but no reliable causal link has been established [2, 3]. Chronic viral infections, which are recognized as important risk factors for various cancers [6], are also implicated as triggering processes in brain tumors. Studies of persistent immunohistochemical detection of cytomegalovirus (CMV) in glioblastoma and the benefit of adding antiviral drugs to the standard treatment protocols are still premature, but importantly remain part of an ongoing debate into the etiology of some gliomas [7, 8].

The term brain tumor applies to cancers that arise in different parenchymal layers and tissues from various cellular origins within the central nervous system. The 2007 WHO classification identifies seven categories of brain tumors, depending on the presumed histogenetic origin: (1) neuroepithelial, (2) cranial and paraspinal nerves, (3) meningeal and mesenchymal, (4) lymphomas and hematopoietic neoplasms, (5) germ cell tumors, (6) sellar region, and (7) metastases [9].

In this chapter we will focus on neuroepithelial tumors derived from central neuroglia, which have an annual incidence of 6.6/100,000 and account for the most malignant forms of primary brain tumors [1]. We review the basic biology of human brain tumors with a special focus placed on the most common primary brain tumors in adults and children including the astrocytoma and medulloblastoma (Fig. 35.1). These and other human brain tumors will be discussed insofar as their relationships to the familial predisposition syndromes,

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_35

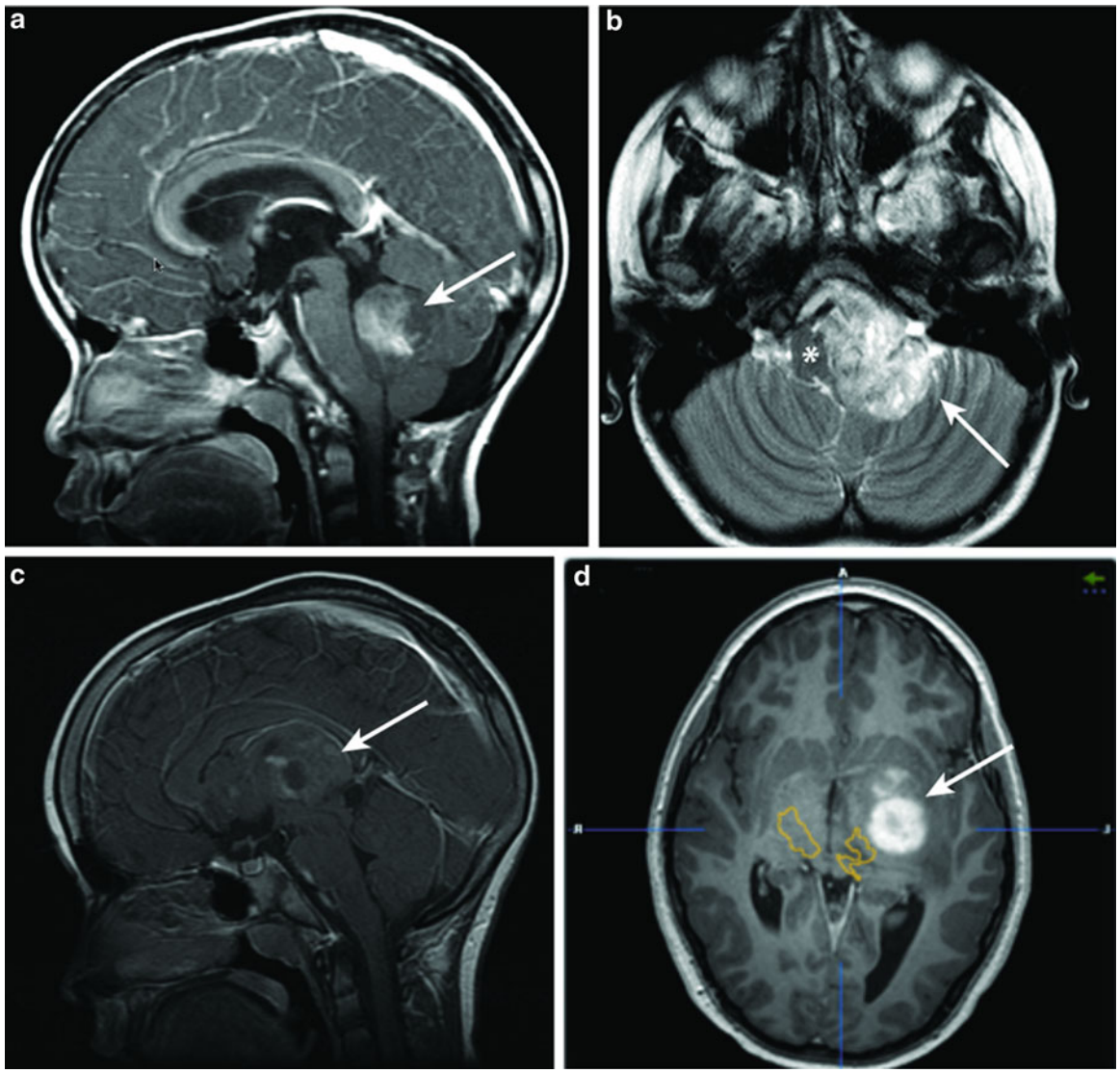


Fig. 35.1 MRI sequences of common primary brain tumors. (**a**) Sagittal T1-weighted and gadolinium-enhanced MR image showing a *medulloblastoma* in the posterior fossa (*arrow*) with compression of the pons and medulla oblongata; (**b**) Axial T2-weighted MR image showing a space occupying e*pendymoma* (hyperintense signaling, *arrow*) in the left paramedian posterior fossa resulting in displacement of the brain stem; (**c**) Sagital T1-weighted and gadolinium-enhanced MR image depicting a slightly and inhomogenously enhancing *low grade astrocytoma* in the thalamic and mesencephalic region (*arrow*); (**d**) Axial T1-weighted and gadolinium-enhanced MR image showing a centrally located Glioblastoma (*arrow*) in the region of basal ganglia resulting in displacement of the pyramidal tracts (*brown* fringe).

cytogenetic alterations, dysregulation of the cell cycle machinery, tumor cell migration and invasion, angiogenesis, developmental signaling pathways, brain tumor stem cells, and preclinical animal models.

The management strategies of human brain tumors rely mostly on histopathological characteristics that are used to predict the clinical behavior and eventual patient prognosis of a diagnosed brain tumor. Features including degree of anaplasia, mitosis, infiltration, necrosis, and neovascularization define the corresponding tumor grade, which ranges from benign, WHO grade I, to the most malignant, WHO grade IV [9]. The advances in molecular diagnostics over the last 5 years however have led to rapid genetic profiling of many brain tumors, which allow us now to recognize remarkable differences in prognosis for tumors harboring similar histopathological patterns [10, 11]. Currently, the utilization of some molecular markers informs individualized treatment decisions for patients with the same histological tumor [10]. Future revisions of brain tumors classification systems need to implement molecular profiles in order to support the differential diagnosis and to be of value in predicting the efficacy of treatment options.

Despite concerted efforts in the field of neuro-oncology, brain tumors represent perhaps the most intimidating and difficult to treat type of cancer. Compared to other neoplasms, patient with malignant gliomas still face disproportionately high rates of morbidity and mortality [12] (Table 35.1). Fortunately, recent advances in molecular biology have

Table 35.1 Clinical characteristics of common primary brain tumors

Tumor	Preferential location	Age	Gender	Annual incidence	WHO grade	Present treatment strategy	Prognosis
Medulloblastoma [1, 424–427]	Cerebellar	Median: 8 y 93 % < 15 y	m: f ~ 1.6:1	0.6/100,000 (age 1–9)	Grade IV	– Maximal safe resection followed by C (<3 y) and RT (>3 y) – High-dose C and autologous stem cell transplantation with or without RT to be considered, especially for *high-risk* group – Re-resection at progression followed by C with or without RT	Pediatric: – *5-y survival* • 42 % (infants < 1 y) • 72 % (1–9 y, including *high-* and *average-risk* (*ar*)) – *10-y survival* for *ar* group: • 81 % Adults: – *5-y survival* • 67 % WNT group correlates with good prognosis (>90 % long-term survivals)
Intracranial ependymoma [1, 208, 428]	Infratentorial (60–70 %), supratentorial (30–40 %)	Median: 8 y	m: f ~ 1.1:1	0.3/100,000	Grade II (~50 %) Grade III (~50 %)	– Maximal safe resection followed by RT – C for Grade III, non-resectable tumors, low EOR and at progression – Re-resection at progression followed by RT with or without C	Pediatric: – *5-y survival* • 66–79 % – *10-y survival* • 55–68 %
Pilocytic astrocytoma [1, 247, 249, 429]	Cerebellar (in children > 50 %), optic pathway, hypothalamus	Median: 18 y 38 % < 15 y 75 % < 20 y	m: f ~ 1:1	0.9/100,000 (age 0–19)	Grade I (>90 %)	– Maximal safe resection – Post-OP: • Usually no additional therapy, depending on EOR • RT optional for non-resectable tumors and at progression • C optional for non-resectable tumors and at progression – Re-resection at progression	Pediatric: – *10-y survival* Cerebellar: 100 % Overall: 97 % Adults: – *5-y survival* Overall: 84 %
Diffuse low-grade glioma (DLGG) – Astrocytoma (A) – Oligodendroglioma – (OD) – Oligoastrocytoma (OA) [107, 430-432]	Frontal (50 %), temporal (25 %) Insular (10 %)	Median: 45 y	m: f ~ 1.3:1	1/100,000 – A 20–50 % – OD 35 % – OA 10–45 %	Grade II	– Maximal safe resection – Post-OP: • C to be considered, especially for OD, non-resectable DLGG, low EOR and at Progression • RT for non-resectable DLGG and at Progression – Re-resection at progression, followed by C and/or RT	*5-y survival* – >95 % After GTR – 78 % if Resection < 50 % *10-y survival* – 90 % After GTR – 54 % if resection < 50 % OD and LOH 1p/19q tend to correlate with better outcome

(continued)

Table 35.1 (continued)

Tumor	Preferential location	Age	Gender	Annual incidence	WHO grade	Present treatment strategy	Prognosis
High-grade/anaplastic (A) glioma – AA – AOD – AOA [121, 206, 433–435]	Parietal, frontal, temporal	Median: 43 y	m: f ~ 1.4:1	0.6/100,000	Grade III	– Maximal safe resection followed by RT or alkylating C (AA, AOD and AOA without LOH 1p/19q) – Maximum resection followed by C with or without RT (AOD and AOA with LOH 1p/19q) – Re-resection and C/RT at progression	*Median survival* – 4.6- 6.5 y (AOD, AOA) *5-y survival* ~50–65 % (AOD, AOA) ~15–50 % (AA) *10-y survival* ~20–45 % (AOD, AOA) ~15–30 % (AA) AOD, IDH1 mutations and LOH 1p/19q correlate with increased sensitivity to RT/C and better outcome
Glioblastoma [433, 436, 437]	Temporal, frontal, parietal	Median: 57 y	m: f ~ 1.7:1	5/100,000	Grade IV	– Maximal safe resection, followed by RT plus concurrent TMZ, followed by adjuvant TMZ (age < 65–70 years) – Maximal safe resection, followed by RT or TMZ with or without RT based on *MGMT* status (age > 65–70 years) – Re-resection and C/RT at progression to be considered	*Median survival* – 14.6 mt (overall) – 23.4 mt with methylated (met) *MGMT* promoter *5-y survival* – 9.8 % (overall) – 13.8 % with met *MGMT* *IDH1/2* mutations and met *MGMT* correlate with better outcome

y Year(s), *mt* months, *m* male, *f* female, *C* chemotherapy, *RT* radiotherapy, *EOR* extent of resection, *GTR* gross total resection

provided a wealth of new information that can now be applied to improving patient treatment strategies and outcome. Modern medicine is on the threshold of achieving many tangible gains in overall patient survival based on the progress that has been made in several areas of experimental neuro-oncology.

35.2 The Familial Central Nervous System Tumor Syndromes

Most CNS tumors occur in adults and arise spontaneously with no previous family history or genealogic pattern of inheritance. In some cases, especially in younger patients, they may present as a familial syndrome and transmitted most frequently in an autosomal dominant (AD) pattern of inheritance. Advances in the identification and characterization of gene mutations have permitted insight into the molecular basis of both familial and sporadic CNS tumors.

Many familial CNS cancer syndromes are associated with mutations in tumor suppressor genes, which mediate functions such as DNA repair and cell cycle arrest. Affected individuals typically inherit a germ-line mutation in one copy of the genetic locus and in the cases of non-AD mutations, are associated with somatic loss of the wild-type allele in order to promote the initiation of tumor growth. One important distinction of the familial syndromes is the predisposition of patients to develop multiple tumors in the CNS as observed in neurofibromatosis 1 (NF1), neurofibromatosis 2 (NF2), von Hippel–Lindau disease, tuberous sclerosis complex, Gorlin syndrome, Li–Fraumeni syndrome, Turcot syndrome, and Cowden syndrome.

35.2.1 Neurofibromatosis Type 1

NF1, also known as von Recklinghausen's disease, is one of the most common inherited monogenetic syndromes predisposing individuals to the formation of CNS tumors, with an incidence of 1 in 3500 individuals and an equal predominance in both genders [13] (Fig. 35.2). NF1 is AD, with marked pleiotropy and variability in clinical expression. Approximately 50 % of newly diagnosed patients have no previous family history, suggesting the occurrence of de novo mutations, with a bias for the paternal germ-line. The disease is caused by loss-of-function mutations of the *NF1* gene. The diagnosis of NF1 is made according to well defined clinical criteria (NIH criteria) [14], which are usually diagnostic by the age of 8 years [15]. Detecting mutations in the *NF1* gene is difficult and complex given the large size of the gene and the lack of established mutation hotspots. Protocols combining PCR and high-performance liquid chromatography to detect germ-line mutations have been developed and have reported the identification of more than 95 % of individuals fulfilling clinical NF1 criteria [16].

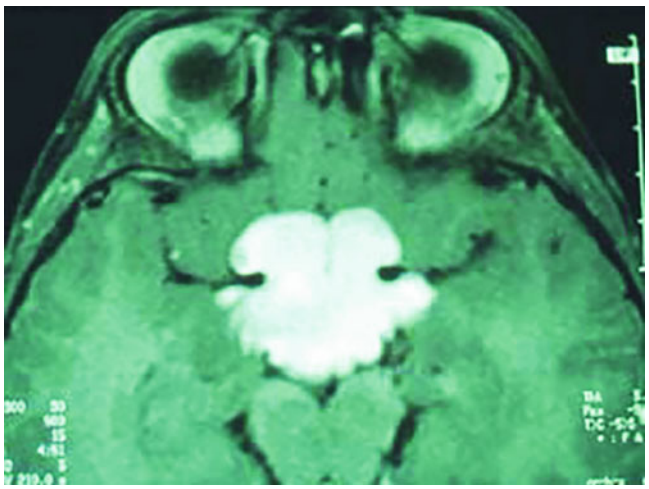


Fig. 35.2 T1-weighted and gadolinium-enhanced MRI sequence showing a Pilocytic Astrocytoma of the optic pathway in a patient with neurofibromatosis type 1.

The disease is characterized by the presence of multiple neurofibromas, pilocytic astrocytoma within the optic nerve pathways, malignant peripheral nerve sheath tumors (MPNST), and susceptibility to other astrocytomas. The optic pathway astrocytoma (OPA) represents the most common CNS tumor in NF1, affecting up to 20 % of all patients and usually arising in children under the age of six [17]. Remarkably, half of the individuals with OPA remain asymptomatic [17].

NF1 is inherited with high penetrance but the phenotypic expression is variable, suggesting a role for other disease-modifying genes and heterogeneous mutations of the *NF1* gene [18–20]. The timing of *NF1* gene inactivation or mutation is also assumed to interfere with disease manifestation [20]. The *NF1* gene is localized to chromosome 17q in 1987 and mapped to the 17q11.2 locus 3 years later [21–23]. The *NF1* gene encodes the protein Neurofibromin and spans at least 350 kilobases (kb) and includes over 60 exons. More than 1000 mutations resulting primarily in Neurofibromin truncations have been identified [24] and complete gene deletion is associated with a severe phenotype [25].

Neurofibromin functions as a tumor suppressor and is a cytoplasmic protein of that is highly expressed in neurons, Schwann cells, oligodendrocytes, astrocytes, leucocytes and the adrenal medulla, while its expression in other tissues is minimal [26]. Neurofibromin harbors a Guanosine Activating Protein (GAP)-related domain, conferring homology to other guanosine triphosphatase (GTP) activating proteins [27]. Ras is a GTP-binding oncoprotein, which promotes cell growth and differentiation. By accelerating the conversion of active GTP to its inactive, guanosine diphosphatase (GDP)-bound form, Neurofibromin acts as a negative regulator of Ras.

Defects in Neurofibromin are thought to increase cell growth and facilitate tumor formation due to loss of Ras regulation. In addition, Neurofibromin regulates other proteins including adenylate cyclase in astrocytes, and potentially plays a role in neural stem cell proliferation, survival, and astroglial differentiation [28, 29].

While most of the neoplasms encountered in NF1 patients are not considered malignant, adults with NF 1 have a 50–100-fold increased risk of high-grade glioma formation [30, 31] and a 20 % lifetime risk to develop a malignant peripheral nerve sheet tumor (MPNST) [32, 33] which are assumed to arise from preexisting neurofibromas [32]. To date, only two genetics alterations have been identified as progression pathways. Mutation in the *TP53* loci and genetic alterations in *INK4A* have been frequently identified in malignant but not benign forms of neurofibromas [30, 34].

Numerous animal models have been employed to explore the molecular basis of NF1 pathogenesis. NF1 heterozygous mice with selective inactivation of *NF1* in Schwann cells develop tumors with histologic and molecular features of human neurofibromas [35]. The generation of astrocyte-specific *NF1* knockout mouse yielded similar findings, with low-grade optic astrocytomas developing only when Neurofibromin expression was ablated in astrocytes of NF1 heterozygous mice [36].

Several therapeutic approaches based on blocking the dysfunctional neurofibromin pathway have been attempted in animal studies [37]. However, most clinical studies have not extended beyond Phase II trials [38]. The mTOR pathway is regarded as a key mediator of optic pathway glioma development. Currently, an ongoing Phase II study is investigating the growth inhibition of NF1 related low-grade gliomas using the mTOR-inhibitor everolimus [39].

35.2.2 Neurofibromatosis Type 2

NF2 is approximately tenfold less common than NF1 and has distinct phenotypic and genetic features. It is an autosomal dominant syndrome that occurs at an incidence of 1 in 25,000–40,000, with a penetrance close to 100 % by the age of 60 [40]. Half of the individuals inherit a germ-line mutation from a parent or acquire a de novo mutation at the stage of embryogenesis [41]. Bilateral vestibular schwannomas are pathognomonic of the disorder, which is also characterized by schwannomas of other nerves (cranial, spinal or peripheral) as well as intracranial and intraspinal meningioma and ependymomas. NF2 is characterized by a variable age at onset, with a majority of patients developing signs in the second to third decade of life, and thus, NF2 schwannomas and meningiomas occur at an earlier age compared to their sporadic counterparts [41]. NF2 is clinically diagnosed according to the 2005 Manchester criteria [42].

The *NF2* gene was mapped to chromosome 22 in 1987 and further localized on locus 22q12.2 [43]. The gene responsible for this disorder was cloned in 1993 by two groups by positional cloning and was notably also found to be mutated or deleted in the majority of sporadic meningiomas and schwannomas [44, 45]. The gene spans 17 exons distributed approximately over 110 kb, and it encodes for a protein of 595 amino acids called merlin (for moesin, ezrin, radixin-like) due to its homology to three proteins of the ezrin, radixin, moesin (ERM) family [45]. Merlin, alternatively named Schwannomin, is expressed in many normal human tissues, including the brain, and functions as a tumor suppressor [46]. Tumors resulting from dysfunction or loss of Merlin activity are mostly benign [47].

Several studies have shown that Merlin controls cell proliferation and cell motility by regulating cytoskeletal organization, but the pathways involved in its mediation of growth suppression are poorly understood [46, 48]. Merlin interacts with F-actin and negatively regulates cell motility through cytoskeletal reorganization. Moreover, it may act with CD44 (a hyaluronic acid receptor), β1 integrin (a transmembrane glycoprotein) and paxillin (a cytoskeletal adaptor protein) to regulate cell motility [48]. Merlin deficiency has also been linked to abnormal activation and overexpression of receptor tyrosine kinases such as EGFR family receptors [49].

A number of NF2 mouse models have been developed. Homozygous mutation of *NF2* results in embryonic lethality, indicating that its function is essential at early stages of mouse development [50]. Heterozygous *NF2* mutant mice develop many neoplasms but do not manifest the classical tumors linked to NF2. However, as observed in NF1 murine models, the model of conditioned Schwann cells for knockout mice show many similarities with NF2 patients [51].

Merlin dysfunction or loss affects various signaling pathways, therefore several therapeutic targets have been proposed. As schwannomas represent the most common symptomatic and limiting tumor in NF2 patients, clinical trials are primarily directed against schwannoma formation [52]. A recent Phase II study in NF2 patients with progressive vestibular schwannomas observed some decrease in tumor volume and improvement in hearing in response to the EGRF inhibitor Lapatinib [53].

35.2.3 von Hippel–Lindau Syndrome

von Hippel–Lindau (VHL) is a rare inherited disease with an AD pattern of transmission and was first described in the early 1900s by Eugen von Hippel and Arvid Lindau [54, 55]. It is characterized by the development of benign hamartomatous tumors in the CNS and adrenal glands including hemangioblastoma and retina angioma, pheochromocytoma, pancreatic neuroendocrine tumors and inner ear

tumors as well as malignant tumors of the kidney such as renal cell carcinoma [56]. Its incidence is estimated at 1 in 36,000–45,000 births per year [57, 58]. Up to 20 % of VHL patients are estimated to derive from de novo mutations [59]. The disease shows variable expressivity with almost 95 % penetrance at the age of 60 years [60]. A combination of characteristic clinical features, with or without a positive family history, permits the establishment of a clinical diagnosis. The most frequent lesion observed is hemangioblastoma, a mesenchymal solid and cystic tumor mainly localized in the brain (mostly located in the cerebellum), which occurs in 60 to 80 % of all VHL patients [61]. Depending on specific mutation in the gene locus, VHL is classified as type 1 or type 2, according to the absence or presence of a pheochromocytoma [56].

The gene responsible for this disease is the VHL tumor-suppressor gene, located on chromosome 3p25-26 [57]. Under normoxic conditions, the gene product targets hypoxia-inducible factors (HIF) for polyubiquitination and proteosomal degradation. Under hypoxic conditions, the accumulation of HIF will induce the transcription of hypoxia-regulated genes such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and transforming growth factor (TGF) -α. This explains the high vascularization of VHL-related tumors. Loss of the VHL gene can also result in aberrant expression of genes that control cell cycle progression and invasion [62, 63]. It has been shown in renal carcinoma cells that loss of the VHL gene results in the accumulation of hepatocyte growth factor (HGF), which interacts with the β-catenin pathway [64]. Loss of VHL protein activity has also been recently correlated to the activation of kinases required for Ras-induced cell transformation, thus representing a possible target for molecular)inhibition [65].

Hemangioblastoma is a mesenchymal-derived hamartomatous tumor and patients with VHL-syndrome are not typically linked with glioma manifestation. However, there is some evidence that VHL expression interferes also with neuroepithelial tumor formation via suppression of HIF-1α/VEGF, which is coupled with inhibition of glioma proliferation and invasion [66].

35.2.4 Tuberous Sclerosis Complex

Tuberous sclerosis complex (TSC) is a multisystem autosomal dominant disorder, affecting 1 in 6000–10,000 individuals [67]. It was first described by Bourneville in 1880 and is characterized by CNS tumors including cortical hamartomas named tubers, subcortical glioneuronal hamartomas, subependymal glial nodules, and subependymal giant cell astrocytomas. TSC patients often suffer from debilitating neurologic disorders, including epilepsy, mental retardation, and autism. Essentially, any organ system can be involved and common features of TSC include dermatologic manifestations such as cutaneous angiofibromas, subungual fibromas, cardiac rhabdomyomas, renal angiomyolipomas, intestinal and visceral cysts, and pulmonary lymphoangiomyomatosis,

The diagnostic criteria consists of a set of major and minor features which appear at distinct points in development, but most patients have manifestations of the disease before 10 years of age [68]. Neurologic disabilities seem to be intimately related to the presence of cortical tubers, which occur in approximately 70 % of patients [69].

The penetrance of the disease is high but the clinical manifestations are considerably variable [70]. There is a high rate of new mutations, accounting for up to 85 % of cases [71]. TSC results from the mutation of one of two genes, *TSC1* on chromosomes 9q34 and *TSC2* located on 16p13.3. Clinical and pathological features caused by mutations in these two genes are indistinguishable, but the prognosis seems to be more severe when the disease is associated with a *TSC2* mutation [72]. The *TSC1* gene encodes a transcript of 8.6 kb called Hamartin, containing 23 exons, whereas the *TSC2* gene encodes a transcript of 5.5 kb called Tuberin, containing 41 exons. Mutations in these genes can be identified in 60–80 % of TSC patients, with a wide spectrum of specific genetic alterations [73]. Hamartin and Tuberin function as a regulatory complex inhibiting the small GTPase RHEB and consequently limiting the activity of the mammalian target of rapamycin complex 1 (mTORC1), which is an intracellular regulator of cell growth and metabolism [74]. In a recent Phase I–II open-label study of patients receiving the mTOR-inhibitor everolimus, a clinically significant reduction in tumor volume of TSC related subependymal giant-cell astrocytoma was shown [75, 76].

35.2.5 Gorlin Syndrome

Gorlin syndrome is also known as nevoid basal cell carcinoma syndrome (NBCCS) or basal cell nevus syndrome (BCNS) and was first described by Goltz and Gorlin in the 1960s. It is a rare AD disorder occurring in 1 of 57,000 live births and characterized by basal cell-carcinoma, skeletal anomalies and a 2–5 % incidence of childhood medulloblastomas (mostly SHH medulloblastomas) [11, 77]. The penetrance is very high but the clinical expression is variable, with up to 30 % of patients presenting with de novo mutations [78]. The clinical criteria are based on the association of multiple basal cell carcinomas and odontogenic keratocysts [79]. Other manifestations include the presence of palm or sole pits, intracranial calcifications in particular along the falx and macrocephaly [77].

The disease results from a mutation of the human homologue of the *Drosophila melanogaster* gene *PTCH* located

on 9q22.3 [80]. It contains 23 exons and encodes for a transmembrane protein (Ptch), which is a receptor and negative regulator for the secreted Hedgehog family proteins such as Sonic Hedgehog (Shh).

35.2.6 Li–Fraumeni Syndrome and TP53 Germ-Line Mutations

Li–Fraumeni syndrome (LFS) is an AD disorder predisposing patients to an early onset of a wide spectrum of neoplasms. The syndrome was defined by Li and Fraumeni after they noticed a high incidence of cancer among family members of children with rhabdomyosarcoma [81]. Often described as a very rare syndrome with only several hundred identified individuals, its effective incidence is probably underestimated [82], in part also due to the existence of different classification schemes [83]. The most frequent tumors observed are breast cancer, sarcomas, osteosarcomas, brain tumors, acute leukemia and adrenocortical carcinomas.

The major genetic abnormality underlying this syndrome is a germ-line mutation of *TP53*, which has been identified in 70 % of LFS cases and in 22–44 % of families with the LF variant form [84]. Importantly, 50 % of the families with a *TP53* germ-line mutation meet the diagnostic criteria of LFS. In the remaining cases of LFS without *TP53* mutation, a germ-line mutation of the *hCHK2* gene involved in G2 checkpoint control has been identified [84]. *TP53* germ-line mutations occur most frequently in exons 5–8, with major hotspots on codons 245 and 248. In the 143 LFS families reported, the most frequently identified mutations are point mutations (85.3 % of cases). Interestingly, low grade and anaplastic astrocytomas of LFS patients harbor somatic *IDH1* mutations at much higher rates compared to sporadic gliomas [85].

35.2.7 Turcot Syndrome

Turcot syndrome is an AD disorder characterized by the occurrence of adenomatous colorectal polyps or colon carcinomas and malignant brain tumors, mainly anaplastic astrocytomas, as well as glioblastomas and medulloblastomas (95 %) [86]. Turcot described the first cases in 1959, where two siblings developed malignant CNS tumors and numerous adenomatous colorectal polyps [87].

There are two major subgroups of Turcot syndrome. The type 1 pattern is characterized by an association of glioblastomas and hereditary non-polyposis colorectal carcinomas (HNPCC). This subgroup results from a germ-line mutation in DNA mismatch repair genes such as *hPMS1* (ch 2q32), *hPMS2* (ch 7p22), *hMSH2* (ch 2p16), or *hMLH1* (ch 3p21) [86]. These deficiencies lead to DNA replication errors and microsatellite instability, which is rare in brain tumors in the absence of Turcot syndrome [88]. In this subgroup, the mean age for occurrence of glioblastoma is lower than in the general population (18 years old versus 40–70 years old). The type 2 subgroup is characterized by association of WNT subgroup medulloblastomas with familial adenomatous polyposis (FAP). These patients tend to have a germ-line mutation in the *adenomatous polyposis coli* (*APC*) gene that lies on chromosome 5q21 [86]. In the type 2 Turcot syndrome subgroup, the median age of occurrence of medulloblastoma is 15 years, compared to 7 years of age in the general population.

35.2.8 Cowden Syndrome and Lhermitte–Duclos Disease

Cowden syndrome (CS) is a rare AD condition associated with multiple benign hamartomatous lesions mostly involving the skin and mucosa, but various organ systems including the cerebellum may be affected [89]. CS predisposes patients to malignant tumors, especially of the breast, thyroid, intestine, and endometrium [89]. The penetrance of CS is high and approaches over 90 % by the age of 20 years [89]. The syndrome was first documented in 1963 by Lloyd and Dennis and named after the first patient [90]. In 1991, more than 70 years after its first description [91], it was recognized that dysplastic cerebellar gangliocytoma or Lhermitte–Duclos disease (LDD) is often a manifestation of CS [92]. The prevalence of CS is estimated at 1:200,000, but the frequency is likely underestimated given that many non-symptomatic mucocutaneous manifestations related to this disease are also common in the general population and thus may not lead to extensive diagnosis [89]. Approximately half of the CS cases are recorded as inherited and half occur spontaneously [93]. The most common germ-line mutation) involves the tumor suppressor gene *PTEN*, located at the chromosome 10q23.3 [94, 95]. Early clinical data including nearly 50 CS families suggest that 85 % of patients with CS harbor a *PTEN* mutation [96, 97] However this may not be universally adopted given that application of risk assessment criteria from the National Comprehensive Cancer Network (NCCN) results in many patients diagnosed as CS despite the absence of *PTEN* mutations [98]. Germ-line mutations downstream of PTEN signaling were found in the PI3K/AKT pathway in up to 10 % of CS patients lacking *PTEN* gene alterations [99]. Not all patients with LDD show clinical manifestation of CS [100] and dysfunction or inactivation of *PTEN* is found in 67–83 % of LDD lesions [100, 101]. Selective inactivation of *PTEN* in neural cells of mice results in impaired migration of granular cell precursors and formation of cerebellar tumors histopathologically resembling LDD [102, 103]. Inactivation of *PTEN* is linked with increased levels of PI3K/AKT, which promotes cell growth and proliferation [102, 103]. Activated AKT regulates various pathways involved in protein synthesis and

cell growth, including the mTOR pathway, suggesting that mTOR inhibitors may be effective in reversing cellular hypertrophy and tumor growth [101].

35.3 Cytogenetics and Molecular Genetics of Neuroepithelial Human Brain Tumors

35.3.1 Glioma (Astrocytoma and Oligodendroglioma)

Gliomas comprise tumors of astrocytic or oligodendrocytic histopathological appearance. Whether these tumors have the same origin of tumor-initiating cells from neural progenitors or arise from dedifferentiated astrodendrocytes/oligodendrocytes is a matter of ongoing debate [104, 105]. The WHO distinguishes between gliomas with more circumscribed growth (WHO Grade I) and gliomas with diffuse infiltration (WHO Grade II: low grade, WHO Grade III–IV: high grade) [9]. Diffuse low-grade gliomas are considered a precancerous disease, as over time they progress to anaplastic, malignant tumors in most instances [106].

Gliomas demonstrate the full spectrum of cytogenetic and molecular abnormalities: From numerical and structural chromosomal alterations, to gene amplifications and overexpression, deletions and small-scale mutations up to and including epigenetic deregulations.

35.3.2 Chromosomal Alterations

Chromosomal number alterations occur with increased frequencies in high grade and adult gliomas, and chromosomal losses are observed more frequently than gains [107]. One of most common numerical autosomal changes seen in high-grade astrocytomas includes gain of chromosome 7, and loss of chromosome 10 [107–109]. Gain of chromosome 7 is seen in up to 83 % of adult high-grade astrocytomas, whereas chromosome 10 is reported to be lost in up to 86 % [109, 110]. In some studies, a combined gain of chromosome 7 accompanied by loss of chromosome 10 ($7^{+}/10^{-}$) occurs in over 80 % of GBM samples [108, 111].

The occurrence of trisomy 7 and monosomy 10 together has often been correlated with short-term survival, although currently available data does not allow a distinct conclusion [112]. Gains of chromosome 7 are also among the most common autosomal changes in low-grade gliomas (LGG), seen in 57 % of cases [110] and loss of chromosome 10 may be correlated with shorter overall survival [113]. Chromosomal alterations commonly observed in secondary GBM include 19q loss (see Box 35.1) [114]. An additional numerical chromosomal abnormality is the presence of *double minute chromosomes*, seen in approximately 20 % of GBM specimens, which can further confer oncogene amplification and drug resistance [115].

Oligodendroglioma (OD) and oligoastrocytomas (OA) constitute a subtype of glioma for which the specific loss of

Box 35.1: Primary Versus Secondary GBM

According to current evidence, there are two different routes of glioblastoma tumorigenesis, both with different genetic alterations but ultimately undistinguishable histology [114]. GBM mostly occurs as a rapidly growing de novo tumor from a glial progenitor or dedifferentiated cell and affecting patients with a mean age around 60 years. This Primary GBM constitutes 90 % of the cases, where no evidence of a less malignant precursor lesion can be detected. Secondary GBMs (10 %) affect typically younger patients around a mean age of 45 years. These tumors progress from a preexisting diffuse low-grade or anaplastic astrocytoma, are more often located frontally, and the overall survival after diagnosis is significantly longer. Histologically, Secondary GBMs show less necrosis and frequent regions of oligodendroglioma-like appearance when compared to the Primary form. From a molecular basis, the Primary tumors often show loss of chromosome 10, *EGFR* amplification and *PTEN* mutation, whereas in Secondary GBM, *TP53* mutations, loss of chromosome 19q and *PDGFRA* alterations are more frequent. Classification of GBM origin has been significantly improved by the recent identification of mutations in the enzyme gene *IDH1/2* (isocitrate dehydrogenase, involved in the citric acid pathway), which are predominantly found in Secondary (80 %) but only rarely (<5 %) in Primary GBMs [132–134]. Consequently, *IDH1/2* mutations are now commonly accepted as molecular markers of Secondary GBM.

Over 90 % of secondary GBM diagnosed through *IDH1/2* mutations belong to the proneural subgroup, which indicates that this tumor subgroup is homogeneous compared to the primary form. *IDH1/2* mutations occur early during progression from low-grade to high-grade glioma given they are detected in over 80 % of grade II and III astrocytoma and oligodendroglial tumors [134, 141]. It is possible *IDH1/2* mutations precede 1p/19q loss in oligodendrogliomas and *TP53* mutation in low-grade astrocytomas [242]. Finally, there is evidence that primary and secondary GBMs derive from different glial progenitor cells. In support of this, CD133 positive cancer stem cells could only be cultured from Primary GBMs, whereas samples from secondary GBMs did not have CD133 positive stem cells within [243].

portions of 1p and 19q (loss of heterozygosity, LOH 1p/19q) is associated with important prognostic implications and is used as a marker to support the histological diagnosis of OD [116, 117]. Approximately 50–80 % of ODs have allelic loss of chromosomes 1p and 19q, whereas OA (mixed oligodendrogliomas, arrangement of cells presenting astrocytic or oligodendroglial differentiation) show this co-deletion 40 % of the time [118–120]. LOH 1p/19q is frequently observed in WHO grade II oligodendric tumors compared to those of grade III, though conflicting data exists in this regard [118–120]. Notably, oligodendric gliomas of the frontal lobe show higher incidence of 1p/19q co-deletion than those of non-frontal origin [119]. Low-grade OD (WHO II) treated by surgical resection and nitrosourea-based chemotherapy without radiotherapy show favorable long-term outcome (10-year overall survival rates over 90 %) irrespective of LOH 1p/19q status [121]. Anaplastic pure and mixed oligodendrogliomas (WHO III) identified with 1p/19q co-deletion are generally associated with marked higher overall survival and chemosensitivity in comparison to non-co-deleted WHO III oligodendrogliomas, where the addition of chemotherapy to radiotherapy did not prove beneficial over radiotherapy alone [120, 122].

35.3.3 Gene Amplification and Overexpression

Gene amplification has been described for several target genes in astrocytic tumors, and appears to be more common in high-grade lesions than low-grade astrocytomas [109, 123]. Genes continuously found to be amplified in high grade gliomas include *EGFR, MET, PDGFRA, MDM4, MDM2, CCND2, PIK3CA, MYC, CDK4,* and *CDK6* [124]. Amplification and overexpression of *EGFR* occurs frequently and predominantly in primary GBMs (60 % of the samples), but rarely in the secondary form [114, 125]. In up to 30 % of the cases this gene has undergone loss of exon 2–7, resulting in the production of a constitutively active, truncated EGFR receptor (EGFRvIII) [109, 126, 127]. At this time, Phase II and III vaccination trials using rindopepimut to stimulate immune response against the EGFRvIII peptide in GBM patients are ongoing [128, 129]

35.3.4 Gene Mutations and Deletions

Tumor suppressor gene silencing events in astrocytomas may be the result of gene deletions, mutations, or promoter methylation events. Primary GBMs are characterized by *CDKN2A* deletion (31-50 %), and *PTEN* mutation (32 %) [109, 130, 131]. While *CDKN2A* deletions occur in primary GBM, *TP53* mutations appear more common in secondary GBM [109, 114]. In the progression of low-grade tumors to secondary GBM, *TP53* mutations are the most common and early-detected genetic abnormality—seen in about 2/3 of precursor low-grade astrocytomas and secondary GBMs derived from them. *TP53* mutations occur in primary GBM but at a lower frequency (35 %) compared to secondary GBM [108, 109, 130]. Additionally, in secondary GBM, 60 % of the *TP53* mutations are clustered in two hot spot codons (amino acids 248 and 273), whereas in the primary form, *TP53* mutations appear more equally distributed within exon 5–8 [108].

Mutations in the gene encoding for NADPH-dependent isocitrate dehydrogenase (*IDH1*) were identified from a large-scale genomic sequencing analysis of astrocytoma [131]. Since the initial description, multiple studies have shown that *IDH1* mutations are common in low-grade diffuse astrocytomas, anaplastic astrocytomas and tumors of oligodendroglial origin [132–134]. Notably, there is evidence that *IDH1* mutations precede *TP53* mutations, suggesting an important role in tumor initiation [114]. A proportion of low-grade astrocytomas that lack *IDH1* mutations (e.g., pilocytic astrocytoma, and ependymoma) contain mutations of the related *IDH2* gene [134]. As *IDH1* mutations are very common in secondary (>80 %) but markedly rare in primary GBM (<5 %), a consensus is that *IDH1* mutation can serve as a molecular marker of secondary GBM, as this form is neither clinically nor histologically distinguishable from the primary form [114]. *IDH1* status is important to clinicians as patients with tumors harboring *IDH1* mutations have a survival benefit [135–139]. *IDH* mutations also seem to correlate with both *TP53* mutational status and 1p/19q deletions, both of which predict survival [137, 140, 141].

35.3.5 Epigenetic Alterations

In addition to aberrant changes in the genome, epigenetic alterations have emerged as important factors in gliomagenesis [142]. Potentially reversible and more amenable to treatment than numerical or structural changes in chromosomes, studies of epigenetic deregulations is a promising strategy in glioma therapy. GBM cells showing high activity level of the DNA repair enzyme *MGMT* (O^6-methylguanine-DNA methyltransferase) are more resistant to therapies including alkylating agent such as temozolomide (TMZ) [143]. About 40 % of GBM have a methylated *MGMT* promoter status resulting in epigenetic silencing of the *MGMT* gene and thus higher sensitivity to TMZ and distinctive longer survival with actual standard therapy [143, 144]. Hence, *MGMT* promoter methylation is a strong prognostic and predictive biomarker.

The increased awareness of the role of metabolic enzymes in astrocytoma pathogenesis has brought about a resurgence of interest in the Warburg Effect [145]. The Warburg effect first described in 1956 by biochemist Otto Warburg promotes the observation that tumors predominantly use a high rate of

aerobic glycolysis to meet metabolic demand [146]. Recently, isoforms of both pyruvate kinase (PK) M1/M2 and hexokinase (HK1/HK2) have shown differential expression in glioblastoma, in which PKM2 and HK2 predominate, promoting glycolysis [147, 148].

35.3.6 Pediatric Versus Adult Gliomas

The genetic changes that are found in pediatric astrocytomas are distinct from those in the adult variant of these tumors. No consistent karyotypic abnormalities have been identified in pediatric low-grade astrocytomas [149, 150]. Even in high-grade gliomas, the number of chromosomal aberrations is generally lower from their adult counterparts with up to 15 % lacking detectably number irregularities [107]. Relatively frequent compared to adult GBM, some pediatric GBMs have gain of chromosome 1q (up to 30 %) and loss of 16p (up to 24 %), while numerical aberrations of chromosome 7 and 10 are relatively rare (<30 %) [151, 152]. Cytogenetic changes recurrently observed in pediatric anaplastic astrocytomas and GBM include gains of 1q, 5q, and loss of 6q, 9q, 10q, 12q, 13q, and 22q [152, 153]. In contrast to adult tumors, amplification of the *EGFR* gene is not common in pediatric astrocytomas and *PDGF*-driven signaling is preferentially activated [151, 154]. Notably, this feature is also prevalent in secondary GBM [155]. Similarly, pediatric astrocytomas rarely exhibit loss or mutation of *PTEN*. Aberrant activation of the *BRAF* proto-oncogene (7q34) by copy number alteration or formation of an abnormal fusion protein is an important marker of pilocytic astrocytoma but not diffuse infiltrating pediatric astrocytomas (see Box 35.2) [156–158]. Aberrant BRAF activation leads to increased signaling through the MAP kinase/ERK pathways, which results in high levels of mTOR activation, and ultimately to increased cell growth [159, 160].

35.3.7 Core Signaling Pathways and GBM Subgroups

The understanding of the molecular basis of astrocytoma has benefited from large-scale genomic profiling approaches from networks such as The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) [109, 130, 131, 161] GBM was the first cancer to be systematically analyzed by TCGA, highlighting the role of oncogenes such as *ERBB2* and *PDGFRA* and suppressor genes such as *NF1* and *TP53* as well as reinforcing core defects in three signaling pathways involved in the pathogenesis: (1) retinoblastoma (RB)-signaling (79 %), (2) p53 signaling (90 %), and (3) PIK(3)K/RAS signaling (88 %) (Fig. 35.3). Large-scale genomic consortia have allowed the stratification of astrocytomas based

Box 35.2: Pilocytic Astrocytoma

Pilocytic Astrocytoma (PA) is a non-infiltrative, slow growing, and often cystic tumor typically classified as WHO grade I. It is one of the most common CNS neoplasms in childhood accounting for ~20 % of all pediatric brain tumors [1]. Almost three-fourths of the patients are younger than 20 years [1]. Approximately half of PAs are located in the cerebellum, but they can occur throughout the neuraxis such as the optic pathway, hypothalamus, and spinal cord (mostly in pediatric patients) as well as in the cerebral hemispheres (mostly in young adults) [244]. Optic nerve PAs are often associated with NF1. Provided that the tumor is accessible and extensive surgical resection is performed, most patients show a favorable outcome with a 10-year survival rate of over 95 % [244–246]. Until recently, the genetic mechanisms underlying this disease were not extensively investigated. Early genomic analysis of tumor samples showed often balanced karyotypes, whereas whole chromosomal gains occurred in about 30 %, frequently affecting chromosomes 5 and 7 [247]. Activating alterations within the MAPK-signaling pathway are considered a hallmark aberration in the pathogenesis of PA, affecting 80–100 % of analyzed tumor samples [248, 249]. Consequently it is hypothesized that PA represents a single-pathway disease [249]. The most frequent genetic event leading to MAPK activation is *BRAF-KIAAI549 (B-K)* fusion, which produces a fusion protein that lacks the regulatory domain of BRAF [249, 250]. In addition, various MAPK pathway-activating fusions)typically involving BRAF are described [249]. The somatic mutation rate is very low (<0.1/Mb) and *BRAF, FGFR1, K/-NRAS, PIK3CA, CDKN2A,* and *NF1* are the main genes found to be mutated or rearranged [245, 249, 251]. Similar to other tumor types, the copy number changes and mutation rates positively correlate with patient age and potentially with an aggressive clinical course [245, 247, 249]. The distribution of oncogenic hits within the MAPK pathway may vary depending on tumor location: *NF1* mutations are more often detected in optic PA, while PAs in other locations are dominated by BRAF activation [248]. BRAF fusions are more common in cerebellar PAs and mutations are typically seen in supratentorial PAs [248]. In adult PAs, *BRAF* V600E mutations appear to be very infrequent compared to the pediatric counterpart [245]. As B-K fusions are particularly rare in diffuse astrocytomas (~2 %), the presence of this event can be considered an adjuvant diagnostic tool for PA [245].

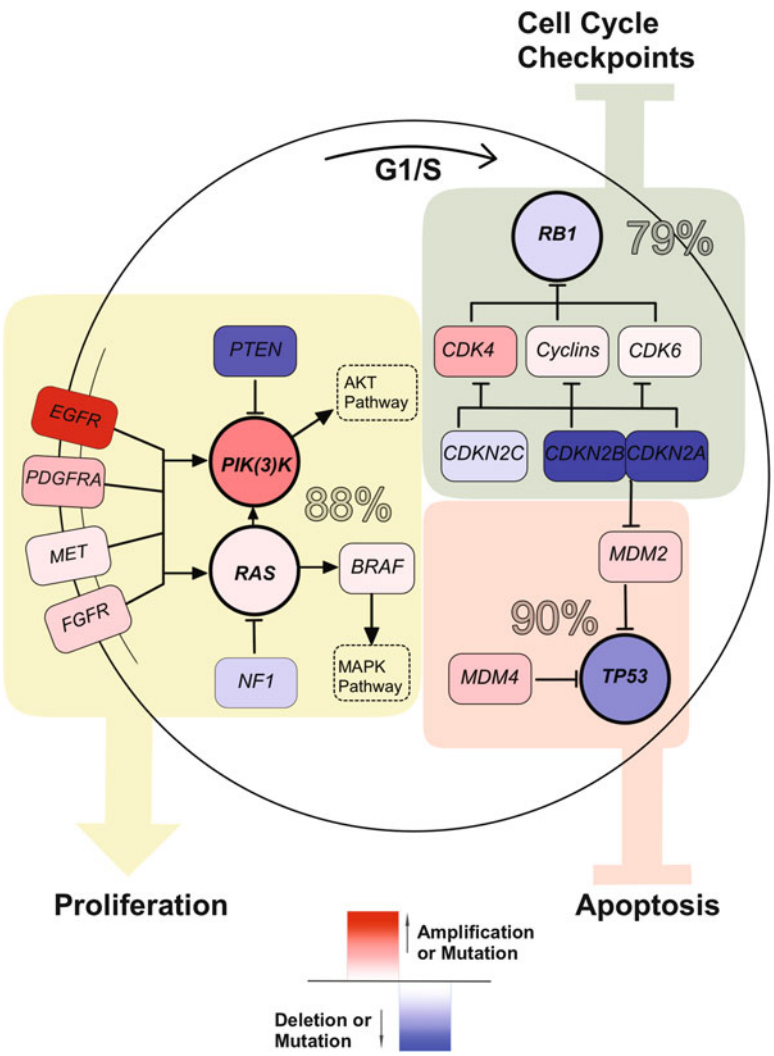


Fig. 35.3 Core defects in three signaling pathways involved in the pathogenesis of Glioblastoma. Overall alteration rate is summarized for the TP53 pathway (eluding apoptosis), PI(3)K/RAS pathway (increasing proliferation), and RB1 pathway (avoiding cell cycle checkpoints).

on genetic profiles giving valuable clinical information. The first subgrouping analysis on high-grade astrocytomas was reported by Philips et al. differentiating three distinct GBM clusters: proneural, proliferative, and mesenchymal, with the proneural subgroup predicting better survival [162]. A recent TCGA based study classified GBM into four molecular subgroups: classical, proneural, neural, and mesenchymal [155]. Genetic alterations in *EGFR*, *PDGFRA/IDH1* and *NF1* characterize the classical, proneural, and mesenchymal subgroups respectively [155]. Classical GBMs are further delineated by amplification of chromosome 7 paired with loss of chromosome 10, while often lacking *TP53* mutations [155]. No distinctive genetic alteration is known that distinguishes the Neural from the other subtypes [124]. It still has to be elucidated to what extent the subgroup defining molecular profile may affect clinical outcome and sensitivity to specific therapeutic agents. DNA methylation profiling of 272 TCGA glioblastomas revealed a subset of tumors with a hypermethylation phenotype that was assigned to the proneural subgroup, further correlated with *IDH1* mutations and in extended analysis also often found in low-grade gliomas [109, 163]. This glioma phenotype is an expression subtype and termed G-CIMP (CpG island methylator phenotype). G-CIMP positive gliomas affect younger patients, are probably more common in secondary GBM, and are associated with improved outcome [163]. In anaplastic oligodendroglioma the G-CIMP phenotype also consistently exhibit *IDH1*-mutations, *MGMT* promoter methylation, and LOH 1p/19q and is used as a predictor for better survival [164].

35.3.8 Medulloblastoma

Medulloblastomas are considered embryonal tumors arising from the dorsal brainstem or the cerebellum. Progenitor cells from the cochlear nucleus, dorsal brainstem, and neuron precursors from the external granular layer of the cerebellar germinal zone are the proposed cells of origin [165].

Medulloblastomas exhibit a high amount of heterogeneity at histomorphological and subcellular level resulting in variable clinical behavior. Histological subtypes comprise classical (70–80 %), nodular desmoplastic/extensive nodular (16 %) and large cell anaplastic (10 %), while all are classified as WHO Grade IV [9, 166]. Over the past few years novel molecular subclassifications have been established that put histopathologic differentiations into perspective.

35.3.9 Chromosomal Alterations

Almost all medulloblastoma samples show various numerical gains or losses of chromosomal regions. The most common specific cytogenetic abnormality seen in this tumor type is isochromosome 17q (iso17q), which occurs in about 50 % of medulloblastomas [167, 168]. The breakpoint is often in the proximal p-arm (17p11), producing a dicentromeric isochromosome. Loss of one arm of chromosome 17p (25–35 %) is often a consequence of isochromosome 17q formation, but may also occur in isolation [169, 170]. In certain cases, smaller deletions are seen, with the minimal deleted region occurring at 17p13.3 [171]. The frequent involvement of 17p in medulloblastoma has resulted in efforts to identify the targeted putative tumor suppressor gene (TSG). *REN(KCTD11)* maps to 17p13.2, and is a candidate TSG for medulloblastoma on chromosome 17p [172]. Aberrations of chromosomal regions have also been evaluated as prognostic markers, whereas iso17q and losses of 10q and 17p are correlated with a poor prognosis and loss of chromosome 6 (monosomy 6) with good outcome across all subgroups [173]. Loss of chromosome 9q, which contains the suppressor gene locus *PTCH 1*, promotes activation of Sonic Hedgehog (SHH) signaling pathway and is used as a co-determinant of the SHH molecular subgroup [11].

35.3.10 Gene Amplification and Overexpression

Gene amplifications are relatively infrequent in medulloblastoma (<10 %), and typically involve the *MYC* and *MYCN* proto-oncogenes [166]. Such amplifications have been observed in the context of double minute chromosomes [174]. Amplification and/or overexpression of *MYC*-family genes are regularly observed in the large cell anaplastic variant of medulloblastoma, and correlate with poor clinical outcome [175, 176]. In addition to gene amplifications, certain genes are aberrantly overexpressed in medulloblastoma such as *ERBB2* [177]. Increased ERBB2 and ERBB4 expression correlates with higher risk of metastasis and is associated with a poor prognosis [178–180].

35.3.11 Gene Mutations and Deletions

Various mutations of tumor suppressor genes important in developmental signaling pathways have been described in medulloblastoma. Mutations in *PTCH1, SMOH*, *Gli2*, and *SUFU* lead to loss of inhibitory factors and thus promote constitutive activation of SHH pathways [181–184]. Germ-line *APC* mutations as found in familial adenomatous polyposis (FAP) syndrome or Turcot syndrome predispose to medulloblastoma formation [86]. The APC-protein is encoded on chromosome 5 and is part of a protein complex inhibiting the WNT signaling pathway. Loss of APC function results in intranuclear accumulation of ß-catenin, which activates transcription factors and promotes the pathogenesis of medulloblastoma. Overall, mutations involving the WNT pathway occur in approximately 10 % of sporadic medulloblastoma cases, including activating mutations in the *CTNNB1* gene [185–188]. In addition, *TP53* mutations have been found in 10 % of medulloblastoma cases (16 % in WNT, 21 % in SHH, virtually absent in Group 3 and 4) and are considered a risk factor for poor outcome in the SHH subgroup [189].

35.3.12 Epigenetic Alterations

Epigenetic alterations such as modification of histone or DNA methylation status are important factors in medulloblastoma pathogenesis and mostly recorded in Group 3 and 4 medulloblastoma [190, 191]. Large-scale genomic analyses have identified *OTX2*, a developmentally regulated transcription factor important for early brain morphogenesis, as a frequently occurring focal genetic gain in medulloblastoma (21 %) [168, 192, 193]. Overexpression of *OTX2* results in impaired demethylation activity of histone modifying proteins. Consequently, levels of trimethylated histone H3 lysine 27 (H3K27me3) are increased which in turn leads to specific chromatin condensation [194]. Medulloblastoma patients with *OTX2* gene amplification (mostly restricted to subgroup 3 and 4) exhibited poorer survival and were more likely to have anaplastic tumors [193]. There is also epigenetic deregulation of DNA methylation as an underlying mechanism of medulloblastoma formation. Subgroup specific patterns of DNA methylation have already been proposed as a biomarker for improving survival prediction in non-WNT medulloblastoma [195, 196].

35.3.13 Subgroups

Advances in next generation DNA sequencing and ultrahigh-resolution genetic mapping over recent years have enabled

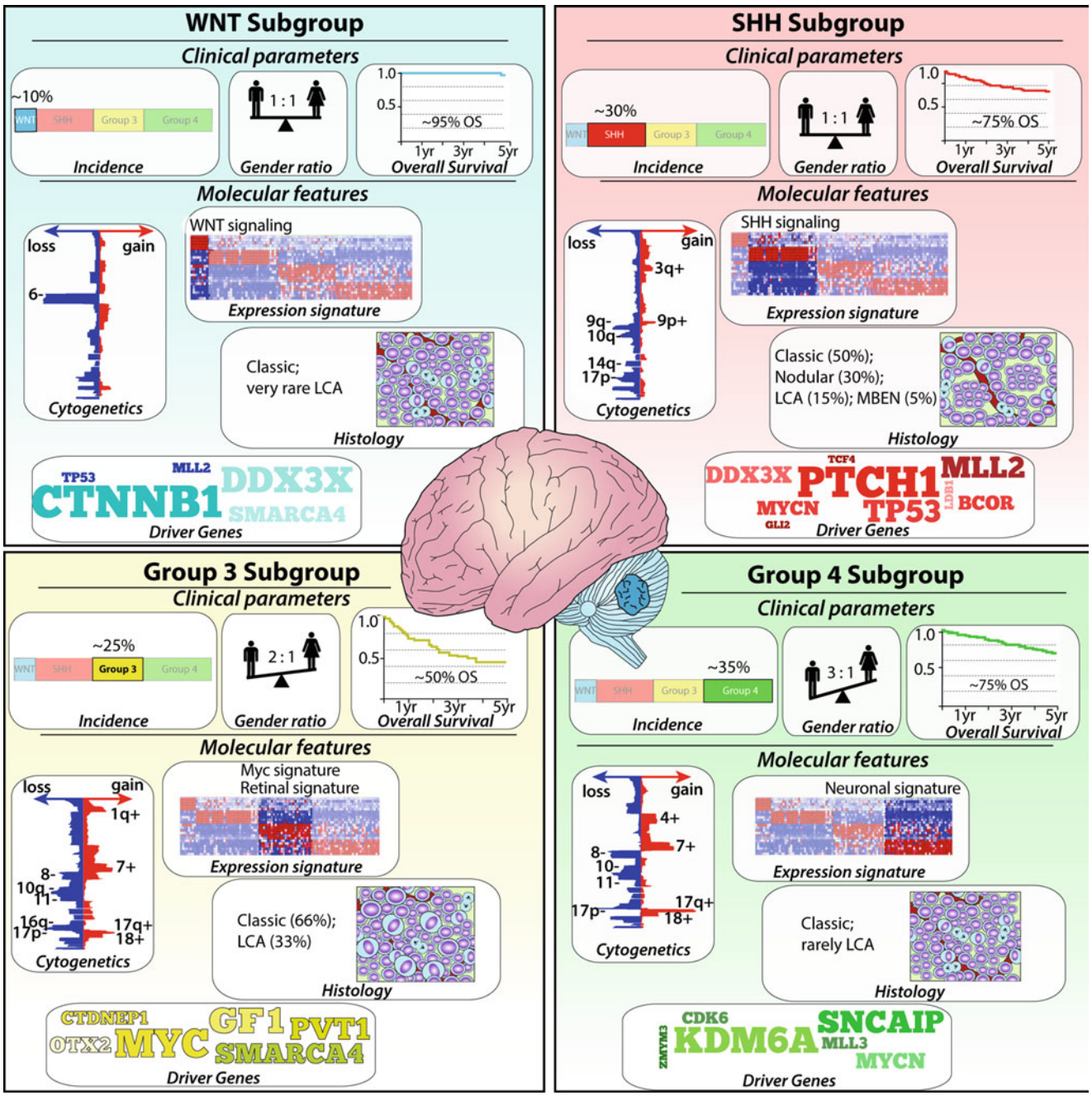


Fig. 35.4 Medulloblastoma Subgroups. Infographic illustrating the incidence, gender ratio, survival, histology, cytogenetic alterations, and common driver genes for each group. *LCA* large cell anaplastic, *MBEN* medulloblastoma with extensive nodularity.

remarkable insight into some of the underlying molecular interactions leading to the observed heterogeneity in clinical behavior. According to current consensus among investigators, four main subgroups can be distinguished: WNT, SHH, Group 3, and Group 4 [11, 197, 198] (Fig. 35.4). The WNT and the SHH group are marked by mutations in the WNT and SHH pathway, respectively. For Group 3 and 4, no single pathway alteration has been identified and the diagnosis relies primarily on transcriptional profile clusters. However, recent studies detected prevalent drivers in particular for Group 3 [199]. These molecular subgroups typically do not correlate with the histological phenotypes. The histopathological classic type occurs in all four medulloblastoma subgroups. The nodular desmoplastic or extensive nodular type falls within the SHH subtype, although it should be mentioned that the SHH subgroup is the only subgroup that may include all of the known major histological variants. Large cell anaplastic tumors are also found in all four subgroups but the majority will be classified to Group 3. The WNT subgroup has mostly classic histology and accounts for 10 % of

all medulloblastomas and has the best prognosis with >90 % long-term survivals. Alterations resulting in increased activity of the WNT pathway results from sporadic somatic mutations of *CTNNB1* encoding ß-catenin or less common in germ-line mutations of the *APC* gene as in Turcot or FAP syndrome. Monosomy 6 and *TP53* mutation are often found. The SHH subgroup (30 %) has a good to intermediate prognosis; almost half of the cases exhibit desmoplastic/nodular histology.

In sporadic SHH tumors, somatic mutation in *PTCH*, *SMO*, and *SUFU* leads to increased SHH activity. In addition, amplifications of *GLI1* and *GLI2* can trigger this pathway. Deletion of chromosome 9q, which includes the *PTCH* gene, is primarily limited to SHH tumors. Hereditary SHH medulloblastomas are found in Gorlin syndrome and are characterized by germ-line mutation of the tumor suppressor gene *PTCH1*. *MYCN* amplification and mutations at *TP53* are regularly observed. Recently, small molecules targeting smoothened (SMO), a positive regulator of the SHH pathway, showed rapid (although transient due to acquired drug resistance) regression of tumor volume in a subset of patients with SHH medulloblastoma [200, 201]. Group 3 medulloblastomas (25 %) primarily demonstrate classic histology but have a high incidence of large cell anaplastic types. They are almost never observed in adults and harbor the worst prognosis of all subtypes. Metastases are frequently found and coupled to poor prognosis. *MYC* and *OTX2* amplification are common features and some tumors show loss of both 5q and 10q and gain of chromosome 1q. Recently, somatic genomic rearrangements resulting in GFI1 and GFI1B activation have been found to occur in approximately one-third of Group 3 patients; thus enhancer hijacking is now considered a prominent mechanism driving Group 3 medulloblastoma [199]. Group 4 is the most common medulloblastoma subgroup (35 %), usually of classic histology and with an intermediate prognosis. Isochromosome 17q is the major cytogenic alteration observed (60–80 % of Group 4 samples). Amplification of *OTX2*, *CDK6*, and *MYCN* are also frequently associated with this subgroup, whereas structural variants associated with GFI1 and GFI1B activation are detected in 5–10 % [199].

Molecular classification will have a large impact on future treatment recommendations and strategies. Given the four subgroups show distinct clinical behavior that does not correlate with histological features, the use of histopathology alone is considered inadequate for the classification of medulloblastoma. It remains to be seen how the revealed diversity in molecular patterns and clinical outcome will influence the WHO Grade IV classification for medulloblastoma. The current treatment modality of surgery, radiation and chemotherapy is highly toxic to the developing brain. Complication and morbidity observed in patients with tumors subgrouped into good prognosis, such as the WNT subgroup, could correspond to a higher extent with iatrogenic interventions than with the natural course of disease. These patients may benefit from a reduction in radiation and chemotherapy, whereas patient with tumors subgrouped into more aggressive lesions may benefit from exhaustive therapeutic strategies.

35.3.14 Ependymoma

Ependymomas originate from the wall of the ventricular system with possible manifestations along the entire craniospinal axis of the CNS [202]. Radial glia cells are presumed to constitute the progenitor cells [203]. In children, over 90 % of ependymomas occur intracranially with 70 % arising in the posterior fossa, whereas in adults the spinal manifestation is more common [204–206]. Based on histopathological features, the WHO divides ependymomas in grade I–III and recognizes four histological types: subependymoma (grade I), myxopapillary (grade I, usually spinal), classic (grade II), and anaplastic (grade III) [9, 202]. However, as for most tumors, molecular characteristics will have to complement and specify future grading systems. Recent studies indicate that ependymomas from different compartments of the CNS display distinct genetic signatures, reflecting their unique origins, despite appearing histologically homogeneous [203, 207].

35.3.15 Chromosomal Alterations

Cytogenetic changes are seen in a significant proportion of ependymomas and are overall more common in adults [206, 208, 209]. About 30 % of sporadic ependymomas have monosomy 22, and deletions or translocations involving chromosome 22 are also observed [210]. The *NF2* gene is located on chromosome 22 (22q12), and NF2 patients are predisposed to develop spinal ependymomas in addition to other CNS tumors [211]. In fact, *NF2* mutations are almost exclusively found in spinal ependymomas [212], thus another tumor suppressor gene on chromosome 22 might be involved in cranial ependymomas [210, 213]. Gain of chromosome 1q is the most common genomic aberration in pediatric intracranial ependymomas, possibly associated with anaplastic histology [206, 214]. Besides numeric chromosomal alterations within the ependymoma genome, translocations are frequently reported to affect chromosomes 1, 11, and 22 [208]. The assumption that tumor location-specific genomic alterations exist in ependymoma has been encouraged by recent revelation of chromothripsis within chromosome 11q13.1 [215]. In more than two-thirds of supratentorial ependymoma samples, a fusion of the oncogene *RELA* to an uncharacterized gene involving 11q13.1 occurs, which is absent in posterior fossa tumors. The aberrant RELA-fusion proteins modulate activation of the transcription factor *NF-kB*. The largest molecular analysis of ependymoma to date, including 583 tissue samples, demonstrated that posterior

fossa ependymomas can be classified in to two subgroups, Group A and B, which differ in their genomics, transcriptomics, location and clinical outcome [209]. Group A ependymomas affect younger patients, are more laterally located and exhibit more frequent recurrence and metastasis with an overall poorer prognosis compared with Group B. The genome in Group A is more balanced, shows increased occurrence of chromosome 1q gains, whereas in Group B the genome is highly unstable with frequent segmental gains of chromosomes 9, 15, 18, as well as losses of chromosomes 6 and 22 [209].

35.3.16 Gene Amplification, Overexpression, Mutations, and Deletions

In ependymoma and especially in children, the overall genetic mutation rate appears to be lower than astrocytic tumors. Expression profiling by microarray analysis on pediatric ependymomas have identified a subset of genes abnormally expressed in this tumor, including increased expression of EGFR, WNT5A, TP53, and many cell cycle, cell adhesion, angiogenesis, and cell proliferation genes, with downregulated genes including *SCHIP-1* and *EB1* [213]. EGFR overexpression has been recorded as a prognostic marker in intracranial grade II ependymomas correlating with reduced progression free and overall survival [216, 217]. Amplification and rearrangement of the *c-erb B* gene has been identified, as well as rearrangements of the *MEN1* gene (11q13) [212, 218]. A large genomic analysis of ependymoma including tumors from 207 samples demonstrated focal amplification of genes involved in stem cell proliferation, pluripotency, and neuronal differentiation such as *THAP11, PSPH, EPHB2, KCNN1, RAB3A, PTPRN2, PCDH* cluster, and *NOTCH1* [207]. This same study identified focal deletions of known tumor suppressor genes (*PTEN, INK4A/ARF)* and novel genes such as *STAG1* and *TNRC6B* [207].

35.3.17 Epigenetic Alteration

Epigenetic modifiers involved in the formation of ependymomas have recently been identified. The methylation status of CpG islands occurs more frequently in posterior fossa Group A (PFA) tumors, leading to transcriptional silencing of the histone methyltransferase *Polycomb repressive complex 2 (PRC2),* which in turn results in repressive effect on expression of cell differentiation genes [219]. This epigenetic mechanism predicts for the more aggressive behavior of Group A tumors. Mice xenograft models implanted with human PFA showed a decreased tumor volume and longer survival after that PRC2 was reactivated through demethylation by administration of established epigenetic drugs that target either DNA or histone methylation [219].

35.4 Cell Cycle Dysregulation and Mitogenic Factors in Human Brain Tumors

The cell cycle can be conceptualized as a circle divided by a number of discrete phases during which nuclear content changes in preparation for cell division (Fig. 35.5). In healthy neural tissue, with advancing differentiation and age, most cells will become quiescent and do not express cell cycle genes due to the presence of cell-type specific repression mechanisms. In human brain tumors, the intrinsic machinery of the cell cycle is the target of mutations or functional derangements leading to release of repression mechanisms and rapid, uncontrolled proliferation—a hallmark of cancer.

35.4.1 General Mechanisms of Cell Cycle Dysregulation

Cyclins (A-E) are a group of proteins whose expression levels are differentially and tightly regulated during the cell cycle. When bound to their respective cyclin dependent kinases (CDKs), an activated complex promotes transcription of products required for a specific stage in the cell cycle [220]. The activity of the various CDKs are coupled to both binding of the cyclins and a series of phosphorylations and dephosphorylations on specific residues by cyclin-dependent kinase activating kinases (CAKs) and cyclin-dependent kinase inhibitors (CKIs) [221, 222].

One of the most important cell cycle pathways known to be perturbed in human brain tumors, especially astrocytomas, is the cyclin D-CDK4-RB-E2F pathway, (RB-pathway) (Fig. 35.5). Here, cyclin D forms an activated complex with CDK4 and/or 6. This active complex partially phosphorylates a previously hypophosphorylated retinoblastoma protein, RB [221, 222]. Partial phosphorylation of RB liberates E2F-DP such that it can act to upregulate target genes involved in the continued regulation of cell cycle, such as cyclin E [221, 222]. Thus, the phosphorylation of RB is a key regulatory step of the G_1–S checkpoint.

Whereas the cyclins and CDKs could be termed accelerators of the cell cycle, inhibitor of cyclin-dependent Kinase 4 (INK4) and Cip/Kip could be termed the brakes of the cell cycle. INK4 is a family of four proteins A-D, $p16^{INK4a}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$. INK4A is encoded from a gene on 9q21. Interestingly, this gene also encodes for a novel second protein, using an alternative reading frame in exon 1 and splicing it to the common exons 2 and 3. This protein is called ARF ($p14^{ARF}$), alternative reading frame, and is also involved in cycle arrest with p53.

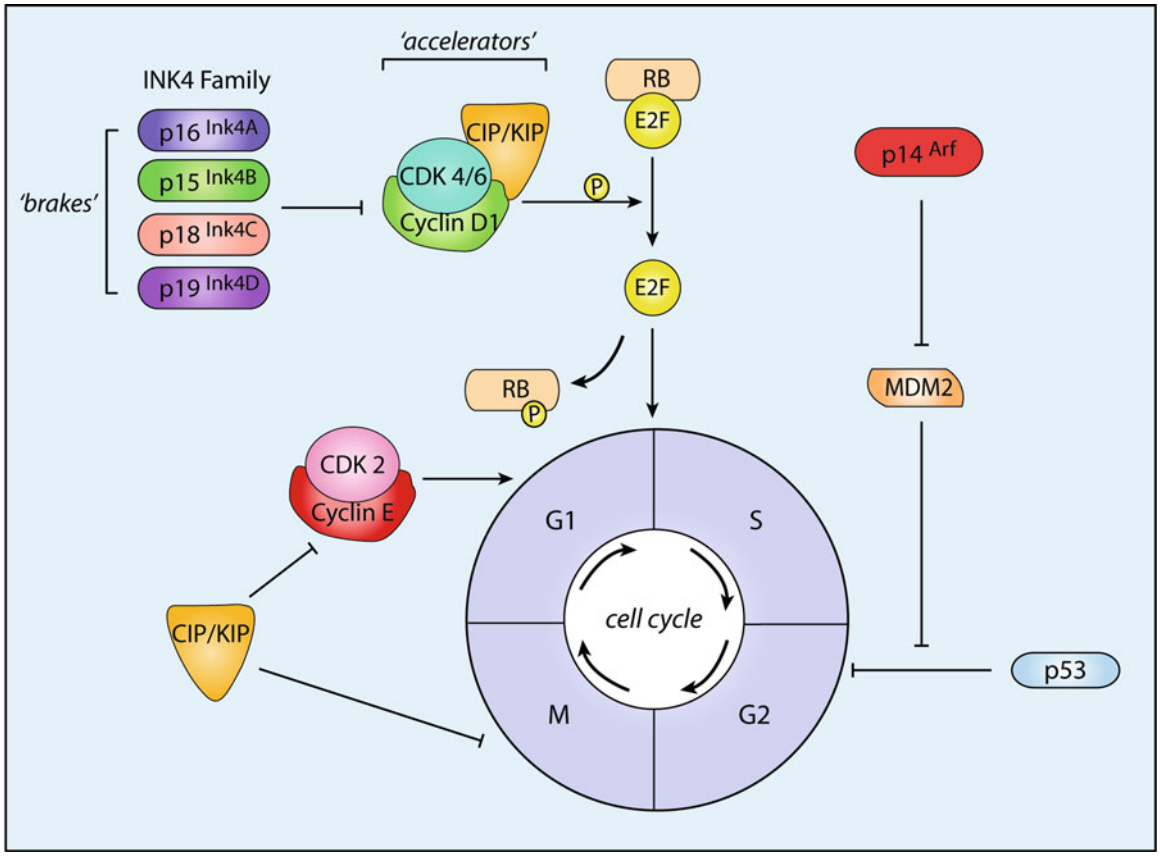


Fig. 35.5 Dysregulation in cell cycle pathways involved in the pathogenesis of astrocytomas.

The Cip/Kip family of CDK inhibitors ($p21^{CIP1}$, $p27^{Kip1}$, $p57^{Kip2}$) can bind and inhibit the G1/S cyclin-CDK complexes, and the mitotic cyclin B/A complexes. They can also act as both positive and negative regulators of the cell cycle. In early G1 phase Cip/Kip family members bind and aid in the formation of cyclin D/CDK4/6 complexes without inhibiting kinase activity and therefore contribute to the formation of hyperphosphorylated RB and cell cycle progression [223]. Once RB is partially phosphorylated and transcription of Cyclin E and Cdk 2 are upregulated, the Cip/Kip members then bind with higher affinity to Cyclin E/CDK 2 complexes and inhibit CDK 2 activity and halt cell cycle progression (Fig. 35.5).

35.4.2 The Importance of p53 and Cell Cycle Control

The *TP53* gene encodes the most well studied tumor suppressor, p53 and to date has been implicated to play a role in almost 50 % of human cancers, including human brain tumors [224]. Mutations in the *TP53* gene can lead to missense or loss of protein expression, whereas the missense mutation is associated with earlier cancer onset and more aggressive tumor profile [225, 226]. P53 has a multitude of stress-induced effects on cellular homeostasis by blocking cell cycle passage and promoting apoptosis. Several cell cycle proteins interact as negative or positive regulators with p53. One such mechanism of regulation of p53 is via the Murine Double Minute-2 protein (MDM2) (Fig. 35.4), which is able to repress p53 activity both at the transcriptional and protein levels [227–229]. MDM2 binds and conceals the p53 transactivating domain and blocks the basal transcriptional machinery for p53. In response to DNA damage both MDM2 and p53 are phosphorylated resulting in conformational changes that incapacitate binding [230, 231]. This allows p53 to be stabilized in the nucleus where it is able to upregulate genes involved in either cell cycle arrest or apoptosis. The ability of MDM2 to block the p53 pathway is reversed by ARF which binds to and sequesters MDM2 in the nucleolus away from p53, as also impairs MDM2's ubiquitin ligase activity [232–234]. This allows for stabilization of p53 levels and downstream activation of p21 and cell cycle arrest.

35.4.3 Mitogenic Signals That Impact on Brain Tumor Growth

The decision of the cell to divide is provided by both internal stimuli and external conditions. External conditions favorable

to cell division, growth, and survival are conveyed via cell membrane tyrosine kinase receptors that typically dimerize upon ligand binding, phosphorylate their cytoplasmic tails and activate downstream signaling molecules. One key growth activator is the small GTPase Ras. It activates multiple mitogenic pathways: RAF-MEF-MAPK, CDC42-RAC-Rho, and PI3K-PTEN-AKT [235]. These downstream signaling molecules subsequently activate cyclin/CDK complexes triggering growth, demonstrating these pathways are commonly targeted during tumor mutagenesis.

Epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), C-Met, and C-kit are all tyrosine kinases implicated in the formation of astrocytomas and other neurological tumors.

35.4.4 Analysis of Cell Cycle and Mitogenic Pathway Disturbances in Brain Tumors: Low and High Grade Astrocytomas

Our understanding of the pathways involved in tumor initiation and progression has been enhanced through molecular genetic analyses of cell cycle and mitogenic stimulation pathways. Genetic alterations of *TP53* are present in low grade, anaplastic and GBMs, and the genetic aberrations are higher for the secondary GBM (up to 80 %) compared to the primary GBM (around 30 %) suggesting a role in astrocytoma initiation [114, 123].

Mutations in the RB pathway of *INK4A, CDK4*, *and RB* represent about 80 % of GBMs and only a small portion of low-grade astrocytomas [109] [236]. There are also differences between primary and secondary GBMs. Deletion of the *CDKN2A* locus leading to reduced CDK4 inhibition is found in over 50 % of primary and 20 % of secondary GBMs [114, 130, 131]. This locus encodes both *INK4A* and *ARF* and therefore disrupts both the p53 and RB pathways indirectly, perhaps contributing to the rapid rate of growth attributed to primary GBMs. Alterations of *RB* itself are found in up to 14 % of GBMs [130, 131]. *CDK4* amplification is present in 14 % of GBMs. Other mutations found to arise in GBMs have been identified in *CDK6*, *cyclin D*, and *cyclin E* [237, 238].

EGFR is amplified or altered in up to 57 % of glioblastomas, mainly detected in the primary and rarely in secondary GBM, which is why this alteration might not be involved early in tumor initiation [109, 239]. A mouse model expressing the mutated *EGFR* alone was insufficient to cause astrocytomas, but expressed in a background of *TP53* +/− or *Ink4A/ARF*−/− astrocytoma formation occurred rapidly [240]. This suggests that EGFR may be responsible for tumor progression but require additional mutations in other genes for tumor initiation.

PDGFRA is a surface tyrosine kinase receptor repeatedly found to be altered in both high and low-grade human astrocytomas [109, 241]. As a growth factor it interacts with the MAPK/RAS pathway, which is implicated in primary GBM formation, however the role of PDGFR in tumor initiation or progression has yet to be elucidated.

The tumor suppressor PTEN acts as a negative regulator of the PI3K-AKT growth control pathway. *PTEN* mutations may be found in 30 % of primary GBMs and up to 8 % of secondary GBMs [114], however these numbers are inconsistent among the different study groups to allow conclusive interpretation regarding order in succession of tumorigenesis.

35.5 Brain Tumor Migration and Invasion

Brain invasion by infiltrative tumor cells is one of the most sinister histopathological hallmarks of malignant brain tumors. Brain tumor invasion is a complex cellular phenomenon including permissive changes in the extracellular milieu and intracellular biomechanical systems. The spread of brain tumor cells within the cerebral tissue involves changes in cell/cell and cell/extracellular matrix (ECM) adhesion, enzyme degradation of the ECM by proteases, and cell motility into normal brain parenchyma [252, 253]. Specifically, cell migration requires a complex balance between extracellular cues and responsive intracellular signals that lead to dynamic regulation of the interactions between actin microfilaments, microtubules, intermediate filaments and associated adhesions [254, 255]. The driving force for cell movement is normally provided by dynamic reorganization of the actin cytoskeleton, which directs protrusions at the front of the cell and retraction at the rear [254, 255]. For a cell to move, it must establish polarity resulting in leading and trailing edges with directionalized forces. One of the first steps in cell migration is the formation of actin-rich structures called lamellipodia at the leading edge of the motile cell [256, 257]. Subsequent attachment of these protrusive structures to the substratum followed by tension across the cell, generated by myosin motor proteins, will lead to the contractile force ultimately required for cell body translocation [256, 257].

Ultimately, this interplay of contractile elements with adhesive matrix increases metabolic demand and triggers enzymes for enhanced glycolysis. As shown recently, GBM cells overexpress glucose-6-phosphatase (G6P) to overcome glycolytic inhibition [258]. By downregulating glucose-6-phosphatase in human derived GBM cells the migration and invasion rate was markedly reduced [258].

