Aamir Ahmad *Editor*

Breast Cancer Metastasis and Drug Resistance

Progress and Prospects

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Preface

Breast cancer is a deadly disease that continues to disrupt the lives of millions of women and their families worldwide, and it is the second leading cause of cancer-related deaths in women in the United States. Breast cancer affects one in eight women in the United States. These statistics are frightening despite decades of innovative research that led to the development of newer targeted therapies. This book attempts to comprehensively summarize breast cancer as a disease, the factors that make it particularly lethal, and the current state of breast cancer research. The contents are broadly divided into five informal sections as outlined in the next few paragraphs.

One factor that particularly makes breast cancer deadly is the enormous heterogeneity associated with it. Cell surface receptors, such as estrogen receptor (ER), progesterone receptor (PR), or HER2/neu (ErbB2) receptor, have been targeted for therapeutic intervention in breast cancers with significant success. However, even this highly successful targeted approach has not been useful for treating 'all' breast cancers, especially those that are negative for these receptors, the triple-negative breast cancers. Chapters 1 through 6 form the first section of this book. These chapters introduce readers to the most up-to-date statistics (Chap. 1) and epidemiological data (Chap. 2) on breast cancer; summarize our current understanding of racial disparity in breast cancer (Chap. 3); introduce the signaling pathways being pursued (Chap. 4); comment on the heterogeneity in breast cancer (Chap. 5) and also brief the readers on the challenges posed by triple-negative breast cancers (Chap. 6).

Not much is known about the factors that may predispose individuals to breast cancer and this has also resulted in debate on the models systems to be evaluated in modern day breast cancer research. The second section in this book, Chaps. 7 through 10, touches upon some of these topics. Included in this section is a chapter that links obesity and diabetes to breast cancer (Chap. 7), followed by a chapter that discusses the clinical and pathological progression of early breast cancer into an invasive disease (Chap. 8). The final two chapters in this section summarize the models available to breast cancer researchers (Chap. 9) and also introduce readers to the state-of-the-art 4-dimensional culture models that have been proposed recently (Chap. 10).

Although the rate of mortality from breast cancer has decreased in developed countries, the incidence of breast cancer has actually risen, all due to early detection. It is estimated that more than 90 % cancer-related deaths are due, directly or indirectly, to cancer metastasis. Bone is one of the earliest and most common sites of breast cancer metastasis. Breast cancer metastasizes to bones in approximately 70–80 % of patients with advanced disease, and similarly brain metastasis of breast cancer is also a very challenging clinical problem. It is believed that 20–40 % of all patients with metastatic cancer end up with brain metastases. We cover these topics in the third section of this book (Chaps. 11, 12). These chapters provide detailed information on our current understanding of the processes of bone (Chap. 11) and brain (Chap. 12) metastases of breast cancer.

In addition to metastatic disease, drug resistance is a major concern for researchers and clinicians, because it is a big hindrance in the successful management of cancer patients. A number of targeted therapies are available for cancer subtypes that are marked by the expression of ER, PR, and overexpression of HER2. Some cancers do not respond to the therapy at all, right from the beginning, and others eventually develop resistance to the targeted therapy. Breast cancers that have acquired drug resistance are usually far more aggressive and difficult to treat. Section 4 of this book, Chaps. 13 through 15, deals with this clinical problem associated with breast cancer. Here, readers are first introduced to clinical problems associated with the resistance to taxanes and anthracyclines in invasive breast cancers (Chap. 13); followed by the problems and current research on tamoxifen resistance in ER expressing breast cancers (Chap. 14), and finally we discuss the resistance mechanisms in HER2 overexpressing breast cancers (Chap. 15).

With a better understanding of breast cancer as a disease and the various challenges it poses, as detailed in the first four sections of this book, we finally showcase the current state of breast cancer research in Sect. 5 (Chaps. 16 through 22). We look at the novel molecular targets/signaling pathways being pursued, and also present the cutting edge approaches to better understand and tackle this disease. We start with a look at some promising novel chemical compounds for therapy (Chap. 16), and then summarize our understanding of Notch signaling pathway in breast cancer (Chap. 17). The next two chapters introduce readers to systems biology approach (Chap. 18) and epigenetics approach (Chap. 19), the two upcoming areas of breast cancer research. We round off by discussing the current understanding of cancer stem cells and miRNAs in breast cancer progression and therapeutics. Chapter 20 introduces readers to these two exciting areas of research, and finally readers are briefed on the therapeutic potential of cancer stem cells (Chap. 21) and miRNAs (Chap. 22) with particular note on how these fields of breast cancer research have advanced in last few years.

It is an honor to be able to work with the experts and leading scientists in individual fields, and be able to compile this very comprehensive volume detailing almost all the aspects of current breast cancer research. I take this opportunity to thank all the authors who, selflessly, worked hard and contributed their knowledge to this book. My special thanks to the publisher, Springer, for entrusting me with this project, with special mention of Fiona Sarne, the editor at the publishing office

for helping me in every way possible. Finally, I cannot thank enough my wife Huma and daughter Nuha for their unconditional love and support throughout.

It is my pleasure to present this volume to the scientific community for a better understanding of breast cancer. I hope this will help spark new ideas and innovative research for the benefit of scores of patients dealing with this deadly disease.

Aamir Ahmad

Contents

Chapter 1
Breast Cancer Statistics

Jiemin Ma and Ahmedin Jemal

Abstract Among U.S. women, breast cancer is the most commonly diagnosed cancer (excluding skin cancers) and the second leading cause of cancer death, following lung cancer. In 2012, an estimated 226,870 new cases of invasive breast cancer and 39,510 breast cancer deaths are expected to occur among U.S. women. Breast cancer rates vary largely by race/ethnicity and socioeconomic status (SES), and geographic region. Death rates are higher in African American women than in whites, despite their lower incidence rates. Historically, breast cancer was recognized as a disease of western countries. However, over the past 20 years, breast cancer incidence and mortality rates have been increasing rapidly in economically less developed regions. According to 2008 GLOBOCAN estimates, half of the new worldwide breast cancer cases (1.38 million) and 60 % of the breast cancer deaths (458,000) occurred in developing countries. This chapter reviews breast cancer incidence and mortality patterns among women in the U.S. and worldwide, and the possible explanations for these patterns.

Keywords Breast cancer · Cancer incidence · Age-standardized rate (ASR) · Cancer mortality · 5-year relative survival · Cancer statistics · Age · Race/ethnicity · Socioeconomic status (SES) · Geographic variation · Trends · Global patterns · Cancer burden

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1.1 Introduction

Among U.S. women, breast cancer is the most common cancer diagnosis (excluding skin cancers) and the second leading cause of cancer death, preceded only by lung cancer. The American Cancer Society estimated that approximately 226,870 new cases of invasive breast cancer and 39,510 breast cancer deaths are expected to occur among U.S. women in 2012 [1]. Over the past 20 years, breast cancer mortality rates have been decreasing in the U.S. and in many other developed countries, whereas increasing incidence and mortality have been seen in most developing countries [2]. In 2008, approximately 1.4 million newly diagnosed breast cancer cases and about 460,000 breast cancer deaths occurred among women worldwide [3, 4]. In this chapter, we review the female breast cancer burden in the United States focusing on incidence and mortality and their temporal trends by race/ethnicity, along with an overview of global burden of this disease. We also briefly discuss possible explanations for the observed patterns and comment on established preventive measures that can reduce breast cancer burden.

1.2 Common Indicators in Cancer Statistics

1.2.1 Incidence

Cancer incidence is the number of new cancer cases occurring in a defined population during a specified time period, usually expressed as the number of cancers per 100,000 persons per year. The numerator only counts new cancers in their primary sites not including metastasized cancers. To facilitate comparing rates between populations that may have different age structures, age-standardized rate (ASR) is routinely reported in cancer statistics, which is a weighted average of the age-specific rates, with each weight being the proportion of persons in the corresponding age groups of a standard population.

1.2.2 Mortality

Cancer mortality is the number of cancer deaths in a specified population during a specific time period, usually expressed as the number of deaths per 100,000 persons per year. As a product of cancer incidence and case fatality (1-survival), cancer mortality is influenced by factors affecting either occurrence, survival, or both. When comparing rates between two populations, mortality rate sometimes can serve as a proxy measure of cancer incidence, under an assumption of equal survival. This approach is reasonable for cancers with high fatality, such as cancers

of the lung and pancreas, but may not be appropriate for breast cancer, of which survival rates vary largely across different populations [5].

1.2.3 Survival

Cancer survival, a measure of cancer prognosis, is the proportion of patients alive at some point subsequent to the cancer diagnosis. In cancer statistics, the most commonly reported survival estimate is relative survival rate, which is an estimate of the percentage of patients who would be expected to survive a specified time period after diagnosis, usually 5 years. It is calculated as the ratio of the observed survival of cancer patients to the expected survival of a comparable group of the general population with respective to age, sex, and calendar time, such that relative survival removes the effect of death causes other than cancer. When relative survival is inestimable (e.g., life-table data are unavailable), cause-specific survival rate could be used as an alternative, which is the probability of not dying of the cancer diagnosed within a specified time period following diagnosis [6].

1.3 Data Sources

1.3.1 Incidence and Mortality Data in the United States

Incidence rates for 2004–2008 were estimated using data from the North American Association of Central Cancer Registries (NAACCR)'s Incidence-CiNa Analytic File [7], which was based on incidence data from the Surveillance, Epidemiology, and End Results (SEER) program and the National Program of Cancer Registries (NPCR). Incidence trend data were from SEER 9 registries for whites and blacks (1975–2008) and from SEER 13 registries for other racial/ethnic groups (1992–2008). Survival data for 2001–2007 were from SEER 17 registries.

The SEER program of the National Cancer Institute (NCI) has been collecting information on patient demographics, tumor morphology and stage at diagnosis, treatment, and follow-up for vital status since 1973. Currently, this program comprises 17 population-based cancer registries covering approximately 28 % of U.S. populations [8]. The NPCR, which is administered by the Centers for Disease Prevention and Control (CDC) and began operating in 1995, has substantially increased population-based cancer registration coverage in the U.S. Currently, the SEER and NPCR together collect data for the entire U.S. population [9].

Mortality data were obtained from the SEER program's SEER*Stat database as provided by the National Center for Health Statistics (NCHS) [10]. For whites and blacks, data are available since 1969, and for other racial/ethnic groups, data are available since 1990. The accuracy of recording breast cancer as an underlying

cause of death in cancer statistics is high in the U.S., with an agreement rate about 92 % between cause of death on the death certificates and breast cancer diagnosis in cancer registries [11]. All rates (both incidence and mortality) for the U.S. were age standardized to the 2000 U.S. standard population.

1.3.2 Worldwide Incidence and Mortality Data

Breast cancer incidence and mortality rates for all countries in 2008 were obtained from GLOBOCAN 2008 published by the International Agency for Research on Cancer (IARC) [12]. The methods used to estimate cancer incidence and mortality rates, which vary country from country according to the availability and the accuracy of data, are described in detail elsewhere [3]. In GLOBOCAN 2008, incidence data are derived from population-based cancer registries, which cover about 21 % of the world population [13]. Mortality data are available for approximately 30 % of the world population. In Asian and African countries, data are often lacking, incomplete, and/or of poor quality.

Breast cancer incidence trend data were obtained from the Cancer Incidence in Five Continents (CI5) series and mortality trend data were obtained from the WHO mortality database. Worldwide incidence and mortality rates were age standardized to the 1960 world standard population. Thus, they cannot be directly compared with the U.S. rates that were age standardized to the 2000 U.S. standard population.

1.4 Breast Cancer Patterns in the United States

During 2004–2008, the age-standardized breast cancer incidence and mortality rates (per 100,000 females) were 121.2 and 23.5, respectively. However, breast cancer rates in the U.S. vary markedly by demographic and geographic characteristics, such as age, race/ethnicity, and state.