Migration along the tight extracellular space requires also considerable adaptation in volume and shape of glioma cells [253, 259], which is mediated by modulation of Cl^- and K^+

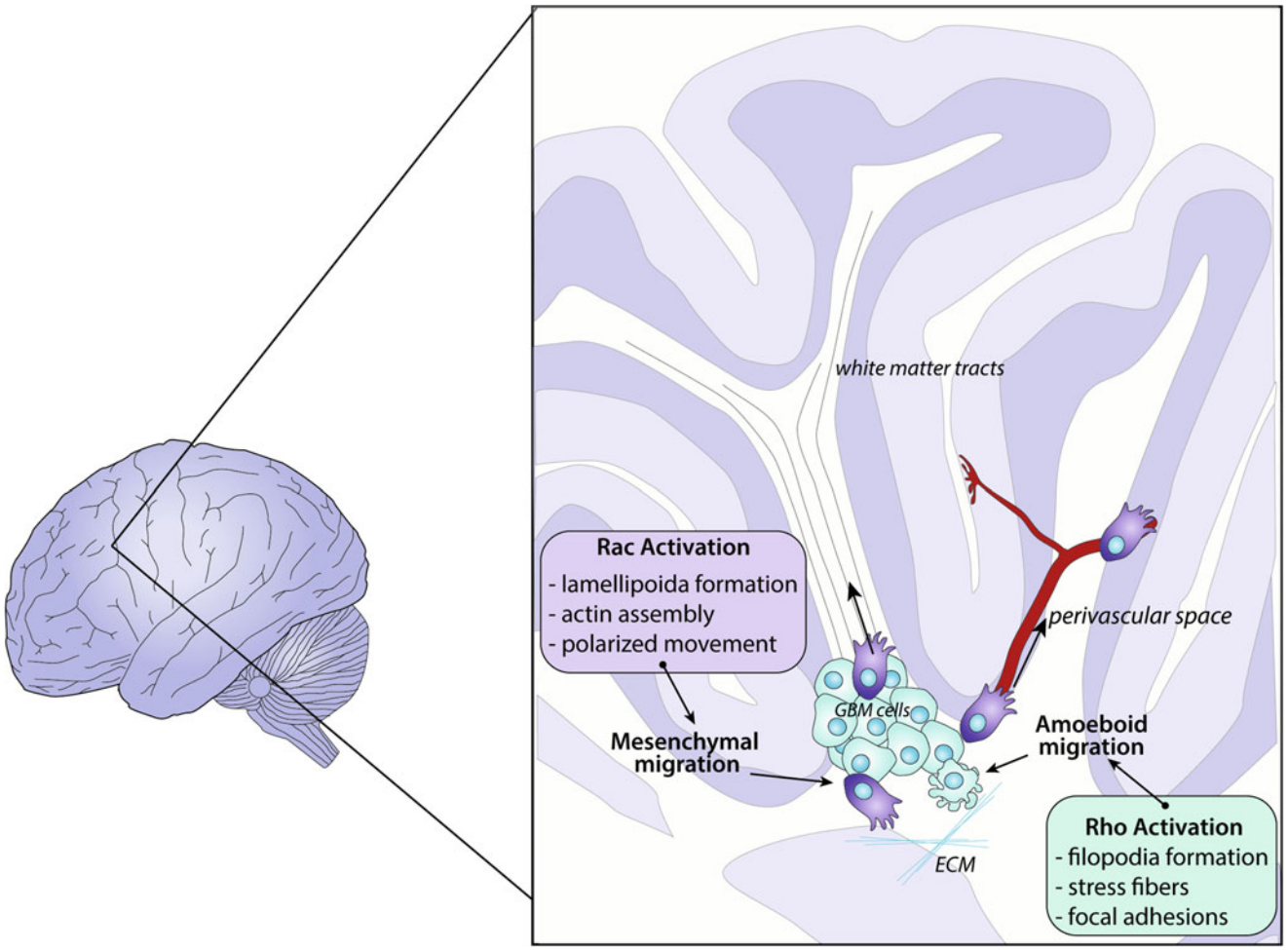


Fig. 35.6 Mechanisms involved in the migration and invasion of Glioblastoma (GBM) cells along the perivascular space and white matter tracts.

channels. The Na^+-K^+-Cl^- co-transporter (NKCC1) is highly expressed in glioma cells, which accumulates intracellular Cl^- leading to opening of Cl^-channels (ClC3) and resulting in volume shrinkage [260]. In recent studies, glioma migration and invasion could be decreased after inhibiting NKCC1 or ClC3 channels [261, 262].

In contrast to solid tumors of other organs, malignant glioma cells usually do not spread through an intravascular route but rather within the perivascular zone. Moreover, lymphatic channels do not exist in the brain. Despite their profound parenchymal invasiveness, metastasis outside the brain is negligible in primary malignant gliomas [263], which is likely due to the blood–brain barrier and an unsuitable environment for growth in other organ systems. The migration is mainly directed into two compartments: the perivascular space and the brain parenchyma, which then results in a spreading of glioma satellites at distant sites from the main tumor mass [253] (Fig. 35.6). The alignment towards existing structures such as white matter tracts, blood vessels and meninges resembles the movement observed in neural progenitor cells during tissue maturation [253].

There have been numerous reviews describing many of the extracellular factors relating to invading brain tumor cells such as the proteolytic degradation by various proteases (MMPs, cathepsins, serine proteases, urokinase plasminogen activator receptor), and ECM deposition and adhesion facilitated by matrix receptors [252, 264, 265]. The increased release of glutamate by glioma cells additionally acts as an autocrine and paracrine ligand supporting Ca^{2+} triggered cell migration and invasion [266]. Indeed, blocking intracellular glutamate uptake through sulfasalazine resulted in reduced tumor spreading in a rodent model [266]. There are also many well characterized genetic lesions in brain tumors which have been shown to affect astrocytoma progression [254, 267, 268], but the understanding of the intracellular and molecular mechanisms that mediate brain tumor invasion is still in its infancy. These molecular pathways include signaling regulated by (1) the non-receptor tyrosine kinases

focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2), (2) members of the Rho family of small GTPases, (RhoA and Rac1) including signaling by lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), and (3) the nuclear factor-kB (NF-kB) family of transcription factors.

The Rho family of small GTPases is essential in cellular migration pathways. RhoA activation mediates formation of stress fibers and focal adhesions in the trailing edge of the cells and leads to a rounded amoeboid form of movement, while Rac1 directs actin assembly in lamellipodia at the leading edge of migrating cells which results in cells moving in an elongated mesenchymal manner [269, 270]. Attempts to reduce cell motility by blocking migration pathways have to take into account that cells can switch between these different modes of migration. CDC42 is a well-studied member of the Rho family and, while not essential for cellular movement, aids in regulating filopodia formation and cellular polarity [271, 272]. Inhibition of Rac1 with siRNA leads to decreased lamellipodia formation and cellular movement [273, 274]. Using a dominant negative Rac1 inhibitor induced death on GBM cells but not normal astrocytes [275]. Additionally, it was shown that downstream inhibition of Rho-Kinase (downstream of RhoA activation) lead to increase motility through activation of Rac1 [276]. Therefore, it may be a balance between RhoA and Rac1 that regulates cellular migration and invasion in astrocytoma cells.

The greatest limitation of brain tumor invasion research to date has been the lack of a reliable and reproducible in vivo animal model of brain invasion. Current applications of existing animal models are currently not optimized or characterized for use in brain tumor invasion research. Induction of invasion in vivo, tracking infiltrative cells and quantitation of brain invasion are the most critical considerations of a live animal model of brain tumor invasion.

35.6 Brain Tumor Angiogenesis

Angiogenesis is defined as the formation of new blood vessels from the sprouting of previously existing vessels. In the fetal brain, angiogenesis begins during embryogenesis when soluble angiogenic factors are secreted to cause capillary sprouting from the extracerebral plexus and neovascularization. In contrast, the adult brain shows very little angiogenic activity due to expression of antiangiogenic factors.

Solid tumors beyond 2 mm in diameter require recruitment of blood vessels for continued growth [277]. In pathological states such as tumors, an angiogenic signaling switch occurs that results in a cascade of events including enzymatic degradation of basement membrane proteins, endothelial migration, endothelial proliferation, and tubular vessel formation. The most important mitogen orchestrating vascular neogenesis in brain tumors is vascular endothelial growth factor, VEGF.

35.6.1 Mechanisms of Angiogenesis in Human Astrocytomas

A distinguishing histopathological feature of astrocytoma is endothelial proliferation, which can be 40 times higher than in normal brain [278]. VEGF overexpression is central to the neovascularization observed in these high-grade lesions such as GBM [279–281]. Monoclonal antibodies directed against VEGF in GBM mice xenografts and withdrawal of VEGF from implanted GBM cell lines in mice resulted in vessel regression underscoring the importance of VEGF in these lesions [282, 283].

VEGF is a 34–45 kDa glycosylated homodimeric protein that acts as a specific mitogen and chemotactic factor for endothelial cells [284]. There are five other VEGF homologs, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PGF) [285]. VEGF, itself, is composed of five splice variants, which have equivalent effects in regards to angiogenesis [286]. Expression of VEGF results in increased permeability of the microvasculature, endothelial production of proteases to break down basement membrane, endothelial survival, and endothelial proliferation [287].

The effects of VEGF are exerted via the tyrosine kinase receptors vascular endothelial growth factor receptor one and two, VEGFR1-2, and neuropilin-1 (Nrp-1). Binding of VEGF to VEGFR-2 promotes endothelial proliferation, whereas binding to VEFGR-1 regulates endothelial cells [288]. VEGF-2 is considered a core driver in complete activation of the angiogenic cascade and thus progression of neovascularization observed in high grade astrocytomas [280].

Central to the expression of VEGF is hypoxia. Increased transcription of VEGF mRNA in vitro can be efficiently induced by hypoxic conditions and upon restoration of oxygen levels, basal VEGF expression is returned [289]. Rapidly proliferating tumors, such as GBMs, quickly outstrip their blood supply creating a hypoxic and necrotic environment. Indeed, VEGF expression of cells at the rim of necrosis shows 50 times basal VEGF expression [290, 291]. This is mediated through hypoxia inducible factor, HIF-1 [292].

HIF-1 is a heterodimer consisting of an α and β subunit. Under normal physiological conditions the α subunit is ubiquitinated by the tumor suppressor von Hippel–Lindau, VHL [293]. As a result of hypoxia, HIF-1α interacts with the hypoxia-response element leading to the increased expression and upregulation of pro-angiogenesis genes, such as VEGF [292]. HIF-1 also cooperates in the stabilization of the VEGF protein and increasing VEGF secretion [294]. VEGF expression

can also be upregulated by fibroblast growth factor (FGF), PDGF, EGF, tumor necrosis factor α, (TNF-α), transforming growth factor β (TGF-β), and interleukins 1 and 6 [287].

Angiopoietins are a four-member family of secreted ligands that bind to the endothelial specific tyrosine kinase Tie-2. The two best understood angiopoietins are angiopoietin 1 (Ang-1), and angiopoietin 2 (Ang-2), which act antagonistically at the Tie-2 receptor. In the presence of VEGF, Ang-2 promotes vessel destabilization in preparation for neovascularization [295]. The combination of VEGF and Ang-2 is found at the hypoxic rim of GBMs [296], whereas Ang-1 stimulation of Tie-2 results in vessel stabilization and is found at the tumor periphery [287].

Bevacizumab, a human monoclonal antibody against VEGF-A, leads to a marked reduction in tumor size and prolongation of progression-free and overall survival in GBM patients in promising preclinical and clinical studies [297, 298]. Two highly awaited and large Phase III randomized trials have been conducted recently (RTOG-0825 and AVAGlio trial) [299, 300]; however neither study demonstrated any benefit in overall survival, albeit a slight increase in progression-free survival was observed.

35.7 Developmental Signaling Pathways and Human Brain Tumors

During normal neuroembryogenesis multiple signaling pathways are active to allow fast proliferation and increased migration of neural progenitor cells. The phase of differentiation and maturation requires a precisely adapted regulation of expression and silencing of the involved signaling networks. In the past decade, an increasing number of genes and pathways involved in this maturation process have been discovered and characterized. As advanced molecular analysis in human brain tumors recurrently revealed mutations and other alterations in these genes, it is assumed that such alterations are involved in the early phase of brain cancer initiation and eventually enable tumor cells to acquire proliferation and migration proprieties similarly found in primitive neural cells. Here we discuss three developmental signaling cascades with proven associations with formation of human brain tumors.

35.7.1 The Notch Signaling Pathway

The Notch signaling pathway mediates cell–cell signaling and is a key developmental pathway regulating cell decisions regarding cell survival, maintenance of stem cell self-renewal, proliferation, differentiation, and apoptosis [301–303]. The Notch protein forms a transmembrane receptor, whereas the extracellular region contains an epidermal growth factor-like ligand domain. When engaged with ligand from the *Jagged* or *Delta* families, the extracellular component gets cleaved by ADAM metalloproteases, which then leads to a second cleavage at the transmembrane region by a *γ-secretase*. This second proteolytic event releases the Notch intracellular domain which promotes transcription of Hes family genes, among others [301] (Fig. 35.7).

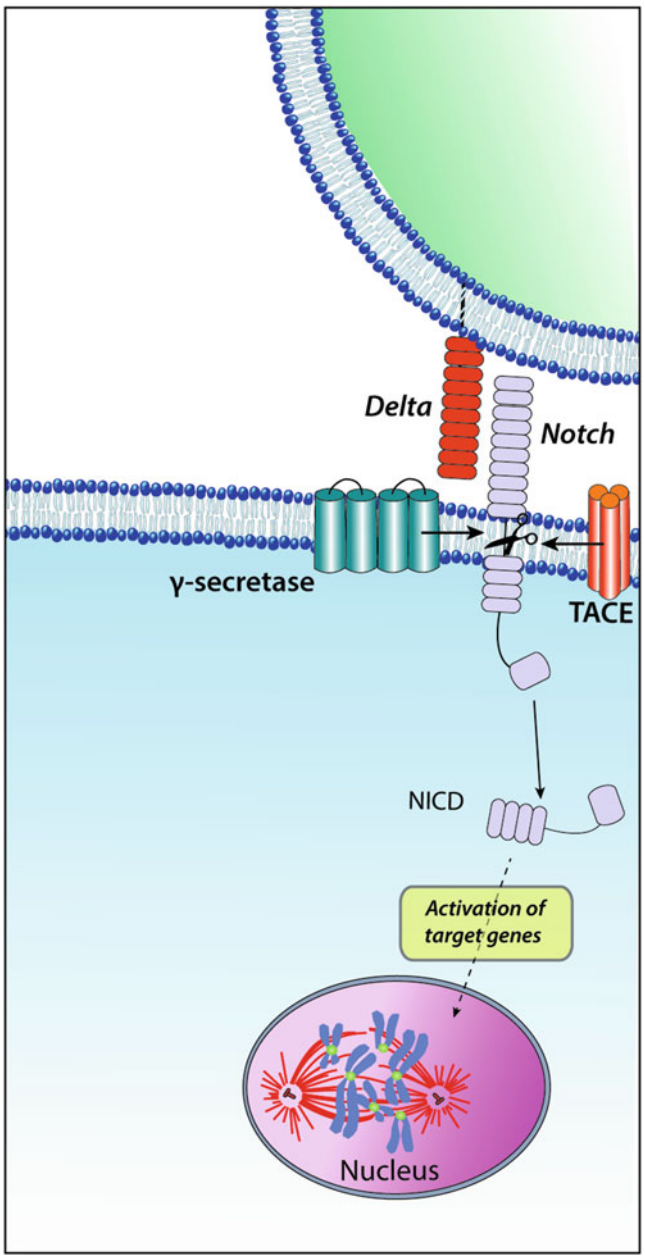


Fig. 35.7 Notch signaling pathway.

The functions of the Notch genes (Notch1-4) are time-dependent and cellular context-dependent, and thus, they may act as an oncogene or a tumor suppressor [304–306]. Activation of Notch signaling is known in various human neoplasms such as cervical carcinomas, T-cell leukemia, pancreatic

cancer, and mucoepidermoid carcinoma [307, 308]. In the nervous system, Notch regulates stem cells in developing and modeling the shape and cytoarchitecture of brain and spinal cord [309, 310]. Notch upregulation was observed in glioma cell lines and primary human glioblastomas resulting in stem cell maintenance and tumor proliferation [311, 312]. Specifically, the self-renewal of stem cell-like GBM cells is sustained in the perivascular stem cell niche by endothelial cells, which provide Notch ligands, promoting radioresistance [313, 314]. Notch inactivation by *γ-secretase* inhibitors (GSI) resulted in increased radiosensitivity through regulation of the PI3K/Akt pathway [314]. GSI induced suppression of Notch can also enhance the antitumoral effect of temozolomide (TMZ) [315]. From the four GBM subgroups, the proneural type particularly shows high Notch pathway activation and sensitivity to GSI in the mouse model [316].

Notch signaling deregulation also occurs in medulloblastomas, primarily within the SHH subgroup, where *Notch 2* amplification and overexpression correlated with tumor progression and poor prognosis [317–319]. Interestingly, inhibiting the Notch pathway in medulloblastoma cell lines using inhibitors of GSI could decrease the number of CD133+ cells and block in vivo engraftments [320]. The role of *Notch* in medulloblastoma genesis and how the Notch pathway interacts with the SHH pathway remains to be elucidated given that recent studies demonstrate that Notch signaling may not be crucial for SHH-driven medulloblastomas [321–323].

Several studies reported an upregulation of the Notch pathway in ependymomas, supporting its potential role in the pathogenesis of these tumors [203, 324, 325]. Taylor et al. identified genetically distinct ependymomas by patterns of gene expression that recapitulated those of radial glial cells in corresponding regions of the CNS [203]. Specifically, it was shown that supratentorial tumors expressed markedly elevated levels of Notch family members.

35.7.2 The Sonic Hedgehog Signaling Pathway

The SHH signaling pathway plays a primary role in morphogenic development of different organs during embryogenesis [326]. The secreted Sonic Hedgehog ligand, encoded on chromosome 7q36, binds and inactivates the transmembrane receptor Patched (PTCH), which results in releasing the inhibition of Smoothened (SMO) [304, 327]. SMO subsequently acts to activate transcription factors of the GLI family to express target genes, while SUFU (Suppressor of Fused) regulates Gli (Gli1–4) proteins to inhibit the SHH pathway [327, 328] A model of SHH signaling is shown in Fig. 35.8.

Dysregulation of the SHH pathway is implicated in cancers of different tissues, including the brain, lung, mammary gland, prostate, and skin [329]. In the nervous system, SHH is crucial for pattering the midline structures in brain and spinal cord as well as controlling of axonal spreading and stem cell proliferation [330]. Proper regulation of SHH is particularly critical for normal development of the cerebellum, explaining the frequent implication of this pathway in medulloblastomas when one or more genes of this pathway are dysregulated [331, 332]. Through analysis of gene expression in medulloblastomas, it has been shown that tumors with mutations in SHH pathway express high levels of Gli1, as well as N-myc and C-myc, cyclin D1 and D2 [333]. Interestingly, sporadic Gorlin's syndrome and medulloblastomas are often characterized by inactivation of *Ptch1* gene or activation of SMO [80, 184, 334]. *Bmi1*, a polycomb group gene, is involved in the clonal expansion of granule cell precursors, and its overexpression is linked to that of Ptch and is ultimately required for SHH driven tumorigenesis [331, 332]. A recent genome sequencing of SHH pathway medulloblastoma showed that the most frequently detected gene mutations were found in *PTCH1* (45%), *SMO* (14%), and *SUFU* (8%) [335]. Interestingly, while *PTCH1* mutations were found at roughly equal distribution in infants, children, and adults, *SMO* alterations were markedly more frequent in adults, and *SUFU* almost exclusively in infants under 3 years [335]. On the other hand, children older than 3 years exhibited strong *MYCN* and *GLI2* amplification together with *TP53* mutations (possibly in correlation with Li–Fraumeni syndrome), while all of these findings were rare in infants and adults [335].

SHH pathway is best studied for the pathogenesis of medulloblastoma, but there is increasing data revealing involvement in glioma incurrence. Amplification and overexpression of *Gli1* has been associated with some low- and high-grade astrocytomas [336, 337], and is assumed that the SHH pathway might have a stronger role in glioma initiation compared to other developmental pathways such as Notch or WNT [338]. Furthermore, it has been demonstrated that $CD133^+$ glioblastoma cancer stem cells (CSC) are promoted by endothelial cells through SHH pathways and inhibition of SHH by SMO knockdown in vitro and in vivo results in reduction of CSC phenotype-appearance [337]. High expression of SHH and Gli1 in glioma patient samples were correlated with both increasing WHO grade and worse prognosis [337].

Therapeutic approaches targeting the SHH pathway have mainly focused on SMO inhibition [335, 339]. Preclinical and clinical studies appeared initially promising with evidence of marked reduction in tumor growth; however, point mutations in *SMO* and *GLI2* amplifications eventually lead to drug inefficacy [340, 341]. Recent xenograft models revealed that SHH medulloblastomas with *PTCH1* mutations were responsive to SMO-inhibition, while tumors with *SUFU* or *MYNC* mutations, which are often found in infants, showed resistance [335]. The addition of PI3K inhibitors to SMO antagonist therapy may be a strategy to delay the development of resistance [340, 341].

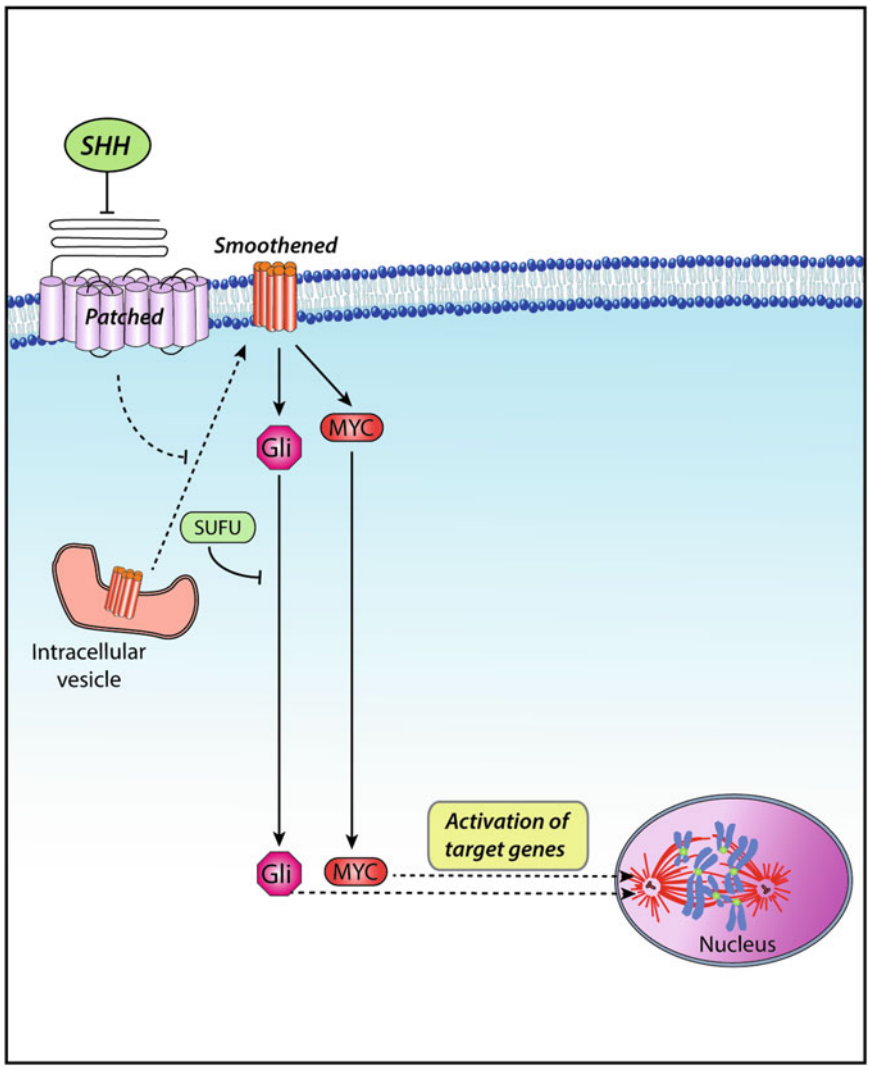


Fig. 35.8 Sonic Hedgehog (SHH) signaling pathway.

35.7.3 The Wingless Pathway

The Wingless (WNT) proteins form a family of secretory molecules that regulate cell–cell interactions during embryogenesis. To date, 19 *WNT* genes and more than 15 associated receptors have been identified in the human genome [304, 342]. WNT binds to the G-protein-coupled receptor Frizzled, which results in intracellular signaling carried out by the phosphoprotein Dishevelled (DSH) and leads to a release of inhibition of the transcription factor β-catenin [343]. With inactive WNT signaling, the cytoplasmic concentration of β-catenin is regulated and decreased by a destruction complex, which mainly is formed by Axin, APC, GCK-3, and CKI [344]. A current model of WNT signaling is shown in Fig. 35.9.

Through its interactions with other families of secreted growth factors, including EGFR, FGF, TGF-β, and the Hedgehog proteins, WNT signaling can regulate diverse processes including cell proliferation, migration, polarity and differentiation [345–347]. The canonical WNT pathway is also a critical regulator of stem cells properties, and its activation has been associated with various cancers [348].

Evidence for the involvement of the WNT signaling pathway in brain tumors has come from studies of Turcot's syndrome, characterized by the development of medulloblastomas and astrocytomas resulting from a germ-line mutation in the *APC* gene [188]. Since then it has been established that WNT signaling influences the proliferation and the renewal of neural stem cells and progenitors [347]. There is increasing evidence that aberrant activation of WNT signaling, resulting in accumulation or increased activity of β-catenin, will lead to the development of sporadic brain tumors, Notably, pediatric medulloblastomas harbor mutations of *β-catenin*, *APC*, and *Axin* [188, 329]. These point mutations are found in approximately 10–15 % of medulloblastomas but are mutually exclusive. For instance, 4 % of sporadic medulloblastomas harbor missense mutations in *APC*, and 1–5 % have *Axin1*

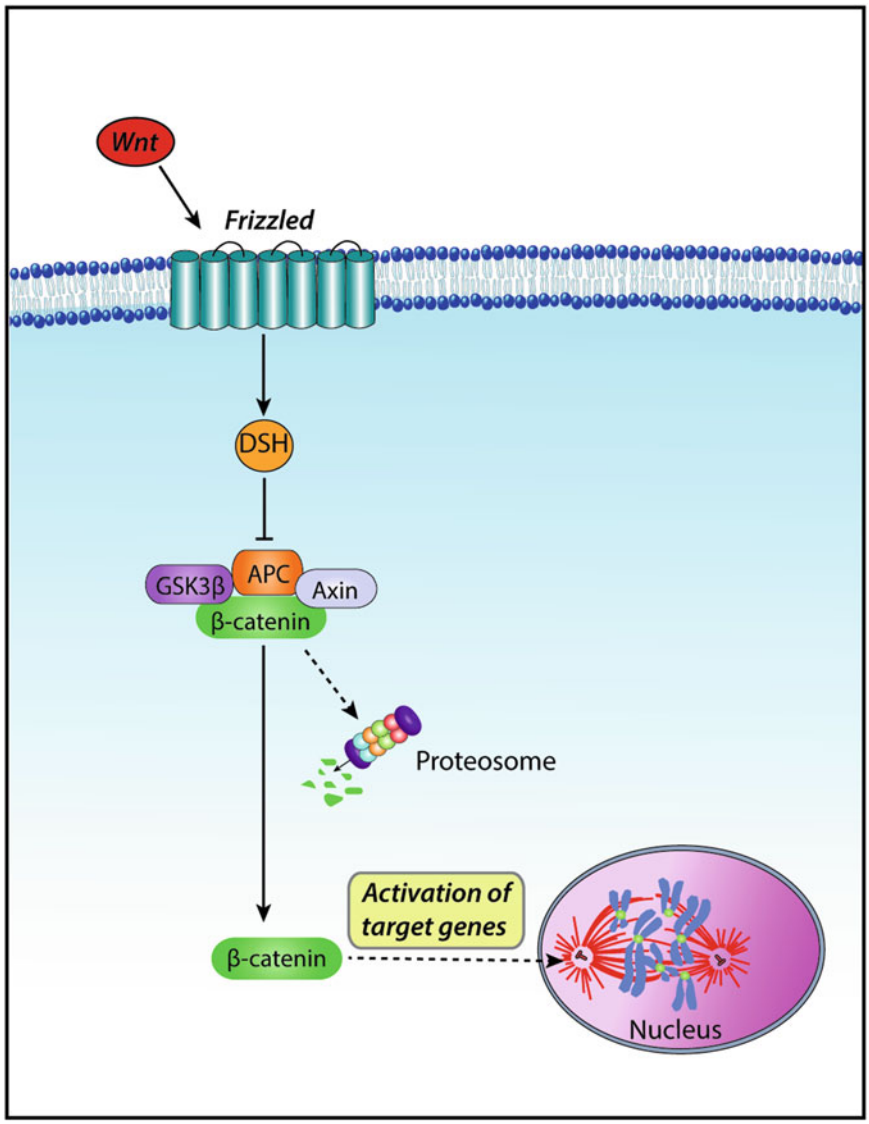


Fig. 35.9 Wingless/Int 1 (WNT) signaling pathway.

point mutations [186, 349]. Interestingly, there is evidence of cross-talk between the WNT and SHH signaling pathways as SUFU has been shown to regulate the activity of β-catenin [350]. *SUFU* mutations in medulloblastomas may lead to the activation of the two pathways: WNT and SHH [351]. The role of WNT signaling in brain tumor formation has primarily been studied in the context of medulloblastoma, but there is increasing evidence that WNT signaling may also modulate the early phase of tumorigenesis in some gliomas [346, 347, 352].

So far, agents targeted against the WNT pathway in medulloblastoma cells or tumors have only been explored in preclinical setting [353, 354]; however given that medulloblastoma patients harboring mutations involving WNT signaling show favorable outcome compared to other subgroups [198], WNT targeted strategies seem to be of lower priority.

35.8 Stem Cells in Brain Tumors

The cancer stem cell (CSC) hypothesis was initially derived from the concept of CSCs giving rise to the hematopoietic malignancies. Since then the CSC hypothesis attempts to explain the development of solid tumors, such as brain tumors. Furthermore, the CSC hypothesis provides important concepts for future brain tumor treatment. The failure or only short lived beneficial effect of chemotherapy drugs and radiotherapy in addition to rapid tumor recurrence for most malignant brain cancers lead to the assumption that actual therapies might not adequately target a subset of dormant or slow cycling cells with abilities of self-renewal and adaptation [105]. Brain tumor stem cells are hypothesized to reside within a nutritive perivascular niche [355] (Fig. 35.10).

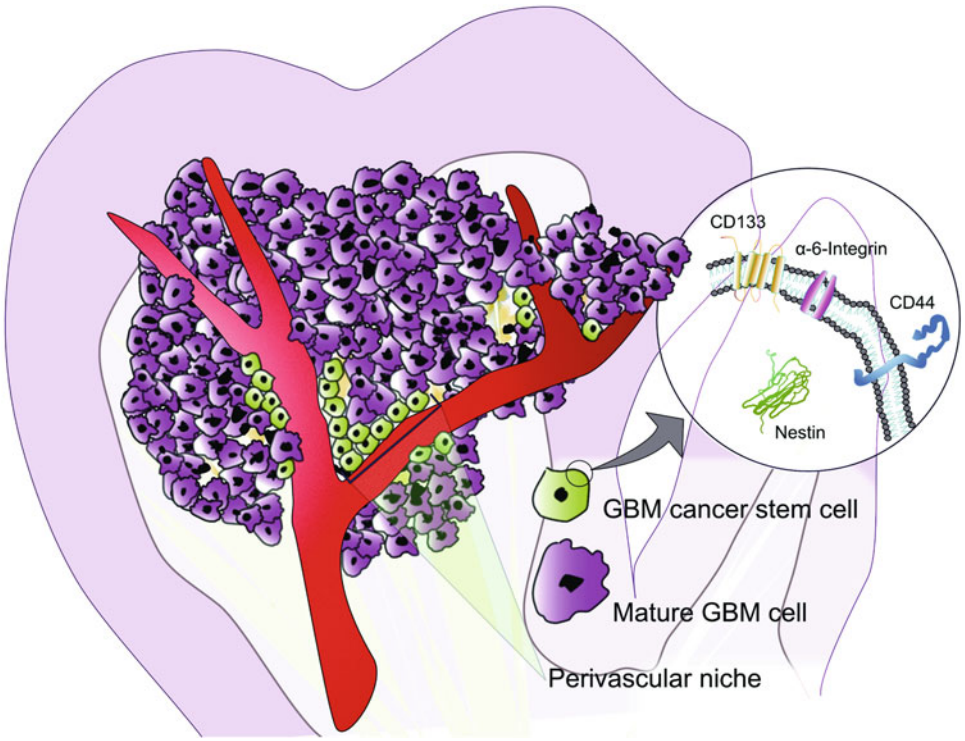


Fig. 35.10 Glioblastoma stem cells and perivascular niche. *GBM* glioblastoma multiforme.

As in normal tissue, tumors are organized in a cellular and functional hierarchy based on stem cells. Neural stem cells (NSC) are defined as cells that have the ability to perpetuate themselves through self-renewal (symmetric division) and to generate mature cells of a particular tissue through differentiation (asymmetric division) [356, 357]. These cells have been isolated from the embryonic brain of humans and persist in the adult in the subventricular zone of the frontal lateral ventricles and dentate gyrus of the hippocampus [105]. Different markers have been proposed to characterize the NSC population, but none have proven to be specific enough to ensure the identification of a pure population. The expression of Nestin is a widely used marker to delineate NSCs, as is the cell surface marker CD133. Additionally, some NSCs have characteristics of glial cells and will express on their surface SSEA-1, CD44, α-6 integrin, BLBP, GLAST, RC2, and GFAP markers [358–361].

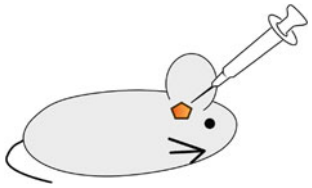
Many genes that have been identified as regulators of neural stem cells properties are also known to be involved in brain tumorigenesis [361]. For instance, Bmi-1 is important for cell proliferation and maintenance, and PTEN acts as a tumor suppressor and important negative regulator of cellular proliferation [362, 363]. The Notch, Sonic Hedgehog, and Wingless pathways have also been implicated in regulating NSCs [348, 364–366]. Importantly, deregulation of these pathways is also strongly implicated in the initiation and progression of CNS cancers when they are deregulated.

To date, CSCs have been identified in many solid tumors, including breast carcinoma, CNS tumors including astrocytomas, ependymomas and medulloblastomas, skin malignancies, and colon cancer [203, 367–373].

Evidence supporting the CSC hypothesis in brain tumors, include finding that these cells have indefinite potential for self-renewal, proliferation, differentiation, and have clonogenic potential based on neurosphere assays. Several studies demonstrate a close link between developmental biology, stem cells and cancer cells. In vitro, CSCs have been shown to express molecular markers of neural precursors as Sox2, Bmi1, Musashi, Notch, Emx2, Pax6, and Jagged1 [374]. These findings suggest that CSCs may have been initially derived from normal stem cells. Several proteins have been proposed as markers of the CDC population in brain tumors including CD133, CD15, α-6 integrin, and ATP-Binding cassette transporters (ABCT) [371, 375–377]. However, other studies have shown that the negative populations of these markers can also give rise to tumors and therefore these markers may only enrich for CSC and not define CSC [378]. Importantly, it is not clear yet if cancer-initiating events occur in the NSC, progenitor cells, or terminally differentiated cells, which reacquire a de-differentiated state [379]. Recent studies have suggested the microenvironment is important in defining the CSC. In melanoma, for example, the complete obliteration of the immune response of the xenograft host lead to a larger fraction of the tumor population exhibiting CSC properties [380].

Using a high-throughput drug screen, Diamandis et al. demonstrated that the functional ground state of neural stem cells may not only be dependent on developmental signaling

Table 35.2 Pros and cons of common animal models of brain tumors

	Brain tumor in vivo model	Pros	Cons
	Orthotopic xenograft	Cell lines can be genetically manipulated prior to transplantation in order to explore specific genetic events Large numbers of animals can be generated in relatively short time and at low breading costs Relative consistent histology and chromosomal profile supports standardization	Brain tumor cell lines generally show genotypic deviation and homogeneity compared to the original patient tumor (applies less to biopsy xenografts, at the cost of longer engraftment time and more variable tumor histology) Mostly demonstrate an expansive growth with only limited infiltration into the perivascular space and occurrence of necrosis or microvascular alterations (better results with sphere cultures compared to monolayer cell lines) Inhibited immunological tumor-host response
	Chemically induced model	Immune competent animals can be used Tumors show relative high degree of invasive growth	Genotype and phenotype of the tumor is not reproducible Histologically present more like gliosarcomas or "glioma-like tumors"
	Genetically engineered mouse model (GEMM)	Tumors arise de novo and in situ within immune competent animals Induction of defined genetic alterations allow to study the role of oncogenes and tumor suppressors in tumorigenesis Intact immune system and distinct molecular characterization of induced brain tumors improves validation of drug testing	Time point of tumor initiation cannot be controlled Biological differences of murine and human malignancies Complex and expensive breading

Transplantable, chemically induced, and genetically engineered mouse models (GEMM)

pathways such as Notch, SHH, and WNT, but also on pathways relating to neural transmission [381].

Although the cancer stem cell hypothesis remains somewhat controversial it offers a unique prospective to examine and understand brain tumor biology. The properties required for a population of cells to become tumorigenic requires a dynamic interplay between genetic aberrations, tumor hierarchy, and microenvironment.

35.9 Animal Models of Brain Tumors

The ideal animal model of a human brain tumor recapitulates the molecular, genetic, histopathologic, and clinical features that are found in the human tumor. While none of the current animal models can fully achieve these requirements, these models are critically important as they can be used to investigate tumor biology, screen novel molecular targets and pave the way towards preclinical trials. In the past 10 years, great progress has been made in establishing several relevant and reproducible animal models of human brain tumors that are based on the molecular genetic alterations that characterize the human tumors. Here we describe transplantable, chemically induced, and genetically engineered models [242] (Table 35.2).

35.9.1 Transplantable Allograft and Xenograft Models

Classic modeling involves transplantation of cultured rodent (allograft) or human (xenograft) cell lines under the skin (subcutaneous) or in the brain (orthotropic) of immunodeficient/immunonaive rodents [382, 383]. These rodent models are highly reproducible in terms of tumor formation, growth, and survival pattern. The cell lines can be genetically manipulated prior to transplantation in order to explore specific genetic events. However, these tumors may not faithfully recapitulate the original tumor histology, and pathobiology [384]. Established brain tumor cell lines generally show genotypic deviation and homogeneity compared to the original patient tumor [385]. One strategy to overcome this is the use of orthotopic serial passaging of primary human brain tumors in mice, as these xenografts often retain the original tumor phenotype. A common drawback of tumor transplants

is that they mostly demonstrate an expansive growth with only limited infiltration into the perivascular space of the brain and rare occurrence of necrosis or microvascular alterations [242]. The advancement in cell selection and preparation of growth media now allows for cultivating cancer stem-like cells which grow as spheres [242, 368]. These sphere cultures closely retain the genetic profile of the patient tumor and are highly tumorigenic, invasive, and angiogeneic [242, 386]. However, *EGFR* amplification is rather rare in neurosphere cultures and some cell populations lose glial and gain mesenchymal features in a process known as mesenchymal drift [387]. The retention of the initial chromosomal profile and tissue architecture of the tumor can be improved when biopsy tissue is directly cultivated as multicellular organotypic spheroids and implanted into immunodeficient rodents [388]. Biopsy xenograft models usually maintain their heterogeneity and show invasiveness, necrotic areas and frequently display *EGFR* amplification [242]. However given that the tumor histology differs between animals and initial engraftment may take up to 1 year, experimental planning becomes problematic [242].

35.9.2 Induction of Brain Tumors via Embryonic Mutagenesis

The second classical method of modeling is inducing tumor formation in the developing brain of embryonic rodents with early exposure to mutagenic alkylating agents (chemically induced model) [389, 390]. These tumors, mostly established in rats (e.g., 9L, C6), show some degree of invasion and necrosis, but histologically present more like gliosarcomas or glioma-like tumors [242]. An advantage over transplanted tumors is that immunocompetent animals can be used, which mimics the situation normally found in humans. However, when cell lines from chemically induced tumors are used as an allograft transplant, immunogenic tumor rejection may still occur and interfere with the interpretation of the effect of experimental antitumoral agents. An essential disadvantage of chemically induced tumors in embryonic animals is that the genotype and phenotype of the tumor is not reproducible, given the inciting genetic event is unknown and reveals little about the molecular etiology of brain tumors in humans.

35.9.3 Genetically Engineered Mouse Models (GEMM)

GEMMs are created by germ-line modifications or somatic cell gene transfer techniques. An advantage over xenografts is that the tumors arise de novo and in situ within immune competent animals. GEMMs have allowed further insight into the molecular events behind tumor initiation and maintenance, although mouse tumors that phenotypically resemble human tumors still behave differently with respect to their genetics and therapeutic response [391].

The generation of transgenic mice involves inserting a DNA construct (usually an oncogene) downstream of a promoter into a fertilized oocyte, which is then implanted into the mouse [392, 393]. All cells of the resulting transgenic offspring homogenously contain the new genetic material. Conditional expression can be conferred using tissue/developmental stage specific promoters, drug response elements such as tetracycline or doxycycline, or recombination techniques (*Cre/lox*) [394, 395]. Strategies employing conditional expression allow the study of genes that would otherwise result in embryonic lethality, and confer tissue and stage specificity.

Knock-out mice or loss-of-function models traditionally involve using homologous recombination to substitute a mutated gene with a selection marker (i.e., antibiotic resistant gene) for a wild type gene. The cells of interest are then selected for and placed into embryonic stem cells, which are then transferred to mouse embryos and generate chimeric mice, which can be mated to generate a homozygous mouse with the inactivated gene [393, 396]. These mice as well as transgenic mice can be mated for a combination of genetic modifications.

Somatic cell transfer techniques allow the infection of only a population of cells in an effort to more closely model the spontaneous genetic events responsible for the majority of brain tumors. These techniques employ the use of viral vectors for the delivery of genetic information. One strategy takes advantage of the Moloney Murine Leukemia Virus (MMLV) and its wild-type helper virus to deliver genes to dividing cells [397]. A second more specific system uses the replication incompetent avian leukosis virus (ALV) to only deliver genes to cells engineered to express the viral receptor tv-a [398, 399]. The ability to study secondary mutations in these systems is related to infection efficiency, as well, the inclusion of large genes in the vectors decreases viral efficiency. However, multiple viral constructs can be coinfected to study multiple genetic hits.

35.9.4 Models of Astrocytoma

Alterations within the three core signaling pathways (p53, RB, RTK/RAS) as characterized by the TCGA are often used to induce astrocytoma formation in GEM models [391]. Transgenic mice with constitutively active H-RAS driven by a GFAP promoter (astrocyte specific) develop Grade II–IV astrocytomas in a dose dependent manner [400]. High H-RAS expressors develop tumors characteristic of GBM and moderate expressors develop low-grade or anaplastic astrocytomas [393, 400].

Mice with tumors characteristic of GBMs can be created by breeding mice with *TP53* deletions with mice with *Nf1*

(the mouse form of the human NF1) deletions [401]. Nf1, as in humans, is a RAS-GAP protein that downregulates RAS activation, therefore, its loss would result in RAS activation.

Transgenic mice with v-src expression driven by a GFAP promoter develop low grade or anaplastic astrocytomas 14.4 % of the time [393, 402]. The src receptor interacts with the EGFR and PDGFR signaling pathways.

Anaplastic astrocytomas were modeled in mice engineered to express the SV40 T-antigen late in development. SV40 inactivates RB and RB family member p107 and p103. These mice develop anaplastic astrocytomas within 10 months in almost 100 % of cases [393, 403]. Interestingly, mutating RB alone does not result in astrocytomas suggesting redundancy between RB family members.

Using the RCAS/tv-a retroviral somatic cell transfer technique to over express K-RAS and AKT in nestin positive CNS progenitor cells results in the formation of tumors characteristic of GBM [404]. Neither K-RAS nor AKT overexpression alone formed tumors. AKT over expression may be analogous to PTEN deletions in human GBM as the inhibitory effects of PTEN are immediately upstream of AKT activation [393]. Marumoto et al. transduced a small number of GFAP$^+$cells heterozygous for *TP53* with lentivirus harboring activated oncogenes H-RAS and AKT in the cortex, subventricular zone and hippocampus of mice [405]. Tumors that recapitulated human GBM arose only from transduced cells in the subventricular zone or hippocampus [405].

Deletions and alterations in tumor suppressor or oncogenes can be combined in order to generate mouse models of GBM subgroups. For example, knocking down p53 and activating RAS signaling resulted in tumors harboring a molecular profile resembling the mesenchymal GBM subtype [104]. Furthermore, the first orthotopic xenograft model of anaplastic oligodendroglioma with mutations in *IDH1* and tumor suppressor genes *FUB1* and *CIC* (both involved in MYC expression and RTK signaling repression) has been reported recently [406].

35.9.5 Models of Oligodendroastrocytoma

Overactive PDGFR has been implicated in the pathogenesis of human oligodendroastrocytoma tumors and as such murine models of this tumor have focused on this abnormality. Holland et al. used the RCAS retroviral system to express oncogenic PDGFB in either nestin or GFAP expressing cells [407]. The nestin group acquired mainly low grade oligodendroastrocytomas approximately 60 % of the time, whereas the GFAP group developed mixed oligoastrocytomas or oligodendroastrocytomas 40 % of the time [407]. When the oncogenic PDGFB mice were crossed with mice lacking the INK4A/ARF gene, the proportion of anaplastic tumors increased the latency to tumor formation [407]. Recently, oligodendroglioma cells from fresh human tissue samples with co-deletion of chromosomes 1p and 19q have been successfully cultivated as permanent cell lines (BT054 and BT088). BT088 was shown to engraft in the brain of immunocompromised mice, and demonstrated extensive infiltration and histology resembling anaplastic oligodendroglioma [408].

35.9.6 Models of Medulloblastoma

Mice heterozygous for mouse *PTCH* deletion develop tumors similar to human medulloblastoma at a rate of 15 % by 10 months [409]. This incidence can be increased to 98 % by 12 weeks if the *PTCH* +/- mice are combined with *p53* null mice [409]. Using the RCAS retroviral and inducing Sonic Hedgehog mis-expression in the cerebellum of fetal or newborn mice resulted in medulloblastoma formation at high rates [410, 411]. The co-overexpression of C-MYC in these mice enhanced the effect [393]. In murine transgenic models, the overexpression of MYC in concert with loss of *TP53* resulted in medulloblastomas that expressed markers typical of Group 3 medulloblastoma [412]. Interestingly, while trying to create astrocytoma models in GFAP expressing cells of mice by deletion of both *TP53* and *RB*, tumors phenotypically similar to medulloblastoma developed in the cerebellum [413]. The WNT subgroup could be recapitulated in mice with activating mutations in the WNT pathway effect protein CTNNB1 [414]. These WNT pathway tumors in the mouse model revealed that this subgroup of medulloblastoma arise from regions in the dorsal brainstem as opposed to other genetically distinct medulloblastoma subgroups such as SHH which arise from the cerebellar hemispheres [414].

35.9.7 Models of Ependymoma

For a long time, preclinical models of ependymoma were lacking [415]. Johnson et al. introduced the first mouse model in 2010 using genetic engineering techniques [207]. After transducing the oncogene *EPHB2* in neural stem cells that were *INK4A/ARF−/−* the mice developed tumors that were histologically indistinguishable from human ependymomas at a rate of 50 %. Advances in optimum medium preparation have permitted ependymoma cell culture derived directly from primary patient material and to create orthotopic xenograft mice models for posterior fossa ependymoma [416]. Recently, an ependymoma stem cell line (*DKFZ-EP1NS*) derived from a patient with metastasizing supratentorial anaplastic ependymoma has been cultivated as

neurospheres and used to establish the first orthotopic animal model for supratentorial ependymoma harboring homozygous deletion for *CDKN2A* [417].

35.10 Conclusion

Continued pursuits in understanding the molecular and genetic events in brain tumor initiation, maintenance and progression will lead to improved therapies in the future. It will one day be possible to analyze the molecular biology of a particular patient's brain tumor and tailor individual therapies based on these findings. The goal of improving patient outcomes in this devastating disease will only be accomplished through the continued application of the latest technologies to the field.

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Cancer Drug Discovery and Anticancer Drug Development

36

James Turkson

36.1 Introduction

Studies of the molecular basis of carcinogenesis have led to the realization that cancer is fundamentally a disease of multiple genetic aberrations. The multiple genetic changes in cancer are predominantly the result of accumulating mutations that have occurred in the genome over time, which in turn lead to gene products with upregulated, repressed, or loss of functions, as well as aberrant signal transduction pathways and dysregulated cellular functions. Bulk of evidence demonstrate that dysregulated signal transduction pathways promote malignant transformation and support the formation, maintenance, and progression of cancers. Numerous investigative studies that cover this area and the compelling observations supporting the genetic basis of cancer, as well as the details of the identified molecular alterations that present themselves in cancer fall outside the scope of this chapter. It is noteworthy to mention that the reality that cancer has underlying multiple fundamental changes at the molecular level ushered in a new era in cancer drug discovery that comprises of the development of a new family of anticancer drugs different from the conventional cytotoxic, chemotherapeutic drugs. This new generation of anticancer drugs is based on the new paradigm known as molecular-targeted therapy, largely reflecting the concept of targeting only the specific key molecular abnormalities or dysregulated molecular entities that exist in cancer cells and that drive tumor progression. The concept of targeted therapeutics is therefore referring to the selective interference with or the thwarting of the functions of the critical, cancer-relevant biological molecules in cancer cells and thereby exerting specific biochemical effects mainly in the cancer cells, and producing a desirable biological outcome. It is also important to note that the altered critical biological molecules may have normal physiological functions in the unaltered state, but by being aberrant or dysfunctional, those functions contribute to promoting the development and the progression of the cancer phenotype. Molecular-targeting has now become the new drug discovery paradigm that drives the development of novel anticancer therapeutic modalities and influences how anticancer drug discovery is approached today.

The multiple genetic changes that are evident in the many human cancers have given rise to several gene products that are aberrant in activity or are defective in function. The underlying general hypothesis is that the abnormal functioning of these molecular entities together lead to the development, maintenance, and the progression of the cancer phenotype. In general, there is substantial and compelling evidence to support this hypothesis, and in a large number of cases, the evidence is strong to demonstrate the all-important requirement for the abnormal molecular entities in the pathological events that drive the cancer. Thus, the premise for the new drug discovery paradigm of molecular-targeting is that those cancer-critical abnormal biological events or molecular entities represent potentially viable therapeutic points or targets for developing effective and safe anticancer therapeutic agents. For this new concept, a large emphasis is placed on the knowledge of those crucial cancer-relevant biological events or molecular entities that are associated with the cancer cells or tissues, their role in the cancer phenotype and how the absence of their functions modulates the cancer phenotype. Without going into much detail here, it is worth mentioning that the genetic alterations that are evident in cancer can be generally classified into two main types: (1) the gain-of-function mutations, and (2) the loss-of-function mutations. Gain-of-function mutations are random mutations that occur in the genes that promote growth and survival and that result in the aberrant activation of the gene products. This type of mutations frequently leads to the dysregulation of critical physiological processes, including cell cycle and cell growth, cell survival, and angiogenesis, or promote events

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_36

that lead to cancer cell migration, invasion, and metastases. Loss-of- function mutations are typically evident in genes whose products function as tumor suppressors, cell cycle inhibitors, or inhibitors of apoptosis. Thus, the loss of the functions for the gene products eliminates the negative regulation and the restrictions on cell growth and survival, or leads to a loss of differentiation. Overall, therefore the evidence indicates the multiple genetic aberrations and the consequent alterations in gene products that are evident in cancer culminate in abnormal biochemical and biological processes, which together contributes to promoting the malignant transformation, tumor formation, and tumor progression. Given these underlying abnormal molecular events in cancer, the focus of molecular-targeted therapy in drug discovery is to design novel agents that directly target those altered molecular entities so as to modulate their abnormal activities and/or rectify their functions, thereby restore the physiological harmony and alter the cancer phenotype.

Given the molecular aberrations in cancer and the propensity of the altered biological processes to promote cancer formation and progression, it is reasonable to conceptualize that cancer is a disease emerging as a result of cell growth and cell survival surpassing cellular differentiation and cell death. Thus, the ultimate goal in the application of molecular-targeted anticancer therapy is to successfully alter the disequilibrium between these opposing processes and to shift the dynamics away from cell growth and survival in favor of cell differentiation and cell death. This will in turn promote the preferential loss of cancer cells that make up the tumor tissue. To accomplish this, the ideal molecular-targeted anticancer agent must be capable of selectively repressing the abnormal molecular events that support cancer cell growth, viability, and survival, while inducing processes that promote cancer cell terminal differentiation or cell death. Furthermore, the ideal anticancer agent will not affect normal cells or their functions so as not to induce toxicity in the patient, although to accomplish this may be idealistic. Certain toxicities have been observed for molecular-targeted therapeutics in patients, which are in part due to the normal functions of the biological targets and the degree to which the inhibition in normal cells affect their functions. However, it needs to be mentioned that many of the currently available anticancer drugs belonging to the category of chemotherapy are purely cytotoxic agents and non-selective, and they mainly promote cell death in the rapidly proliferating tumor and normal cells. It is expected that molecular-targeted therapeutic approach will not have the same level of toxicity that is associated with chemotherapy as these drugs are designed to selectively and preferentially target cancer, but not normal cells, thereby reducing the extent of toxicity in the patient. Other challenges associated with molecular-targeted therapy is the extent to which targeting of a single entity alone might be sufficient to alter the tumor phenotype. Thus, the clinical outcome of the few molecular-targeted anticancer agents has not lived up to expectations. This may be explained in part by the well-structured architecture of intracellular signaling pathways comprised of an array of proteins and other macromolecular structures that are uniquely arranged to promote signal cross-talk and provide redundancies. Thus, the less than a complete appreciation of the targets' functional relationship with other molecular entities that exist in the network of molecular and biochemical processes poses a challenge. In light of this, it is becoming increasingly clear that the targeting of a single biological molecule or pathway might be insufficient and that multiple molecular-targeted therapeutics might be needed to eliminate the cancer cells. In presenting a discussion on the development of new anticancer drugs that target specific cancer-relevant pathways under the new molecular-targeting paradigm, the discovery and the development of cytotoxic chemotherapeutic agents will not be discussed [1–6].

36.2 Molecular Targeting: A Modern Day Concept to Anticancer Drug Discovery and Development

Non-specificity of effects and severe toxicity to diverse organ systems and tissues in patients are some of the limitations of conventional chemotherapy. These problems further compound the already difficult health condition of the patient and frequently lead to patient non-compliance or the unwillingness to proceed with the chemotherapy. In contrast to conventional chemotherapeutic agents, which exert effects via acting on multiple processes that affect highly proliferating cells of both normal and tumor tissues, and which also lack specific tumor-selective targets of action, the molecular-targeted anticancer drug design and therapeutic strategies represent a shift in paradigm by focusing on specific cancer-relevant molecular targets. The concept of molecular-targeting includes a focus on the identification and the targeting of specific altered molecular entities of relevance to cancer. The molecular abnormalities may be specific biological processes or molecular entities whose functions are critical to the underlying events for initiating and developing the tumor and for tumor maintenance. Those causal events become the target areas for candidate molecular-targeted therapeutic agents.

Although the ideal candidate drug will have a well-defined target and mechanism(s) of action, the discovery and development process for new candidate drugs does not always begin with the knowledge of the target. The present-day drug discovery and development process is rather a lengthy one with a high failure rate. For the drug discovery process, with its objective to identify a molecule that is selectively active and potent against the cancer phenotype and that exerts lim-

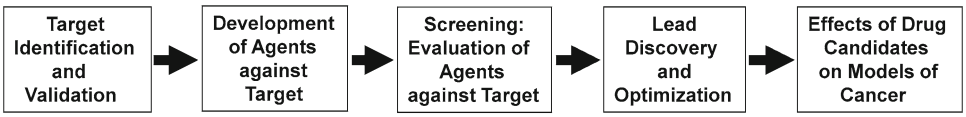

Fig. 36.1 Schematic representation of target-based drug discovery and development process. Drug discovery process typically begins with the identification and the validation of the target in vitro and in vivo studies. The next stages involve the choosing of the agents, compound libraries, or assessment of the approaches for obtaining agents to be screened. Also involved at these stages are the assays and their development, the choice of which must be based on the target, and the evaluation of agents against the target, lead discovery and optimization, with medicinal chemistry, and ultimately the evaluation of candidate drugs against suitable in vivo models of the type of cancer.

ited undue toxicity to normal cells, and which produces clinical benefits with minimal toxicity in the patients, there is no set rule or way to reach this goal, as long as it is accomplished. There is no more higher emphasis on the manner in which to begin the drug discovery and development process than there is on accomplishing the goal of delivering an effective and safe drug, although a particular strategy might offer certain advantages and may be preferable to an organization based on logistical and technical issues. Aside of these, two main models may be used to describe the present-day drug discovery and development process. With model one, as illustrated in Fig. 36.1, an agent may be identified and designed against a known target, which through rigorous experiments has been validated and established to have a critical role in carcinogenesis and tumor formation. Ultimately, the modulation of the target by the identified agent is expected to inhibit growth of cancers in an experimental setting. This model is described as the forward, target-based, hypothesis-led approach to drug discovery. With model two, the alternative, reverse approach may be taken that initially focuses primarily on finding drug candidates that have the ability to alter specific biological events relevant to the cancer phenotype without knowledge of the potential target(s) or the molecular mechanisms of action of the drug candidates. The antitumor properties of the candidate agents will then be pursued at a later stage in studies that focus on the identification of the target(s), validation of target(s) in regard to the cancer phenotype, and the determination of the molecular mechanism(s) of action of the candidate drugs in the context of the biological response of the cancer cells and the effect of the candidate drugs on the target. Both of these models have been utilized with positive results. Each approach has its advantages and disadvantages, which together with other factors will influence the decision of an organization in how the drug discovery process is initiated and carried out. Although the two models are different in the order and sequence of pursuing the objective of identifying new anticancer drugs, in both cases the target and the target identity remain important parameters and the need to ultimately solidify the validity of a candidate agent as an anticancer drug remains. Drugs that are discovered and developed based on these approaches, called molecular-targeted drugs, are aimed at suppressing dysregulated biological molecules with functions that are critical to the cancer. We will start by discussing the target-based model and the important concepts, such as cancer drug target, and their significance [1, 2].

36.3 Target-Based Drug Discovery and Development Approach

Molecular-targeted drugs are designed with the aim of modulating the cancer phenotype by interacting with and altering the properties of specific biological molecules. Those biological molecules are altered in such ways that make them crucial for the initiation of cancer, and for the survival of the cancer cells and are therefore important for maintaining the cancer phenotype. The target of any molecular-targeted drug may be cell surface entities (extracellular domains of receptors, membrane phospholipids, integrins, and adhesion molecules), membrane-anchored proteins (receptors), or intracellular, cytoplasmic entities (intracellular domains of receptors, enzymes and signaling proteins, RNA, microRNA, and DNA). They are typically dysregulated in some fashion in tumor cells, compared to normal tissue and are expected to be specifically modulated by the novel anticancer agent. Therefore the forward, target-based approach to drug discovery builds on the premise that the biological molecules that make up the target(s) have potential roles in terms of their aberrant functions and thereby contribute to the development and the progression of the cancer phenotype. However, it needs to be stressed that what constitutes an important target for consideration for a drug development project is a crucial question that should not be taken lightly. In that context, it needs to be stressed that the mere association of a biological molecule or event with malignant transformation and tumorigenesis does not constitute sufficient evidence for target status nor does it necessarily make the biological molecule a good target. Although a biological molecule or event may have an association with the disease phenotype, one ought to be open-minded, but critical going into the drug discovery project, especially at the initial phase of the discovery process and recognize the need for a thorough examination of the target-worthiness of the biological entity prior to making any commitment to pursue the biological entity. In that regard, there are key issues that need to be considered

following the initial observations of an association of a biological entity with the disease phenotype, but prior to deciding to focus on the biological entity as a target in terms of designing, adopting, and applying a strategy for evaluating and identifying compounds that modulate the biological entity. We will now look at these important issues and discuss their significance within the context of the forward, target-based approach to drug discovery [7–9].

36.3.1 Target Identification, Selection, and Validation, and Criteria to Establish Validity

36.3.1.1 Target Identification and Selection

Any type of disease condition will likely be associated with several abnormal molecular entities and pathological events. For discovering and developing molecular-targeted therapeutics against a disease condition, it will be important to determine which of those altered molecular entities and biological events are essential to the disease onset and to the underlying pathological mechanism(s) that initiate, support, and maintain the disease condition, and also promote its progression. The knowledge of this information is crucial for determining the suitability of the biological entity as a target and for defining the significance of the target to the disease. Target identification thus represents the key first step in the target-based discovery approach. A cancer drug target represents a biological entity with molecular and biochemical functions that are critical to the key biological processes underlying the existence of cancer. The altered biological entity may be a genetic material, a gene or gene product, or a molecular or biological mechanism present within cancer. The identity or the knowledge of the critical target also provides a basis for defining some aspects of the biological and molecular underpinnings of the disease. A defined target may be distinctive to the particular cancer type, representing part of the properties that define the cancer phenotype, as well as distinguish the cancer from other cancer types or subtypes, or the cancer cells from their normal counterparts from which the tumorigenesis events may have originated. It provides a means of characterization of the cancer in terms of differences in specific molecular and biological properties that are the foundation of the disease and for which the target is critically important. Identifying and defining a target is also an important step toward setting priorities regarding the specific aspects of the target that need to be focused on that might be relevant to function. Selecting the target influences the decision on what approach to take in order to maximize the potential to achieve the highest level of selectivity and specificity of effects for the candidate drug. The knowledge of the target is also useful for making predictions about the potential to regulate specific tumor types with minimal toxicity to the patient. It could be said that a defined target for a tumor type provides the platform for predicting the potential clinical outcome when the new drug is used in a patient against the target, which is aberrantly regulated in some fashion, although it needs to be stressed that this prediction is no guarantee of the clinical success of the candidate drug. Other important considerations in determining target identity that impacts significance are the cellular location and the expression profile of the target, molecular status in normal versus tumor cells, the functional status in relation to the tumor phenotype, as well as other physical/biochemical and biological properties of the target.

The functional status of the candidate target should be considered relative to the mechanism of carcinogenesis and the development of the cancer phenotype. The important questions to address here are what role the target has and in what manner, as well as how important the target's role is to the disease phenotype. Determining the answers to these key questions provides the framework for making the critical decision on whether to focus on the target for drug discovery purposes. These questions are addressed experimentally to ensure that the selected candidate target sufficiently meets certain minimum conditions or set guidelines. Information from those studies becomes the basis for making critical decisions on moving forward. Other important issues include druggability and how well drugs can be designed and developed against the target, as well as the tractability of the target or the difficulty of inhibiting the target. It is proposed that of the genes in the human genome, only a small percentage is druggable and is associated with diseases. This is even more important when the disease phenotype is linked to several similar targets, and the question becomes which one of these possible targets should be pursue in the discovery program. It is worth stating that what constitutes a good target may be regarded as an ongoing debate and this debate and the evaluation of the target should continue throughout the drug discovery process to ensure suitability. In that regard, what is now emerging is a sense of value that the potential target presents and this influences the "go-no-go" decision. Moreover, emphasis is placed on target value for the sake of patentability and intellectual property matters, which are important considerations from financial standpoint, given how much of resources would need to be invested and how much efforts would be allocated for pursuing the target in the discovery program, and the overall cost associated. It is a common practice for one target to be pursued by many organizations, particularly if it is highly relevant to the cancer type and its druggability is high. Overall, the concept of target is generally accepted as one of the most critical factors to consider in the anticancer drug discovery process [10–14].

36.3.1.2 Target Validation

The concept of target represents one of the most important issues in anticancer drug discovery. Granted the multiple genetic alterations, which are associated with the cancer type, lead to a myriad of functional aberrations in the gene products with altered biological outcome, it is reasonable to expect many factors to become associated with any particular cancer phenotype. While such associations are significant and may suggest some level of importance for the altered entities in relation to the carcinogenesis and the tumor formation processes, rigorous investigative studies are required before any major conclusions can be made regarding target validity. The point needs to be made that an observation that a biological molecule is altered in some fashion in cancer cells, while suggesting an association with the cancer phenotype, should not necessarily be taken as an indication the altered entity is essential for the events that led to the cancer phenotype or that it is important within the underlying mechanisms leading to carcinogenesis, tumor formation, and tumor progression. Ideally, to be considered as potential targets, altered proteins, or biological molecules and their functions should have critical roles in the underlying events leading to the cancer formation. Adopting this viewpoint is to safeguard against selecting and focusing on spurious biological entities that may not be essential to the basis of the disease phenotype, but may be readily available for targeting for reasons other than a requirement for the cancer formation. This brings us to the issue of the cause and effect. This is an important consideration, which indicates the need to establish the causal relationship between the altered molecular entity (and its function), as a potential target, and the biological or pathological events underlying the cancer phenotype. It will be ideal to envision that the altered molecular entity will have a causal role in the induction of the cancer phenotype in order to represent a validated and credible target for drug discovery and development.

Although the validation of a putative target may be viewed as a complex and long process, when successfully done, it authenticates the drug discovery process and has the potential to improve the chances of success in terms of the clinical outcome in patients for the drug candidate that emerges from targeting the altered biological entity. The question of what makes a good target for anticancer drug design and development may not be answered in one study. The attempt to address this question may take the form of a number of observations made from a series of studies throughout the drug discovery and development process. The significance of these studies is primarily in terms of establishing the nature of the relationship between the candidate target and the disease. The type of relationship will have a strong influence on the potential response of the drug candidate in the treatment of the disease based on the effect of the drug on the target. Thus, a high importance should be placed on defining the importance of the altered biological entity, as a target, in relation to the cancer. In that regard, there ought to be a set of criteria with minimum requirements that need to be fulfilled by the putative target in regard to its consideration as the target of choice for the cancer phenotype, and to the justification to develop agents that modulate its activity or function. The validation does not only make certain that the target is critical to the cancer type in terms of the viability and the survival of the cancer cells, but also serves to minimize the chances of toxicity by ensuring that the potential drug candidate would exert specific effect preferentially on tumor cells without indiscriminate effect on normal cells. Overall, target validation is an important consideration in the drug discovery process, and establishing the causal role for a target in the disease phenotype is a good beginning of a potentially viable anticancer drug discovery process. However, target validation should be perceived as an ongoing process pursued at all stages of the drug discovery and development process. By its very nature, the validation therefore represents a complex process that will require diverse experimental approaches and important decision-making steps in order to accomplish and establish a tractable and viable drug target [10, 14].

36.3.1.3 Criteria for Validating a Target

An important issue in considering a candidate target for drug discovery is whether an entity hypothetically associated with a disease necessarily represents an appropriate point for new drug intervention. It is vital to establish the credibility of the hypothetical target as it relates to its activity in the context of the disease and the target's mechanism(s) of action, and to establish a cause and effect relationship between the disease and the candidate target. There are no standard processes for validating and establishing the credibility of a target, and different organizations may consider different approaches for accomplishing this goal. Notwithstanding, the main goal is to present a clear definition of the position of the target in relation to the disease phenotype and the molecular and biological events leading to cancer. Achieving this goal will require using various experimental models of the disease and diverse approaches. It should be pointed out that disease-relevant systems that are available for experimental use outside of humans may not be accurately reflective, but nonetheless serve the purpose at this stage of the discovery process. While the establishment and the use of appropriate disease models for target validation present some challenges, the lack thereof for evaluating a potential target for credibility will only compromise the pharmacological usefulness of the emerging drug candidate. Given the objective, which is to verify the credibility of the potential target in relation to its role in the cancer phenotype, one could propose some minimum conditions and lay out a set of criteria that need to be

fulfilled by a molecular entity that is being considered or evaluated as a potential target for cancer drug discovery:

1. There should be evidence in cell culture and whole animal models that changes in the function of the candidate target alone is sufficient to alter the relevant disease phenotype. Here, studies need to establish that the elevation of the expression or function of the putative target alone, in the case where it is stimulatory, is sufficient to induce the cancer phenotype. Where the function of the putative target is suppressive, there should be evidence that its overexpression alone is sufficient to abrogate the malignant phenotype. Such studies when appropriately conducted will provide information to build confidence in the target and to establish the causal relationship between the putative target and the cancer type.
2. Definitive proof from studies in disease-relevant models of cell culture, and whole animals, and in clinical models that modulating the function of the molecular entity as a target alters the disease phenotype. Complementary to the studies conducted in response to the requirements in the criterion (1) above, it is important to employ the appropriate disease models, both in vitro and in vivo, to investigate the underlying critical role of the potential target in the incidence and the progression of the cancer phenotype. Granted that the putative target has a critical role in the disease, the modulation of the candidate target will be expected to result in a corresponding change in the cancer phenotype that is consistent with the purported role of the target in the disease.
3. Finally, the molecular mechanism(s) of oncogenesis must be clearly outlined and defined in accordance with the assertion that a potential biological entity as target has a critical role in the development of the cancer type. One needs to ascertain how the function of the biological molecule is essential to the events that ultimately lead to the cancer phenotype. This will ensure that targeting this biological entity modulates some critical underlying mechanism(s) that support(s) the maintenance of the tumor.

These target-focused requirements are important considerations in determining the validity of a target for developing novel anticancer drugs for therapeutic purposes. The ultimate test of the validity of the target in relation to the cancer type is the clinical outcome in the appropriate cancer patients who are given the drug. An example is seen in the good clinical activity of Bevacizumab, the humanized anti-VEGF monoclonal antibody as standalone or used in drug combination. Various genetic, molecular, biological, and pharmacological approaches are frequently used to evaluate and validate a putative target and its relevance to cancer. While the experimental approaches to evaluate the potential for a molecular entity or biological event to make a good target may vary from one organization to the next, the basic premise remains the same and that is to establish the credibility of the putative target for the disease phenotype. The emphasis is on ensuring that a particular biological entity has all the necessary properties and fulfills the criteria for its consideration as a valid drug target. It suffices to mention that even when a target is validated, its credibility as a good target continues to be evaluated at every stage throughout the drug discovery process and finally at the level of clinical response when the drug is administered to patients [8, 10, 11, 14–18].

36.3.2 Lead Identification and Optimization, and the Development of a New Drug

The successful identification and validation of a target pave the way for the next step in the discovery process, which could be classified as designing and identifying leads that target the biological entity or entities and modulate their function (Fig. 36.1). This is an important phase in the discovery and development process and thus carrying out the studies in this phase may take several months to complete. Also, many small-molecule drug discovery approaches do not result in clinical candidates in a large because of the lack of a good lead [19, 20], thus securing a suitable lead for the drug discovery and development process is critical for the success of the program. There are no specific set of guidelines regarding how this phase may proceed. However, further studies defining and expanding on the understanding of the molecular and biochemical bases for the induction of the target could be incorporated into this phase so as to facilitate identifying specific key aspects of the target's properties that may be amenable to exploitation for the design of lead molecules as modulators and potential anticancer agents. To some extent, studies here will also serve as proof-of-concept testing in order to establish the validity and viability of the strategy for generating suitable lead compounds. These studies may also test the therapeutic potential of the candidate drug in modulating the target(s) and the function of the target(s). The work at this stage includes the development of assays, both in vitro and in vivo, the screening of compounds, which may be on a high throughput screening (HTS) platform to identify leads, and a medicinal chemistry component with lead optimization steps, and subsequent further evaluation of derivatives or analogs. Where appropriate, these steps may be interspersed with the evaluation of activity, with the expectation of progress in terms of enhancement and improvement over previous generations of agents.

It should be noted that there are variations in the sequence of the conduct of these studies. The use of the lead compounds in studies to further provide proof-of-concept could also be conducted, and different types of assays can be

performed simultaneously to evaluate multiple targets. Because each of the steps involved in the evaluation in this phase of the discovery and development process is crucial and will impact the final outcome, there are key considerations. These include the assay development and factors driving the assays and the screening strategy, such as specificity to the target, robustness, and the reproducibility of results, with minimum inherent errors or tendency for false positives, high sensitivity, and the cost-effectiveness. It is also important to establish criteria for the consideration of compounds for the initial screening and for the next stage of the evaluation following the first screening. The availability of set criteria allows to define and select suitable agents with optimum characteristics that lend themselves to the drug discovery process [21].

36.3.3 Specific Strategies to Identify and Develop Novel Anticancer Drugs

We will now examine some specific models of anticancer drug targets and the strategies taken to develop agents that modulate the functions of these targets. The discovery, identification, and validation of a molecular entity as a critical cause for the development, maintenance, and progression of cancer, and as a target are all important initial steps in the development of a new drug. Those initial investigations and evaluations together represent the rationale for initiating a discovery process with the focus on the target, and serve as the proof-of-concept in the support of the development of new anticancer agents that are directed at that target. Many of the critical biological entities that are altered in cancer and are considered as targets are signal transduction mediators or signaling intermediates, although there is also focus on nucleic acids as targets for developing anticancer therapeutics. Frequent genetic aberrations, including activating-mutations that lead to hyperactive behavior of the target proteins, suppressive mutations that result in repressed or lost functions, gene silencing by methylation that causes loss of functions, and abnormal post-translational modification patterns that create altered functions of proteins altogether serve as the driving force for the altered cellular phenotype and tumorigenesis. The abnormal functional properties of those altered biological molecules contribute to the dysregulation of the cell growth and survival processes, and induce angiogenesis, migration, invasion and metastasis, and the repression of host immune surveillance.

Several strategies have been taken for developing effective agents against validated cancer targets or tumor processes. The objective is to develop a product that selectively modulates the cancer target in question and alters its function in the context of the tumor processes promoted by the target, and thereby eliminate the support of the target for the tumor maintenance and progression. The drug candidate should be able to affect the target and its function and undermine the survival of the tumor cells without exerting undue effects on unrelated biological entities and their functions or on normal cells. Given these requirements, limitations, and expectations, let us look at specific examples of novel molecular-targeted therapeutics that target specific signaling pathways, or modulate biological processes or macromolecular structures. This is a brief discussion on the subject matter and readers are encouraged to consult the cited papers for more detailed information.

36.3.4 Modulation of Signal Transduction Pathways

In the past few decades, there has been an increased interest in exploring signal transduction pathways for novel targets for anticancer drug discovery due to their importance in regulating diverse tumor processes. This is based on the compelling evidence demonstrating multiple aberrations in signal transduction networks in many human cancers. Numerous research studies provide evidence that the abnormalities in signal transduction serve to provide the necessary foundation for the biological events that drive tumorigenesis, maintain the tumors, and induce tumor progression. Multiple mutational events are detected in ligands, receptors, and signaling intermediates, which result in their aberrant functions. These events consequently lead to and support uncontrolled cell growth and survival, and promote angiogenesis and other events that lead to tumor metastasis, and additionally compromise host immune competence. In the end, the aberrant signaling pathways become a necessary requirement for tumor cell survival, and for the maintenance and the progression of the tumors. The implication is that targeting any of those corrupted molecular entities in the signal transduction pathways or the dysregulated signaling intermediates will undermine the support for the tumor cells, and thereby induce tumor cell death, and eradicate tumors. While details of the signal transduction pathways and their importance to cancer are discussed elsewhere in this volume, herein is outlined specific examples of the molecular-targeted therapeutic approaches that are based on signal transduction pathways [22–24].

36.3.4.1 Inhibitors of Receptor and Non-receptor Tyrosine Kinases

Aberrations occur very frequently in receptor and non-receptor tyrosine kinases (TKs) and are associated with human cancers. Thus, the receptor tyrosine kinases (RTKs) represent a group of biological molecules that are most widely explored as targets for anticancer drug discovery. The rational for targeting the growth factor RTKs and the

non-receptor TK pathways is based on a strong evidence of frequent activating gene mutations and/or over-expressions in these proteins, which are common in many human tumors. Fundamentally, growth factor receptor-mediated signaling, including the Human Epidermal growth factor Receptor (HER or ErbB) family, c-Met/hepatocyte growth factor receptor (HGFR), insulin-like growth factor receptor (IGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and fibroblasts growth factor receptor (FGFR), and the receptors for cytokines, such as IL-6 and other polypeptide ligands are all important in cell growth and proliferation, survival, development, inflammation and immune responses, and other cellular and physiological processes. As a result, studies have shown that the aberrations in the receptors or their ligands lead to uncontrolled growth and survival of tumor cells, the induction of tumor angiogenesis, and the promotion of tumor metastasis. In particular, the molecular-targeting and the inhibition of the members of the EGFR family is presently one of the most explored approaches in anticancer drug design, and inhibitors of the HER family as anticancer therapeutic agents are currently in various stages of development or in clinical application. Among the strategies, antibody and protein-based modalities that block the extracellular surface of the receptor were some of the first to be considered in the development of targeted therapeutics for human tumors. In addition, small-molecule inhibitors (SMI) of the EGFR TK have been explored. Both the antibody-based and SMI approaches against growth factor receptors have resulted in viable therapeutic agents that are in the clinic or candidates that have the potential to become agents for the treatment of many solid tumors. Monoclonal antibody (Mab) therapies are presently approved for the treatment of human breast and lung cancers, diseases in which the ErbB family of receptors, including ErbB2 (HER2/neu) are validated to be essential. Small-molecule EGFR TK inhibitors include Gefitinib and Erlotinib, which compete at the adenosine triphosphate (ATP) binding site of the catalytic domain of the receptor kinase and block the EGFR signaling and are a consideration for the treatment of non-small cell lung cancer, pancreatic cancer, ovarian, and head and neck cancers. Another small-molecule inhibitor in clinical application is Imatinib (Glevec), which targets the TK activity of the breakpoint cluster region (BCR)-Abelson (ABL) fusion protein that results from the chromosomal abnormality, Philadelphia chromosome and causes the chronic myelogenous leukemia (CML). Other kinases of interest in the novel drug discovery and development include Src (a tyrosine kinase), the protein serine-threonine kinase, Aurora kinase, and cyclin-dependent kinases, as well as other non-receptor protein and lipid kinases. Consideration is also now being given to agents that inhibit multiple targets, as is the case with Sorafenib, that inhibits PDGFR family, Flt-3, Kit, RET family, VEGFR2, VEGFR3, and Raf protein kinase family, Dasatinib that inhibits Abl, Fyn, Src, Lck TKs, Sunitinib, that inhibits Flt-3, Kit, VEGFR2, and PDGF TKs, and Lapatinib, which inhibits EGFR and ErbB2 TKs [25–56].

36.3.4.2 Inhibitors of Protein:Protein Interactions

In the past, protein-protein interactions have posed a significant challenge to researchers for developing effective disrupting agents as therapeutics. These interactions were considered intractable in a large part due to the large surface areas presented by the proteins at the interface and hence, the difficulty of achieving sufficient interference using small-molecules. The focus on disrupting protein-protein interactions as drug discovery approaches has now emerged and there are several efforts in this arena from both the pharmaceutical industry and academia. Approaches to evaluate and target protein-protein interactions for drug discovery purposes range from traditional medicinal chemistry to non-traditional strategies. The strong rationale for targeting protein:protein interactions is the incidence in many human tumors of aberrations that occur in the proteins that are engaged in protein:protein interactions and how the altered functions of these proteins promote tumor processes. Details about signal transduction involving proteins that engage in protein-protein interactions, their importance to normal physiological processes and their involvement in the molecular events that contribute to malignant transformation and tumor progression are discussed in detail elsewhere in this volume and will not be presented here. Among the targets being pursued for drug discovery are the interaction of the tumor suppressor, p53 and its natural antagonist, MDM2 or the human congener, HDM2, the Bcl-2 or Bcl-xL/BH3, the XIAP/Caspase (and XIAP/Smac), the Myc/Max, and the Stat3:Stat3 dimerization, all of which are critical mechanisms that regulate cell growth and survival and are important for tumor-associated processes. Diverse approaches that are under consideration for modulating interactions involving these proteins include peptides and peptidomimetics that mimic the displayed recognition surface, and non-peptide analogs, natural products, oligonucleotide-based approaches, terphenyl, and other alpha-helix mimetics, chalcones, piperidines, piperazines, fused indoles, isoindolinones, spiro-oxindoles, cis-imidazolines (nutlins), quinolinol, and benzodiazepines [17, 57–77].

36.3.4.3 Protein:DNA-Binding Complex Inhibitors

Another approach to designing antitumor agents focuses on modulating protein:DNA complexes and disrupting their functions in tumor cells. Topoisomerase inhibitors, such as anthracyclines, and camptothecin and analogs (irinotecan and topotecan) mediate their antitumor cell effects by this mechanism. Here, the complex between the protein

(example Topoisomerase enzymes) and DNA becomes the target for the binding by the drug, thereby stabilizing an otherwise cleavable complex and leading to DNA lesions and apoptosis. Inhibitors of protein:DNA complexes, such as Topoisomerase I and II inhibitors are currently in clinical use or are being considered for clinical use in a variety of human cancers, including ovarian and lung cancers [78–83].

36.3.4.4 Modulation of Protein Synthesis

The inhibition of protein expression at the translation level is one of the ways for modulating aberrant proteins and that can be exploited for the design and development of novel therapeutic agents against cancer. Recent reports have shown the therapeutic potential of the molecular modulation of the complex formation of the set of initiation factors (eIFs), factors required to dissociate the 40S and 60S ribosomal subunits, and to recruit the mRNA and the initiator tRNA to the 40S subunit, and to promote the joining of the 60S subunit for elongation to begin. In that regard, studies were recently reported that showed that the inhibition of the association between eIF4A and eIF4G (eIF4A:eIF4G complex) and the promotion of the formation of a stable ternary complex between eIF4A and eIF4B by a natural product, or the disruption of eIF4E:eIF4G complex by a small-molecule, identified through a high throughput screening, which ultimately modulated the translation and the expression of oncogene proteins. The antitumor cell effects of the modulation of those molecular events in multiple cancer cell lines are a positive sign of the potential to explore this approach in drug discovery and development. More recent approaches are also focusing on the deregulation of microRNA and the application of siRNA or antisense oligonucleotides to target the protein expression at the level of translation. For microRNAs, these short non-coding RNA molecules control the gene expression of their respective target mRNAs and in doing so control different physiological and developmental processes. The deregulation of microRNA expression and function is frequently observed in many human cancers, with studies showing that the aberrant expression and function of certain microRNAs modulates tumor cell proliferation, survival, angiogenesis and metastasis, which makes them suitable targets for the development of therapeutic approaches [84–94].

36.3.5 Reverse Approach to Drug Discovery: Beginning with Modulating a Biological Response and a Cancer Phenotype

In contrast to the forward, target-based approach, the reverse approach to drug discovery and development process is initiated by focusing on the identification of agents that modulate general biological or biochemical processes that contribute to malignant transformation without the initial consideration to what constitutes the drug target. With this strategy, the knowledge of the potential targets that are critical for malignant transformation and tumor progression or the molecular mechanisms underlying the cancer phenotype is not essential at the initial stage of the drug discovery process. Instead, the emphasis may be on the modulation of a specific response that is consistent with antitumor cell effects of the candidate agent. Such readouts may be in terms of biological responses, biochemical activity or may be within the context of the changes in molecular property, but in none of these cases is the knowledge of the potential target that is being modulated by the candidate drug necessarily required. Thus, rather than focusing on the target at the beginning phase of the discovery process, the reverse approach will identify an active candidate agent based on the potential to produce a defined desirable and tumor-specific biological or molecular response. The emphasis on the biological response as a readout is based on the principle that a desirable change in the biology of cancer cells or in the cancer phenotype, which is consistent with the ultimate goal of any anticancer therapeutic modality, is the result of modulation of specific biochemical events or molecular entities in the cancer cells. Thus, by focusing the initial screening on biological response or the modulation of the cancer phenotype as readouts to identify a potential drug candidate, the reverse approach serves to assess the antitumor cell activity of the agent at the very early stage. This approach provides an indication of the potential for the candidate agent to induce antitumor cell effects prior to acquiring any knowledge of the mechanism(s) of action and/or what the target(s) might be. Illustrated in Fig. 36.2 is a model of the reverse approach showing an initial screening of compound libraries or natural products using biological response as readout. A variety of assays have been employed for evalu-

Fig. 36.2 Flow chart of the reverse (non-target-based) approach to anticancer drug discovery. The approach is initiated with the screening of compounds or libraries using assays that may be cell-based to identify active compounds that induce a well-defined biological response or a response within a molecular context. Subsequent steps include structure-activity relationship studies and medicinal chemistry efforts for the identification of lead compounds. The next stages involve the evaluation of the activity of select compounds in biological assays, and the identification, characterization, and validation of target(s) relative to the candidate compounds and the disease model, and ultimately the identification of drug candidates.

ating compounds in this approach. In most cases, the measurable response is biology-based, which examines a change in phenotype, such as tumor cell growth inhibition or induction of apoptosis, or evaluating and selecting active agents that inhibit angiogenesis, migration and invasion, or tumor growth. Additional biological responses that have been considered or used are autophagy (the dynamic process of subcellular degradation) and tumor metastasis. In the case of a non-biological response, this will involve the induction of changes within specific molecular mediators, which may be critical for tumor processes, malignant transformation, and tumor progression. Such biochemical or molecular analyses include gene or protein array technology for screening to identify active agents that modulate cancer-relevant molecular entities prior to any knowledge of the potential target for the agent. The completion of the human genome project provides a suitable platform for evaluation and to identify genes of relevance to human cancers.

Overall, the objective at the initial screening stage is simply to identify agents that modulate specific tumor processes, or produce biological or molecular responses relevant to malignant transformation and tumorigenesis. While the focus at the initial stage of the drug discovery process is shifted away from determining what the target entities or pathways are for the active agents, it is presumed that those agents that modulate tumor cell phenotype do so by interacting with specific molecular entities or pathways that have functions crucial to the cancer phenotype. Targets may include cancer-specific cell surface entities, extracellular domains of receptors or other membrane-bound molecules, intracellular entities, or a combination of these targets. In accordance with the objectives of this approach and therefore by the nature of the initial assay design, the candidate compound(s) may interact with one or multiple entities or pathways, thereby modulating multiple aspects of cellular functions and producing the measurable biological response. Thus the measured biological response can be presumed to be due to some combination of the molecular modulations that are induced by the candidate drug of the specific biological entities or pathways important to the cancer cell and essential for maintaining the cancer phenotype. The overall response of the drug will be expected to be influenced by the level of expression, as well as the functional importance of each of those molecular entities, which are modulated by the candidate agent and that contribute the measured biological response [2, 95–102].

From the initial screening stage, compounds that are identified as possessing bioactivity and are selected will progress through the drug discovery process to the next stage, which is to characterize the compound in more detail in terms of activity, potency, and selectivity, determination of the identity of the target, and then target validation, and also to perform studies of the interaction of the drug candidate with the putative target. Also important at this stage are studies of the structure-activity relationships that are directed at establishing a correlation between the activity of the compound and the chemical moieties, thereby identifying the chemical functionalities that confer activity, and medicinal chemistry and lead optimization processes to further enhance the activity and the potency of the candidate agent and to derive more suitable drug candidates. Optimized drug candidates will ultimately be evaluated for in vivo pharmacological properties and antitumor efficacy in tumor models.

36.4 Summary

Until recently, the potential targets for drug discovery has been limited to a select few molecular entities that evidence has established have a critical role in cancer, including the Ras small G protein. New biological entities emerged as potential new targets following the increased understanding of the molecular basis of cancer. Those entities that were confirmed to be highly critical in malignant transformation and tumor progression became the focused areas for targeting in the drug discovery arena. Moreover, the completion of the human genome project has further opened the way for identifying novel cancer-relevant genes and gene products and hence, increased the potential to identify new targets. It is expected that substantial information will be derived from the analyses of the human genome that will be useful in many areas, including identifying important cancer-related genes and gene products, and this possibility has generated a strong excitement from both academic institutions and the pharmaceutical industry. Such information would facilitate the identification of novel tractable and druggable targets and help drive the discovery and development of more effective and safe drugs for a variety of human diseases, including cancer. We are already seeing an upsurge of information from the genomics and proteomics analyses. The large volume of information demands substantial and suitable evaluation procedures and diverse analytical tools in order to make insightful deductions and meaningful decisions on what constitute druggable and tractable targets, thereby promote the development of novel effective anticancer drugs. Moving forward therefore in the era of genomics, proteomics, and other omics approaches, it is expected that appropriate data mining and chemical genetic approach will continue to expand and improve the drug discovery and development process. The expectation is also that as advancement in technology occurs, this will enhance the ability to design and generate more effective tools and approaches for identifying good candidate lead anticancer agents and drive the drug discovery and development process. New analytical tools also enhance the drug discovery process in many ways, including the identification of new targets, and in assay

development and screening, and lead identification and optimization. For example, the availability of molecular modeling and the in silico virtual screening strategies in combination with structural biology have all enriched the modern-day drug discovery and development process in more ways than it ever was in the past. There is therefore increased chance to find new drugs that are focused on cancer that will be more effective and safe, and which will provide a much need relief to the many cancer patients who receive them [21, 97, 98].

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Biological Principles and Clinical Application of EGFR Inhibitors in Cancer

37

Zhuo (Georgia) Chen, Suresh Ramalingam, and Dong M. Shin

37.1 Introduction

Epidermal growth factor receptor (EGFR), an important oncogenic protein, has attracted intensive research efforts for over 40 years. In the 1960s, epidermal growth factor (EGF) was discovered by Stanley Cohen as a factor from submaxillary glands that induced precocious eyelid opening in newborn mice [1]. EGF was purified in the year 1962 and identified in 1975 as a mitogenic polypeptide and an initiator of cellular response to epithelium proliferation and growth through its cell surface receptor [2, 3]. Like other growth factors, the binding of EGF to its specific receptor EGFR triggers a series of signal transductions and is essential in cellular regulation for normal physiological activities. In the 1980s, EGFR was purified as a transmembrane protein from A431 epidermoid carcinoma cells [4]. The cDNA of EGFR was then isolated and the coding sequence of this protein was determined [5, 6].

The oncogenic function of EGFR was first suggested by Downward et al. [7], who described a close similarity between EGFR and the avian erythroblastosis virus (AEV) v-*erbB* transforming protein [7, 8]. In fact, EGFR overexpression has been documented extensively in a wide variety of malignant tumors, supporting its oncogenic function and making it an appropriate molecular target for cancer treatment. The importance of EGFR is underscored by the publication of over 10,000 papers since its discovery that described EGFR signal transduction pathways and interaction partners [9]. These studies have resulted in the development of EGFR inhibitors, which have shown clinic efficacy in the treatment of several solid tumor types.

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This chapter first summarizes the structure, biological function, and signal transduction pathways of EGFR and then provides an overview of EGFR inhibitors, their preclinical studies, and clinical applications in cancer treatment. Recent advances in understanding cancer cell response to EGFR inhibitors by using biomarkers are discussed at the end of the chapter.

37.2 Biological Characteristics of EGFR

37.2.1 EGFR Gene Structure and Localization

EGFR is encoded by a gene located on chromosome 7p12 [10]. Wild-type EGFR contains 1186-amino acids translated from a 5.8 kb mRNA, which was first identified in A431 cells [5, 11–13]. Studies of A431 cells illustrated that human EGFR has a striking homology to v-erbB, particularly in the amino acid residues beginning at the junction of the cytoplasmic and transmembrane domains to the carboxy-terminus of EGFR. Ninety-five percent of the residues in this region, which includes an ATP-binding domain, are conserved, indicating the functional significance of this portion of the receptor. The amplification and variation of EGFR encoding sequences were also found to result from chromosomal translocation (M4) involving chromosome 7 [5, 11, 14]. Currently, there are over 600 publications reporting *EGFR* gene structures in the National Center for Biotechnology Information database.

37.2.2 EGFR Protein Structure and Function

The mature EGFR is a 170 kDa single polypeptide belonging to the erbB family [6, 15, 16]. Four receptor proteins have been identified as members of the erbB family. They are EGFR or erbB1, Her2/neu or erbB2, erbB3, and erbB4 [15, 17, 18]. EGFR consists of several featured domains, including an extracellular domain (residue 1–621), a hydrophobic

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_37

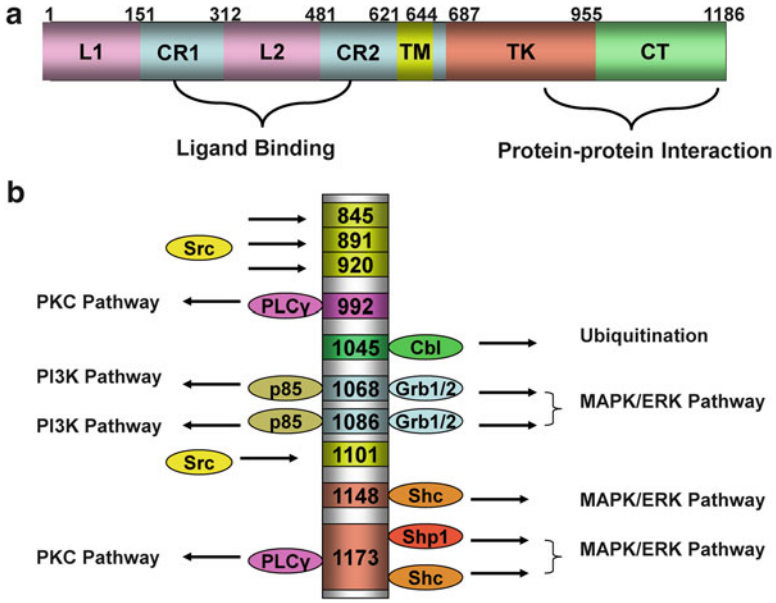

Fig. 37.1 Epidermal growth factor receptor structural and functional domains. (**a**) Schematic representation of EGFR sequence. *L and CR*, ligand-binding and cysteine-rich domains, *TM* transmembrane domain, *TK* tyrosine kinase domain, *CT* carboxyl-terminal domain. (**b**) Tyrosine-phosphorylation sites of EGFR and the bound signaling protein mediators.

transmembrane domain (residue 622–644) and a cytoplasmic domain (residue 645–1186) (Fig. 37.1a). The extracellular domain of EGFR is responsible for binding EGF and other EGFR-specific ligands with high affinity. Structurally, this domain of the receptor contains 10–11 N-linked oligosaccharide chains which are required for translocation of EGFR to the cell surface [19, 20], and 10 % comprises half-cystine residues. The ligand-binding domain lies between the two half-cysteine-rich clusters.

The cytoplasmic domain of the receptor is characterized by a highly conserved tyrosine kinase (TK) domain. This region contains an ATP-binding pocket which is essential for the intrinsic TK activity of EGFR. The substrates of TK are several tyrosine residues next to the ATP-binding region or outside the ATP-binding pocket near the C-terminus of the receptor. Autophosphorylation of tyrosine residues induces conformational change of the protein, creating a docking structure for EGFR interaction with its downstream signaling mediators. In addition, seven serine and threonine residues bordering the TK domain are substrates for phosphorylation by non-receptor kinases, such as protein kinase C. Phosphoserine and phosphothreonine are also required for protein-protein interactions and have been inferred to be important for the receptor downregulation processes.

37.2.3 EGFR Ligands and Its Activation

EGFR activation is triggered by binding of its specific ligand. Since the discovery of EGF, several other ligands containing EGF-like domains have been identified as specific EGFR binding partners [9, 21, 22], including transforming factor α (TGF-α), amphiregulin, epiregulin, and β-cellulin. The soluble ectodomain of EGFR binds ligand, and this formation of a ligand-receptor complex on the cell surface induces the dimerization of EGFR [23, 24]. A 2:2 complex is formed in which the affinity of EGF or TGF-α to EGFR is 100–500 nM [25–27].

In the absence of ligand binding, EGFR exists on the cell surface as either monomer or dimer. However, ligand-induced dimerization is required for activation of the intrinsic TK activity [28–30]. Studies of the kinetics of stimulated and unstimulated EGFR have shown that ligand binding doubles the V_{max} parameter and decreases the K_m parameter for ATP by tenfold [31]. Binging to ligand increases the proportion of dimerized EGFR and reorientates the kinase domains in a way that increases the affinity for ATP binding due to conformational change, thus enhancing the enzymatic activity.

EGFR is autophosphorylated on several C-terminal tyrosines, including Tyr 992, Tyr 1045, Tyr 1068, Tyr 1086, Tyr 1148, and Tyr 1173 (Fig. 37.1b). The role of this autophosphorylation is to create a specific docking structure and facilitate the formation of protein complexes with EGFR downstream signal transduction partners. The formation of these protein complexes requires specific ternary configurations depending on autophosphorylation at specific tyrosine sites, leading to phosphorylation-dependent activation of major intracellular signaling pathways.

37.3 Biological Function and Related Signal Transduction Pathways of EGFR

Depending on the functional diversity of the proteins that EGFR complexes with, or is phosphorylated by, stimulation by EGFR ligands results in simultaneous activation of

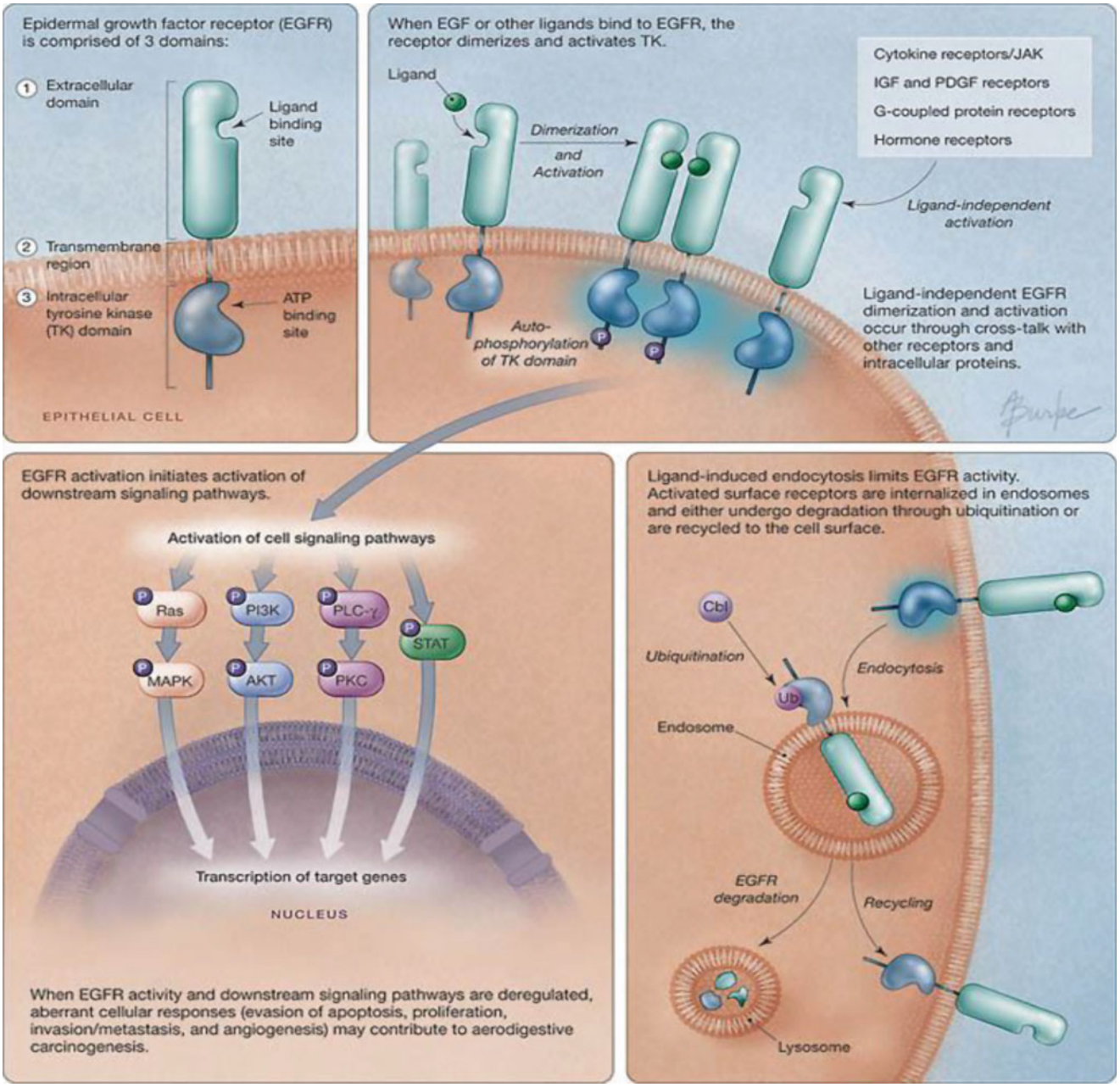

Fig. 37.2 Epidermal growth factor receptor activation, processing, and signaling. *ATP* adenosine triphosphate, *Cbl* a ubiquitin E3 ligase, *IGF* insulin-like growth factor, *JAK* Janus kinase, *MAPK* mitogen-activated protein kinase, *P* phosphate, *PDGF* platelet-derived growth factor, *PI3K* phosphatidylinositol-3-kinase, *PKC* protein kinase C, *PLC* phospholipase C, *STAT* signal transducers and activators of transcription, *TK* tyrosine kinase, *Ub* ubiquitin (adapted from Karamouzis, M. V. et al. JAMA 2007;298:70–82).

multiple signal transduction pathways (Fig. 37.2). Thus, activation of EGFR induces many biological activities, including evasion of apoptosis, promotion of cell growth, and cell migration. At the same time, ligand binding induces a rapid internalization of the receptors and enclaves them in endosome [32–34]. The internalized EGFR either undergoes degradation by locating to lysosomes or is recycled back to the cell membrane.

37.3.1 Ras/Mitogen-Activated Protein Kinase (MAPK) Pathway

RAS/MAPK is one of the major signaling pathways activated by EGFR. The formation of a protein complex between EGFR and Grb2 initiates signal transduction through the RAS/MAPK pathway. Grb can associate with EGFR either directly at Tyr 1068 and 1086 sites, or indirectly by binding

to EGFR-associated Shc, which recognizes phosphorylated EGFR at Tyr1148 and 1173 [35, 36]. It can constitutively bind to Ras exchange factor Sos, resulting in the exchange of Ras-bound GDP for GTP and thus Ras activation. The activated Ras then activates the serine/threonine kinase Raf-1 [37, 38]. Activated Raf-1, through MEK, contributes to the phosphorylation, activation, and nuclear translocation of ERK1/2 (MAPK). ERK1/2 is responsible for phosphorylation of several nuclear transcription factors, such as c-fos and C/EBPs, which regulate cell proliferation and cell cycle progression [39, 40]. Activated MAPK also provides a negative feedback loop for this pathway by promoting dissociation of the Grb2/Sos complex and a reduction in the strength of signal transduction.

37.3.2 Phosphoinositide-3-Kinase (PI3K)/AKT Pathway

PI3K is a major regulatory protein that is involved in a variety of cellular activities, including cell proliferation, survival, adhesion, and migration [41–43]. Among its three classes, only Class Ia is activated by a tyrosine kinase receptor. One of the PI3K subunits, p85, can directly interact with Grb1/2 through its SH2 domain when Grb1/2 associates with EGFR at Tyr 1068 and Tyr 1086 [44]. Although the major binding partner of p85 is not EGFR, but ErbB3 [45, 46], activation of PI3K is found in response to the EGFR ligand through EGFR/ErbB3 heterodimers. Activated PI3K-Ia produces phosphatidylinositol-3,4,5-trisphosphate (PIP_3). One of the well-known targets of this second messenger is the ser/thr kinase AKT [47]. Phosphorylated AKT can prevent apoptosis through targeted inhibition of Bad, a pro-apoptotic member of the Bcl-2 family and caspase 9, an enzyme involved in the Fas-mediated death pathway [48, 49]. PI3K/AKT also regulates the mammalian target of rapamycin (mTOR), which is responsible for the activation of translational machinery [50] and regulation of angiogenesis [51].

37.3.3 Phospholipase C-γ (PLCγ) and Protein Kinase C (PKC) Pathways

EGFR signaling also affects cellular phospholipid metabolism. In addition to PI3K, it is found that PLCγ is regulated by EGF stimulation [52]. PLCγ binds directly to EGFR at Tyr 992 and 1173 and can be phosphorylated by EGFR at Tyr 771 and Tyr 174 [53]. The activated PLCγ catalyzes the hydrolysis of PtdIns [4, 5]-P_2 to yield the important secondary messengers 1, 2-diacylglycerol (DAG) and inositol 1,3,5-triphosphate (IP_3). IP_3 mediates calcium release and at the same time affects the Ca^{2+}-dependent enzyme PKC, which uses DAG as its cofactor. Thus, through PLCγ, EGFR can activate the Ca^{2+}-dependent PKC pathway, which plays important roles in cell differentiation, proliferation, migration, and invasion [54, 55].

37.3.4 Janus Kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) Pathways

STATs are identified as signal transducers upon activation by cytokine receptors [56, 57]. There are at least seven STAT genes that have been identified in mammals, including STAT-1 to STAT-4, STAT-5a, STAT-5b, and STAT6. Homo- and heterodimerization of STAT proteins allows them to translocate into the nucleus and work as transcription factors for activation of gene expression in response to cell growth and proliferation. STATs, particularly STAT-1, STAT-3, and STAT-5, have been implicated in EGFR signaling. However, their mode of activation is significantly different from that by cytokine receptor. First, the EGFR ligand-stimulated phosphorylation of STATs does not require cytokine receptor JAK kinases. Second, STATs do not bind to the C-terminal phosphotyrosines of EGFR; instead they are constitutively associated with EGFR [58, 59]. Activation of STAT transcription activity strictly depends on EGFR tyrosine kinase activity [60, 61]. Moreover, Src kinase may play a role in EGF-dependent STAT activation [59, 62].

37.3.5 Src Pathway

Src and its family members have been extensively reported to support EGFR activity [63–65]. Overexpression of Src proteins strongly enhances EGF-mediated proliferation and transformation of fibroblasts and epithelial cells [66, 67]. Src does not bind to the major autophosphorylation site of EGFR, but phosphorylates EGFR in its kinase domain at Tyr891 and 920 sites [68]. These two phosphotyrosines bind the SH2 domain of Src and may provide a docking site for the p85 subunit of PI3K. In addition, two other Src-dependent phosphorylation sites were identified at Tyr 845 and Tyr 1101 in the EGFR cytoplasmic domains [69]. Phosphorylated Tyr 1101 facilitates Src binding to EGFR, while phosphorylation of Tyr 845 is required for Src mediated STAT5b binding [62]. It is possible that Src serves as a signal transducer downstream of EGFR or a contributor to EGFR activation.

37.3.6 Transactivation of EGFR

In addition to ligand binding, EGFR can be functionally activated through cross-talk with other cell surface receptors. G-protein-coupled receptors [70, 71], such as prostaglandin

E2 receptors and gastrin-releasing peptide receptor, platelet-derived growth factor receptor [72], insulin-like growth factor receptor [73], and some hormone receptors [74] also transactivate EGFR-mediated signal transduction pathways.

37.4 Oncogenic Activities and Expression of EGFR in Human Cancer Cell Lines and Tissues

37.4.1 In Vitro and In Vivo Studies of EGFR

The oncogenic activity of EGFR is evident from its ability to transform normal cells and promote cell proliferation. EGFR, by itself, is insufficient to cause transformation, but the addition of EGF, TGF-α, or other EGFR-specific ligands, or the existence of activation mutations is necessary for manifestation of the transformation phenotype [8, 75–77]. In addition to cell line studies, the use of transgenic animals has allowed the study of whether overexpression of EGFR or its ligands is indeed causative for hyperproliferation or tumor formation. Using TGF-α as an example, transgenic mice that overexpressed TGF-α driven by a keratin promoter show hypertrophy and hyperkeratosis accompanied by alopecia or stunted hair growth [78, 79]. TGF-α transgene expression is also linked to the appearance of papillomas following irritation or wounding. Targeted expression of TGF-α in the mammary gland results in hyperplasia, cystic expansion, and papillary adenoma following multiple pregnancies and lactation [80]. Therefore, overexpression of TGF-α is responsible for hyperproliferative responses but does not generally lead to tumors in rodents, suggesting that the TGF-α/EGFR signaling pathway provides the initial step in multistage carcinogenesis. Neoplastic transformation requires the overexpression or mutation of other proteins in the target tissues, such as c-Myc oncoprotein. This notion is supported by studies using mice with double transgenes of TGF-α and c-Myc that show development of mammary tumors [81].

Constitutively activated EGFR with no requirement for ligand binding can result from mutations that are found in many tumor tissues. A well-known example of a naturally occurring EGFR deletion mutant is EGFRvIII (Δ2-7) [82, 83], although other classes of EGFR ectodomain mutations EGFRvI and vII have also been discovered [84]. The EGFRvIII mutant has been detected in 40–50 % of grade VI glioblastomas [85], in up to 70 % of medulloblastomas and a small proportion of breast, ovarian, prostate, lung, and head and neck carcinomas [86–90]. Due to the deletion of exons 2–7, EGFRvIII lacks most of the extracellular domain, thus cannot be activated by ligand. Instead, it is constitutively activated and not internalized, resulting in constitutive long-term signaling which supports cell proliferation. A similar situation is observed in breast cancer, in which the Her2/neu or erbB2 oncogene was identified [91]. Her2/neu has a truncated extracellular domain and a point mutation in the transmembrane region, which activates the protein by inducing its dimerization in the absence of ligand. This receptor is a major oncogene and is overexpressed in 20–25 % of breast cancer tissues and associated with shortened survival [92].

Other activating mutations of EGFR are commonly found in lung cancers, and also observed in esophageal and head and neck cancers [93–96]. A deletion in exon 19 and nucleotide substitution in exon 21, such as L858R and L861Q, are the most common EGFR mutations in lung cancer cells. These genotypes have been identified as EGFR-TK inhibitor (TKI)-sensitizing mutations, because tumor cells harboring these mutations showed greater sensitivity to an EGFR-TKI (gefitinib) than those maintaining the wild-type gene [97, 98]. The mutations were suggested to stabilize the binding of EGFR with ATP or its competitive inhibitors. These mutations also induce anti-apoptotic pathways mediated by STAT-3 and AKT, strengthening the oncogenic activity of EGFR. However, a recent study using samples from the NCIC CTG trial showed that the presence of an EGFR mutation may increase responsiveness to EGFR-TKI, but is not indicative of a survival benefit [99].

37.4.2 EGFR Expression in Solid Malignancies

Since EGFR activation results in cell proliferation, inhibition of apoptosis, and the formation of neo-vasculature, several studies have evaluated the impact of EGFR activation on the clinical outcomes of patients with various solid malignancies. These include evaluation of ligand expression/concentrations, receptor expression, and activation of downstream signals such as MAP kinase.

EGF is a potent mitogen in the gastrointestinal tract and has been shown to increase proliferation in glandular cells in the colon [100]. TGF-α, another EGFR ligand, is also a growth factor in colonic epithelial cells [101]. Varying levels of receptor overexpression have been noted in colon cancer. Although overexpression of the receptor is noted in tumor tissues of approximately two-thirds of patients with colon cancer, the impact on prognosis is less certain [102–104]. EGFR expression may increase the propensity to develop hepatic metastasis [105]. EGFR is also frequently over expressed in aerodigestive malignancies. In patients with non-small cell lung carcinoma (NSCLC), EGFR overexpression is noted in approximately 40–80 % of the tumors [106–109]. In some studies, a higher level of EGFR expression has been linked to more advanced stages of disease at the time of diagnosis, and also to a poor prognosis [110–112]. More recently, aberrant activation of the EGFR pathway has been

shown to result from mutations in the tyrosine kinase binding domain of EGFR [93]. Such mutations are noted in approximately 10–15 % of Caucasian patients. EGFR mutations are noted more frequently in women, patients with adenocarcinoma histology, those who have never smoked and in patients of Asian ethnicity [94]. High copy numbers of the EGFR gene also appear to have an impact on prognosis and may also predict heightened sensitivity to treatment with EGFR inhibitors [113, 114]. However, there does not appear to be a clear correlation between protein expression, gene copy number, or mutational status in NSCLC.

EGFR overexpression is also noted in a vast majority of tumor specimens from patients with head and neck carcinoma [115–117]. A higher level of EGFR expression in the tumor tissue relative to surrounding normal tissues has been noted by several investigators [118]. In many of these reports, EGFR overexpression was associated with an inferior clinical outcome and higher rate of disease recurrence following definitive local therapy. Aberrant activation of EGFR has also been noted in other solid malignancies such as breast, ovarian, pancreatic, and prostate cancers [102].

Based on reports of aberrant activation of EGFR in a variety of epithelial cancers, several agents that inhibit EGFR pathway activation have been tested in preclinical and clinical studies. Overexpression of the receptor in the tumor tissue relative to surrounding normal tissue provides the opportunity for cancer-selective therapy. Overexpression of EGFR and its ligands and the expression of constitutively active EGFR mutants support diverse cellular biological activities utilized by cancer cells to progress, including cell cycle progression, cell growth, differentiation, angiogenesis, invasion, and metastasis [9, 119, 120]. Elucidating the effects of EGFR on these biological activities has been greatly accelerated by the development of EGFR inhibitors in recent years.

37.5 Development of EGFR Inhibitors

The crucial roles that EGFR plays in tumor progression make this receptor an ideal target for the development of anti-cancer therapeutics [121–127]. There are several potential approaches to blocking EGFR signaling pathways. The receptor can be inhibited through its extracellular domain by antibodies and at the TK domain by small molecules [128]. In addition, antisense DNA/RNA, siRNA, or ribozymes can also be used to reduce expression of the receptor [129–131]. Table 37.1 summarizes EGFR-targeting agents that are currently being developed or are used in the clinic.

Many synthetic and semi-synthetic compounds have been identified as EGFR-selective TK inhibitors [128, 132–134], such as gefitinib (ZD1839, Iressa) [135, 136], CI-1033 [137–139], and erlotinib (OSI-774, Tarceva) [140]. These compounds are orally bioavailable and work as competitive inhibitors in the ATP-binding domain of EGFR. Both in vitro and in vivo preclinical studies show that they have various effects on tumor cells expressing EGFR. EGFR-TKIs inhibit cell growth of several human tumor cell lines by blocking phosphorylation of EGFR, ERK, and AKT, which may result from G1 arrest of the cell cycle by reduction of cyclin D1 [141] and upregulation of cyclin-dependent kinase inhibitors p21 and $p27^{kip1}$ [142, 143]. These inhibitors also downregulate the secreted growth factors TGF-α, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF), which are important autocrine/paracrine factors stimulating both tumor cell growth and angiogenesis [144, 145]. Thus, it is no surprise that EGFR-TKIs can provide significant growth inhibition of human tumor xenografts in animal models [144, 146–148]. Particularly, they markedly enhance the efficacy of cytotoxic agents including cisplatin, paclitaxel, and other platinums in the treatment of human tumor xenografts in nude mice [149–151]. Furthermore, EGFR-TKIs show synergistic inhibitory effects when they are combined with other targeted agents [152, 153]. For example, studies from our group and the others illustrate that the combination of EGFR-TKIs with cyclooxygenase-2 inhibitors (COX-2Is) either additively or synergistically reduces the growth rate of several types of cancer cells [154–157]. In addition, EGFR-TKIs have been combined with inhibitors of IGF-1R [158–160], mTOR [161, 162], and VEGF/VEGF-Receptor [144, 163–165], and antisense oligonucleotides against Bcl-2 [166] and MDM2 [167]. Simultaneously blocking the expression and/or activity of both EGFR and these proteins results in effective induction of cell cycle regression and apoptosis in vitro and in vivo.

Table 37.1 EGFR inhibitors in clinical use

Agent	Disease	Indication (FDA approved)
Gefitinib	Non-small cell lung cancer	Treatment of advanced NSCLC following prior chemotherapy
Erlotinib	Non-small cell lung cancer Pancreatic cancer	For advanced stage NSCLC, following 1 or 2 prior chemotherapy regimens In combination with gemcitabine for previously untreated advanced stage pancreatic cancer
Lapatinib	Breast cancer	In combination with capecitabine for HER2-positive breast cancer
Cetuximab	Colorectal cancer Head and neck cancer	For advanced stage colorectal cancer, in combination with irinotecan or as monotherapy (EGFR-positive tumors) In combination with radiation or as monotherapy
Panitumumab	Colorectal cancer	Monotherapy following failure of prior chemotherapy for advanced stage colorectal cancer (EGFR-positive tumors)

Monoclonal antibodies (mAbs) directed against the extracellular domain of EGFR are another class of therapeutic agents that has been well-studied in recent years. Among the available anti-EGFR mAbs, the one currently used in the clinic is the chimeric human:murine mAb cetuximab (IMC-225, Erbitux) [168]. This antibody binds to EGFR with high affinity (K_d = 0.39 nM), thereby competing with EGFR ligand binding and preventing ligand-induced EGFR activation [169–172]. In addition, cetuximab and other anti-EGFR antibodies can induce EGFR dimerization, resulting in receptor internalization and downregulation [173, 174]. Cetuximab also induces G1 arrest because of elevated levels of $p27^{kip1}$ [175], followed by apoptosis [176]. Blockade of EGFR by cetuximab decreases tumor cell production of angiogenic factors, such as bFGF, VEGF, and interleukin-8 [177, 178], leading to a significant reduction in microvessel density and enhancement of apoptotic endothelial cells in human xenograft tumors. Cetuximab and similar anti-EGFR mAbs also inhibit the expression and activity of several matrix metalloproteinases (MMPs). The antibody-mediated reduction of MMPs results in inhibition of tumor cell invasion in vitro and metastasis in vivo [178–180]. Similar to EGFR-TKIs, cetuximab markedly augments the antitumor effects of chemotherapeutic agents such as cisplatin and doxorubicin in human xenograft tumor models [181, 182]. However, the functions of EGFR-TKIs and anti-EGFR mAbs are not completely overlapping. Anti-EGFR antibodies, but not EGFR-TKIs, have the ability to induce receptor internalization, an important mechanism for attenuating receptor signaling. Accordingly, the combination of an EGFR-TKI with cetuximab demonstrated a synergistic inhibitory effect on tumor cell growth in an in vitro study [183].

Recently, high-throughput screening approaches and rational drug design have been used to develop new EGFR inhibitors. These methods allow the identification of novel EGFR inhibitors that can be further modified chemically to target a tumor population that is resistant to current EGFR-targeting agents [184]. Structure-based rational drug design utilizes nucleic acid-directed gene silencing molecules including antisense oligodeoxynucleotides, RNA interference, and ribozymes to prevent EGFR translation from its mRNA [185]. Although these approaches are still in the early phase of development, they have great potential to enhance our ability to block EGFR and its related signaling pathways in a clinically applicable manner.

37.6 Clinical Application of EGFR Inhibitors

37.6.1 Non-small Cell Lung Cancer

EGFR inhibitors are now in routine clinical use for the treatment of advanced stage NSCLC. Gefitinib was the first drug among this class of agents to demonstrate anti-cancer activity in NSCLC. Two doses of gefitinib (250 and 500 mg/day) were compared to each other in patients with advanced stage NSCLC following progression with standard therapy [186, 187]. The objective response rate was 10–19 % and an additional 30–40 % of the patients experienced disease stabilization. Improvements in quality of life and lung cancer symptom scores were also noted in many patients. No difference in efficacy was noted between the two doses, though the lower dose was associated with a more favorable tolerability profile. This led to the approval of gefitinib for the treatment of advanced NSCLC by the Food and Drug Administration (FDA). However, a subsequent phase III study that compared gefitinib with placebo failed to demonstrate a survival advantage when gefitinib was used in second or third line treatment settings for advanced NSCLC [188]. Despite a response rate of 9 %, and modest disease stabilization rate, the lack of improvement in progression-free survival and overall survival with gefitinib effectively halted the use of this agent for the treatment of advanced stage NSCLC. However, there were selected sub-groups of patients, including never-smokers and those of Asian ethnicity, who did experience superior survival with gefitinib over placebo. This is suggestive of a role for this agent in clinically-selected or molecularly-selected subsets of patients with NSCLC.

Erlotinib, another EGFR-TKI, is also FDA approved for the treatment of advanced NSCLC. Following the demonstration of its anti-cancer activity in a phase II study [189], erlotinib was compared with placebo in a randomized phase III study (2:1 randomization) for advanced NSCLC patients. Those who progressed following one or two prior chemotherapy regimens were eligible for the study [190]. The median survival was superior with erlotinib (6.7 months vs. 4.7 months). The response rate was 9 %, and 35 % of patients experienced disease stabilization with erlotinib. Improvements were also noted in time to symptomatic deterioration for patients treated with erlotinib. The main side effects were skin rash (75 %) and diarrhea (54 %). The data from this study led to the approval of erlotinib for use as either second line or third line therapy of advanced stage NSCLC.

Cetuximab, a monoclonal antibody against EGFR also has efficacy as monotherapy, although the activity appears to be relatively modest. In a phase II study for patients with previously treated advanced NSCLC, cetuximab therapy demonstrated a response rate of 5 % and disease stabilization rate of 30 % [191]. The incidence of skin rash was more frequent, but diarrhea was uncommon with the antibody.

Following the demonstration of efficacy of EGFR inhibitors as monotherapies, randomized clinical trials have been conducted to study them in combination with chemotherapy. This approach was supported by preclinical data that demonstrated supra-additive effects when an EGFR inhibitor was co-administered with chemotherapeutic agents in NSCLC cell lines. However, there was no improvement in overall survival and progression-free survival for the combination of

an EGFR-TKI and standard platinum-based chemotherapy over that with chemotherapy alone in patients with advanced stage NSCLC [192, 193]. Although the reasons behind the conflicting pre-clinical and clinical findings are not entirely clear, further development of combination regimens with EGFR-TKIs and chemotherapy has been discontinued.

On the contrary, the monoclonal antibody cetuximab was associated with promising response rates and survival duration when combined with chemotherapy in phase II studies for patients with advanced stage NSCLC [194–196]. This prompted phase III studies of chemotherapy with or without cetuximab in patients with chemotherapy-naïve advanced stage NSCLC. The results have been conflicting, with one study that combined cetuximab with chemotherapy (carboplatin and a taxane) showing no improvement in progression-free survival over chemotherapy alone, whereas another study demonstrated improved survival in patients with EGFR-expressing tumors who were treated with cetuximab in combination with chemotherapy (cisplatin and vinorelbine) (Press release, Imclone Pharmaceuticals, Inc., September 2007). The detailed results of this trial are yet to be reported. Of note, the positive study with cetuximab was unique in selecting patients for therapy based on EGFR expression status.

The clinical development of panitumumab, a fully human monoclonal antibody against EGFR, was discontinued based on data from a randomized phase II study (carboplatin and paclitaxel administered with or without panitumumab) [197]. There was no improvement in any of the efficacy parameters evaluated in the study with the addition of panitumumab to chemotherapy. Presently, the reasons behind the differential interaction of EGFR-TKIs and monoclonal antibodies with chemotherapy are unclear. It is conceivable that antibody-mediated cellular cytotoxicity (ADCC) might play a role. Furthermore, it can be hypothesized that the non-target effects of EGFR-TKIs may be responsible for their unfavorable interaction with chemotherapy.

EGFR inhibitors are now being evaluated for the treatment of earlier stages of NSCLC. This includes combination approaches with external beam radiation for locally advanced, surgically unresectable NSCLC and in the adjuvant therapy setting for patients with surgically-resected early stage NSCLC. Appropriate patient selection, using molecular and clinical factors is a key focus of ongoing research efforts.

37.6.2 Head and Neck Cancer

Several studies have documented the efficacy of EGFR inhibitors for the treatment of head and neck cancer. Both gefitinib and erlotinib have demonstrated single agent activity in this setting. Administration of gefitinib to patients with advanced head and neck cancer following progression with one prior chemotherapy regimen was associated with a response rate of 11 % and an overall disease control rate of 53 % [198]. In a small subset of patients who underwent sequential tumor biopsies, no consistent correlation was noted between response to therapy and changes in various biomarkers. Erlotinib was also associated with objective tumor responses in patients with advanced head and neck cancer [199]. Some recent studies have evaluated the combination of gefitinib or erlotinib with chemotherapeutic agents. A phase I/II study of erlotinib with cisplatin found response and disease stabilization rates of 21 and 49 %, respectively, in previously untreated patients with advanced head and neck cancer [200]. In another study that evaluated the combination of erlotinib, cisplatin, and docetaxel, a high response rate of 67 % was noted [201].

The monoclonal antibody cetuximab has received FDA approval for the treatment of patients with locally advanced head and neck cancer, either as a single agent in patients with advanced platinum-refractory disease, or in combination with radiation for localized disease. Since EGFR activation is a survival pathway for irradiated cells [202], the combination of radiotherapy with an EGFR inhibitor was evaluated in a randomized phase III study in patients with locoregional head and neck cancer [203]. Concurrent administration of external beam radiation with cetuximab resulted in near doubling of the median survival over that with radiation alone. The median duration of local control was 24 months with cetuximab and radiation compared to 15 months with radiotherapy alone. Notably, there was no increase in radiation-related toxicities with the addition of cetuximab, with the exception of skin rash. These results led to the evaluation of cetuximab in combination with radiation in the treatment of other solid malignancies.

When given to patients with advanced head and neck cancers as monotherapy following failure with prior platinum-based therapy, cetuximab was associated with a response rate of 13 % and a disease stabilization rate of 46 % [204]. Cetuximab has also been shown to overcome resistance to platinum-based chemotherapy. The administration of cetuximab in combination with cisplatin to patients who failed prior platinum-based therapy resulted in a response rate of 10 % and disease control rate of 53 % [205]. The favorable tolerability profile and anti-cancer activity associated with the combination of cetuximab and platinum-based chemotherapy was further substantiated by a phase II study by Herbst et al. [206]. These findings were confirmed by a phase III study, which demonstrated a superior response rate with cetuximab and cisplatin when compared with cisplatin alone (26 % vs. 10 %) and a non-significant trend towards improved progression-free survival with the combination (4.2 months vs. 2.7 months) [207]. Another phase III trial conducted recently confirmed the utility of cetuximab in first line therapy of head and neck cancers. The median survival associated with cetuximab in combination with platinum-

5-fluorouracil was superior to that of chemotherapy alone in patients with recurrent or metastatic squamous cell carcinoma of the head and neck (10.1 months vs. 7.4 months) [208]. EGFR inhibitors are now being evaluated in combination with other targeted agents and also in earlier stages of head and neck cancer as adjuvant or neo-adjuvant therapy.

37.6.3 Colorectal Cancer

Cetuximab is efficacious in the treatment of advanced colorectal cancer, both as a monotherapy and in combination with chemotherapy. In a large phase II study ($n=346$ patients), cetuximab was administered as a monotherapy to patients who had progressed with standard chemotherapeutic regimens [209]. Expression of EGFR in the tumor (immunohistochemistry positive) was mandated as an entry criterion. The response rate (12 %) and median overall survival (6.6 months) were suggestive of single agent activity for cetuximab in this setting. Subsequently, a phase III study compared cetuximab with best supportive care in patients with EGFR-expressing colorectal cancer, following progression with standard chemotherapy [210]. The response rate was 8 % with cetuximab and there was a significant improvement in progression-free survival and overall survival. Patients treated with cetuximab reported less deterioration in global health status at 8 weeks and 16 weeks of therapy, compared to that with supportive care alone.

Cetuximab has also undergone extensive evaluation in combination with chemotherapy for the treatment of advanced colorectal cancer. In a study for irinotecan-refractory colorectal cancer, patients were randomized to treatment with cetuximab alone or in combination with irinotecan [211]. The combination was associated with a higher response rate than that with monotherapy (23 % vs. 11 %). The median time to progression (4.1 months vs. 1.5 months) and overall survival (8.6 months vs. 6.9 months) were also superior for the combination. Interestingly, anti-cancer activity was also noted when irinotecan was added to cetuximab after progression with monotherapy. This was consistent with preclinical studies that demonstrated reversal of resistance to irinotecan by the addition of cetuximab [212]. This notion was further substantiated by a non-randomized study ($n=1123$ patients) that treated irinotecan-refractory, EGFR-expressing colorectal cancer patients with the combination of cetuximab and irinotecan [213]. The median progression-free survival rate of 34 % at 24 weeks met the primary endpoint of the study.

Recent studies have focused on the evaluation of cetuximab in patients who had not received any prior chemotherapy for advanced colorectal cancer. A large phase III study randomized EGFR-expressing advanced colorectal cancer patients to treatment with 5-fluorouracil, leucovorin, and irinotecan (FOLFIRI) with or without cetuximab [214]. The addition of cetuximab to chemotherapy resulted in a superior response rate (47 % vs. 39 %) and a modest improvement in median progression-free survival (8.9 months vs. 8 months). Cetuximab also improved the efficacy of oxaliplatin-based chemotherapy regimens. In a randomized phase II study, the addition of cetuximab to 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) resulted in a higher response rate than with FOLFOX alone (46 % vs. 37 %) [215]. These studies suggest a role for cetuximab in combination with chemotherapy as front-line therapy for advanced colorectal cancer. Cetuximab also appears to enhance the efficacy of other molecularly-targeted agents. Bevacizumab, a monoclonal antibody against the vascular endothelial growth factor, is commonly used in combination with standard chemotherapy for advanced colorectal cancer. In a randomized phase II study, the combination of cetuximab and bevacizumab resulted in a response rate of 23 % in patients with irinotecan-refractory colorectal cancer [216]. Thus, in addition to its routine use in patient care, cetuximab is being extensively studied in various settings for the treatment of colorectal cancer.

Panitumumab has also demonstrated anti-cancer activity in advanced colorectal cancer. A phase III study randomized patients with EGFR-positive, chemotherapy-refractory colorectal cancer to panitumumab monotherapy or supportive care [217]. The response rate with panitumumab was 10 %. There was a modest improvement in median progression-free survival for patients treated with panitumumab. This led to the FDA approval of panitumumab for advanced colorectal cancer in the setting of progressive disease following standard chemotherapy. This agent is now being evaluated in combination with chemotherapy.

In contrast to the single agent activity in colorectal cancer noted with EGFR monoclonal antibodies, the small molecule inhibitors appear less active as monotherapies. In a phase II study of erlotinib for advanced colorectal cancer, no objective responses were noted although some patients experienced disease stabilization [218]. Consequently, gefitinib and erlotinib are now being studied in combination with standard chemotherapy regimens. A phase II study of gefitinib in combination with 5-fluorouracil, leucovorin, and oxaliplatin in patients with previously treated colorectal cancer demonstrated a response rate of approximately 30 %. Similarly, the combination of erlotinib with oxaliplatin and capecitabine has been demonstrated to be safe and efficacious in the treatment of advanced colorectal cancer [219]. Randomized studies are currently underway to confirm the promising results from phase II studies with these agents.

37.6.4 Pancreatic Cancer

Erlotinib has recently been approved by the FDA for the treatment of pancreatic cancer in combination with gemcitabine. Its efficacy was established in a phase III study that randomized patients with advanced pancreatic cancer to treatment

with gemcitabine alone or in combination with erlotinib [220]. There was a statistically significant improvement in median overall survival in patients treated with the combination (6.2 months vs. 5.9 months). There was no difference in response rate between the two treatment arms. While the clinical significance of the magnitude of additional benefit with erlotinib has been called into question, this is the first randomized study to establish improved survival for any combination regimen in patients with pancreatic cancer. Cetuximab, which demonstrated promising activity in combination with gemcitabine in a phase II study, did not improve overall survival in a confirmatory phase III study [221]. The median survival duration was 6 and 6.5 months, respectively, for gemcitabine and the combination of cetuximab with gemcitabine.

37.6.5 Breast Cancer

Lapatinib is a novel dual kinase inhibitor that targets both EGFR (HER1) and HER2. It has recently been shown to be an active drug for the treatment of metastatic breast cancer. Initial phase II studies with lapatinib demonstrated a response rate of approximately 20 % in HER2/*neu*-positive breast cancer patients following progression with trastuzumab-based therapy [222]. Subsequently, a phase III study was conducted for patients with HER2/*neu*-positive breast cancer with randomization to capecitabine alone or in combination with lapatinib. The study was restricted to patients who had progressed following prior therapy with trastuzumab-based regimens. Median time to progression, the primary endpoint, was superior for patients treated with the combination (8.4 months vs. 4.4 months), without a significant increase in serious toxic events. Lapatinib is now being studied in combination with hormonal therapy and in earlier stages of breast cancer.

37.7 Predictive Biomarkers for Patient Selection

As EGFR inhibitors are tested and used for the treatment of a variety of solid organ malignancies, it is clear that their clinical benefit is confined to a sub-group of patients. Methods to identify this sub-group have led to the evaluation of several clinical and molecular biomarkers as part of clinical trials (Table 37.2).

Table 37.2 Molecular predictive markers under evaluation for EGFR inhibitors

Marker	Agent	Disease	Comment
EGFR protein expression	Erlotinib Gefitinib Cetuximab Panitumumab	NSCLC Colorectal cancer	EGFR positivity linked with favorable outcome
EGFR gene copy number	Erlotinib Gefitinib Cetuximab	NSCLC Colorectal cancer	Higher gene copy number linked with favorable outcome
EGFR gene mutation	Erlotinib Gefitinib	NSCLC	Exon 19 and 21 mutations associated with high response rate
EGFR polymorphisms	Erlotinib Gefitinib Cetuximab	NSCLC Colorectal cancer	Gene polymorphisms may account for differential sensitivity
MALDI-TOF proteomics analysis	Erlotinib Gefitinib	NSCLC	Ability to identify patients with a favorable outcome

37.7.1 Clinical Predictive Markers

Initial studies conducted in patients with NSCLC identified certain patient sub-groups that had a higher likelihood of achieving objective responses. These included women, patients with adenocarcinoma histology, those of Asian ethnicity and patients who were life-long never-smokers. Although the reason behind this was initially unknown, the higher prevalence of EGFR mutations in these subsets of patients might account for the greater efficacy of EGFR inhibitors. The preliminary results of a recent phase II study in chemotherapy-naïve patients with advanced NSCLC demonstrated the feasibility of patient selection based on clinical factors [223]. The eligibility criteria for the study were female sex, former or never-smoking status, and adenocarcinoma histology. The median progression-free survival was 5.6 months and the median overall survival was greater than 23 months.

Skin rash has been correlated with improved outcome for patients treated with EGFR inhibitors in a variety of malignancies [189, 224, 225]. In metastatic colorectal cancer, patients who develop a skin rash upon treatment with cetuximab have a better survival outcome than those without a rash. Even among patients with a rash, the severity of the rash has been correlated with efficacy: the more severe the rash, the better the outcome [189]. The reasons behind the correlation between development of skin rash and survival are not clear. It has been postulated that the skin rash is a surrogate marker of the degree of EGFR inhibition in the tumor. Some have hypothesized that skin rash indicates the extent of immune response, which might also contribute to antitumor efficacy. Intra-patient dose escalation of the EGFR inhibitor until the development of skin toxicity is now being tested in prospective studies.

37.7.2 Molecular Predictive Markers

37.7.2.1 EGFR Protein Expression

Several studies have evaluated the correlation between EGFR expression in the tumor tissue and treatment outcome. In colorectal cancer, initial studies were conducted only in patients with EGFR-expressing tumors. Subsequent studies

have demonstrated efficacy of cetuximab even in patients with EGFR-negative tumors [226]. Similarly in NSCLC, some of the initial studies were conducted only in patients with EGFR-expressing tumors [189, 191]. However, subsequent studies have enrolled patients regardless of EGFR status, based on the lack of clear correlation between EGFR expression and response. In the BR 21 study that led to the approval of erlotinib for NSCLC, a retrospective analysis was conducted to evaluate the correlation between outcome and EGFR expression status. Tumor tissues were available for immunohistochemistry (IHC) analysis from approximately one-third of the participating patients. The survival benefit with erlotinib was seen in IHC-positive patients, whereas for IHC-negative patients there was no advantage over placebo [190]. The variation in results across studies could be due in part to the technical difficulties associated with the assessment of protein expression by IHC in a consistent manner. The low sensitivity associated with IHC, inter-observer variability in scoring, and the variation in EGFR expression both within the same tumor and between metastatic sites [227] are all potential challenges in the use of EGFR protein expression by IHC for patient selection.

37.7.2.2 EGFR Gene Copy Number

The number of EGFR gene copies has been linked with treatment outcomes with EGFR inhibitors [113, 228]. Amplification of the EGFR gene is noted in approximately 10–15 % of patients with NSCLC [114]. There does not appear to be a clear correlation between EGFR gene amplification and protein expression. In a predictive algorithm developed by Hirsch et al, higher numbers of gene copies, assessed by fluorescent in situ hybridization (FISH), was associated with improved outcome for patients treated with gefitinib or erlotinib for advanced NSCLC. FISH positivity was defined as the presence of >4 copies of the EGFR gene in at least 40 % of the tumor cells studied. Based on this definition, the investigators were able to identify patients who were more likely to experience improved survival. In a retrospective analysis conducted on tumor specimens from patients who participated in the ISEL study, the hazard ratio for survival with gefitinib for EGFR FISH-positive patients was 0.61, compared with 1.16 for patients with low copy numbers. A similar observation has been noted in a retrospective analysis of the BR 21 study [99]. Based on this finding, prospective studies are now underway to evaluate the predictive potential of EGFR gene copy number for treatment with EGFR inhibitors in NSCLC. However, planned subset analyses of two recently reported studies in NSCLC have yielded conflicting results. In the randomized phase III INTEREST study, gefitinib was compared with docetaxel as second or third line therapy. The study met its primary endpoint of non-inferiority for gefitinib in this unselected patient population. However, a pre-planned subset analysis in EGFR FISH-positive patients failed to demonstrate superiority for gefitinib over docetaxel [229]. Similarly, another randomized study (INVITE) that compared gefitinib to vinorelbine in elderly NSCLC patients demonstrated a lack of improved outcome for FISH-positive patients with EGFR inhibitor therapy [230]. Therefore, until results from ongoing prospective studies are available, testing for gene amplification cannot be recommended for routine clinical practice.

37.7.2.3 EGFR Mutations

The presence of specific mutations in the tyrosine-binding kinase domain of EGFR is associated with a higher likelihood of response to treatment with EGFR-TKIs. Either in-frame deletions with the removal of 4 amino acids in exon 19, or point mutations that caused the substitution of an amino acid in exon 21 of the EGFR gene were noted in tumors of NSCLC patients who had robust responses to EGFR-TKIs [93]. These mutations are not present in other tumor types besides NSCLC. These EGFR mutations do not confer constitutive activity, but result in heightened responsiveness to receptor activation. The prevalence of EGFR mutations in Caucasian patients is approximately 10–15 %, whereas a higher proportion of patients with Asian ethnicity harbor them [94]. Other patient subsets that have a higher prevalence of EGFR mutations are those of female gender, never-smoking status, and adenocarcinoma histology. Elevated levels of EGFR mutations might account for the higher response rates noted in these patient subsets. Contrary to this hypothesis, a retrospective analysis of the BR 21 study failed to demonstrate a survival advantage for erlotinib over placebo in patients with EGFR mutations [99]. This study was limited by the small number of patients whose tumor tissues were available for analysis. Recently, the results from prospective studies have suggested very high response rates and prolonged times to progression with EGFR-TKIs in patients with EGFR mutations. In a study by the Spanish Lung Cancer Group, 38 patients with EGFR mutations were given erlotinib monotherapy [231]. The response rate was 82 % and the median progression-free survival was >12 months. Of interest was the observation that the response rate was higher in patients with the exon 19 mutation than the exon 21 mutation. This is suggestive of differential biological effects of the two mutations. This observation has been confirmed further by other studies conducted in patients with EGFR mutations [232, 233].

Although TKIs are active in patients with EGFR mutations, the role of monoclonal antibodies against EGFR are unclear in this setting. In preclinical studies, the TKIs are more potent against mutant EGFR-bearing NSCLC cell lines compared with the monoclonal antibodies [234]. Furthermore, the question remains as to whether EGFR mutations have a prognostic or predictive effect in patients with NSCLC. An analysis of tumor tissues from patients who participated in a phase III study that compared chemotherapy with or without erlotinib noted better outcomes with chemotherapy alone even in patients with EGFR-mutated

tumors [235]. Therefore, the utility of EGFR mutations in patient selection for treatment with EGFR-TKIs remains a subject of ongoing investigation.

Recently, a secondary mutation in the EGFR, located in exon 20, has been reported. This mutation confers structural changes in the tyrosine kinase binding domain and results in resistance to therapy with EGFR-TKIs [236]. It is commonly found in the tumors of patients with an exon 19 or 21 mutation, following treatment with an EGFR-TKI. Agents specific for tumors with a secondary EGFR mutation are now under clinical evaluation. Alternative mechanisms of resistance to EGFR inhibition such as activation of the C-MET pathway have also been described in NSCLC [237].

37.7.2.4 Serum Proteomics

Mass spectroscopic analysis of serum samples has recently been demonstrated to enable the identification of patients who are likely to derive a survival benefit from treatment with EGFR-TKIs [238]. First, a predictive algorithm based on matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) was developed based on sera from 130 patients treated with EGFR-TKIs. Eight specific protein peaks were noted to differentiate patients with a good outcome with EGFR-TKI therapy from those with a poor outcome. Subsequently, the algorithm was tested in a cohort of patients treated with EGFR-TKIs. The median survival was 307 days in patients with a good profile compared to only 107 days in those with a poor profile (hazard ration 0.41). The serum samples were analyzed in two independent laboratories and were found to have a concordance rate of 97 %. The predictive algorithm appears to be specific for EGFR-TKIs, since the outcomes in patients treated with chemotherapy were not different between those with a good vs. poor profile. The predictive potential of serum proteomics analysis to select patients for treatment with EGFR-TKIs is now being tested in prospective clinical trials. If proven useful, this could be an inexpensive, non-invasive tool to select the most appropriate patients for therapy with EGFR inhibitors.

37.8 Conclusions

Targeting the EGFR pathway is now a proven strategy for the treatment of a variety of solid organ malignancies. The development of this class of agents illustrates the complex and methodical process that is necessary for target identification, validation, and clinical evaluation for successful drug discovery efforts. Although these agents do have non-target effects that contribute to adverse events, they have a high degree of selectivity for cancer cells. There are still a number of unanswered questions regarding EGFR inhibitors. It is unclear why the tyrosine kinase inhibitors are effective in certain tumor types in which the monoclonal antibodies are less active, and vice versa. The association between skin rash and efficacy has yet to be understood clearly. Furthermore, optimal patient selection methods continue to be evaluated, and will hopefully result in the use of these agents in a truly targeted setting. Nonetheless, the incorporation of EGFR inhibitors into clinical practice has resulted in meaningful improvements in symptoms and survival for cancer patients.

The current crop of clinical trials will address the role of EGFR inhibitors in the treatment of earlier stages of cancer. EGFR inhibitors may also be useful in the chemoprevention setting in patients with high-risk pre-neoplastic conditions. The combination of EGFR inhibitors with other molecularly-targeted agents is a promising strategy that will be tested in the near future in clinical trials. The administration of anti-angiogenic agents in combination with EGFR inhibitors is already in advanced phase clinical trials in colorectal and non-small cell lung cancer. Such novel strategies will undoubtedly contribute to improved outcomes for patients with cancer.

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Targeting Oncoproteins for Molecular Cancer Therapy

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38.1 Turning Chemical Warfare into Cancer Therapy

Cancer and efforts to treat cancer are described in Ancient Egyptian documents dating back to 1600B.C. The first successful cancer treatments were arsenic-based therapies for leukemias, with the first reported application to cancer in the nineteenth century [1]. However, nitrogen mustards are often accredited as the first modern chemotherapy. Originally intended as a chemical warfare agent in World War I, nitrogen mustard was stockpiled by several countries. During World War II, Axis bombers sunk a ship containing large quantities of nitrogen mustard and killed numerous Allied sailors. Autopsies revealed that most of the victims' white blood cells were depleted, suggesting that the nitrogen mustard destroyed these cells or inhibited cell division of these cells. This observation birthed the hypothesis that nitrogen mustards might prevent the rapid division of cancer cells, one of the few properties of cancer understood at that time. Today, the hallmarks of cancer as recently redefined by Hanahan and Weinberg [2] include several complex and connected cellular properties that allow for this phenotype: resistance to cell death, sustained angiogenesis, limitless ability to replicate, self-sufficiency in growth factor signaling, unresponsiveness to anti-growth factor signaling, genomic instability and mutation, deregulating cellular energetics, evading immune-mediated destruction, oncogenic inflammation, and invasiveness and metastasis [3]. The identification and understanding of these hallmarks is a direct result of our molecular understanding of cancer that has surfaced relatively recently. Each of these hallmarks is determined by a host of molecules which together represent distinct therapeutic opportunities to target molecules that give rise to these defining properties of cancer (Fig. 38.1).

The National Cancer Act of 1971, signed by President Richard Nixon, declared "…*war on cancer*…" and announced full Congressional and presidential support to eradicate the disease. As a result of increased funding and technological advancements, our understanding of cancer biology on the genetic and molecular level has exploded. Today, cancer phenotypes are associated with genetic and molecular culprits along with complex networks of various regulatory mechanisms that together cause and sustain cancer. Superficially, the genes capable of inducing carcinogenesis are divided into two categories. The first is a tumor suppressor, which is a gene that if inactivated, restricts cell division. Genes that confer pro-survival changes if activated are oncogenes. Intuitively, tumor suppressors such as p53 are commonly inactivated in cancer while oncogenes such as myc are activated and/or overexpressed. These carcinogenic changes manifest themselves by a number of avenues including overexpression, mutations, deletions, loss or gain of alleles, epigenetic modifications that alter genomic structure, alternative splicing, interference with the translation and transcription of the gene, chaperone-mediated protein folding, protein degradation processes, and posttranslational modifications that modulate localization, protein-protein interactions, and/or activity of the protein. Whether some of these changes occur simultaneously, sequentially, or otherwise is highly context-dependent and debated. Furthermore, the functional consequences of these alterations have a wide range of effects through crosstalk in cell signaling pathways. In the background of cancer-associated genomic instability, these wide-spread alterations and the functional redundancy of various genes provide a breeding ground for therapeutic resistance and make targeting cancer cells at the molecular level a challenging feat.

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_38

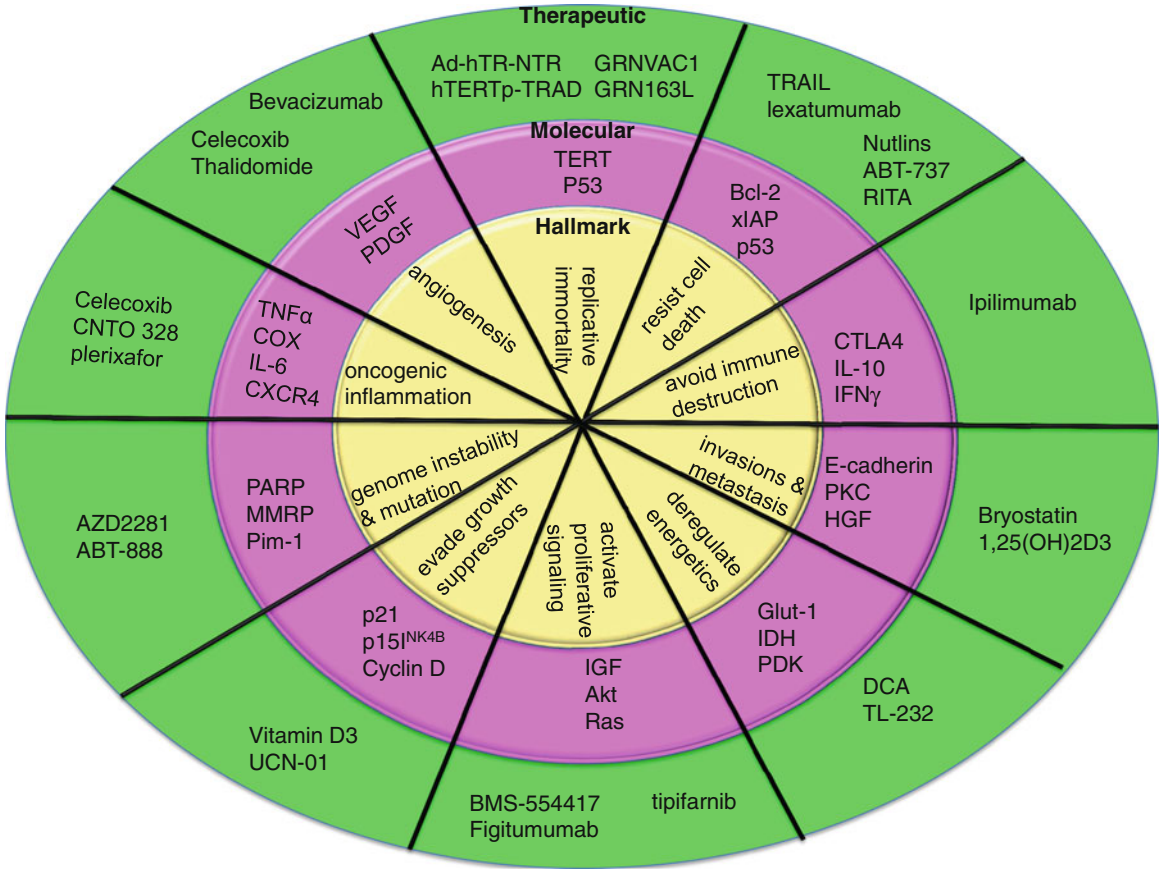

Fig. 38.1 Molecular therapies with targets associated with the hallmarks of cancer. The *inner yellow circle* shows the hallmarks of cancer as defined by Hanahan and Weinberg. The *middle purple ring* contains proteins that govern these hallmarks. The *outer green circle* contains exemplary molecular therapies that explicitly such molecules.

Radiation, surgical resection, and chemotherapy still comprise the vast majority of first-line cancer therapy today. While chemotherapy has yielded enormous patient benefit it is often accompanied by side effects that limit dose and therefore efficacy. Traditional chemotherapy is based on the notion that cancer cells divide more rapidly than normal cells and consequently will be differentially affected by an inhibitor of cell division. However, many types of normal cells need to divide for normal function. Our increased understanding of cancer has yielded numerous molecular targets for cancer therapy that may be less necessary for normal cell function and therefore may be less toxic. For instance, most normal cells divide a finite number of times, which is called the Hayflick limit. When cells divide, the ends of their chromosomes termed telomeres shorten as result of the DNA replication process, a phenomenon called the end replication problem. Upon reaching a critical telomere length, cells stop dividing and enter a dormant state referred to as cellular senescence. Cancer cells must evade senescence as they need to propagate indefinitely. Cancer cells escape this phenomenon by activating telomerase, an endogenous enzyme capable of elongating telomeres. As this oncogenic process is essential to cancer cells but not essential to most types of normal cells, telomerase is an attractive target for cancer therapy. Several types of inhibitors targeting various aspects of the telomerase molecular machinery and function are being investigated as a novel cancer therapy. There have been and continue to be efforts to discover therapies that alter the function of cancer-specific targets such as this. The rise of targeted therapies over the past two decades is a result of the rich marriage of our modern tools to understand cancer and our ancient desire to cure it. This chapter details the discovery, translation, development, and exemplified therapeutic concepts of several novel cancer therapies that specifically target oncoproteins and have molded how we discover and develop novel therapies today (Fig. 38.2).

38.2 Small Molecules with Big Consequences

A narrow library of atomic arrangements make up the relatively small number of molecular building blocks of cellular life such as nucleic acids that comprise DNA and RNA as

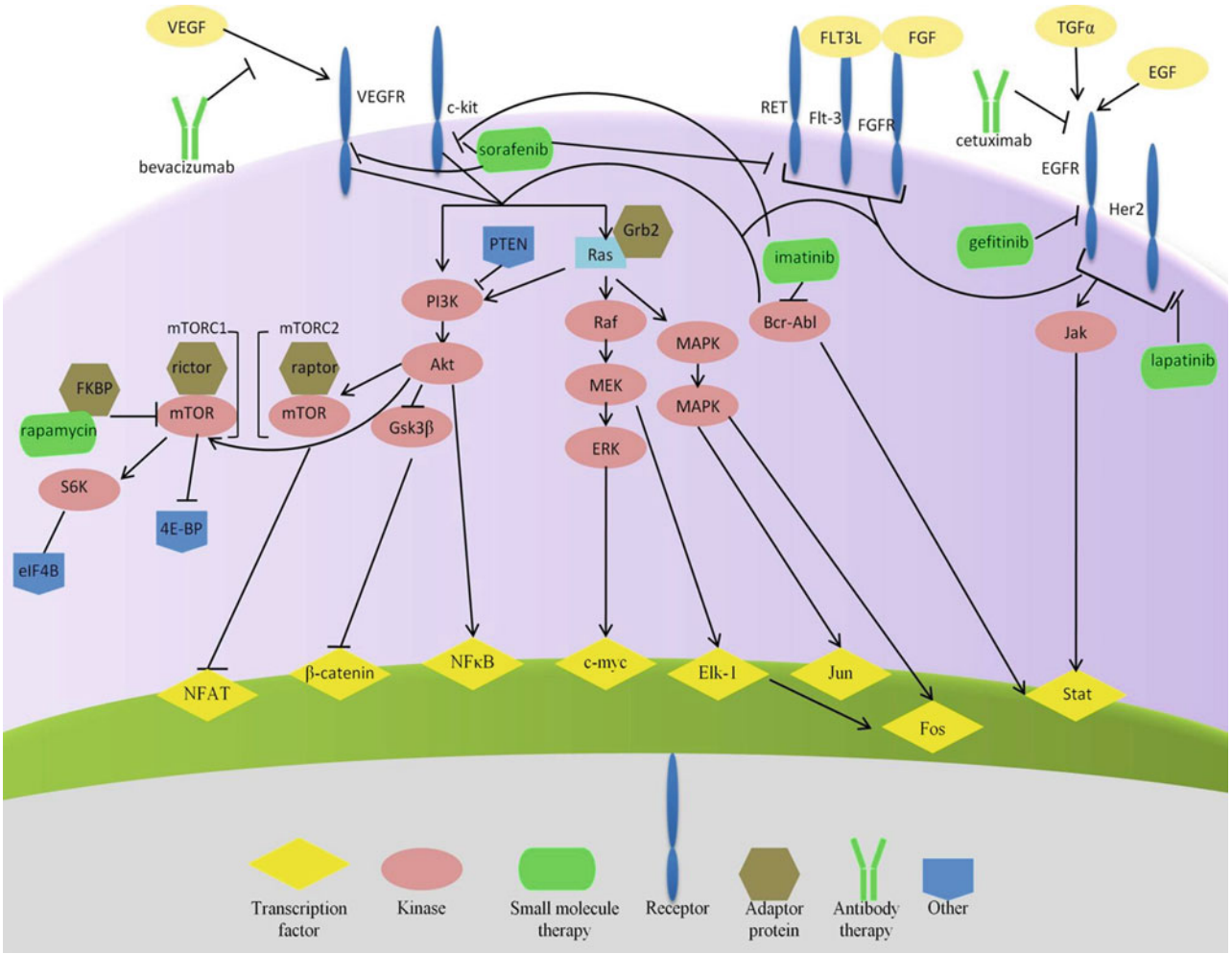

Fig. 38.2 Outline of exemplary molecular therapies targeting oncoproteins and their representative signaling networks.

well as amino acids that the end product, proteins, are made of. These building blocks are themselves synthesized from other molecules during cellular uptake and metabolism. Life has evolved to find chemical means for cells to convert small molecules to other molecules that are more useful for them. In a sense, we attempt to do the same for cancer therapy through medicinal chemistry to make more effective therapies from lead compounds. Small molecules offer a vast range of activity: sucrose sweetens foods and beverages, sodium pentothal is lethal, and amoxicillin can cure many bacterial infections. Our expanding knowledge in chemistry enables us to modify small molecules in nature or our synthetic libraries and create new molecules with altered functions, just as a cell does. Our understanding and ability to produce biological molecules on a therapeutic scale has been a relatively recent endeavor, therefore chemotherapies are almost entirely composed of small molecules. The availability, diversity, synthetic amenability, cost, and size of synthetic and natural compounds make small molecules an irreplaceable source of therapies.

38.2.1 A Rational Success Story: Imatinib

Chronic myelogenous leukemia (CML) is a cancer that causes increased amounts of white blood cells and is almost always associated with a specific translocation of chromosomes 9 and 22. The resulting shorter chromosome 22 is known as the Philadelphia chromosome, a tribute to the city housing the researchers who identified this in 1960s. The Philadelphia chromosome encodes a fusion of the two genes that results in the production Bcr-Abl. Abl is a tyrosine kinase that becomes constitutively active in the gene fusion product. This unregulated kinase activity causes oncogenic cell signaling shown to be sufficient to induce leukemia in mice. As a poster-child for translating modern cancer knowledge to clinical benefit, imatinib has significantly improved CML patient response rates. The FDA approved imatinib in 2001 for the treatment of CML as the first cancer therapy to target an intracellular molecule. Imatinib is a direct result of medicinal chemistry performed on a molecule identified as a protein kinase C (PKC) inhibitor [4]. Chemical modification

of the PKC inhibitor altered the specificity of the molecule and rendered the derivative a potent inhibitor of v-abl [5], c-kit, and platelet-derived growth factor receptor (PDGFR) [6]. All of these proteins are receptor tyrosine kinases (RTKs) that bind extracellular factors and transduce the signal by phosphorylating specific protein substrates. The observation that imatinib inhibits Abl and BCR-Abl led to preclinical development and ultimately clinical trials of imatinib as a cancer therapy for CML. The concept of taking the currently available knowledge of a particular target and identifying a way to alter its function for therapeutic benefit is known as rational drug design.

Structural biology has played a key role in understanding how molecules look, how they move, how they interact with other molecules, and how all of these things change in different environments. Computational molecular docking in concert with an X-ray crystallographic structure of the catalytic domain of Abl bound to imatinib revealed that imatinib binds to the ATP-binding site of Abl preferentially when the protein is in the inactive form (Fig. 38.3) [7, 8]. The crystal structure also showed that a chemical group added to increase solubility also forms hydrogens bonds with two residues of Abl. The insight gained from such structures provides an understanding of how and where a drug binds, what types of interactions are formed, and what role each residue or functional group plays. Due to the relatively facile excision and crystallization of the catalytic domains of many kinases, several atomic structures of these proteins are freely available in the Protein Data Bank (PDB). This provides fertile ground for numerous applications of computational chemistry to foster drug discovery and development. In addition to structural biology, medicinal chemistry also provides insight into the function of particular atoms of a molecule while searching for a more therapeutically potent derivative. The drug development process typically begins by identification of lead molecules using various screening techniques that search for a desired effect. Validation of these leads and optimization by medicinal chemistry ensues to identify the most promising compound to continue to develop. The process of lead optimization and elucidative structural biology intrinsically discerns the role of particular parts of drug and target molecules in the activity of the drug, giving rise to structure–activity relationships (SARs).