1.4.1 Age

Age is the strongest risk factor for breast cancer in women. Incidence of breast cancer increases sharply with increasing age among premenopausal women (aged $\leq$ 50 years) and then increases at a slower rate among postmenopausal women (aged $>$ 50 years) until age of 80 years (Fig. 1.1). This pattern largely reflects the influence of reproductive hormones on breast cancer occurrence [14]. The decline after age 80 may be due to decreased rates of mammography screening in this age group. During 2004–2008, the incidence rate among U.S. women ranged

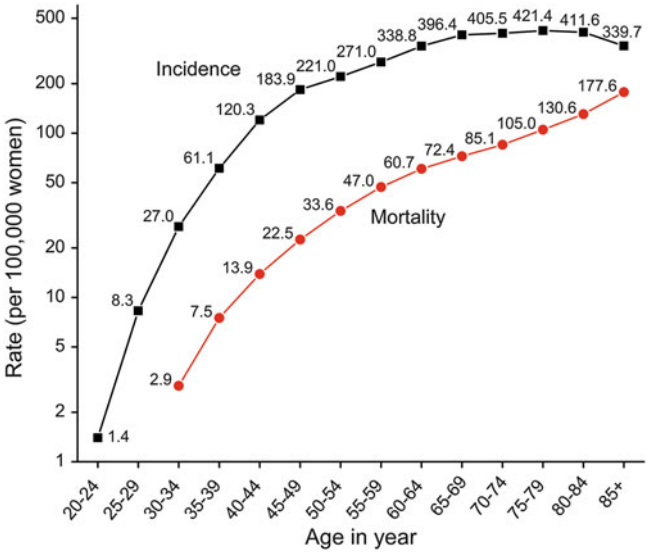

Fig. 1.1 Breast cancer incidence and mortality rates (rates are age adjusted to the 2000 U.S. standard population) by age, U.S., 2004–2008

from 1.4 per 100,000 women for ages 20–24 to 421.4 per 100,000 women for ages 75–79 (Fig. 1.1); the median age at diagnosis of breast cancer was 61 years, with approximately 22 % new cases occurring under age 50, 36 % between ages 50 and 64, 29 % between ages 65 and 79, and 16 % at age 80 or above [15].

In contrast to the incidence patterns, mortality rate increases monotonically with increasing age without interruption (Fig. 1.1). This pattern may partly reflect the poorer survival of breast cancer diagnosed after age 75 [15]. During 2004–2008, breast cancer mortality rate increased from 2.9 per 100,000 women for ages 30–34 to 177.6 per 100,000 women for ages $\geq$ 85; the median age at death from breast cancer was 68 years, with approximately 13 % deaths occurring under age 50, 30 % between ages 50 and 64, 31 % between ages 65 and 79, and 26 % at age 80 or above [15].

1.4.2 Race/Ethnicity

Breast cancer incidence rates vary markedly by race/ethnicity in the United States (Fig. 1.2). During 2004–2008, the age-standardized incidence rate of breast cancer was highest among non-Hispanic whites (125.4/100,000 women) and lowest among Asian Americans/Pacific Islanders (84.9/100,000 women). The high incidence rate among whites may reflect combined effects of early menarche, late child bearing, fewer pregnancies, greater use of menopausal hormone therapy, as well as increased detection through mammography [16, 17]. Although breast

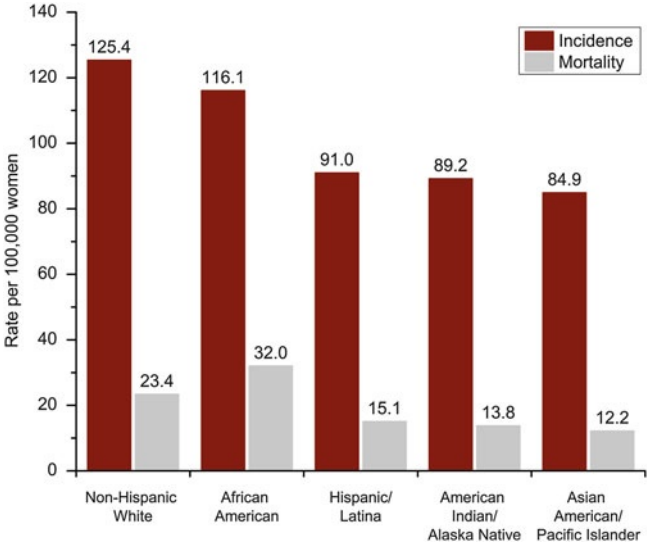

Fig. 1.2 Breast cancer incidence and mortality rates (rates are age adjusted to the 2000 U.S. standard population) by race and ethnicity, U.S., 2004–2008

cancer incidence rates are higher in non-Hispanic white women than in black women for most age groups, African American women have a higher incidence rate before age 45 [15].

Breast cancer mortality rates also varied substantially across different racial/ethnic groups in the United States (Fig. 1.2). Despite lower incidence rate than non-Hispanic whites, African Americans have the highest death rate (32.0/100,000 women during 2004–2008). The higher mortality rate among African Americans is in part due to later stage at diagnosis as a result of poorer quality of screening and delayed follow-up for abnormal mammography findings, as well as due to poorer stage-specific survival rates as a result of delayed treatment [18–20]. In addition, African American women are more likely to be diagnosed with breast cancers with predictors of poor prognosis, such as triple-negative tumors [21, 22]. As observed in incidence, Asian Americans/Pacific Islanders also have the lowest breast cancer death rates (12.2/100,000 women during 2004–2008) among the five major racial/ethnic groups (Fig. 1.2). The racial disparity in breast cancer is discussed in more detail in Chap. 3.

1.4.3 Socioeconomic Status

Unlike most other diseases, the risk of developing breast cancer is positively associated with socioeconomic status as measured either by income or education [23, 24]. This association may partly be explained by the established reproductive risk factors for breast cancer, such as less parity and later age at first child birth

[25]. Women with high SES are often to have fewer children and a later full-term pregnancy than women with low SES. However, due to better survival, women with high SES do not necessarily have higher breast cancer mortality rates than low SES women. In fact, women in affluent areas (poverty rate < 10 %) had a 7 % lower risk of breast cancer death than those in poor areas (poverty rate > 20 %) during 2003–2007, although breast cancer death rates were lower in poor areas than in affluent areas before 1990 [26]. Socioeconomic disparities in breast cancer related factors have been thought to be a major driving factor for racial/ethnic disparities in breast cancer burden in the Unites States [27].

1.4.4 Geographic Variation

Moderate geographic variations in breast cancer incidence and mortality exist in the United States (Table 1.1). During 2004–2008, breast cancer incidence rate for all races combined was highest in Connecticut (136.2/100,000 women) and lowest in Arizona (106.7/100,000 women); for non-Hispanic white women, the incidence rates ranged from 110.8 per 100,000 women in Arkansas to 140.4 per 100,000 women in California and the District of Columbia; among African American women, the incidence rate was lowest in New Mexico (73.2/100,000 women) and highest in Delaware (131.0/100,000 women).

During 2004–2008, District of Columbia had the highest breast cancer death rate for all races combined (27.6/100,000 women) and Hawaii had the lowest rate (17.8/100,000 women) (Table 1.1). Among non-Hispanic white women, breast cancer death rates ranged from 20.9 per 100,000 women in Montana to 27.4 per 100,000 women in New Jersey. In contrast, breast cancer death rates among African American women ranged from 23.1 per 100,000 women in Colorado to 36.8 per 100,000 women in Tennessee. The state variations in breast cancer rates are partly explained by the differential prevalence of known risk factors associated with socioeconomic status [28–31]. State differences in mammography screening may also contribute to the state variation in breast cancer incidence, in part because of early detection and over diagnosis [26, 32].

1.4.5 Trends in Breast Cancer Incidence

During the early 1980s, breast cancer incidence rate increased sharply by 4.0 % per year (Fig. 1.3). This rapid increase largely reflected increased diagnosis due to the introduction of mammography screening [33]. Changes in reproductive patterns including delayed childbearing and less parity may also have contributed to this trend. The rates stabilized during 1987–1994 and then increased again at a relatively lower rate (1.7 %) till 1999. This decelerated increase may be due to combined effects of leveled screening rates, increased use of postmenopausal

Table 1.1 Breast cancer incidence and mortality rates (rates are per 100,000 and age adjusted to the 2000 U.S. standard population) by race/ethnicity and state, U.S., 2004–2008

State	All races		Non-Hispanic white		African American	
	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality
Alabama	117.2	24.5	117.2	22.3	115.8	32.1
Alaska	130.4	21.7	132.6	22.6	122.1	[a]
Arizona	106.7	21.0	112.6	21.9	95.8	27.2
Arkansas	109.0	24.0	110.8	22.9	101.5	32.0
California	122.4	22.5	140.4	25.2	121.0	33.0
Colorado	122.3	20.5	125.0	21.4	103.5	23.1
Connecticut	136.2	23.2	139.4	23.7	112.8	26.4
Delaware	126.6	24.3	125.5	24.6	131.0	24.8
District of Columbia	127.0	27.6	140.4	23.6	122.4	31.6
Florida	113.6	21.9	118.6	21.9	102.3	29.9
Georgia	119.2	23.2	121.2	21.5	118.5	29.9
Hawaii	122.4	17.8	136.3	23.4	78.9	[a]
Idaho	116.5	21.2	118.6	21.6	[a]	[a]
Illinois	123.9	24.7	128.7	24.0	119.5	36.0
Indiana	115.1	24.0	115.1	23.7	113.8	33.6
Iowa	122.5	22.1	123.7	22.3	110.3	32.5
Kansas	124.4	23.1	124.7	22.9	127.0	30.9
Kentucky	120.5	23.5	120.2	23.3	128.3	31.2
Louisiana	118.2	26.8	118.5	23.6	122.3	35.9
Maine	128.9	21.5	128.7	21.4	[a]	[a]
Maryland	123.4	25.6	127.3	24.2	117.8	32.1
Massachusetts	133.4	22.3	136.6	22.8	109.0	25.6
Michigan	120.3	24.4	120.1	23.3	119.2	34.5
Minnesota	126.4	21.6	127.3	21.7	109.0	29.0
Mississippi	112.8	25.5	111.7	21.8	115.4	34.0
Missouri	120.6	25.4	120.9	24.9	125.6	33.5
Montana	120.0	20.7	119.6	20.9	[a]	[a]
Nebraska	125.0	22.0	126.1	21.9	129.1	28.9
Nevada	110.8	23.5	115.7	25.8	104.4	25.7
New Hampshire	132.2	22.8	132.5	23.1	[a]	[a]
New Jersey	129.7	26.5	138.8	27.4	111.9	31.6
New Mexico	110.5	21.5	124.4	23.3	73.2	[a]
New York	124.3	23.1	133.5	23.4	106.7	27.4
North Carolina	123.3	24.4	124.5	22.7	122.3	32.8
North Dakota	124.2	22.3	123.7	21.6	[a]	[a]
Ohio	119.8	25.9	119.4	25.2	120.7	34.8
Oklahoma	125.6	24.1	125.1	24.2	125.3	35.4
Oregon	130.3	22.5	129.9	23.1	93.4	24.3
Pennsylvania	124.8	24.8	124.9	24.5	125.5	32.0
Rhode Island	132.5	22.2	136.1	22.8	118.8	[a]
South Carolina	119.9	24.3	121.5	22.0	114.5	31.2
South Dakota	117.4	21.8	118.3	22.0	[a]	[a]
Tennessee	117.2	24.5	117.3	22.8	116.4	36.8

(continued)

Table 1.1 (continued)

State	All races		Non-Hispanic white		African American	
	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality
Texas	113.7	22.6	121.6	22.7	117.1	34.4
Utah	109.5	22.1	112.1	22.7	75.7	[a]
Vermont	130.1	21.7	131.5	22.0	[a]	[a]
Virginia	124.2	25.1	125.8	23.6	126.4	34.7
Washington	129.8	22.4	131.6	23.5	117.7	26.5
West Virginia	112.6	23.9	113.3	23.8	98.9	35.1
Wisconsin	123.4	22.1	123.4	22.2	113.0	27.1
Wyoming	114.6	22.1	116.3	22.2	[a]	[a]

[a] Statistic not displayed due to fewer than 25 cases or deaths

hormone therapy, and rising obesity epidemic [34]. After peaking in 1999, incidence rate started to decrease and sharply dropped by 7 % during 2002–2003 [35]. This dramatic decline is thought to be a result of decreased use of menopausal hormones following the publication of the results of the Women's Health Initiative trial in 2002, which linked hormone use with increased breast cancer risk [36]. This trend has also been attributed to a reduced pool of prevalent cases as a result of widespread screening [34, 37]. Since 2003, breast cancer incidence rates have remained relatively stable [38].