All of the kinases in the human genome, called the kinome, share a high degree of sequence and structural homology. This means that identifying inhibitors that are specific for a given kinase is challenging and often kinase inhibitors have multiple targets. Imatinib is no exception, as it inhibits autophosphorylation of c-KIT, platelet-derived growth factor (PDGF), and ARG [9] kinases in addition to BCR-ABL. c-KIT is a receptor tyrosine kinase that is almost ubiquitously mutated in gastrointestinal tumors (GISTs) during the transformation of interstitial cells of Cajal located in the gastrointestinal tract. This mutation typically occurs in exon 11 of the c-Kit gene, which results in constitutive autophosphorylation of the protein that continually activates downstream pro-survival signaling that is oncogenic. The observation that imatinib inhibits c-KIT lead to preclinical development and clinical trials with the treatment of GISTs. Seven years after its approval for treatment of advanced CML, the FDA approved imatinib for treatment of GISTs following surgical removal of the tumor.

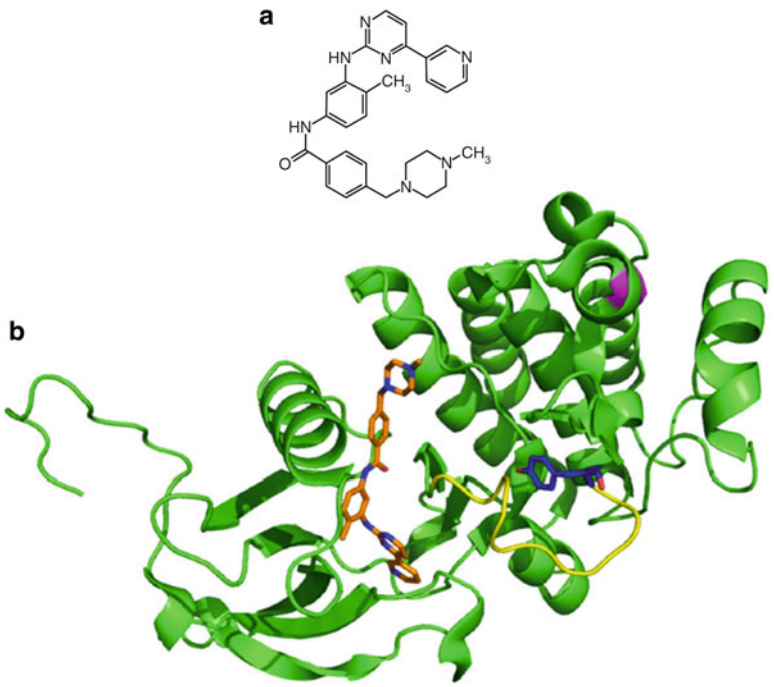

Fig. 38.3 Crystal structure of imatinib bound to Abl kinase. The Abl kinase (*green*) is represented as a secondary structure cartoon with its activation loop highlighted in *yellow*. The confirmation is in an inactive kinase state. PBD accession 1IEP.

This extension of clinical applications demonstrates a few key concepts in cancer therapy. Firstly, exclusive specificity of a cancer drug for a molecular target is a virtue rooted in our movement toward targeted therapy. We know that without specificity, effects on normal cells can yield side effects that are deleterious. However the distinction should be made that while cancer therapies should target cancer-specific properties, this does not have to be accomplished by targeting a single molecule. It is conceivable that evolving therapeutic resistance is easier against a single target rather than an array of molecules. Furthermore, different types of tumors seem to rely on alterations in multiple genes and so multiple targets may allow for broader spectrum and more potent antitumor activity. The clinical extension of imatinib to GISTs also underscores the importance of identifying and understanding the molecular targets of therapies and how they fit with our molecular understanding of cancer. The application of a therapy used in distantly related clinical settings is a concept that continues today. Such applications are often a direct result of rational drug design and typically have an expedited timeline for starting clinical trials as they have already been tested in humans. The time and cost associated with development of cancer therapies is astounding, taking an average of over 14 years and $2 billion to reach FDA approval of a cancer drug with a success rate of just over 7 % [10]. Clearly, reducing the time spent in early phase trials profiling safety of the drug would reduce cost and expedite evaluation and patient benefit.

Therapeutic resistance is frequent in cancer. Cancer is defined by its uncontrollable cellular division and therefore is a disease of evolution governed by natural selection. While our cells copy our genome during cell division with amazing fidelity, the molecular machinery that performs this task is not completely error free. This endogenous source of mutations, environmental mutagens such as UV radiation or tobacco, and the genomic instability associated with cancer provide a sufficient source of heritable variability. Cancer treatments serve as a selective force for the cancer cells. Imagine yourself looking at population of millions of CML cells circulating in the blood that have the oncogenic Bcr-Abl fusion gene. Now introduce imatinib into the blood stream, which inhibits Bcr-Abl, and watch as the CML cells begin to die due to their dependency on the function of this oncogenic protein. If any single cell out of these millions of CML cells evolved into cancer by a process not involving BCR-ABL or can continue to divide using other oncogenic alterations, the cell will survive and continue to divide. The entire offspring of such cells will not rely on BCR-ABL to propagate and thus the patient will now not respond to therapies that target BCR-ABL. The enhanced sources of genetic variability and the solitary goal to divide more rapidly give rise to heterogeneity of tumors. This heterogeneity has significant implications as to how cancer is diagnosed and managed.

There are several ways that cancer cells get around therapeutic road blocks in intracellular signaling to keep propagating. In the case of imatinib, mechanisms of resistance include increasing the amount of Bcr-Abl to saturate the drug, mutating Bcr-Abl to still be constitutively active in the presence of imatinib, bypassing BCr-Abl and activating its downstream targets to achieve the same end goal, or simply getting rid of the drug altogether. Following the first route, cell culture and patient data revealed that overexpression of Bcr-Abl by gene duplication occurred in refractory patients and that this was sufficient for imatinib resistance [11–14]. Additionally, several patients were found to have mutations in the BCR-ABL that allowed for sustained signaling in the presence of imatinib. Due to the availability of the drug-bound crystal structure of the Abl catalytic domain, the effects of these point mutations were rationalized at the molecular level. Another intriguing avenue of drug resistance is getting rid of drugs by upregulating molecular efflux pumps localized at the cell membrane. The most characterized member of this family of proteins that induce multidrug resistance is p-glycoprotein (PgP), exhibiting broad substrate specificity to include several chemotherapies such as vinblastine, doxorubicin, and paclitaxel. Upregulation of PgP was also found in imatinib-resistant clones and in advanced CML patients. Pharmacological inhibition of multidrug resistance proteins is being explored in clinical trials in combination with chemotherapy with mixed success [15–19]. Strategies to circumvent imatinib resistance in the clinic include increasing the dose, changing to other investigational therapies, and administering alternative Bcr-Abl inhibitors such as nilotinib and dasatinib. Nilotinib and particularly dasatinib also target Sarc-family kinase (Src) and have significantly improved patient outcome after imatinib failure [20–29]. Treating patients that are resistant to first-line therapies is a challenge in clinical oncology and is a major barrier in getting an investigational drug approved as these are often tested in these refractory patients.

Ten years after the discovery of imatinib, it was approved in the US, Japan, and Europe as the first-line therapy for CML. The story of imatinib is a testament to our modern molecular understanding of cancer. Computational biologists, chemists, cell biologists, translational oncology researchers, and clinical oncologists from various countries around the world collaborated to discover and translate this therapy. Such interdisciplinary integration is a recipe for success in modern drug discovery and development and is a theme found throughout molecular targeted cancer therapy and beyond.

38.2.2 Gefitinib

Growth factor signaling is intimately involved in tumorigenesis and propagation and is involved in two of the hallmarks of cancer. Growth factor signaling typically involves the binding of an extracellular factor by a transmembrane receptor on the cell surface, which triggers intracellular signaling events. These events such as substrate phosphorylation ultimately allow for cellular proliferation through various mechanisms such as turning on transcription factors that activate genes necessary for cell cycle progression. One group of receptors that mediate several of such signals is the epidermal growth factor receptor (EGFR) family and as a consequence, this family is commonly altered by mutations and/or overexpression in a wide range of solid tumors. These receptors form homo- or hetero-oligomers upon binding various ligands to trigger a range of intracellular signaling events via Ras/Raf/MAPK, PI3K/Akt, STAT, or Src kinase pathways. EGFR is one member of this family that homodimerizes upon binding and induces proliferation through ERK, PI3K/Akt, Ras, and STAT signaling pathways. Due to the frequency of their alteration in a variety of cancers and its plethora of potent downstream oncogenic targets, the EGFR family members have successfully targeted by a number of therapeutic approaches over the past two decades.

Gefitinib is an orally active EGFR inhibitor identified by Astra Zeneca and first reported in 1996. A quinazoline derivative was found to be an ATP-competitive inhibitor and highly specific to EGFR over its related family members. Cell-based events in accordance with the inhibition of EGFR activation were observed such as autophosphorylation, upregulation of the CDK inhibitor $p27^{Kip1}$, and transcriptional inhibition of the transcription factor c-Fos [30, 31]. Preclinical studies found that gefitinib had cooperative to synergistic combinations with several chemotherapies in EGFR-overexpressing cancer cell lines [32]. Interestingly, another group reported similar effects with gefitinib-chemotherapy combinations but in cancer cell lines with low EGFR expression [33]. Oral and intravenous administration of gefitinib in rats and dogs found the bioavailability of the drug to be ~50 % and that the drug was well distributed throughout the body [34]. Pharmacokinetic (PK) studies indicated that oral administration of gefitinib at 100–700 mg/day was well tolerated, had a terminal half-life between 1 and 2 days, and reached serum concentrations that inhibited 90 % of EGFR activity in vitro [35–39]. The first phase I trial of gefitinib was a dose escalation study in patients with various solid tumors that reported objective partial responses in NSCLC patients at oral doses ranging 300–700 mg per day on a 14 days-on, 14 days-off schedule [40]. The observed dose range with antitumor activity was below the dose-limiting toxicity (DLT) reported and was corroborated with another phase I trial [41]. These clinical trials used high-performance liquid chromatography (HPLC) with mass spectrometry (MS) to monitor serum concentrations of gefitinib [42].

HPLC along with complementary molecular identification techniques are often employed in clinical trials involving small molecules. HPLC allows for the separation and quantification of molecules based on their absorbance and interactions with a solid matrix. Molecular properties such as size and charge along with instrument and solvent parameters determine how the molecule interacts with this matrix. These interactions determine how long the molecule takes to migrate through the matrix column. HPLC conditions are optimized to allow for quantitative identification of a given molecule based on this empirically determined elution time, called retention time. The calculated serum concentrations can then be used to determine a plethora of pharmacokinetic parameters. These parameters are particularly important in guiding dosing schedules of new therapies and rationalizing patient responses. This technique is often coupled with mass spectrometry to allow for further verification that the molecule identified at a particular retention time is indeed the target molecule. Mass spectrometry is an electromagnetic separation method that distinguishes molecules based on their size to charge ratio of ionized forms of the molecule, which are generated by molecular collisions. Together, these ionized fragments yield a unique fingerprint for each molecule. Coupling HPLC and mass spectrometry has been instrumental in increasing the accuracy and reliability of pharmacokinetic data and identifying metabolites of drugs. Direct and indirect modifications of drugs often occur once delivered due to the staggeringly diverse mixture of molecules presence in the blood, digestive system, etc. As for gefitinib, HPLC-MS identified desmethyl-gefitinib as a metabolite of gefitinib that is inactive in vitro and in vivo [43]. Identification of metabolites is important for understanding and monitoring the various molecular species responsible for therapeutic activity as well as guiding chemical optimization.

Phase II trials with gefitinib again yielded some patient benefit in NSCLC along with low toxicity [44, 45]. With data available from phase III clinical trials, the FDA granted accelerated approval for gefitinib as a third-line therapy in NSCLC in 2003. This type of approval is granted based on promising clinical evidence of efficacy when there is no current therapy for a particular clinical setting. However, this approval is temporary and full approval is contingent on a more complete clinical data set. In the few years following accelerated approval of gefitinib, several studies found responses in the overall population of NSCLC patients [44–46]. However, a small subset of responders was identified amongst these trials with the following characteristics: female, Asian, never-smokers, adenocarcinoma, and mutant EGFR [46–48]. Several other studies confirmed efficacy of

gefitinib in NSCLC patients with EGFR mutations [49–52] though no benefit was found in NSCLC patients with EGFR gene amplification [53]. A breakthrough study published in 2004 found that the majority of gefitinib responders had EGFR deletions or point mutations clustered at the ATP-binding site of EGFR, which results in a ten-fold increase in sensitivity to gefitinib [54]. Others studies corroborated these EGFR alterations in gefitinib-responsive patients [55, 56]. These genetic alterations were structurally modeled and rationalized using the crystal structure of the human EGFR kinase domain bound to gefitinib (Fig. 38.4) [57]. These response-determining mutations occur at the active site of the kinase domain in structural regions that are responsible for autoregulation of kinase activity. Further studies found that these particular mutations in EGFR stabilize the active form of the kinase and shift its affinity from ATP toward gefitinib [58].

One study mandated by the accelerated FDA approval of gefitinib and another phase II clinical trial found no benefit with gefitinib in NSCLC refractory to first-line or second-line therapies. This led the FDA to restrict its usage to patients who have previously benefited or are currently benefiting from gefitinib in 2005. Subsequent clinical trials have supported gefitinib following chemotherapy resistance [59, 60]. Together, the clinical trials comparing gefitinib and chemotherapy are confusing as they conclude significant to no benefit of gefitinib over chemotherapy. These conflicting results have been ascribed to fundamental differences in the therapeutic mechanisms of chemotherapy and targeted therapy, differences in patient populations, and biomarker selection and technique accuracy [61]. Recent studies have supported gefitinib as a first-line therapy due to demonstrated superiority over standard of care therapy in mutant EGFR NSCLC patients [62–64]. This data is anticipated to extend the restricted use label of gefitinib to its use as a first-line therapy in NSCLC patients with mutant EGFR. The use of targeted therapy based on molecular determinants surfaces later in the chapter and is increasingly integrated into FDA approval stipulations as personalized medicine emerges in practice.

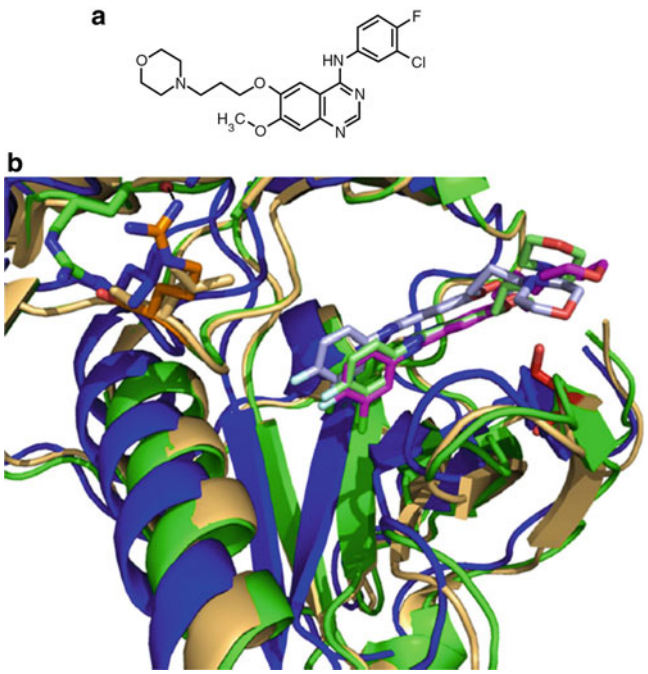

Fig. 38.4 Molecular structure of gefitinib and gefitinib bound to EGFR. (**a**) Molecular structure of gefitinib. (**b**) Crystal structure of gefitinib bound to EGFR kinase domain. Overlay of wild-type (*blue*), L858R (*green*), or G719S (*beige*) EGFR bound to gefitinib shown in *gray*, *light green*, and *magenta*, respectively. The L858R point mutation is shown in orange and its hydrogen as a *black dashed line* while the G719S point mutation is shown in *red*. PBD accessions 2ITY, 2ITZ, 2ITO.

38.2.3 Two Birds with One Stone: lapatinib

As discussed elsewhere, the EGFR family is overexpressed in numerous cancers. One particular EGFR member, Her2, does not bind ligands but can form heterodimers with other ligand-bound family members to transduce oncogenic signaling through the Ras/Raf/MAPK cascade. By 2001, several recent EGFR family-targeted molecular therapies had demonstrated significant clinical efficacy in cancer including the EGFR-targeted therapies gefitinib, erlotinib, and cetuximab along with anti-Her2 monoclonal antibody herceptin. Her2 and EGFR are well-established therapeutic cancer targets based on these clinical successes, the prevalence of EGFR family alternations in cancer, and the highly homologous and druggable ATP-binding site shared by EGFR family members. A collaboration of private companies including GlaxoSmithKline launched a large synthetic effort to simultaneously target these two proteins [65–67]. Published results of these efforts detail the synthesis, specificity, and biological activity of several quinazoline or pyridopyrimidine compounds. One initial compound, GW2974, produced tumor stasis given orally at a dose of 30 mg/kg in squamous cell head and neck carcinoma and breast cancer xenografts. A later report found yet another compound, GW572016, to be a potent, reversible inhibitor of EGFR and Her2 even in the presence of excess EGF [68].

The full length 185 kDa Her2 protein can be proteolytically cleaved to shed its extracellular domain and give rise to its truncated form, $p95^{Her2}$. $p95^{Her2}$ is constitutively active which causes autophosphorylation, has a high oncogenic transformation ability, and correlates with lymph-node positive metastasis and poor therapeutic response [69–76]. $p95^{Her2}$ was found to be insensitive to trastuzumab, preferentially dimerize with ErbB3, and be regulated by the ErbB3 ligand heregulin [77]. Lapatinib blocked baseline autophosphorylation and downstream signaling events induced by

$p95^{Her2}$. Her2 was found to contribute to androgen receptor transcriptional activity [78, 79]. Accordingly, lapatinib cooperated with the small molecule estrogen receptor-agonist tamoxifen to reduce estragon receptor-dependent transcriptional activity and inhibit the growth of a tamoxifen-resistant xenograft [80]. Lapatinib-resistance clones were generated by chronic exposure of cancer cell lines and were enriched in androgen receptor signaling events, suggesting the importance of this signaling in lapatinib response [81]. Combining lapatinib with anti-Her2 antibodies such as trastuzumab enhanced downregulation of the anti-apoptotic protein survivin and apoptosis in Her2-overexpressing breast cancer cells [82, 83] and trastuzumab-resistant cells [84]. These preclinical observations served as the basis for combining lapatinib with hormone therapy and trastuzumab.

Phase I studies in healthy volunteers found orally administered lapatinib to reach peak serum concentrations at 3 h and achieve steady state concentrations after 1 week [85]. High fat content of the patient significantly increased bioavailability of the drug, highlighting yet another clinical variable impacting therapeutic response [86–88]. EGFR-overexpressing and Her2-overexpressing metastatic carcinoma patients receiving lapatinib showed significant clinical responses and tolerated oral daily doses up to 1600 mg [89]. Lapatinib was also safe and effective as a first-line monotherapy in Her2-amplified advanced or metastatic breast cancer [90]. The agent has shown preliminary efficacy in head and neck squamous cell carcinoma [91, 92], but not in NSCLC [93] or prostate cancer [94].

Lapatinib has been combined with a variety of chemotherapies, hormone agonists, and trastuzumab. Addition of lapatinib to the FOLFOX4 or FOLFIRI chemotherapy regimen was safe [95, 96] and is now being explored for efficacy. Based on the clinically active combination of trastuzumab with capecitabine, studies with this combination were conducted on Her2-overexpressing advanced or metastatic breast cancer patients [97, 98]. The study found that the time to progression doubled when lapatinib was added to capecitabine without significant additional toxicity. Efficacy within these $Her2^+$ patients was not limited to a subgroup [99], but a separate study found lapatinib to be effective in $Her2^+$ but not $EGFR^+/Her2^-$ inflammatory breast cancer patients [100]. This suggests that Her2 inhibition is a key mediator of antitumor efficacy in this malignancy. However, a higher lapatinib was recently found in preclinical models to have EGFR-independent and Her2-independent effects on death receptor upregulation that enhances efficacy when combined with TRAIL and TRAIL-receptor antibodies that bind to these receptors [101]. This rationalizes the clinical exploration of higher doses of lapatinib to gain increased efficacy via off-target mechanisms.

In 2007, the FDA approved trastuzumab and chemotherapy-resistant, for the treatment of $EGFR^+$ advanced or metastatic breast cancer. An increase in progression-free survival (PFS) from 3 to 8.4 months was observed with the addition of lapatinib to letrozole in estrogen receptor (ER)-positive metastatic breast cancer relative to letrozole monotherapy [102]. Based on this data, the FDA extended the indication for lapatinib to its use with letrozole in $Her2^+ER^+$ metastatic breast cancer in 2010. Addition of lapatinib to tri-weekly paclitaxel as a first-line therapy yielded a significant increase in time to progression of metastatic breast cancer patients that was restricted to the $Her2^+$ patients [103]. A recent study has also demonstrated the safety and potential efficacy of combination with weekly paclitaxel [104]. In agreement with lapatinib and trastuzumab combinatorial preclinical data, PFS was significantly prolonged in trastuzumab-resistant $Her2^+$ metastatic breast cancer with the combination of trastuzumab and lapatinib [105]. Lapatinib demonstrates the power of multitargeted therapies over single-targeted therapies such as trastuzumab and continues to be explored with various therapeutic combinations as a breast cancer therapy.

38.2.4 Rapamycin: From the Ground Up

The PI3K/Akt/mTOR pathway is a complex signaling network that controls cell survival, death, and division in response to a variety of stimuli such as hypoxia and growth factor deprivation. The elucidation of this pathway started in 1975, when rapamycin was first isolated as an antifungal agent from bacteria in a soil sample from the Polynesian island of Rapa Nui, where the compound got its name [106, 107]. Follow up studies in rats found that rapamycin was a potent immunosuppressant [108] but the molecule lost attention in the scientific literature. Over a decade later, a high-profile immunosuppressant macrolide, FK506, was found to inhibit the proliferation of activated T-cells [109]. The same investigators that reported this observation also noticed structural homology between FK506 and rapamycin, which prompted a comparison study of their effects. Interestingly, rapamycin and FK506 antagonized the biological effects of each other. Experiments with radiolabelled FK506 found that rapamycin could directly compete with FK506 in cells for its undescribed binding target [110], which turned out to be FK506 binding protein (FKBP) [111–114]. However, the two molecules had different effects on signaling events involved in T-cell activation such as the transcriptional activity of NFAT or induced IL-2 transcription [115]. The structure of FKBP alone was determined by NMR [116] and in complex with FK506 by X-ray crystallography [117] which together revealed a rather unique drug binding site and revealed a strong conformational shift in FK506 and to a lesser extent in FKBP. Rapamycin was later found to bind to the same site but did not demonstrate a significant conformational shift itself (Fig. 38.5) [118].

Genetic studies in yeast found two homologous genes to be determinants of rapamycin-toxicity and as such were

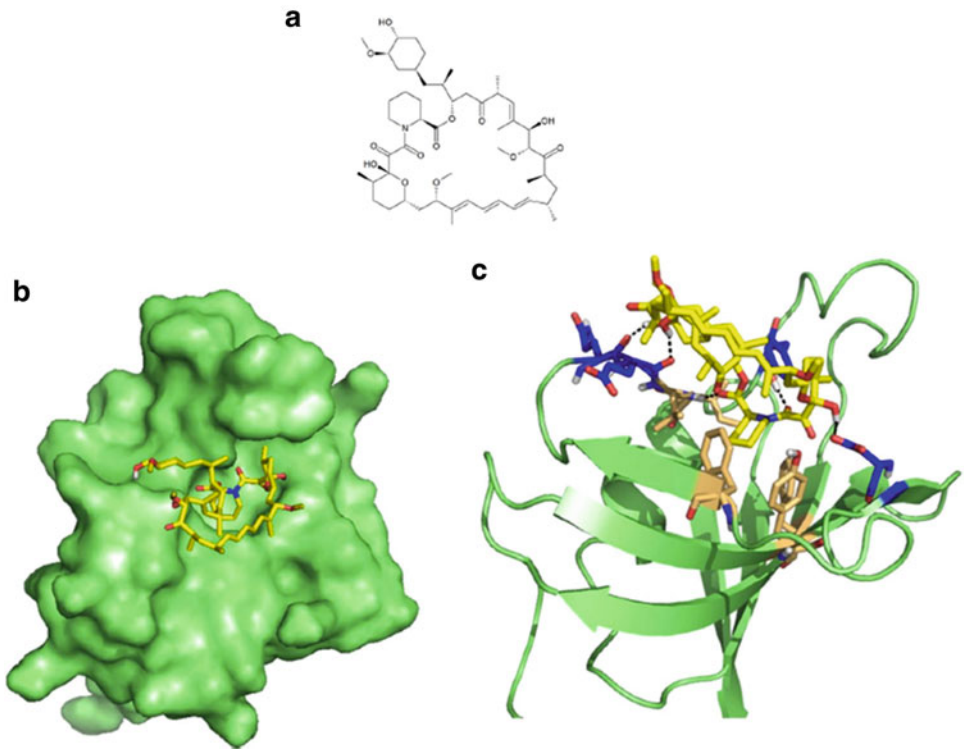

Fig. 38.5 Rapamycin and its association with FKBP. (**a**) Molecular structure of rapamycin. (**b**) Surface representation of rapamycin (*yellow*) bound to FKBP (*green*) at a hydrophobic pocket. (**c**) Key interacting residues of FKBP involved in hydrophobic interactions (*beige*) and hydrogen bonds (*blue*) indicated with *black dashed lines*. PBD accession 1FKB.

called targets of rapamycin (TOR1 and TOR2) [119]. Soon after, several reports identified a mammalian homologue of the proteins present in complex with FKBP that was dependent on the presence of rapamycin [120–122]. Furthermore, this mammalian target of rapamycin (mTOR) was required for the G1-arrest induced by rapamycin, which had been widely reported [110, 115, 123–126]. Rapamycin inhibits the function of the Akt-substrate mTOR [127–129], a serine/threonine kinase that phosphorylates $p70^{S6K}$, which mediates growth factor signaling in response to cytokines such as interleukin-2 [125, 130–132]. mTOR was also found to impact EIF4E, a protein that inhibits translation by binding to the 5′ end of mRNA [133–136]. In the absence of rapamycin, mTOR can associated with adaptor proteins rictor or raptor to form mTORC1 or mTORC2, respectively. These complexes have different substrate specificity and cause distinct downstream signaling events.

In 1999, the FDA approved rapamycin as an immunosuppressant to prevent graft rejection in combination with cyclosporine A and steroids. However, the properties of rapamycin extend beyond this applicaiton. Phenotypic evaluation of growth inhibition in a particularly rapamycin-sensitive fungus, *Candida albican*, found nucleotide degradation and inhibition of synthesis as a primary mechanism of action in 1979 [137]. Nucleotide synthesis is a target of several clinically effective chemotherapies such as methotrexate and fluorouracil. This observation caught the eye of the National Cancer Institute (NCI). NCI experiments and an independent report found rapamycin to have antitumor activity comparable to that of cyclophosphamide and 5-FU in several solid malignancies and leukemia [138]. Other studies supported anticancer activity of rapamycin B-cell lymphoma [139], small cell lung cancer (SCLC) [140], rhabdomyosarcoma [141], melanoma [142], and pancreatic cancer [143]. Rapamycin was also found to inhibit angiogenesis under hypoxia [144] by causing transcriptional inhibition of VEGF [145], a process detailed later in the discussion of bevacizumab. Furthermore, rapamycin sensitized promyelocytic leukemia [141] and ovarian cancer [146] cell lines to cisplatin-induced apoptosis and inhibited transformation by PI3K or AKT. However, the clinical trial data generated in early phase trials of rapamycin as an immunosuppressant uncovered a poor pharmacokinetic profile [147, 148]. To overcome this problem, a large array of rapamycin analogues were created that are collectively called rapalogues.

Temsirolimus (CCI-779) is one of the first rapalogues and is a water soluble, chemically stable derivative of rapamycin (also called sirolimus) developed by Wyeth Pharmaceuticals. Preclinical studies found temsirolimus to have PTEN-dependent antitumor activity in a number of cancers [149–155] and like rapamycin, bound FK506bp and inhibited phosphorylation of S6K and 4EBP-1 [156, 157]. Phase I evaluation of temsirolimus found reversible mucositis or skin-related toxicity, no immunosuppressive functions, and that the major metabolite of temsirolimus was rapamycin [158]. Another study reported a linear correlation of time to progression and $p70^{S6K}$ kinase activity as measured in peripheral blood mononuclear cells, thus providing a pharmacody-

namic marker [159]. Temsirolimus had similar toxicities and response rates at a dose of 25, 75, and 250 mg in renal cell carcinoma. A multicenter phase III study in renal cell carcinoma (RCC) demonstrated extended overall survival (OS) and PFS in patients relative to interferon A but found no support for the combination of these therapies [160]. In parallel with the publication of this study, temsirolimus became the first FDA-approved cancer therapy to explicitly target mTOR and joined sorafenib and sunitinib for the treatment of advanced RCC. A comparison of temsirolimus with other approved therapies for CML as chosen by the investigator found superiority of temsirolimus in terms of improving PFS and OS [161]. Combinations with anti-angiogenic therapies such as sorafenib, sunitinib, and bevacizumab have yielded additional toxicity and largely no benefit [162–164]. Temsirolimus anticancer activity is also being clinically explored in breast cancer [165], gynecological malignancies [166], multiple myeloma [167], glioma [168–170], small cell lung cancer [171], and neuroendocrine carcinomas.

Everolimus and ridaforolimus are two other rapalogues that are being investigated in clinical trials. Unlike temsirolimus, everolimus retains the immunosuppressive properties of rapamycin and was approved in 2010 for organ rejection prophylaxis. Clinical trials found oral everolimus to be safe at an oral dose of 10 mg/kg [172–175]. A phase III study in metastatic (RCC) patients who had failed sorafenib, sunitinib, or the combination demonstrated an increased PFS with everolimus [176]. This resulted in FDA-approval of everolimus in 2009 for this indication. Based on promising early clinical data, this agent further received accelerated approval in 2010 for subependymal giant-cell astrocytoma patients with tuberous sclerosis who are not eligible for surgery [177]. Everolimus has promising preliminary efficacy in Hodgkin's lymphoma [178], metastatic gastric cancer [179], refractory NSCLC in combination with docetaxel [180] or gefitinib, and in breast cancer as a monotherapy [181] or in combination with letrozole [182, 183] or trastuzumab [184]. Interestingly, a phase II study with everolimus in refractory CLL reported partial responses along with an unexpected increase in the absolute lymphocytic count [185]. This has important implications for therapeutic sensitization as these cancer cells are likely to be more sensitive intravenous therapies when in circulation rather than in situ, however there is no clinical data to support this. Everolimus along with best supportive care was recently found to double PFS in patients with pancreatic neuroendocrine tumors [186, 187]. Ridaforolimus is in earlier clinical development but has demonstrated some partial responses and acceptable toxicity profiles in as a monotherapy [188–190] and in combination with capecitabine [191] and paclitaxel [192] in solid and hematological malignancies.

These three rapalogues work by virtually the same mechanism of action and have been developed separately by pharmaceutical companies. As the development of these rapalogues has happened in a relatively short time span, one is left wondering if these therapies have the same clinical efficacy. This is a difficulty inherent in the drug development process as clinical trial design dictates combinations with approved therapies. Combined with conflicting private interests, discerning the efficacy and unique roles of competing therapies with a similar mechanism of action is challenging. A recent phase II study in advanced pancreatic cancer attempted to compare temsirolimus and everolimus but toxicity and lack of objective response in any treatment group confounded this comparison [193]. Nevertheless, the discovery and development of rapamycin and rapalogues has extended the life of numerous cancer patients across several malignancies, prevent transplanted rejections, and elucidated a critical cell signaling pathway. At a conceptual level, the rapamycin story highlights the ability of existing therapies to be applied to other medicinal situations, the insight that can be gleaned from mechanistic studies of pharmaceuticals, and the power of medicinal chemistry.

38.2.5 Sorafenib

Raf is a serine/threonine kinase that is the apical member of the MAPK signaling cascade, which mediates a variety of cellular processes such as cell death, proliferation, and differentiation in response to extracellular stimuli. Aberrant Raf is observed in about 30 % of human cancers and correlates with the progression of prostate cancer to androgen insensitivity [194]. This increased signaling can result from alterations in upstream members such as Ras or in one of the three isoforms of Raf1 (A-Raf, B-Raf, and C-Raf). The V600E mutation in B-Raf is seen commonly in melanoma and NSCLC [195]. C-Raf overexpression has been noted in hepatocellular carcinoma [196] and validated in preclinical models as a potent drug target for ovarian cancer [197]. Earlier reports found direct evidence for Raf in determining tumorigenicity and sensitivity to radiation that posed Raf as a drug target [198, 199]. Bayer Pharmaceutical and Onyx Pharmaceutical jointly performed a high-throughput screen for Raf1 inhibitors. The screen found 3-thienyl as a lead compound with a Raf1 IC_{50} of 17 μM and that adding a methyl group at a particular position results in a tenfold decrease in IC_{50}. A follow-up screen with a library of analogues yielded 3-amino-isoxazole with an IC_{50} of 1.2 μM [200]. Substitution of the phenyl group of this compound for a 4-pyridyl moiety lowered the Raf1 IC_{50} to 230 nM as well as increased aqueous solubility. This compound was found to be orally active, inhibit signaling downstream of Ras through MEK and ERK, and inhibit cancer cell growth in vitro and in xenografts [201]. Based on SARs found throughout this process, further chemical modifications were explored and ultimately yielded sorafenib with a Raf1 IC_{50} of 6 nM (Fig. 38.6) [202].

Sorafenib reduces MAPK signaling by inhibiting numerous oncogenic kinases: wild-type and V600E B-Raf, the

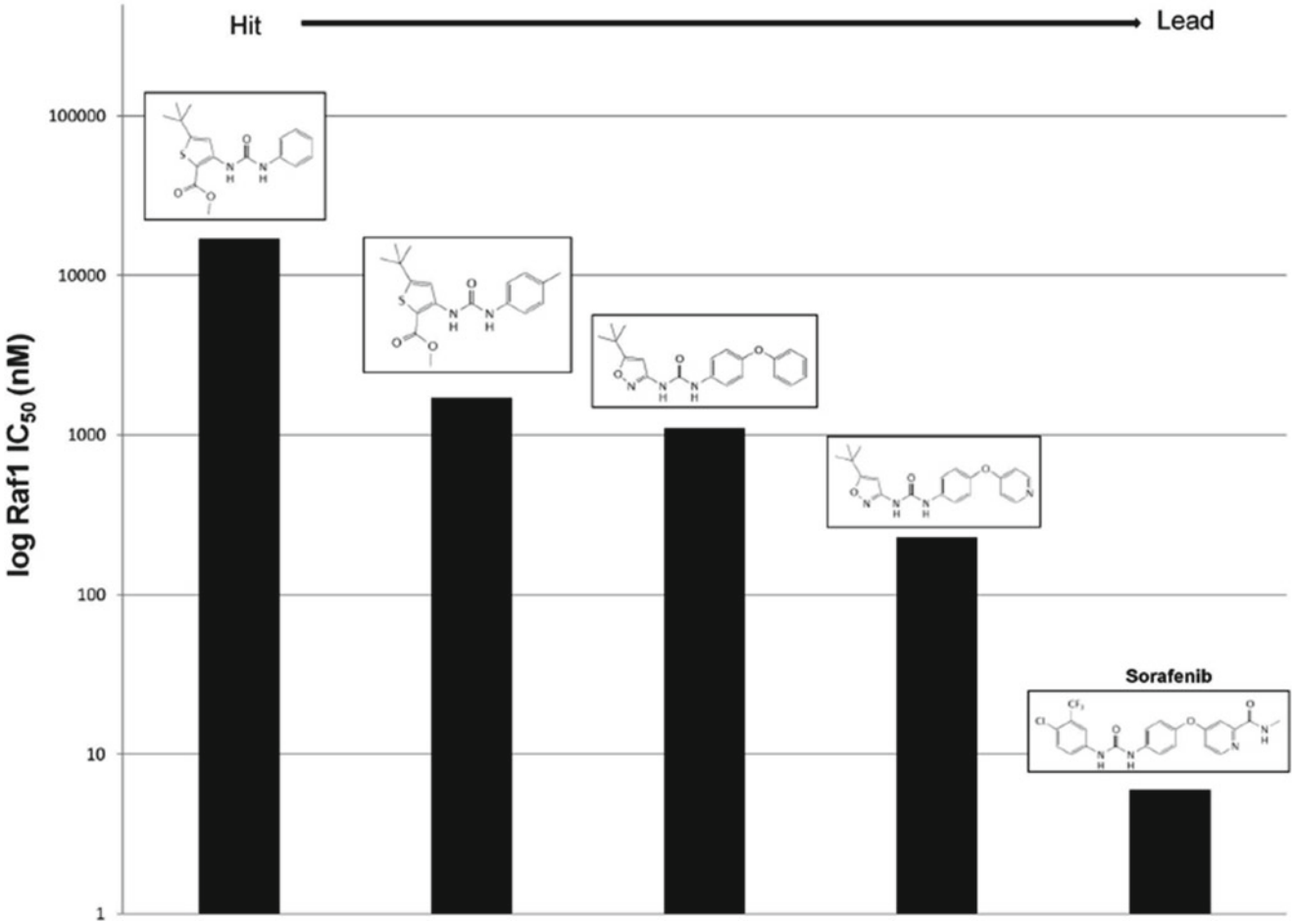

Fig. 38.6 Structure–activity relationships of Raf1 inhibitors explored during hit to lead development of sorafenib.

angiogenic VEGFR family, platelet-derived growth factor receptor-β (PDGFRβ), fibroblast growth factor receptor 1 (FGFR1), the neurotrophin receptor RET, and the cytokine receptors c-Kit and Flt-3 [203, 204]. The growth of several xenografts were inhibited by sorafenib though in a few cases, no change in MAPK signaling was detected but was rationalized by its anti-angiogenic effects of VEGFR inhibition [203]. Crystal structures of wild-type and V600E B-Raf in complex with sorafenib revealed key interactions with residues conserved in c-Raf [205]. The pyridyl ring occupies the ATP-binding pocket while the trifluoromethyl phenyl ring occupies a proximal hydrophobic pocket. Interestingly, the nitrogen of the pyridyl group forms a hydrogen bond with B-Raf and rationalizes the potent increase in activity following substitution of the pyridyl group for the phenyl group. The urea group bridging the rings was found to form a hydrogen bond network with protein, explaining its conservation throughout the analogue search and development. In this case, the structural data rationalized the previously found SARs. However, structural data can conversely be used to guide the exploration of analogue and can also potentiate in silico screen that computationally models the binding of a virtual library of ligands to protein structure. It should be noted that crystal structures do not provide a complete platform for computing binding affinities. While the biophysical details of ligand binding are beyond the scope of this chapter, the affinity of two molecules is determined by the change in enthalpy and entropy. As crystal structures represent a single average molecular conformation across the crystal lattice, they do not reflect the motions of the molecule. Therefore crystal structures cannot provide direct entropic information themselves and by extension do not fully represent binding affinity.

Since sorafenib inhibits a multitude of kinases, preclinical and early clinical trials explored a variety of malignancies. Several phase I trials found dose-dependent responses with an optimal oral dose of 400 mg [206–208] and addition of sorafenib to a variety of standard of care chemotherapy regimens did not increase toxicity profiles [209–214]. Renal cell carcinoma (RCC) is particularly resistant to the majority of chemotherapies and until 1997 was treated with interferon that causes significant toxicities and limited responses. Due to the poor clinical outcomes with standard of care therapies, preclinical efficacy of sorafenib, and an encouraging phase I

result in a metastatic RCC patient, phase II studies were enriched with RCC patients. This study found a strong response in RCC patients [215, 216] and led to a large-scale phase III study that reported a doubled PFS and ~40 % increase in OS [216, 217]. These results gained sorafenib FDA-approval at the end of 2005 for RCC. A subset analysis of this phase III trial found similar clinical benefits regardless of previous cytokine therapy [218] and a follow-up >1 year from treatment initiation found sustained efficacy and a well-tolerated toxicity profile [219]. Liver transplant has traditionally been the only treatment option available for hepatocellular carcinoma. However, many patients become ineligible while waiting for the transplant as a result of disease progression. Human xenografts of liver cancer cell lines showed partial tumor regression from sorafenib and inhibition of ERK and EIF4 [220]. A phase II trial found moderate efficacy and that time to progression correlated with phospho-ERK levels [221]. A multicenter phase III trial demonstrated an unprecedented increased OS in HCC and solicited the FDA to extend the indication of sorafenib to unresectable HCC [222].

Due to the multitargeted nature of sorafenib, it is being explored as a monotherapy and in combination with chemotherapies in a variety of malignancies, though biomarkers are difficult to find. A recent trial in metastatic melanoma found no correlation between clinical responses and BRAF V600E mutation status, cyclin D1, or the proliferation marker Ki-67 [223]. Early evidence was promising with sorafenib, carboplatin, and paclitaxel in melanoma [224] but a recent phase III study in advanced melanoma failed to demonstrate any benefit as second-line therapy [225]. A phase III trial in advanced HCC doubled PFS and OS with sorafenib plus doxorubicin compared to doxorubicin alone [226]. Other promising efficacy of sorafenib has been seen as a neoadjuvant in advanced RCC [227], as a monotherapy [228] or with erlotinib [229] or gefitinib [230] in NSCLC, metastatic RCC with gemcitabine and capectibaine [231]. Sorafenib has had limited to no clinical efficacy in malignant mesothelioma [232], prostate cancer [233, 234], uterine cancer [235, 236], sarcomas [237], advanced and metastatic squamous cell carcinoma [238], with paclitaxel and carboplatin in NSCLC [239, 240], or as a neoadjuvant in advanced ovarian cancer [241], sunitinib-refractory metastatic RCC [242].

38.2.6 Vemurafenib

Melanoma is particularly dependent on signaling through the c-Kit/NRAS/BRAF/MEK/ERK signaling axis. This solicits the clinical application of imatinib to melanoma as it inhibits c-Kit among other targets. However, targeting c-kit has been clinically limited in melanoma as several patients have gene amplification or have oncogenic alterations downstream in this signaling axis. Targeting BRAF in melanoma was explored during the clinical development of sorafenib but ultimately proved ineffective as a monoagent and did not improve OS in combination with carboplatin and paclitaxel in a phase III placebo-controlled trial [225]. Several reasons have been proposed for this failure such as unsaturated MAPK inhibition at the MTD of sorafenib [243] and furthermore, the ability sorafenib to target BRAF in vivo has been challenged [244].

The V600E activating mutation in BRAF is commonly observed in melanoma and results in resistance to therapies targeting upstream molecules. Using structural biology, vemurafenib was developed as a selective inhibitor of $BRAF^{V600E}$ [245, 246], though other targets have been uncovered such as CRAF, ACK1, SRMS, and MAP4K5 [247]. Phase I studies with Vemurafenib reported objective responses [248] that were corroborated in phase II studies and found pERK to be a valid correlative response marker [249]. A large phase III study in therapy-naïve patients ineligible for resection was recently reported an increased PFS and OS relative to dacarbazine [250]. In 2011, the FDA approved vemurafenib for the treatment of unresectable or metastatic melanoma with BRAFV600E.

While verumafenib is clearly a clinical oncology success, patients with wild-type BRAF still need better treatment options, biomarkers are needed to preemptively identify unresponsive patients with melanoma harboring $BRAF^{V600E}$, and many patients relapse despite the improvements in OS [251]. Verumafenib-resistant melanoma cells appear to upregulate PDGFR, NRAS, or MAPK signaling in vitro and in tumors [252, 253]. Preclinical evidence suggests that targeting MEK, PI3K, and mTORC in vemurafenib-refractory patients may be an effective combinatorial strategy [254]. Other preclinical reports have suggested the combination of vemurafenib with metformin [255], immunotherapy [256], or a monoclonal antibody targeting chondroitin sulfate proteoglycan 4 [257].

38.3 Antibodies

While evolution has allowed cells to use small molecules to remain viable, it has allowed organisms to protect themselves from disease through the immune system. Antibodies are large proteins used by the immune system to recognize, respond to, and remember foreign biological material. Antibodies contain a region that is highly specific for a protein target associated with the biological material known as the hypervariable region (Fv), which allows for a high degree of diversity and specificity. The constant region (Fc) of the antibody consists of a particular immunoglobulin that determines the class of antibody and ultimately the type of immune response mounted. Several factors including the

complexity of these large proteins have curtailed our ability to synthesize these highly specific binding molecules. However in 1975, medicine was forever changed by discovery the ability to harness cells to make antibodies [258]. This process consists of immunizing a mouse with the desired antigen, fusing splenic cells from the immunized mice with mouse myeloma cells using Sendai virus, selecting and growing desired clones. This capability has revolutionized modern medicine and biomedical research by offering an unparalleled ability to recognize any given protein with unparalleled specificity. Oncogene pathways mediated by soluble or cell-surface proteins naturally lend themselves as cancer therapeutic targets within the reach of antibodies.

38.3.1 Rituximab

CD20 is a B-cell-specific cell surface protein and as such, is expressed in B-cell cancers such as Non-Hodgkin's lymphoma (NHL). While the function of CD20 remains unclear, it is known that the protein is not secreted or cleaved from the cell surface [259] nor is it internalized following antibody binding [260]. An early study found that administration of murine anti-CD20 in malignant B-cell lymphomas produced a 90 % reduction of circulating malignant cells within four hours in humans [261]. Variable regions of the murine antibody were cloned in to an expression vector to allow for an antibody with human constant regions and would later gain the name rituximab [262]. Rituximab demonstrated a binding affinity for CD20 of 5 nM and resulted in a near complete depletion of peripheral blood cells and a 40–70 % depletion of B-cells in lymph nodes that began recovery around 2 weeks after administration. How does sticking an antibody to a surface molecule that does not have an obvious functional importance potently inhibit cancer? The currently understood answer is that rituximab induces three modes of cell death mediated by immune and cancer cells (Fig. 38.7). Unlike its murine counterpart, this hybridized antibody bound C1Q in vitro and furthermore induced cell lysis in the presence of serum as a source of complement. C1Q is part of a large protein complex that is found in serum and binds IgG or IgM to trigger a series of intra-complex cleavage events that ultimately form a transmembrane complex, called the membrane attack complex, to induce osmotic lysis of the antigen-expressing cell. This process is known as compliment-dependent cytotoxicity (CDC). CDC appears to be a key aspect of the antitumor activity of rituximab as rituximab-resistant patient samples were associated with CD59, which negatively regulates this process [263, 264]. Restoration of compliment has been shown to reverse resistance in small-scale patient studies [265, 266] though this has been challenged in preclinical models [267, 268] and follicular NHL [269].

In addition to CDC, effector cells such as macrophages and natural killer cells (NK) express a family of activating and inhibitory receptors called FcγR that bind the constant region (Fc) of IgG. A study in mice showed that blocking these various receptors mediated anti-CD20 mAb-induced B-cell depletion and was isotype-specific [270]. FcγR bound to IgG present on the surface of a cell can result in phagocytosis by

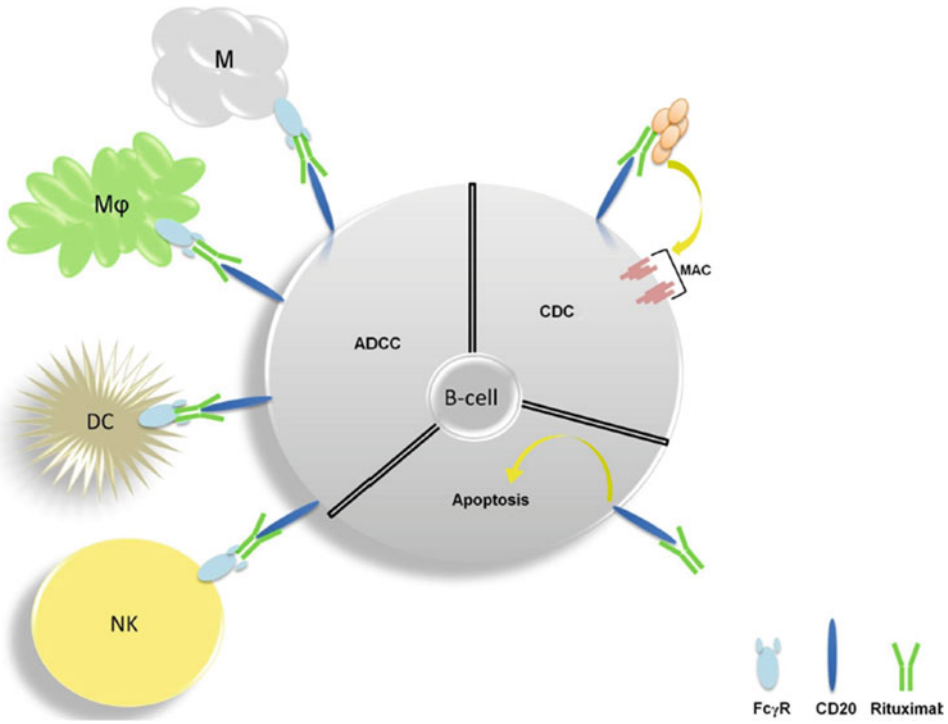

Fig. 38.7 Antitumor mechanisms of rituximab. Antibody-dependent cytotoxicity (ADCC) is mediated by natural killer cells (NK), dendritic cells (DC), macrophages (Mφ), and mast cells (M). Complement-dependent cytoxicity (CDC) involves a series conformational changes and cleavage events upon binding IgM or IgG that ultimately leads to the formation of the membrane attack (MAC) which induces osmotic lysis. Induction of apoptosis directly by CD20 also occurs but is less clear in mechanism.

effector cells but these events are determined by the affinity and balance of activating and inhibitory FcγR molecules [271–273]. Accordingly, a follicular NHL study found significantly higher rituximab responses in patients that harbor the FcγR 158V allotype compared to the 158F allotype, which has a relatively weaker affinity for IgG1 [274]. *Ex vivo* studies demonstrated that rituximab causes NK-mediated cell lysis [275–277] in a dose-dependent manner that was determined by the FcγR allotype [275]. There is also evidence that CD20 has direct effects. Early studies found that antibodies against CD20 significantly mediated RNA synthesis and cell cycle progression [278–280]. More recent evidence has found that cross-linking CD20 with antibodies in some, but not all, B-cell lines causes caspase-mediated, Bcl-2-independent apoptosis, and effects the tyrosine kinases Src, Jnk, and p38 [281–284]. The contribution of these different mechanisms appears to be highly context dependent and is likely to be a dynamic process varying between patients or even within a single patient.

Early clinical data found rituximab to have an average half-life of 18.5 days [285] and similar efficacy and favorable toxicity in relapsed NHL patients relative to CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone) [285–289]. A small-scale study of rituximab found antitumor activity concentrated in the follicular subtype of NHL, though the population size prevented any significant conclusion [290]. Other studies corroborated the significant monotherapy efficacy of rituximab in follicular lymphoma [291, 292]. Adding rituximab to chemotherapy yielded an additive benefit and did not augment the toxicity of standard chemotherapy for NHL [293, 294]. Furthermore, polymerase chain reaction (PCR) of NHL patients with follicular histology treated with this combination showed a depletion of the chromosome 14 and 18 translocation often associated with follicular NHL [295, 296]. Another study found the disappearance of this translocation a year after a 4-week course of rituximab [292]. This translocation induces a sustained transcriptional upregulation of Bcl-2, a protein that prevents mitochondria-mediated apoptosis carried out by many tumor suppressors and inhibited by oncogenes. In 1996, rituximab became the first antibody approved by the FDA as a cancer therapy and was indicated for relapsed or refractory low-grade or follicular, $CD20^+$, B-cell NHL. Three phase III studies (E4494, GELA, and MiNT) that were highly enriched in therapy-naïve, large diffuse cell NHL patients found a significant increase in OS at a 2 year follow-up with the addition of rituximab to CHOP or other anthracycline-based chemotherapies. Analysis of biopsies from E4494 patients found p21 expression as a rituximab-specific, independent predictor of clinical outcome [297].

NHL patients who previously received at least a single 4-week course of rituximab therapy had equivalent efficacy and toxicity after an additional course with a median internal of 14.5 months [298]. The combination of rituximab and interferon yielded no significant additional benefit in the short term in follicular NHL [299]. Today, rituximab is also approved as a first-line therapy for low-grade or follicular cell $CD20^+$ NHL with CHOP and large diffuse B-cell $CD20^+$ NHL with CVP (cyclophosphamide, vincristine, and prednisone). Initial studies of rituximab were conducted with intravenous administration for a 4-week cycle. However, exploration of alternative dosing schedules has provided efficacy in small lymphocytic lymphoma [300] (SLL) and chronic lymphocytic leukemia (CLL) patients [300, 301].

The addition of rituximab to fludarabine and cyclophosphamide in CLL was found to be safe [302–307] and result in unprecedented clinical responses in CLL patients [305]. A phase III study found that this combination increased the amount of patients without disease progression (65 to 45 %) and overall survival (87 to 83 %) [308], resulting in the extension of its indication to this malignancy in 2010. Shortly after, it was also approved as a maintenance therapy following a response with chemotherapy and rituximab in $CD20^+$ NHL based on phase III evidence of prolonged PFS with 1 weekly dose [309]. Rituximab and the proteasome inhibitor bortezomib have been shown to synergistically induce apoptosis in preclinical cancer models [310, 311] and a phase II supported the combination but noted significant neurological toxicity [312]. Also targeting CD20, the monoclonal antibody ocrelizumab was approved in 2009 for CLL refractory to fludarabine and the anti-CD52 antibody alemtuzumab. Other anti-CD20 antibodies are in clinical development for lymphoma including ocrelizumab, veltuzumab, AME-133V, PRO131921, GA101. As seen with rapamycin, the ongoing elucidation of the mechanism of action of rituximab has strongly augmented our biological understanding within and beyond cancer.

38.3.2 Outcompeting Growth Factors with Cetuximab

A number of recent cancer therapies target the EGFR and its family members by inhibiting the intracellular tyrosine kinase domain. An alternative approach is to inhibit EGFR signaling by blocking the extracellular binding of the growth factor through use of antibodies. Cetuximab was FDA-approved for the first-line treatment of metastatic colorectal cancer in combination with irinotecan in 2004, making it the first monoclonal antibody approved by the FDA for this type of cancer. The discovery of cetuximab started with the observation that antibodies secreted from mouse hybridoma cells that target EGF receptors were able to block EGF-induced signaling events and proliferation [313]. A follow up study found that these antibodies potently inhibited the growth of human cancer cells in a murine xenograft [314]. Further studies of a particularly potent antibody against EGFR, mab

225, found that it competes directly with EGF-binding [315], is internalized after two hours in cells [316], blocks EGFR autophosphorylation, induces G1 cell cycle arrest by $p27^{KIP1}$ induction [317–319], induces caspase-8-mediated apoptosis [320], and preferentially accumulate in EGFR-expressing tumors [321]. The latter was found using a radiolabeled form of the antibody that was subsequently used for the first clinical trial of mab 225. This trial found mab 225 to be safe in squamous cell lung carcinoma patients and profiled the overall and liver drug uptake, serum clearance, and whole-body clearance as monitored radiographically [322]. Noninvasive molecular imaging of drugs and therapeutic targets remains an active area of research and informs on the distribution, concentration, and kinetics of therapies, their targets, and/or therapeutic response markers in their clinical setting.

As with rituximab, mab 225 was engineered into a chimeric human/mouse antibody. This process retains residues in the binding region (F_v) of the antibody from the mouse and replaces other residues not specific for the antigen (F_c) with related human residues. This substitution in the F_c region reduces side effects resulting from immune responses to foreign, e.g. mouse, immunoglobulins. The mab 225 chimeric antibody, called cetuximab and marketed as Erbitux, was used for further studies and clinical trials. An early phase II trial with cetuximab in combination with irinotecan, a topoisomerase I inhibitor, demonstrated objective responses in EGFR-expressing solid cancers that had become resistant to first-line therapy consisting of leucovorin, 5-fluorouracil (5-FU), and irinotecan [323]. Combining cetuximab with this treatment regimen also proved effective in a first-line therapy setting [324–326] and moderate efficacy as a monotherapy [327–330]. Safety and efficacy was reported with cetuximab in FOLFOX6 [331, 332] and FOLFOX4 [333–335] treatment regimens which consist of leucovorin, 5-FU, and oxaliplatin. A phase III trial also found cetuximab to be effective as a monotherapy and in combination with irinotecan in irinotecan-refractory, $EGFR^+$ colorectal cancer patients [329]. Based on the rational design and specificity of cetuximab, most of these clinical trials exclusively included patients that had EGFR-expressing tumors as determined by immunohistochemical assays. Interestingly, the intensity of EGFR express did not correlate to cetuximab clinical response and another trial has shown responses in $EGFR^-$ colorectal cancer patients [336]. This unexpected finding underscores the complex nature of therapeutic responses in the clinic and the principle that while rational design has well-evidenced benefits, it only allows for hypotheses that ultimately must be tested.

However, the notion that certain patients will respond better than other patients based on certain characteristics is gaining importance in clinical trial design and interpretation. As required by the drug development process, testing therapies in large-scale clinical trials results in considerable patient diversity. Imagine a clinical trial is conducted to examine the efficacy of a new cancer therapy in 1000 patients where 500 patients receive FOLFOX4 and 500 patients receive FOLFOX4 plus the new therapy. The results show no benefit by clinical response or overall survival. However, let us say that ten patients within this group showed a clinical response and improved overall survival due to a unique genetic characteristic. These patients comprise 2 % of the investigational therapy population and therefore will not impact response parameters with any level of statistical significance. Redesigning the clinical trial to include patients that harbor this response-determining characteristic may now show a strong therapeutic benefit otherwise concluded as ineffective. The obvious challenge in patient stratification in clinical trial design is determining what characteristic(s) confer therapeutic response. Clinical trials are sometimes analyzed retrospectively to find such biomarkers. Some of biomarkers have even been added as stipulations for FDA-approved cancer therapies, cetuximab being no exception.

Two years after FDA approval in 2004, a study found that among downstream targets of EGFR signaling, particular KRAS mutations were significantly associated with lack of response to cetuximab [337]. Other cetuximab trials also found KRAS to be a strong predictor of cetuximab response [330, 338, 339], which led ImClone, the company marketing cetuximab, to petition the FDA to add WT KRAS as a requisite for cetuximab treatment [340]. In 2009, the FDA updated the indications and usage label of cetuximab in colorectal cancer to include that cetuximab is not recommended for patients harboring KRAS mutations in codon 12 or 13.

38.3.3 Bevacizumab

Cell division is an active energy-dependent process and therefore cancer cells must gain alterations that provide more energy to allow for proliferation. While several mechanisms of harvesting intracellular energy are available, cellular respiration is a mainstay that relies primarily on oxygen. The oxygen source for cells is the blood stream and perhaps it is unsurprising that generation of new blood vessels to supply additional oxygen is seen in tumors. A number of pathways and proteins mediate this process of generating new blood vessels in for tumors, particularly the vascular endothelial growth factor (VEGF) family. VEGF is transcriptionally upregulated in a variety of solid tumors particularly in hypoxic regions due to stabilization of the transcription factor HIF1α that is stabilized under hypoxia. VEGF has four family members, VEGF-A being the prototypical member and itself has four isoforms as a result of alternative splicing which determines its localization and binding properties. Soluble VEGF can bind to VEGF receptors (VEGFR1, VEGF-R2) and its coreceptors (NRP-1,NRP-2) that are

expressed on the surface of endothelial cells. Secretion of VEGF ultimately causes the formation of new blood vessels by endothelial cell recruitment and proliferation. Due to the involvement of VEGF in several disease and disorders such as cancer and macular degeneration, antibodies designed to bind VEGF and thereby prevent cognate receptor binding were generated and first described in 1992 [341]. A follow up study found that one of these mouse monoclonal antibodies, A4.6.1, had potent antitumor activity in vivo but not in vitro [342]. Interestingly, xenografts of human cancer cells in mice found that introducing soluble VEGF receptors that bind both human and mouse VEGF was superior to a receptor binding only the human or mouse VEGF [343, 344]. Together, these findings indicate that both the tumor and other cells in the tumor microenvironment induce participate in generating a new vasculature system through VEGF. Over the last decade, the tumor microenvironment has increasingly garnered attention as a dynamic and strong influence on aspects of tumor biology including therapeutic response, growth rates, and metastasis through a variety of mechanisms.

Due to the potent antitumor activity of the A4.6.1 antibody, a humanized form of the antibody was generated using site-directed mutagenesis of the variable region of a human antibody to that of the murine A4.6.1 while maintaining a similar binding affinity for VEGF [345]. This humanized version of A4.6.1, known as bevacizumab, is specific for all isoforms of VEGF-A and its cleaved products that result from extracellular proteins such as matrix metalloprotease-9 (MMP9) present in the tumor microenvironment. A crystal structure of the antigen-specific fragment of bevacizumab bound to VEGF identified a critical residue for binding and specificity (Fig. 38.8) [346]. Bevacizumab was evaluated for safety in cynomolgus monkeys due to the complete conservation of VEGF isoforms between humans and monkeys [347]. After several weeks of administration at doses up to 50 mg/kg, adverse effects on ocular, ovarian, and uterine angiogenesis-dependent processes were evident but were dose-dependent and reversible. A phase I clinical trial found no additional toxicity associated with adding bevacizumab to various chemotherapies and a terminal half-life of 2–3 weeks [348, 349]. The following year, several phase II trials were conducted in a variety of solid tumors [350–354]. Particularly encouraging results were found in RCC as a first-line-therapy and metastatic colorectal cancer in combination with standard chemotherapy. A phase III trial in metastatic colorectal cancer with bevacizumab plus standard chemotherapy increased overall survival, progression-free survival, and objective response rate [355].

The addition of bevacizumab to a paclitaxel-carboplatin treatment regimen increased median survival and PFS but also increased treatment-related deaths, including pulmonary hemorrhage [356]. Based on the significant improvement in OS, bevacizumab in combination with carboplatin and paclitaxel was approved for first-line therapy in unresectable, locally advanced, recurrent, or metastatic NSCLC. Bevacizumab has now been approved for Her2-negative breast cancer, metastatic RCC, NSCLC, glioblastoma, and metastatic colorectal cancer. However, bevacizumab seems continually surrounded by controversy from a variety of perspectives including clinical, economic, and ethical issues. A highly debated meta-analysis of 15 clinical trials with bevacizumab found a significant increase in venous thrombosis [357–361]. A separate analysis also

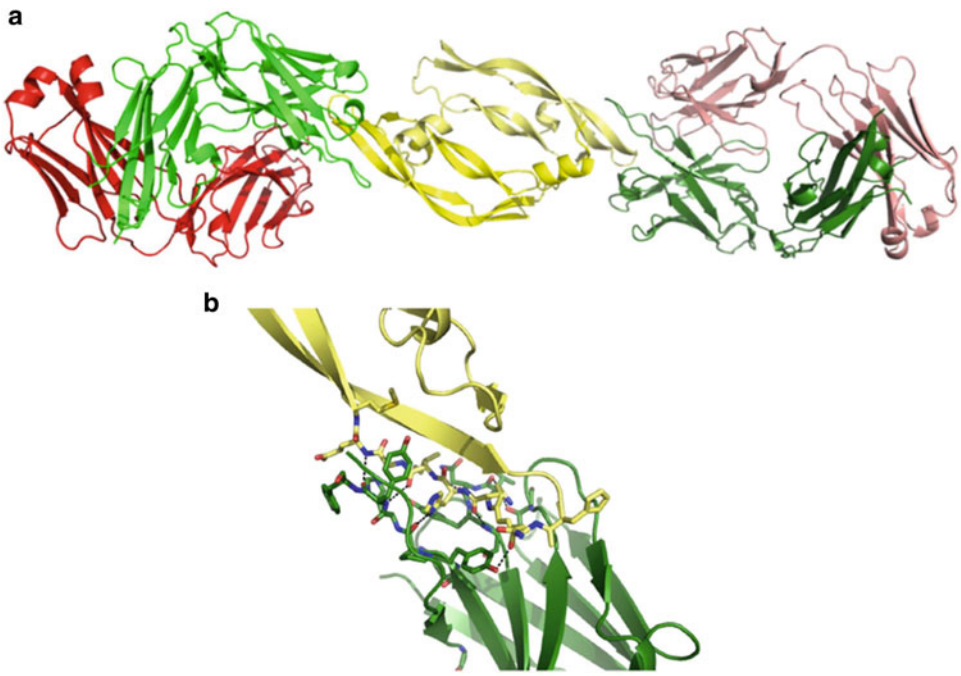

Fig. 38.8 Binding interface of bevacizumab Fab and VEGF. (**a**) Symmetric assembly of VEGF dimmers and Fab fragments of bevacizumab. There are two monomers of VEGF in the center (*light* and *dark yellow*) along with four flanking monomers of bevacizumab. (**b**) Binding interfaces of bevacizumab (*light yellow*) and VEGF (*green*) with interacting residues displayed and hydrogen bonds represented as *dashed lines*. PDB accession 1BJ1.

found an increased risk of high-grade bleeding [362]. At the end of 2010, the FDA decided to revoke its approval for bevacizumab in metastatic breast cancer based on the results of three large clinical trials (E2100 [363], AVADO, and RIBBON1). These trials found that while PFS was prolonged, this magnitude was variable among trials and life-threatening adverse events were increased without any change in OS. On the same day, the European Medicines Association decided to maintain its approval in metastatic breast cancer but only in combination with paclitaxel based on the same clinical trials.

Bevacizumab was granted accelerated approval in metastatic breast cancer based on promising data indicating prolonged PFS but was never shown to increase OS. Traditionally, OS has been the key parameter used to decide whether or not a new therapy receives FDA approval. This brings up difficult questions. Will the therapy provide a net benefit to the patient? How do you quantify this? These answers will be unique for every patient as each has a unique situation that determines how benefit is defined. Obviously, most patients want to live longer but quality of life is also a consideration, which raises yet another patient-specific question: what is quality of life? Clinically, fewer adverse events and prolonged PFS and OS are quantifiable parameters that may act as a partial surrogate definition but this consideration is more complex. For bevacizumab, even this incomplete surrogate definition is unclear as both adverse events and prolonged PFS have been reported in multiple large-scale clinical trials and the magnitudes of these are highly variable among these trials. These discrepancies and lack of improvement in OS have led to a range of decisions from removal to full approval by medical agencies across the globe. The U.K. National Institute of Health and Clinical Excellence (NICE) denied the approval of bevacizumab for metastatic breast cancer, citing no evidence in improvement of survival or quality of life.

One less obvious consideration of a therapy like bevacizumab is the possibility of its use as a neoadjuvant, i.e. shrinking tumors to a size where resection is then possibility. This type of consideration is difficult to evaluate in early clinical trials typically compare an investigational therapy to other therapies in patients where such data would be excluded. Proper examination of this nuance and its effect on OS is warranted in this case. In summary, bevacizumab significantly extends PFS to varying degrees in several solid cancers, may shrink tumors to allow for resection, does not increase OS, and significantly increases adverse events. Cost is the elephant in the room. The cost of bevacizumab treatment varies by country, about $90,000 per year in United States [364]. In addition to rejecting bevacizumab for metastatic breast cancer, NICE has also denied its approval in metastatic colorectal cancer and RCC. Cost was clearly an issue in renal cell cancer, as the benefits of bevacizumab with interferon was similar to sunitinib, a multi-kinase inhibitor already approved for RCC and available at a much lower cost. The various agencies that make the approval decisions on these therapies explain their decisions on the grounds of clinical parameters but patient and social burdens of treatment cost is clearly an underlying influence. Off-label explorations, more clinical trials, and altered clinical trial design are likely to yield more information on the utility of bevacizumab. Nevertheless, this controversial therapy highlights difficult questions lurking in the background of the clinical management of cancer: How do we determine quality of life? Should we consider costs in approval decision? What price is too high for a given therapeutic benefit? How do you weigh concomitant risks and benefits?

38.3.4 Ipilimumab

The immune surveillance of cancer is an endogenous mechanism of tumor suppression that is lost during cancer progression. Inactivating the RAG-2 gene, which is intimately involved in recombination events required for the activation and specificity of immune responses, renders mice more susceptible to carcinogenesis [365]. T-cells are part of the adaptive immune system that modulates the immune response to antigen threat. The immune escape of cancer may occur by multiple mechanisms such as altering proteins involved in antigen presentation or enriching for regulatory T-cells that secrete immune-inhibitory cytokines such as IL-10 [366]. Regulatory T-cells have other mechanisms of immune suppression such as elevated expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) [367]. CTLA4 is an inducible cell surface receptor that binds CD80 and CD86, which are expressed on the surface of antigen-presenting cells and function in concert to activate naive T-cells through CD28. CD28 is a costimulatory receptor expressed on naïve T-cells that cooperatively acts with CD4 as part of the major histocompatibility complex to activate T-cells in response to antigen. Thus, CTLA4 plays a key function in regulatory T-cells by inhibiting T-cell activation as an inhibitory receptor for ligands that are essential for T-cell activation. Accordingly, blocking CTLA4 with antibodies in mice reduces T-cell response and increased tolerability to immunogenic tumors [368].

Ipilimumab is a fully human antibody developed by Medarex Inc. that targets CTLA4. Cynomolgus monkey studies found that ipilimumab induces a humoral response without autoimmunity [369]. Early human studies with ipilimumab in melanoma and ovarian cancer reported tumor necrosis and lymphocyte infiltration in ipilimumab-responsive tumors, though several patients experienced grade III/IV adverse events and some developed T-cell reactivity to normal melanocytes [370, 371]. Autoimmunity was noted in other clinical studies with ipilimumab but was found to correlative with

response [372]. Enterocolitis was the most frequent adverse event, suggesting that CTLA-4 plays a critical role in protection from immune-mediated enterocolitis [373]. Similar efficacy and toxicity events were seen in metastatic RCC [374]. The investigation of single-nucleotide polymorphisms (SNPs) in the CTLA4 gene amongst ipilimumab-treated melanoma patients revealed a response-associated and nonresponse-associated haplotype comprised of seven single-nucleotides in the CTLA4 gene.

A phase II study of ipilimumab in previously treated melanoma reported that 30 % of patients were alive at 2 years in the highest dose cohort of 10 mg/kg [375]. Another phase II study in melanoma evaluated the safety and efficacy of ipilimumab at 3 mg/kg in combination with dacarbazine, noting a preliminary increase in objective response rate with the combination. The pivotal phase III study compared ipilimumab, the gp100 peptide vaccine, and the combination [376]. The overall survival as 10.1 months in the combination cohort, 10.0 months in the ipilimumab alone cohort, and 6.4 months in the gp100 alone cohort. This study corroborated survival adverse event observations noted in previous trials and was revered as the first trial to ever show survival benefit in metastatic melanoma. Another phase III study measured the efficacy of adding ipilimumab (10 mg/kg) to dacarbazine as a first-in-line therapy [377]. Adding ipilimumab to dacarbazine increased the overall survival from 36 to 47 %, though adverse events were increased by the combination.

Based on these phase III trials, the FDA recently approved ipilimumab for the treatment of unresectable or metastatic melanoma at 3 mg/kg every 3 weeks for four doses. Ipilimumab is a first-in-class cancer therapy that reactivates the adaptive immune system to restore antitumor immunity and provides unprecedented patient benefit in melanoma. The clinical efficacy and toxicity of ipilimumab highlights the potential benefits and dangers of immunotherapy as it clinically emerge as a valid new modality of cancer treatment. Despite frequent and severe adverse events associated with ipilimumab, it has been approved based on its unparalleled efficacy in a clinical situation that desperately needed better treatment options. Future trials with ipilimumab are exploring other malignancies and the combination of ipilimumab with other approved agents including chemotherapy and the recently approved vemurafenib.

38.3.5 Lexatumumab

Cell death is a very tightly controlled process that may be initiated by various inputs inside and outside of the cell. TNF-related apoptosis-inducing ligand (TRAIL) is an endogenous mammalian protein utilized by the immune system in the immune surveillance of cancer to potently induce apoptosis in cancer cells while exerting little toxicity to normal cells [378]. TRAIL binds four transmembrane receptors in humans at similar affinities that results in a homotrimeric receptor–ligand complex [379]. These receptors include two pro-apoptotic death receptors, DR4 and DR5, and two decoy receptors, DcR1 and DcR2. The ratio of decoy receptors to death receptors along with mediators of downstream signaling events are thought to determine TRAIL sensitivity and be utilized by normal cells to afford protection from TRAIL-mediated apoptosis. The two decoy receptors compete for TRAIL binding with the death receptors at similar binding affinities.

TRAIL-induced trimerization of the death receptors colocalizes their intracellular death domains, which recruit Fas-associated death domain (FADD) and procaspase-8, forming death inducing signaling complex (DISC). At the DISC, procaspase-8 is activated by autocatalytic cleavage to form caspase-8 which can cleave effector caspase-3, caspase-6, and caspase-7 to induce apoptosis by the extrinsic death pathway. Alternatively, caspase-8 can initiate the intrinsic death pathway by cleaving Bid to tBid, which primarily interacts with Bax and Bak at the mitochondrial membrane. This interaction induces oligomerization of Bax and Bak to promote cytochrome c release. In the cytosol, cytochrome c binds to apoptotic peptidase activating factor 1 (Apaf-1) and caspase-9 to form the apoptosome, which initiates the caspase cascade. While TRAIL directly targets death receptors that generally play a tumor suppressor role, several oncoproteins mediate this process and serve as potent resistance mechanisms. One of the most striking examples is Mcl-1, an anti-apoptotic member of the Bcl-2 family that interacts with pro-apoptotic family members such as Bax to inhibit mitochondrial-mediated apoptosis. Notably, this resistance mechanism can be overcome by combining TRAIL with sorafenib [380].

The ability of TRAIL to selectively induce apoptosis in tumors cell has led to the clinical trials with recombinant TRAIL. However, TRAIL has a short serum half-life and cytotoxic resistance can result from elevated decoy receptor expression. As a result, TRAIL-agonist antibodies targeting either of two pro-apoptotic death receptors were created and are currently in clinical trials [381]. Lexatumumab is one of the most developed DR5 agonist antibodies. A phase I study with lexatumumab in previously treated advanced solid tumors reported a MTD of 10 mg/kg when given once every 21 days, a second-phase serum half-life of ~16 days, and stable disease in approximately a third of the treated patients [382]. Another phase I study found lexatumumab to be safe at 10 mg/kg once every 14 days [383].

Several preclinical studies have suggested the use of lexatumumab in combination with other therapies to increase efficacy such as radiation [384], paclitaxel [385], bortezomib [386–388], HDAC inhibitors [389], doxorubicin [390], and cisplatin [391]. Phase Ib studies evaluated lexatumumab in

combination with gemcitabine, pemetrexed, doxorubicin, or FOLFIRI [392]. Severe adverse events potentially attributable to lexatumumab included anemia, fatigue, and dehydration. Tumor regression was noted in patient cohorts receiving the combination of lexatumumab and doxorubicin or FOLFIRI. Numerous other DR5-agonst antibodies including TRA-8, LBY135, Apomab, and Conatumumab and the DR4-agonist mapatumumab are also currently in clinical trials.

38.3.6 Custom Cures

We still have not won the war on cancer. However, we have greatly expanded our understanding and gained some highly effective therapies in the course of battle. Cancer was once understood as a simple clonal expansion of a cell that gained a growth advantage by a stepwise loss or gain of function of a few critical genes. This previously linear pathway of carcinogenesis is now a multinodal, interconnected network of events and we have begun to appreciate the immense heterogeneity of tumors and the dynamic parameters that govern their biology and therapeutic response. So how much has all of this research investment helped cancer patients? Clearly some types of cancer have benefited from new targeted agents such as NHL and breast cancer. Chemotherapy, radiation, and surgery remain the cornerstones of treatment regimens in many malignancies and none of these were developed with a molecular understanding of their mechanism of action. Serendipity has played a key role in the discovery of many cancer therapies still used today. Nevertheless, it has been our hypotheses and observations built upon our prior knowledge base that has guided the application of these serendipitous discoveries. The discovery of nitrogen mustards was certainly not found in a quest to cure cancer but it was applied to cancer by a hypothesis generated from the current understanding of cancer. It is the application of our knowledge to what is right under our nose that can yield new solutions, quite literally in the case of rapamycin. The more we understand how cancer works, the better we understand what we are looking for and how we can use the tools we already have.

Oncogenes, tumor suppressors, epigenetics, immunology, the tumor microenvironment, and several other factors play critical roles in tumor biology. Considering this multifactorial and complex nature of cancer and the level of effort put forth throughout history to cure it, it is likely that there will be no magic bullet that cures cancer. The future of cancer therapy lies in continued drug discovery, explorations of multimodal and combinatorial therapy, improved agent targeting, and personalized medicine. Combinations of existing therapies can yield antagonistic to synergistic responses in the clinic but is often difficult to interpret due to the heterogeneity of patient responses. Interpretation of inter-patient and intra-patient responses will also play a critical role in the future of cancer therapy and could benefit from the use of biomarkers and molecular imaging. It is important that we understand why a therapy fails in a certain situation so that successful situations can be clearly identified, future failures may be avoided, and other successful treatment options can be discovered.

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Therapeutic Agents That Inhibit Angiogenesis

39

Francesco Torino, Roberta Sarmiento, Raffaelle Longo, and Giampietro Gasparini

39.1 Introduction

Angiogenesis is a complex multistep process leading to the formation of new blood vessels from the existing vascular network, tightly regulated by pro-angiogenic and antiangiogenic growth factors. Several potential pathways for tumor-induced angiogenesis have been proposed: (1) secretion by tumor cells of endothelial growth factors which may stimulate tyrosine kinase activities in endothelial cells (ECs), being vascular endothelial growth factor (VEGF) the most specific and potent pro-angiogenic agent; (2) downregulation of naturally occurring inhibitors of angiogenesis; (3) circulating CD34-positive EC precursors may contribute to vasculogenesis, by presently unknown mechanisms mainly involving genetic regulations.

Targeting the molecular pathways involved in tumor progression by biologically designed treatments is a new therapeutic paradigm aimed to reach cancer growth control. Inhibition of angiogenesis presents certain advantages on conventional therapies, such as the direct accessibility from the circulation, the potential low rate of drug resistance and the favorable toxicologic profile.

A recently proposed classification of antiangiogenic agents is based on their mechanisms of action, and include three categories: (1) direct antiangiogenic drugs acting by targeting the endothelial cells and their functions involved in angiogenesis (proliferation, migration, formation of new vessels); (2) indirect antiangiogenic drugs that inhibit the production of angiogenic factors by tumor and microenvironment cells, and/or interfere with extracellular processes; (3) mixed antiangiogenic drugs that may be able to interfere with both endothelial and tumor cells [1].

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A functional classification, based on the potential targets for inhibition of angiogenesis, includes drugs that are directed towards activated endothelial cells, pericytes, hypoxia pathways, and nitric oxide [2].

- Endogenous antiangiogenic agents such as angiostatin, endostatin, caplostatin, and thrombospondin-1 (TSP-1) selectively block the proliferation and migration of intratumoral vascular endothelium and induce prolonged tumor dormancy in several experimental models [2]. In 2005, rh-endostatin was approved in China for the treatment of advanced non-small-cell lung cancer (NSCLC) based on the positive results of the phase III trial by Sun et al. [3]. Moreover, the recent identification and characterization of the endothelial progenitor cell (EPC) and its capability to migrate from bone marrow into circulation and then into other tissues where it stimulates angiogenesis may also open new selective anti-EPC therapeutic strategies [4–6].
- Pericytes are mural cells differentiated from pools of c-kit+sca-1+VEGFR-1+ perivascular progenitor cells mobilized from bone marrow in response to the platelet derived growth factor (PDGF)-BB. When PDGF is overexpressed, tumor microvasculature is covered by a high number of mural cells and tumor growth is accelerated. Drugs targeting PDGFR-β inhibit the recruitment of pericytes, induce dilation of tumor vessels and stimulate endothelial cells to enter apoptosis. The combined block of both VEGFR and PDGFR-β by multitarget tyrosine kinase inhibitors, such as sunitinib, increases the antiangiogenic effect, even in late stage solid tumors [7–10].
- Among angiogenesis promoting molecules identified and purified up to now, VEGF is a key regulator of angiogenesis, overexpressed in the majority of human tumor types. The most advanced in the clinic, among the anti-VEGF inhibitors, are the humanized monoclonal antibody bevacizumab and a number of VEGFRs selective tyrosine kinase inhibitors (Table 39.1). Additional compounds targeting VEGF in clinical development include a VEGF trap and antibodies against VEGFR-2,

W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_39

Table 39.1 Angiogenesis inhibitors approved for clinical use

Date approved	Drug	Place	Disease
May 2003	Bortezomib	USA (FDA)	Multiple myeloma
December 2003	Thalidomide	Australia	Multiple myeloma
February 2004	Bevacizumab[a]	USA (FDA)	Colorectal cancer
November 2004	Erlotinib	USA (FDA)	Lung cancer
December 2004	Bevacizumab[a]	Switzerland	Colorectal cancer
December 2004	Macugen[a]	USA (FDA)	Macular degeneration
January 2005	Bevacizumab[a]	EMEA	Colorectal cancer
September 2005	Endostatin[a]	China	Lung cancer
December 2005	Sorafenib[a]	USA (FDA)	Kidney cancer
December 2005	Revlimid	USA (FDA)	Myelodysplastic syndrome
January 2006	Sunitinib[a]	USA (FDA)	Gastric (GIST), kidney cancer
June 2006	Lucentis	USA (FDA)	Macular degeneration
October 2006	Bevacizumab[a]	USA (FDA)	Lung cancer

[a]"Pure" antiangiogenic agents

VEGFR-1. Interestingly, anti-VEGF agents not only arrest proliferation and migration of endothelial cells, but also induce regression of existing vessels by inducing apoptosis and by suppressing the mobilization of EPCs from bone marrow [11–13].

- Oxygen limitation is central in regulating angiogenesis, glucose metabolism, survival, and tumor growth [2]. A major key factor is the hypoxia-inducible factor (HIF) which is required transcriptional factor in nutrient stress signaling. HIF is a pleiotropic factor that controls the expression of VEGF-A and angiopoietin-2 [14]. In the nucleus, HIF links the hypoxia-response-elements that control oxygen tension via oxidizing enzymes and hydroxylases. Moreover, HIF also transcriptionally regulates the expression of MET promoter that is a key regulator of invasive growth by driving cell motility and metastasis [2].

Nitric oxide (NO) is a multifunctional gaseous molecule and a highly reactive free radical that regulates several vascular functions, being capable to mediate VEGF and angiopoietin-1-induced angiogenesis in vivo [15]. Inhibition of NO signaling is another potential antiangiogenic therapeutic strategy. A number of compounds interfere with NO production, such as cavtratin, a caveolin-1 derived peptide, L-NNa, and NO-donating nonsteroidal anti-inflammatory drugs (NSAIDs) [16–18].

More than 50 antiangiogenic agents with different mechanisms of action have been discovered up to now. They include tyrosine kinase inhibitors (TKIs), monoclonal antibodies, small molecule inhibitors and transcription inhibitors. Recently, nanobodies have been introduced into the antiangiogenic armamentarium.

39.2 Anti-VEGF Targeted Therapies

39.2.1 Bevacizumab

Bevacizumab is a humanized monoclonal antibody against VEGF. In February 2004, bevacizumab received the Food and Drug Authorization (FDA) approval for first-line therapy of colorectal cancer (CRC) in combination with an IFL-based (irinotecan, fluorouracil and leucovorin) regimen. In CRC, a randomized multicenter study evaluating the clinical benefits of bevacizumab combined with a bolus IFL in front-line treatment of CRC showed an advantage in median duration of overall survival, median duration of progression-free survival (PFS), response rate (RR) and median duration of response [19]. The most significant clinical toxicities reported in bevacizumab-treated patients were thrombosis, hypertension, proteinuria, and bleeding. In addition, gastrointestinal perforation (1.5 %) as well as an increase in diarrhea, leukopenia, and hypertension was also described. Results from the Three Regimens of Eloxatin Evaluation (TREE)-2 trial study in patients with metastatic CRC showed that the addition of bevacizumab to an oxaliplatin/fluorouracil regimen in first-line therapy improves RR and time to progression (TTP) with acceptable tolerability, and no unexpected toxicity [20]. Several other trials studying the clinical benefits of bevacizumab combined with different anti-EGFR agents, such as erlotinib, panitumumab, or cetuximab are ongoing.

The clinical utility of bevacizumab in metastatic RCC was investigated in a randomized phase II trial in which 116 patients with metastatic clear-cell RCC refractory to immunotherapy were randomized to receive placebo, low-dose (3 mg/kg) bevacizumab, or high-dose (10 mg/kg) bevacizumab given intravenously every 2 weeks. Patients treated with bevacizumab experienced a significant prolongation of TTP compared with the placebo group [21]. There were four partial responses (10 % ORR), all in the high-dose bevacizumab arm. There were no life threatening toxicities or deaths attributable to bevacizumab. Common toxicity included hypertension and proteinuria, more commonly seen in the high-dose bevacizumab arm. All side effects were reversible with cessation of therapy. Grade 1 or 2 hemoptysis was observed in two patients receiving bevacizumab and two patients receiving placebo. No thromboembolic events were reported in any arm.

In a double-blind phase II trial comparing bevacizumab plus erlotinib versus bevacizumab plus placebo, data showed that the combination was safe and well tolerated, although adding erlotinib did not improve efficacy [22]. However, PFS of 8.5 months observed in patients treated with bevacizumab [23] appears to be more favorable than what is reported with the use of IFN-α, suggesting a potential clinical benefit of bevacizumab in RCC. Given the promising activity of bevacizumab, several clinical trials investigating

the clinical advantages of bevacizumab with IFN-α2b therapy (CALGB 90206; $n=600$) (Rini = 72) or in addition to erlotinib plus imatinib are currently in progress [24].

The addiction of bevacizumab to carboplatin/paclitaxel regimen caused a higher RR (31.5 % in the high-dose arm versus 18.8 %) and longer median TTP (7.4 months in the high-dose arm versus 4.2 months) than chemotherapy alone in NSCLC patients [25]. Bevacizumab was generally well tolerated. However, severe haemoptysis episodes among patients with squamous cell histology and central tumor mass were observed. Another study evaluated the clinical benefits of bevacizumab with the carboplatin/paclitaxel regimen as first-line therapy in 842 patients with NSCLC. This US cooperative group phase III trial (E4599) reported a higher RR, longer PFS and increased survival in the bevacizumab/chemotherapy arm compared with the chemotherapy-alone arm [26]. In a trial combining bevacizumab with erlotinib in patients with recurrent NSCLC, preliminary data showed a promising antitumor activity, with partial response achieved in 20 % of patients and stable disease in 65 % [27]. The most common adverse events reported ranged from mild-to-moderate and included rash, diarrhea and proteinuria. Bevacizumab in combination with erlotinib is still under investigation in recurrent or refractory NSCLC [28].

A phase II study assessed the combination of bevacizumab plus gemcitabine in 52 patients with stage IV pancreatic cancer [29]. The objective RR was 21 %, the median PFS 5.4 months and the 6-month OS of 77 %. Adverse events included hypertension, thrombosis, and bleeding episodes. The promising efficacy prompted two ongoing trials: the European phase III trial (BO17706) and the US Cooperative Group phase III trial (CALGB 80303), investigating both the therapeutic benefits of bevacizumab when added to gemcitabine alone or with erlotinib.

Zhu et al. reported encouraging results from a phase II study investigating the therapeutic benefits of bevacizumab combined with a gemcitabine and oxaliplatin regimen in patients with advanced hepatocellular carcinoma [30]. Data suggested that this combination was generally well tolerated, with the most common grade 3–4 adverse events being fatigue, transient elevation of transaminases, nausea/vomiting and hypertension. Although no patients achieved a complete response, the overall response rate was 20 % and the rate of PFS at 6 months 48 %.

In a phase III metastatic breast cancer (mBC) trial, the addition of bevacizumab to capecitabine in a cohort of taxane-refractory and-anthracycline-refractory patients failed to show an improvement in PFS and OS [31]. The addition of bevacizumab to a carboplatin/albumin-bound form of paclitaxel and trastuzumab regimen showed promising antitumor activity among patients with HER2-positive mBC [32]. Of nine evaluable patients, eight achieved a major clinical response. The clinical activity of bevacizumab alone or in combination is being examined in ovarian cancer, recurrent cervical cancer, hormone-refractory prostate cancer, refractory or relapsed AML and malignant melanoma, as well as head and neck cancer. Bevacizumab is also being evaluated as adjuvant treatment of CRC in combination with different regimens including FOLFOX4, FOLFOX6, or XELOX (capecitabine/oxaliplatin) in the National Surgical Adjuvant Breast and Bowel Project (NSABP) C08 trial, the AVANT (AVastin adjuvANT) trial, and the Intergroup Rectal Adjuvant trial [33].

39.2.2 HuMV833

HuMV833 is a humanized monoclonal antibody that recognizes VEGF121 and VEGF165 isoforms. Results from a phase I trial showed that HuMV833 was safe and well tolerated in patients with solid tumors [34]. Infusions were well tolerated without any grade 3 or 4 toxicities. The most common adverse events included fatigue/asthenia, nausea, vomiting, gastrointestinal symptoms, and rash. Recently, phase II/III clinical trials of HuMV833 have begun in Europe.

39.2.3 VEGF-Trap

VEGF-Trap is a novel, high-affinity molecule to VEGF molecule, generated as a fusion molecule of the VEGF receptor extracellular domain and the Fc portion of immunoglobulin G1. Data from phase I studies showed the clinical feasibility of these agents, which are currently being investigated in phase II/III trials [35, 36]. In particular, VEGF-Trap R1R2 is a derivative of the soluble form of VEGFR-1, which irreversibly binds to VEGF. This VEGF blocker is a chimeric fusion molecule composed of the second immunoglobulin domain of VEGFR-1 and combined with the third immunoglobulin domain of VEGFR-2 [37]. VEGF-Trap R1R2 was engineered to minimize interactions with the extracellular matrix, maintaining its potent affinity for VEGFR-2. Preclinical studies showed tumor growth inhibition in various models [38, 39]. In a phase I study, VEGF-Trap administered to patients with solid tumors did not induce antibody response with evidence of biological activity [40]. At present, a phase I clinical is investigating the side effects of VEGF-Trap R1R2 in patients with relapsed or refractory advanced solid tumors or non-Hodgkin's lymphoma.

39.3 Anti-VEGF Receptors Targeted Therapies

39.3.1 IMC-1C11

Among VEGF family receptor, the VEGFR 2/kinase-insert-domain-containing receptor (KDR) appear to be significantly upregulated during tumorigenesis. IMC-1C11, a chimeric

monoclonal anti-KDR antibody, blocks the binding of the ligand to the receptor and subsequently inhibits downstream events such as VEGFR and MAPK activation, inhibiting VEGFR-induced endothelial cell proliferation [41]. This agent is presently being examined in a phase I trial in patients with liver metastases from CRC carcinoma. IMC-1C11 appears to be safe and well tolerated, although 7 out of 14 patients experienced detectable levels of antibodies against IMC-1C11 [42]. A fully human anti-VEGFR-2 has been produced as a second-generation agent to be less immunogenic for chronic administration as monotherapy or in combination with chemotherapy or radiotherapy [43].

39.3.2 Sunitinib Malate

Sunitinib is an oral inhibitor of VEGFR-1, VEGFR-2, stem-cell factor receptor (c-KIT), PDGFRα, PDGFRβ, and fetal liver tyrosine kinase receptor 3 (FLT3) [44–46]. A phase I study investigating the biological activity of sunitinib in patients with AML showed that the phosphorylation of FLT-3 was inhibited in 50 % of patients with wild-type FLT-3 and in 100 % of patients with mutated FLT-3. The majority of gastrointestinal stromal tumors (GISTs) harbor mutations in the receptor tyrosine kinase KIT or PDGFR-A and are responsive to imatinib. Unfortunately, tumors develop resistance due to amino acid mutations in the kinase domain of the targeted receptor, thus preventing or weakening the interaction with the inhibitor. In vitro studies have demonstrated that sunitinib potently inhibited various imatinib-resistant KIT variants [47, 48]. In phase I studies sunitinib showed a manageable toxicity and the most common side effects reported were fatigue, hypertension, sore mouth, skin, gastrointestinal and hematological toxicity [49, 50]. Among side effects, an increased incidence of hypothyroidism was reported, related to a reduced drug-induced vascularity of thyroid gland [51]. Sunitinib demonstrated activity in patients affected by NSCLC, neuroendocrine and other tumor types, although most data are available for patients affected by GIST and RCC [52, 53].

The therapeutic advantages of sunitinib were examined as second-line therapy in patients with RCC. RCC is one of the most resistant tumor types, and, cytokines were the only moderately active agents. Two phase II clinical trials evaluated the activity of sunitinib (50 mg daily administered for 4 weeks followed by 2 weeks off) in patients with RCC after failure of previous immunotherapy [54, 55]. In the first study of Motzer et al. ($n=63$) 40 % of partial responses were reported and TTP was 8.7 months. In addition, 27 % of patients demonstrated stable disease lasting more than 3 months. The most common reported adverse event was fatigue (grade 3 in 11 % of patients). Therapeutic activity was confirmed by a further open-label study single-arm clinical trial as second-line treatment of mRCC that had progressed despite previous cytokine therapy. A partial response was observed in 36 of 106 patients (34 %) and the median progression-free survival was 8.3 months. Recently, Motzer et al. compared IFN-α to sunitinib as first line therapy in metastatic RCC in a phase III randomized clinical trial. Sutininib showed a statistically significant higher response rate (31 versus 6 %) and PFS (11 versus 5 months) as compared to IFN-α (HR 0.42; $p<0.001$) [9].

This agent was also investigated in a phase III trial in 312 patients affected by GISTs who progressive after imatinib therapy. The trial was unblinded because a planned interim analysis showed significant better outcome in the sunitinib arm [56]. Median time to tumor progression was significantly longer in the sunitinib group compared to placebo (27.3 versus 6.4 weeks, respectively; HR: 0.33; $p<0.0001$). The confirmed objective response rate was 7 % in the sunitinib group versus 0 % in the placebo group ($p=0.006$). The survival benefit could be underestimated as a result of the cross-over of patients receiving placebo.

Clinical activity of sunitinib is being investigated in NSCLC and preliminary data have shown that stable disease and partial response have been achieved [52]. Adverse events were generally mild-to-moderate and included fatigue, diarrhea and nausea. On the basis of these clinical results, in January 2006, sunitinib was approved by FDA for the treatment of advanced RCC refractory to cytokine therapy and for imatinib-resistant or imatinib-intolerant GIST.

39.3.3 Sorafenib

Sorafenib is an oral inhibitor targeting multiple kinases including VEGFR-2, VEGFR-3, PDGFR, FLT-3, c-KIT, c-Raf1, and B-Raf [57]. In a phase I study sorafenib showed activity in various tumor types and a manageable toxicity profile, both in monotherapy and in association with chemotherapy [58, 59]. Dose limiting toxicity was associated with diarrhea, fatigue, hypertension, skin rash, and hematological toxicity. The dose of 400 mg twice daily was recommended for phase II evaluation, showing interesting antitumoral activity in patients with various types of solid tumors, especially renal cell carcinoma (RCC) [60–63]. Data from a large phase II randomized discontinuation trial of sorafenib in patients with mRCC demonstrated significant disease-stabilizing activity in the arm receiving the active drug compared with placebo group. During the run-in period, 73 out of 202 patients had tumor shrinkage of >25 % and median overall PFS was 29 weeks for the overall population [64]. The most common drug-related adverse effects included rash, hand–foot syndrome, and fatigue. Grade 3/4 drug-related events occurred in 47 % of patients, with the most common being hypertension (24 %), hand–foot syndrome

(13 %), and fatigue (5 %) No patient died from sorafenib-induced toxicity. A subsequent phase III study including 769 patients with advanced or metastatic cytokine-refractory RCC, demonstrated antitumoral activity of sorafenib in this population and significant clinical benefit [65]. Median PFS was indeed 24 weeks in the sorafenib arm versus 12 weeks in the placebo group (HR = 0.44; $p < 0.00001$). Common side effects were diarrhea (33 %), rash (34 %), hand–foot skin reactions (27 %), fatigue (26 %), and hypertension (11 %). Updated results recently reported demonstrated a survival advantages as compared to placebo (median OS of 19.3 versus 15.9 months, respectively. HR = 0.77; $p = 0.015$) [66]. Based of these results sorafenib was approved by FDA for the treatment of advanced or metastatic RCC refractory to cytokine therapy. Feasibility of combination therapy with Interferon-α (IFNα) is under evaluation in phase II trials, as well as direct comparison between these two agents in patients with RCC [67–69]. Additional investigations are underway to better define role of sorafenib in combination with other targeted agents or cytokines in RCC and as adjuvant therapy following a nephrectomy. Ongoing phase III studies are evaluating the activity of sorafenib for treatment of HCC, melanoma, and NSCLC.

39.3.4 PTK-787/ZK-222584

Vatalanib (PTK787/ZK 222584) is a small molecule orally active TKI of all known VEGFRs, PDGFR tyrosine kinases and the c-kit protein tyrosine kinase. In phase I studies the most common side effects reported were fatigue, hypertension, nausea, vomiting, dizziness, and transaminases elevation [70–72]. Dose identified for further examination was 1250 mg daily. In phase II studies vatalanib showed clinical activity in several solid tumors [73–75]. Phase III trials did not show conclusive results in patients affected by mCRC. In fact CONFIRM-1 study, including 1168 patients with mCRC, demonstrated no beneficial effect of adding vatalanib (1250 mg once a day) to chemotherapy (FOLFOX-4) in first line treatment [76]. Adverse events attributable to PTK/ZK were generally reversible and similar to other antiangiogenic agents. In the phase III placebo-controlled CONFIRM-2 study, enrolling 855 patients with mCRC, adding valatinib to the same chemotherapy regimen (FOLFOX-4) in second line of treatment, demonstrated a longer PFS versus chemotherapy alone (5.5 months versus 4.1 months; HR: 0.83; $p = 0.026$). Response rate was similar in the two arms (18.5 % in the PTK/ZK arm versus 17.5 % in the placebo arm) and survival advantage was not reported (OS was 12.1 months in the PTK/ZK arm and 11.8 months in the placebo arm. HR: 0.94; $p = 0.511$) [77]. Recently, a meta-analysis of these two studies was performed, based on the preliminary results from CONFIRM trials that showed a greater clinical benefit and improvement of PFS in patients with high LDH levels. This analysis confirm that the effect of vatalanib in improvement of PFS is strong in high LDH population (HR 0.65, $p < 0.001$), as compared to overall population (HR 0.85, $p = 0.005$) [78].

A report of preliminary results from a phase II clinical trial investigating the therapeutic benefits of PTK-787/ZK-222584 as a single second-line agent for NSCLC showed that disease control was achieved in 58 % of patients [75]. Other clinical trials investigating PTK-787/ZK-222584 in metastatic neuroendocrine tumors and imatinib mesylate-resistant metastatic GIST are currently ongoing [74, 79].

Our group designed a dose finding phase I study with PTK787/ZK in combination with chemotherapy and trastuzumab, involving patients affected by HER-2/*neu* positive mBC who progressed after trastuzumab and anthracycline-based and/or taxane-based chemotherapy for metastatic disease. The schedule of study treatment includes vinorelbine (day 1-8-15, q 28 days, dose ranging from 25 to 30 mg/mq), weekly trastuzumab and daily PTK787/ZK (dose ranging from 500 to 1250 mg). Four dose levels are planned: the primary objective of the present study is to determine the maximum tolerated dose (MTD) and the pharmacokinetics interactions of PTK787/ZK with the cytotoxic agents. The secondary objective of the study is to evaluate the tolerability and the optimal schedule of the schedule.

39.3.5 AMG-706

AMG 706 is an oral small molecule multi-kinase inhibitor with both antiangiogenic and direct antitumor activity that selectively targets VEGF, PDGF, and Kit receptors [80]. It has demonstrated antiangiogenic and antitumor activity. Data from a phase I study presented at the 2005 American Society of Clinical Oncology (ASCO) meeting showed that AMG-706 in patients with advanced solid tumors was safe at doses up to 125 mg/day [81, 82]. Preliminary data were promising, as they indicated vascular changes and stable disease in the majority of patients. Most frequently reported adverse events were hypertension, fatigue, diarrhea, headache, and nausea. AMG-706 is currently undergoing several phase I/II clinical trials alone or in combination with chemotherapy in CRC, GIST, NSCLC and thyroid cancer. Among these studies, a phase I clinical trial evaluating the safety and clinical activity of AMG-706 in thyroid cancer showed that this agent was well tolerated and objective response was achieved in 43 % of patients [83]. AMG-706 is also being investigated in combination with panitumumab, an anti-epidermal growth factor receptor (EGFR) monoclonal antibody, in NSCLC, and preliminary data revealed that this regimen was safe and exhibited clinical activity [84].

39.3.6 ZD-6474 (Zactima, Vandetanib)

ZD6474 is a dual-kinase inhibitor that inhibits VEGFR-2, but also has moderate anti-EGFR activity and RET receptor. In phase I evaluation, ZD6474 was well tolerated at daily dose of 100–300 mg/day and adverse events commonly reported were diarrhea, rash, fatigue and asymptomatic QT-prolongation [85]. Preclinical studies have yielded data consistent with a potent inhibition of the VEGF signaling pathway, suggesting potential use in a broad range of tumors including colon, lung, prostate, breast, and ovarian cancers [86]. However, most clinical data in phase II studies are available in NSCLC. Adding of ZD6474 to carboplatin/paclitaxel in first line chemotherapy is safe and did not significantly increase treatment toxicity. In a phase II randomized study performed by Heymach et al., patients with locally advanced or metastatic NSCLC after failure of first line platinum-based chemotherapy were randomized to receive docetaxel plus ZD6474, either at dose of 100 mg 300 mg, or docetaxel alone. Median PFS was higher in patients receiving the combined therapy (19 versus 17 versus 12 weeks, respectively) [87]. Recently, a double-blind phase II randomized trial compared ZD6474 300 mg with gefitinib 250 mg in 168 advanced previously treated NSCLC patients, with the option of crossover at the time of progression. Preliminary data showed a response rate of 8 % in the ZD6474 arm and 1 % in the gefitinib arm and a statistically significant longer PFS with ZD6474 was reported (11.9 versus 8.1 weeks, respectively; $p=0.011$) [88]. These positive results were not confirmed in BC: in 44 patients refractory to anthracycline/taxane, ZD6474 either at dose of 100 mg or 300 mg did not show clinical activity [89]. ZD6474 displayed also promising evidence of activity in patients with hereditary medullary thyroid carcinoma; in a phase II trial in 15 evaluable patients, three had partial response and ten stable disease [90].

39.3.7 Axitinib (AG-013736)

Axitinib is an oral multitargeted TKI with inhibitory effects against VEGFR-2, VEGFR-3, and PDGFR-β, evaluated in a phase I clinical study among patients with advanced solid tumors, AG-013736 demonstrated clinical activity in RCC, adenoid cystic cancer and NSCLC [91]. Dose-limiting toxicities included hypertension, hemoptysis, and stomatitis [92], manageable with appropriate medication or dose reduction. A phase I study identified maximum tolerated dose of 5 mg twice daily. Rini and colleagues evaluated axitinib in a phase II trial in 52 patients with advanced RCC refractory to one prior cytokine-based therapy. Partial response was reported in 24 patients (46 %) and stable disease in a further 40 %. Median TTP has not been reached after 12- to 18-month follow-up in all patients. Most common serious adverse events (grade 3–4) were diarrhea (8 %), hypertension (15 %), and fatigue (8 %) [93]. Axitinib has been also evaluated in 32 patients with advanced thyroid cancer, refractory to or not suitable candidates for Iodine therapy. Although response assessments are still ongoing, partial response was achieved in three patients [94].

39.3.8 Other Multitargeted TKIs in Clinical Development

GW-786034 is another oral TKI with activity against VEGFR that has demonstrated antitumor and antiangiogenic activity in vitro and in vivo [95]. It is currently undergoing phase II clinical trials on refractory multiple myeloma, soft tissue sarcoma, ovarian cancer, and RCC.

CP-547,632 has shown antitumor and antiangiogenic activity against VEGFR-2 in several preclinical models [96]. This agent is currently in a phase II clinical trial for recurrent or persistent small-volume ovarian cancer.

The agent AEE-788 is a dual family EGFR/ErbB2 and VEGFR TKI [97], showing interesting antitumor activity in various models [98, 99]. In phase I studies AEE788 was generally well tolerated and most of the adverse events were generally mild or moderate diarrhea, asthenia, anorexia, rash, nausea, and vomiting [100–102]. Dose-limiting toxicities were observed at the 500–550 mg dose levels. Enrollment in clinical trials is ongoing.

XL647 is an orally bioavailable small molecule with inhibitory effect on EGFR, HER-2, VEGFR2/KDR and EphB4. In a phase I study XL647 showed a satisfactory toxicity profile. The maximum tolerated dose was established in 4.68 mg/kg orally administered for 5 consecutive days every 2 weeks [103].

CEP-7055 is a novel orally active inhibitor of all three VEGFR kinases with broad preclinical antitumor and antiangiogenic activity [104], now entering a phase I clinical study.

39.3.9 RPI-4610

RPI-4610 belongs to a new class of drug termed chemically stabilized ribozymes, synthesized to target and cleave a specific mRNA sequence. RPI-4610, administered intravenously, targets the VEGFR-1 mRNA [105]. A phase I clinical study in patients with refractory solid tumors showed grade 1/2 infusion reactions as the most common toxicities [106]. A phase II clinical trial is currently investigating the effectiveness of RPI-4610 in patients with metastatic RCC.

39.4 Targeted Therapies Interfering with Metalloproteinases

The involvement of proteolytic activity of metalloproteinases (MMPs) in angiogenesis is well established. The extracellular matrix (ECM) surrounding endothelial cells must be broken down to allow cell migration and proliferation. MMPs belong to a family of proteolytic enzymes that degrade ECM components and contribute through this mechanism to the angiogenic process. Aberrant MMP expression contributes to the invasive growth and spread of a variety of solid malignancies [11, 107, 108]. It has been suggested that the control of MMPs activity through inhibitors has been considered a potential target for anticancer therapy, but clinical trials yielded disappointing results. Incyclinide, an oral MMP inhibitor (MMPI), showed biological activity in AIDS-related Kaposi's sarcoma [109]. The most common adverse events were photosensitivity and rash. BMS-275291, a broad spectrum MMPI, failed to achieve partial or complete tumor responses in patients with advanced or metastatic cancer [110]. The most frequently reported adverse events were joint toxicity, rash, fatigue, headache and nausea. In a recent study evaluating two different doses, this agent demonstrated limited clinical activity in hormone-refractory prostate cancer with bone metastases [111]. Other MMPIs, such as BAY-129566 or BB-2516, have failed to show therapeutic activity in human malignancies despite preclinical antimetastatic and antiangiogenic activity. The reasons for the disappointing results observed with MMPIs in cancer therapy remain unclear. The negative results of MMPIs reported in several studies have definitely raised serious concerns about the opportunity to continue the evaluation of MMPIs as a therapeutic anticancer strategy.

39.5 Targeted Therapies Directly Interfering with Endothelial Cells

Thrombospondin-1 (TSP-1) is a naturally occurring inhibitor of angiogenesis that limits vessel density in normal tissues and curtails tumor growth. ABT-510, a promising new agent, is a TSP-1 analogue. A phase I study in patients with advanced solid malignancies showed a favorable toxicity profile, with the most common toxicities observed being injection-site reactions and fatigue. Stability of disease was observed in a significant number of patients, warranting further clinical trials [112]. A phase II trial testing the clinical benefits of ABT-510 in head and neck cancer is currently underway. An additional avenue being explored is integrin protein, which plays an essential role in cell–cell and cell–matrix adhesion. Integrins are cell surface adhesion molecules coupling the extracellular environment to the cytoskeleton, as well as receptors for transmitting signals important for cell migration, invasion, proliferation and survival. Deregulation of adhesion can lead to pathological processes, including tumor metastasis, either by disrupting the normal anchorage, thereby altering cell movement and regulatory signaling, or by promoting inappropriate adhesion. One member of the integrin family, αvβ3-integrin is overexpressed in tumor cells Supporting evidence for the integrin involvement in tumor angiogenesis was recently reported by Nikolopoulos and colleagues who demonstrated that the subunit of integrin promotes endothelial migration and invasion [113]. Therefore, agents targeting integrins are currently being evaluated as potential therapeutic options to treat tumors. Such agents include abegrin, a monoclonal antibody, and cilengitide (EMD-121974), a cyclic peptide. Both interfere with the αvβ3-integrin and are under investigation in phase I/II studies.

Another antiangiogenic drug tested in human malignancies is thalidomide, although its exact mechanism of action is still unclear. Thalidomide was once notorious for producing severe deformities in the arms and legs of newborn babies whose mothers were given the drug during pregnancy. Off the market for decades, it has recently emerged as a somewhat effective treatment for several cancers. Thalidomide and its immunomodulatory analogues are being investigated in several phase II/III trials for treating various tumors including multiple myeloma, RCC, prostate cancer, and hepatocellular cancer [114]. Finally, endostatin, an endogenous angiogenesis inhibitor, represents an additional target for cancer therapy [115]. Clinical trials evaluating the safety of r-hu endostatin in patients with advanced solid tumors showed that this agent has a good safety profile, but modest antitumor activity [116], with positive results only in NSCLC.

39.6 New Strategies for Antiangiogenic Therapy Combinations

39.6.1 Broad-Spectrum Targeting

Recently Folkman proposed the paradigm to block tumor growth indefinitely by the use of a broad-spectrum single multitarget agent capable simultaneously to interfere with several angiogenesis pathways or, alternatively, by combinations of different highly selective drugs. Two examples of the first strategy are the multitarget tyrosine kinase inhibitors sunitinib and sorafenib.

Examples of the second strategy include the combinations of bevacizumab and anti-EGFR agents taking into account that angiogenesis is linked to other tumor molecular pathways. Preclinical studies demonstrated that interactions between EGFR and VEGF signaling pathways sustained tumor growth and progression. They exert effects

both directly and indirectly on tumor cells, and combining drugs targeting both the targets confer additional clinical benefit. EGFR has been detected in the endothelial cells of tumor vasculature preclinically [117]. Co-expression of EGFR and TGFα has been correlated with increased vascularity in invasive BC [118]. VEGF is also downregulated by EGFR inhibition [119, 120] and a recent study suggests that blockade of VEGF may also inhibit the EGFR autocrine signaling [121].

A number of preclinical studies investigating the antitumor activity of combined anti-EGFR and anti-VEGF agents suggest promising activity [122–126].

Furthermore, VEGF blockade is critical in preventing resistance to EGFR inhibition. The use of agents such as erlotinib and bevacizumab targeting different signaling pathways and affecting different cell types (tumor cell and endothelial cell) may abide by different rules than standard cytotoxic chemotherapy because of the significant cross talk between the pathways in numerous cell types. These encouraging data have led to the initiation of a number of clinical studies evaluating the combination of erlotinib with bevacizumab in a number of tumor types, including phase II trials in RCC [127] and mBC [128] and a phase I study in patients with HNSCC [129].

Herbst et al. [130] evaluated the combination of bevacizumab and erlotinib in 40 patients (34 patients at phase II dose) with previously treated NSCLC. Eight patients (20.0 %) had partial response and 26 (65.0 %) had stable disease as their best response. The median OS for the 34 patients treated at phase II dose was 12.6 months, with a PFS of 6.2 months. The most common adverse events were mild to moderate rash, diarrhea and proteinuria.

In another multicenter phase II trial, 63 patients with metastatic RCC were treated with bevacizumab 10 mg/kg intravenously every 2 weeks and erlotinib 150 mg orally daily. Fifteen (25 %) of 59 assessable patients had objective response and an additional 36 patients (61 %) had stable disease after 8 weeks of treatment. The median and 1-year PFS was 11 months and 43 % respectively. After a median follow-up of 15 months, median OS has not been reached: survival at 18 months was 60 %. Treatment was generally well-tolerated: only two patients discontinued treatment because of toxicity (skin rash); grade 1/2 skin rash and diarrhea were the most frequent treatment-related toxicities [131].

Encouraging results have been reported with the combination of two different monoclonal antibodies with or without chemotherapy. The BOND II Trial evaluated the combination of cetuximab/bevacizumab +/– irinotecan in mCRC patients after irinotecan failure. Preliminary data showed a better efficacy in the experimental arm in terms of response rate (37 % versus 20 %) and median TTP (7.9 months versus 5.6 months) [132].

Based on preclinical results showing co-upregulation of VEGF and HER-2/neu in breast cancer, a recent phase I/II trial explored the combination of trastuzumab and bevacizumab in relapsed mBC HER-2 positive [133].

39.6.2 Metronomic Chemotherapy

Another therapeutic strategy is based on the use of antiangiogenic schedules of cytotoxic agents (i.e., metronomic chemotherapy) aimed to block endothelial cells proliferation. The most studied cytotoxic agent is oral cyclophosphamide, which is more active combined with selective antiangiogenic agents such as the TSP-1 peptide ABT-510, thalidomide and celecoxib, or anti-VEGF agents [134].

A recent phase II study in pediatric cancer patients with recurrent or progressive poor prognosis tumors for which no curative therapy remained, Kieran et al. [135] tested the continuous administration of oral thalidomide, celecoxib with alternating etoposide and cyclophosphamide. Such a therapy was well-tolerated and 40 % of patients had clinical benefit with 25 % of all cases continuing to be progression-free for more than 123 weeks. Elevated circulatory levels of TSP-1 correlated with prolonged response.

39.6.3 Bidirectional Block of Angiogenic Balance

At a certain time the angiogenic activity of a tumor is the result of the net balance between pro- and contra-angiogenic molecules. It is reasonable to hypothesize that the concurrent use of an anti-pro-angiogenic molecule (i.e., anti-VEGF) and a naturally occurring angiogenesis inhibitor (i.e., endostatin) should be more effective than the use of each single class of agents alone. Such a strategy is now feasible in the clinic due to the approval in China of therapy for NSCLC with rh-endostatin, a tumor type for which also bevacizumab is active and approved [3].

39.6.4 Nanobodies

A new attractive category of targeted agents is that of nanobodies, consisting of the smallest functional fragment of a single-chain antibody, with interesting pharmacodynamic and pharmacokinetic characteristics such as: high stability, alternative routes of administration (intranasal, pulmonary, oral, transdermal, etc.), high tissue penetration (properties more similar to small molecules) and high affinity/potency, to the target, and low inherent toxicity. In addition, nanobodies are easier to of manufacture in bacteria and yeasts, have low

immunogenicity and a wider range of epitopes including cavities. The BIV nanobody (Ablynx) has shown a high affinity against ~100 targets, one of them being VEGF-A; it has been demonstrated his activity as a potent antagonist in vitro against ~20 targets. In vivo his efficacy has been shown in over ten animal models [136]. Preclinical development is ongoing at the moment and phase I/II clinical trials are planned to be started in 2007.

39.7 Conclusions

From 2004 antiangiogenic agents improved the antitumor therapeutic armamentarium. Up to now four inhibitors of angiogenesis has been approved by FDA. Bevacizumab significantly improved clinical outcome of patients with advanced CRC, NSCLC, and BC as compared with standard chemotherapy. Two multitargeted TKI compounds, namely sunitinib and sorafenib, changed the natural history of advanced RCC, a tumor type characterized by its poor responsiveness to cytotoxic and immunomodulatory agents.

Taking into account that tumor angiogenesis is a multistep process very complex, novel strategies based on the use of broad-spectrum antiangiogenic compounds, schedules of multitargeted agents (i.e., anti-VEGF + anti-EGFR or antimTOR) as well as the development of new highly active and selective drugs (i.e., nanobodies) are expected to further ameliorate the efficacy of antiangiogenic therapy in future years.

With the promising results obtained in patients with advanced, metastatic disease it is likely to be reproduced and enhanced in the adjuvant setting. Because the angiogenic switch is an early event in tumor growth, the capability to develop orally active and safe angiogenic inhibitors may open the opportunity to develop novel strategies of chemoprevention for high-risk subjects.

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Small Molecule Inhibitors

40

Xin Chen and Jian Jin

40.1 Introduction

Before the biomedical community had had any understanding of the molecular mechanisms that drive tumorigenesis, the discovery of cancer chemotherapy exclusively focused on the development of novel cytotoxic compounds targeting DNA processing and cell division including DNA alkylating and cross-linking agents, antimetabolites, topoisomerase inhibitors, and anti-tubulin agents [1]. Although these drugs can be very efficacious in killing tumor cells, serious side effects accompanied due to the lack of selectivity for tumor cells versus normal cells. The side effects, such as bone marrow suppression and gastrointestinal, cardiac, hepatic, and renal toxicities, significantly limit their use. In addition, drug resistance was frequently observed after initial stabilization or regression of the disease [2].

Starting from 1980s, the biomedical research community has begun to identify key molecular changes responsible for malignant transformation and understand the etiology of cancer at the molecular level [3]. For example, numerous cancer-causing oncogenes and tumor-suppressor genes that normally hold cancer in check have been discovered. Oncogenes are frequently activated by inherited or spontaneous gain-of-function mutations or fusions with other genes. They can be aberrantly expressed due to amplification, increased promoter activity or protein stabilization, thus have integral roles in the genesis of human tumors. The inherited or spontaneous loss of function of tumor-suppressor genes resulting from inactivating mutations or epigenetic events (for example, promoter hypermethylations) has also been implicated in tumorigenesis. As a result, the focus of anticancer drug discovery and development shifted from nonspecific chemotherapeutics to rationally designed drugs that target cancer-specific pathways or proteins that are unique to or upregulated in cancer cells. Such agents are typically less toxic than conventional cytotoxic anticancer drugs because they would spare normal cells and thereby offer improved safety benefits over standard chemotherapeutics and provide a higher therapeutic index.

Much of the pioneering work in the targeted anticancer drug discovery field has been carried out via targeting signaling pathways. Cells use a wide variety of intracellular and intercellular mechanisms to signal for processes, including growth, apoptosis, and intracellular protein degradation. Molecular and genetic approaches have uncovered the entirely new signaling networks that regulate cellular activities such as proliferation and survival. Many of these networks are found to be radically altered in cancer cells. Therefore, inhibition of these altered pathways via signal transduction inhibitors or secondary messenger inhibitors can lead to anticancer effect.

Research in the protein kinase area has led to 33 FDA-approved kinase inhibitor drugs for the treatment of various cancers to date [4]. Additionally, more than 100 kinase inhibitors are presently in various phases of clinical development for treating cancers [5]. In this chapter, rather than covering every FDA-approved kinase drug and every drug candidate in clinical development, we describe discovery stories of a number of representative kinase inhibitor drugs as examples to illustrate the tremendous success in this area. In addition to these kinase drug discovery stories, we describe the discovery of inhibitors of other proteins or protein families as targeted cancer therapeutics including inhibitors of poly(ADP-Ribose) polymerases (PARPs), DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and heat shock protein 90 (HSP90).

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_40

40.2 Small Molecule Inhibitors as Targeted Cancer Therapeutics

40.2.1 Kinase Inhibitors

Kinases, also known as phosphotransferases, are a class of enzymes that transfer a phosphate group from high energy donor molecules, such as adenosine triphosphate (ATP), to specific substrates. The largest group of kinases is protein kinases, which constitute one of the largest protein families in the human genome, accounting for about 1.7 % of the human genome-encoded proteins [6]. Protein kinases modulate the signaling systems necessary for cell division, growth, survival, and migration. As many as 120 kinases, out of more than 500 kinases encoded in human genome, have been found to be present in various cancers [7]. Small molecule inhibitors that block the actions of protein kinases and the associated signaling pathways can thus have significant impact on the proliferation, differentiation, apoptosis, and migration of cancer cells.

40.2.2 Classification of Protein Kinases

By attaching a phosphate group to a serine, threonine, or tyrosine residue of various proteins, the process called phosphorylation, protein kinases play a primary role in the complex signaling systems that transfer information between and within cells via switching on or off a cascade of cellular events. There are two main ways to categorize protein kinases.

(a) Based on their specificity for amino acid residues:
- Serine or threonine-specific kinases: catalyze the phosphorylation of serine or threonine residues.
- Tyrosine-specific kinases: catalyze the phosphorylation of tyrosine residue.
- Mixed function kinases: catalyze serine, threonine, or tyrosine phosphorylation.

(b) Based on their structure and cellular localization:
- Receptor kinases: contain an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and a cytoplasmically located kinase domain. The extracellular ligand-binding domain is typically glycosylated and conveys ligand specificity. Epidermal growth factor receptors (EGFRs), platelet-derived growth factor receptors (PDGFRs), and vascular endothelial growth factor receptors (VEGFRs) are the three major families. Other well-known receptors include insulin-like growth factor 1 receptors (IGF-1Rs), Mast/stem cell growth factor receptor (SCFR, also known as proto-oncogene c-Kit or tyrosine-protein kinase KIT) and mutant FMS-like tyrosine kinase 3 (FLT-3) receptors.
- Non-receptor kinases: have no transmembrane or extracellular domains and may be associated with the cytoplasmic surfaces by membrane localization via a lipid modification that anchors them to the phospholipid bilayer or by non-covalent binding to a membrane receptor. Abelson tyrosine kinase (ABL), Janus kinase (JAK), focal adhesion kinase (FAK), and sarcoma (Src) kinases belong to this category.

40.2.3 General Mechanisms of Protein Kinase and Kinase Inhibitors

Protein kinases help transmitting information from a factor outside a cell into the cell plasma and nucleus while the initiating factor does not have to across the cell membrane. This mechanism allows the cellular function such as proliferation, cell–matrix adhesion, cell–cell adhesion, movement, apoptosis control, transcription, and membrane transport to be regulated by extracellular stimuli. There are two ways through which the signals from an extracellular factor can be transferred into the interior of the cell with the aid of kinases. First, ligand binding to receptor tyrosine kinases (RTKs) induces receptor dimerization or oligomerization, leading to the activation of kinases [8]. Secondly, non-receptor kinases are similarly activated in response to appropriate extracellular stimuli via interacting with other membrane-bound receptors such as G-protein coupled receptors; however, dimerization may or may not be necessary for activation [9]. In either case, the key step in transferring the signal across the membrane is the activation of the cytoplasmic kinase domain. The activated kinases then initiate a cascade of phosphorylation reactions resulting in the activation of other proteins, as well as the production of secondary messenger molecules that transmit the signal initiated by the extracellular factor to the nucleus [10].

Protein kinases play major etiologic roles in the initiation of malignancy and may contribute to the uncontrolled proliferation of cancer cells, tumor progression, and development of metastatic disease [11]. It is thought that cancer cells depend almost entirely on signaling by protein kinases for their continued proliferation [6], whereas normal cells rarely invoke these pathways [12]. Many protein kinases are mutated [13] or overexpressed [9] in tumor cells. Recent sequencing efforts in a wide variety of tumors have identified mutations in numerous RTKs, which are now collected in the COSMIC (Catalogue of Somatic Mutations in Cancer) database [14].

There are four main types of kinase inhibitors [15]. Type 1 inhibitors constitute the majority of ATP-competitive

inhibitors and recognize the active conformation of the kinases. Type 2 kinase inhibitors recognize the inactive conformation of the kinases and are still ATP-competitive. Type 3 kinase inhibitors bind outside the ATP-binding site, at an allosteric site, and modulate kinase activity in an allosteric manner. Therefore, this class of inhibitors is not competitive with ATP. Type 4 inhibitors are capable of forming an irreversible, covalent bond to the kinase active site, most frequently by reacting with a nucleophilic cysteine residue. A detailed description of these four types of kinase inhibitors was summarized in this excellent review article [15]. A very significant amount of drug research and development activities has been devoted to the discovery of small molecule inhibitors of the protein kinases thought to be important in the initiation and growth of tumors. These efforts have led to the approval of 33 kinase inhibitor drugs by the FDA. Most of these FDA-approved drugs and the drug candidates that are currently in clinical development are either type 1 or type 2 kinase inhibitors. We use the following examples to illustrate how small molecule kinase inhibitors as targeted cancer therapeutics were discovered.

40.2.4 BCR-ABL Inhibition: The Discovery of Imatinib (Gleevec™)

Being the first small molecule kinase inhibitor approved by FDA for treating cancers (approved in 2001 for chronic myeloid leukemia (CML) and in 2002 for gastrointestinal stromal tumors (GISTs)), imatinib (Gleevec™, Novartis) which is also known as STI-571, is widely acknowledged to be the classic example of kinase targeted drug discovery and the drug that validated the strategy of signal transduction inhibition. The molecular target of imatinib is BCR-ABL (breakpoint cluster region—Abelson), a result of genetic abnormality known for a long time as chromosomal translocation that creates an abnormal fusion protein (i.e., the BCR-ABL kinase) that signals aberrantly and leads to uncontrolled proliferation of the leukemia cells in CML [16].

CML is a malignant myeloproliferative disorder of hematopoietic stem cells [17]. It is characterized by a chromosomal abnormality, known as the Philadelphia chromosome, discovered by Nowell and Hungerford and named after the location of discovery [18], which results from a reciprocal translocation involving the Abelson (ABL) oncogene from chromosome 9 being transferred to a region on chromosome 22 termed the breakpoint cluster region (BCR) [19]. The resulting fused BCR-ABL gene produces a constitutively active tyrosine kinase that initiates multiple signaling pathways and causes perturbed interaction with extracellular matrix and bone marrow stroma, enhanced cell proliferation and reduced growth-factor dependence and apoptosis [16]. This leads to an increase in the number of granular leukocytes in the peripheral blood and bone marrow [20].

It has been well established that BCR-ABL is the causal to the pathogenesis of CML, and that constitutive tyrosine kinase activity is central to the capacity of BCR-ABL to transform hematopoietic cells in vitro and in vivo [21, 22]. The fact that it proved difficult to identify essential components downstream of BCR-ABL indicates that BCR-ABL is an attractive drug target from the therapeutic standpoint. The clear difference in the function of BCR-ABL between normal and leukemic cells further supports this view.

The discovery of imatinib represents an excellent example of rational drug design in cancer drug discovery [23, 24]. Starting in the late 1980s, scientists at Ciba Geigy (now Novartis), under the direction of N. Lydon and A. Matter, initiated projects on the identification of compounds with inhibitory activity against protein kinases. From a screen for inhibitors of protein kinase C (PKC), a phenylaminopyrimidine was identified as a hit (Fig. 40.1) [25, 26]. Although this compound had low potency and poor kinase selectivity, it had promising "lead-like" properties [27] and a high potential for structural modifications, allowing rapid generation of more potent and selective inhibitors. Adding a 3′-pyridyl group to the parent compound resulted in compound **1A** with significantly improved cellular activity. The introduction of a benzamide group on the phenyl ring (compound **1B**) provided inhibitory activity against tyrosine kinases such as BCR–ABL in addition to PKC. Further structure–activity relationships (SAR) exploration revealed that a substitution at the position 6 of the diaminophenyl ring abolished PKC inhibitory activity completely and the introduction of a simple flag-methyl at this position (compound **1C**) retained or even enhanced the activity against BCR–ABL. However, compound **1C** had poor oral bioavailability and low aqueous solubility. Further exploration of this series revealed that the attachment of a highly polar side chain such as *N*-methylpiperazine could significantly improve both solubility and oral bioavailability. In addition, the introduction of the CH_2 spacer between the phenyl ring and the nitrogen atom avoided the mutagenic potential of the aniline moiety. Compound **1D** (originally named as STI571, now known as imatinib), the most promising compound in this series, was selected for clinical development.

Binding of imatinib occurs at the ATP-binding site as indicated by docking studies [28] and X-ray crystallography [29]. It has also been shown from the crystal structure that imatinib inhibits the kinase by binding with high specificity to an inactive form of the kinase. Thus, imatinib is a type 2 kinase inhibitor. Binding to this unusual kinase conformation might contribute to the high selectivity of this compound. Surprisingly, the *N*-methylpiperazine group (added to increase aqueous solubility and oral bioavailability) was

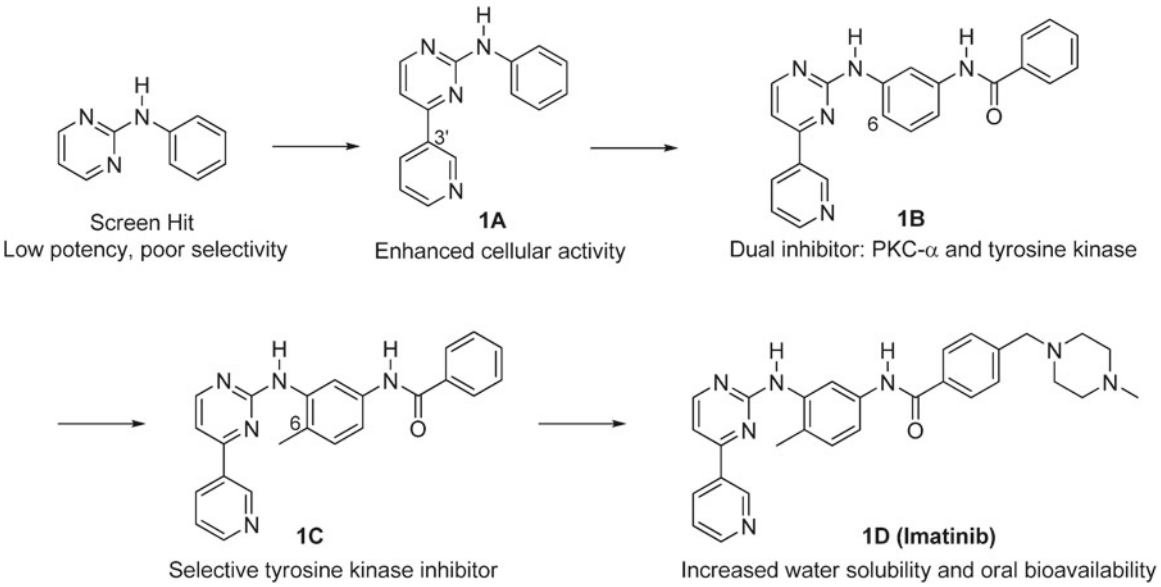

Fig. 40.1 Discovery of imatinib.

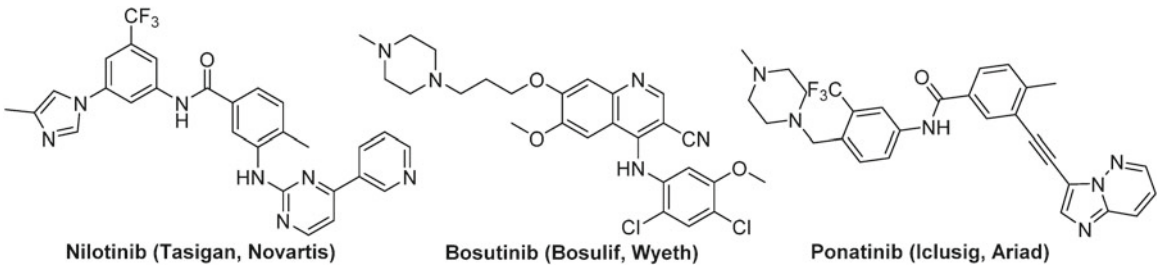

Fig. 40.2 FDA-approved BCR-ABL inhibitors.

found to have strong interaction with the protein via hydrogen bonds to the backbone carbonyl groups.

In addition to being orally bioavailable, thus more convenient for patients, imatinib lacks some of more serious side effects commonly observed with cytotoxic agents [30]. Imatinib has now been approved in numerous countries for treating the chronic phase of Philadelphia chromosome-positive CML. Through its use in clinic, it was discovered that, in addition to inhibiting BCR-ABL, imatinib effectively inhibited ABL, PDGFR, and SCFR (c-Kit) [31]. In particular, the activity against c-Kit manifested as remarkable clinical activity in c-Kit-positive unresectable or metastatic malignant GISTs, for which it is now also licensed.

In patients with newly diagnosed CML in the chronic phase, daily oral administration of imatinib has demonstrated impressive event-free survival (81%), freedom from progression (93%), and overall survival (86%) rates [32]. However, many patients eventually develop intrinsic or acquired resistance to this first-line therapy [33]. To overcome resistance in CML, a number of second generation inhibitors have been developed [34]. These include imatinib-like inhibitors nilotinib (Tasigna™, Novatis, Approved 2007) (Fig. 40.2) [35], dual SRC/ABL inhibitors dasatinib (Spreycel™, Bristol-Myers Squib, Approved 2006) [36], and bosutinib (Bosulif™, Wyeth, Approved 2012) [37], which have been approved for the treatment of adults in all phases of CML with resistance or intolerance to imatinib. Unfortunately, there are still subsets of mutants remain resistant even with these second-generation compounds, particularly a common BCR-ABL^{T315I} mutant at the gatekeeper position which represents ~15–20% of all clinically observed mutants [37–40]. To address this unmet need, several programs targeting development of potent BCR-ABL^{T315I} inhibitors have been established and the progress has been summarized in a number of reviews [41–44]. Ponatinib (AP24534, Iclusig™, Ariad) was developed as a "pan-BCR-ABL" inhibitor which is potently active against BCR-ABL^{T315I} and all other tested BCR-ABL variants and suppresses emergence of resistant mutations in a cell-based screen [45]. In addition, ponatinib was also found to be a multitargeted inhibitor with activity against FLT3, FGFR, VEGFR, c-Kit, PDGFR, Eph, Src family kinases, RET, and Tie2. Ponatinib was approved by FDA for the treatment of T315I positive CML and Philadelphia chromosome positive

ALL as well as CML or Philadelphia positive ALL that are resistant or intolerant to prior tyrosine kinase inhibitor therapy in December 2012 through FDA Accelated Approval program. Serious adverse reactions have been reported with ponatinib, with vascular occlusion, heart failure and hepatotoxicity prompting the US FDA to issue boxed warnings. Therefore, clinicians need to consider whether the potential benefits of therapy will outweigh the risks before starting treatment.

40.2.5 EGFR Inhibition

Because of their fundamental role in regulating key cellular functions including cell proliferation, differentiation, metastasis and survival, epidermal growth factor receptor (EGFR) signaling pathways have been a main focus of research for novel targeted anticancer agents [46]. EGFR is a member of the EGFR subfamily known as human epidermal growth factor receptor (HER) family, which contains 4 closely related receptors that include itself (as known as ErbB-1), HER2/neu (as known as ErbB-2), HER3 (as known as ErbB-3) and HER4 (as known as ErbB-4). EGFR is a 170-kDa glycoprotein that is expressed in most human tissues and is highly expressed in many human solid tumors [47].

EGFR is a receptor tyrosine kinase, which has an extracellular domain, a transmembrane domain and an intracellular tyrosine kinase domain. The activation of the EGFR pathway is initiated when an appropriate ligand (e.g., EGF, transforming growth factor-α or TGF-α, heparin-binding EGF, amphiregulin, betacellulin, epiregulin, or neuregulin G2b) [48] binds to the extracellular domain of the inactive single unit of the receptor. This causes receptor dimerization with another identical EGFR receptor to form a homodimer or with a nonidentical receptor from the same EGFR subfamily (e.g., HER2/neu) to give a heterodimer. This dimerization process activates the tyrosine kinase enzyme located in the intracellular domain, and that leads to the transphosphorylation of both intracellular domains. This, in turn, initiates a cascade of phosphorylation events that eventually results in a signal arriving at the nucleus [46]. The magnitude of EGFR signaling is influenced by several cellular mechanisms including receptor mutations, heterodimerization with other members of the HER family, increased expression of autocrine ligands, and alterations in molecules that control receptor signaling output [49].

The work from several research groups has provided strong rationales for EGFR as an anticancer target. EGF was first discovered by Stanley Cohen in the early 1960s [50]. As one of the first growth factors isolated, its discovery opened up a research field that has been crucial to the development of modern anticancer and other medical treatments. A decade later, human EGF4 was isolated [51]. It took another 5 years to demonstrate the link between EGFR and the malignant transformation of cells [52]. An autocrine mechanism was found to be involved in that transformation, involving autostimulation of EGFR in cancer cells by ligands such as EGF and TGF-α, which are produced by the cancer cells themselves. The ligand binding to EGFR activates a cyclic-AMP-independent phosphorylation system through an inherent tyrosine kinase located within the receptor [53]. This up-regulated EGFR-mediated signaling can help move cells into a continuous and uncontrolled stated of division, thus leading to greater numbers of malignant cells and an increase in tumor size. Additional compelling evidence for the role of EGFR in cancer pathogenesis came from reports that many human solid tumors, such as head and neck, lung, and colorectal tumors, express high levels of EGFR, which frequently correlates with poor prognosis [54], in particular, with an increased risk of metastasis and decreased survival. The high expression levels of EGFR can also protect cancer cells from hormone therapies, cytotoxic agents, and radiotherapy [55], thus reducing the effectiveness of these treatments. Furthermore, many tumors that express EGFR also produce one or more EGFR ligands, which further supports the hypothesis that autocrine growth-stimulatory mechanisms are involved in EGFR-mediated tumorigenesis [56]. In addition, it has been shown that EGFR signaling not only increases cell proliferation, but also regulates a range of processes that are essential for tumor progression, including cell motility, cell adhesion, tumor invasion, cell survival, and angiogenesis [57].

40.2.6 The Discovery of Gefitinib (Iressa™)

These strong target validation and disease association results have prompted the research community to pursue small molecule EGFR-TK (tyrosine kinase) inhibitors as potential anticancer therapeutics. These small molecule inhibitors can compete with ATP binding to the TK domain of the receptor, which inhibits TK activity and subsequently block signal transduction from the EGFR. Gefitinib (Iressa™), an 4,6,7-trisubstituted-4-quinazolinamine discovered by AstraZeneca in the mid- to late-1990s, was one of the first agents in this new family [49].

The discovery of gefitinib started from a phenylaminolquinazoline (compound **3A**, Fig. 40.3), which was discovered from the SAR study of the 4-anilinoquinazoline class as EGFR-TK inhibitors [58, 59]. This compound was found to be a potent inhibitor of EGFR-TK (IC_{50} = 5 nM) and EGF-stimulated human cell growth (IC_{50} = 50 nM (KB oral carcinoma cells)]. However, compound **3A** had a short half-life (approximately 1 h) and appeared to undergo rapid oxidative metabolism. Substitution of the methyl group with chlorine and introduction of fluorine at the *para*-position of

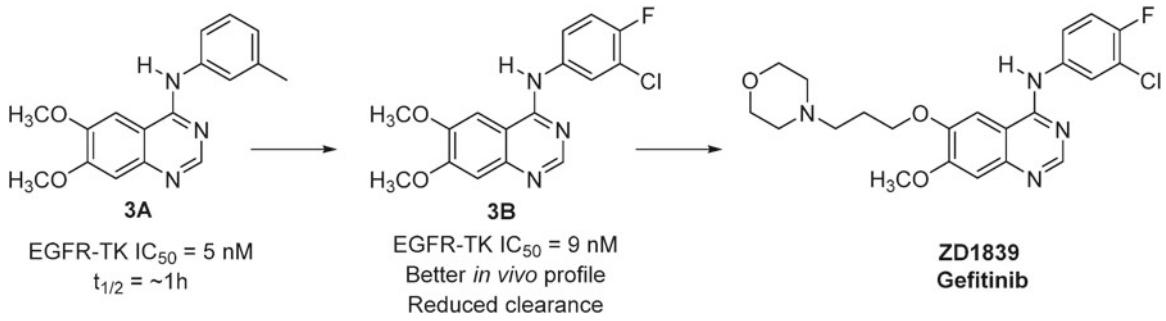

Fig. 40.3 Discovery of gefitinib.

the aniline gave compound **3B**, which was resistant to the oxidative metabolism. Although this compound showed a slight loss of potency in vitro (EGFR-TK, IC_{50} = 9 nM; EGF stimulated KB cell growth, IC_{50} = 80 nM) compared to compound **3A**, it had improved efficacy in in vivo models via oral dosing and better pharmacokinetic (PK) properties (e.g., reduced clearance, longer half-life—approximately 3 h). The approach aimed at further improving in vivo activity, which eventually proved successful, involved modification of the alkyl group of the methoxy side chain. Introduction of a basic group into the alkoxy side chain resulted in improved physical chemical properties such as solubility and further improved PK parameters. After profiling a series of compounds with different aminoalkoxy side chains, gefitinib (ZD1839) (Fig. 40.3) was chosen as a development candidate based on its excellent in vitro and in vivo profiles. Although gefitinib was not the most potent compound synthesized, it achieved high and sustained blood levels over a 24-h period and had good oral bioavailability in preclinical species [60]. Gefitinib is selective against structurally closely related ErbB2, the receptors of VEGF, FMS like tyrosine kinase (FLT1; VEGFR1), kinase insert domain-containing receptor tyrosine kinase (KDR; VEGFR-2), and a number of serine/threonine kinases [61]. Gefitinib inhibits the growth of a broad range of human solid tumor xenografts in a dose-dependent manner with marked regressions seen in some tumors. Treatment for up to 4 months in nude mice was well tolerated. Gefitinib has demonstrated a long half-life in humans compatible with once-daily oral dosing. The FDA approved gefitinib as a second-line treatment for non-small-cell lung cancer in May 2003.

40.2.7 The Discovery of Afetinib (Gilotrif™)

As exemplified by etitinib and erlotinib (Tarceva™, OSI), the first-generation small molecule EGFR tyrosine kinase inhibitors were shown to be effective against lung tumor cells harboring mutations in the kinase domain of EGFR, most commonly small in-frame deletions in exon 19 or the L858R missense mutation in exon 21 [62]. However, despite the initial response, patients almost invariably develop resistance to these inhibitors and relapse after several months [63]. A secondary mutation, T790M, in exon 20 of the EGFR kinase domain is account for about half of cases with acquired resistance to first-generation EGFR inhibitors [64, 65]. T790M EGFR exhibits elevated enzymatic and transforming activity, both alone and in combination with primary alterations in exon 19 or 21 [66–69], indicating a need for increased therapeutic efficacy of the next generation of EGFR inhibitors. Ideally, these new small molecule tyrosine kinase inhibitors should be more broadly active against ErbB receptor tyrosine kinases, yet retain the exquisite overall selectivity of first-generation EGFR TKIs within the human kinome that has afforded an acceptable drug safety and tolerability profile.

Irreversible inhibitors that covalently modify EGFR and/or HER2 exhibited increased efficacy against mutants resistant to gefitinib and erlotinib in cell-based assays [64, 69–73]. One such inhibitor, afetinib (BIBW2992) (Fig. 40.4), is a new irreversible dual EGFR/HER2 inhibitor derived from the anilino-quinazoline chemical series that was designed to covalently bind to Cys 773 of EGFR and Cys 805 of HER2 via a Michael addition [74]. Afetinib was found to potently suppress the kinase activity of wild-type and activated EGFR and HER2 mutants, including the most common mutations, L858R and deletion-19, and the exon 20 gatekeeper T790M mutations, albeit at lower potency [74, 75]. It has received regulatory approval for use as a treatment for non-small-cell lung cancer in 2013 although there is emerging evidence to support its use in other cancers such as breast cancer [76].

40.2.8 The Discovery of Lapatinib (Tykerb™)

Among the four members (EGFR, HER2, HER3, and HER4) of the EGFR family, only HER2 is unknown to bind ligands [77]. However, HER2 is the preferred heterodimerization partner for EGFR, HER3, and HER4. Ligand binding to the extracellular domain of members of this family causes receptor homodimerization or heterodimerization and tyrosine kinase activation. The resultant receptor autophosphorylation

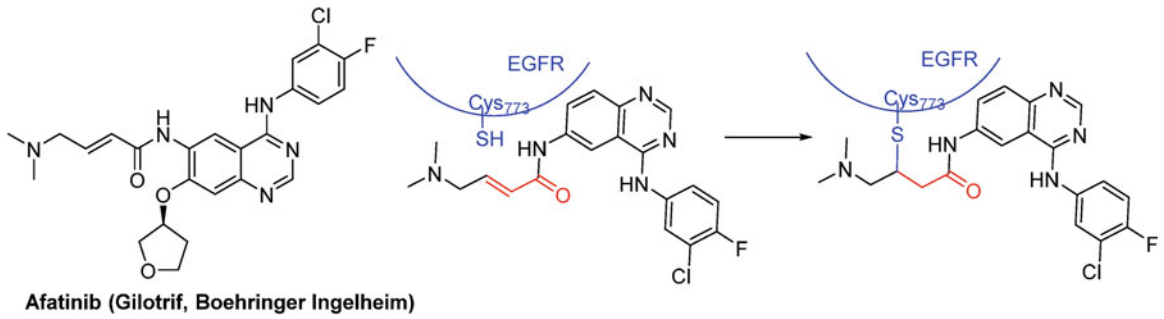

Fig. 40.4 Afatinib and its inhibitory mechanism.

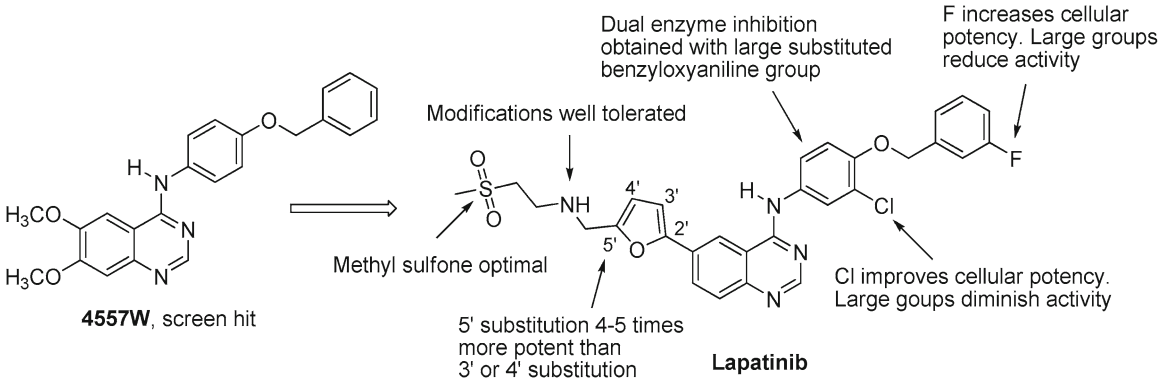

Fig. 40.5 Discovery of lapatinib.

leads to the activation of various signaling cascades that are involved in cell proliferation and survival [78].

EGFR and HER2 have been shown to promote the growth and survival of various types of epithelial malignancies. EGFR is overexpressed in up to 30 % of human breast cancers [79], and HER2 is amplified and overexpressed in up to 20–25 % of primary human breast cancers [80]. Importantly, overexpression of HER2 is associated with aggressive disease and shortened disease-free survival and overall survival [81]. Abundant evidence suggests that EGFR and HER2 work in synergy to produce oncogenic effects [82]. In particular, HER2 dimerization with EGFR increases the EGF binding affinity to EGFR [83], and the binding of EGF to EGFR subsequently increases the activation of HER2 [84]. It was therefore hypothesized that agents that inhibit both EGFR and HER2 might be more effective at inhibiting the activation of the PI3K-Akt and Ras-Raf-mitogen-activated protein kinase signaling cascades and could have more effective anticancer activity than those that target either kinase alone. Efforts on developing such molecules led to the discovery of lapatinib (Tykerb™, GlaxoSmithKline) [85].

The discovery of lapatinib (originally known as GW572016) started with the screen hit 4557 W (Fig. 40.5) [86], which was a potent inhibitor of both HER2 (IC_{50} = 0.079 μM) and EGFR (IC_{50} = 0.020 μM). Initial SAR study demonstrated that large anilino substitutions were needed to retain both EGFR and HER2 inhibition activity. The most potent dual enzyme inhibition could be obtained when the aryl group was the substituted benzyloxyaniline group, and the optimal substitution pattern involved a *para*-benzyloxy group. Other notable SAR include that although chlorine at 3-position afforded insignificant increases in enzyme potency, it improved cellular activity. Fluorine at 2′-position yielded compounds with greater potency in cellular assays. Larger groups at 3-position or 2′-position generally diminished activity.

For the left-hand side capping group, the simple methyl sulfone group appeared to be optimal. Study on the regiochemistry of the furan ring showed that the 5′ substituted analog was 4–5 times more potent against the tumor cell lines than the 3′ or 4′ regioisomer. In addition, a diverse set of amine substitutions was tolerated, presumably due to the binding mode of these inhibitors. However, the extra synthetic steps and increased molecular weight were deemed nonbeneficial for optimizing physical chemical properties.

The medicinal chemistry optimization ultimately led to the discovery of lapatinib, which possessed the desired enzyme potency, cellular activity in a panel of tumor cell lines, and excellent selectivity [87]. In March 2007, FDA approved lapatinib for treating breast cancer.

40.2.9 VEGFR Inhibition: The Discovery of Pazopanib (Votrient™) and Axitinib (Inlyta™)

Angiogenesis plays a critical role in the growth and metastasis of solid and hematologic malignancies [88]. It is considered as the rate-limiting step in tumor development because without blood supply, a solid tumor is limited to a maximum size of 1–2 mm [89]. This complex and highly regulated process involves numerous different cell types and mediators.

VEGF is the growth factor most often associated with angiogenesis [90]. Oxygen-deficient cells use VEGF in key signaling pathways to promote the growth of new blood vessels. VEGF interacts with VEGFRs on endothelial cell surfaces, directing them to build new blood supplies [91]. The cells respond to this message by producing specialized protease enzymes to penetrate the basal lamina, so that they can migrate to oxygen-deficient regions. Once there, the cells replicate and form into tubes, thus creating new capillary pathways. Elevate VEGF levels have been correlated with increased microvessel counts and poor prognosis in many cancer types [92].

As a principal subfamily of RTKs, VEGFRs are specifically expressed in vascular endothelial cells. They include FMS like tyrosine kinase-1 (FLT-1; VEGFR-1), kinase insert domain-containing receptor tyrosine kinase (KDR; VEGFR-2), and FLT-4 (VEGFR-3) [93]. Activation of VEGFR family RTKs and, in particular, VEGFR-2 by VEGF plays a primary role in tumor angiogenesis. VEGF mediated VEGFR-2 signaling induces a series of endothelial responses such as proliferation, migration, and survival and ultimately leads to new vessel formation and stabilization [94].

The therapeutic hypothesis that inhibiting the VEGF/VEGFR pathway can interrupt the angiogenesis process and subsequent tumor growth was first clinically validated by Bevacizumab (Avastin™; Genentech), a monoclonal antibody to VEGF, which is approved by FDA for the treatment of both metastatic colorectal cancer in combination of 5-fluorouracil [95], and for non-small-molecule lung cancer in combination with carboplatin and paclitaxel [96]. Efforts on discovering small molecule inhibitors of VEGFRs [97] led to the FDA approval of pazopanib (Votrient™, GlaxoSmithKline), N^4-(2,3-dimethyl-2H-indazol-9-yl)-N^4-methyl-N^2-(4 methyl-3sulfona midophenyl)-2,4-pyrimidinediamine, as an oral treatment for renal cell carcinoma (RCC).

The discovery of pazopanib started from a screening for agents active against VEGFR-2 [98]. Initial screening hit compound **6A** (Fig. 40.6), which inhibited VEGFR-2 with an IC_{50} of ~400 nM, was optimized to compound **6B** using a homology model of the kinase domain as the crystal structure was not known at that time. Compound **6B** inhibited VEGFR-2 with an IC_{50} of 6.3 nM, almost a 100-fold potency improvement compared to **6A**. In cellular assays, compound **6B** inhibited VEGF induced proliferation of human umbilical vein endothelial cells (v-HUVEC) with an IC_{50} of 0.54 μM. It also had an IC_{50} of 3.5 μM against basic fibroblast growth factor (b-FGF). However, the pharmacokinetic profile of this compound was poor (moderate to high clearances, 28–83 mL/min/kg, and low oral bioavailability, <11 %) in mice. Incorporation of a 3-methylindazole moiety into this series yielded indazolylpyrimidine (compound **6C**) with desired cellular potency, and selectivity in VEGF and bFGF driven HUVEC, with IC_{50}'s of 0.13 and 13 μM, respectively. **6C** had moderate plasma clearance (40 mL/min/kg) and oral bioavailability

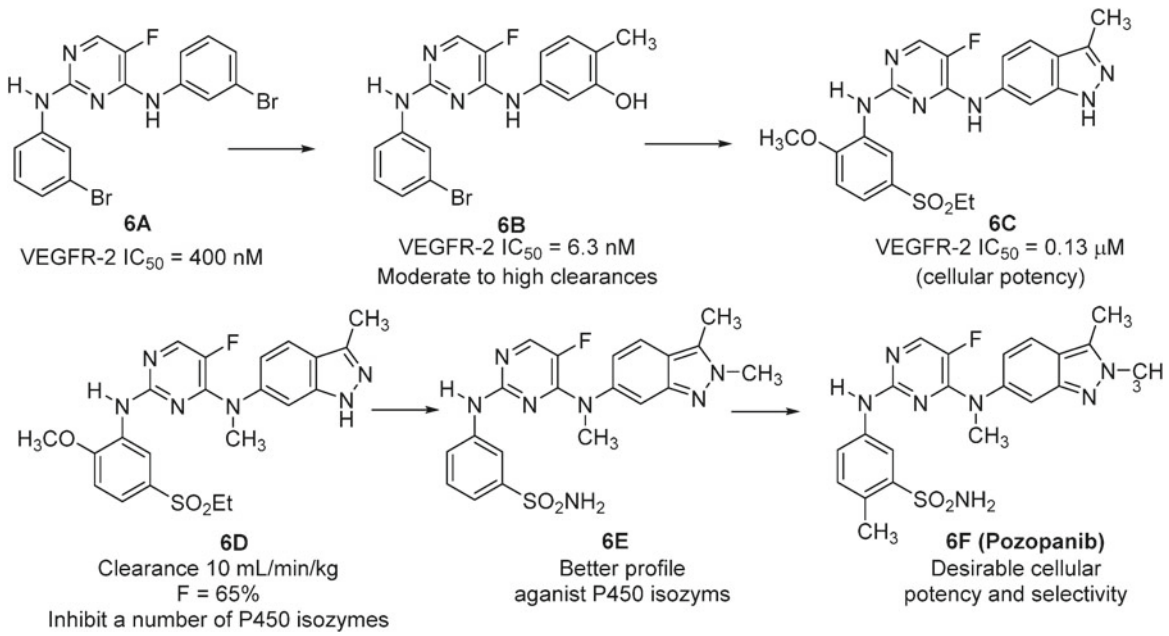

Fig. 40.6 Discovery of pozopanib.

(28 %) in rats. Introducing a methyl group into the C-4 amino nitrogen resulted in compound **6D**, which showed an improved PK profile with lower clearance (10 mL/min/kg) and higher oral bioavailability (65 %) while providing comparable enzymatic potency to its non-alkylated analog **6C**. Although compound **6D** possessed both good in vitro potency and cross-species PK profiles, significant inhibition ($IC_{50} < 10$ μM) was observed against a number of cytochrome P450 isozymes possibly due to the binding of the nitrogen on the indazole to the heme iron of the cytochrome P450 enzymes. Compound **6E** resulting from the addition of a methyl group at the 2-position of the indazole ring, which increases steric hindrance of the heterocycle ring, had an improved profile against the CYP2C19, CYP2D6, and CYP3A4 isozymes as well as improved cellular efficacy against v-HUVEC cells. With the preferred right-hand side moiety identified, a second aniline survey at the C-2 position of the pyrimidine resulted in the discovery of compound **6F** (pazopanib), which possesses a desirable combination of excellent in vitro potency, selectivity, cellular activities, PK, and developability properties. Pazopanib was potent against all human VEGFR receptors with a respective IC_{50} of 10, 30, and 47 nM for VEGFR-1, -2, and -3. Significant activity was also seen against the closely related tyrosine receptor kinases PDGFRα, PDGFRβ, c-Kit, FGF-R1, and c-fms with IC_{50}'s of 84, 74, 140, and 146 nM, respectively. In cellular assays, pazopanib had a desirable combination of cellular potency and selectivity in VEGF and bFGF driven HUVEC, with IC_{50}'s of 0.021 and 0.72 μM, respectively. It also potently inhibited VEGF induced phosphorylation of VEGFR-2 in HUVEC cells with an IC_{50} of 8 nM. Pazopanib had good PK parameters including low clearances and good oral bioavailability in preclinical species. The cytochrome P450 profile was also significantly improved with $IC_{50} > 10$ μM against the isozymes tested, with the exception of CYP2C9 ($IC_{50} = 7.9$ μM). On the basis of its favorable PK profiles and in vivo efficacies, the mono-HCl salt of compound **6F** (pazopanib) was progressed into clinical development [98] and approved by the FDA for the treatment of renal cell carcinoma in October 2009.

Axitinib (AG013736, Inlyta™, Pfizer) (Fig. 40.7), approved by FDA in January 2012, is another ATP-competitive VEGFR inhibitors for patients with metastatic renal cell carcinoma (mRCC) after failure of one prior systemic therapy [99]. This small molecule indazole derivative is a potent, orally bioavailable multitargeted tyrosine kinase receptor inhibitor, which selectively inhibits vascular endothelial growth factor receptors (VEGFR)-1, -2, and -3 at subnanomolar concentrations, in vitro [100]. The structure-based drug design of axitinib allows strategic optimization of critical binding elements, with the tight fit of axitinib into the deep pocket conformation of the kinase domain of VEGFRs resulting in high potency and selectivity (Fig. 40.7). In vitro, axitinib inhibits VEGFR-1, -2, and -3 autophosphorylation

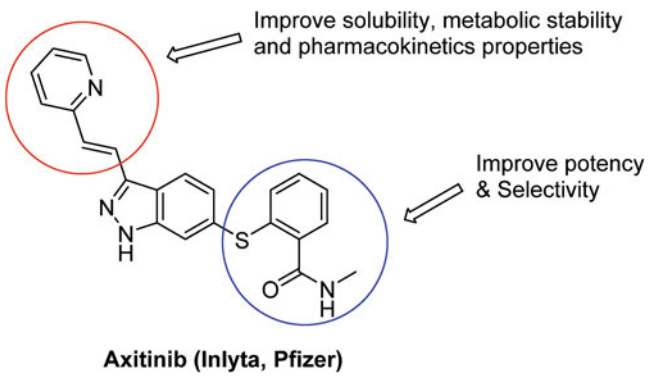

Fig. 40.7 Drug design strategy for axitinib.

at picomolar concentrations. Axitinib is tenfold more potent for the VEGF family receptors than for RTKs of other family receptors [100]. Additionally, axitinib did not significantly inhibit other receptor kinases including colony-stimulating factor (CSF)-1R, fms-like tyrosine kinase (Flt)-3, fibroblast growth factor receptor (FGFR)-1, ret proto-oncogene (RET), epidermal growth factor receptor (EGFR), and met proto-oncogene encoding hepatocyte growth factor (c-Met) [100].