The overall trends in breast cancer incidence largely reflected the trend for women 50 years of age and older, among whom the incidence rates increased annually by 5.4 % during 1982–1987, stabilized from 1987 to 1993, then increased again at a slower rate (1.9 % per year) during 1993–1999, then declined by 2.6 % per year from 1999 to 2005, and have since stabilized. In contrast, after a rapid increase (3.2 % per year) during 1980–1985, the incidence rates for women younger than 50 years have since remained almost constant (Fig. 1.3).

The temporal trends in breast cancer incidence were generally similar between white and black women from 1980 to the early 1990s (Fig. 1.3). However, different patterns between these two racial groups have been observed since then, partly due to differences in the use of mammography screening and menopausal hormone therapy. During 1994–1999, the rates for white women increased annually by 2.0 %, then decreased by 2.4 % per year during 1999–2004 with a dramatic decline between 2002 and 2003, and have remained relatively stable since then. In contrast, the rates in black women have remained relatively stable since 1992, although they are slightly increasing in the most recent time period.

1.4.6 Trend in Breast Cancer Mortality

In contrast to some dramatic changes in incidence, trends in breast cancer mortality rates have evolved gradually over time (Fig. 1.4), which reflected combined effects of trends in underlying risks of breast cancer occurrence, changes in

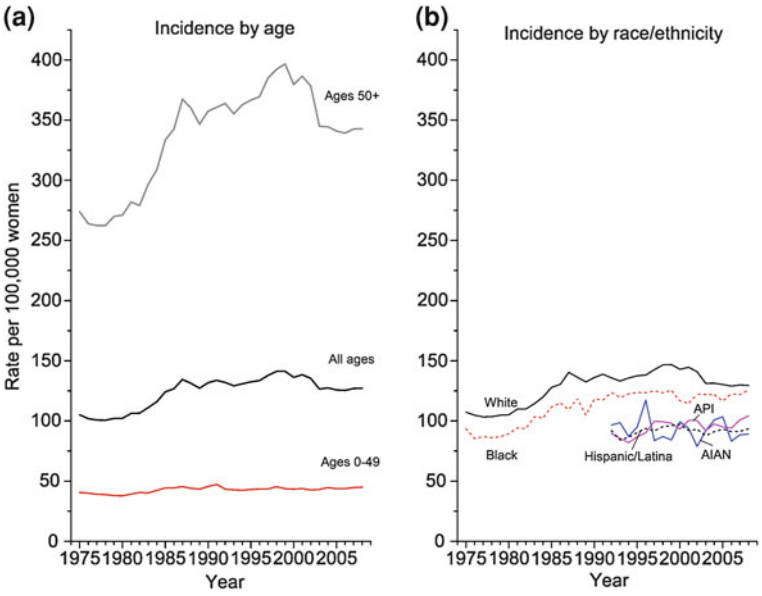

Fig. 1.3 Breast cancer incidence rates (rates are age adjusted to the 2000 U.S. standard population) by age and race/ethnicity (data for whites and blacks are from the SEER 9 areas; data from other races/ethnicities are from the SEER 13 areas; incidence data for AIAN are based on contract health service delivery area (CHSDA) counties), U.S., 1975–2008. Abbreviation: *AIAN* American Indian/Alaska Native, *API* Asian American/Pacific Islander

screening practices, and advances in cancer treatment. From 1975 to 1990, breast cancer death rates slowly increased by 0.4 % per year and then decreased annually by 2.2 % from 1990 to 2008. The recent decline in death rates has been attributed to both improvements in treatment and early detection. Researchers in the Cancer Intervention and Surveillance and Modeling Network (CISNET) estimated that screening and adjuvant treatment equally contributed to the reduction in breast cancer mortality rates in the United States [39].

The overall trends in breast cancer mortality mask some important variations by race. Historically, breast cancer death rates were slightly higher among white women than among black women. After converging in the late 1970s, the rates for white and black women diverged rapidly (Fig. 1.4). Specifically, breast cancer death rates for white women increased slowly by 0.3 % per year from 1975 to 1990, and then decreased annually by 2.3 % from 1990 to 2008. Among black women, in contrast, death rates increased rapidly by 1.5 % per year from 1975 to 1992 and then declined annually by 1.4 % from 1992 to 2008. The differential trends between whites and blacks have resulted in a widening black-white disparity in breast cancer death rates in the U.S. since 1980. By 2008, breast cancer death rates were 43 % higher in black women than in white women (Fig. 1.4). This difference is thought to reflect differences in access to care as well as survival.

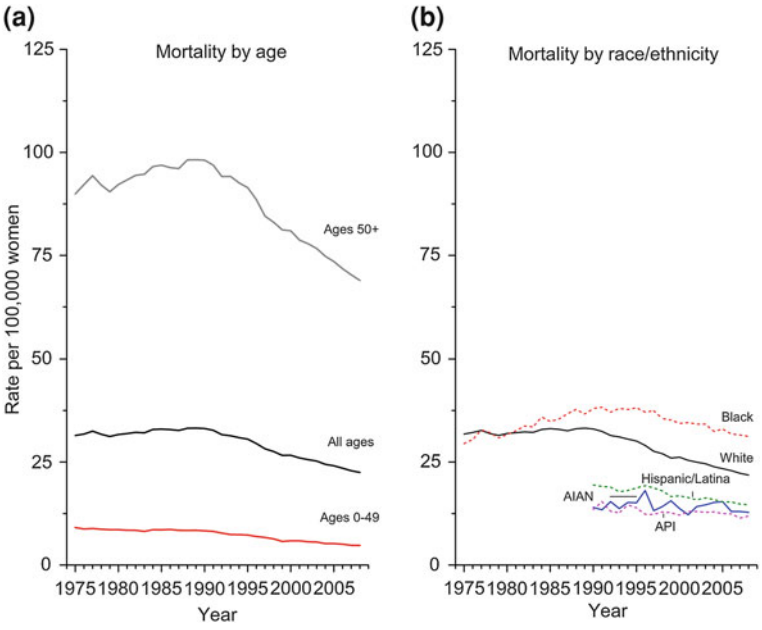

Fig. 1.4 Breast cancer mortality rates (rates are age adjusted to the 2000 U.S. standard population) by age and race/ethnicity, U.S., 1975–2008. Abbreviation: *AIAN* American Indian/Alaska Native, *API*, Asian American/Pacific Islander

1.4.7 Survival

Survival of breast cancer has improved greatly over the last 30 years in the U.S. Based on data from SEER 17 registries, the 5 year relative survival rate was 90.0 % for cancers diagnosed in 2001–2007 [15]. Because of this relatively good prognosis as well as the high incidence, breast cancer is by far the most prevalent cancer among women in the U.S., with an estimated 2.6 million women with a history of breast cancer in 2008 [15].

Stage at diagnosis is an important predictor of breast cancer prognosis. For cancers diagnosed in 2001–2007, the 5 year survival rate was 98.6 % for localized (confined to primary site), 83.8 % for regional (spread to regional lymph nodes), and 23.3 % for distant (cancer has metastasized) disease. Survival of breast cancer is also associated with age at diagnosis, with a lower 5 year survival rate for cancers diagnosed at either a younger or an older age [15]. Tumors diagnosed at younger age tend to be more aggressive and or/less response to treatment [40]. Compared with white women, black women are more likely to have poorer breast cancer survival rates at all ages of diagnosis [15], due to both later stage at diagnosis and poorer stage-specific survival among black women (Fig. 1.5).

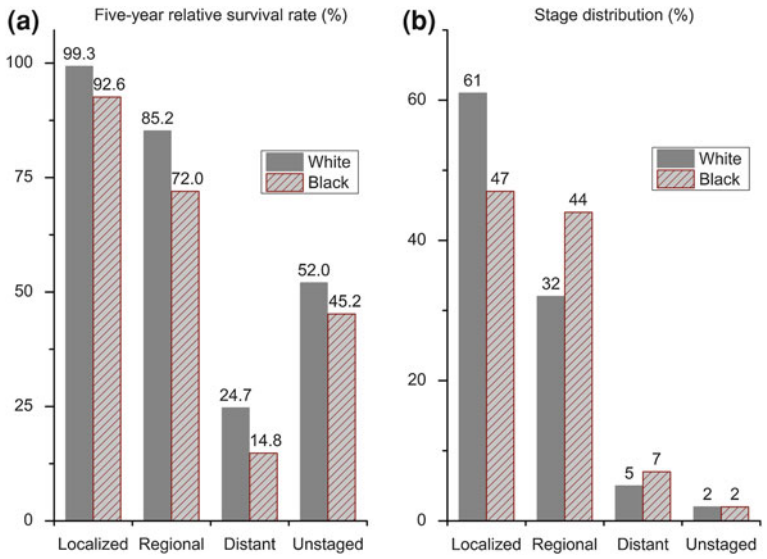

Fig. 1.5 Five-year relative survival rate and stage distribution of breast cancer, U.S., 2001–2007

1.5 Global Patterns of Breast Cancer Incidence and Mortality

Worldwide, breast cancer is the most common cancer and the leading cause of cancer death among women, with approximately 1.38 million new cases and 458,000 deaths in 2008 [3, 4]. It was estimated that in 2008, there were about 5.2 million women alive who were diagnosed with breast cancer in the previous 5 years [41]. Worldwide, the burden of breast cancer varies substantially across regions and countries. The geographical and temporal patterns in breast cancer incidence and mortality are described below.

1.5.1 Global Variations in Incidence and Mortality

In general, breast cancer incidence rates are highest in Western and Northern Europe, North America, and Australia/New Zealand; intermediate in Southern and Eastern Europe, South America, the Caribbean, and Northern Africa; and lowest in sub-Saharan Africa and Asia (Figs. 1.6, 1.7). According to GLOBOCAN 2008 [12], age-standardized (1960 world standard population) breast cancer incidence rates ranged from 19.3 per 100,000 women in Eastern Africa to 89.7 per 100,000 women in Western Europe (Fig. 1.6); the incidence rate in more developed regions (66.4/100,000 women) was 2.5 times as high as in less developed regions

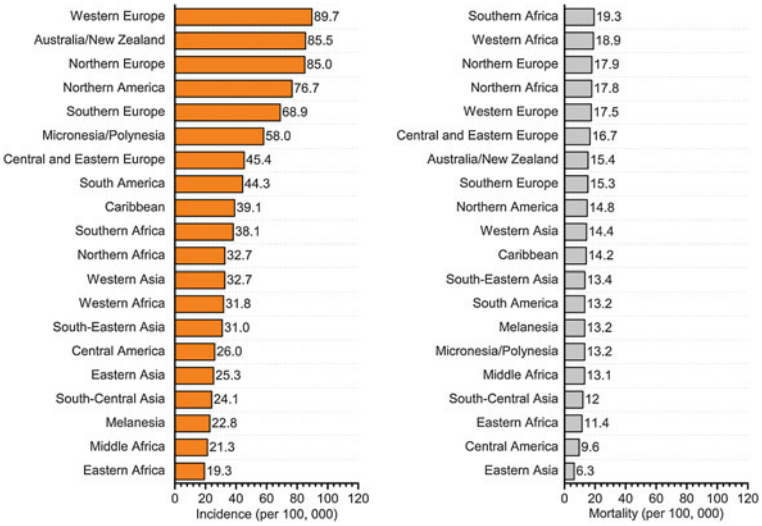

Fig. 1.6 Breast cancer incidence and mortality rates (rates are adjusted to the 1960 world standard population) by world region, 2008

(27.1/100,000 women). Between countries, the rates varied about 10-fold, with the highest rates in some Northern and Western European countries and the lowest rates in some Eastern African countries [12]. Results from migrant studies suggest that international variations in breast cancer incidence largely reflect differences in environmental or lifestyle factors rather than genetic differences [42, 43].

Wide variations in breast cancer incidence rates were also seen within regions and countries [12]. For example, the incidence in Southern Africa (38.1/100,000 women) was twice as high as in Eastern Africa (19.3/100,000 women); the incidence in Singapore was 55.9 per 100,000 women, which is much higher than the average rate of Asian populations (26.0/100,000 women); in China, the 1993–1997 incidence of breast cancer in Shanghai was 27.2 per 100,000 women, compared with 10.0 per 100,000 women in the more rural Qidong county [44]. These variations were likely due to differences in population make-up, health resources, and/or lifestyle factors. A notably high breast cancer incidence (96.8/100,000 women) was found in Israel, in part due to the high prevalence of BRCA1 and BRCA2 mutations in the Ashkenazi Jewish population [45].