Axitinib, orally administrated at a dose 2–10 mg twice daily, has been shown to reduce vascular permeability, tumor vascularization and tumor volume, and has demonstrated antitumor activity as a single agent in patients with cytokine- and/or sorafenib-refractory mRCC. The activity range of axitinib is the highest compared with other drugs currently approved for use in mRCC. Axitinib also has a favorable and non-cumulative tolerability profile associated with manageable adverse effects, which are generally mild to moderate in severity [101]. Based on the promising results from clinical trials, FDA approved axitinib for the treatment of mRCC after failure of one prior systemic therapy in 2012.

40.2.10 Multitargeted Tyrosine Kinase Inhibition

In search for more effective anticancer therapeutics, the multitargeted approach has emerged as a new paradigm [102]. Malignant tumors are complex tissues involving interplay between cancer cells and surrounding or supportive cells, thus a multitargeted therapeutic approach that affects supportive tissues and cancer cells may be of clinical benefit. Such agents can simultaneously target the tumor and surrounding or supportive cells and thereby interact with the complex multimolecular lesions that drive tumor growth and survival. A key advantage of the multitargeted approach is that it potentially reduces drug resistance. Due to mutations [72], overexpression of key components of signaling pathways [103], drug-efflux systems and/or signaling bypass

[104], tumors will likely develop resistance to single-targeted kinase inhibitors rapidly [105]. This drug resistance will be less likely to arise with multitargeted agents. In addition to multitargeted agents, the use of a combination of multiple kinase inhibitors has been investigated, which could offer similar benefits as multitargeted agents. In this section, we use sunitinib and dasatinib as examples to illustrate the success of multitargeted kinase inhibitor agents.

40.2.11 The Discovery of Sunitinib (Sutent™)

Growth factors including VEGFs and PDGFs are important pro-angiogenic factors. Their activity is mediated by binding to specific cell-surface receptor tyrosine kinases. There are four PDGF ligands (PDGFA to PDGFD), which bind to the receptor tyrosine kinases PDGFRα and PDGFRβ. PDGFB and PDGFRβ have been suggested to have crucial roles in tumor-vessel stability by recruiting pericytes to newly formed vessels [106]. The multitargeted tyrosine kinase inhibitor sunitinib (Sutent™, Pfizer) was designed to inhibit both VEGFRs and PDGFRs. Sunitinib, which demonstrated activity in RCC and other types of cancers, has been approved by the FDA for the treatment of kidney cancer and GISTs in January 2006.

Sunitinib (Sutent™, formerly SU11248, named from Schlessinger and Ullrich who created Sugen, a biotech company later acquired by Pharmacia then Pfizer) [107] is an orally active oxindol that has antiangiogenic and antitumor activities. It inhibits VEGFR-1, VEGFR-2, VEGFR-3, PDGFRα, PDGFRβ, KIT, FLT3, glial cell line-derived neurotrophic factor (GDNF) receptor, and colony stimulating factor 1 (CSF1) receptor (CSF1R) [108]. Sugen initially discovered SU5416 (Fig. 40.8) as a potent and selective VEGFR inhibitor [109]. Both in vivo and in vitro studies demonstrated its antiangiogenic potential [110]. Unfortunately, SU5416 failed in clinical trials due to its limited solubility and high toxicity. SU6668, a close analog of SU5416, was identified as a potent and selective PDGFRβ inhibitor [111, 112]. However, this compound had inadequate PK properties for clinical development. To broaden the kinase activity spectrum of this scaffold and to optimize PK properties, diversification at the C-4′ position on the pyrrole ring of SU5416 had been explored. It was found that modifications at the C-4′ position could lead to compounds with different kinase inhibition profiles for VEGFR2 and PDGFRβ. The cocrystal structure of SU6668 with the catalytic domain of fibroblast growth factor receptor 1 (FGFR1) kinase [111], which has high sequence similarity to the ATP-binding pocket of VEGFR-2, revealed that the substitution at the C-4′ position on the pyrrole ring was positioned close to the opening of the binding pocket and could be exposed to solvent. Thus, substitution at this position might serve as a handle for improving physical chemical properties of the core structure. Optimization of this region resulted in the discovery of sunitinib (SU11248), a more potent inhibitor of VEGFRs and PDGFRs. Comparing to SU6668, SU11248 was about 30-fold more potent against VEGFR-2 and PDGFRβ in biochemical assays, over ten-fold more potent in cellular kinase assays, and significantly more soluble under neutral (20-fold) and acidic (>500-fold) conditions [112]. Additional kinase selectivity study revealed that SU11248 was also a good inhibitor of KIT and FLT-3 [108, 113]. Furthermore, SU11248 had excellent oral bioavailability, was highly efficacious in a number of preclinical tumor models, and was well tolerated at efficacious doses [108]. Based on the satisfactory results from clinical trials, sunitinib was approved by the FDA for the treatment of RCC/GISTs in January 2006.

40.2.12 The Discovery of Dasatinib (Sprycel™)

Dasatinib (BMS-354825, Sprycel™; Bristol Myers Squibb) is a potent, second-generation, multitarget kinase inhibitor of BCR-ABL and the Src (sarcoma) family of kinases [114]. In addition to inhibiting BCR-ABL and Src kinases such as Fyn (Proto-oncogene tyrosine-protein kinase), Yes (Proto-oncogene tyrosine-protein kinase), Src, and Lyk (tyrosine-

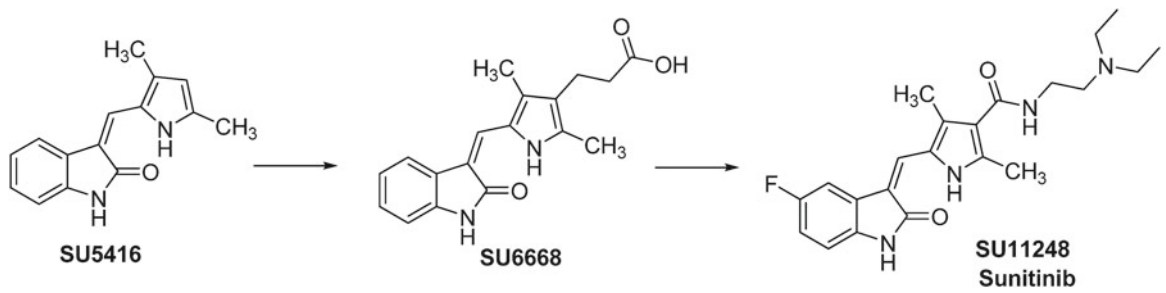

Fig. 40.8 Discovery of sunitinib.

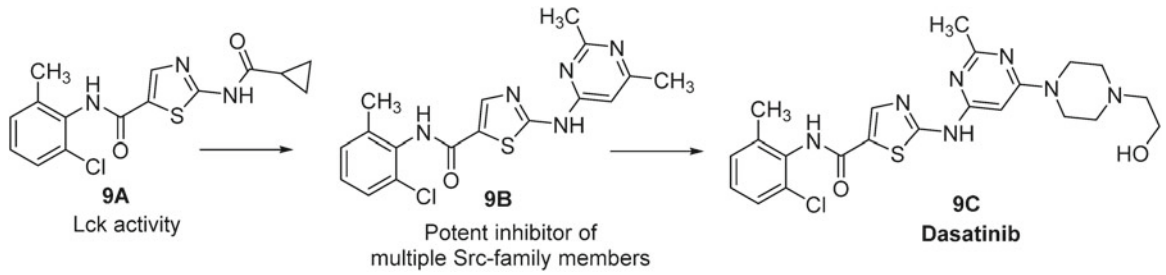

Fig. 40.9 Discovery of dasatinib.

protein kinase), dasatinib was found to inhibit ephrin receptor A2 (EphA2), PDGFRs, c-Kit, mitogen-activated protein kinases, and discoidin domain receptor 1 (DDR1) [115].

Src proto-oncogene is believed to play a role in the development of several human cancers, contributing to cellular proliferation, adhesion, invasion, and motility [116]. Src-family kinases such as Src and LYN (V-yes-1 Yamaguchi sarcoma viral related oncogene homolog) modulate signal transduction by phosphorylating tyrosine residues of molecules such as EGFRs, PDGFRs, fibroblast growth factor receptors (FGFRs), and VEGFRs. Src kinases have also been found to have oncogenic activities in cancer cell lines from tumors involving the colon, breast, pancreas, lung, and brain [117]. Thus, it was hypothesized that blocking signaling through the inhibition of the kinase activity of Src would be an effective means of modulating aberrant pathways that drive oncologic transformation of cells.

The discovery of dasatinib started from 2-acylamino-5-carboxamidothiazoles **9A** (Fig. 40.9), a Lck (lymphocyte-specific protein tyrosine kinase) kinase inhibitor [118]. Replacing the 2-acyl functionality with a variety of heterocycles led to the identification of compound **9B** [119], which was a potent inhibitor of multiple Src-family members including Lck, Fyn, Src, and Hck (hemopoietic cell kinase). In order to improve cellular activity of this series, a number of **9B** analogs were synthesized. These efforts resulted in the discovery of the piperazinyl ethanol **9C** (dasatinib), which has broad spectrum antiproliferative activity, favorable circulating plasma levels following oral dosing, long half-life, and favorable in vivo efficacy [120].

In addition to inhibiting multiple Src-family kinases including Lck, Fyn, Src, and Hck, dasatinib is a BCR-ABL inhibitor that blocks both the active and inactive conformations of ABL. Dasatinib is active against wild-type and almost all BCR-ABL mutant cell lines that are resistant to imatinib, except those carrying the T315I mutation [36]. The activity of dasatinib against ABL kinase mutations is likely due to the fact that it does not require interaction with some of the residues involved in those mutations. Dasatinib is also 325-fold more potent than imatinib against native BCR-ABL in in vivo studies [121] and is slightly more potent against Src family kinases ($IC_{50} = 0.5$ nM) compared to ABL ($IC_{50} = 1$ nM) [120]. It also inhibits c-KIT, PDGFR and EphA2 at nanomolar concentrations [115]. Potent Src inhibitory activity of dasatinib in CML progenitors involves BCR-ABL-dependent and BCR-ABL-independent Src activity [122]. Dasatinib also inhibits downstream signaling pathways in CML progenitors, as well as P-mitogen-activated protein kinase (MAPK), P-protein kinase B (Akt), and P-signal transducer and activator of transcription 5 (STAT5) levels in CML progenitors in the absence of growth factors. In addition, dasatinib significantly suppresses CML colony-forming cells and long-term culture-initiating cells [122]. In June 2006, the FDA approved dasatinib for the treatment of chronic phase, accelerated phase, or blastic phase CML, resistant or intolerant to imatinib, and for the treatment of Philadelphia chromosome-positive acute lymphoid leukemia that was resistant or intolerant to prior therapy.

40.2.13 Other Potential Kinase Targets

Encouraged by the rapid progress and tremendous success achieved in the fields of cancer molecular biology and kinase drug discovery to date, the academic research community and the pharmaceutical and biotech industry are targeting many other kinases for achieving potential clinical benefits. For examples, inhibitors of integrin-linked kinase (ILK), a focal adhesion serine–threonine protein kinase, are being developed for treating anaplastic thyroid cancer [123]. Focal adhesion kinase (FAK) has been shown to be an important mediator of cell growth, cell proliferation, cell survival, and cell migration [124]. Cell division cycle 7-related protein kinase (Cdc7) is a conserved serine–threonine kinase, which has been shown to be required for the initiation of DNA replication [125]. Other areas of research include the hepatocyte growth factor receptor (MET) [126], and the Ras/ERK1-2 (extracellular signal-regulated kinase 1-2) [127], Akt [128] and STAT (signal transducer and activator of transcription) [129] pathways. In this section, we describe the discovery of PD0325901, ARRY-142886, and trametinib (GSK1120212), three inhibitors of mitogen-activated protein kinase kinases (MEK), to highlight recent progress in this new area.

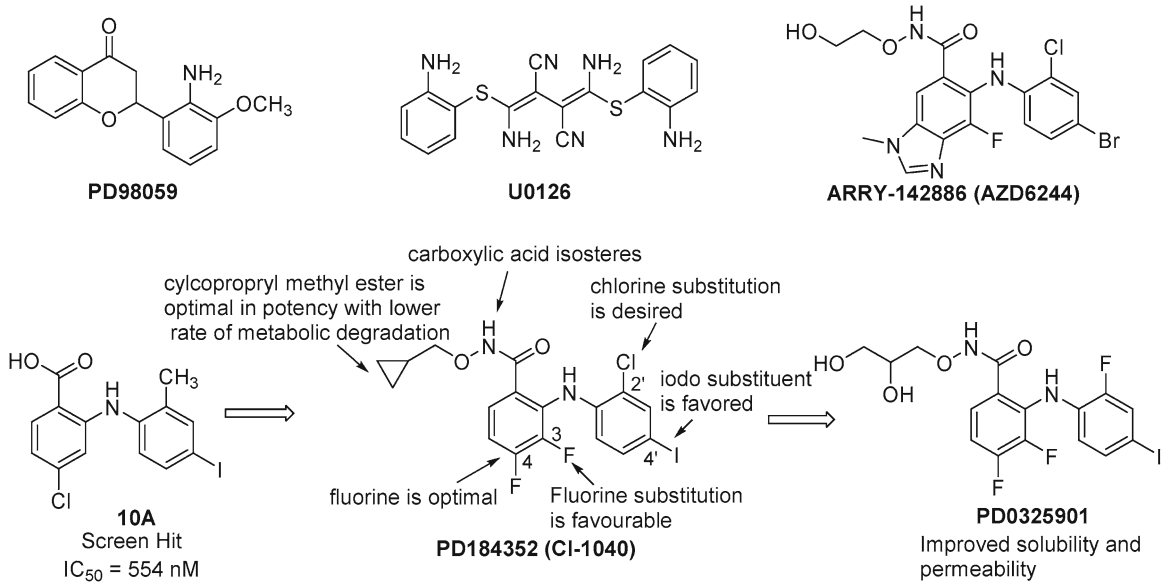

Fig. 40.10 Discovery of MEK inhibitors.

40.2.14 MEK Inhibition

The signaling pathway comprising mitogen-activated protein kinase (MAPK) kinases (MEK) /extracellular signal-regulated kinases (ERK) is activated in most human tumors [130]. Activation of the ERK pathway involves the guanosine triphosphate (GTP)-loading of Ras at the plasma membrane, and the sequential activation of a series of protein kinases. Initially, activated Ras recruits the Raf family of kinases such as Raf-1 to the plasma membrane, where it is then able to phosphorylate and activate MEK1/2 by serine phosphorylation. MEK1 and 2 then phosphorylate and activate their only currently known substrates, ERK1 and 2. When activated, ERK1 and 2 phosphorylate various downstream substrates involved in a multitude of cellular responses such as cell proliferation, cell differentiation, cell survival and cell motility [131].

Because the Raf-MEK-ERK kinase signaling module is activated as a consequence of several gene amplifications and genetic mutations identified in human cancer, it is an attractive target for small-molecule intervention [127]. Selective MEK1/2 inhibitors could be valuable tools to evaluate the role of this signaling axis because MEK1 and 2 have extremely unique characteristics among the components of the ERK pathway. Although Raf-1 is the major activator of MEK1/2, these kinases are activated also by several other kinases such as Mos, A-Raf, and B-Raf. However, no substrates for MEK1/2 have been identified other than ERK1 and ERK2. In addition, MEK1 and 2 are dual-specificity kinases that phosphorylate both tyrosine and threonine residues and can integrate many mitogenic signaling pathways into the ERK pathway. All these very prominent features including unusually restricted and unique substrate specificities and the integrating role of mitogenic signaling pathways highlight MEK1/2 as excellent targets for developing inhibitors against the ERK pathway [127]. Currently, a number of potent and selective MEK1/2 inhibitors have entered clinical development [132]. Most of them are ATP noncompetitive which means they do not directly compete for the ATP-binding site. Rather they bind to a unique allosteric site adjacent to the ATP site. This makes the noncompetitive MEK inhibitors highly specific [133].

From a screening campaign using an in vitro cascade assay, PD98059 (Fig. 40.10) was identified as the first pharmacological inhibitor of MEK1/2 [134]. Meanwhile, U0126 (Fig. 40.10) was discovered by screening a total of 40,000 compounds in a cell-based reporter assay [135]. Because of their poor PK and developability properties, these two early MEK1/2 inhibitors were not progressed into clinical development. Nevertheless, these two compounds were widely used as in vitro tools to elucidate the role of the ERK pathway in a variety of biological processes. The results from these studies have strengthened the hypothesis that the ERK pathway represents an attractive target for developing anticancer drugs.

The discovery of PD0325901 began with the high throughput screening hit **10A** (Fig. 40.10), a potent inhibitor of MEK with an IC_{50} of 554 nM [136]. However, this compound had poor cellular potency likely due to poor cell membrane permeability resulting from the carboxylic acid moiety. In order to improve cellular potency, carboxylic acid isosteres such as benzohydroxamic acid esters were investigated. A variety of small alkyl and cycloalkylmethyl esters showed comparable potency with cyclopropyl methyl ester

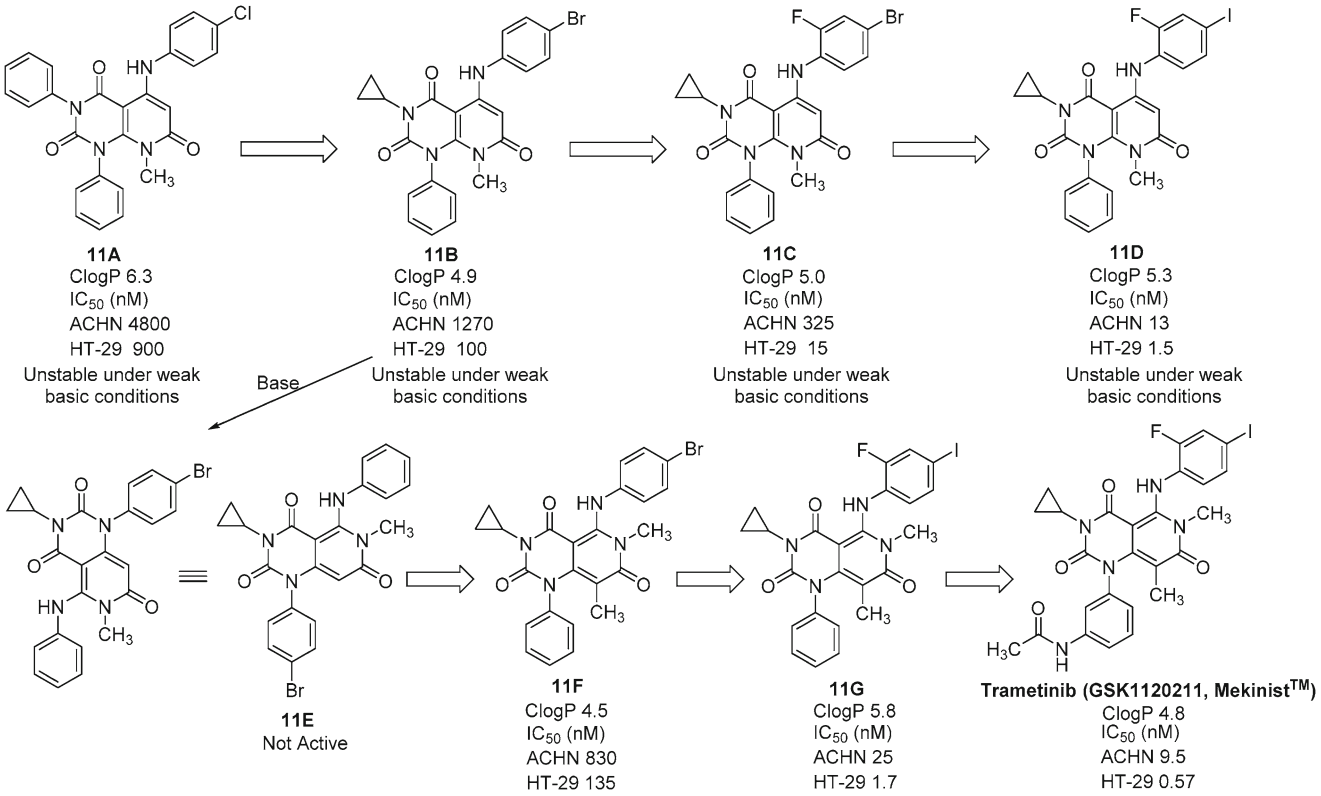

Fig. 40.11 Discovery of trametinib.

being the best. SAR studies also showed that the iodo substituent was favored at the 4′-position and a chloro substituent was desired at the 2′-position. Additionally, a halo substituent at the 4-position, in particular, a fluorine was optimal as the MEK1 X-ray cocrystal structure revealed that the fluorine had a dipolar interaction with the amide backbone [137]. Substitution of fluorine at 3-position gave moderate potency enhancement. These SAR studies resulted in the discovery of PD184352 (CI-1040), which demonstrated in vivo activity in a mouse tumor model following oral administration [138]. This compound was advanced into Phase II clinical trial, but failed for inadequate efficacy likely due to insufficient systemic exposure. The solubility of PD184352 is less than 1 μg/mL in pH 6.5 phosphate buffer, suggesting that low in vivo exposure is at least partly due to solubility limitations. In order to improve aqueous solubility of PD184352, replacing the cyclopropylmethoxy group with a (*R*)-dihydroxypropoxy group and the 2′-chloro substituent with a 2′-fluoro substituent led to the discovery of PD0325901, a second-generation MEK inhibitor. This compound has markedly improved potency, oral bioavailability, and half-life. Excellent anticancer activity has been demonstrated for PD0325901 against a broad spectrum of human tumor xenografts [136]. In an open-label, phase II study, patients with progressive, recurrent, or advanced NSCLC were treated with 15 mg PD-0325901 twice daily [139]. There were no objective responses during the trial period. Due to a lack of responses coupled with the safety issues, the trial was closed after the first stage [132].

In addition, ARRY-142886 (AZD6244, selumetinib, Fig. 40.10), a benzimidazole derivative of PD0325901, was discovered as a potent inhibitor of MEK1/2 with IC_{50} values of 12 nM in an in vitro assay and 8 nM in a cell-based assay [140]. ARRY-142886 is noncompetitive with ATP—potentially explaining its excellent selectivity for MEK1/2 as in the case of other MEK inhibitors. ARRY-14886, which is orally bioavailable and has low metabolic clearance, has been shown to exhibit promising antitumor efficacy in several human xenograft models [141]. Tumor growth inhibition by this compound correlates well with decreased phospho-ERK levels. ARRY-14886 is currently being evaluated in Phase III clinical trials for NSCLC.

Trametinib (GSK1120212, Mekinist™, GlaxoSmithKline) (Fig. 40.11), a selective inhibitor of MEK 1 and 2, has emerged as the first MEK inhibitor to show favorable clinical efficacy in a phase III trial [142] and has been approved by FDA for the treatment of Melanoma in May 2013.

Compound **11A** was identified as a hit via high throughput screening for compounds that can induce expression of the cyclin-dependent kinase (CDK) 4/6 inhibitor $p15^{INK4b}$ (Fig. 40.11) [143]. It was later confirmed that it had an antiproliferative activity against human cancer cell lines ACHN (renal adenocarcinoma) and HT-29 (colorectal adenocarcinoma) with IC_{50} values of 4800 and 990 nM, respectively

[144]. A medicinal chemistry campaign aiming at optimizing these antiproliferative effects led to the discovery of orally bioavailable GSK1120212 (trametinib), which has demonstrated selective inhibition of the proliferation in various BRAF mutant cancer cell lines. Later it was confirmed that this compound was a highly potent and selective inhibitor of MEK1/2 [144].

Compound **11A** has high hydrophobicity (ClogP 6.3). Therefore, the first step in chemical modification was to reduce the hydrophobicity of **11A** by replacing each aromatic ring with a small alkyl group. This resulted in compound **11B**, which contains a cyclopropyl group at the 3-position (upper left) instead of the phenyl group (Fig. 40.11). Compound **11B** exhibited reduced hydrophobicity (ClogP 4.9) and improved potency. Introducing a fluoro group at the 2-position of the aniline ring (compound **11C**) led to ca. four-fold potency improvement. Further exploration of the substitution at the 4-position at the aniline ring yielded compound **11D**, which was significantly more potent than compound **11C**.

Unfortunately, all the above compounds, possessing the pyrido[2,3-d]pyrimidinedione core, were unstable under weak basic conditions. For example, treatment of **11B** with potassium carbonate in methanol/chloroform at ambient temperature provided compound **11E**, which has a pyrido[4,3-d]pyrimidinedione core. Compound **11E** was totally inactive. However, it was hypothesized that compound **11F** could be chemically stable and highly active. Indeed, compound **11F** restored the inhibitory activity up to the level of **11B** but with much more chemical stability. Next, 2-F,4-I aniline moiety was introduced in the same substitution fashion as **11D** and the resulting **11G** had much improved potency as expected. To reduce high hydrophobicity of **11G**, polar substituents at the lower phenyl ring were investigated. GSK1120211, which has an acetamide group, was found to be the best compound.

40.3 Inhibitors Targeting DNA Repair

Numerous DNA repair mechanisms can protect DNA from damage by carcinogens, radiation, and viruses and therefore reduce the efficacy of DNA-interactive anticancer agents [145]. DNA repair is mediated by a remarkable set of enzymes that first recognize the damage and then signal to other enzymes to repair the lesion [146]. A number of distinct repair pathways have been identified with mechanisms ranging from simply removing an extra methyl group to completely excising the damaged segment of DNA and resynthesizing a new one [146]. Agents that target these pathways and mechanisms, thus inhibit DNA repair could potentially enhance the efficacy of DNA-interactive anticancer drugs [145].

40.3.1 Poly(ADP-Ribose) Polymerase Inhibition: ABT-888

Poly(ADP-ribose) polymerase-1 (PARP-1) is the most abundant and well characterized member of the PARP family of nuclear enzymes [147]. Among the 18 members of the PARP family identified and characterized to date, PARP-1 is the most thoroughly studied and PARP-2 has the highest homology to PARP-1 [148]. Highly evolutionarily conserved in all advanced eukaryotes, PARP-1 accounts for >90 % of the ADP-ribosylation within the cell [149]. Only PARP-1 and PARP-2 can be activated by DNA damage. The importance of these two family members in genomic integrity and cell survival has been indicated by the fact that PARP-1 knockout mice are viable, while double PARP-1 and PARP-2 knockout mice are embryonically lethal [150]. Because of the structural similarity between PARP-1 and PARP-2, most PARP-1 inhibitors also inhibit PARP-2.

By sensing and binding to breaks mainly in single-strand DNA, the zinc-finger DNA-binding enzyme PARP-1 becomes activated. It then initiates DNA repair by recruiting and ADP-ribosylating nuclear proteins, such as the scaffold protein X-ray repair cross-complementing 1 (XRCC1), which can, for example, direct DNA polymerase b (polb) to replace a damaged nucleotide. Nuclear proteins associated with apoptosis may also be recruited [151]. Therefore, the PARP-1 mediated poly(ADP-ribosyl)ation of nuclear proteins transforms DNA damage into signals that lead either to cell death or activation of the base excision repair pathway [150].

Since the discovery of PARP-1 about 40 years ago, many inhibitors have been developed both as potential adjuvants for use with DNA-interactive agents in anticancer therapies and as tools for investigation of PARP-1 function [152]. There is now convincing biochemical evidence at the cellular level, demonstrating that PARP inhibition can induce an enhanced sensitivity to ionizing radiation, topoisomerase I inhibitors, and DNA-alkylating agents [148]. The inhibition of PARP-1 has two potential therapeutic applications. The first application is as a chemopotentiator for many DNA targeting anticancer therapeutics as the PARP-1 mediated repair pathway is one major mechanism for the drug resistance and continued tumor growth [153]. Hence, PARP-1 inhibition together with DNA damaging chemotherapeutics or radiation would compromise the cancer cell DNA repair mechanisms, which results in genomic dysfunction and cell death [148]. The second application is as a standalone cancer therapy. It has been recently discovered that PARP-1 can be used as a mono-therapy for tumor types that are already deficient in certain types of DNA repair mechanisms [154, 155].

ABT-888 (Veliparib) was discovered starting from 3-aminobenzamide (**12A**, Fig. 40.12), a starting point for many PARP-1 inhibitors [156]. The very small size and good potency of the benzimiazole carboxamide core (**12B**,

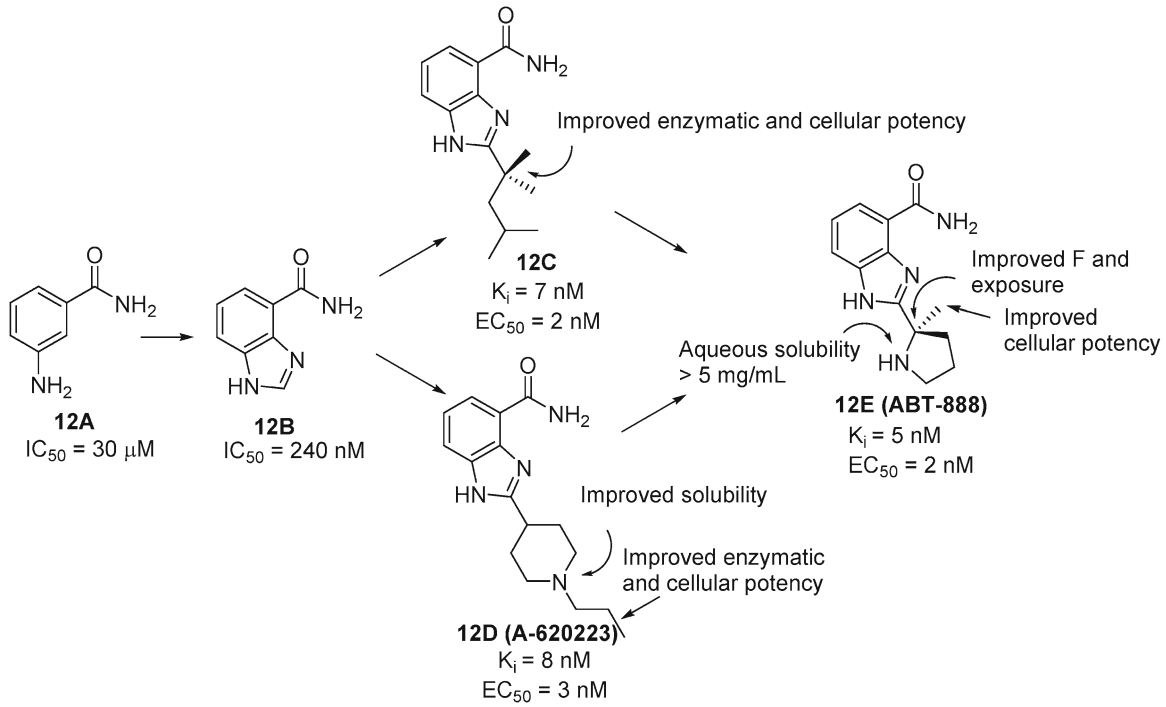

Fig. 40.12 Discovery of ABT-888.

IC_{50} = 240 nM, MW = 161) had attracted the Abbott group to synthesize several hundreds of 2-alkylamino derivatives. As a result, two closely related preclinical candidates **12C** (Ki = 7 nM) [157] and A-620223 (**12D**, Ki = 8 nM) [158] were identified. It was found that a quaternary carbon in the 2-alkyl side chain of the benzimidazole (**12C**) was beneficial for both enzymatic potency and cellular efficacy. Compound **12C** had a relatively short intravenous (iv) half-life across species (from 0.6 h in mice and monkeys to 2.8 h in dogs) and exhibited variable oral bioavailability (from 13 % in monkeys to 82 % in mice) [157]. Because of the variability of PK parameters, this compound was not advanced into human clinical trials. It was also found that adding an amino group provided adequate solubility for the core structure (>5 mg/mL for **12D**), and in the case of **12D**, also improved the cellular penetration and potency in a peroxide induced DNA damage cellular assay (EC_{50} = 3 nM). Compound **12D** showed good oral bioavailability across species (32–82 %) and half-lives of 1.2–2.7 h in the same species. This compound displayed excellent chemopotentiation activity with temozolomide (TMZ) and cisplatin in two subcutaneous, murine tumor models [158]. ABT-888 (**12E**, Ki = 5 nM, EC_{50} = 2 nM), was subsequently discovered from further optimization of this series [159]. The quaternary carbon adjacent to the benzimidazole ring was found to be necessary for high enzymatic potency and cellular efficacy and potency. While the enzymatic potency of **12E** and its (*S*)-enantiomer were identical (Ki = 5 nM), stereochemistry played an important role in both the oral bioavailability and exposure of the compounds. The (*R*)-enantiomer (ABT-888) was selected as the clinical candidate, which demonstrated excellent oral bioavailability across species (56–92 %) and a comparable half-life to preclinical leads **12C** and **12D** (1.2–2.7 h). Moreover, ABT-888 displayed moderate brain penetration (~1:3 brain/plasma ratio in rodents), a factor that influenced the eventual clinical path and its potential use in treating brain cancer. Based on its excellent chemopotentiation activity in preclinical xenograft models with TMZ and carboplatin [159], ABT-888 has been progressed to clinical development and is currently in several phase I, phase II and three phase III studies including neoadjuvant treatment of triple-negative breast cancer, non-small-cell lung cancer and HER2-negative, BRCA1, and/or BRCA2-positive breast cancer.

40.4 Inhibitors Targeting Epigenetic Mechanisms

Epigenetics is defined as heritable changes in gene function without modifications to the DNA sequence itself. Through modifications of chromatin that is primarily made up of DNA wrapped around octamers of histone proteins, these so-called epigenetic changes are present from birth to death and are involved in the first crucial steps that govern embryonic development, and also in influencing the expression and silencing of genes in human diseases [160].

The post-translational modifications of histones and DNA include: histone lysine methylation, arginine methylation,

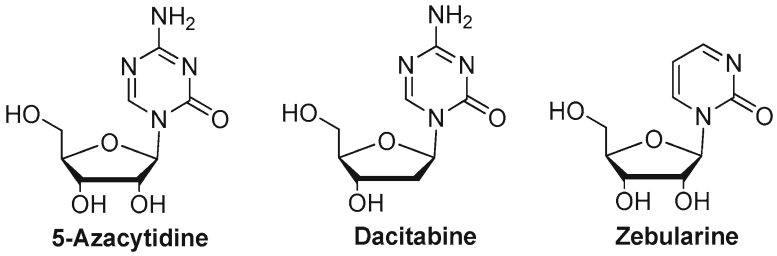

Fig. 40.13 Nucleoside DNMT inhibitors.

lysine acetylation, sumoylation, ADP-ribosylation, ubiquitination, glycosylation and phosphorylation, and DNA methylation [161]. Given the widespread importance of chromatin regulation to cell biology, the "writers" (the enzymes that produce these modifications), the "readers" (the proteins that recognize them), and the "erasers" (the enzymes that remove them) are critical targets for manipulation to further understand the histone code [162, 163] and its role in human diseases.

Epigenetic targets have been considered as attractive targets for therapeutic interventions because of the reversibility of epigenetic modifications [164]. Unlike genetic mutations, which are passively inherited through DNA replication, epigenetic mutations must be actively maintained. Therefore, faulty modification patterns could be corrected by pharmacologic inhibition of certain epigenetic modifications and thus directly change gene expression patterns and the corresponding cellular characteristics. Among the writers, readers, and erasers of the histone code, DNA methyltransferases and histone deacetylases have been studied most. In this section, we describe small molecule DNA methyltransferase inhibitors [165] and histone deacetylase inhibitors [166] approved by the FDA or in clinical development.

40.4.1 DNA Methyltransferase Inhibitors

DNA methylation can inhibit the binding of control proteins, such as transcription factors, to promoters, thus directly switching off gene expression. It is an essential epigenetic modification required for normal mammalian development, gene regulation, genomic imprinting, and maintaining chromatin structure [167]. However, human tumor cells frequently show altered patterns of DNA methylation, particularly at CpG islands, which are DNA sequences rich in CpG dinucleotides often found close to gene promoters [168]. Methylation within islands appears to be associated with transcriptional repression of the linked genes. Genes involved in all phases of tumor development and progression can become methylated and epigenetically silenced [169]. Re-expression of such silenced genes could lead to suppression of tumor growth or sensitization to anticancer therapies [170].

DNA methylation occurs at the C-5 position of cytosine and is carried out by a family of DNA methyltransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B [171]. DNMT1 acts primarily as a maintenance methyltransferase by copying existing methylation patterns following DNA replication [172]. DNMT3A and DNMT3B exhibit de novo methyltransferase activity and are required for establishing new methylation patterns during embryonic development [173].

The prototypical inhibitor 5-azacytidine (5-AzaC; Mylosar™) (Fig. 40.13) has shown promising response rates in myelodysplastic syndrome (MDS) patients and has recently been approved by the FDA. It is a simple derivative of the nucleoside cytidine described more than 40 years ago [174]. Its demethylating activity was discovered later because of its ability to influence cellular differentiation [175]. Inside the body, 5-azacytidine can be chemically modified into a deoxyribonucleoside triphosphate and this triphosphate is able to be incorporated into DNA. When DNMTs methylate the 5-azacytosine residues in DNA, a covalent, irreversible protein–DNA adduct is formed between DNMTs and 5-azacytosine residues, which leads to DNMTs being trapped and inactivated [176]. Consequently, cellular DNMTs are rapidly depleted. As the DNA replication continues, the genomic DNA is demethylated. However, before converted into deoxyribonucleoside triphosphate, a portion of 5-azacytidine can be converted to a ribonucleoside triphosphate and incorporated into RNA, which affects a variety of RNA functions. Therefore, 5-azacytidine has cellular consequences independent of demethylation [177].

5-Aza-2′-deoxycytidine (decitabine, Fig. 40.13), a deoxyribose analog of 5-azacytidine, is another DNMT inhibitor [178]. Because this compound already possesses a deoxy sugar moiety and does not need to be modified before converting to the triphosphate form, it can be more directly incorporated into DNA. Therefore, Decitabine should be theoretically more specific and less toxic than 5-azacytidine. Indeed, greater inhibition of DNA methylation and antitumor activity in experimental models was achieved with this drug [177]. Decitabine, as a single agent, showed activity in myeloid malignancies [169, 179], including myelodysplastic syndrome (MDS), acute myelogenous leukemia, and chronic myelogenous leukemia and has been approved by the FDA for treating all MDS subtypes, though it is used most commonly to treat higher-risk MDS. However, substantial toxic effects were also found with Decitabine, in particular myelosuppression with neutropenic fever [180]. These toxic effects

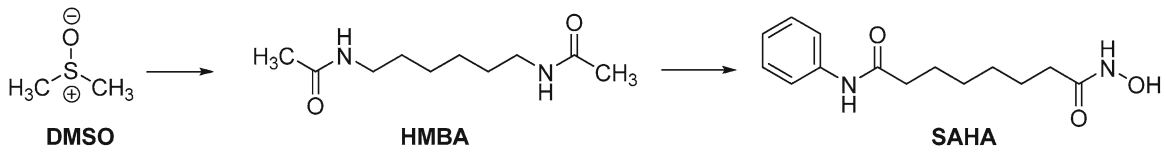

Fig. 40.14 Discovery of ™HDAC inhibitor SAHA.

are likely due to forming the covalent adducts between DNA and the trapped DNMTs [181].

More recently, zebularine (Fig. 40.13), another derivative of 5-azacytidine, has been discovered as a nucleoside DNMT inhibitor [182]. Similar to 5-azacytidine and Decitabine, Zebularine is incorporated into DNA as a cytosine analog after several chemical modifications. It is more stable than 5-azacytidine or decitabine. However, because of the low oral bioavailability in monkeys, Zebularine has not been evaluated in clinical trials [183]. Because of the similar mechanism of action as other nucleoside DNMT inhibitors, zebularine also sufferes from the toxicity resulting from covalent enzyme trapping.

The inherent cytotoxicity of nucleoside DNMT inhibitors poses considerable limitations on their use as therapeutic agents. Non-nucleoside DNMT inhibitors, on the other hand, are likely less toxic because they are not incorporated into DNA. A significant amount of research efforts are devoted to discovering non-nucleoside DNMT inhibitors [165]. Progress in this area is keenly awaited.

40.4.2 Histone Deacetylase Inhibitors

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) control the level of acetylation of lysine residues in histone N-terminal tails [162]. The positive charge on histone lysine residues is masked by the addition of an acetyl group and therefore the affinity of the histones for DNA is decreased (e.g., chromatin opening), which generally facilitates transcriptional activation. Removal of the acetyl group by HDACs reconstitutes the positive charge of the lysine residues and consequently makes that the affinity of histones for DNA is increased, which leads to transcriptional repression (e.g., chromatin closing). HATs and HDACs exist in multi-protein complexes, where they work together to maintain fine control of transcriptional activation and repression [184].

HDACs have been pursued as anticancer targets based on results from numerous target validation studies [185]. It has been shown that aberrant recruitment of HDACs is associated with the development of certain human cancers [186]. By inhibiting the deacetylation of histone lysine residues, HDAC inhibitors (HDACIs) maintain the neutralized charge state on the histones to open up the phosphate backbone of the DNA and therefore facilitate the transcription of many genes, including tumor suppressor genes silenced in cancer. Moreover, acetylation of histones destabilizes DNA–nucleosome interaction and renders DNA more accessible to transcription factors [163]. In addition to inducing growth arrest and activating differentiation pathways, HDACIs have also been shown to be efficient inducers of apoptosis in several cellular systems [187].

There are four classes of HDACs in human with altogether 18 HDAC enzymes [188]. Class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), and class IV (HDAC11) HDACs are zinc-binding enzymes [189]. The class III HDACs, the so-called Sir2 family, represent a structurally and mechanistically distinct class of NAD^+-dependent hydrolases [190]. Most of the HDACIs developed or being developed to date mainly target class I and II HDACs.

Suberoylanilide hydroxamic acid (SAHA, Vorinostat, Zolinza™) (Fig. 40.14) is the first HDACI approved by the FDA [191]. The discovery of HDACIs began with the initial observation by Charlotte Friend. When dimethylsulfoxide (DMSO) was placed with supertransfect murine erythroleukemia cells (MELCs) in culture, Charlotte Friend, in 1971, observed that many of these cancer cells turned red—suggesting the presence of hemoglobin [192]. Later on, it was found that the polar group of DMSO was required to induce the differentiation of MELCs, and simple polar amides were better than DMSO [193]. Compounds with two amides were found to be more potent, presumably because of the chelating effect that makes stronger binding. This led to the discovery of hexamethylene bisacetamide (HMBA), which was 6- to 20-fold more potent than simple acetamide [194]. HMBA was found to be able to induce growth arrest and differentiation of various transformed cells [195] and selectively alter gene expression [196, 197]. Extensive SAR study of this series led to the discovery of bishydroxamic acids with as much as two orders of magnitude more potent than HMBA in inducing transformed cell growth arrest and cell death [198, 199]. Among them, SAHA was active at low μM concentrations in causing MELC growth arrest and differentiation. The X-ray co-crystal structure of SAHA in complex with a histone deacetylase-like protein (HDLP) has revealed that SAHA binds to the catalytic site of the enzyme [200]. The hydroxamic acid group doubly coordinated to a zinc atom at the bottom of the catalytic cavity. The left-hand side

phenyl group of SAHA interacts with the hydrophobic surface of the enzyme while the polymethylene chain extends down a relatively narrow channel.

SAHA inhibits all class I and II HDACs at about 50 nM and arrests cell growth of a wide variety of transformed cells in culture at 2–5 μM [201–203]. It selectively alters the transcription of expressed genes in transformed cells, which includes the upregulation of pro-apoptotic genes and the downregulation of a constellation of anti-apoptotic genes [204–207]. In October 2006, SAHA was approved by the FDA for the treatment of cutaneous T cell lymphoma (CTCL).

Another FDA approved HDAC inhibitor is Romidepsin (Istodax™) [208] which is a natural product obtained from the bacteria *Chromobacterium violaceum* [209]. It was approved for the treatment of CTCL in October 2009. Most currently, two new HDAC inhibitors, belinostat (Beleodaq™, approved by FDA in July 2014) and panobinostat (Farydak™, approved by FDA in Feburary 2015) have also been approved for the treatment of peripheral T-cell lymphoma and multiple myeloma after at least 2 previous treatments, including bortezomib and an immunomodulatory agent respectively.

40.5 Inhibitors of Emerging Targets

Many emerging targets and novel approaches are being pursued by the academic research community and the pharmaceutical and biotech industry in order to develop novel, more effective targeted cancer therapeutics. In this section, we use the discovery of inhibitors of heat shock protein 90 (HSP90) [210] as an example to highlight the progress made in these novel approaches.

40.5.1 Inhibitors of Heat Shock Protein 90 (HSP90)

The function of the heat shock proteins (HSPs) is to fold and maintain the proper conformation of other proteins, called clients, so that they can function properly. HSPs are therefore molecular chaperones. They are overexpressed when a cell encounters transient temperature increase or other stresses, such as oxidants or radiations, to assist in refolding denatured client proteins [211]. In addition, HSPs play a routine homeostatic role in regulating client protein folding under non-stressed conditions [212]. They account for 5–10 % of all proteins in the cell, and are among the most abundant of cellular proteins. One protein of this type, HSP90, which even under basal, non-stressed conditions comprises approximately 1 % of the cellular protein population [213], is particularly important because it is a master protein that controls a series of other HSPs. It is also crucial for the folding of several client proteins, many of which are highly relevant to cancer including mutated p53 [214], BCR-ABL [214], HER2/neu, Raf-1, VEGFR, Cyclin-Dependent Kinase 4 (CDK4), c-Met, Polo-1, Akt, Src, FLT-3, human telomerase reverse transcriptase (hTERT), and steroid hormone receptors [215–220].

Inhibition of HSP90 causes client proteins to adopt aberrant conformations, and these abnormally folded proteins are rapidly eliminated by cells via ubiquitination and proteasome degradation. Because cancer cells typically have a large number of mutated proteins that might not fold properly, HSP90 is often overexpressed in cancer cells so that even mutated proteins can be folded sufficiently well to avoid disposal by the proteasomes, which allows cancer cells to survive [221]. In general, HSP90 inhibitors display remarkable selectivity for cancer cells compared to normal cells [222]. As HSP90 is involved in the correct functioning of so many oncoproteins and pathways (including signal transduction and transcription), inhibition of HSP90 should be able to block multiple oncogenic pathways in cancer cells, which is more preferable compared with targeting a single point of vulnerability [223]. Therefore, this type of "combinatorial blockade" of oncogenic targets has a greater chance to inhibit most if not all of the hallmark traits of malignancy, has the potential for broad-spectrum clinical activity across multiple cancer types, and could diminish the potential of the tumor acquiring resistance to any single therapeutic pathway.

Among all the 16 HSP90 inhibitors advanced to clinical trials to date [224], SNX-5442, an orally bioavailable and efficacious prodrug of SNX-2112, was developed by Serenex (acquired by Pfizer in 2008) and has been in clinical development since May 2007 [225]. The discovery of SNX-5442 started from compound **15A** (Fig. 40.15), a screening hit with low binding affinity to HSP90 (K_d = 3.7 μM) [226]. Although it was inactive in multiple cellular assays (IC_{50} > 50 μM), compound **15A** was considered a tractable starting point because of its relatively high selectivity for HSP90, high ligand binding efficacy, and chemically novel scaffold that is amenable to a broad range of structural modifications.

Initial SAR studies revealed that the C-2 (*ortho*) substitutions on the phenyl ring with an NH linker increased HSP90 binding affinity. By optimizing this 2-amino moiety, compound **15B** with much improved binding affinity was identified. However, this compound had poor cellular activity. To improve cellular potency, the indol-4-one moiety was modified. It was found that moving the C-2 methyl group to the C-3 position (**15C**) significantly improved cellular potency and activity. However, **15C** was neither orally bioavailable nor effective in mouse xenograft tumor models following intraperitoneal (ip) dosing. In addition, the electron rich trimethoxyaniline ring could potentially be metabolized to reactive quinone species. To address these problems, the tri-

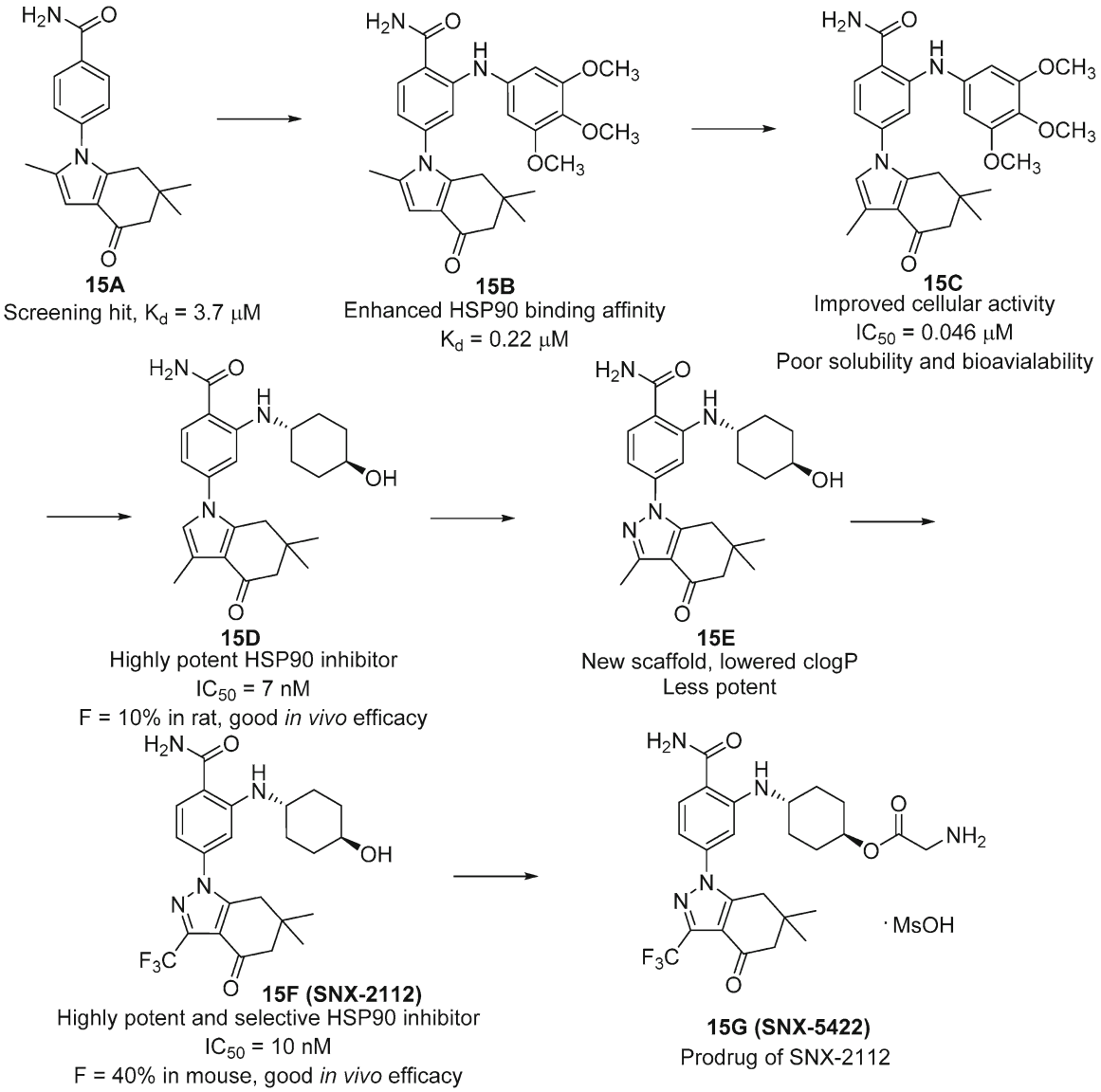

Fig. 40.15 Discovery of HSP90 inhibitor SNX-5422.

methoxyaniline ring was replaced with a trans-4-hydroxycyclohexylamino group, which resulted in compound **15D** with high potency, measurable oral bioavailability (ca. 10 % in rat), and good in vivo efficacy in mouse xenograft tumor models (ip dosing) [226]. Replacing the metabolically liable pyrrole moiety (**15D**) with the pyrazole moiety (**15E**) increased stability and polarity, thus aqueous solubility. However, compound **15E** was less potent than compound **15D**. Further exploration of this new scaffold afforded compound **15F** (SNX-2112), which was equally potent as **15D**. The amorphous form of compound **15F** displayed good oral bioavailability (40 %, mouse) and in vivo antitumor efficacy in mouse xenograft models following oral dosing. Compound **15F** was highly selective for HSP90 over a panel of 75 enzymes and receptors and did not exhibit significant inhibition against a panel of 9 cytochrome P450 enzymes. However, the crystalline forms of **15F** were not orally bioavailable, and their aqueous solubility at physiological pH (7.4) was reduced 25-fold to 6 μM. A simple ester prodrug strategy was explored and resulted in the discovery of the glycine ester **15G** (SNX-5442). SNX-5442 was crystalline, stable, and reasonably soluble (~1 mg/mL, pH 4–6). Excellent exposure of the parent compound **15F** can be achieved following oral and intravenous dosing indicating that the conversion of ester **15G** to alcohol **15F** was rapid and complete in vivo. SNX-5442 was selected as a clinical candidate based on its superior oral bioavailability and in vivo efficacy. Later, the development of SNX-5422 was discontinued during phase I clinical trial based on reports of ocular toxicity and the potential for irreversible retinal damage [227].

40.6 Conclusion

Over the last several decades, discovering and developing targeted cancer therapeutics has been one of the fastest growing areas in the cancer research field. The drug discovery examples described above illustrate the tremendous success in this exciting and fast moving area. As this field continues gaining momentum, one eagerly awaits the arrival of the next groundbreaking discoveries and lifesaving medicines.

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Molecular Detection and Diagnosis of Cancer

41

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41.1 Introduction

The incidence of cancer is increasing worldwide, owing to the trends toward increased lifespan and adaptation to western lifestyle [1]. Although progress has been made in therapeutic and diagnostic strategies contributing to a slight reduction in mortality, cancer still remains a serious health condition and is often fatal. Many cancer deaths occur because the disease is usually diagnosed at advanced stages and has spread to distant organs with lymph node involvement, where most of the treatment options fail. It has been well demonstrated that if the disease is detected earlier the chances of 5-year cancer-free survival and reduction in mortality are better. For example, colon cancer survival rates for localized disease are 82–93 %, compared with only 5–8 % for cases with distant disease [2]. Similarly, significant mortality reduction in cervical cancer cases is mainly due to the availability of effective screening strategies [3]. Some cancers such as ovarian, pancreatic, and lung remain asymptomatic and are diagnosed at advanced stages with poor survival [4–6].

Cancer detection and diagnosis is critical to population screening, cancer staging, prognosis, and consideration of appropriate measures for treatment. Current cancer diagnostic strategies rely mostly on imaging, tumor size, histology, lymph node involvement, and other clinical parameters. Unfortunately, imaging modalities suffer from lack of sensitivity and fail to detect tumors smaller than 1 mm. Mammography, used to screen breast abnormalities, suffers from lack of sensitivity and leads to a large number of false-positive rates [7]. Malignant tumors are repeatedly much smaller than can be detected by these approaches. For example, colonoscopy is commonly considered the gold standard for detecting colon cancer, yet it fails to detect some of the flat adenomas that are highly malignant [8–10]. Moreover, these approaches do not provide detailed information on molecular phenotypes of cancer. Breast cancer staging and mammography fall short of giving information on personalized treatment choices that are based on molecular analyses such as estrogen receptor expression or human epithelial growth factor receptor (Her-2) expression [11]. There is a clear need for better diagnostic tools that will enable improved prognostic prediction and therapeutic strategies.

41.2 Cancer Is a Molecular Disease

Cancer is a heterogeneous molecular disease and carcinogenesis is a multistep process wherein normal cells evade normal growth regulatory and inhibitory signals and enter a state of uncontrolled cell growth and proliferation leading to malignant transformation. The first step, termed initiation, involves changes at the genetic level and mutations leading to DNA malfunction. This is usually rapid and irreversible with no appreciable morphologic changes. However, it may involve mutational changes in oncogenes and tumor suppressor genes. The second step in the neoplastic transformation is promotion, which involves increased cell growth and proliferation. This step is typically reversible and involves a mixture of genetic and epigenetic cellular changes leading to alterations in protein expression and metabolic pathways. The third step, progression leads to ultimate malignant transformation with a carcinogenic phenotype. The process of progression is very long, often proceeding over many years, and disrupts cellular processes and communication pathways leaving unique cellular and molecular profiles for cancer. These molecular and cellular signatures present great opportunities for the development of clinical tools and assays to diagnose cancer and to complement existing diagnostic strategies.

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_41

The systematic study of the cancer genome has led to the discovery of mutations of key members of gene families involved in signal transduction, which are essential for the physiological regulation of gene expression, cell cycle progression, and programmed cell death, and associated with different stages of oncogenesis. The genome-wide analysis of the protein-encoding genes in breast and colorectal cancer has helped define the genetic landscape of these two types of human cancer [12] and such studies are currently extended to other types of human cancer. Pancreatic cancer is an aggressive disease with poor prognosis. The currently 5-year survival rate, despite aggressive therapy is still less than 5 %. Activating mutations of the K-Ras oncogene are highly prevalent in pancreatic cancer (>80 %) [13], and are assumed to occur during the initial stages in its development [14, 15]. Pancreatic cancer is also associated with high rate of inactivation of three tumor suppressor genes: TP53 (~50 %), $p16^{INK4A}$ (80–95 %), and SMAD4 (~50 %) [16–19]. Based on the understanding of the temporal sequence of acquisition of these mutations, a progression model of pancreatic cancer development emerges, in which genetic mutations occur in a temporally ordered sequence. While some function as gatekeepers in the initiation of neoplastic growth, such as the activating K-Ras mutation, others ($p16^{INK4A}$, TP53) allow tumor growth and malignant progression (Fig. 41.1). This illustration is provided only as an example and does not encompass many of the molecular pathways that are associated with different types of cancers and have been extensively studied and reported elsewhere [20–25]. The delineation of the different pathways whose function is altered in different types of cancer provides the scientific basis for the development of novel biomarkers (Table 41.1).

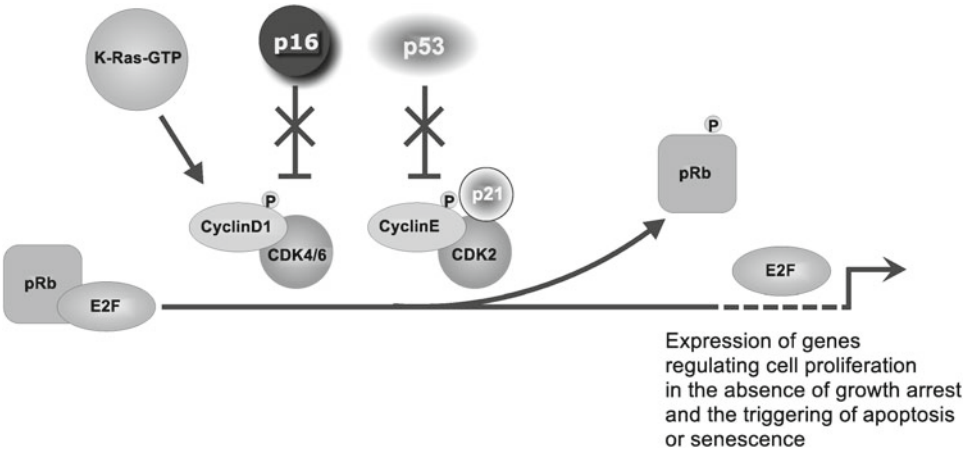

Fig. 41.1 Activating mutations in K-Ras occurring early on during pancreatic cancer development lead to higher proliferating rates of affected cells. While this may trigger the cell surveillance system that may cause cell growth arrest and apoptosis or senescence, the additional loss of expression of $p16^{INK4A}$ and/or mutation of TP53 allow the higher proliferating potential of the cells and the progression toward malignancy.

Table 41.1 Candidate biomarkers for cancer detection, risk assessment, and diagnosis

Type/marker name	Cancer type (organ)	Biospecimen	Utility	Reference
Epigenetic	Kidney	Tissue	Risk assessment	[97]
VHL	Pancreas	Pancreatic fluid	Risk assessment	[18]
$p16^{INK4a}$	Colon	Tissue	Disease recurrence	[98]
TIMP3	Lung	Sputum, Plasma	Diagnosis	[98]
Gene methylation panel				
Genetic	Pancreas	Pancreatic/bile fluids	Early detection	[99]
K-Ras (mutation, amplification)	Colon (HNPCC)	Tissue	Diagnosis	[97]
Bethesda microsatellite panel	Bladder	Urine	Early detection	[98]
Microsatellite assay (MSA)	Esophagus	Tissue	Disease prediction	[98]
FISH panel				
Genomic	Breast	Tissue	Disease prediction	[70]
OncotypeDx	Breast	Tissue	Disease prediction	[80]
Mammaprint				
Proteomic	Liver	Serum	Early detection	[94]
Des-γ-carboxyprothrombin (DCP)	Liver	Serum	Early detection	[94]
Alpha-fetoprotein (AFP)-L3	Liver	Serum	Early detection	[98]
MALDI 6-peak profile	Ovary	Serum	Early detection	[95]
Yale 6-protein marker panel	Breast	Tissue	Disease prediction	[84]
COX-2/Ki67/$p16^{INK4a}$	Breast	Tissue	Disease prediction	[100, 101]
MMP1/CEACAM6/HYAL1	Pancreas	Serum	Early detection	[98]
Mucin protein panel assay				

Identified new targets can be utilized for the design of novel strategies for screening for early cancer detection and molecular diagnosis, and for the development of novel preventive and therapeutic modalities.

41.3 Early Detection Versus Diagnosis

A cancer biomarker is defined as any molecular or biochemical alteration that can be measured and effectively used in a clinical scenario for cancer detection, diagnosis, prognosis, and prediction of therapeutic response. Cancer detection usually involves clinical testing of asymptomatic healthy subjects as in population screening or following stratified high-risk cohorts for the presence of preclinical/early disease. It has several implications in the clinical context, such as enabling further investigation to determine the extent of disease, or taking measures to contain the initial disease by surgical removal, limiting therapeutic treatment, or adapting preventive care. Ideally, a cancer biomarker for detection is present in easily accessible body fluids such as saliva, blood, urine, upper digestive tract effusion, and the material that can be extracted by fine needle aspirates and other minimally invasive procedures. It enables easy screening of a larger population without requiring extensive training and clinical expertise. However, biomarkers for diagnosis need to provide extensive information about the tumor and its microenvironment. For this reason, tissue biopsies and other invasive procedures are also used when necessary to enable critical decisions.

To be useful for detection, a cancer biomarker ideally should be able to distinguish normal individuals from cancer patients with 100 % accuracy. Sensitivity and specificity determine how accurately a biomarker can predict the presence of a disease and its ultimate utility in clinical practice. Sensitivity is defined as the ability of the biomarker to detect the cancer cases (true positive); while specificity is defined as the ability of the marker to identify the healthy cases (true negative). A useful biomarker should be able to detect cancer with high precision, allow early detection of the premalignant lesions and ultimately reduce mortality due to cancer. Unfortunately, most of the current biomarkers do not have sufficient diagnostic power to be clinically useful if used alone. The widely used clinical marker prostate-specific antigen (PSA) has only 70 % sensitivity and 59–97 % specificity [26–28]. When used in population screening, PSA, due to its low specificity, leads to many false positives and triggers unnecessary biopsies and clinical follow-up. CA-125, the biomarker for the detection of ovarian cancer, has only 70 % sensitivity and 87 % specificity and is not currently recommended for population screening [29–32].

In contrast, diagnosis of cancer requires more confirmatory tests with high precision and sensitivity to enable more critical decisions, such as surgical removal of vital organs, decisions related to suitable chemotherapy, preserving unaffected organs and follow-up. Moreover, it is important to differentiate different types of cancers that affect the same organ to enable additional diagnostic and efficient treatment strategies. *BRCA1* and *BRCA2* mutations affect the incidence of genetically predisposed breast cancers. Genetic screening of breast cancer patients for these mutations is complex and time-consuming owing to the large size of both genes. It is desirable to have more understanding of both the phenotype and the morphological and molecular characteristics of the tumors underlying these mutations. *BRCA1*-associated tumors are usually poorly differentiated, infiltrating ductal carcinomas and show frequent morphological features of atypical medullary carcinomas. *BRCA2*-associated tumors tend to be of higher grade than sporadic cancers. Additionally, *BRCA1* tumors are usually estrogen receptor and progesterone receptor negative and p53 positive, whereas such changes are not seen in *BRCA-2*-associated tumors. Both *BRCA1*-associated and *BRCA2*-associated breast tumors show a low frequency of Her-2 overexpression compared to sporadic cancers, in addition to various other molecular abnormalities [11]. Such information can be especially useful in making decisions in appropriate screening and therapeutic choices. However, the current imaging modalities only provide spatial resolution of tumors, but not their molecular composition. As such, there is a continuing need for multiple molecular markers to enable accurate detection and diagnosis of the disease.

41.4 Molecular Biomarkers of Cancer

The biology of carcinogenesis provides a large window of opportunity to study the range of molecular events that can provide information on malignant transformation. Over the last 30 years, there has been a tremendous explosion in the understanding of cancer biology. For example, it has been well characterized that Ras and Raf mutations and alterations in downstream MAP kinase pathways frequently form the early events in malignant transformation and have been explored as potential anticancer targets in a variety of cancers [33]. Epidermal growth factor receptor (EGFR) mutations have been implicated in the progression of breast, lung, and other cancers. Epigenetic changes and *P53* mutations have been indicators of late events of progression [33–35]. While many of the alterations in these pathways could provide molecular signatures that oncologists can reliably use in the detection and diagnosis of cancer, none of them have actually translated well into clinical application. One of the main problems in molecular diagnosis of cancer is that it is a cellular proliferation disease and many of the cancer-related proteins and changes are part of normal pathophysiological

phenomena and not specific to cancer. No abnormal molecular changes are effectively detected that indicate malignant transformation. Many of these events are viewed as part of the body's normal inflammatory responses. For example, it has been demonstrated that many of the initial changes are usually confined to the tumor microenvironment, making them inaccessible for noninvasive molecular diagnostic approaches [36]. The magnitude of the malignant changes associated with minute tumors by and large go undetected mainly because these changes are very small compared to variations in normal cellular and physiological processes. Confounding this problem is that most of the molecular detection approaches lack sufficient analytical sensitivity to detect such subtle changes. The detection sensitivity of currently used ELISA and other clinical assays is 1–2 pg/ml, which fails to detect molecular markers associated with cancer that are secreted at much lower levels (fg/ml) [37–40]. Detection systems with much lower detection limits (in Pico- and Femto-molar concentrations) are needed to detect such abnormal proteins.

41.5 New Technologies for Detection and Diagnosis Marker Discovery

Genome-wide DNA sequence analysis has led to a surge in the enthusiasm for the application of distinct high-throughput approaches to analyze multiple biomolecules giving rise to novel omic technologies. Using these technologies, it is now possible to analyze multiple molecular changes in DNA (genomics), RNA (transcriptomics), proteins (proteomics), or metabolites (metabolomics) in a limited sample size in a single platform, thereby saving time, efforts, and costs.

Current cancer research has taken a multidisciplinary approach to address the gaps in the translation of cancer molecular biomarkers into the clinic. This approach may lead to the identification of novel biomarkers that conventional approaches have long failed. High-throughput molecular analytical techniques combined with the latest tissue acquisition techniques, such as laser-aided microdissection enable global molecular profiling with very high precision. Both targeted and untargeted approaches are employed giving rise to information on either known molecular species or molecular profiles, such as in mass spectrometry. The resulting data are analyzed using state-of-the-art computational analytical tools for pattern recognition allowing the differential diagnosis of cancer. It is generally unnecessary to identify the molecular nature of the peaks (black box approach). With computational methods, it is possible to integrate the information from diverse markers to improve their clinical sensitivity and specificity for cancer detection and diagnosis. This has opened the door to a novel paradigm of cancer diagnosis in which a single marker for a single disease is no longer the norm. Instead, multiple markers for a single disease are the mode of choice. It is assumed that such a strategy allows accurate measurement of unique biological processes measured simultaneously and integrated for improved diagnosis.

41.6 Role of Specimens in Biomarker Development

Most of the molecular analysis approaches rely on the availability of freshly collected tissues as compared to fixed tissue and archived specimens. Some of the molecular approaches demand highly defined and precise cellular species. The introduction of laser scanning capture microdissection (LSCM) techniques into clinical specimen collection has improved the quality of the specimens for analysis. The need for immediate sample processing, fresh-frozen tissues, and concomitant specimen storage conditions, and the effect of the length of storage time make the molecular diagnostic approaches difficult to adapt in clinical practice. These issues undermine the utilization of existing repositories for biomarker development. However, some recent development efforts focused on the utilization of fixed tissues by high-throughput molecular approaches are improving this situation. The collection of specimens for molecular analysis, such as nipple aspirate fluid and ductal lavage for breast cancer detection and pancreatic effusion fluid for pancreatic cancer detection, poses further challenges in molecular diagnostics, and the development of special collection methods is needed.

41.7 High-Throughput Analysis and Biomarker Development

One of the main issues in high-throughput technological analysis is analytical sensitivity. Depending on the sensitivity of the technology employed, the numbers of molecular species resolved can vary widely. For example, 2D gel electrophoresis has a limited resolution of only 200–300 proteins per analysis and mainly focuses on the resolution of highly abundant proteins. More advanced mass spectrometric analyses, however, allow the resolution of hundreds to thousands of proteins ranging from MW 2000 to 30,000. As such, it may not always be possible to resolve both high-abundant and low-abundant proteins on the same technology platforms but it is usually difficult to compare proteins resolved on different platforms. It is possible to identify some of the low-abundant proteins (functional peptidomics), using more advanced mass spectrometric techniques combined with time-of-flight (TOF) techniques, and exoprotease digestion techniques [41]. Mass spectrometric techniques routinely suffer from inconsistencies in reproducibility mainly because of the nature of instrumentation, mass calibration shifts and

factors related to baseline correction in mass spectrometric analysis [42, 43]. Another confounding factor in reproducibility is the batch variations in protein chips; it is normally difficult to overlay the sets of features from different runs of the same specimens and to validate these molecular features as biomarkers.

High-throughput approaches, such as mass spectrometry, yield patterns and profiles that are subjected to computational analyses to simplify the dimensionality of the data and to identify meaningful patterns that may be clinically significant. Two major types of computational approaches, namely supervised and unsupervised methods, are applied to analyze the data. In the supervised methods, the information is organized in a predetermined fashion as to which molecular changes are expected to group together and the resulting patterns are analyzed collectively for their ability to differentiate cancers. When applying supervised approaches, it is difficult to analyze the patterns without established classification rules. Depending on the size of the training set, the test results give diverse patterns that may not be reproducible. With unsupervised methods, the data are grouped based on their inherent expression patterns and the resulting profiles are tested for their ability to group together and predict the outcome. Unsupervised approaches rely on imposition of algorithms that tend to fault depending on the method. Thus, the method of data analysis plays an important role in biomarker development. One of the confounding aspects with high-dimensional data analysis is the signal-to-noise ratio. Many genes undergo changes in expression that are not necessarily specific and related to cancer. These changes are recorded by microarray and other high-throughput approaches, thereby resulting into complex patterns and enormous volumes of data and leading to erroneous over-fitting of the data.

Another critical factor in the application of high-throughput technologies in biomarker development is that the platforms for discovery and development may not be the same as for clinical application, where more directed, simpler, and specific tests are needed. Typically, more than one approach can be applied to detect a molecular marker. For example, three molecular tests for human epidermal growth factor receptor-2 (Her-2/neu) oncogene expression have been approved by the Food and Drug Administration (FDA): one to monitor the treatment response of breast cancer with herceptin, a second to determine the sensitivity to doxorubicin therapy, and the third for the early detection of recurrence and to determine the prognosis of breast cancer [44–46]. Her-2 expression can be detected by various methods at the protein level (by immunohistochemistry; IHC), at the DNA level [using fluorescence in situ hybridization (FISH) analysis and polymerase chain reaction (PCR) amplification] or at the messenger RNA level. The FDA-approved tests include a FISH-based molecular assay and two antibody-based assays. However, a number of antibodies with varied sensitivity are commercially available, which together with kit to kit variability they make the detection of Her-2 a complicated task with many false-positive cases and misleading results. While FISH analysis is very sensitive, its high cost and marginally superior results make it less attractive as a routine monitoring tool [46]. Thus, the choice of the test method and its application depends on the analytical sensitivity and the cost-effectiveness.

The molecular profiles or signatures that arise from mass spectrometric and other high-throughput approaches may have clinical significance and could be successfully applied in the clinic if they have high sensitivity and specificity (even without prior knowledge of the molecular species contributing to the signatures). However, the negative results are difficult to interpret; hence, the absence of information on the peaks can often lead to inconclusive results. These molecules therefore should be further characterized using more specific analytical methods such as southern, northern, and western analyses or quantitative polymerase chain reaction (qPCR) and IHC, which can allow easier application of the molecular tests in a clinical setting [47].

Approaches to detect LMW proteins include the use of nanoparticles conjugated to molecular baits. These enable selective LMW protein binding and release on harvest platforms with nanoparticles such as nanobarcodes with distinct physicochemical properties [48].

One of the impediments in cancer biomarker development is that the tumor-specific proteins and cytokines are secreted at very low concentrations below the analytical sensitive range of most clinical methods. The real challenge is to identify such low abundant proteins that can provide critical information on the nature of a tumor. A variety of approaches are applied to detect such proteins either on sandwich arrays, where a pair of antibodies is used to detect each protein, or a label-based system, where target proteins are labeled to enable detection after they bind to the arrays. These approaches are highly sensitive and specific and assays are generally easy to develop [49, 50]. There are also other approaches that may significantly improve the sensitivity, accuracy, and reproducibility [50]. These include the two-color rolling-circle amplification antibody arrays, which amplify the signals from labeled proteins. Such methods allow detection of multiple proteins from two different sets of samples simultaneously. Two sets of samples are covalently labeled with biotin and digoxigenin individually and incubated with antibody arrays. The immobilized labeled proteins are detected using antibodies against biotin and digoxigenin that are covalently conjugated to two separate primers. The primers are amplified by complementary circular DNAs tagged to two different colors (cy5 and cy3) by DNA polymerase. This results in signal amplification in two colors for two different sets of proteins. These approaches allow acquisition of expression profiles of multiple proteins

from diverse samples and require very small biological specimens with minimal processing, making them attractive analytical tools. Although still in the developmental stage, these tools are beginning to show promising results when applied to clinical specimens [50].

Host immune systems play a critical role in surveying infectious and foreign bodies, including tumor antigens, and responding by eliciting antibodies, thereby paving the way for novel detection strategies for early detection of cancers. Autoantibodies are promising tools for detecting cancers as they are elicited in response to low concentrations of circulating tumor antigens; and the production of a large repertoire of antibodies makes the detection highly sensitive. Unlike tumor antigens, which are usually degraded by proteases, the antibody response persists for a long time even after the antigen has been removed from the circulation. They are more stable with a relatively long half-life in circulation. Assorted targeted antibody array approaches are attempting to identify cancer-specific autoantibodies that have promise for cancer detection, diagnosis, and therapeutics. These approaches make use of antigen libraries created on a variety of biological vectors such as phages, yeast, and others created from cancer cell lines, or by expressing protein in situ using DNA vectors and in vitro translation methods [51, 52]. The protein arrays are challenged with sera from healthy individuals and cancer patients to identify cancer-specific autoantibodies that interact with tumor antigens present on the arrays. Although the field is still emerging, growing evidence indicates that this approach may have important implications in cancer detection and diagnosis [51, 52]. Other emerging biomarker discovery modalities include the application of molecular species-specific flow cytometry to study pathological signal networks at the single cell level. Danna et al have used this approach to study molecular signaling alterations in acute myeloid leukemia [53].

MicroRNAs are small non-coding highly conserved RNAs that are derived from pre-miRNA precursors by cytosolic RNase action. They play an important role in diverse cellular functions including proliferation, differentiation, and death. Currently, more than 400 miRNAs have been identified. The relatively small numbers of miRNAs and their role in cellular functions make them promising candidates as biomarkers for diagnosis and detection. Despite the fact that the miRNA field is still in its infancy, evidence suggests miRNAs can provide critical information on the diagnosis, prognosis, and therapeutic targets of cancer [54]. Using miRNA microarray analysis, Bloomston et al identified several miRNAs that are differentially expressed among pancreatic adenocarcinoma, benign pancreatic tissue, chronic pancreatitis, and normal pancreas [55]. Using the Predictive Analysis of Microarrays method, they identified a subset of 21 miRNAs overexpressed and four miRNAs underexpressed that could discriminate pancreatic cancers from normal pancreatic tissue with 90 % accuracy. In the same study, they identified a set of 15 miRNAs overexpressed and eight miRNAs underexpressed which could identify pancreatitis from pancreatic cancers with 93 % accuracy. A set of 15 overexpressed miRNAs and two underexpressed miRNAs could differentiate pancreatitis from normal pancreas. These early results need further study, but these studies demonstrate the potential role of miRNAs as candidate diagnostic markers. miRNAs are being explored in many cancers for their diagnostic ability [54].

Other omic technologies that hold promise for the development of novel cancer biomarkers are metabolomics, the study of small molecules or molecular profiles in tissues or body fluids. Metabolomics broadly applies to nuclear magnetic resonance (NMR) and mass spectrometric technologies to measure a variety of metabolites or their profiles, which leads to enormous amounts of data. The relatively small number of metabolites compared to the number of proteins or genes present in the human body makes them attractive candidates for disease detection, diagnosis, and other clinical applications. However, the metabolome or metabolic profiles of a physiological system is dynamic and subject to influences such as environmental, pharmacological exposure, temporal, dietary, physiological, age, sex, and genetic predisposition. The technology has great promise and its potential is only beginning to be explored in cancer research. Recently, metabolomics technologies were applied to the differential diagnosis of ovarian cancer using differences in spectral patterns of healthy and affected women [56]. In this study, sera from 12 women with benign ovarian cysts, 38 ovarian cancer patients, 19 pre-menopausal, and 32 post-menopausal healthy women was subjected to ^{1}H-NMR spectroscopy and the resulting data were analyzed using both supervised and unsupervised methods. Demonstrating the promise it holds for future molecular diagnosis, researchers were able to identify a pattern that could discriminate between ovarian cancer and healthy subjects. Moreover, these patterns allowed discrimination between benign cases and ovarian cancer patients. Using fine needle aspirate biopsies from 140 patients with breast lesions analyzed by ^{1}H-NMR spectroscopy and pattern recognition analysis, Mountford et al were able to classify benign cases from malignant cases with 93 % sensitivity and 92 % specificity [57].