Similar to the observed patterns for incidence, breast cancer mortality rates were higher in more developed regions (15.3/100,000 women) than in less developed regions (10.7/100,000 women) [12]. Across different geographic regions, breast cancer mortality rates ranged from 19.3 per 100,000 women in Southern Africa to 6.3 per 100,000 women in Eastern Asia in 2008 (Fig. 1.7). Across countries, the rates varied about 5-fold, with the highest rates in some European countries and the lowest rates in some Eastern Asian countries [12]. The

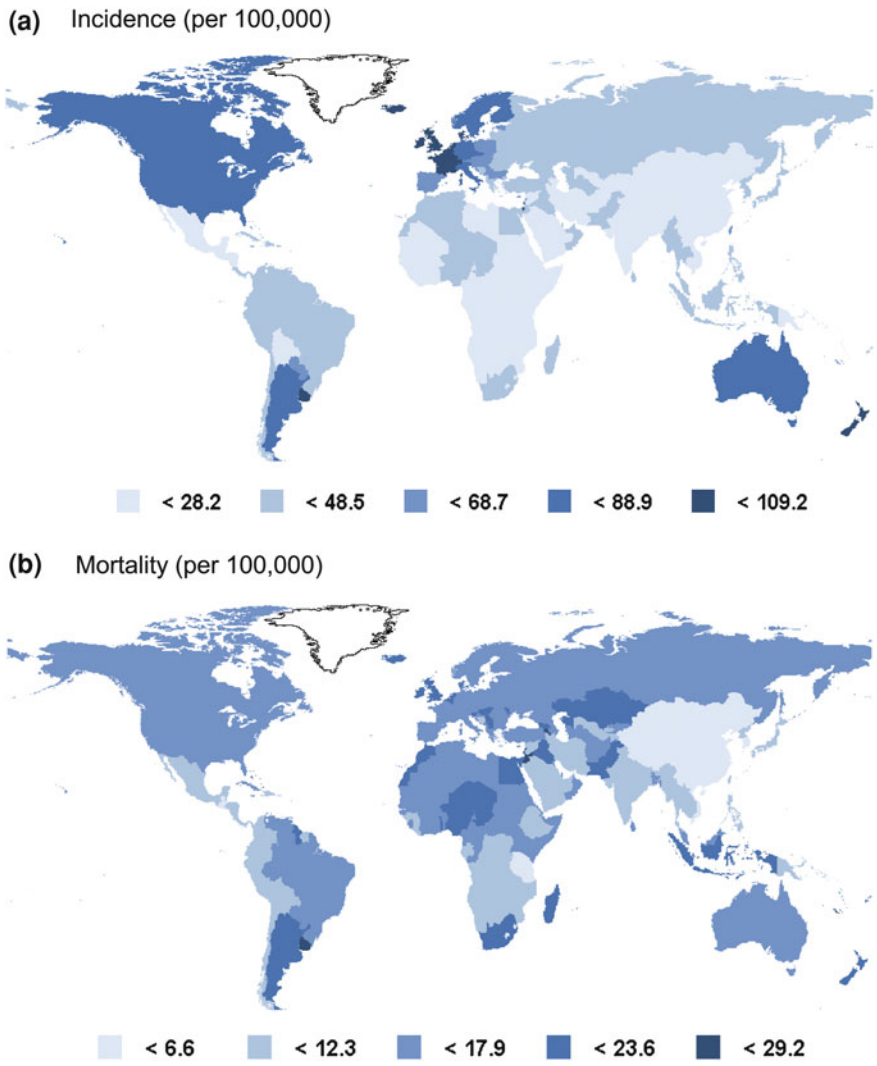

Fig. 1.7 Breast cancer incidence and mortality rates (rates are adjusted to the 1960 world standard population), world map, 2008

smaller geographic variations in mortality than in incidence are due to more favorable survival of breast cancer in countries with higher incidence rates (more developed countries). For example, only 40 % of women in Campinas (Brazil) and Setif (Algeria) survive 5 years after a diagnosis of breast cancer, compared with 83 % of women in Canada, 80 % of women in Finland, and 81 % of women in Australia [5].

1.5.2 Global Trends in Incidence and Mortality

Over the past two or three decades, breast cancer incidence has increased rapidly in countries that historically had a low incidence rate (e.g., several developing countries and Japan). For example, incidence rates increased by 140 % in Miyagi (Japan) from 1973–1977 to 1998–2002, by 40 % in Chennai (India) from 1983–1987 to 1998–2002 [2, 41], and by 4.5 % per year in Kampala (Uganda) from 1991 to 2006 [46]. The rapid increases in these countries are widely attributed to the 'westernization' of lifestyles, such as late childbearing, less parity, increased exogenous hormonal intake, and reduced physical activity [47]. In developed countries, the incidence rates of breast cancer increased substantially from the 1980s through the middle or late 1990s. However, since the early 2000s, a downward trend in breast cancer incidence has been seen in the United States and many other western countries, which has been partly attributed to the reduced use of menopausal hormone therapy [48–50].

In contrast to the trends in incidence, breast cancer mortality rates have been decreasing in North America, Western Europe, Australia, and New Zealand over the past two or three decades. For instance, the mortality rate from breast cancer decreased from 29.4 per 100,000 women in 1986 to 17.4 per 100,000 women in 2009 in the United Kingdom [2, 41]. The decreasing trends in these countries have been attributed to improved breast awareness, extended use of mammographic screening, intensified early clinical diagnosis, and advances in both primary and adjuvant treatments for breast cancer [51]. In most developing countries and Japan, however, breast cancer mortality rates continued to increase. For example, breast cancer mortality rates in the Philippines increased from 9.0 per 100,000 women in 1992 to 16.8 per 100,000 women in 2008; in Japan, the rates increased from 4.4 per 100,000 women in 1970 to 8.9 per 100,000 women in 2009 [41].

1.6 Summary

Breast cancer is the most common cancer (excluding skin cancers) among U.S. women and kills more women than any other cancers except lung cancer. There are large variations in breast cancer rates across racial/ethnic and socioeconomic groups. The incidence rates are high among whites and high SES populations, whereas African American and low SES women have high mortality rate. Eliminating breast cancer disparities between different population segments has been an overarching goal of government and private public health agencies in the United States.

Over the last few decades, breast cancer incidence and mortality rates have been increasing rapidly in developing countries, in part due to a wide adoption of western lifestyles, which are characterized by delayed childbirth, reduced parities, physical inactivity, and early-menarche-causing dietary habits. In 2008, the majority of breast cancer deaths occurred in developing rather than developed

countries, although incidence rates remained high in more developed regions (except Japan). The wide spread of population-based mammographic screening programs and extensive use of adjuvant therapy have led to large decreases in breast cancer mortality in the U.S. and many other developed countries. However, challenges remain to curb the growing burden of breast cancer in many low- and middle-income countries, where limited health and financial resources hinder the adoption of these effective but resource-demanding strategies. Alternatively, raising breast awareness among the public and medical communities and promoting clinical breast examination, as an early detection strategy, should be a viable approach to reduce breast cancer burden in these countries [52]. In addition, national and international collaborations between governments, non-governmental organizations, research institutes, and biochemical or pharmaceutical companies are needed to improve the accessibility and affordability of early detection services and treatment among populations with limited resources.

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Chapter 2
Epidemiology of Breast Cancer in Women

Steven S. Coughlin and Yasmin Cypel

Abstract Epidemiologic studies have contributed importantly to current knowledge of environmental and genetic risk factors for breast cancer. Worldwide, breast cancer is an important cause of human suffering and premature mortality among women. In the United States, breast cancer accounts for more cancer deaths in women than any site other than lung cancer. A variety of risk factors for breast cancer have been well-established by epidemiologic studies including race, ethnicity, family history of cancer, and genetic traits, as well as modifiable exposures such as increased alcohol consumption, physical inactivity, exogenous hormones, and certain female reproductive factors. Younger age at menarche, parity, and older age at first full-term pregnancy may influence breast cancer risk through long-term effects on sex hormone levels or by other biological mechanisms. Recent studies have suggested that triple negative breast cancers may have a distinct etiology. Genetic variants and mutations in genes that code for proteins having a role in DNA repair pathways and the homologous recombination of DNA double stranded breaks (*BRCA1*, *BRCA2*, *XRCC2*, *XRCC3*, *ATM*, *CHEK2*, *PALB2*, *RAD51*), have been implicated in some cases of breast cancer.

Keywords Incidence · International trends · Risk factors · Anthropometric factors · Mammographic breast density · Ionizing radiation exposure · Environmental exposures · Genetic factors · Gene mutations · Genetic polymorphisms · Family history · Race

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2.1 Introduction

The global burden of breast cancer in women, measured by incidence or mortality, is substantial and rising in several countries [1, 2]. Breast cancer is the most commonly diagnosed invasive cancer in the United States for women of all racial and ethnic groups, with an estimated 230,480 new cases diagnosed in 2011 [3]. Breast cancer accounts for more cancer deaths among United States women than any site other than lung cancer. Breast cancer also occurs in men [4], but the disease is rare among men and there is a pronounced female-to-male disparity in breast cancer incidence. This chapter provides a summary of the distribution and determinants of breast cancer in women including both the descriptive epidemiology of the disease and an up-to-date review of risk factors identified in epidemiologic studies.

2.1.1 Incidence and Mortality Rates in the US

Breast cancer incidence and death rates increase with age; about 95 % of new cases occur in women 40 years of age and older [3]. Breast cancer incidence rates in the United States continue to rise after menopause and are highest in the older age categories. Age-standardized incidence rates are higher among white women than black women, although black women in the United States have a higher mortality rate than white women. Incidence rates for Asian/Pacific Islander, American Indian/Alaska Native, and Hispanic women in the United States are generally lower than those for white or black women [5, 6] (Fig. 2.1).

The incidence of breast cancer in the United States increased until about 2000 then decreased from 2002 to 2003 [7]. Most of the decrease in that period was among women with estrogen receptor positive cancers [8].

2.1.2 International Trends in Breast Cancer Incidence and Mortality

Worldwide, an estimated 1.4 million women were diagnosed with breast cancer in 2008 and about 458,400 women died from the disease that same year [2]. Breast cancer incidence rates tend to be higher among more affluent women, both within countries and internationally (Fig. 2.2). More than two-thirds of breast cancer cases are diagnosed in women aged 50 years and older; the majority of these cases are in developed countries [9]. For women aged 15–49 years, twice as many breast cancer cases are diagnosed in developing countries than in developed countries [9]. Between 1980 and the late 1990s, breast cancer incidence rates rose about 30 % in westernized countries [2]. This trend was likely due to changes in

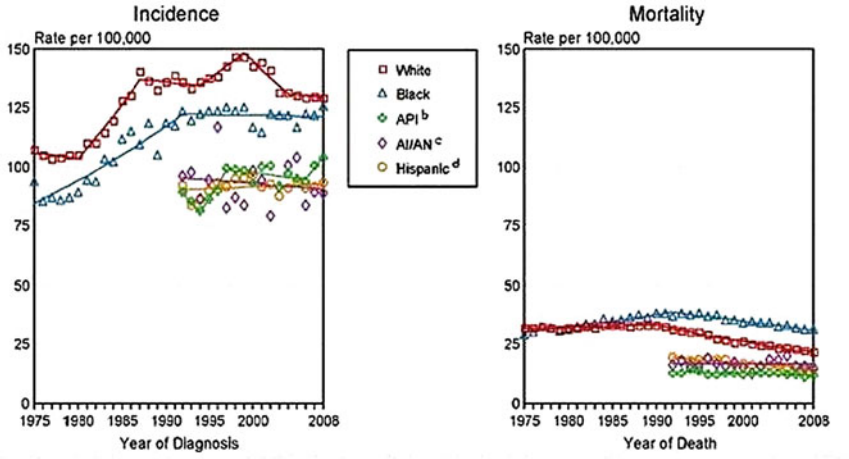

Fig. 2.1 SEER incidence and US death rates, cancer of the female breast, joinpoint analyses for whites and blacks from 1975 to 2008 and for Asian-Pacific Islanders, American lndians/Alaska Natives, and Hispanics from 1992 to 2008 (http://sees.cancer.gov)

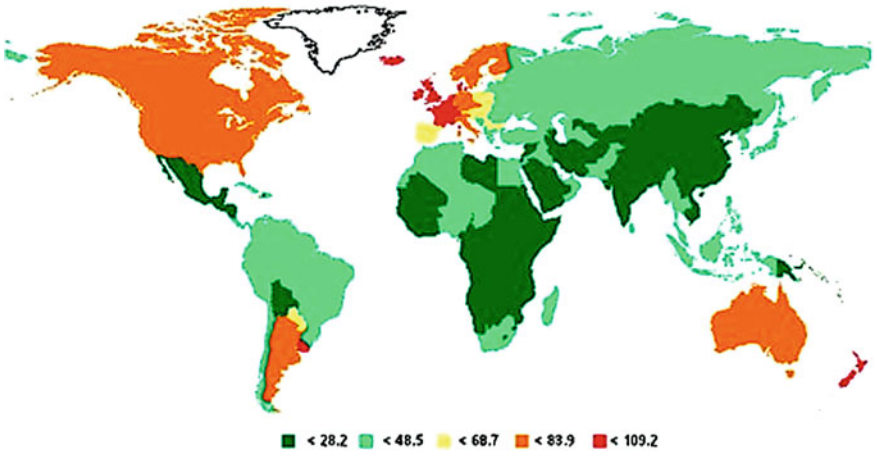

Fig. 2.2 Estimated age-standardized breast cancer incidence rate per 100,000 women, worldwide (http://globoscan.iarc.fr)

reproductive patterns and increased screening. In the last decade, breast cancer incidence rates rose in many Asian and African countries [2]. In countries where mammography is available or affordable, adherence to recommendations for routine screening is associated with reduced mortality from breast cancer. Over the past two decades, breast cancer mortality has been stable or decreasing in many countries in Europe and North America [2].

2.2 Risk Factors

A variety of risk factors for breast cancer have been well-established by epidemiologic studies carried out to date, in addition to increasing age and female sex. These risk factors include nonmodifiable factors such as race, ethnicity, and genetics, as well as modifiable exposures related to diet, physical inactivity, exogenous hormones, and certain female reproductive factors. Circulating levels of endogenous sex steroid hormones such as estradiol have been associated with increased risk of breast cancer among postmenopausal women [10]. Sex hormone levels are strongly associated with some risk factors for breast cancer (for example, obesity and higher alcohol consumption) and may mediate the effects of these factors on breast cancer risk [11].

2.2.1 Race

Several factors may account for racial differences in breast cancer mortality including socioeconomic factors, access to screening mammography and timely treatment, and biological factors. In the United States, Hispanic ethnicity and black race have been associated with later stage at breast cancer diagnosis [12]. Compared with white women in the United States, black women tend to have more aggressive breast cancers that present more frequently as estrogen receptor negative tumors [13]. Among premenopausal women, tumors that are estrogen receptor negative, progesterone receptor negative, and HER2 negative ("triple negative" tumors) are more common among black women than among white women.

2.2.2 Age at Menarche, Parity, and Age at First Live Birth

Younger age at menarche, parity, and older age at first full-term pregnancy are well-established risk factors for breast cancer. These risk factors may influence breast cancer risk through long-term effects on sex hormone levels in premenopausal women, through long-lasting changes in breast tissue, or by other biological mechanisms [14]. Reproductive hormones may influence breast cancer risk by increasing cell proliferation and increasing the likelihood of damage to DNA or by promoting cancer growth [3]. In a pooled analysis of control group data from 13 studies of postmenopausal women, circulating levels of estradiol were 6 % lower in women who had menarche at ages 14 years or older than in women who had menarche before 12 years [11].

Nulliparity increases breast cancer risk in older women [15]. Results from a cohort study of Norwegian women indicated that nulliparity and obesity may have a synergistic effect on breast cancer risk among older women [16]. In the Black

Women's Health Study in the United States [17], higher parity was associated with a reduced risk of estrogen receptor positive/progesterone receptor positive breast cancer (hazard ratio = 0.53, 95 % CI 0.39–0.73 for 3 + versus 0 births, p(trend) = 0.0002). Pregnancy may reduce breast cancer risk by bringing about persistent changes in the mammary gland that make the breast less susceptible to carcinogenic factors [16]. Younger age at first live birth is protective.

2.2.3 Breast Feeding

Breast feeding reduces a woman's risk of breast cancer and is an important modifiable preventive behavior. Longer duration of breast feeding has been associated with a greater reduction in breast cancer risk. The higher incidence of estrogen receptor negative/progesterone receptor negative breast cancer among black women in the United States may be partly explained by their lower prevalence of breastfeeding relative to white women [17].

2.2.4 Menopausal Status and Age at Menopause

Older age at menopause is also a well-recognized risk factor for breast cancer. Both early menarche and older age at menopause increase lifetime exposure of breast tissue to hormones. Menopause hormone therapy is discussed below in Sect. 2.4.6.

2.2.5 Oral Contraceptives

Epidemiologic studies of oral contraceptive use and breast cancer risk have generally shown little or no increased risk [18]. Recent use of oral contraceptives may slightly increase the risk of breast cancer [3]. In an analysis of data from a multicenter, population-based case–control study, Marchbanks et al. found that breast cancer risk did not vary by oral contraceptive formation [18]. No formulation was significantly associated with an increased risk of breast cancer.

2.2.6 Hormone Therapy

Results from observational studies and the Women's Health Initiative Randomized Trial indicate that hormone replacement therapy after menopause increases breast cancer risk [19–21]. Use of a regimen that includes both estrogen and progesterone has been associated with a higher risk of breast cancer than the use of estrogen

alone [19]. Studies of breast cancer incidence in the United States, Canada, and European countries showed a 5–10 % decline in breast cancer incidence following reductions in hormone therapy (HT) use after 2002 [22]. In several countries, however, temporal changes in screening mammography are also likely to have played a role in the decline in breast cancer incidence. Women who do not currently use HT may also undergo screening mammography less frequently [22, 23].

2.2.7 Diet

A wide variety of dietary factors have been examined as potential breast cancer risk factors in case–control and prospective studies, including increased consumption of alcohol [24–26], red meat, processed meat, and animal fat, and decreased consumption of fruits and vegetables, calcium, vitamin D, soy, and antioxidants such as beta-carotene and other carotenoids, vitamin C, and vitamin E [27, 28]. The ratio of omega-3 to omega-6 fatty acids has also been examined in relation to breast cancer risk. Although initial studies suggest that a higher ratio of omega-3 to omega-6 fats may reduce breast cancer risk, more research is warranted [29]. For most dietary factors, epidemiologic studies of breast cancer have provided inconsistent or inconclusive results. A notable exception is alcohol consumption, which is discussed separately below.

Foods with a high glycemic index and glycemic load and dietary carbohydrates, which can influence blood glucose and insulin concentrations, have also been examined in relation to breast cancer risk [30–33]. The glycemic index is an indicator of the blood sugar response of the body to a standardized amount of carbohydrate in food. The glycemic load takes into account the amount of food consumed [29]. A meta-analysis by Mulholland et al., which focused on cohort study results, showed no overall association between postmenopausal breast cancer risk and glycemic load intake (RR = 1.03, 95 % CI 0.94–1.12) [34].

In a recent meta-analysis of prospective studies (14 studies of breast cancer incidence and 4 studies of breast cancer recurrence), Dong and Qin found that soy isoflavones consumption was inversely associated with breast cancer risk (RR = 0.89, 95 % CI 0.79–0.99). However, the protective effect of soy was only observed among studies conducted in Asian populations [27].

2.2.8 Alcohol

An increasing number of epidemiologic studies have implicated alcohol consumption as a risk factor for breast cancer [24–26]. Studies have shown a linear dose–response relation between alcohol consumption and breast cancer risk. Chen et al. examined the association of breast cancer with alcohol consumption among 105,986 women enrolled in the Nurses' Health Study, of whom 7,690 developed

invasive breast cancer over the period 1980 through June 2008. Alcohol consumption was significantly associated with increased breast cancer risk even at levels as low as 5.0–9.9 g per day, or about 3–6 drinks per week (RR = 1.15, 95 % CI 1.06–1.24). Cumulative average alcohol consumption over long periods of time was found to be the most relevant measure [24]. The possible biological mechanisms include alcohol's effects on circulating estrogen levels.

2.2.9 Physical Activity

There is considerable evidence from epidemiologic studies that high levels of physical activity reduces breast cancer risk in women. The possible biological mechanisms include the influences of physical activity on body composition, insulin resistance, and circulating levels of sex steroid hormones [35]. In the Women's Health Initiative Cohort Study, which involved 74,171 women aged 50–79 years recruited by 40 United States clinical centers, women who engaged in regular strenuous physical activity at age 35 had a 14 % decreased risk of breast cancer (RR = 0.86, 95 % CI 0.78–0.95) compared to inactive women [36]. Similar but attenuated findings were observed for strenuous physical activity at ages 18 years and 50 years. The study results also indicated that longer duration of physical activity provides the most benefit [36].

2.2.10 Anthropometric Factors

Anthropometric factors such as body height, weight, and adiposity have been extensively studied in epidemiologic studies of breast cancer [37, 38]. Body fat provides a substrate for the production of estrogen from androgen in adipose tissue [39]. In the Cancer Prevention Study II cohort (n = 495,477 women), Calle et al. found that women with higher values of body mass index had an increased risk of dying from breast cancer and certain other cancers [40]. Although overweight and obesity are important modifiable risk factors for breast cancer among postmenopausal women, epidemiologic studies have shown that high body mass index and other measures of adiposity are associated with a reduced risk of breast cancer among premenopausal women [41, 42]. The age at which body mass or adiposity is assessed (childhood, adolescence, or adulthood) is important. In some studies, body mass index at age 18 years and body fatness during youth have been inversely associated with breast cancer risk in both pre- and postmenopausal women [42].

Obesity and physical inactivity are important determinants of hyperinsulinemia and insulin resistance. Hyperinsulinemia with insulin resistance has been reported to be an independent risk factor for breast cancer [43].

Obesity influences the amount of free insulin-like growth factor I (IGF-I) available to cells. Breast cancer has been related to cell proliferation in response to

growth factors such as IGF-I and sex hormones [44]. Increases in serum or plasma levels of IGF-I have been observed in some epidemiologic studies of premenopausal breast cancer [45], but results to date have been inconsistent. The relationship between prediagnostic IGF-I and insulin-like growth factor binding protein-3 (IGFBP-3) levels and breast cancer risk was examined in a meta-analysis of data from 17 prospective studies conducted in 12 countries [46]. The overall odds ratio for breast cancer for women in the highest versus the lowest quintile of IGF-I concentration was 1.28 (95 % CI 1.14–1.44). The positive association with IGF-I, which was not substantially modified by IGFBP-3 or menopausal status, was limited to estrogen receptor positive breast cancers.

In general, results from epidemiologic studies do not support an association between IGFBP-1 and breast cancer risk. Although results from some epidemiologic studies support an association between IGFBP-3 and risk of breast cancer among younger women, results to date have been inconsistent. Rinaldi et al. conducted a pooled analysis of data from three prospective studies in New York, Northern Sweden, and Milan, Italy [47]. Statistically nonsignificant, positive associations were observed between IGF-I and IGFBP-3 and breast cancer risk among younger women.

2.2.11 Mammographic Breast Density

Breast density is one of the strongest established risk factors for breast cancer. Women with more extensive mammographic density have over a 4-fold increased risk of breast cancer [48]. Mammographic density likely reflects the amount of epithelial and stromal cells in the breast and the proliferation of these cells but does not indicate any histological abnormality [49]. Mammographic breast density is less extensive in women who are parous and in those with a larger number of live births, and changes in response to exposure to hormones [49]. Mammographic breast density decreases throughout menopause and increases with combined hormone therapy [50]. Longitudinal epidemiologic studies have shown that mammographic density declines as women get older [51]. The change in mammographic density with age reflects a reduction in glandular tissue and increase in fat [49]. Although influenced by changes in exposure to hormones, mammographic density is also a heritable quantitative trait [50].

2.2.12 Environmental and Occupational Exposures

Exposure to ionizing radiation (as a result of nuclear explosions, diagnostic fluoroscopy, or radiotherapy in adolescence) is an established breast cancer carcinogen [52, 53]. The biological mechanism is likely to be induction of DNA mutations. The risks of breast cancer associated with a wide variety of environmental exposures

were recently reviewed by the Institute of Medicine at the request of Susan G. Komen for the Cure [54]. The IOM concluded that the evidence associating individual chemicals with breast cancer risk is not conclusive, and also recognized the need for further research in this area. The IOM noted that exposure to chemicals with estrogenic or other properties relevant to sex steroid activity, such as bisphenol A (BPA), polybrominated diphenyl ethers (PBDEs), and certain dioxins or dioxin-like compounds, may possibly influence breast cancer risk. The risk of breast cancer from exposure to 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been reviewed by several authors and expert panels with no consistent evidence of an increased risk [55]. Despite the lack of conclusive evidence from epidemiologic studies, exposures to chemicals with estrogenic or other properties relevant to sex steroid activity could influence breast cancer risk if the exposures occur at critical life stages or in combination with exposure to other similar chemicals [54].