41.8 Molecular Imaging in Cancer Detection and Diagnosis

Functional magnetic resonance (MR) techniques such as dynamic contrast-enhanced MR imaging (DCE-MRI) and magnetic resonance spectroscopic imaging (MRSI) combine the virtues of conventional imaging with molecular profiling. Besides the spatio-temporal resolution of tumors, these enable additional novel molecular information on tumor metabolism, and provide more sensitive and accurate strategies for molecular diagnosis. Using this strategy, van Dorsten

et al have shown that the diagnostic sensitivity of prostate tumor regions can be significantly enhanced by adding metabolic profile information to the quantitative DCE-MRI [58]. It was shown that the ratio of (choline + creatine)/citrate was elevated in prostate cancer compared to healthy zones peripheral to the tumor and in the central gland. A ratio above 0.68 is a reliable indicator of cancer in determining the cancer-affected regions. Such an approach is feasible in clinical practice and has excellent clinical implications. Positron emission tomography (PET) imaging is another modality that has been approved by the FDA to determine cancer staging. It provides useful information on spatio-temporal resolution of the tumors. With the aid of specialized metabolic analogs, it is possible to visualize the tumor metabolism to distinguish between indolent and malignant cancers and normal tissues. The most frequently used PET agent is [^{18}F] fluorodeoxyglucose (FDG), a glucose analog taken up by highly metabolizing tumor cells, which allows the study of tumor metabolism. Many contrast agents have been approved by FDA for clinical applications that are used in molecular imaging. This approach has been approved for tumor staging of several cancers including breast, lung, and colon [59–62].

Other molecular imaging modalities for cancer detection utilize optical probes that can be triggered by cellular proteases that are specifically activated in cancers. Such approaches have been used in mouse models to detect lung tumors smaller than 1 mm diameter [63]. Similarly, fluorescent-based imaging agents in combination with endoscopic confocal microscopy allow detection of precancerous lesions that are not identified by conventional imaging methods [64]. Such imaging modalities have much higher sensitivity and provide high spatial resolution that is not possible with conventional modalities. The use of magnetic nanoparticles can provide information on cancer staging and have been applied to detect nodal metastasis in patients with occult cancers [65]. A functionalized nanoparticle-related approach allows the study of cellular functions, including cellular trafficking and receptor-based targets such as prostate-specific membrane antigen (PSMA), HER-2/neu, estrogen, progestin, and androgen receptors [65]. Animal models have been used extensively in molecular imaging for the evaluation of both the contrast agents and the imaging technologies [65].

41.9 Role of Animal Models in Biomarker Discovery

Animal models provide great biological tools for cancer biomarker development. Genetically, engineered mouse models based on genetic mutations mimic human disease conditions and provide a great deal of information on molecular pathogenesis. This provides a unique opportunity for the discovery of diagnostic biomarkers [63]. Animal models allow homogenous genetic background and controlled environmental exposures to reduce variability and control for other confounding factors among samples, thus providing samples from the same mice before and after onset of malignancy. Mouse models based on conditional activation of genetic mutations provide information on spatio-temporal regulation of carcinogenesis. These models can be explored further to identify novel blood-based biomarkers and to augment other strategies. Genetically engineered MIN mice, which harbor a germ-line truncation in the adenomatous polyposis coli (APC) gene, produce colon cancer similar to human colon cancers, and the genetically predisposed human condition known as familial adenomatous polyposis (FAP) syndrome. These mice have been used to study the novel optics technology of four-dimensional elastic light scattering finger printing (ELF) at several points in the neoplastic process. They have also been used to study the responsiveness of chemoprevention strategies and the efficacy of chemopreventive agents for the early detection of intestinal tumorigenesis before the clinical manifestation of the disease [66].

41.10 Translation of Cancer Biomarker Discovery to the Clinic

Cancer detection and diagnosis play a critical role in the management of disease treatment. Application of cancer biomarkers in clinical use will revolutionize cancer screening and open new avenues for personalized medicine. Recent molecular technologies offer great potential for discovery and development of novel biomarkers that will enable effective cancer screening and detection. Discovery is important, but for biomarkers to reach clinical application they need to be critically evaluated and validated both analytically and clinically. A five-phase biomarker development process has been proposed that lays out a systematic approach to bring putative biomarkers from discovery to ultimate clinical application for cancer detection (Fig. 41.2) [67]. These phases include: preclinical discovery, clinical and analytical validation, retrospective and longitudinal evaluation, prospective screening, and finally, cancer control for the utility of the marker. A similar approach can be adopted for validating diagnostic biomarkers. It is usually necessary to conduct pre-validation studies before determining the need for a successful validation study. Such an effort will help eliminate the gap between translational efforts.

Randomized prospective trials of biomarkers will prove their ultimate utility for clinical prediction of cancer detection and diagnosis. However, such trials are expensive and typically time-consuming. Validation of biomarkers requires a coordinated, multidisciplinary, and multi-institutional effort. Advances in novel methodologies of data analysis, data modeling, and integration of information from panels of

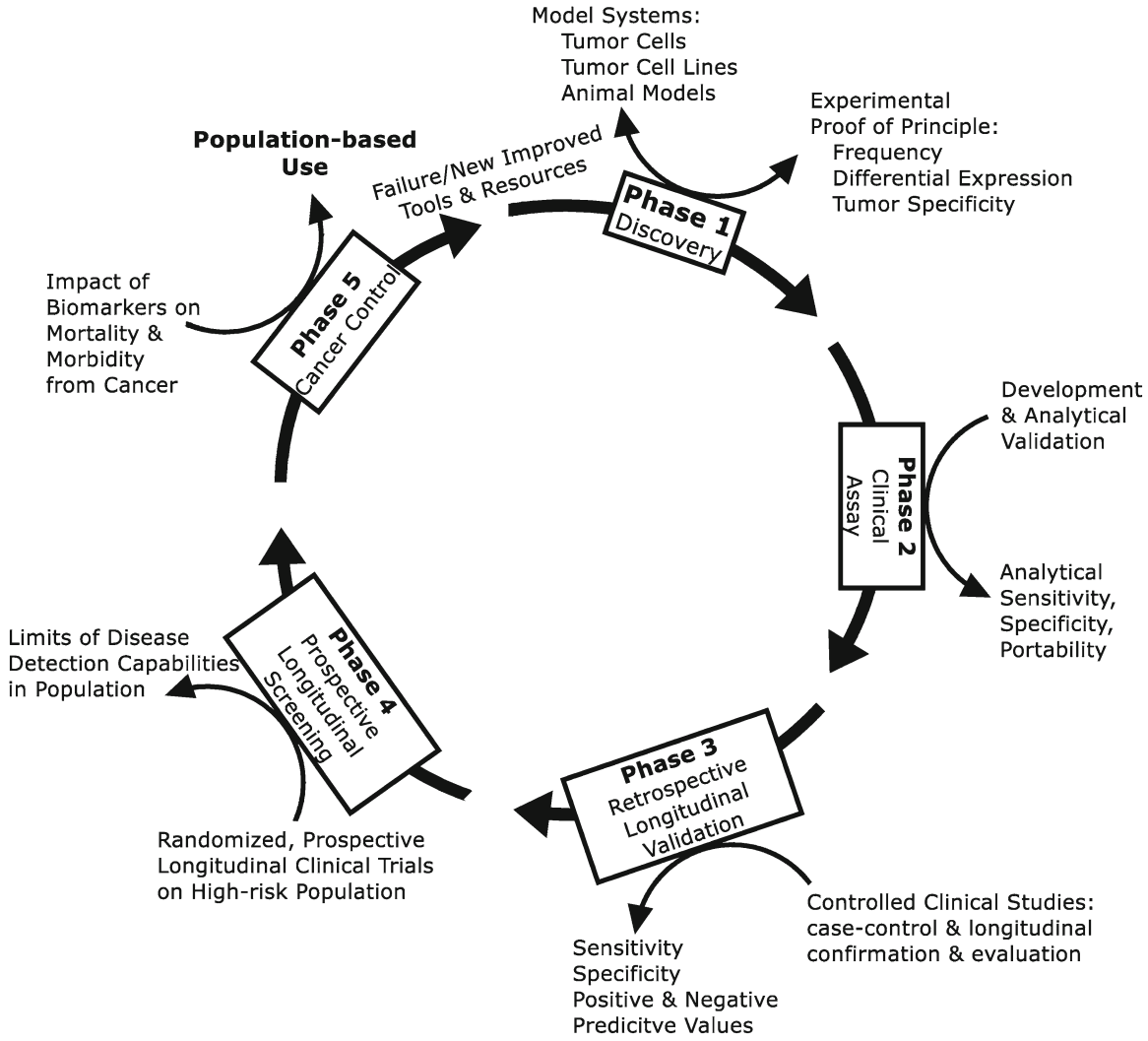

Fig. 41.2 A 5-phase, cyclic process of development of molecular markers for early detection and diagnosis. Adapted with modifications from [102].

biomarkers and algorithms to predict successful recognition patterns for biomarker profiles are necessary. Well annotated specimens and well characterized reference sets for triaging putative markers are critical. Novel high-throughput technologies require well developed standardized protocols and procedures and ways to translate them into clinically applicable assay methodologies.

While thousands of biomarkers are discovered, very few sustain the validation process to be approved by FDA for clinical use [68]. One reason for this is the discordance between academic investigators and diagnostic companies. It is considered that biomarker development is not as exciting as discovery. Conversely, efforts to advance potential markers into clinical application have been painstakingly slow even though the importance of biomarkers in cancer screening is highly recognized [69]. The general perception is that support from the federal agencies is essential to advance biomarkers across the translational architecture. To meet this challenge, the Department of Human Health Services has adapted many approaches. In 2002, FDA set up an exclusive regulatory unit, the Office of In vitro Diagnostic Devices, to expedite the approval process for biomarker and diagnostic devices. FDA and the National Cancer Institute (NCI) created a Critical Path Initiative to bridge the gaps in translational efforts in biomarker development. The Center for Medicaid and Medicare Services has been forthcoming in approving promising biomarkers and tests that can be applied in a research setting before they can be successfully approved by the FDA. For example, Oncotype DX™, a 21-gene panel which holds promise for predicting recurrence of breast cancer, was recently approved for use in a research setting [70]. NCI initiated the Early Detection Research Network (EDRN) program to coordinate translational biomarker developmental efforts [71–74]. The efforts are expected to expedite the validation process and fill a key gap in biomarker research [75, 76].

Cancer biomarkers will be able to detect early cancers and pave the way for personalized choices for cancer treatment and subsequently bring down the costs associated with it. Today, cancer detection and diagnosis strategies heavily depend on imaging modalities. Mammography is the method of choice for screening women above age 40 for breast cancer and colonoscopy is the screening strategy for people above 50 for colon cancer. It is unlikely that biomarkers will be able to eliminate existing imaging modalities. However, they will be able to complement them in providing additional information that may not be possible by current screening. The ultimate utility of the cancer biomarker is its ability to reduce cancer-associated mortality. The best biomarkers will be able to detect cancer early enough to allow intervention, reduce costs associated with cancer treatment, and reduce cancer-related mortality and morbidity. It appears that no single biomarker will have the necessary sensitivity and specificity to predict cancers accurately. Instead, panels of genes, proteins, or other candidate signatures obtained from novel high-throughput technologies will collectively provide useful information on cancer development and progression.

41.11 New Era of Molecular Detection and Diagnosis

Despite unresolved issues, high-throughput technologies are already providing evidence as promising tools for biomarker development. While few of these molecular tests have entered clinical practice, increasing evidence suggests they will play a critical role in aiding decisions related to personalized medicine for treating and managing disease. In this section, examples are discussed in which molecular diagnostic techniques may soon be applied in the clinic for numerous cancers to improve clinical diagnosis, monitoring of treatment, and management of disease. A small number have been approved for early detection of cancer and they provide excellent evidence and promise for the future development of molecular diagnostic tests.

41.11.1 Organ-Specific Biomarker Effort

Breast cancer is a heterogeneous disease with diverse clinical outcomes. Over the last two decades, there has been a documented decrease in mortality rate largely due to improvements in early detection and therapeutic strategies [77, 78]. However, the clinical outcome and the therapeutic course of an individual patient are customarily uncertain. Molecular profiling of breast cancer not only sheds light on different subtypes of breast tumors, but provides information clinically applicable for the prognosis. Several prognostic indicators based on clinical and histopathologic variables, such as tumor size, lymph node status, and hormone receptor status, already are used in clinical practice. Yet, these indicators still fail to determine the specific risk for recurrence associated with each patient. Several groups have developed a range of prognostic profiles using microarray approaches that provide specific information about breast cancer prognosis using both supervised and unsupervised analytical methods [79]. They have been validated using independent patient populations. Among them, the Amsterdam 70 gene profile popularly known as Mammaprint has been developed using microarray profiling of fresh-frozen tumors from two distinct populations, one with and one without distant metastasis. Using a supervised analytical approach, they identified a 70-gene set that appeared to correlate with the clinical outcome. The 70-gene panel, which was approved by FDA, was validated using retrospective analysis of 295 patients. It was able to predict prognosis regardless of lymph node involvement and acts as an independent risk factor for distant metastasis [80]. The 21-gene recurrence score indicator popularly known as Oncotype Dx, developed from a literature and database search, selected a 16-cancer-gene panel and a 5-reference-gene panel from 250 candidate genes. Their expression profiles were later established using fixed tumor samples and externally validated in patient samples collected for the National Surgical Adjuvant Breast and Bowel project, and then validated further in a number of studies [70]. The Recurrence Score provides reliable and reproducible information on the recurrence and therapeutic response. The Rotterdam 76-gene signature was developed by the expression profiling of more than 200 lymph node negative patients with both ER- and ER+ breast cancer, which outperformed clinical variables in predicting prognosis [81, 82]. Several other prognostic signatures still under study may be of high significance in clinical application. About 65,000 women are diagnosed annually with breast ductal carcinoma in situ (DCIS). All patients are treated similarly with lumpectomy and additional radiation or tamoxifen. However, only ~15 % of them will recur within 5 years and only half of the recurrent disease (~6.2 %) will be an invasive breast cancer (ICA) [83]. In a preliminary study, three protein markers were identified, whose individual expression pattern in DCIS lesions can provide information for patient risk stratification for a subsequent recurrent malignant event (ICA) [84]. While patients at a higher risk of ICA may undergo more aggressive preventive treatment, those at low risk may be spared the morbidity of unnecessary treatments. A large case–control study is currently being initiated by EDRN investigators at the University of California San Francisco to validate the clinical utility of the 3-marker panel.

Bladder cancer is the sixth leading cause of cancer deaths and as a matter of course it results in high recurrence rates. Cystoscopy, which is highly invasive, serves as the gold standard for monitoring the recurrence and diagnosing bladder

cancer with a sensitivity of 73 % and specificity of 37 % [85]. Urine cytology, another promising diagnostic tool, also suffers from low sensitivity and specificity leading to high number of false-positive rates. There is a need for a noninvasive, easy to administer test with reasonable performance characteristics that can either complement or be used alone. Numerous tests have been discussed in detail elsewhere, some of which have been recently approved by the FDA [85]. A variety of urinary proteins show promise as diagnostic biomarkers. Nuclear matrix protein 22 (NMP22) is a protein expressed by a variety of cell types and is found in the urine of bladder cancer patients at high levels. It has been approved by FDA as an inexpensive and sensitive tool that can be used as an adjunct to current diagnostic methods [86, 87]. Basement membrane antigen (BTA) has a sensitivity comparable to urine cytology and is approved as both qualitative and quantitative tests [88]. The ImmunoCyst test, based on sialylated carbohydrate on a mucin protein and 19A211 cell surface glycoprotein and the LDQ10 antigens by immunofluorescence microscopy, is also approved as an adjunct test for detecting bladder cancer. While tests are proposed and approved for diagnosing and managing bladder cancer, most suffer from low specificity and high false-positive rates, mainly because of interference due to other urological conditions such as inflammation and urinary infections and stones. Abnormal increased copy number of chromosomes 3, 7, and 17, and homozygous deletions of the p16 at 9p21 have been used to develop a FISH-based assay with high sensitivity (81 %) and specificity (96 %), which is approved by FDA as an adjunct diagnostic test. A microsatellite instability assay (MSA) has been recently developed by EDRN investigators at Johns Hopkins University, which can detect bladder cancer in urine sediment with an accuracy of >90 %, frequently 6–8 months prior to a positive cystoscopy [89, 90]. A large-scale validation study is currently being conducted to confirm the detection power of the MSA test.

Pancreatic cancer poses a serious health problem causing death in more than 98 % of the affected populations. Currently, pancreatic cancer is diagnosed at advanced stages. There are no approved diagnostic markers. CA-19-9, a sialylated Lewis blood group antigen, was approved for monitoring treatment, but it has very low specificity due to other confounding health conditions involving the pancreas. Efforts are underway to develop biomarkers for pancreatic cancer using a variety of approaches. Among them, global gene expression profiling of pancreatic cancer and normal pancreatic tissues has been used in identifying candidate markers. Efforts are underway to further characterize the candidate serum protein markers, namely macrophage migration inhibitory factor-1, osteopontin, and CA-242 [91, 92]. While none of them have been validated for clinical use, a strong indication exists that in combination they significantly improve the performance of CA-19-9.

Hepatocellular carcinoma (HCC) is one of the most common solid malignancies worldwide and its incidence in the United States is increasing [93]. Despite all advances in treatment, the 5-year survival of patients with HCC still remains at 5 %, which may be largely due to diagnosis at late stages of the disease. Hence, biomarkers for its early detection are really needed. Since 90 % of HCC patients have underlying cirrhosis, these are good candidates for HCC surveillance. Currently, HCC surveillance tests are ultrasound evaluation of the liver with or without measurement of serum alpha-fetoprotein (AFP). These tests are inadequately sensitive and specific (41–78 %) for use in screening. Various other biomarkers for HCC have been evaluated, including des-gamma carboxyprothrombin (DCP) and the fucosylated variant of AFP (AFP-L3). DCP was significantly better than total AFP or AFP-L3 in differentiating HCC from cirrhosis, with a sensitivity of 86 % and specificity of 93 % [94]. Currently, EDRN investigators at the University of Michigan Ann Arbor are conducting a large-scale study to confirm the validity of DCP alone or in combination with AFP or AFP-L3 as a test for early clinical diagnosis of HCC among cirrhotic patients.

Ovarian cancer is the fifth leading cause of cancer-related deaths in the United States. More than 20,000 women are diagnosed with ovarian cancer each year and more than 15,000 die annually from the disease. The high mortality rate of ovarian cancer is mainly due to the advanced stage at diagnosis of most cases and the current lack of a screening strategy for the detection of the disease at an early stage when treatment is more successful. Transvaginal ultrasound (TVS) and serial measurements of the biomarker CA-125 are being used for the surveillance of the high-risk population. The sensitivity and specificity of CA-125 are, however, very low (<60 % and PPV ~20 %) to justify its use as a population screening biomarker, given the overall relatively low incidence of ovarian cancer. Recently, a combination of six serum markers that also includes CA-125 was shown to detect early- and late-stage ovarian cancer significantly better than CA-125 alone. With sensitivity and specificity at 95.3 and 99.4 % respectively, the accuracy of the test is ~99 % [95]. The EDRN investigators together with LabCorp, Inc. are currently in the process of validating this test in Prostate-Lung-Colon-Ovarian (PLCO) screening trial sera of ovarian cancer patients and healthy controls that were collected up to 2 years prior to diagnosis. If its accuracy determined with clinical specimens is also validated with the PLCO pre-diagnostic samples, this test will be of great importance both for surveillance of the high risk as well as the general population.

State-of-the-art proteomic technologies are attempting to address issues in the management of common but not so life-threatening cancers. Thyroid cancer is on the rise in the United States in the last three decades and a large population

needs constant surveillance to manage the disease. The current test approved to monitor the disease, thyroglobulin, fails to accurately distinguish between benign and malignant tumors. The other diagnostic modalities tend to be highly invasive and expensive, and require more adaptable tests for making surgical decisions. Villanueva et al employed a serum peptidomic approach where serum peptides are captured and concentrated on magnetic, reverse-phase beads and analyzed with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer to generate a peptide signature [41]. They propose that the low molecular weight (LMW) peptide biomarkers are the result of ex vivo action by proteinase-mediated enzymatic cleavage as part of the coagulation and complement activation pathways. It is claimed that the fragments of endogenous blood proteins generated ex vivo serve as a substrate pool for disease-specific proteinases that arise from the tumor itself or from the tumor microenvironment. Using this approach, they identified a 12-member peptide panel thyroid cancer signature that was able to distinguish between thyroid cancers and normal subjects in an independent validation set, thus indicating that the technology indeed has promise for clinical application. Currently, they are characterizing the peptides in the panel and optimizing the set by expanding the study population. While this approach seems fascinating and may provide promising molecular markers, it calls for a tight coordination of efforts from nurses, phlebotomists, messenger service staff, and clinical technicians and for strict adherence to the standard protocol, which may be impractical in clinical application [41, 96].

41.12 Conclusion

Cancer detection and diagnosis efforts are constantly evolving because of recent applications of high-throughput omic technologies to cancer biology. Advances in bioinformatics and computational tools augment the process by allowing data analysis in myriad ways. These approaches may result in promising biomarkers that will enable personalized choices in cancer treatment. Potential biomarkers need to be critically validated both clinically and analytically before they can be successfully applied in the clinic. The actual value of a biomarker lies in its ability to reduce cancer-related mortality and morbidity by enabling cancer detection and subsequent early intervention and reducing the costs associated with late-stage treatment. A multidisciplinary, coordinated effort seems necessary to enable biomarker validation. Federal agencies are making provisions to expedite biomarker development. Molecular markers are likely to revolutionize the state-of-the-art in cancer detection and diagnosis and complement imaging strategies.

Acknowledgements The authors sincerely thank Dr. Paul Wagner for helpful discussions during manuscript preparation.

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Gene Therapy in the Treatment of Human Cancer

42

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42.1 Introduction

Cancer can be considered a disease caused by mutations and/or epigenetic changes in tumor suppressor genes and oncogenes that populate the host genome [1, 2]. It is well established that most of the genetic events in cancer result from a series of accumulated, acquired genetic lesions [3, 4]. These genetic events either inactivate tumor suppressor genes or activate oncogenes. With an enhanced understanding of the genetic lesions associated with malignant transformation and progression in a wide variety of human cancers, different therapeutic approaches are being identified. In this regard, gene therapy is emerging as a method of preventive and therapeutic intervention against cancer targeted at the level of cellular gene expression [2]. In this approach, altering the complex cancerous pathophysiologic state is achieved by delivering nucleic acids into cells. These nucleic acids may be genes, portions of genes, oligonucleotides, or RNA. In conventional therapeutics, as in pharmacotherapy, altering a cell or tissue phenotype is accomplished by altering cell physiology or metabolism at the level of protein expression. In contrast, in gene therapy this is accomplished by changing the pattern of expression of genes whose products may correct defects in cellular phenotype.

42.2 Why Gene Therapy?

In the treatment of human disease, gene therapy strategies may offer the potential to achieve a much higher level of specificity of action than conventional drug therapeutics by virtue of the highly specific control and regulatory mechanisms of gene expression that may be targeted. Additionally, interceding at an earlier, upstream point in disease pathogenesis may offer greater potential to induce fundamental changes in phenotypic patterns of disease, with a more favorable clinical outcome. The availability of gene transfer systems, or vectors, for permanent or long-term genetic modification of cells and tissues allows for definitive therapeutic or preventive interventions. Furthermore, gene transfer can be accomplished in a limited loco-regional context, producing a high concentration of therapeutic molecules in the local area. Thus, potential undesired systemic effects of specific therapeutic molecules are avoided. Lastly, using the host to produce therapeutic proteins has a practical advantage of its own [5]. Briefly, limitations associated with manufacture, stability, and duration of effect after administration of drugs based on, for example, synthetic peptides are completely avoided. From the same pharmacological point of view, designer drugs designed from selective small molecules, currently under intensive investigation, cannot yet serve as an efficacious therapeutic alternatives or substitutes for the protein products of tumor suppressor genes.

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Table 42.1 Potential contributions of gene therapy against the obstacles for curing cancer

Obstacles to curing cancer imposed by tumor cells	Potential contribution of gene transfer
1. Tumors are genetically unstable, and thus they are extraordinarily adaptable to environmental changes (This is arguably the biggest obstacle, and has never been approached directly)	Gene transfer of DNA repair or cell cycle checkpoint genes that restore DNA stability
2. Tumors are heterogeneous in many respects, including genetic mutations, expression of oncoproteins, immunogenicity, response to environmental changes, etc	Targeting of "genetically homogeneous" tissues, such as the tumor vasculature; genetic immunopotentiation
3. As a consequence of obstacles #1 and #2, tumors have, or acquire, resistance to cellular toxins and to many other therapeutically induced cellular insults	Strategies above, associated with chemotherapy or radiotherapy or with the transfer of genes that sensitize tumor cells to drugs or radiation
4. Tumors can have a low cellular growth fraction; therefore they are less susceptible to mitotic toxins and to gene transfer vectors that require dividing cells	Use of vectors that do not require cellular division for gene delivery and expression (adenovirus, herpesvirus, lentivirus, chimeric systems); repeated administration of non-immunogenic vectors
5. Tumors form metastases, which have to be reached systemically to eradicate the tumor	Use of targetable, injectable vectors (tropism-modified viruses, cellular vehicles); genetic immunopotentiation
6. Tumors do not express specific tumor antigens or immune costimulatory molecules; alternatively, tumors downregulate immune recognition, induce tolerance, and inhibit the immune response	Transfer of genes encoding costimulatory molecules and cytokines; genetic modification of antigen-presenting cells; induce inflammatory reactions that activate antigen presentation; transfer of genes blocking tumor-secreted inhibitors of the immune response
7. The spontaneous behavior of human tumors is somewhat different from that of malignant cells in vitro, and from that of experimental tumors in animal models	Development of better animal models, including tumor models in transgenic mice
8. Tumors are diagnosed in advanced stages, when billions of tumor cells exist in the body, frequently widely disseminated	Development of amplification vector systems (replicative viral vectors); use of targetable, injectable vectors; genetic immunopotentiation
9. The understanding and treatment of cancer requires the contribution of very diverse fields of basic knowledge, biotechnology, and medical practice	De facto multidisciplinary recruitment of gene therapy researchers

In the treatment of advanced human malignant tumors, several obstacles limit the effectiveness of currently available therapies preventing definitive cures (Table 42.1). It is apparent that new chemotherapeutic drugs, higher drug doses, cytokines, novel modalities of radiotherapy, and more ambitious and sophisticated surgeries can achieve incremental improvements in cancer treatment. But these therapies do not address critical biological obstacles, and thus will not bring the much-needed essential radical advances in the implementation and success of cancer treatments. Gene therapy, in contrast, offers an ability to overcome many of these fundamental barriers (Table 42.1).

42.3 Gene Therapy Vectors

Different vectors (both viral and nonviral) are employed for gene therapy. The six most frequently used viral vectors include those derived from adenovirus, retrovirus, poxvirus, adeno-associated virus, herpes simplex virus and lentivirus while the nonviral approaches includes calcium based molecular compounds, lipofection, and direct injection of naked DNA or RNA (Table 42.2). These vectors must meet certain basic criteria to be used to deliver therapeutic genes. In this regard, the vectors are based upon their fundamental ability to deliver therapeutic nucleic acids into relevant target cells. Further, the delivered genes must be expressed at an appropriate level and for an adequately prolonged period of time. Finally, the delivery and expression of the therapeutic genes must not be deleterious to the surrounding normal tissue, nor to the individual as a whole [6, 7]. In practice, two general approaches have been employed to meet these gene vector criteria: an ex vivo approach and an in vivo approach. In the former method, target cells are removed from the host and transduced with the genetic vector extracorporally, followed by reinjection or implantation. Though this approach has allowed reasonably efficacious transduction of target cells and has also allowed characterization of modified cells, the number and types of parenchymal cells that can be modified in this manner are quite limited. An alternate approach to achieve therapeutic gene delivery has been the in vivo administration of vectors into target parenchymal cells directly in their natural location. In this regard, both viral and nonviral vectors of diverse types have been employed to achieve in situ transduction of relevant target endodermal, ectodermal, and mesenchymal-derived cells (Table 42.2).

Table 42.2 Gene transfer systems used clinically against cancer[a]

Type	Vector system	Duration of expression	Distinguishing features
Nonviral	Liposomes	Transient	Repetitive and safe administration feasible, inefficient gene delivery, transient expression
	Naked DNA or RNA (injection, gene gun, electroporation)	Transient	Easy preparation, inefficient gene delivery, transient expression
	Molecular conjugates	Transient	Flexible design, inefficient gene delivery, transient expression, unstable in vivo
Viral	Retrovirus	Prolonged	Integrates into the chromosome of dividing cells, unstable in vivo
	Adenovirus	Transient	Highly efficient in vivo, production in high titer, tropism can be modified, induces potent inflammation and immunity, replicative vectors available
	Poxvirus (vaccinia)	Transient	Extensive clinical experience with parent virus, large insert capacity, induces potent inflammation and immunity
	Adeno-associated virus	Prolonged	Nonpathogenic, low insert capacity, difficult to scale up
	Herpes simplex virus	Transient	Highly efficient in vivo, large insert capacity, cytotoxic, replicative vectors available
	Chimeric vectors (e.g., Ad/Retro)	Prolonged	Combine features of component genetic vectors
	Lentivirus	Prolonged	Integrates into the chromosome of *both* dividing and nondividing cells, well-characterized production system not yet established

[a]Registered at Recombinant DNA Advisory Committee (RAC), Office of Biotechnology activities at NIH (http://oba.od.nih.gov/rdna/rdna.html)

42.4 Gene Therapy Strategies

A number of strategies have been developed to accomplish cancer gene therapy. Broadly, these approaches include (1) mutation compensation, (2) molecular chemotherapy, (3) genetic immunopotentiation, and (4) virus-mediated oncolysis. Briefly, for mutation compensation, gene therapy techniques are designed to correct the molecular lesions that are etiologic of malignant transformation, or to avoid the contribution by tumor-supporting normal cells. For molecular chemotherapy, methods have been developed to achieve selective delivery or expression of a toxin gene in cancer or tumor stromal cells to induce their eradication. Also, attempts have been made to deliver genetic sequences that protect normal bone marrow cells from the toxic effects of standard chemotherapeutic drugs, thus allowing the administration of higher drug doses without reaching otherwise limiting myelosuppression. Genetic immunopotentiation strategies attempt to achieve active immunization against tumor-associated antigens by gene transfer methodologies. Both tumor cells and cellular components of the immune system have been genetically modified to this end. Viral mediated oncolysis is a relatively new therapeutic strategy in the treatment of cancer involving conditionally replicating viral vectors. As their name implies, these viruses conditionally replicate uniquely in tumor cells, thereby exhibiting oncolysis by direct cytopathic effects. These viral vectors are either genetically engineered (e.g., Herpes simplex virus type 1 or HSV-1, adenovirus), naturally attenuated (e.g., Newcastle disease virus), or nonpathogenic in humans (e.g., reovirus), so they replicate selectively in tumor cells, but do not harm normal tissues [8, 9]. Importantly, each of these strategies has been rapidly translated into human gene therapy clinical trials (Table 42.3).

In general, a common finding in many of these studies, including many clinical trials, has been the disparity noted between the in vitro and in vivo gene transfer efficiencies of these vector systems, and the suboptimal tumor transduction that presently available vector systems can achieve. In addition, the promiscuous tropism of current vectors may potentially allow for genetic modification of a number of normal non-transformed cells besides target cells. Furthermore, this non-selective gene transfer impedes the administration of vectors to tumor cells by the systemic route. Thus, important limitations of current approaches used for implementation of gene therapy for cancer have been noted. Although many potentially effective strategies exist to affect the molecular treatment of cancer, gene delivery issues currently limit the definitive evaluation of these methods.

In this regard, we examine the lessons learned from the results of the early attempts to apply gene therapy in human cancer. This will show both the rationale of gene therapy and the problems encountered in its development. We emphasize in our discussion the general biological concepts of each therapeutic strategy, and suggest comprehensive reviews for readers interested in detailed discussions. Finally, we illustrate prospects for overcoming the obstacles to gene therapy by novel methods that are currently being refined.

Table 42.3 Clinical trials of gene therapy for the treatment of cancer

Strategy	Clinical trials[a]	Molecular mechanism of anticancer effect
Mutation compensation	11	Inhibition of expression of dominant oncogenes, Augmentation of deficient tumor-suppressor genes, single chain antibodies, antisense, dominant negative mutation
Molecular chemotherapy	6	Pro-drug/HSV-TK and ganciclovir, chemoprotection of normal tissues during high-dose chemotherapy, radiotherapy
Genetic immunopotentiation	48	In vitro transduction—augmentation of tropism or cell killing capacity of tumor-infiltrating lymphocytes; genetic modification of irradiated tumor cells
	50	In vivo transduction—administration of costimulatory molecules or cytokines; immunization with virus encoding tumor-associated antigens
Viral-mediated oncolysis	14	Tumor cell lysis by viral vector replication

[a]"Active" Clinical Trials registered at Recombinant DNA program, Office of Biotechnology activities at NIH in February 2011. (http://oba.od.nih.gov/rdna/rdna.html)

42.5 Mutation Compensation

Gene therapy techniques based on mutation compensation are designed to rectify either the molecular lesions in the cancer cell, the etiology of malignant transformation or the associated changes in stromal cells that support cancer progression. The genetic lesions in cancer cells that are linked to the pathogenesis of the malignancy may be thought of as a critical compilation of two general types; loss of expression of tumor suppressor genes (TSG) or aberrant expression of dominant oncogenes [3, 4]. In addition, contribution of other associated genes to either the individual cancer cell or its local microenvironment are critical during neoplastic progression. Angiogenesis, cellular motility, invasion, and metastasis are some examples of such phenotypic processes modulated by these associated genes. A knowledge of the major role that growth factors, signaling molecules, cell cycle regulators, and determinant factors of key steps along the metastatic cascade play, have positive implications for gene therapy, and therapeutic approaches have been proposed to achieve correction of each of these processes (Table 42.4).

42.5.1 Replacement of Tumor Suppressor Genes

Mutations of more than two dozen TSGs have been described in numerous cancers. Their functions are diverse and include maintaining cellular structure and signaling of intercellular junctions and receptors (*APC*, *DCC*, *DPC4*, *NF1*), components that regulate the transcription apparatus (*p53, VHL*, *RB1*, *WT1*), and DNA mismatch (*hML1*, *hMHS2*, *hPSM1*, *hPMS2*) or excision (*XPA*, *XPB*, *XPC*, *XPD*, *XPG*) repair. Thus, the inactivation of tumor suppressor genes contributes to the neoplastic phenotype by abrogating critical cell cycle checkpoints and DNA repair mechanisms. To approach this loss of function, a logical intervention is replacement of the deficient function with its wild-type counterpart gene [10]. Many of the TSGs have been shown to induce apoptosis or cell cycle arrest in cancer cells. Among these are p*53* [11], RB (Retinoblastoma) [11, 12], $p16^{INK4a}$, [13, 14], PTEN (Phosphatase and Tensin Homolog) [15], *mda7*/IL-24 (melanoma differentiation associated gene-7/IL-24^{New}) [16–21], BRCA1 (Breast Cancer 1 gene) [22], BRCA2 (Breast Cancer 2 gene) [23], APC (Adenomatosis Polyposis Coli gene) and OPCML (Opiod binding Protein/cell-adhesion molecule like) [24] genes, many of which are being targeted by gene therapy approaches (Table 42.4). Of these TSGs, *p53*, *RB1*, *BRCA1*, and *p16* ^{INK4a} have advanced to being administered in clinical trials as replacements for their mutated counterparts. *mda*-7/IL-24 represents a novel TSG that is not mutated in cancers, but when reintroduced into cancers of diverse origin induces programmed cell death (apoptosis) [16]. Additional attributes of *mda*-7/IL-24 that bode well for its therapeutic applications as a gene therapy for cancer include its ability to inhibit tumor blood vessel formation (angiogenesis), promote killing of distant tumor cells (a bystander anti-tumor effect), induce anti-tumor immune responses and synergize with other conventional modes of therapy (including chemotherapy, radiation and monoclonal antibodies) [16, 25]. Moreover, many of these properties have been persevered following intratumoral injection of a replication incompetent adenovirus expressing *mda*-7/IL-24 (Ad.*mda*-7; INGN 241) in advanced cancers including melanoma [26–29]. These studies confirmed that *mda*-7/IL-24 is safe and documented significant clinical anti-tumor activity.

Mutations in the p*53* tumor suppressor gene are observed in more than half of all malignancies [30]. Correction of such mutations may be particularly relevant due to the central role played by *p53* as guardian of the genome and regulator of apoptosis [31]. An additional antiangiogenic [32, 33] mechanism of action of *p53* has also been suggested. In this regard, *p53* has been the most frequently studied tumor suppressor gene [34] and is widely used in different gene therapy clinical trials. Replacement of the mutated p53 with its wild-type

Table 42.4 Mutations compensation strategies used clinically

Target[a]	Strategy	Vector	Tumor type
p53[b]	Replacement of tumor suppressor gene	Adenovirus	Varied cancer
RB[b] *(retinoblastoma)*	Replacement of tumor suppressor gene	Adenovirus	Bladder cancer
BRCA-1[b]	Replacement of tumor suppressor gene	Retrovirus	Ovarian cancer
Insulin-like growth factor 1[b]	Blockade by antisense	Cationic liposome complex	Glioblastomas
k-ras[b]	Blockade by antisense	Retrovirus	Non-small cell lung cancer
c-myc[b]	Blockade by antisense	Retrovirus	Breast and prostate cancers
TGFβ[b]	Blockade by antisense	Plasmid and electroporation	Glioblastoma
Bcl-2[a]	Blockade by antisense	Cationic liposome complex	Varied cancer
Clusterin[a]	Blockade by antisense	Cationic liposome complex	Prostate cancer
Telomerase[a]	Blockade by antisense	Cationic liposome complex	Varied cancer
erBb-2[b]	scFv	Adenovirus	Ovarian cancer

scFv single-chain intracellular antibody
[a]From [351]
[b]Registered at Recombinant DNA program, Office of Biotechnology activities at NIH (http://oba.od.nih.gov/rdna/rdna.html)

counterpart allows phenotypic correction (usually with subsequent apoptosis) both in vitro and in vivo in a variety of tumors. In particular, several authors have shown, in murine models employing human cancer xenografts, that intratumoral delivery of the wild-type *p53* gene via recombinant viruses can prolong survival by inducing apoptosis in the tumor cell population [35]. Importantly, nontransformed cells can tolerate exogenous administration of *p53*, thereby providing an optimal therapeutic index for this intervention. It should be emphasized that restoration of wild-type *p53* expression in cells with a mutant or deleted gene has been shown to be sufficient to cause apoptosis or growth arrest, despite the presence of multiple additional genetic abnormalities in the tumor cell. This fact has established the rationale for human clinical gene therapy trials designed to achieve mutation compensation through restoration of *p53* in several cancers. In a pioneering study, Roth et al administered intratumoral injections of a *p53*-encoding retrovirus to patients with non-small cell lung cancer (NSCLC) [36]. One-third of patients in this group had tumor regression, while another third had stabilization; none presented high grade toxicity directly related to the treatment, thereby demonstrating the safety and feasibility of this gene therapy approach [37, 38]. In another trial involving 19 patients with chemoradiation resistant NSCLC, recombinant adenovirus was used to deliver the p*53* transgene; 63 % of these patients had no residual viable tumor by biopsy. Stated radiologically, 58 % showed partial response and 5 % showed complete response to treatment as evaluated by Computed Tomography (CT) and bronchoscopy [39]. Various Phase I and II clinical trials for carcinoma of the breast [40], esophagus [41], colorectum [42], and several other sites [43], have also shown similar clinical feasibility, low toxicity, and indications of tumor regression either alone or in combination with chemotherapy/radiotherapy.

Replacement of *RB1* and *BRCA1*, and forced expression of *mda-7*/IL-24 have shown similar preclinical experimental results, and are also currently undergoing clinical testing. Attempts to restore wild-type *RB* have been described in prostate, retinoblastoma, osteosarcoma, breast, bladder, and non-small cell lung cancers [44]. Of note, and perhaps not surprisingly, some *RB*-deficient tumors have shown persistent tumorigenicity and proliferation after successful restoration and expression of wild-type *RB*, a phenomenon referred to as tumor suppressor resistance [45]. Thus, restoration of the *RB* tumor suppressor gene in certain tumors may not effect complete reversion of the malignant phenotype. *BRCA1* is only occasionally affected in spontaneous tumors, and its function has not been completely characterized. Its clinical use has, therefore, been somewhat controversial [46]. In contrast, *mda*-7/IL-24 provoked apoptosis in human tumors with multiple genetic defects, including wild type, mutant or null for p53 and/or mutations in RB1 and Ink4a, and in patients that had received prior therapies (including radiation, chemotherapy and/or immunotherapy) and presumably contain multiple mutations.

42.5.2 Repression of Dominant Oncogenes

In addition to mutations and epigenetic mechanisms that cause the loss of normal tumor suppressor functions, most tumors exhibit dysregulated oncogenes. For dominant oncogenes, it is the aberrant expression of the corresponding gene product that elicits the associated neoplastic transformation. In this context, molecular therapeutic intervention is designed to ablate expression of the dominant

oncogene. Inhibition of oncogenic function has been attempted at all three levels of molecular regulation, i.e., transcription, translation, and post-translation. (1) At the first level, transcription of the oncogene can be inhibited using triplex-forming DNA binding antisense oligonucleotides. Also, transcriptional silencing by epigenetic regulation of CpG islands in the oncogene promoter has been attempted in vitro. (2) Translation of oncogene mRNA can be blocked by using sequence specific small interfering RNA (siRNA) or ribozymes. (3) And finally at the posttranslational level, (a) mobilization of the nascent oncoprotein to its final location can be blocked using intracellular antibodies that intercept and interfere with the processing of the oncoprotein or (b) its function inhibited at its final cellular location by the heterologous expression of mutant protein that can inhibit the function of the native oncoprotein. Oncogenes implicated in cancer and used as targets in clinical trials include genes encoding: (1) growth factors, such as insulin-like growth factor 1 (IGF-1), and transforming growth factor β1 (TGF-β1); (2) growth factor receptors, such as erbB-2; (3) proteins involved in cell signaling, such as K-ras; (4) and transcription factors, such as c-myc.

42.5.2.1 Transcriptional Inhibition of Genes

The most universally employed methodology to achieve proto-oncogene ablation is the utilization of antisense oligodeoxynucleotides (ODN) with sequences complementary to that of target genomic DNA. These molecules are thus designed to specifically target sense sequences to achieve blockade of the encoded genetic informational flow. In this approach, ODNs bind to genomic DNA by Hoogsteen hydrogen bonding which leads to the formation of non-functional triplex DNA, thereby blocking its transcription [47]. This technique has been utilized both in in vitro and in vivo model systems for targeting the c-*myc*, c-*ras*, *bcl-2, IGF-1,* and c-*erbB-2* oncogenes. Since there are no known naturally existing pathways to direct the ODNs to their target gene, the efficiency of inhibition of oncogenes by this technique is limited. Along with the discovery of the RNAi pathway, the utility of ODNs as a method to suppress an oncogene has largely been replaced with siRNAs.

Another method utilized to ablate dominant oncogenes is by inhibiting its promoter regulatory sequence. It has been shown, for instance, that the K1 mutant of the viral SV40 large T antigen inhibits the human c-erbB-2 promoter in human ovarian cancer cells. Moreover, liposome-mediated K1 gene transfer decreases the p185 erbB-2 protein level in these cancer cells, and significantly prolongs survival in an in vivo orthotopic animal model [48]. Although not a human gene, the E1A gene of adenovirus serotype 5 exhibits tumor suppressor functions in cancer cells over expressing the c-erbB-2 oncogene. E1A inhibits transcription of the human c-erbB-2 promoter by inhibiting the p300/CREB-binding protein (CBP) and accordingly suppresses the tumorigenicity and metastatic potential induced by the oncogene [49]. Studies have shown that both cationic liposomes and an adenoviral vector can efficiently deliver E1A into ovarian tumor cells in mice, resulting in suppression of tumor growth and significantly longer survival of treated animals [50]. A phase I clinical trial of intraperitoneal administration of a cationic lipid complex containing the E1A gene in patients with ovarian cancer showed the safety and efficacy of this approach [51]. Currently, phase II clinical trials are being undertaken for solid tumors overexpressing c-erbB-2.

42.5.2.2 Translational Inhibition of Genes

Specific antisense binding to transcribed RNA sequences may interrupt the flow of genetic information through several mechanisms including RNA degradation, translational arrest by steric hindrance and less probably through the impaired transport of target mRNA across the nuclear membrane. These inhibitory interventions may be accomplished by (1) an RNAi approach (siRNAs, miRNA), or by (2) ODNs that possess additional catalytic activity to accomplish cleavage of target sense sequences, called ribozymes [52, 53].

RNAi Approach

RNAi refers to a group of related gene silencing techniques with a terminal effector molecule of siRNA. Posttranscriptional inhibition of gene expression was revolutionized with the discovery of the RNAi pathway first in transgenic plants [54] and then in *Caenorhabditis elegans* [55]. Briefly, double stranded RNA (dsRNA) or a short hairpin RNA (shRNA) homologous to a gene, when introduced into a cell are processed by a cellular molecule, Dicer to generate short 19–26 nt RNA duplexes with 3′ overhangs called siRNA. The antisense strand of this siRNA is subsequently incorporated into an RNA-induced silencing complex (RISC) and guides the RISC complex to its complementary mRNA for cleavage. siRNA has been used to downregulate the oncoprotein product of the Philadelphia chromosome (BCR/ABL gene) found in almost all chronic myeloid leukemia (CML) patients and in one-third of acute lymphoblastic leukemia (ALL) patients. In vitro studies in K652 cells (a human CML cell line) showed the specificity of knocking down the BCR/ABL gene by siRNA without affecting other genes, yielding the desired phenotype of increased susceptibility to apoptosis [56, 57]. Similar in vitro studies of siRNAs for different oncogenes like *K-ras* [58] and *bcl-2* [59] as well as receptor including *EGFR* [60] and oncogenic fusion proteins like AML/MTG8 [57] and EWS/Fli-1 [61] have shown specific knock-down of respective genes. In vivo knockdown of oncogenes in tumor xenografts models using siRNA are currently being undertaken [62, 63] and a phase I clinical trial targeting safety and efficacy of siRNA treatment for CML is underway.

Ribozyme Approach

A variety of experimental models, both in vitro and in vivo, have demonstrated the potential utility of an ODN approach as an anticancer therapeutic [64–67]. ODNs can block the translation of mRNA by steric hindrance [68] while an RNA–DNA hybrid can be degraded by RNAase H [69]. ODNs used in a clinical setting have either a phosphorothioate [70] or morpholino [71] backbone modification to improve biodistribution and stability. The specificity of antisense molecules has been convincingly shown in the case of K-*ras*. ODN methylphosphonates directed at either normal human Ha-ras p21 or ras p21 mutated at a single base at codon 61 have been examined for their efficacy and specificity as inhibitors of p21 expression. Mixed cultures of cells expressing both forms of p21 have been treated with ODN complementary to the normal or point-mutated p21. Each of the ODNs specifically inhibited expression of only the form of ras p21 to which it was complementary and left the other form of p21 unaffected. Thus, in general the antisense approach offers the potential to achieve targeted disruption of specific genes in human cancer. Table 42.4 shows a list of clinical trials which use antisense oligonucleotides to treat various cancers. Several cancer associated molecules including *bcl-2, clusterin*, *survivin,* and *eIF4E* are being targeted using this antisense approach.

Despite the potentially novel therapeutic strategies offered by the antisense approach, this methodology in practice is associated with severe limitations [65, 72]. These practical constraints have limited wide employment of this technology in protocols of human anticancer gene therapy. What is most disconcerting is that, there are no universal rules for quantifying the efficacy of a given antisense oligonucleotide for achieving specific gene inhibition. An array with 1938 oligonucleotides ranging in length from monomers to 17-mers has been built to measure the potential of the oligonucleotides for heteroduplex formation with rabbit β-globin mRNA [73]. The oligonucleotides were complementary to the first 122 bases of the mRNA. Surprisingly, very few oligonucleotides gave significant heteroduplex yield and no obvious features in the mRNA sequence or the predicted secondary structure could explain this variation. In fact, despite the utility of antisense inhibition in selected contexts, attempts to achieve antisense blockage of many cancer-related genes have failed. In addition, delivery of the antisense molecules has been highly problematic. The tumor environment is deleterious to these unstable molecules, and it is often difficult to maintain effective intracellular levels. To circumvent this problem, a number of design modifications of the antisense molecules have been developed to enhance their in vivo stability. In addition, a number of vector approaches have been explored for effective cellular delivery. Despite these various maneuvers, the overriding limitations to the employment of this still promising therapeutic modality remain the idiosyncratic efficacy of specific antisense molecules for a given target gene and its suboptimal delivery to date.

42.5.3 Posttranslational Inhibition of Genes

42.5.3.1 Single-Chain Antibodies

Dominant oncogenes have also been targeted at the posttranslational level. Techniques have been developed to allow the derivation of recombinant molecules that possess antigen-binding specificities expropriated from immunoglobins [74]. In this regard, single-chain immunoglobin molecules, denoted by scFv, retain the antigen-binding specificity of the idiotypic region of the immunoglobin from which they were derived, but lack other functional domains characterizing the parent molecule. The encoded scFv may be expressed in the target cell and localized to specific, targeted subcellular compartments by incorporating appropriate signal molecules. Based on this, an approach leading to oncogene suppression has been developed in our laboratory. It was shown that if an anti-erbB-2 scFv was localized to the endoplasmic reticulum (ER) of cancer cells, the nascent, newly synthesized erbB-2 protein would be entrapped within the ER and therefore unable to achieve its normal cell surface localization. It was also shown that this intracellular entrapment would prevent the erbB-2 product, a transmembrane receptor, from interacting with its ligand, abrogating the autocrine growth factor loop driving malignant transformation in erbB-2 overexpressing cell lines. We showed that intracellular expression of anti-erbB-2 resulted in the following cellular effects: (1) downregulation of cell surface erbB-2 expression; (2) marked inhibition of cellular proliferation; (3) marked reduction in survival of neoplastic cell clones; and (4) selective cytotoxicity in tumor cells expressing the proto-oncogene target [75]. In addition, scFv-mediated erbB-2 ablation induced additional phenotypic alterations in tumor cells, including chemosensitization and radiosensitization [76]. In addition, the ability to accomplish selective abrogation of erbB-2 expression has been shown to be effective in the eradication of primary human ovarian cancer cells [77]. This shows the feasibility of such a therapeutic approach for tumor cell eradication by utilizing intracellular immunoglobulins to achieve targeted disruption of dominant oncogenes, thereby accomplishing reversion of the malignant phenotype, chemosensitization, radiosensitization, and initiation of cell death. To this end, we have translated the strategy into an approved human clinical gene therapy protocol for ovarian carcinoma [78, 79]. The feasibility of this strategy against other oncogenes including ras and c-myc has also been shown in animal models [80–82]. In spite of its rapid refinement in a relatively short time frame, scFv application in cancer gene therapy has not yet been fully realized. Although oncogenes have been the primary target of scFv therapy, targeting of the gene therapy

vectors to specific cell surface receptors are now first being attempted by the construction of bifunctional adapter proteins. Among these are sCAR-CD40 (the ecto-domain of the coxsackievirus-adenovirus receptor (sCAR) with an scFv against human CD40) for efficient transduction of dendritic cells [83] and sCAR-MFE (sCAR with an scFv against human CEA) for targeting selective cancer cells expressing high levels of CEA [84].

42.5.3.2 Transdominant Molecules

Alternatively, oncogenes can be inactivated at the protein level by the heterologous expression of mutant proteins that inhibit the function of the native version of oncoproteins, the so called dominant-negative mutation strategy. For instance, transdominant mutants of c-Jun have been shown in vitro to possess potent suppressive effect on colorectal cancer cells [85]. The feasibility of efficient delivery and production of adequate levels of mutant protein in vivo has been shown in a mouse model of human lung cancer in which the dominant negative mutant of mitogen activated protein kinase kinase 4 (MKK4) [86] has been delivered. Currently, further refinement of this strategy is underway to translate this approach into clinical practice.

42.5.4 Phenotypic Expression of Neoplastic Progression Dependent on Associated Genes

42.5.4.1 Angiogenesis

Folkman in 1971, proposed that angiogenesis is required for tumor growth [87]. The development of new blood vessels is now known to be a critical factor in the growth, progression, and metastatic spread of both solid and hematopoietic tumors. Despite heterogeneity in virtually all other respects, all tumors share a *universal* feature, i.e., they depend on the vasculature to maintain their viability and to sustain their growth and dissemination. Extensive experimental data supports this contention [88–91]. Furthermore, numerous clinical studies have shown the correlation between the development of intratumoral microvessels and the prognosis of individual cases in a variety of cancers [92–94]. Vessel targeting, therefore, should be useful for the treatment of most kinds of cancer [95–98]. Importantly in this regard, the genetic stability of endothelial cells, admittedly different one from the other, by site, should help minimize the appearance of resistance to molecular therapeutic interventions targeted to these cells [99]. This hypothesis has indeed been confirmed in a cancer animal model of treatment with a natural inhibitor of angiogenesis; endostatin [100]. An additional advantage of targeted killing of endothelial cells is the highly amplified killing effect over large numbers of tumor cells when deprived from its blood supply.

In the last decade, antiangiogenic drugs targeted to the proliferating endothelium of tumors and other diseases have been applied in the clinical setting and have entered clinical trials. In addition, the association of chemotherapy or radiotherapy with antiangiogenic agents has been shown to produce an enhanced antitumor effect in preclinical models. Notably, combined treatments can achieve cures that are not observed with either treatment alone [101]. Thus, molecular therapeutic interventions against the tumor and its vasculature are not only strongly appealing on theoretical grounds for their use in a variety of clinical contexts, but their utility is also being tested clinically [102]. Based on this, genetic modification of the endothelium lining the tumor vasculature has been proposed as an alternative therapeutic modality [103]. With this genetic strategy, many of the problems of previously failed tumor/vessel approaches can potentially be overcome. For instance, local production of high levels of therapeutic proteins can be induced, thus obviating or diminishing the difficulties associated with systemic toxicity while other pharmacological issues, such as large-scale manufacture, bioavailability, and the cost of ordinary drugs can be circumvented. In addition, the ability to continuously release a gene-encoded product may be relevant in certain cases, such as the appropriate antiangiogenic effect of interferon-γ against selected hypervascular solid tumors.

Both suppression of angiogenic cellular signals and augmentation of natural inhibitors of angiogenesis have proven to be feasible strategies in in vivo tumor models. Examples of effective genetic interventions for the suppression of angiogenesis factors include the downregulation of vascular endothelial growth factor (VEGF) by antisense [104], blockade of VEGF functionality by delivery of mutant versions of one of its cognate membrane receptors, Flk-1 (VEGF-R2) [105–107], or of a secreted soluble version of its other receptor, Flt-1 (VEGF-R1) [108–111]. Downregulation of VEGF receptor (VEGF-R) by siRNA [112, 113], or ODNs in models of glioma [114, 115] and liver cancer [116] have also shown similar effects. Conversely, the replacement or supplementation of inhibitors of angiogenesis has been tried by using vectors that encode soluble platelet factor 4 [117], angiostatin [118], and endostatin [119, 120]. However, none of these strategies has been clinically tested in patients using similar gene therapy approaches and major issues remain to be solved. Most obvious is the probable need to assure long-term expression of the therapeutic antiangiogenic genes to keep the tumor deprived of its growth-enabling vascularization. In addition, the current lack of targetable, systemically injectable vectors impedes the simultaneous application of anti-angiogenesis gene-based strategies to multiple foci of tumor that characterize disseminated cancer. Lastly, different combinations of endothelial growth factors and its receptors are altered in different tumors, and may even change in single tumors during different stages of progression. Thus,

despite its powerful rationale, the successful clinical implementation of antiangiogenesis gene therapy will require additional major advances.

42.5.4.2 Invasion and Metastasis

Increasingly, genes and proteins involved in phenotypic aspects of tumors, in addition to disordered proliferation, are being described and identified as potentially useful therapeutic targets. In this regard, besides angiogenesis, one fundamental component of the metastatic cascade is the local invasion of the extracellular matrix by tumor cells. Studies in animal models have begun to show that modulation by gene transfer of molecules involved in degradation of extracellular matrix (ECM), cellular motility, and cellular adhesion has the potential for inhibiting tumor cell spread [121]. Gene therapy approaches have targeted the inhibition of pro-metastatic genes like uPA, LIM kinase-2 and replacement of mutated anti-invasive, anti-metastatic genes like *p202*, PA-1 and 2, *MKK4/SEK1* have all been championed to prevent the invasion and metastasis of cancer cells.

Urokinase-type plasminogen activator (uPA) is a protease involved in the processes of tissue remodeling, tumor invasion and cell migration in vitro. Plasminogen activators are thought to degrade ECM proteins and cellular basement membranes and thus allow local tumor invasion and access to the vascular system for neoplastic cells destined for metastasis [122]. The inhibitors of plasminogen activation, PA-1 and PA-2, have also been described in association with different types of cancer. The levels of both uPA and its receptor uPAR are elevated in ovarian, prostate, glioma and other tumor cell types [123–125] and correlate with the clinical stage of disease [124–126]. Furthermore, it has been shown that inhibition of uPA receptor expression by siRNA [127] or ODNs can cause regression of preestablished tumor and abrogate the spread of human glioblastoma in both in vitro [128] and in vivo model systems [129, 130]. In an in vivo model of uveal melanoma, an adenoviral vector has been used for the transfer of plasminogen activator inhibitor type 1 (PAI-1) cDNA [131]. Intraocular injection of the vector resulted in a 50 % reduction in the number of animals developing liver metastases and a reduction in the metastatic tumor burden in animals that eventually developed metastases. These results support disruption of uPA function through gene transfer as an experimental strategy for preventing metastases and prolonging host survival. LIM kinase-2 is another such pro-metastatic gene which when inhibited by ribozyme caused a reduction in the metastatic potential of the cancer cell [132].

Metastatic suppressor genes by definition suppress tumor metastasis without affecting primary tumor growth. Among these metastatic suppressor genes are p202 [133], KAI1 [134] and MKK4/SEK1 [135]. When ectopically expressed in AT6.1 Dunning rat prostate cancer cells, these metastatic suppressor genes have been shown to reduce the lung metastatic capabilities of the cell line in xenograft mouse models in vivo. Multiple classes of metastatic suppressor genes are now in preclinical studies to assess their efficacy in reducing the invasion and metastatic potential of tumors.

42.5.5 Obstacles to Mutation Compensation

Although the strategies currently used for restoration and ablation of mutant genes have offered in-depth insights into the molecular biology involved in carcinogenesis and tumor progression of cancer, they face critical problems for allowing their use in the clinic. Human tumors are remarkably heterogeneous in the patterns of expression of relevant oncogenes. Thus, therapeutic targeting of a single molecular abnormality may have only an inconsequential impact on the management of the disease, both for the population as a whole and for individual patients. In addition, several mutated genes produce molecules with transdominant effects, thus requiring the blocking of their effects and not only the mere supplementation of the gene with a wild-type version. Furthermore, because these strategies modulate intracellular responses, nearly every tumor cell must be targeted for these approaches to be clinically effective. The current state of development of gene therapy vectors, both viral and nonviral, makes this feat unachievable within nontoxic margins of vector dose. Clearly, breakthrough developments in vector technology are needed for these obstacles to be overcome. Lastly, approaches such as molecular chemotherapy or immune system augmentation that exhibit an amplified regional or systemic effect hold the promise of tackling, by their own design, some of the aforementioned limitations.

42.6 Molecular Chemotherapy

A number of distinct approaches to accomplish molecular chemotherapy for cancer have been developed. These include the administration of (1) toxin genes to eliminate tumor cells and the stromal cells that support them, or (2) drug resistance genes to protect the bone marrow from myelosuppression induced by chemotherapy, or (3) genes that enhance the effect of conventional anticancer treatments. Initially, the molecular chemotherapy approach was designed to achieve selective eradication of neoplastic cells via expression of a toxin gene. This is similar to conventional chemotherapy, where pharmacological agents are employed. However, in order to effect a reduction in the neoplastic cell burden, patient's normal cells, tissues and organs have to be exposed to potentially harmful doses of the drug., Molecular chemotherapy is designed to circumvent this limitation by selectively targeting delivery of

the toxin into cancer cells on the basis of more specific tissue or transformation-associated marker targeting, thus reducing the potential for nonspecific toxicity.

42.6.1 Toxin Genes: Gene-Directed Enzyme Prodrug Therapy (GDEPT)

In this approach, a nontoxic prodrug which requires enzymatic activation to be transformed into a toxic metabolite is administered systemically. The gene for that enzyme is then selectively delivered and expressed in cancer cells, wherein the prodrug is locally converted to cytotoxically active drug that ultimately leads to cancer cell death [136–140]. This approach carries the moniker gene-directed enzyme prodrug therapy (GDEPT) or suicide gene therapy. A variety of prodrug–enzyme combination has been shown to work effectively both in in vitro and in vivo model systems with many of them being translated into multicenter phase III clinical trials [141]. Herpes simplex virus thymidine kinase/ganciclovir (HSV-*tk*/GCV), bacterial cytosine deaminase/5-fluorocytosine (CD/5-FC), bacterial nitroreductase/CB1954 (NTR/CB1954), and cytochrome P450/cyclophosphamide are all GDEPT systems which have been or are now in clinical trials. P450 reductase/tirapazamine, carboxypeptidase/CMDA, horseradish peroxidase/indole-3-acetic acid or paracetamol are some of the other GDEPTs which are currently in the preclinical experimental phase of development.

42.6.1.1 Thymidine Kinase

The most common molecular chemotherapy system utilized to date to accomplish cell killing has been the herpes simplex virus thymidine kinase (HSV-*tk*) gene given in combination with the prodrug ganciclovir (GCV) [142]. The selectivity of the HSV-*tk* system is based on the fact that, contrary to normal mammalian thymidine kinase, HSV-*tk* preferentially monophosphorylates GCV, rendering it toxic to the cell. GCV is then further phosphorylated by cellular kinases to triphosphates that are incorporated into cellular DNA. The incorporation of the triphosphate form of GCV causes inhibition of DNA synthesis and of RNA polymerase, leading to cell death [138]. In vitro studies have shown that the HSV-*tk* increases the sensitivity of the cell to GCV by up to 2000-fold [143] and thus, tumor cells (or any other cell undergoing mitosis) transduced to express the viral *tk* gene have enhanced sensitivity to cell killing after exposure to GCV. Normal cells transduced with HSV-*tk* after intravenous [144] or intrahepatic [145] administration of adenoviral HSV-*tk* vector have also shown high sensitivity to GCV, leading to liver degeneration and low survival in mice. The absence of toxicity of GCV after intravenous administration of a control adenovirus, or subcutaneous administration of an adenovirus encoding HSV-*tk,* suggests that the toxicity is specifically liver-associated. Phase I/II clinical trials for HSV-*tk* have demonstrated the safety and efficacy of this system in many tumors including ovarian cancer [146, 147], retinoblastoma [148], colorectal cancer [149], malignant gliomas [150–152], melanoma [153], and prostate carcinoma [154]. But the clinical efficacy of this system is still poor. A multicenter phase III clinical trial with HSV-*tk*/GCV in 248 glioblastoma multiforme patients showed no significant difference between conventional treatment (surgical resection followed by radiotherapy) and conventional treatment with adjuvant gene therapy [155]. Poor transduction of the cancer cells and the use of non-migratory fibroblasts as retroviral delivery vehicles were considered to be the possible reason for the trial failure. Approaches of increasing the transduction efficiency using an adenoviral vector system have shown an improved mean survival time of patients with recurrent glioblastoma tumors [156].

42.6.1.2 Bystander Effect

While the benefits of selectively eradicating tumor cells are obvious, an important limitation associated with molecular chemotherapy (as noted earlier) is the inability to target 100 % of the tumor cells with the toxin gene. However, this may prove not to be as severe a limitation as initially believed due to the phenomenon known as bystander effect, whereby eradication of HSV-*tk* transduced cells elicits a killing effect upon the surrounding non-transduced tumor cells. That not all of the tumor cells need to contain the HSV-*tk* gene for obtaining complete eradication of the tumor was an observation of early experiments employing the relatively inefficient retroviral vectors in brain tumors [157]. This occurrence was later confirmed in a variety of other tumor model systems [158–161]. Our laboratory has previously established the necessary biological properties to elicit bystander-mediated cell killing in a murine model of ovarian cancer [162].

Both local and distant bystander effects have been described in in vitro and in vivo models. In this regard, the basic biological mechanisms that underlie the bystander effect have been hypothesized. They include (1) exchange of toxic metabolites through gap junctions between cells [163, 164], (2) phagocytosis of apoptotic bodies containing toxic metabolite by neighboring cells [158, 161], (3) tumor vasculature necrosis due to toxicity of the active drug to dividing endothelium [165–168], and (4) an immune response to GCV-exposed HSV-*tk* transduced tumor cells [167, 169, 170]. Both the local and distant bystander effects, observed in vivo in distant foci of untreated tumors, are accompanied by the regional induction of cytokines [169] and an immune cellular response against the tumor [170–173]. A phase I/II clinical trial showed this bystander effect in human glioma patients and a better prognosis (time of progression of disease, survival) in patients who had a bystander effect than those who did not [150]. Nevertheless, the authors were

guarded in considering it as an indicator for treatment effectiveness and outcome because the patients with bystander effect had other favorable prognostic factors including a younger age and smaller tumor volume.

42.6.1.3 Other Toxins

In addition to the HSV-*tk*/GCV system, several additional combinations of enzyme/prodrug have been developed to improve the efficacy of molecular chemotherapy (Table 42.5). Features of these combinations have caused investigators to hypothesize that they might overcome the limitations of HSV-*tk*/GCV. For example, some of them induce toxic effects not only in cycling but also in non-cycling cells (carboxypeptidase G2, nitroreductase, purine nucleoside phosphorylase). With others, the bystander effect is stronger (purine nucleoside phosphorylase) or does not require cell contact (cytosine deaminase, nitroreductase) (Table 42.5).

The combination of cytosine deaminase (CD) and 5-fluorocytosine (5-FC) has been the first of these alternative systems to be tested clinically [174]. Studies both in vitro and in vivo have shown that transfer of the microbial CD gene sensitizes cells to the innocuous antifungal drug 5-FC. This effect is induced by metabolizing 5-FC into the toxic antitumor agent, 5-fluorouracil (5-FU) [175]. By administering high doses of the nontoxic 5-FC, intratumoral activity of the enzyme can provide increased intratumoral concentrations of the active drug, without its accompanying systemic toxicity. Potential weaknesses of the system are its dependence on cellular proliferation, and its complex metabolism, which facilitates acquired resistance by selective tumor cell population.

With some notable exceptions, single drugs in standard chemotherapy do not cure cancer. Historically, effective treatments were developed when drugs with different mechanisms of action were used in combination. Extending this concept to molecular chemotherapy, several combinations of enzyme/prodrug have been shown to induce synergistic killing effects in vitro [176, 177]. Combination schemas have also achieved higher rates of tumor regression and cure in animal models [178, 179]. The application of classical principles for designing drug combinations would recommend the use of prodrug/enzymes that target both dividing and nondividing cells, that elicit different mechanisms of bystander effect, and that have nonoverlapping toxicities.

42.6.2 Drug-Resistance Genes

In a second molecular chemotherapy approach, the host's tolerance to higher doses of standard chemotherapeutic drugs is increased by transducing bone marrow cells, known to be highly sensitive to chemotoxicity, with genes that confer drug resistance [180, 181]. In this context, retroviral vectors have been the vectors of choice for the in vitro derivation of stably transduced cells, due to their capacity for integration in host cell chromosomes. It was shown that when mice transplanted with bone marrow cells containing a transferred multiple drug resistance (MDR1) gene were treated with the

Table 42.5 Prodrug–enzyme combinations for molecular chemotherapy

Enzyme (origin)	Prodrug system		In vitro bystander effect	Target cell	Reference
	Prodrug	Release drug			
Thymidine kinase (herpes simplex virus)	Ganciclovir and other modified pyrimidine nucleosides	Monophosphate nucleotide analogs	Yes	Dividing cells	[138]
Cytosine deaminase (*E. coli*)	5-fluorocytosine	5-fluorouracil	++ (Independent of cell contact)	Dividing and possibly nondividing cells	[174, 175, 352]
Nitroreductase (*E. coli*)	CB1954 and analogs	Alkylating agents, pyrazolidines	+++ (Independent of cell contact)	Dividing and nondividing cells	[353–356]
Cytochrome P-450 isoenzyme (rat liver)	Cyclophosphamide, isophosphamide	Alkylating agents	Yes	Dividing cells	[357]
Carboxylesterase (rabbit liver)	CPT-11 (irinotecan)	SN-38	+++	Dividing cells	[358, 359]
Carboxypeptidase G2 (bacterial)	CMDA	CMBA	+++	Dividing and nondividing cells	[360]
Deoxycytidine kinase (human)	Cytosine arabinoside	Cytosine arabinoside monophosphate	+ (Dependent on cell contact)	Dividing cells	[361, 362]
Purine nucleoside phosphorylase (*DeoD* gene of *E. coli*)	Purine nucleosides, flutarabine	6-Methylpurine, 2-fluoroadenine	+++	Dividing and nondividing cells	[363]
Xanthine-guanine phosphoribosyl transferase (*gpt* gene of *E. coli*)	6-thiopurines	6-thiopurine nucleoside	++ (Independent of cell contact)	Dividing cells	[364–366]

CB1954: 5-(aziridin-1-yl)-2,4-dinitrobenzamide; CMBA: 4-[(2-chloroethyl)(2-mesyloxyethyl)aminobenzoic acid; CMDA: 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid; SN-38: 7-ethyl-10-hydroxy-(20S)-camptothecin. Transduced cells needed for complete cell growth inhibition in cell mixing experiments: 10 % or less: +++; from 10 to 50 %: ++; more than 50 %: +

cytotoxic drug taxol, a substantial enrichment of transduced bone marrow cells was observed [180, 181]. The MDR1 gene encodes an ATP dependent plasma membrane efflux pump called P-glycoprotein (P-gp). Hydrophobic drugs like vinca alkaloids, taxol, anthracyclines, colchicine, actinomycin D, taxotere, and the epipodophyllotoxins are pumped out of the cell by P-gp, thereby rendering the cell resistant to these drugs. The demonstration of positive selection in an in vivo model system established the ability to amplify clones of transduced hematopoietic cells and suggested possible applications for human therapy. Patients with breast cancer [182–184], lymphoma [185], other advanced neoplasms [186], and germ cell tumors [187] have undergone clinical trials with autologous CD34 expressing hematopoietic cells transduced with retroviral encoded MDR1 [183]. Taken as a whole, this series of work has shown a selective expansion of MDR1 transduced hematopoietic cells in patients treated with drugs which can be effluxed out by P-gp. Another study [187] observed the expression of a drug resistance transgene for more than 1 year in 4 out of 9 patients in the trial. Currently a phase I clinical trial utilizing another drug resistance gene, methyl-guanine methyl transferase (MGMT) whose product O^6-alkylguanine-DNA-methyltransferase (AGT) provides resistance to O^6-benzylguanine (BG) is underway for patients with advanced solid tumors or non-Hodgkin's lymphoma. Some potential problems with this strategy are, however, apparent. These include (1) failure to demonstrate that higher chemotherapy doses translate into improved patient survival, (2) very low transduction efficiency of the target human hematopoietic cells with retrovirus vectors, (3) the dose-limiting effects determined by other non-hematological toxicities, and (4) the fact that cancer cells in the marrow could be transduced with the drug-resistance gene, which could rapidly give rise to clones of treatment-resistant tumor cells.

42.6.3 Chemosensitization and Radiosensitization

A third molecular chemotherapy approach seeks to modulate the level of expression of a variety of genes that influence the sensitivity of the cell to toxic stimuli, including conventional chemotherapeutic drugs and radiotherapy. Genetic chemosensitization can be achieved by modulating apoptosis, inhibiting tumor cell resistance, or enhancing the intratumoral production of cytotoxic drugs. To facilitate apoptosis, genes such as *p53* may be administered to tumor cells to enhance the mechanisms of apoptosis induced by chemotherapeutic agents [188]. In vitro studies with the human oral squamous cell carcinoma cell lines HSC4 and SAS have shown that replacement of wild type *p53* concomitantly with X-irradiation had a better cell killing effect than either of the two approaches separately [189]. A phase II clinical trial with Ad-*p53* and radiotherapy in patients with non-small lung cell cancer showed that 63 % of the patients showed no viable tumor 3 months post-therapy [39]. Our group has also shown that downregulation of Bcl-2 protein levels by an intracellular anti-Bcl-2 single-chain antibody increased drug-induced cytotoxicity [190]. Analogously, genetic downregulation of cellular factors related to chemoresistance has been shown to enhance chemosensitivity. Again, our group has been able to show that single-chain antibody-mediated abrogation of the c-erbB-2 oncoprotein can significantly mitigate intrinsic chemoresistance in erbB-2 overexpressing ovarian cancer cells. This allows for augmented sensitivity to the DNA-damaging drug cisplatin [76]. Alternatively, genes can be administered intratumorally that enhance metabolic conversion of conventional chemotherapeutic agents. Studies have shown that transfer of a liver cytochrome P450 gene, CYP2B1, into human breast cancer cells greatly sensitized these cells to the cancer chemotherapeutic agent cyclophosphamide as a consequence of the acquired capacity for intratumoral drug activation. This effect produced a substantially enhanced antitumor activity in vivo [191]. Lastly, combinations of conventional chemotherapeutic agents and molecular chemotherapy can serve the established rule of administering cytotoxic drugs with different mechanisms of action and toxicities.

Several drugs are proven radiosensitizers, a fact that is commonly exploited in the clinic. One of these drugs is 5-fluorouracil (5-FU), which is the product of the CD suicide gene. In this regard, molecular chemotherapy based on CD has been shown to enhance the effects of radiation therapy in animal models of gliosarcoma [192] and cholangiocarcinoma [193]. Thus, strategies to alter both chemosensitivity and radiosensitivity by gene transfer appear to have potentially wide applicability in many tumor contexts.

42.6.4 Obstacles to Molecular Chemotherapy

With all of its promise, molecular chemotherapy also bears some practical limitations. To date, the strategy of molecular chemotherapy has been mainly used in loco-regional disease models. In these in situ schemas, a vector encoding the toxin gene is administered intratumorally or into an anatomically closed compartment containing the tumor mass. The goals of this delivery method are to achieve high local vector concentration in order to favor tumor transduction and to limit vector dissemination. However, transduction efficiencies of presently available vectors have been shown to be inadequate. Even in closed compartment delivery contexts, it has not been possible to modify a sufficient number of tumor cells to achieve a relevant tumoral response in clinical models [194–198]. Furthermore, although transduction with HSV-*tk* followed by ganciclovir treatment reduces tumor burden and prolongs survival in various model systems, including those

utilizing intratumoral and intraperitoneal administration, the required increased doses of viral vector needed for obtaining quantitative tumor cell transduction is associated with limiting toxicity. In fact, substantial toxicity and experimental animal death have been noted [144, 145, 197]. Thus, the small therapeutic index of currently available vectors in the context of in situ administration is a critical limiting factor for the purpose of gene therapy of cancer. Furthermore, and most importantly, a well-known limitation of conventional chemotherapy is also to be expected with the use of molecular chemotherapy, i.e., the appearance of drug-resistant tumor subpopulations (Table 42.1). In conclusion, vector limitations and well-known barriers to classical cytotoxic maneuvers impede the full exploitation of the promise of a more selective eradication of carcinoma cells via expression of a toxin or protective genes.

42.7 Genetic Immunopotentiation

The development of clinically evident tumors in patients implies the obvious failure of the host immune system to recognize and eliminate tumor antigen(s). Involvement of the immune system or lack thereof, in the development of cancer has been known for eons with the earliest record of cancer immunotherapy in its rudimentary form being attempted in ancient Egypt around 2600 BC [199]. Simply stated, cancer cells have or acquire the ability to escape the immune system. Factors that can explain this failure of the immune system to recognize nonself in the cancer patients include: (1) inadequate immunogenicity of the tumor or (2) a deficiency of the immune system to recognize, respond, and reject. Genetic immunopotentiation strategies attempt to achieve active immunization against tumor-associated antigens by gene transfer methodologies applied either directly to tumor cells to increase their immunogenicity or to cellular components of the immune system to recognize the tumor cells more efficiently. To this end, insights into the pathophysiology of tumor escape from the immune system surveillance offer guidance for designing new therapeutic strategies [200].

42.7.1 Genetic Modification of Tumor Cells

One of the strategies for augmenting the antitumor immune response is to genetically modify tumor cells, or to manipulate their components, to facilitate a robust immune response. Thus, it has been hypothesized that a formerly tolerant host may revert from its immune status resulting in either tolerance or anergy, ultimately leading to tumor rejection. In other words, it is hypothesized that the host can be vaccinated against the tumor by exposing tumor antigens to the immune system in a more favorable context [201–203]. Most clinical experience with antitumor vaccines to date has been obtained with melanoma patients. For years, irradiated tumor cells, either autologous or allogeneic, have been administered in combination with different adjuvants, such as Bacille Calmette-Guérin (BCG) back into stricken patients. Later, as our molecular knowledge of tumor-associated antigens progressed, this allowed the testing of vaccines based on individual antigenic determinants delivered to the patient in the form of peptides or DNA. More recently, tumor cells themselves have been genetically modified to increase their immunogenicity by transfer of a variety of genes, including cytokines such as IL-1, IL-2, and GM-CSF, along with MHC molecules, costimulating signal molecules such as B7, and tumor associated antigens themselves. A common requirement, not yet adequately accomplished, is to introduce the gene of interest into tumor explants or cultured cells with high efficiency.

42.7.1.1 Cytokine Gene Transfer

The utility of antitumor vaccines based on whole tumor cells has been poor when analyzed in all (randomized) clinical trials to date. However, it has been argued that the genetic modification of tumor cells with carefully selected cytokine genes will eventually lead to an augmentation of the immune response against the tumor [204]. One possible intervention is to induce, by intratumoral gene transfer, cytokines that, once secreted by the tumor cell, can activate directly the response of cytotoxic T-lymphocytes (CTL), and thus increase the awareness of the immune system cells to tumor cells in the microenvironment. Cytokines tested to this end include: IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IFN-γ, TNF, G-CSF, and GM-CSF [205–219]. In some cases, intense inflammatory infiltrates have been observed surrounding cytokine-secreting tumor cells, with the type of infiltrate varying with the particular cytokine utilized. In animal models, immune-mediated tumor regressions are consistently observed with this strategy. Also, specific immunity mediated by CTLs against subsequent exposure to unmodified tumor cells is commonly observed in treated animals. A phase I clinical trial using adenoviral vector to deliver IL-12 intratumorally in patients with advanced gastrointestinal tumors showed the safety of the approach [220]. Under the experimental conditions, tumor growth was seen to stabilize in 30 % of the patients in this clinical trial. Another phase I clinical trial using IL-2 showed similar effects in patients with prostate cancer. The tumors were excised and pathological analysis showed intense necrosis and T-lymphocyte infiltration [221]. Other clinical trials using IL-24 (melanoma differentiation antigen gene-7 [*mda*-7]) [26, 27, 29], GM-CSF [222–224], TNF-alpha [225, 226] and γ-IFN [227, 228] have also shown the safety and efficacy of the approach in phase I clinical trials. Even though phase I trials have been successful, cytokine gene therapy as a single treatment approach for cancer has not advanced given the recognized

complexity of immunological intervention and its potential host toxicities.