Results from several studies support an association between shift work and disruption of the circadian rhythm with breast cancer risk. In the Nurses' Health Study [56] a moderate increase in breast cancer risk was observed among women who worked 1–14 years (adjusted RR = 1.08, 95 % CI 0.99–1.18) or 15–29 years on rotating night shifts (adjusted RR = 1.08, 95 % CI 0.90–1.30). Levels of serum melatonin, which may have a protective effect, decrease when people are exposed to light at night. In experimental studies, the disruption of the nocturnal melatonin signal has been shown to activate human breast cancer growth, metabolism, and signaling [57].

Epigenetic changes such as DNA methylation have been associated with breast cancer in epidemiologic studies [58]. DNA methylation, which has been associated with environmental exposures such as cigarette smoke and persistent organic pollutants, may play a role in cancer causation by silencing genes through hypermethylation or, conversely, by activating genes through hypomethylation [58].

2.3 Risk Factors According to ER, PR, and HER2 Expression

As detailed in other chapters in this book, breast cancer subtypes are biologically distinct and may have distinct etiologies [59, 60]. This includes cases that express estrogen and/or progesterone receptors and those that overexpress the tyrosine kinase human epidermal growth factor receptor-2 (HER2) due to amplification of its encoding oncogene *ERBB2*. Using data from the Breast Cancer Surveillance Consortium (n = 743,623 women), Phipps et al. examined associations between reproductive history and breast cancer cases classified according to tumor marker expression: estrogen receptor (ER) positive (n = 8,203 cases), ER negative/progesterone receptor (PR) negative/HER2 positive (n = 288), or ER negative, PR negative, and HER2 negative (triple negative, n = 645). Nulliparity was most strongly associated with risk of ER positive breast cancer (hazard ratio = 1.31, 95 % CI 1.23–1.39). Late age at first birth was most strongly associated with risk of ER negative/PR negative/HER2 positive disease (hazard ratio = 1.83, 95 % CI

1.31–2.56). Neither parity nor age at first birth was associated with triple negative breast cancer. Studies have shown that female reproductive factors such as early age at menarche, nulliparity, and older age at first live birth are most clearly associated with hormone receptor positive tumors, suggesting that triple negative breast cancer may have a distinct etiology [61]. Recent studies, including emerging areas of research, have focused on central obesity and the metabolic syndrome as predictors of triple negative breast cancer [62].

2.4 Genetic Factors

Population-based epidemiologic studies and family-based studies have identified a number of low-penetrance genetic variants and rare, moderate-to-high penetrance genetic mutations including *BRCA1* and *BRCA2* gene mutations. As discussed in other chapters in this book, breast cancer is a heterogeneous disease and genetic factors likely account for pathological subtypes and much of the heterogeneity of the disease [63].

2.4.1 Family History of Cancer

Having a positive family history of breast cancer is an established risk factor for the disease. Women who have one first degree relative with breast cancer have about a two-fold increased risk of developing breast cancer [64, 65]. Risk increases the younger the relative was at the time of diagnosis and with increasing number of first-degree relatives with breast cancer [3]. About 20 % of breast cancer patients have a family history of the disease in a first degree relative. Only about 5–10 % of breast cancer cases associated with a family history of the disease in a first-degree relative are inherited in an autosomal dominant fashion. These cases have features such as bilaterality, early age at onset, and occurrence in multiple generations [66]. Most breast cancer cases are sporadic and not associated with high penetrance gene mutations.

2.4.2 Genetic Polymorphisms

Genetic polymorphisms may account for why some people are more sensitive than others to environmental carcinogens such as exogenous estrogens and alcohol. A large number of genetic variants have been reported to be associated with breast cancer risk but relatively few low-penetrance polymorphisms have been consistently associated with the disease [67]. Most breast cancer susceptibility loci identified in candidate gene studies have not been confirmed [63]. Single

nucleotide polymorphisms (SNPs) of the *XRCC2* and *XRCC3* genes, which code for proteins that play a role in the homologous recombination of DNA double strand breaks, have been shown to influence breast cancer risk. These include *XRCC2* rs3218536 and rs3218536 [67–69]. A variant of the caspase 8 gene (*CASP8*) has been convincingly associated with breast cancer risk [63]. Caspase 8 is a protease that is involved in the initiation of programmed cell death (apoptosis) following DNA damage [70].

2.4.3 BRCA Gene Mutations

Mutations in the *BRCA1 gene*, which is located on chromosome 17q, have been identified as causes of predisposition to breast, ovarian, and other cancers. The *BRCA2* gene is located on chromosome 13q. BRCA1 and BRCA2 are expressed in breast, ovarian, and other tissues and play a key role in the repair of double-stranded DNA breaks in the cell nucleus. *BRCA1* and *BRCA2* mutations account for about 15–20 % of familial breast cancers [71]. Women who carry *BRCA1* and *BRCA2* mutations have an estimated 40 % to 87 % risk of breast cancer by age 70, although these risks are modified by other factors [72, 73]. There is considerable variability in the age of onset of cancer and the site of cancer across populations [74]. Most of the deleterious mutations in the *BRCA1* and *BRCA2* genes are small deletions or insertions that result in the translation of a truncated protein [63].

Genetic variants and gene–gene interactions that account for inter-individual variation in DNA repair capacity influence risk of breast cancer [74]. These include variants in the *CHEK2*, *PALB2*, and *ATM* genes, which, like *BRCA1* and *BRCA2*, play a role in DNA repair mechanisms and help to maintain chromosomal stability [63]. Studies have suggested that genomic variation at multiple loci modify breast cancer risk in women who carry *BRCA1* mutations [75]. Some of these loci are known to encode proteins that interact biologically with *BRCA1* [63]. Candidate gene studies suggest that homozygosity for the *RAD51* 135G > C allele is associated with breast cancer risk in women who carry *BRCA2* gene mutations [76]. Interacting with *BRCA1*, *BRCA2*, and *ATM* at the cellular level, *RAD51* is part of a protein complex that plays a role in the repair of double strand DNA breaks. Genome-wide association studies carried out in general populations have identified additional genetic variants that are associated with breast cancer risk among *BRCA1* and *BRCA2* mutation carriers.

Other high-penetrance genetic mutations that increase breast cancer risk, and which are rare in the general population, include *TP53* germ-line mutations (found in Li-Fraumeni cancer syndrome), *PTEN* mutations (Cowden syndrome), and *STK1* mutations (Peutz-Jegher syndrome) [63].

2.5 Summary and Conclusions

This chapter has summarized the substantial epidemiologic literature on environmental and genetic risk factors for breast cancer in women. Breast cancer risk factors that have been well-established by epidemiologic studies include race, ethnicity, family history of cancer, and genetic variants, as well as modifiable exposures such as increased alcohol consumption, physical inactivity, exogenous hormones, and certain female reproductive factors such as younger age at menarche, nulliparity, and older age at first full-term pregnancy. There is increasing evidence that breast cancer is a heterogeneous disease and that subtypes such as triple negative breast cancers may have a distinct etiology. Epidemiologic studies, family studies, and genome-wide association studies have identified several genetic variants and rare but moderate-to-high penetrance gene mutations that account for some cases of breast cancer. These include genetic variants of genes involved in DNA repair and the homologous recombination of DNA double-stranded breaks. However, the etiology of many breast cancer cases in the population remains unknown.

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Chapter 3
The Complexities of Racial Disparity in Breast Cancer

Joy Tang, Aamir Ahmad and Fazlul H. Sarkar

Abstract Breast cancer is one of the most common types of cancers as well as a leading cause of cancer-related deaths in women in the United States. Although there has been a recent decline in breast cancer mortality, certain ethnic groups continue to suffer from higher mortality rates. The causes of racial disparities in breast cancer patients are still unclear, but understanding the molecular mechanism(s) and associated factors that may contribute to racial disparity will help in improving the treatment outcome of patients in such minority groups in the future. The disparity in breast cancer statistics between African American (AA) women and European American (EA) women has particularly been a topic of much discussion and investigation. Previous studies have focused on breast cancer mortality rates, but more recent studies are addressing the racial and ethnic disparities specific to breast cancer. In the future, a deeper understanding of the racial disparities in breast cancer will lead to improved cancer preventative care, diagnosis and treatment. Here, we summarize the social factors that are known to contribute to racial disparity in breast cancer.

Keywords Breast cancer mortality rates · European American women · African American women · Racial disparity · Social factors · Attitudes · Tobacco control · Family history · Poverty · Racial discrimination · Social discrimination · Household income · Racial influence

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3.1 Introduction

Breast cancer is the most common malignant disease among women and one of the leading causes of cancer-related deaths among women in the United States. As many as 1 in 8 women will develop breast cancer during the course of her lifetime [1]. Although screening with mammograms has improved the fatality of breast cancer if detected at earlier stages, there is still increasing disparity between several factors in breast cancer patients [2]. Various studies have examined potential influences, such as diagnosis, histology, and race, which have all affected mortality rates among population groups [1]. Though it is still unclear as to why there is racial disparity among different ethnic groups, recent research has found new patterns and developed deeper understandings to variant causes of the disparities. For example: Are African Americans (AA) at a greater risk for breast cancer mortality than European American (EA) women? Many aspects, such as social, economic and cultural barriers, also contribute to the racial disparities. Barriers to health care resources also contribute to the widening mortality rate gap between EA and the ethnic minority groups, especially AA women with breast cancer. The purpose of this chapter is to investigate the underlying causes of racial disparity in breast cancer patients, further discussing the results and preventative measures that could be incorporated in the future for the management of patients toward improving their survival outcome, focusing on the elimination of racial disparity. In this chapter, we will restrict our discussion on the social and behavioral aspect and exclude the genetic and associated molecular mechanism of racial disparity among the different racial groups, which should be an interesting topic for future discussion.

3.2 Family History

Diagnosing cancer as early as possible is essential for the first steps in treating a patient. Thus, understanding racial disparities in cancer screening by family history risk could be critical in proactively diagnosing breast cancer. Patients who have a family history of breast cancer are at a greater risk of developing it in comparison to patients who have no family history [3, 4]. Screening rates for breast cancer are higher for the 8 % of the population who report a family history than for the regular, average-risk individuals [4]. Although the average individual understands the importance of screening for cancer if they have a family history risk, less information is known in regards to racial disparities in screening for cancer. Investigating patients who are at a higher risk for breast cancer could provide critical information for patients and thereby allow the healthcare provider time for interventions specific to racial groups in order to help reduce cancer mortality in such minority populations.

Furthermore, the importance of investigating family history is important for many ethnic minorities in order to develop appropriate preventative strategies by

changing the behavior of the subject and adopting an appropriate management plan in consultation with the attending physician. Recent evidence suggests that many ethnic minorities underestimate their cancer risk and are less likely to recognize family history as a potential risk factor [4, 5]. Moreover, research indicates that many ethnic minorities, in comparison to their EA subject counterparts, are less likely to discuss cancer-related issues because it is a cultural stigma [6]. Understanding racial disparities in individuals with a family history would therefore help to educate ethnic minorities about cancer screening.

By evaluating the 2005 California Health Interview Survey, a diverse set of ethnicities was analyzed, demonstrating how family history affects screening behavior. Results have shown that there were no significant racial or economic disparities in mammography use among women with a family history risk [4]; however, further studies should examine racial groups in other states to support the data obtained from California.

3.3 Black–White Disparities

3.3.1 AA Suffer from Higher Breast Cancer Mortality Rates

Although the overall mortality rate from breast cancer has decreased since the 1980s, there is still a growing disparity between black and white women. Despite higher rates of prevalence among EA women, AA women face a significantly higher mortality rate due to breast cancer [7, 8]. A recent study done in Chicago showed that compared to AA, EA mortality rates have been consistently declining, but the rate has remained steady for AA, which has led to an increased disparity over time [9]. Furthermore, AA women are also disadvantaged in regards to early detection of the cancer.

The Medical College of Georgia Tumor Registry studied the overall survival rate of AA and EA patients after breast cancer diagnosis and analyzed various factors that may affect the disparity in mortality rate. 1,178 women with breast cancer were examined, of which 41.5 % were AA and 56.9 % were EA; the remaining samples were disregarded due to the small percentage of other minority groups [2]. Their research shows that AA women were more likely to be younger at diagnosis, have later-stage disease upon diagnosis, and were less susceptible to hormonal therapy [2, 10]. On average, the EA women survived for 8.8 months longer after treatment than the AA women and had a 5 year survival rate increased by 8.8 % [1]. This data demonstrates that AA women overall experience lower survival after being diagnosed with breast cancer in comparison to EA woman.

More studies have also suggested that AA women struggle more for survival after being diagnosed with breast cancer in part due to socioeconomic reasons. However, in a study representing only underinsured AA and EA patients at the Wishard Memorial Hospital, the results showed that AA women still had worse

overall survival rates after diagnosed with breast cancer in comparison to the EA women [11, 12]. Even though both racial groups had equal socioeconomic status and had equal access to the same health care system, the AA women faced higher mortality rates. These results suggest that even though socioeconomic factors may influence racial disparity in terms of early diagnosis and preventative measures, they do not affect the survival rate of those who have already been diagnosed with cancer [12]. In this study, both the AA women and the EA women were unlikely to have access to mammography, so they were in equal social positions. The time from diagnosis to the breast cancer surgery was similar for both ethnic groups, but AA women were still more likely to face advanced breast cancer.

3.3.2 Many Barriers Lead to Health Disparities

3.3.2.1 Poverty

Poverty is a primary contributing factor to health disparities and associated with worse breast cancer outcomes to all racial groups in the United States [13, 14]. Individuals at a lower socioeconomic status are associated with decreased rates of cancer screening, increased probability for advanced stage at diagnosis, and face higher mortality rates from breast cancer [15, 16]. Those dealing with poverty typically lack access to a primary care physician and do not live in geographical areas that allow easy access to primary care clinics [17]. Lower income individuals generally have inadequate health insurance, which increases their chances of being diagnosed with advanced stage breast cancer, and they usually lack the knowledge about breast cancer and how to be proactive to implement preventive strategies. Recent findings suggest that AA women are less likely to schedule follow-up appointments than EA women, which leads to delays in diagnosis [18, 19]. However, it has been observed that delayed diagnosis of breast cancer of only 3 months is associated with lower survival in comparison to a prompt follow-up appointment [20]. Thus, it is important to improve cancer screening efforts for younger AA women [21]. Furthermore, cancer preventative measures are not a high priority for those who feel daily pressure to meet other survival needs, such as obtaining food, shelter, and security. More AA women than EA women are paid based on an hourly rate, which means that taking time off from work will result in lesser earnings [16]. The economically disadvantaged minority groups struggle with other priorities that are more important than the risk of being diagnosed with breast and other cancers [22, 23].

3.3.2.2 Cultural Issues

There is an increasing amount of research that points towards cultural factors that also contribute to racial disparities. Some cultural aspects include spirituality,

perceived susceptibility to breast cancer, and cultural beliefs and attitudes. Spiritual practices often encourage groups, such as the AA community, to believe that they can restore health through religious ceremonies [16]. Rather than seeking treatment in a hospital or medical facility, the spiritual will rather put their faith in God. In other cases, women may believe that they are not vulnerable to breast cancer or have some cultural fear about breast cancer or screening practices [16]. Women may feel embarrassed, pain, or even fear when approached with a mammography procedure. In some communities, there is even a general mistrust on the health care system and many women will instead place their faith in spiritual guidance or home remedies [16]. Cultural influences typically will affect an individual's decision for breast cancer screening and diagnosis, which is essential for early intervention with preventive care.

3.3.2.3 Racial/Social Discrimination

Racial discrimination and other forms of social injustice are another foundation that link to disparities in health. Results from the Black Women's Health Study suggest that there is a connection between racial discrimination and prevalence of breast cancer [24]. Women who reported dealing with increased racial discrimination on a daily basis were at a greater risk for developing breast cancer, which suggests that such discrimination may actually promote negative health consequences. Furthermore, racial discrimination from physicians may also encourage ethnic minorities not to proactively seek breast cancer screening. The quality of care that a physician provides is often affected by the physician's perceptions of his or her patient's race and socioeconomic status [14]. A recent study found that physicians rated AA patients with coronary artery disease as less educated, more likely to abuse drugs and alcohol, and less likely to comply with cardiac rehabilitation than the EA patients [25]. The negative patient perceptions may result in lower quality of patient care, but further research is needed to support whether racial prejudice affects racial disparities in breast cancer mortality.

3.4 Examining Underlying Causes

3.4.1 City-Level Analysis

Although there is much discussion on racial disparity in breast cancer mortality, there is relatively little analysis at the city-level. Thus, the Metropolitan Chicago Breast Cancer Task Force was established, comprising of 100 individuals and 74 organizations, with the aim to approach racial disparity through a multifaceted approach [26]. A city-level understanding of the phenomenon could offer comprehension to breast cancer issues at a local level and even offer insight on socioeconomic influences. By examining the 25 largest cities in the United States

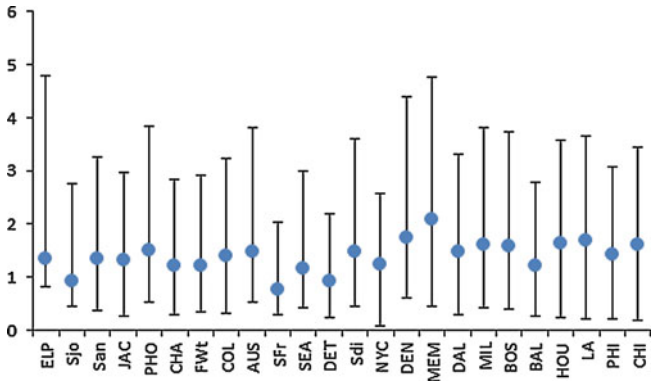

Fig. 3.1 The graph shows the comparative disparity rate ratio between NHB and NHW women for 24 of the largest cities in the United States, from 2005 to 2007, in accordance to the index of disparity, which is arranged in increasing disparity, from least to greatest as documented in a recent publication [9]

and comparing the non-Hispanic Black (NHB) women and non-Hispanic White (NHW) women for breast cancer mortality rates, several very interesting patterns were found.

The study examined the breast cancer mortality rate by comparing the NHB:NHW ratio in order to closely analyze the relationship in each city. A ratio of 1.00 indicates that there is no disparity between the black women and white women, while any ratio above 1.00 indicates that the NHB rate is higher than the NHW rate and a ratio below 1.00 indicates that the NHB rate is lower than the NHW rate [9]. Results showed that between the years of 2005 and 2007, out of the 25 largest cities in the United States, only three cities indicated a rate ratio less than 1.00, and none were significantly lower than 1.00 [9] (Fig. 3.1 and Table 3.1).

Based on research done by Whitman et al., two main variables—the median household income and the Index of Dissimilarity, which is a measure of segregation,—appeared to relate most significantly to the disparity rate ratio. The median household income was found to be lowest for Detroit ($29,100), highest for San Jose ($76,400), and then second-highest for San Francisco ($65,500), of which three cities also had the three lowest rate ratios, all less than 1.00 [9]. Though poverty is often a large contributor in poor health and may contribute to racial disparities, there is not enough evidence to make a conclusive statement. The Index of Disparity indicates the fraction of black patients who would have to move to another census tract in order to perfectly integrate with the white patients, or vice versa [9]. The Index of Disparity is smaller in certain cities, such as El Paso, because there are fewer white and black populations living there. Segregation of ethnic groups has been linked to poor health in ethnic minority groups, and thus contributes to disparities in health [9, 27].

Another observation was that other influences may have affected the research findings, notably that some breast cancers in black women may be more aggressive

Table 3.1 Whitman et al. studied estimates from 2005 to 2007 for breast cancer mortality disparity between NHB and NHW women from the 25 largest cities in the United States [9]. The rates are expressed per 100,000 females based on the US 2000 standard population data

City, State	NHB rate	NHW rate
New York City, NY	31.2	25.2
Los Angeles, CA	46.5	27.4
Chicago, IL	37.8	23.4
Houston, TX	47.3	28.7
Philadelphia, PA	35.8	25.1
Phoenix, AZ	32.9	22.0
San Antonio, TX	36.8	27.0
San Diego, CA	36.7	24.7
Dallas, TX	37.5	25.3
San Jose, CA	27.2	28.9
Detroit, MI	35.2	37.3
Indianapolis, IN	–	–
Jacksonville, FL	37.1	28.1
San Francisco, CA	19.6	25.2
Columbus, OH	36.6	26.1
Austin, TX	33.1	22.2
Memphis, TN	44.6	21.3
Baltimore, MD	31.6	25.7
Fort Worth, TX	29.8	24.6
Charlotte, NC	32.3	26.3
El Paso, TX	24.9	18.4
Milwaukee, WI	29.6	18.4
Seattle, WA	30.0	25.9
Boston, MA	34.6	21.7
Denver, CO	30.8	17.7

and result in a lower survival rate, regardless of location [28–30]. However, the low rate ratios in cities, such as Baltimore and New York, suggest that even if biological differences in cancer aggressiveness are present, it would still not account for the city differences [9]. Further studies in this area could help in improving local patient care and long-term health status, particularly for those who are likely to be diagnosed with breast cancer.

3.4.2 Racial Influences

According to past research, AA women have a higher mortality rate from breast cancer than any other major ethnic group [1]. Although it is still not clear as to the specific causes of the racial disparity, one aspect to consider is the advanced stage of breast cancer at the time of diagnosis, which is more common in AA patients [1]. Many studies have indicated that early detection is one of the best ways to improve

prognosis for patients diagnosed with breast [28, 31]. Many minority groups experience longer time intervals for diagnostic testing and also are less likely to abide by follow-up screening examinations. Further research indicates that there is also racial disparity in the time between a first abnormal breast examination and the result of the final status. The AA patients are significantly less likely to follow-up on a mammographic workup than EA patients after initial abnormal diagnosis [32]. The delayed response contributes to the higher mortality rate for AA patients. Moreover, due to the advanced stage, the cancer in the AA women is more likely to present with a larger tumor size and higher grade, which are well known factors that contribute to overall poor survival [1, 33].

Genetics also affects the susceptibility of patients to breast cancer and its treatments. Breast cancers would be typically treated with anti-estrogen therapy; however, AA patients, along with certain other minority groups, are more likely to have the estrogen receptor-negative (ER−) disease, which makes the therapy less effective [1]. Case control studies show that for subjects diagnosed between 1990 and 2002, breast cancer mortality rates decreased over time for patients with estrogen receptor-positive (ER+) tumors than in patients with estrogen receptor-negative tumors [34]. With fewer appropriate therapeutic options of treatment, AA patients typically face with increased risk of developing advanced stages of disease, which contributes to overall poor survival.

3.4.3 Tobacco Control

Cancer control is associated with two main components—reducing tobacco use and systematic screening coupled with timely treatment [35]. Tobacco smoking is known to contribute to at least 15 types of cancer [36]. Eliminating tobacco smoking would help decrease the development of breast cancer. Studies show that between the early 1990s to 2003, the overall cancer death rates between the AA and the EA was reduced, which was attributed to decreased tobacco use [35]. However, EA women were also shown to more likely receive appropriate treatment for breast cancer due to socioeconomic status [35]. The AA women are more affected by poverty, and thus have less access to cancer screening procedures and treatments.

3.5 Improving Health Disparities

3.5.1 Using Community-Based Participatory Research

Although health disparities have been studied throughout the past few decades, means to ameliorate racial disparities have mostly been ineffective [37]. Recent evidence, however, supports a new method in reducing the racial disparity in

health care. Community-engaged research has improved the survival rates of racial minority groups and has the potential to help reduce many health-related disparities [38]. The method engages a collaborative effort between researchers and community members to better understand health problems in a local setting. By pairing scientific investigators with community voices, researchers were able to improve project aims and formulate new conclusions [37]. Community-engaged research has the potential to reduce racial disparities in screening, incidence, mortality, survivorship, and treatment of breast cancer by developing ideas that are culturally specific to ethnic minority groups.

3.5.2 Using Simulation Models to Eliminate Racial Disparities

Statistically, there is a general pattern of poorer health, including mortality from breast cancer, which exists for racial minority groups. Poverty and lack of health insurance both affect the type of care that patients will receive. Though many reasons have been suggested for the increasing racial disparities in health care, one causative factor is the disparities in the *quality* of health care, which refer to the differences in levels of quality provided to the patients [39]. Research from the Commonwealth Fund and the Health Resources and Services Administration (HRSA) suggests that a patient's ethnicity can be associated with the quality of health care plan [39].