42.7.1.2 Induction of MHC Expression and Transfer of Allogeneic MHC

Recognition of a tumor-associated antigen by CTLs requires its simultaneous presentation with major histocompatibility complex (MHC) class I molecules on the surface of tumor cells. Hindering this approach is the evidence for a decrease in MHC class I expression levels in numerous tumors samples derived from patients [229]. To complicate matters further, in vitro co-culture experiment of cancer cell with CTLs demonstrated the evasion of these cancer cells (with low MHC class I molecule levels) from cytotoxicity [230, 231]. In this regard, gene therapy approaches directed at replacing the MHC class I antigen on the tumor cells surface have been attempted. Interestingly, tumor transfer of allogeneic MHC genes can generate CTLs reactive not only against the treated tumor mass, but also against non-modified tumor cells. This results in widespread tumor regression [232]. On this basis, phase I and II clinical trials have been performed by transferring the HLA-B7 gene, by intratumoral injection into tumors that do not express the molecule [233, 234]. The results of a recent multicenter phase II trial utilizing plasmid DNA expressing HLA-B7/β2-m mixed with cationic lipid has shown minimal adverse effects while exhibiting biological activity at the given dose (9.1 % of the patients had a complete or partial response and another 23.4 % had stable disease) [235]. Other cytokines like Interferon-γ (IFN-γ) and tumor necrosis factor (TNF) also stimulates the expression of MHC molecules, and can, therefore, make tumor cells more recognizable to the immune system [236]. In vitro studies have confirmed that the transfer of the interferon-γ gene increases MHC expression. These results were the foundation for establishment of a phase I clinical trial using this approach [237]. A theoretical risk of upregulating MHC class I antigen is the inhibition of the antitumor activity of NK cells, which recognize and attack specifically MHC class I-negative cells.

42.7.1.3 Costimulatory Molecules

Rejection of a tumor by CTLs requires not only the presence of cell surface tumor antigens and their appropriate display in association with MHC class I molecules (signal 1) but also an array of *costimulatory signals*, provided by antigen-presenting cells (APCs) (signal 2). A costimulatory signal promotes the clonal expansion of antigen-specific T cells, cytokine release and their differentiation into effector and memory cells. These costimulatory signals also reduce the threshold for T-cell activation especially when recognizing weak antigens like tumor associated antigens [238]. The efficacy of increased costimulation of T lymphocytes for antitumor immunotherapy has been shown in mice vaccinated with tumor cells expressing genes encoding several costimulatory molecules. For instance, tumor cells transfected with B7.1 (also known as CD80, a ligand of CD28 in lymphocytes and distinct from HLA-B7) potently stimulate an immune response, which is not observed with unmodified cells. When injected into syngeneic animals, B7.1 expressing cells are rejected, whereas unmodified cells are not. Furthermore, B7.1 expressing cells induce a potent immune response against unmodified cells in distant regions. The reason is that the presence of B7.1 increases the activation of T lymphocytes, but the cytolytic activity of differentiated CTLs does not depend on B7.1. These results in animals led to the development of clinical trials, in which tumors from patients were cultured, genetically modified, irradiated, and finally reintroduced into the same patient as vaccines. This strategy, although time-consuming, technically demanding, and costly, could be effective even when no tumor antigens had been identified. A phase I clinical trial utilizing recombinant vaccinia virus to deliver B7.1 for the treatment of melanoma showed only minimal side effects, i.e., low grade fever, myalgia, and vitiligo. Of the 12 patients in the trial, partial response to treatment was observed in one patient and disease stabilization in two more. Local immunity at the tumor site suggested tumor regression and an increase in the expression of CD8 and IFN-γ. Immune ignorance of primary tumor sites due to a lack of mobilization of activated T lymphocytes towards the original tumor localization can severely limit the utility of strategies based on costimulatory molecules and may thus explain the lack of effectiveness to date in patients treated by this methodology.

Other molecules involved in costimulation are formed by the intercellular adhesion molecules ICAM-1, ICAM-2, ICAM-3 and the lymphocyte functional antigen (LFA-1). Many tumors do not express ligands of the ICAM family efficiently, thus decreasing their ability to costimulate or be targets of the immune response. Again, the transfer of the corresponding gene could conceivably be useful to augment the effect of antitumor vaccines, and that seems to have been the case in several animal models [239, 240]. CD40, present in APCs, and its ligand in lymphocytes, CD40L, form a third system of costimulatory molecules of unclear importance with respect to the systems mentioned above.

42.7.1.4 Vaccination with Tumor-Associated Antigens

There are five general *categories of tumor antigens*. First, antigens coded by genes that are silent in normal cells and expressed almost uniquely in tumors, such as the MAGE family of antigens in melanomas. Second, antigens resulting from mutations in normal proteins, such as p53, MUC-1, MUM-1, CDK4, and beta-catenin. Third, differentiation antigens, present normally in the cells from which the tumor is thought to originate, such as melanoma antigen recognized by T cells (MART-1), gp75, gp100, and tyrosinase, again in melanoma and melanocytes. The fourth category includes antigens encoded by normal genes that are dysregulated in

tumor tissues, such as erbB-2. Finally, antigens encoded by genomes of oncogenic viruses, such as the human papillomavirus E6 and E7 gene products round out the tumor antigen family.

Antitumor vaccines have been developed based on tumor-associated antigens. Patients have been vaccinated with these tumor-associated antigens combined with adjuvants that increase their immunogenicity. The rationale is the probability that existing T-lymphocytes with antitumor specificity can be activated and specifically stimulated by the vaccine. Immunization could further be performed by direct administration of the peptides, by viral vectors encoding the antigens, by cellular lysates obtained after infection of tumor specimens with vaccinia vectors encoding the antigens, or by naked DNA.

One limitation of vaccines based on peptides is that it is improbable that these peptides could efficiently replace other non-immunogenic peptides already present and associated with MHC molecules in the surface of tumor cells in the patient. Furthermore, extracellular proteins are presented to the immune system via MHC class II molecules, which activate $CD4^+$ helper lymphocytes but not cytotoxic $CD8^+$ lymphocytes. In contrast, DNA vaccines are better stimulators of CTLs resulting in expression of antigens from within cells, where they are associated with MHC class I molecules and are thus presented in the appropriate manner at the tumor cell surface.

The MUC-1 mucin is a transmembrane glycoprotein expressed by both normal and malignant cells. Hypoglycosylated forms of MUC-1 are associated with adenocarcinomas having exposed the cryptic epitopes which are recognized by the immune system of the host [241]. Unlike other tumor associated antigens, the CTLs specific for MUC-1 exhibit both MHC restricted and unrestricted recognition of cancer cells expressing MUC-1, thereby making it an excellent target for immunotherapy [242, 243]. Phase I clinical trials with MUC-1 in combination with IL-2 in patients with advanced prostate cancer [244] and other MUC-1 positive advanced forms of cancer [245] showed its safety and efficacy. Four of the 13 patients in the latter study had stabilization of their disease for approximately 6–9 months. Other particularly attractive candidate antigens (as they are shared by multiple tumors) include the melanoma antigens MAGE-1, MART-1, and gp100, which are currently being used in several ongoing clinical trials.

42.7.2 Genetic Modification of Immune Effector Cells

So far we have seen gene therapy approaches targeting tumor cells to increase their immunogenicity and thereby facilitating the immune system to clear the tumor cells from the host. Alternatively, the host immune effector cells themselves can be modulated by gene therapy techniques to effectively increase their efficiency in removing cancer cell populations from the patient.

42.7.2.1 Tumor Infiltrating Lymphocytes (TIL)

Several therapeutic maneuvers have been based in trying to modify the cells of the immune system. Tumor infiltrating lymphocytes (TIL) are derived from mononuclear cells obtained from leukocytes infiltrating solid tumors. In the early 1990s, it was hypothesized that TILs could be an enriched source of NK cells and CTLs specific for tumor antigens and could also have tropism towards systemic tumor foci. On this basis, technology for their expansion in culture was developed and TILs were the first immune cells to be genetically modified and applied in a human gene therapy clinical trial against cancer [246]. It was soon observed that while TILs do include CTL and NK activated cells, only a few of these cells in these mixed populations are specific against the tumor from which they are isolated. Furthermore, reinfused TILs localized poorly into tumors.

Three strategies have been applied to improve treatments based on TILs [247]. First, the IL-2 gene has been transferred into TILs to increase lymphocyte number and survival when in the host. Second, to boost antitumoral efficacy, the tumor necrosis factor (TNF) gene has been transferred into TILs ex vivo previous to reinfusion. Thirdly, to improve localization into tumor foci several novel cellular receptors have been engineered in lymphocytes by genetic and immunological means. In this regard, Steven Rosenberg, who pioneered clinical trials with TILs at the National Cancer Institute, has reported rare clinical responses, with notable toxicity from the IL-2 treatment frequently added to maintain the requisite number and functionality of TILs. Localization of TILs in tumor biopsies is, as mentioned, modest. Targeted lymphocytes expressing chimeric T-cell receptors against tumor antigens have been developed with the aim of improving that barely exploitable tropism, and are currently undergoing clinical testing in colorectal and ovarian cancer, and melanoma.

42.7.2.2 Dendritic Cell (DC) Targeted Gene Therapy

Dendritic cells, with their unique ability to prime naïve T cells to initiate immune response are the most powerful antigen presenting cells (APC) identified to date [248]. In contrast to T cell stimulation, which is based on distinction between self and nonself antigens, DC stimulation has been hypothesized to be dependent on a danger signal as perceived by DCs [249]. For e.g., pro-inflammatory signals associated with microbes will lead to DC activation which in turn activates specific T cells, while a steady state condition would not cause activation of DCs and hence would maintain a state of peripheral tolerance. The tumor microenvironment comes under the latter category with no

DC activation, as the immune system does not perceive it as possible threat [250].

DC Maturation Strategies

Poor DC differentiation and maturation has been observed in many tumors, and a decrease in DC infiltration of the tumor microenvironment is considered as a poor prognostic factor among different cancers [251, 252]. Therefore gene therapy approaches targeted to correct the DC maturation in cancer are underway. Different cytokines are known to modulate the differentiation and maturation of DCs. The most commonly used cytokine in this regard is GM-CSF, which stimulates DC maturation [253]. In vivo studies in mouse models of cancer showed an increase in the anti-tumor immunity following GM-CSF transfer [254–257]. Other cytokines used for this purpose include IL-4 [258], TNF-α [259], IFN-α [260], and IL-18 [261].

DC Targeted Polynucleotide Immunization

Alternatively, the mature DCs are transferred with the genes for tumor associated antigens (TAA) either ex vivo or in vivo to potentiate the immune response of the host T cells to TAAs and to eliminate the tumor. The most common ex vivo approach involves the generation of DCs in vitro from monocytes, followed by TAA transduction and adoptive transfer. Though the transduction efficiency can be controlled by this approach, it is a tedious, time consuming procedure and also may serve as a possible source of infection when introduced into the patient. In vivo targeted delivery of TAA to DCs has, however, demonstrated tumor rejection in mouse studies. But the route of administration has to be considered carefully to target DCs, with intradermal injection of the vector with TAA being the preferred route. Also, the efficacy of this approach depends on the DC-mediated activation of CTLs. Targeted transduction of DCs in vivo is being attempted by using different targeting molecules including DC-SIGN [262–265], CD1 molecules [264], TNF and TNF receptor superfamilies. A number of phase I clinical trials utilizing viral vectors to deliver different TAA to DCs have shown the safety of this method, but there was no objective clinical response reported [266–268].

42.7.3 Obstacles to Genetic Immunopotentiation

The main advantage of genetic immunopotentiation is the possibility of enlisting physiological mechanisms for a potentially vast amplification of the therapeutic maneuver. To this end, even modest levels of gene transfer can theoretically be followed by clonal expansion and systemic spread of effector immune cells and mediators. Thus, efficiency of gene transfer here is not critical, given the relatively low amounts of cells and gene products needed to obtain a potentially powerful response from the immune system. There are, however, other more important obstacles that perhaps explain the *poor* results obtained to date by tumor immunotherapy in humans. Antigenic heterogeneity and plasticity, redundancy of immune system regulation, and well-established tolerance to natural tumors are the more evident barriers.

42.7.3.1 Antigenic Heterogeneity and Plasticity

During the last two decades numerous reports have confirmed both in vitro and in vivo that expression of tumor cellular antigens in different tumor types is heterogeneous, with variability being found not only between different patients with the same tumors, but between different regions of a single tumor and even in single cell clones [269, 270]. Moreover, this variability changes with time. This fact, first confronted by monoclonal antibody therapists, may clearly limit the impact of vaccines against single tumor-associated antigens, including those based on cultured, homogeneous populations of tumor cells.

42.7.3.2 The Redundant Phenomenology of the Immune System

The destructive power of the immune system, occasionally needed in its entire exuberance, obligingly requires a complex network of balances and counterbalances to control the pathways of activation and termination of the immune response. Interventions directed to supplement or inhibit single mediators will most probably obtain partial physiological and therapeutic results in the best case, may frequently yield no result at all, and occasionally will produce effects opposed to those desired. Increasingly, combinations of cytokines are being used to try to control the complexity of the immune response against tumors. Current therapeutic interventions for inducing organ graft tolerance successfully prolong organ survival by blocking multiple effector cells and mediators of the adaptive and innate immune systems. Similarly, it is conceivable that breaking tumor tolerance will require a strategy of multiple interventions against several simultaneous target cells and cytokines.

42.7.3.3 Lack of an Immune Response (Tumor Tolerance)

Current knowledge in tumor immunobiology establishes that T cells able to recognize tumor-associated antigens can be found in vivo and are inducible (with varying degrees of difficulty) in vitro. Thus, the lymphocyte repertoire against these epitopes has not been fully defined. However, either tolerance to these (tumor) self-antigens has been induced or,

in the absence of costimulatory signals, peripheral T cells simply have ignored these antigens [271]. This phenomenon should be an early event in tumor progression [272], but may have been totally missed in many animal models employed to date, which are based on tumor grafts. In this regard, studies with transgenic mice that develop spontaneous tumors have shown that vaccination with tumor cells transduced with cytokines fail to inhibit tumor onset and progression, whereas the same cells are able to immunize non-transgenic mice subsequently grafted with tumors [273]. Thus, the failure of naturally established tumors to efficiently present antigens, and to attract and activate tumor-specific T cells at the tumor site may impede successful vaccination against cancer antigens. Furthermore, ignorance by the immune system can abort most of the immunotherapy maneuvers being tested.

42.8 Oncolytic Viruses for Cancer Therapy

The anticancer effects following natural viral infections have been recognized for more than a century. Numerous examples of remission of cancers, including acute lymphoblastic leukemia [274] and Burkitt's lymphoma [275], following varicella or measles virus infections have been documented since the 1950s. However, due to the toxicity associated with using wild type virus [276] and lack of techniques to upscale and purify viruses, this approach for treating cancer did not take off until 1990s, when recombinant DNA technology revolutionized the field of molecular biology. Oncolytic viruses (Onco: cancer, lysis: killing) are replication competent viruses, which can selectively replicate and hence kill the cancer cells by their cytopathic effects. The cancer cell selective replication of these viruses may be due to genetic engineering of wild type virus (e.g., HSV-1, adenovirus), attenuation of natural virus (e.g., Newcastle disease virus) or use of nonpathogenic viruses in humans (e.g., reovirus) that replicate specifically in tumor cells [8]. Since the first description of genetically engineered conditionally replicating virus (CRV) 15 years ago [277], the field of oncolytic virotherapy has significantly advanced with the approval of H101—an oncolytic adenovirus treatment for head and neck cancer in China [278]. Table 42.6 shows the list of oncolytic viruses which are currently being used in a clinical setting. We shall briefly discuss the tumor selective replication mechanism for oncolytic Herpes Simplex Virus (HSV) and conditionally replicative adenovirus (CRAds) and also discuss double-armed CRVs which are essentially oncolytic viruses carrying additional therapeutic genes (such as pro-apoptotic genes) to enhance cell killing.

Table 42.6 Conditionally replicating oncolytic viruses commonly used in clinical trials

	Parental strain	Agent name	Genetic alteration in virus resulting in selectivity	Target molecules in cancer cells rendering virus replicative
Engineered virus	Adenovirus	ONYX-015	(1) Δ E1b-55K, (2) Δ E3b deletion	Mutated *p53* molecule
		Ad-Δ24	Δ Amino acids 121–128 of E1a gene	Mutated *Rb* gene product
		H101	(1) Δ E1b-55K, (2) Δ E3	Mutated *p53* molecule
		CN706	E1a gene under PSE element	Prostate tissue specific expression of E1a gene product
		AvE1a041	E1a gene under AFP promoter	HCC and testicular carcinoma specific expression of E1a gene product
	Herpes simplex virus (HSV)	G207	(1) Δ ICP6 (ribonucleotide reductase subunit), (2) Δ *γ34.5* gene	Activated Ras pathway and decreased PKR
		1716	Δ *γ34.5* gene	Loss of neurovirulence
		G47Δ	(1) Δ ICP6 (ribonucleotide reductase subunit), (2) Δ *γ34.5* gene, (3) Δ *α47*	Activated Ras pathway, decreased PKR and upregulation of MHC I molecules with HSV-1 antigens
		G92A	*ICP4* gene under albumin enhancer/promoter	Liver tissue (HCC) selective replication
Non-engineered virus	Reovirus		None	Activated Ras pathway
	Newcastle disease virus (NDV)	NDV (73-T)	Unknown (serial passage in cancer cells)	IFN resistance of tumors
	Vesicular stomatitis virus		None	IFN resistance of tumors

AFP α-fetoprotein, *HCC* hepatocellular carcinoma, *ICP4, ICP6* infected cell protein 4 and 6, *IFN* interferon, *MHC I* major histocompatibility complex I, *PKR* protein kinase R, *PSE* prostate-specific enhancer

42.8.1 Oncolytic Herpes Simplex Virus

HSV is a naturally occurring neurotropic virus which initially was genetically engineered to have a single mutation in a nonessential gene associated with either viral virulence or viral DNA synthesis, to make it conditionally replicative in cancer cells [279, 280]. This first generation vector was shown to be an effective oncolytic agent, but concerns that a further single mutation could revert it to wild-type phenotype led to the development of second and third generation oncolytic HSVs that have more than one mutation. G207, a second generation oncolytic HSV, with deletion of both copies of the γ34.5 gene—the major determinant of neurovirulence [281], showed its selective oncolytic potential. The *γ34.5* gene antagonizes the double stranded RNA-dependent protein kinase R (PKR) response seen following HSV-1 infection that, in turn, leads to the shut down of cellular protein machinery [282]. Since Ras signaling is activated in tumor cells with inhibition of PKR, the *γ34.5* gene deleted G207 could be seen to replicate selectively in tumor cells [283, 284].

There is another insertional mutation of the *Escherichia coli lacZ* gene, in the infected-cell protein 6 (ICP6) coding region, which inhibits ribonucleotide reductase—a key enzyme for viral DNA synthesis in nondividing cells [285]. These double mutations (the *γ34.5* gene deletion and *lacZ* gene insertion) greatly minimizes the chances of G207 reverting to the wild type phenotype. Additionally, the presence of the *lacZ* gene can be utilized to detect G207 infected cells histochemically. R3616, 1716, and NV1020 are other second generation oncolytic HSVs with the same *γ34.5* gene deletion [281, 286]. Preclinical in vitro and in vivo studies have shown the selective replication of G207 in cancer cells with inhibition of tumor growth and prolonged survival in mouse models of human glioma [287, 288]. Since HSVs are neurotropic viruses, G207's neurotoxic effects were evaluated in nonhuman primates. Results showed normal brain tissues at necropsy with no infectious particles or viral DNA detected outside the brain [289, 290]. These safety profile data led to phase I clinical trials using G207 and 1716 oncolytic viruses. The dose escalation phase I trial for G207 in patients with recurrent gliomas showed a maximum tolerated dose of 3×10^9 plaque forming units (pfu) [291]. Of the 21 patients studied at two centers, two of them had seizures and one patient had brain edema. All patients showed disease progression but histology of the brain samples at autopsy in five patients showed no evidence of encephalitis or other inflammatory changes and all tissues were negative for HSV-1 immunoreactivity. NV1020, another oncolytic HSV was used in a phase I clinical trial in patients with colorectal cancer with liver metastasis. It showed similar tolerable doses with minimal adverse effects [292]. G47Δ is a third generation oncolytic HSV, which has an additional mutation in the *α47* gene, is also being currently tested in both in vitro and in vivo studies. The *α47* gene product normally inhibits a key transporter associated with antigen presentation and hence the G47Δ virus infected cells present higher levels of MHC I molecules and were better at stimulating cytotoxic T-lymphocytes. However, since the interaction of the *α47* gene product with the transporter is species specific, it is not possible to test this virus in in vivo tumor models [293].

42.8.2 Oncolytic Adenovirus

When a cell is infected with adenovirus (Ad), the cellular *p53* and *Rb* genes activate the cell's apoptotic pathway and inhibit cellular proliferation respectively, to contain the viral spread to other cells. Adenovirus in turn has evolved to antagonize the function of these TSGs by expressing certain proteins, which interact with the components of the *p53* and *Rb* protein pathway, thereby promoting cellular proliferation and inhibiting apoptosis. E1a, E1b, E4-orf6/7, and E4-orf6 (viral early proteins) are the major proteins encoded by Ad for this purpose. E1a [294, 295] and E4-orf6/7 [296] inhibits the *Rb* protein product, thereby promoting cellular proliferation, while the E1b 55K protein [297] and E1b 19K protein [298] in a *p53* dependent pathway, and E4-orf6 protein product [299–301] in a *p53* independent pathway prevents the premature apoptosis of the infected cell. In this regard, genetically engineered Ads with mutations in either of these genes can replicate only in cells with a defective *p53* or *Rb* pathway (cancer cells). E1a and E1b are the most commonly modified viral genes in derived CRAds.

ONYX-015 is a E1b-55K gene deleted virus [302] and was shown to be selectively replicative in tumor cells with a dysregulated *p53* pathway. Subsequently it was determined that the deletion of the E1b-55K gene affected the late viral protein synthesis regardless of the *p53* status of the cell [303, 304]. This led to the development of other mutants which did not affect the late stage of viral replication (e.g., ONYX-051 with a R240A point mutation in the E1b-55K region of ONYX-015). Irrespective of that observation, ONYX-015 was tested in a number of phase I/II clinical trials and shown to have a very good safety profile [305–311]. Ad-Δ24, an oncolytic adenovirus which has a deletion of amino acids 121–128 in the E1a region has also been shown to be effective in inducing cytopathic effects selectively in cells with a dysregulated *Rb* pathway [294]. Table 42.6 provides a list of the most commonly used oncolytic viruses currently being used in clinical studies.

42.8.3 Double Armed Conditionally Replicative Virus

In spite of their great potential, oncolytic viruses do not achieve complete lysis of all cells within a tumor tissue because of tumor cell heterogeneity. The introduction of a therapeutic gene, be it one that codes for a cytokine or a pro-apoptotic gene

greatly enhances their oncolytic potential. But therapeutic genes selected should not interfere with the replicative properties of the virus they exist within. Further, they should be expressed in the late phase of the viral replication. In this way the local concentration of the therapeutic gene product is increased many fold due to the replication capacity of the vector, when compared to its delivery via a replication defective virus. Cytokines like IFN [312, 313] and TNF [314], pro-apoptotic genes like *Bcl-xs* [315], *p53* [316], and *mda*-7/IL-24 [317, 318], and prodrug conversion enzymes like HSV-*tk* [319, 320] and CD/uracil phosphoribosyl transferase [321] have all been used within CRAds, complementing their effects both in vitro and in vivo while demonstrating a greater cytopathic effect than seen with either of them alone.

42.8.4 Obstacles to Oncolytic Viral Cancer Therapy

One of the major issues for achieving an acceptable therapeutic index in patients is the selective replication of oncolytic virus in tumor cells avoiding nonspecific replication in noncancer cells. Adenoviruses, due to their natural hepatotropism, have to be untargeted from liver to avoid hepatotoxicity. To achieve this liver-off, tumor-on replication pattern, different approaches including transductional targeting (redirecting Ad away from its natural coxsackie-adenovirus receptor [CAR] to different receptors [322], transcriptional targeting (transcription of E1a gene under tumor or tissue specific promoters [323, 324], translational targeting (translation of E1a gene only in tumor cells [325]) and liver-untargeting by hexon swapping (which decreases coagulation factor X binding to Adenovirus and hence decreased liver tropism) [326] have been attempted. Our Center had recently undertaken the first phase I clinical trial in the USA utilizing a CRAd, specifically the Ad-Δ24, which has a RGD (Arginine–Glycine–Aspartic acid) peptide modification in its fiber [327] that binds integrins overexpressed in cancer cells (transductional targeting), for the treatment of patients with ovarian cancer [328]. Transcriptional targeting of oncolytic viruses utilizing different tissue or tumor specific promoters (such as CXCR4, survivin, midkine [329], rodent progression elevated gene-3 [330], or modified E2F-1 promoter [331]) are also being evaluated in both in vitro and in vivo model systems for their tumor specific oncolytic functions. Our lab has also recently shown the ability to target the replication of CRAd by translational regulation of E1a in addition to its transcriptional control [325]. A long and highly structured 5′ untranslated region (5′ UTR) element from fibroblast growth factor-2 (FGF-2) mRNA was inserted upstream of the E1a region. Eukaryotic translation initiation factor (eIF4E) which is over-expressed in solid tumors is needed to unwind this highly structured 5′ UTR element upstream of the E1a transcript. This leads to the regulation of translation of E1a mRNA only in cells over expressing eIF4E (cancer cells) and thereby allowing replication in cancer cells only [325].

42.9 Novel Strategies to Overcome Current Limitations of Gene Therapy

Gene transfer therapies are remarkably successful in in vitro and in vivo animal model systems. In effect, we already know that the malignant phenotype can be reverted in tumor cell lines by knocking out or knocking in certain genes; that tumors can be eradicated by delivery of cytotoxic genes followed by treatment with appropriate prodrugs; and that tumors can be cured in murine models by making the tumor cells either more immunogenic or by making the immune system cells more responsive, via the expression of cytokines, costimulatory, or immunogenic molecules. However, overriding limitations have been made apparent in preclinical experiments and in the first human gene therapy clinical trials against cancer, as emphasized by the Orkin and Motulsky Report to the NIH [332]. Most difficulties in obtaining clinically relevant benefits come from the inefficiency of current gene vectors in transducing tumor or immune cells and their inability to access selectively target cells distributed systemically. In this regard, each of these limitations undermines the implementation of a particular gene therapy strategy. Several avenues for improvement have been proposed, and some will be succinctly reviewed.

42.9.1 A Successful Gene Therapy Approach Requires Targeting

Less than optimal transduction levels of vectors may require the employment of gene vectors at higher magnitudes of infectivity, leading to target cell cytotoxicity. Thus, for direct in situ infection of selected organs, improvements in basic gene transfer efficiency may be required. In addition, and more specifically, the promiscuous tropism of the vector may potentially allow ectopic transduction of non-tumor cells with therapeutic genes. Therefore, strategies to enhance the efficiency of the vector as well as methods to enhance the specificity of target cell transduction will be necessary to render gene delivery optimal for cancer gene therapy purposes. In this regard, studies have demonstrated the feasibility of creating tropism-modified retroviral and adenoviral vectors to achieve cell-specific targeting (Table 42.7). To this end, immunological and genetic strategies for retargeting vectors to nonviral specific cellular receptors have been designed. Modifications of the adenovirus vector to alter native viral tropism in order to achieve selective transduction of target cells have proved to be feasible [333–336], in contrast with the more structurally demanding characteristics

Table 42.7 Targeting of cancer gene therapy

Principle	Basis	Strategies	Examples of applications
Selective delivery (transductional targeting)	Anatomically directed administration	Intratumoral, intravascular, or body compartment injection of vector	Injection of a plasmid encoding the immunostimulatory molecule B7 in foci of malignant melanoma [367]; intra-arterial infusion of a plasmid encoding VEGF in an ischemic vascular tree [368]; intraperitoneal administration of adenovirus expressing the suicide gene *tk* in model of ovarian cancer [369]
	Target cell physiology	Exploit cell cycle differences: gene transfer by retroviruses occurs only in dividing cells	Brain tumors (tumors are surrounded by neural cells, which are non-mitotic) [370]
		Exploit cell cycle differences: Herpes virus deleted in *tk* or other genes replicates only in cells undergoing division	Injection of virus is followed by lytic replication in liver metastasis [371] and brain tumors [372] (tumors are surrounded by cells that are non-mitotic)
		Exploit differences between transformed and normal cells: adenovirus deleted in E1B gene replicates selectively in p53 defective tumor cells	Injection of virus is followed by lytic replication in head and neck tumor cells [297]
	Specific receptors in target cells	Retrovirus: pseudotyped retroviruses (built with heterologous envelope proteins that confer novel tropism); retroviruses with genetically, chemically, or immunologically modified envelope proteins	Retrovirus with the genome and core of murine leukemia virus and the envelope protein of vesicular stomatitis virus have wider tropism [373] Retrovirus with modified envelope protein that includes a fragment of erythropoietin [374] or a single-chain variable region directed against known epitopes such as MHC class I molecules [375] or CEA [376]
		Adenovirus: genetic modifications in the fiber; immunologically mediated attachment of cellular ligands	The modification of the adenovirus fiber is feasible [377, 378]. Adenovirus with folate [378] or the fibroblast growth factor (FGF) attached to the virus via anti-knob Fab antibody fragments in ovarian tumors
		Molecular conjugates that combine a DNA binding domain and a cellular ligand	Asialoglycoprotein-based conjugates target hepatocytes [379]; adenovirus enhances transduction efficiency of conjugates [380] transferrin-based conjugates target leukemic cells [381]
		Liposomes modified with antibodies specific against cellular receptors	Liposomes coupled with antibody G22-MCA against glioma cells have higher transduction efficiency [382]
Selective expression (transcriptional targeting)	Tumor specific promoters	A suicide gene is administered under the control of a promoter sequence that is active on tumor cells	Adenoviruses expressing *tk* under the alpha-fetoprotein promoter in hepatoma [340], or DF3 (MUC1) in breast cancer [337]. Also shown with cells stably expressing the CD gene under the CEA promoter in colon cancer [341], and with cells transfected with the SLPI promoter, which is expressed in several carcinomas, to direct transcription of *tk* [338]. Same principle shown with promoter of erbB-2, overexpressed in breast and pancreatic tumors [339]. PEG-Prom displays broad spectrum cancer-specific expression [330]
	Tissue specific promoters	A suicide gene is administered under the control of a promoter sequence that is active in a particular tissue	Tyrosinase (melanocytes) directs expression of *tk* into malignant melanoma [343] and other melanocytes; surfactant protein-A drives *tk* expression in lung cancer cell lines [342]

CD cytosine deaminase, *SLPI* secretory leukoprotease inhibitor, *tk* thymidine kinase, *VEGF* vascular endothelial growth factor

of retroviruses. Apart from the transductional targeting, the expression of the transgene can be transcriptionally targeted by the use of tissue or tumor specific promoters. These novel constructs have been used in in vitro and in vivo studies and shown to be effective [337–343].

42.9.2 Prolonged Transgene Expression: Immune Tolerance to Viral Vectors

Under certain conditions as with mutation compensation strategies, the expression of the therapeutic gene is needed for an extended duration. However, gene delivery via adenoviral vectors has been associated with the induction of both characteristically intense inflammatory and immunological responses when employed in vivo. A number of specific cellular and humoral immune effector mechanisms, together with nonspecific defense mechanisms, eliminate the infecting virus [344–346]. This process has been associated with attenuation of expression of the transferred therapeutic gene based, at least in part, on loss of the vector transduced cells. Based on an understanding of the biology of this phenomenon, specific strategies have been developed to mitigate this process.

Maneuvers to minimize the immune response against viral vectors include manipulations of both the vector and the host. Firstly, recombinant viral vectors are genetically engineered to delete viral genes encoding highly immunogenic or cytotoxic viral proteins. New generations of deleted viral vectors are, however, more difficult to propagate while still not completely devoid of immunogenic properties. Alternatively, different serotypes have been employed or, in regard to adenoviruses, either chimeras composed of mixed fiber and knob components of different serotypes or unique viruses from of other species such as canine [347, 348] or ovine Ads [349] have been proposed to minimize the stimulus for an immune response. Secondly, vectors have been modified to *express* immunomodulatory molecules. It has been hypothesized that this could create a locally privileged environment for the vector. Some of these engineered molecules are viral genes that interfere with the apparatus of antigen presentation, such as the adenoviral glycoprotein 19K or the herpes simplex virus (HSV) immediate early protein ICP47. Others are recombinant molecules designed to abrogate antigen presentation, such as antisense oligonucleotides or single-chain antibodies against MHC class I and II proteins, or have the ability to block costimulation, such as CTL4IgG.

Intervention of the immune system of the host has been adopted from common practices in the field of organ transplantation. In this regard, virally transduced cells have been considered to behave, to some extent, as allogeneic cell transplants. Thus, antibodies or drugs are employed that inhibit the cellular immune response, among these are anti-CD4 antibodies, cyclosporine, dexamethasone, and FK 506. In addition, drugs that decrease the humoral immune response, including cyclophosphamide and deoxyspergualin, have been used. Several groups have shown transient and more specific immune blockade with inhibitors of T cell costimulation, such as anti-CD40 ligand, CTL4IgG, and anti-LFA-1. Unfortunately, the required chronic administration of these immunosuppressive drugs affects systemic immune function and results potentially in a number of complications, not the least of which are life-threatening infection and malignancy. This makes them markedly less attractive in principle for clinical application, although short-term treatment in patients should be feasible. Lastly, induction of tolerance to adenovirus vectors by oral ingestion of adenoviral antigens has been described, but this approach needs further characterization. Thus, although inflammatory and immunological issues have limited the overall utility of vectors for gene therapy applications, many of the aforementioned strategies appear promising and may ultimately allow overcoming these limitations.

42.10 Conclusion

The delineation of the molecular basis of cancer provides unique opportunities for specific intervention at the molecular level for therapeutic purposes. To this end, four main gene therapy approaches have been developed to treat cancer: mutation compensation, molecular chemotherapy, genetic immunopotentiation, and viral oncolysis. For each of these conceptual approaches, human clinical protocols have entered testing in Phase I and II to assess dose escalation, safety, and toxicity issues, and more recently to evaluate efficacy, respectively. In fact, the People's Republic of China has conducted a phase III clinical trial with oncolytic virus (H101) for the treatment of head and neck cancer [350]. However, major problems remain to be solved before these approaches can become effective and a common place strategy for the treatment of neoplasia. Principal among these issues is the basic inability to deliver therapeutic genes quantitatively, and specifically, not only into tumor cells but also into tumor supporting tissues and effector cells of the immune system. As vector technology more fully fulfills these stringent requirements, it is anticipated that the promising results already observed in preclinical studies will translate quickly into the clinic for the amelioration of life-threatening disease. In addition, the complex heterogeneity of cancerous tissues should call for a multimodality approach whereby combinations of gene therapy with conventional therapies including chemotherapy and radiotherapy exist to effectively eradicate the transformed cells in the host. That is where the future of gene therapy stands with regard to anticancer therapy.

Acknowledgements The authors are supported by National Institute of Health grants P01CA104177, R01CA121187, P50CA101955, P30AR046031, P30DK074038, R01DK081463, P30DK079337, R01CA097318, R01CA108520, R01CA127641, R01DK052825, R01CA141703, R01CA150214. Additional support was provided by the National Foundation for Cancer Research (NFCR) and the Samuel Waxman Cancer Research Foundation (SWCRF).

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Personalized Medicine for the Treatment of Human Cancer

43

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43.1 Introduction

The concept of personalized medicine is being introduced into routine patient care at all levels of healthcare. The Human Genome project has been a catalyst for this new age of medicine by fulfilling initial promises to provide better tools for diagnostic, prognostic, and therapeutic interventions [1, 2]. New and more rapid drug development, reclassification of tumor types, better diagnostic assays, and novel technologies are a few of the benefits we are now experiencing from this heroic effort [3–6]. For the cancer patient, these new diagnostic approaches will improve such things as tumor classification, mechanisms for monitoring disease, evaluations for susceptibility to cancer, prognostic markers, indicators of tumor cell response to therapeutics with less adverse reactions, and the development of novel therapeutics. As all of this comes to fruition, preventive medicine through our understanding of pharmacogenomics will lead to more personalized therapy and better overall outcomes.

Pharmacogenomics, as an underlying principle, represents the role that genes play (pharmacogenetics) in the processing of drugs by the body (pharmacokinetics) and how these drugs interact with their targets to give the desired response (pharmacodynamics) [7–9]. While other environmental factors also play a role in this selection, an individual's genetic makeup provides new insights into the metabolism and targeting of commonly prescribed, as well as, novel targeted therapies. Current medical practice often involves a trial-and-error approach to select the proper medication and dosage for a given patient. This can lead to adverse drug reactions and more often decreased efficacy of a therapeutic management strategy. While this may be the standard of practice, this approach cannot be sustained by our healthcare system at a time when the cost of novel therapeutics can exceed $10,000 per cycle of treatment and knowing that only a subset of the patient population will experience substantial benefit from that therapy. The overall aim of pharmacogenomic testing is to decrease adverse responses to therapy and increase efficacy by ensuring the appropriate selection and dose of therapy.

43.2 Historical Perspective

Although Fredrich Vogel did not coin the term pharmacogenetics until 1959, the concept of pharmacogenetics existed long before [10]. The concept was recognized and first documented in 510 BC by Pythagoras who observed that some individuals developed hemolytic anemia with fava bean consumption [11]. In 1914, Garrod expanded upon these early observations by documenting the fact that enzymes detoxify foreign agents so that they may be excreted harmlessly and that some people lack these enzymes and experience adverse effects [12]. Hemolytic anemia due to fava bean consumption was later determined to occur in glucose-6-phosphate dehydrogenase deficient individuals [11].

Pharmacogenomics evolved throughout the twentieth century as investigators combined Mendelian genetics with observed phenotypes. In 1932, Snyder performed the first global study of ethnic variation and deduced that taste deficiency was inherited. As such, the phenylthiourea non-taster phenotype was an inherited recessive trait and the frequency of occurrence differed between races [13]. Subsequently, genetic deficiencies in other enzyme systems were documented and shown that metabolism of drugs played a critical role in a patient's response as well as risk for development of

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2_43

adverse drug reactions (ADRs) [14–16]. The introduction of molecular technologies to examine an individual's DNA for genetic variants that are responsible for these phenotypes propelled the field of pharmacogenomics forward. Genotype:phenotype correlations could now confirm ones predisposition to adverse events and allow simpler and more rapid analysis of patient samples in a clinical setting.

43.3 Genotyping Technologies

The evolution of molecular technologies and their introduction into the clinical laboratory setting has been a remarkable transformation for diagnostic testing. Our ability to detect single base variants in any region of the three billion base human genome has set an unprecedented paradigm for technical, clinical, and social implications of a laboratory test. From a technical perspective, we can now identify many different types of genetic variants, including copy number variants (CNV), insertions/deletions, chromosomal rearrangements, and single nucleotide variation, with a high degree of sensitivity and specificity using various available technologies, including quantitative PCR, Sanger sequencing, pyrosequencing, PCR/RFLP, allele-specific PCR, high-resolution melting curves, somatic mutation arrays, chromosomal microarrays, and others [17–22]. Recent advancements in next-generation DNA sequencing technologies have allowed for parallel sequencing of multiple genomic targets from multiple patients in one clinical test. The rapid incorporation of this technology into the clinic has resulted in the development of a variety of applications ranging from the detection of common mutations in tumor-specific genes (hotspot panels) to the detection of all variations across the entire genome. The ability to readily identify all relevant genomic variations in one clinical test has the potential to revolutionize the personalized treatment of the modern-day cancer patient.

Genotyping methods are generally easier to perform and more cost-effective than the more traditional phenotyping methods. Because an individual's genotype is not expected to change over time or during the course of treatment, most genotyping applications need only be performed once in an individual's lifetime. This is commonly the case with variants associated with drug metabolism and transportation. However in cancer the disease-causing (oncogenic) mutation may change over the course of the disease requiring the need for repeated genotyping depending on responsiveness of initial treatment.

43.4 Pharmacogenomics and Drug Metabolism

Many of the enzymes involved in drug metabolism are members of the cytochrome P450 (CYP450) superfamily [23, 24]. CYP450 enzymes are mainly located in the liver and gastrointestinal tract and include greater than 30 isoforms. The most polymorphic of these enzymes responsible for the majority of biotransformations are the CYP3A, CYP2D6, CYP2C19, and CYP2C9 isoforms. Benign genetic variants in these genes, including single nucleotide polymorphisms (SNPs), insertions/deletions and duplications, can lead to the following phenotypes. Poor metabolizers (PM) have no detectable enzymatic activity; intermediate metabolizers (IM) have decreased enzymatic activity; extensive metabolizers (EM) are considered normal and have at least one copy of an active gene; ultra-rapid metabolizers (UM) contain duplicated or amplified gene copies that result in increased drug metabolism. The following are several examples of polymorphic drug metabolizing enzymes that can affect response to therapy.

In an effort to standardize genetic polymorphism annotation of pharmacologically important genes, star allele nomenclature is used. Briefly, *1 is usually the first sequence described that codes for a functional protein, and all other alleles are compared to this sequence. Each novel functional variant identified is assigned a unique number. Nonfunctional variants thought to be associated with a named star allele are not given new numbers but are defined by additional letters, and the principal star-allele is designated with an "A" (e.g. CYP2D6*14A, CYP2D6*14B) [25].

43.4.1 CYP2D6

CYP2D6 is the most widely studied member of this supergene enzyme family. The CYP2D6 gene is localized on chromosome 22q13.1 with two neighboring pseudogenes, CYP2D7 and CYP2D8 [26]. More than 50 alleles of CYP2D6 have been described of which alleles *3, *4, *5, *6, *7, *8, *11, *12, *13, *14, *15, *16, *18, *19, *20, *21, *38, *40, *42, and *44 were classified as nonfunctioning and alleles *9, *10, *17, *36, and *41 were reported to have substrate-dependent decreased activity. CYP2D6 alone is responsible for the metabolism of 20–25 % of prescribed drugs. Screening for CYP2D6*3, *4, and *5 alleles identifies at least 95 % of poor metabolizers in the Caucasian population. Based on the type of metabolizer, an individual can determine the response to a therapeutic drug. The U.S. FDA has approved the Luminex xTAG® CYP2D6 assay for clinical genotyping.

CYP2D6 has become an enzyme of interest for a specific oncology application in regards to the metabolism of one of the more commonly used therapies for breast cancer. All newly diagnosed breast cancers are evaluated for the expression of the estrogen receptor (ER) (Fig. 43.1). ER-positive breast cancers are then treated with hormonal therapies that mimic estrogen. One of these estrogen analogs is Tamoxifen (TAM), which itself has now been shown to have altered metabolism due to CYP450 genetic polymorphisms [27, 28]. TAM is used to treat all stages of ER-positive breast cancers

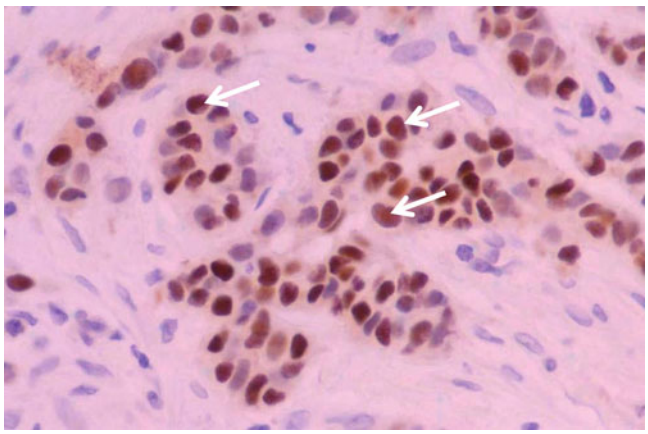

Fig. 43.1 *Immunohistochemical staining for the estrogen receptor (ER) in breast cancer.* The ER is detected as a *brown* color in the nucleus of the tumor cells (*arrows*).

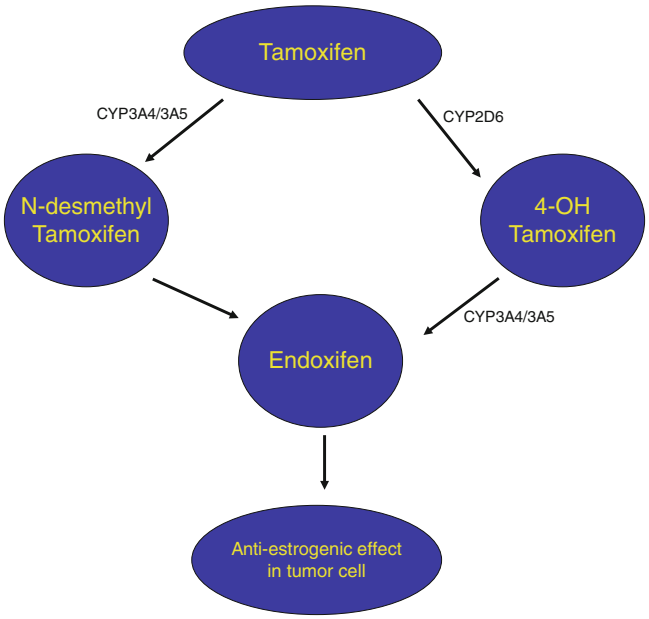

Fig. 43.2 Simplified schematic diagram showing metabolism of tamoxifen by CYP450 enzymes with the resulting production of the active metabolite, endoxifen.

yet it is not effective in all ER-positive breast cancer patients and it is frequently associated with side effects, such as hot flashes. TAM and its metabolites compete with estradiol for occupancy of the estrogen receptor, and in doing so inhibit estrogen-mediated cellular proliferation. Conversion of TAM to its active metabolites occurs predominantly through the CYP450 system (Fig. 43.2). Conversion of TAM to primary and secondary metabolites is important because these metabolites can have a greater affinity for the estrogen receptor than TAM itself. For example, 4-OH *N*-desmethyl-TAM (endoxifen) has approximately 100 times greater affinity for the estrogen receptor than tamoxifen. Activation of tamoxifen to endoxifen is primarily due to the action of CYP2D6. Therefore, patients with defective *CYP2D6* alleles may derive less benefit from TAM therapy than patients with functional copies of *CYP2D6*. The most common null allele among Caucasians is *CYP2D6*4*, a splice site mutation (G1934A) resulting in loss of enzyme activity and therefore, lack of conversion of TAM to endoxifen. Patients with lower CYP2D6 activity and lower levels of endoxifen have been shown to have a significantly shorter time of disease-free progression [29]. This would result in significantly decreased response to this commonly used anti-hormonal therapy. Common polymorphisms such as this make it feasible to accurately genotype patients so that treatments may be optimized.

43.4.2 UGT1A1

The uridine diphosphate glucuronosyl transferases (UGT) superfamily of endoplasmic reticulum-bound enzymes is responsible for conjugating a glucuronic acid moiety to a variety of compounds, thus allowing these compounds to be more easily eliminated. A member of this family catalyzes the glucuronidation of bilirubin, allowing it to be excreted in the bile.

As irinotecan therapy for advanced colorectal cancers became more widely used, it was observed that patients who had Gilbert syndrome (defects in UGT leading to mild hyperbilirubinemia), suffered severe toxicity [30]. Irinotecan is converted to SN-38 by carboxylesterase-2, and SN-38 inhibits DNA topoisomerase I activity [31, 32]. SN-38 is glucuronidated by uridine diphosphate glucuronosyl transferase (UGT), forming a water-soluble metabolite, SN-38 glucuronide, which can then be eliminated (Fig. 43.3). The observation made in Gilbert syndrome patients revealed that SN-38 shares a glucuronidation pathway with bilirubin. The decreased glucuronidation of bilirubin and SN-38 can be attributed to polymorphisms in the *UGT1A1* gene. The wild-type allele, *UGT1A1*1*, has six tandem TA repeats in the regulatory TATA box of the *UGT1A1* promoter. The most common polymorphism associated with low activity of *UGT1A1* is the *28 variant, which has seven TA repeats. Severe diarrhea and myelotoxicity can be found in those individuals who are homozygous for the *28 allele [33, 34]. In August 2005, the FDA amended the irinotecan (Camptosar®) package insert to recommend genotyping for the UGT1A1 polymorphism and suggested a dose reduction in patients homozygous for the *28 allele.

43.4.3 TPMT

Thiopurine S-methyltransferase (TPMT) is a cytosolic enzyme that inactivates thiopurine drugs such as 6-mercaptopurine and azathioprine through methylation. Thiopurines are frequently used to treat childhood acute lymphoblastic leukemia and

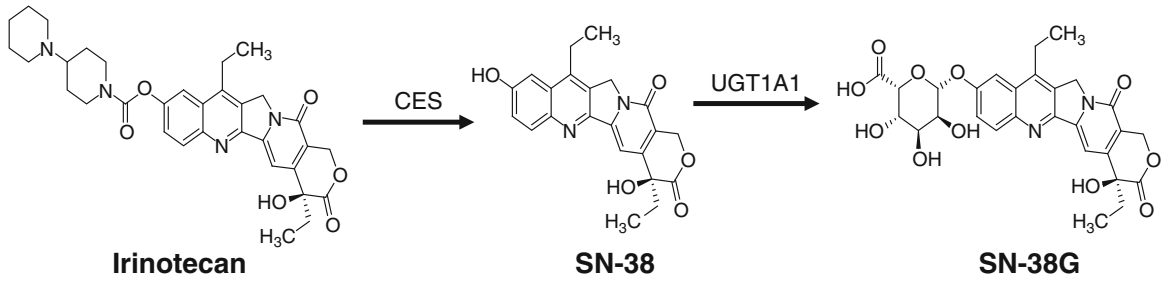

Fig. 43.3 *Schematic diagram of irinotecan metabolism.* The prodrug is converted to the active metabolite, SN-38, which is inactivated by UGT1A1.

various other diseases including inflammatory bowel disease (IBD), and rheumatoid arthritis [35, 36]. They are also used as immunosuppressants in solid organ transplants. Thiopurines are metabolized partly by TPMT whose gene contains polymorphisms that result in null or decreased enzyme activity. These polymorphisms have been associated with increased risk of myelosuppression and toxicity.

Variability in activity levels of TPMT enzyme function exists between individuals, and it has been found that this variability can be attributed to polymorphisms of the *TPMT* gene [37, 38]. The most common variant alleles, *TPMT*2*, *TPMT*3A*, and *TPMT*3C*, account for 95 % of TPMT deficiency. Molecular technologies provide an accurate method for assessment of TPMT enzyme function in patients before treatment with thiopurines. Approximately 90 % of patients have the *TPMT**1/*1 (wild-type) genotype and thus have normal *TPMT* enzyme activity. Up to 14 % of patients have been shown to be heterozygous for a *TPMT* genotype and possess one *TPMT* variant allele with lower enzyme activity. These patients may experience moderate to severe myelosuppression such that drug dose reduction may be warranted. Low TPMT activity levels could put a patient at risk for developing toxicity, since too much drug would be converted to 6-thioguanine nucleotides (6-TGNs), the cytotoxic active metabolite incorporated into DNA. On the other hand, a patient with high TPMT activity levels would need higher than standard doses of a thiopurine drug to respond well to the therapy, since a large amount of the drug is being inactivated before it can be converted to 6-TGNs. TPMT genotyping represents yet another example where molecular techniques can provide adequate information to assess drug-related risk of toxicity.

43.5 Pharmacogenomics and Drug Transporters

Although the genes that code for drug metabolizing enzymes have received more attention in recent years as markers for pharmacogenomic testing, the genes that code for proteins used to transport drugs across membranes also need to be considered when discussing a genetic basis of drug efficacy. These drug transporter proteins move substrates across cell membranes, bringing them into cells or removing them from cells. These proteins are essential in the absorption, distribution, and elimination of various endogenous and exogenous substances including pharmaceutical agents.

Several groups of drug transporters that may be significant in the field of pharmacogenomics exist, including multidrug resistance proteins (MDRs), multidrug resistance-related proteins (MRPs), organic anion transporters (OATs), organic anion transporting polypeptides (OATPs), organic cation transporters (OCTs), and peptide transporters (PepTs). ABCB1 (MDR1) is a member of the multidrug resistance protein family and is one example of a drug transporter protein important in the field of cancer pharmacogenomics.

43.5.1 ABCB1

ABCB1 is a member of the ATP-binding cassette (ABC) superfamily of proteins. Also known as P-glycoprotein (P-gp) or MDR1, it is a 170 kDa glycosylated membrane protein expressed in various locations including the liver, intestines, kidney, brain, and testis [39]. Generally speaking, ABCB1 is located on the membrane of cells in these locations and serves to eliminate metabolites and a wide range of hydrophobic foreign substances, including drugs, from cells by acting as an efflux transporter [40, 41]. Due to the localization of ABCB1 on specific cells, ABCB1 aids in eliminating drugs into the urine or bile and helps maintain the blood–brain barrier.

Like other eukaryotic ABC proteins, the ABCB1 protein is composed of two similar halves, each half containing a hydrophobic membrane-binding domain and a nucleotide-binding domain ([39]. The membrane-binding domains are each composed of six hydrophobic transmembrane helices and the two hydrophilic nucleotide-binding domains are located on the intracellular side of the membrane where they bind ATP. ABCB1 was first identified in cancer cells that had developed a resistance to several anticancer drugs because of an overexpression of the transporter. When expressed at normal levels in noncancerous cells, ABCB1 has been shown to

transport other classes of drugs out of cells including cardiac drugs (digoxin), antibiotics, steroids, HIV protease inhibitors, and immunosuppressants (cyclosporin A).

Genetic variations in the ABCB1 gene expressed in normal cells have been shown to have a role in interindividual variability in drug response [39]. Although many polymorphisms have been detected in the ABCB1 gene, correlations between genotype and either protein expression or function have only been described for a few of the genetic variants. Most notable among these is the 3435 C>T polymorphism, found in exon 26, which has been found to result in decreased expression of ABCB1 in individuals homozygous for the T allele. The possible importance of the 3435 C>T is particularly interesting considering the mRNA levels are not affected by this polymorphism which is found within a coding exon. Although the correlation between the 3435 C>T polymorphism and ABCB1 protein levels may be due to linkage of this polymorphism to others in the ABCB1 gene, a recent study showed that this polymorphism alone does not affect ABCB1 mRNA or protein levels but does result in ABCB1 protein with an altered configuration. The altered configuration due to 3435 C>T is hypothesized to be caused by the usage of a rare codon which may affect proper folding or insertion of the protein into the membrane, affecting the function, but not the level of ABCB1 [42]. Genetic variations affecting the expression or function of drug transporter proteins, such as ABCB1, could drastically alter the pharmacokinetics and pharmacodynamics of a given drug.

43.6 Pharmacogenomics Applied to Oncology

Cancer represents a complex set of deregulated cellular processes that are often the result of underlying molecular mechanisms. While there is recognized inter-individual variability with respect to an observed chemotherapeutic response, it is also apparent that there is considerable cellular heterogeneity within a single tumor that may account for this lack of efficacy. It is becoming clear that molecular genetic variants significantly contribute to an individual's response to a particular therapy and part of this response is due to polymorphisms in genes coding for drug-metabolizing enzymes as previously described. However, in the cancer patient the acquired genetics of the tumor cell must also be taken into account. Unlike traditional pharmacogenomic testing, testing for the cancer patient must be able to identify acquired or somatic genetic alterations that deviate from the underlying genome of the individual.

Cancer patients exhibit a heterogeneous response to chemotherapy with only 25–30% efficacy. Pharmacogenomics can improve on chemotherapeutic and targeted therapy responses by providing a more informative evaluation of the underlying molecular determinants associated with tumor cell heterogeneity and by integrating information on drug responsiveness due to alterations in molecular biomarkers. The advancement of genotyping methods (i.e. next-generation sequencing) has allowed for the simultaneous identification of this heterogeneity. In large part these methods have illuminated genotyping as a major pharmacogenomics limitation. However, to date, only a handful of these mutations are deemed clinically actionable and have FDA-approved therapy associated with improved patients outcomes (Fig. 43.4) [20]. As research in clinical pharmacogenomics identifies more genotype specific therapies, the potential of these advanced genotyping methods in personalized medicine will be fully realized. Thus, the therapeutic management of the cancer patient can be tailored to include a specific therapy or a combination of therapies targeted to the patient's tumor genotype.

Most therapeutic drugs act on targets to elicit the desired effects. These targets include receptors, enzymes, or proteins involved in various cellular events such as signal transduction, cell replication, etc. Knowing if the target is present or being able to identify polymorphisms in these targets that

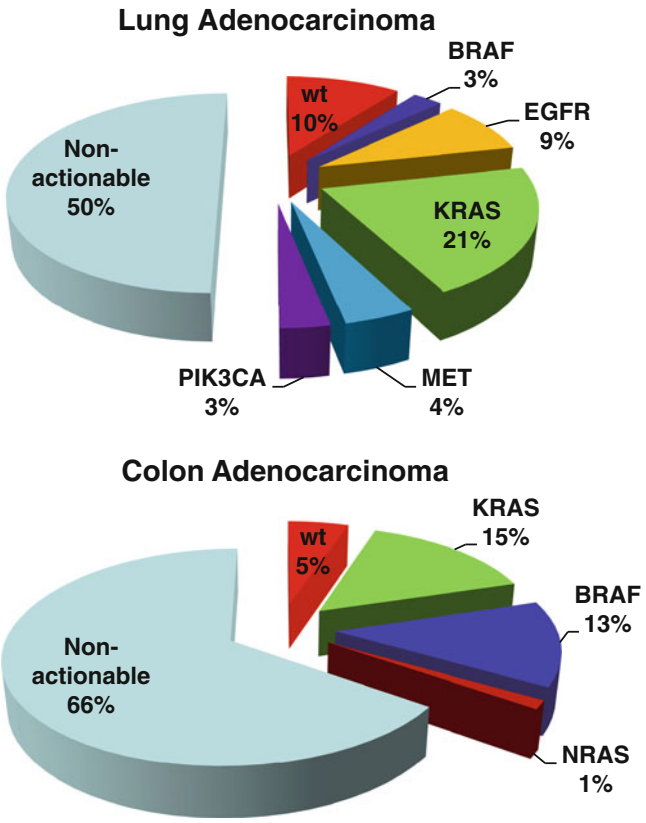

Fig. 43.4 *Next-generation sequencing of cancers identifies actionable mutations.* Types of mutations (actionable and non-actionable) identified in (**a**) lung adenocarcinomas and (**b**) colon adenocarcinomas by next-generation sequencing.

either sensitize or render them resistant to the particular therapeutic agent is critical to patient management (Table 43.1). The following sections highlight several examples of characterized drug targets whose presence in a normal or variant form could affect the outcome of the therapeutic response.

43.6.1 FLT3

FLT3 is a receptor tyrosine kinase expressed and activated in most cases of acute myeloid leukemia (AML) which has a relatively high relapse rate due to acquired resistance to tra-

Table 43.1 List of common mutation associated with the activation or resistance to FDA-approved pharmacogenomic therapies

Cancer type	Activity	Genes	Mutation	Therapeutic agent
AML	Resistance	*FLT3*	G835X	General TKl (sorafenib)
Breast	Activating	*HER2*	Amplification	HER2 antibodies (trastuzumab peruzumab) TKls (lapatinib)
CML	Resistance	*BCR-ABL1*	Y253H	ABL1 Tkl (lmatinib, nilotinib)
			E255K	
			E255V	
			V299L	
			F359C	
			F359l	
			F359V	
			T315A	ABL1 Tkl (imatinib, dasantinib)
			T315l	
			F317C	
			F317I	
			F317L	
			F317V	
CRC	Resistance	*KRAS*	G12X	EGFR antibodies (cetuximab, panitumumab)
			G13X	
			Q61X	
			K117N	
			A146X	
			G12X	
		NRAS	Q61X	
		BRAF	V600E	
	Activating	*BRAF*	V600E	BRAF Tkls (vemurafenib, dabrafenib)
Melonma	Activating	*BRAF*	V600E	BRAF Tkls (vemurafenib, dabrafenib)
NSCLC	Resistance	*ALK-EML4*	L1196M	ALK/MET TKls (crizotinib)
			G1202R	
			S1206Y	
			1151Tins	
			L1152R	
			C1156Y	
			G1269A	
		EGFR	T700M	EGFR Tkls (erlotinib, gefitinib)
	Activating	*EGFR*	Exon 19 insertions	EGFR Tkls (erlotinib, gefitinib) EGFR/HER2 Tkls (afatinib)
			Exon 19 deletions	
			Exon 20 insertions	
			L858R	
			G719X	
			L861Q	
		BRAF	V600E	BRAF Tkls (vemurafenib, dabrafenib)

ditional chemotherapies. An internal tandem duplication (ITD) mutation in the *FLT3* gene is found in up to 30 % of AML patients while a point mutation at D835 has been shown to account for approximately 5 % of refractory AML [43, 44]. The *FLT3*-ITD induces activation of this receptor and results in downstream constitutive phosphorylation in STAT5, AKT, and ERK pathways. This mutation in *FLT3* is a negative prognostic factor in AML.

FLT3-ITD-positive AML has become a clinical dilemma due to the poor results obtained with standard chemotherapy. One potential therapeutic option for patients who are at risk for relapse is allogeneic stem cell transplant [45]. In addition, randomized phase III trials using *FLT3* inhibitors in combination with chemotherapy in patients with *FLT3*-positive AML are ongoing. Two promising multitargeted receptor tyrosine kinase inhibitors (TKIs), sorafenib and ABT-869, have been developed to suppress signal transduction from constitutively expressed kinases such as a mutated *FLT3* [46]. The investigational FLT3-specific TKIs, quizartinib (AC220) and crenolanib, have also shown some recent success in a phase II trial in patients with FLT3-ITD+ AML with some evidence for increased activity against the D835 resistance mutation [47].

43.6.2 ALK

The fusion of the anaplastic lymphoma kinase (*ALK*) gene with the echinoderm microtubule-associated protein-like 4 (*EML4*) gene was first identified in 2007 in Japanese non-small-cell lung cancers (NSCLCs) [48]. The *EML4-ALK* fusion gene is the result of an inversion on the short arm of chromosome 2 that joins exons 1–13 of *EML4* to exons 20–29 of *ALK* [49]. The fusion gene can be detected by use of break-apart probes and FISH technology (Fig. 43.5).

The *EML4-ALK* fusion gene represents a novel biomarker and potential molecular target in NSCLC and translocations or rearrangements of the *ALK* gene with other genes have been reported in several other human cancers [49, 50]. Approximately 3–5 % of NSCLCs found in North America harbor the *EML4-ALK* fusions. The fusion of these two genes results in constitutive ALK tyrosine kinase activity which has significant oncogenic function. Because of its oncogenic role, the EML4-ALK fusion product has been a target for tyrosine kinase inhibitors. Several ALK kinase inhibitors have been developed and FDA-approved for treatment of NSCLC, such as crizotinib (Xalkori) and certitnib (Zykadia) [49, 51]. Crizotinib is a first-line oral inhibitor of ALK tyrosine kinase activity which has shown high response rates in patients with *EML4-ALK*-positive NSCLC [52]. However, a large number of patients treated with crizotinib develop resistance to treatment, many by acquiring secondary mutations in the *ALK* tyrosine kinase domain, driving relapse [53]. These patients are now eligible for treatment with a newly approved ALK TKI, ceritinib, which has exhibited a response rate of greater than 70 % in those patients resistant to crizotinib [54]. In addition, there are numerous phase II trials investigating the efficacy of heat shock protein 90 (HSP-90) inhibitors to treat patient who are positive for ALK fusions. Early results suggest a significant response to treatment with these HSP-90-targeted inhibitors [55]. The identification of these resistance mutations and the successful development of second- and third-generation therapies to combat them exhibit the utility in fully characterizing the genotypes of therapy-resistant tumors.

43.6.3 BCR-ABL1

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by three distinct clinical courses and a well characterized chromosomal translocation. CML consists of a chronic indolent phase, an accelerated phase, and a terminal blast crisis. In addition to this heterogeneous clinical presentation, CML represents one of the most significant human malignancies with respect to characterization of a disease-causing genetic variant and now biomarker for targeted treatment options. The Philadelphia chromosome represents a reciprocal translocation between chromosomes 9 and 22, t(9;22), which creates a fusion gene product between the *ABL1* gene on chromosome 9 and the *BCR* gene on chromosome 22. This results in constitutive activation of the ABL tyrosine kinase which is thought to induce the leukemia [56].

The management of CML with small-molecule drugs began with the introduction of the TKI imatinib (Gleevec), which significantly improved outcomes for Philadelphia chromosome-positive CML patients [57]. The progression of CML patients receiving this first-line TKI therapy is measured by evaluation of hematologic response, cytogenetic response, and molecular response. It became evident that the sensitivity of molecular monitoring would be needed to follow patients through their treatment course and for the detection of minimal residual disease as traditional cytogenetics and FISH testing are not capable of this level of sensitivity. Using real-time quantitative PCR assays, clinical laboratories can monitor the level of Philadelphia-positive leukemic cells in bone marrow and peripheral blood samples [58]. A major molecular response (MMR) is defined as at least a 3-log reduction in the *BCR-ABL1* level relative to a control gene. After achieving MMR, the goal is to achieve a complete molecular response (CMR), defined as undetectable *BCR-ABL1* levels.

Despite these improved outcomes observed in CML patients, a significant percentage of patients can show partial response and/or drug resistance due to secondary mutations

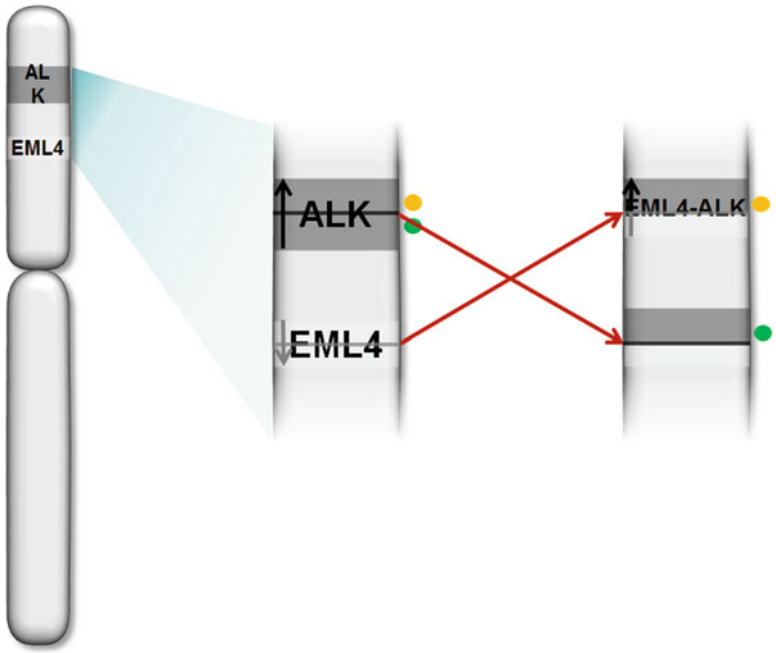

Fig. 43.5 *EML4-ALK FISH.* Diagram of translocation and location of break-apart probes (*colored dots*).

in the fusion gene [59]. This led to the development of new TKIs of BCR-ABL1, including dasatinib, nilotinib, ponatinib, and bosutinib [60–62]. These second- and third-generation TKIs have been shown to have an increased potency against BCR-ABL1 kinase activity and have varying effectiveness in treating CML after the accusation of imatinib-resistant mutations. The National Cancer Comprehensive Network (NCCN) now provides tailored treatment guidelines based on the BCR-ABL1 secondary mutations present in an individual's cancer. Therefore, in addition to monitoring *BCR-ABL1* levels, it has become important to screen imatinib-resistant CML cases for mutations in the fusion gene.

43.6.4 HER2

The human epidermal growth factor receptor 2 (ERBB2 or HER2) is the second member of the EGFR gene family. This gene is amplified and/or overexpressed in up to 30 % of all breast cancers [63, 64]. While this gene can be expressed in low levels in a variety of normal epithelia, amplification of the HER2 gene is the primary mechanism of HER2 overexpression that results in increased receptor tyrosine kinase activity. HER2 amplification is an indicator of poor prognosis and predicts resistance to hormonal therapies and other chemotherapeutic agents. HER2 status can be utilized to determine sensitivity to anthracycline-based chemotherapy regimens. However, its main clinical utility is as the first FDA-approved companion diagnostic for Trastuzumab (Herceptin). As the first humanized monoclonal antibody against the HER2 receptor, this therapeutic was approved by the FDA in 1998. Introduction of this therapeutic into routine use made it necessary for laboratories to determine the HER2 status in breast cancer cells before patients would be eligible for treatment (Fig. 43.6). In addition to trastuzumab, in 2007 the FDA approved lapatinib for use in combination with capecitabine for treatment of advanced HER2-positive breast cancers. Thus, there is increasing need for accurate HER2 testing strategies for appropriate management of breast cancer patients.

The importance of accurate HER2 status determination was highlighted again in October 2010, when the FDA approved trastuzumab for use in combination with cisplatin and either capecitabine or 5-fluorouracil in patients with metastatic gastric or gastro-esophageal junction cancer (GC/GEJ) who had not previously received treatment for metastatic disease. This approval was granted following the results of the ToGA (Trastuzumab for Gastric Cancer) phase III, international, randomized controlled trial which were published in 2010 [65].

Several FDA-cleared tests for immunohistochemical detection of HER2 protein and fluorescence in situ hybridization (FISH) detection of gene amplification are commercially available and approved as companion diagnostics for Herceptin therapy [66]. An approved set of guidelines have been published and recently updated for performing and interpreting HER2 testing [67–69]. These guidelines attempt to standardize HER2 testing by addressing pre-analytical, analytical, and post-analytical variables that could lessen result variability due to technical and interpretative subjectivity.

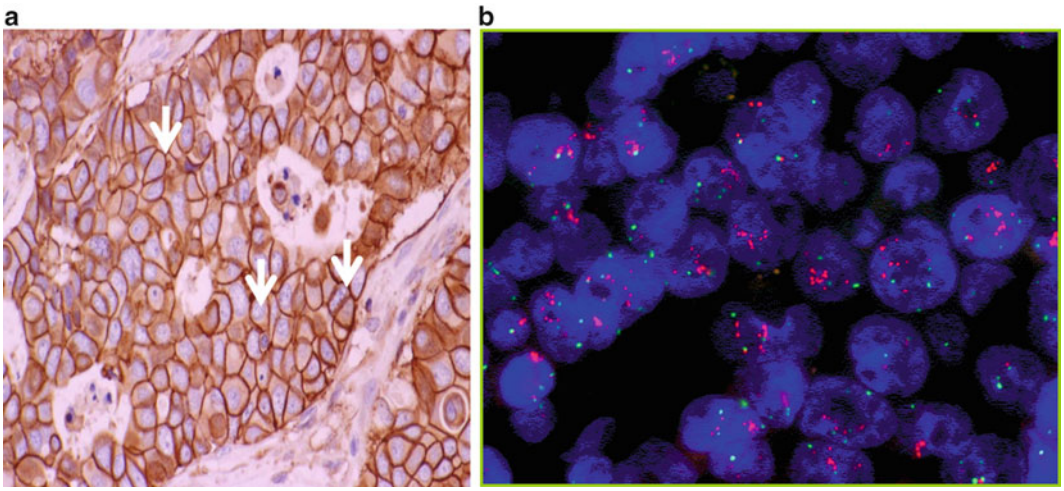

Fig. 43.6 *Assessment of HER2 status in breast cancer.* Determination of HER2 status in breast cancer cells using immunohistochemistry for detection of membranous HER2 receptor staining (*arrows*) (**a**) and by fluorescence in situ hybridization (FISH) to detect gene amplification (*orange* signal) (**b**).

43.6.5 EGFR

The epidermal growth factor receptor (EGFR or HER1) is one of the best-characterized receptors and signal transduction pathways to date. As a member of the ErbB family of tyrosine kinases, EGFR joins HER2, HER3, and HER4 as unique proteins that undergone homo- or hetero-dimerization upon binding of their respective ligands. Once activated, these receptors initiate signaling to downstream pathways such as PI3K/AKT and RAS/RAF/MAPK, which in turn regulate cell proliferation and apoptosis [70].

Recently, the EGFR TK has been the target of both monoclonal antibody and tyrosine kinase inhibitor therapies as it is expressed in many human cancer types, including lung cancer [71]. It has been shown that tumors that respond to these TKIs contain somatic mutations in the EGFR TK domain [70–72]. The most common of these mutations found in non-small-cell lung cancers (NSCLC) include in-frame deletion within exon 19 and a single-point mutation in exon 21 (T2573G). These mutations are known as activating or sensitizing mutations as they are associated with a favorable response to the new TKIs. However, other EGFR mutations are associated with a poor response and resistance to these new therapeutics [70, 73].

Mutation analysis of the EGFR gene represents a new application for molecular diagnostics as some mutations confer response to therapy while others confer resistance. It has become routine practice for all NSCLC to be tested for these mutations so that the management of the NSCLC patient can be optimized.

43.6.6 BRAF

The *BRAF* gene, located on chromosome arm 7q34, is a member of the *raf* family of serine/threonine protein kinases in the RAS/RAF/MAPK signaling pathway [74] (Fig. 43.7). With respect to the signaling cascade, *BRAF* is found downstream of *KRAS*. Once activated, BRAF phosphorylates and activates MEK and ERK. The *BRAF* gene consists of 18 exons and the most common activating mutation is found in exon 15. The T1799A point mutation is a transversion that replaces valine with glutamic acid at amino acid 600 ($BRAF^{V600E}$) (c.1799T>A[p.Val600Glu]) and results in constitutive activation of the kinase activity. BRAF and KRAS signal through the same pathway and thus far there is mutual exclusivity to their mutation status.

The $BRAF^{V600E}$ mutation has been shown to be present in greater than 60 % of melanomas. Because of this high prevalence, selective BRAF inhibitors have been explored. Sorafenib was one of the first multi-kinase inhibitors which targeted BRAF but showed minimal activity in melanoma. Vemuranfenib (Zelboraf) was later developed to selectively inhibit the V600E mutant BRAF. In 2011, the FDA approved vemuranfenib for treatment of late-stage metastatic melanoma alongside a companion diagnostic detecting the activating BRAF V600E mutation. This was largely due to the fact the clinical trials showed up to 70 % objective response rates in patients with BRAF mutant positive [75, 76]. Clinical studies examining the efficacy of this therapy in additional tumor types harboring the V600E mutation are ongoing.

In colorectal cancer, *BRAF* mutation testing is also used to differentiate sporadic colon cancer from Lynch Syndrome,

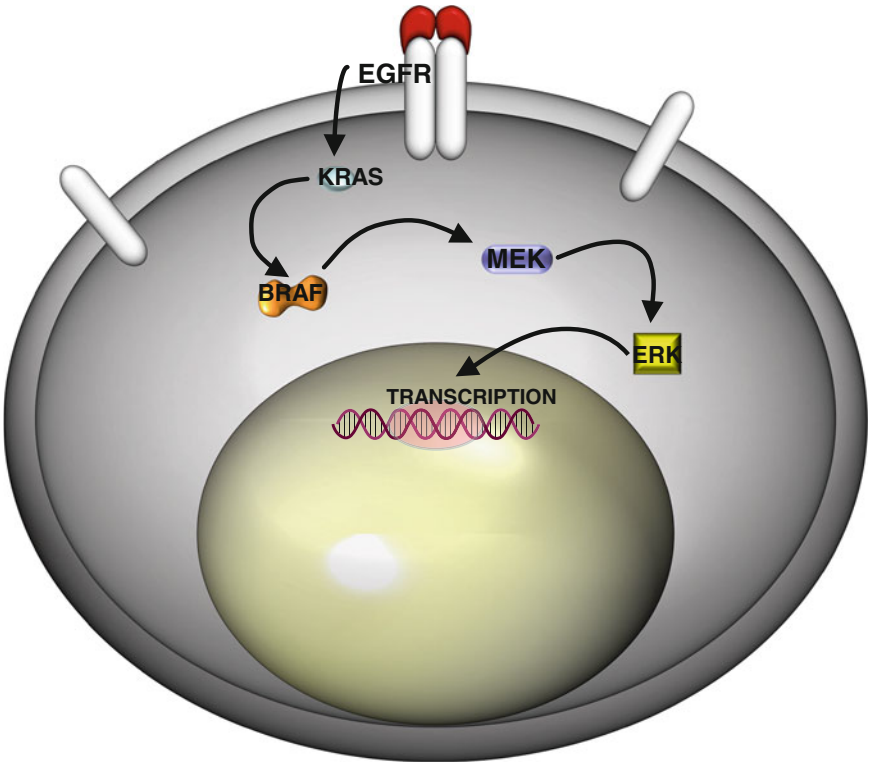

Fig. 43.7 Schematic of the RAS/RAF/MAPK signal transduction pathway.

to identify those tumors that are BRAF mutation negative and thus more likely to respond to EGFR tyrosine kinase inhibitors, and as a prognostic indicator in microsatellite stable colorectal cancers [77]. Several investigators have shown that the V600E mutation is associated with shorter progression-free survival and overall survival in metastatic colon cancer chemorefractory patients treated with anti-EGFR mAbs [78–80]. So while the presence of the BRAF V600E mutation results in improved efficacy of some BRAF TKIs, it can also be indicator of resistance for alternative therapies in a tumor-specific manner.

43.6.7 KRAS

The *KRAS* gene is one of the most commonly mutated proto-oncogenes in human cancers. *KRAS* is a signaling molecule downstream of the epidermal growth factor receptor (EGFR), a transmembrane receptor for extracellular signaling, and upstream of RAF in the RAS/RAF/MAPKs signaling pathway [81, 82] (Fig. 43.7). Wild-type KRAS regulates signal transduction through its GTPase activity by switching the GDP bound inactive form to the GTP bound active form. Point mutations at codons 12 or 13 in exon 2 of *KRAS* convert its GTPase activity into a constitutively expressed active form, irrespective of upstream signaling [82].

Mutations in *KRAS* occur in approximately 35–40 % of all colorectal cancers. In patients with colorectal cancer carrying one of seven known point mutations in *KRAS* codons 12 or 13, inhibition of upstream EGFR activation with monoclonal therapeutics against EGFR, such as cetuximab, will not affect the constitutively activated mutant *KRAS*. Its effects on the downstream signaling pathways will continue to lead to the uncontrolled proliferation of tumor cells.

Target-specific treatments, such as cetuximab and panitumumab, monoclonal antibodies directed against EGFR, improve progression-free survival and the quality of life in patients with colorectal cancer that have not responded to traditional chemotherapies. However, patients with metastatic or recurrent colorectal cancers harboring *KRAS* mutations do not benefit from these anti-EGFR therapies [83–87]. In 2010, De Roock et al. showed that those colorectal cancers that harbor a specific codon 13 mutation (p.G13D) showed better overall survival when treated with cetuximab than those with other KRAS mutations [88]. This demonstrates the need not only to identify a tumor as positive or negative for mutation but also to identify accurately which mutation is present. It is clear that the routine detection of KRAS mutations in colorectal cancer patients is of critical importance to the management of these patients.

43.7 Conclusion

Our knowledge base of pharmacogenomics and applications to clinical oncology are progressing at record speeds. Technology is allowing us to perform these tests routinely in the clinical laboratory from specimen types that include

whole blood, bone marrow, and formalin-fixed paraffin-embedded tissues. In the cancer patient, our ability to detect polymorphisms and mutations associated with responses to newly developed small-molecule targeted therapies is critical to patient management. The ultimate goal of these efforts is to truly provide a personalized medicine approach to patient management for the purpose of eliminating ADRs, selecting more efficacious therapeutics, and improving the overall well-being of the patient.

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W.B. Coleman, G.J. Tsongalis (eds.), *The Molecular Basis of Human Cancer*, DOI 10.1007/978-1-59745-458-2