A simulation model was created to establish a plan to eliminate health care disparities. The method suggests that from a business aspect, employers should focus on ethnic disparities in health care. The idea is that purchasers can play an active role in improving the disparities in health care by establishing health care plans based on a variety of regular reports that analyze the purchaser-supplier relationship in order to improve clinical practice [39]. The model can be used to predict the benefits to both employers and health care members by analyzing the medical care costs paid by employers and the effects of absenteeism and productivity after improving known racial disparities in mammography screening [39]. One simulation model suggests that there are financial benefits for both parties in eliminating the disparity in asthma medications and mammography rates [39]. However, it is clear that further comprehensive approach in research is required to understand the complexities of racial disparity of breast cancer among the different racial groups.

3.6 Conclusions

The causes of racial disparities in breast cancer are very complex and still unclear, which suggests that further in-depth research is warranted in order to eliminate racial disparity of breast cancer. Although many studies suggest that certain ethnic

groups are at higher risk for breast cancer mortality, the molecular mechanism for such a disparity is unknown and was not the subject of our discussion in this chapter, which focused mainly on social aspects. Emerging evidence suggests that racial disparities in cancer-related deaths are often closely linked to socioeconomic factors that could hinder individuals from access to cancer prevention, early detection, and access to high quality care. Together these factors hinder quality patient care, and thus improvement in this area will certainly help to reduce breast cancer mortality. Most studies have emphasized the importance of early detection and preventative measures to reduce breast cancer mortality rates in any racial group. Recommendations for improving breast cancer care include: providing better primary prevention care and addressing socioeconomic barriers, such as poor access to health care or inadequate health insurance. Patient education is also an important aspect, and thus encouraging women to schedule regular cancer screening procedures and attending appropriate follow-up visits would be the key for reducing the existing racial disparity of breast cancer. Further studies could also be conducted on related areas of interest to support current findings. Although much research has been focused on EA and AA racial disparities in breast cancer, there still lacks sufficient information on other ethnic minority groups. Moreover, there are other variables related to racial disparities that must be investigated for making conclusive arguments, such as analyzing disparities at the state level or by metropolitan or rural areas as well as the underlying molecular mechanism of racial disparity. Overall, it appears that social, cultural, and economic factors play important roles in racial disparities in breast cancer in the United States. Thus targeted elimination of these weaknesses should become the highest priority toward reducing racial disparities in breast cancer patients, especially because genetic and molecular mechanism associated with racial disparities would be harder to control, which would be important for further discussion in the future.

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Chapter 4
Major Signaling Pathways Involved in Breast Cancer

Saba Wasim Aziz and Moammir Hasan Aziz

Abstract Breast cancer is the leading cause of cancer-related mortality among women worldwide. Significant advancement has been made recently in delineating the cellular processes and signaling pathways involved in breast cancer. Cross-communication between different pathways allows cells to identify and respond appropriately to the extracellular environment. Cancer development is a gradual and complex process resulting from any disruption in these pathways that ultimately generates signals defining the required biological response. The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases represents both key regulators of normal cellular development as well as critical players in the development of a variety of cancers including breast cancer. The aim of this book chapter is to give a broad overview of signal transduction networks such as Ras/Raf/MEK/ERK and the PI3K/AKT pathways that are controlled by the EGFR superfamily of receptors. The elucidation of these signaling pathways will further provide new insights in understanding the pathogenesis of breast cancer and targeting these pathways to combat against breast cancer development, progression, and metastasis.

Keywords Breast cancer treatment · Cross-communication · Epidermal growth factor receptor (EGFR) · Signaling pathways · Human epidermal growth factor receptor-2 (HER-2) · Ras signaling pathway · Class I phosphoinositide 3-kinase (PI3Ks) · Class II phosphoinositide 3-kinase (PI3Ks) · Class III phosphoinositide

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3-kinase (PI3Ks) · Receptor tyrosine kinases (RTKs) · Phosphatase and tensin homolog PTEN · Protein kinase B (AKT)

Abbreviations

AKT	Protein kinase B
AREG	Hereregulins
BTC	Betacellulin
CREB	CAMP response element-binding
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPI	Epiregulin
EPG	Epigen
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Kinase
Gata-1	Globin transcription factor 1
GPCRS	G protein coupled receptors
HB-EGF	Heparin-binding EGF-like growth factor
HER-1/2/3/4:	Human Epidermal Growth Factor Receptor-1/2/3/4
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
mTOR	Mammalian target of rapamycin
NRG	Neuregulin
PKC	Protein Kinase C
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RTKs	Receptor Tyrosine Kinases
TGF-α	Transforming Growth Factor alpha

4.1 Introduction

Breast cancer is the leading cause of cancer-related mortality among women worldwide. In United States, the incidence of breast cancer in women was reported to be 230,000 with approximately 40,000 breast cancer related deaths in 2011 alone, according to the American Cancer Society [1]. Significant advancement has been made recently in delineating the cellular processes and signaling pathways involved in breast cancer. Cross-communication between different pathways allows cells to identify and respond appropriately to the extracellular environment. Cancer development is a gradual and complex process resulting from any disruption in these pathways. Several integrated signaling pathways are involved in breast cancer as they subsequently impact cellular responses such as cell survival, proliferation, migration, differentiation, and apoptosis [2–8]. In this chapter, we

have focused on Epidermal Growth Factor Receptor (EGFR) and two major signaling pathways regulated by EGFR, the Ras/Raf/MEK/ERK and the PI3K/AKT pathways, which play significant role in breast cancer development. The knowledge of these signaling pathways further helps in understanding the pathogenesis of breast cancer and targeting these pathways to combat against breast cancer development, progression, and metastasis.

4.2 Epidermal Growth Factor Receptor Family

The epidermal growth factor receptor (EGFR), a 170 kDa glycoprotein, is a transmembrane receptor tyrosine kinase of the ErbB family that is abnormally activated in several tumors including breast cancer [9]. The ErbB family consists of four related receptors which share considerable sequence homology to each other: the epidermal growth factor receptor (EGFR/HER-1/ErbB-1), HER-2 (ErbB2/c-neu), HER-3 (ErbB3), and HER-4 (ErbB-4) [10–18]. These receptors are made of three major functional domains: two cysteine-rich extracellular domains, which are critical for ligand binding region, a hydrophobic transmembrane domain, and a cytoplasmic tyrosine-kinase-containing domain. The extracellular ligand-binding domain is the N-terminus of ErbB receptors which binds a variety of ligands [19, 20]. The ligands of ErbB family receptors can be divided into three groups based on their affinities for various receptors: group 1 consists of the epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), and amphiregulin which bind to EGFR [20–23], group 2 consists of betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), and epiregulin (EPI) which can interact with both EGFR and HER-4 [22, 24–26] and group 3 consists of tomoregulins, hereregulins (AREG), epigen (EPG), and neuregulins (NRG-1, NRG-2, NRG-3, NRG-4) which bind to HER-4, NRG-1 and NRG-2 also bind to HER-3 [27, 28]. There is no known ligand for HER-2, but it is the preferred hetero-dimerization partner for other members of the ErbB receptor family [19] (Table 4.1).

The ligand binding to the extracellular domain activates EGFR which subsequently mediated by either homo-dimerization or hetro-dimerization with other family members, undergoes autophosphorylation at the tyrosine kinase domain leading to activation of EGFR regulated several downstream signaling pathways including components of the Ras/Raf/MAPK/ERK, the PI3K/AKT, signal transducer and activator of transcription (STAT), and protein kinase C pathways [3, 29–35]. Aberrant regulation of EGFR is often observed in association with cell proliferation, differentiation, apoptosis, invasion, and angiogenesis eventually leading to carcinogenesis [28, 36–38]. Thus, in depth understanding of various EGFR-mediated signaling pathways and their dysregulation in breast cancer will provide better and more effective treatment strategies for breast tumors.

Table 4.1 ErbB family and their ligands

Receptors	Ligands
EGFR(ErbB/HER1)	Epidermal growth factor (EGF), transforming growth factors-α (TGF-α), epiregulin (EP), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF)
HER-2 (ErbB2/c-Neu)	Unknown
HER-3 (ErbB-3)	Neuregulin (NGR-1)/heregulin(HRG) isoforms, NRG-2α and β
HER-4 (ErbB-4)	NRG-1/HRG isoforms, NRG-2α and β, NRG-3, NRG-4, tomoregulinHB-EGF, BTC, EP

4.2.1 Human Epidermal Growth Factor Receptor-2 (HER-2) and Breast Cancer

Studies suggest that as many as about 1 in 4 of the more than 180,000 breast cancers diagnosed in the US each year are labeled HER-2-positive [39–41]. In recent years it has evolved to become an important biomarker and target of therapy for breast cancer [42–45]. HER-2, also known as c- ErbB2/c-neu, is a transmembrane glycoprotein receptor that appears on the surface of some breast cancer cells [16, 40, 45]. This protein is key molecule in the regulation of cell growth, apoptosis and survival in breast cancer [46–48]. About 25–30 % of breast cancers produce an excess amount of the HER-2/neu protein, which makes the cancer more aggressive [18, 49, 50]. Recent studies have shown that women with HER-2 positive breast cancer have more aggressive cancer, which spreads more readily, and is less responsive to hormonal therapy and chemotherapy [51]. These cancers have also greater likelihood of recurrence, poorer prognosis, and decreased survival compared to women with HER-2-negative breast cancer [51].

Studies suggest that genetic alteration in the HER-2 gene is one of the reasons that produces an increased amount of the growth factor receptor protein on the tumor cell surface [52–55]. HER-2 has no known ligand, and therefore relies for activation on heterodimerization with other HER receptors, or homodimerization with itself when it is expressed at very high levels on the cell surface [56–58]. The overexpression of HER-2 is associated with overactive HER-2 dimerization, abnormal signaling (such as proliferation, survival, differentiation, angiogenesis, invasion, and metastasis) and ultimately tumor growth [56–58]. The paired receptor molecules phosphorylate one another on tyrosine residues on their intracellular domains. Growth and survival signals stimulated by activated HER-2 are largely mediated via PI3K/Akt [59–62] and Ras/MAPK signaling [63]. Preclinical studies in HER-2 positive breast cancer have also demonstrated promising antitumor efficacy with associated downregulation of Signal transducer and activator of transcription (STAT) signaling pathways [64, 65]. Induction of HER-2 leads to up-regulation of anti-apoptotic proteins survivin and Bcl-2 in breast cancer cells [66–68]. In addition, HER-2 regulates survivin in part through PI3K-dependent effects on serum-and glucocorticoid-induced kinases (SGK) and/

or phospholipase γ [66, 67, 69, 70]. Targeting HER-2 signaling pathways will help to treat the patients with tumors that are dependent on HER-2 induced signaling pathways for their survival. HER-2 status has been shown to be predictive for response to HER-2 targeted therapies tratuzumab {Herceptin}, pertuzumab, and lapatinib (Tykerb), a small molecule oral tyrosine kinase inhibitor directed specifically to the HER-2 receptor [39, 43, 46, 48, 56, 59, 60, 71–73].

4.3 EGFR and Ras Signaling Pathway in Breast Cancer

Target-based therapies are widely considered to be the future of cancer treatment. Recently much attention has been focused on targeting the Ras/Raf/MEK/ERK signaling pathway and its upstream activators in breast cancer [74–77]. Evidences suggest that this pathway is aberrantly activated in breast cancer with overwhelming frequency, particularly by upstream activation of EGFR [78, 79]. The Ras/Raf/MEK/ERK signaling pathway consist of a kinase cascade that is regulated by phosphorylation and de-phosphorylation by specific kinases, phosphatases as well as GTP/GDP exchange proteins, adaptor proteins and scaffolding proteins [80]. Both Ras and Raf members belong to multiple gene families and there are three human *ras* genes (*Ha-*, *N-*, and *Ki-Ras*) and three Raf members (B-Raf, Raf-1/c-Raf, and A-Raf) [81, 82]. Raf phosphorylates serine/threonine (S/T) residues of mitogen activated protein kinase kinase-1 (MEK1), which in turn phosphorylates ERK1 and 2 at specific T and Y residues. Activated ERK1/2 kinases further phosphorylate and activate a variety of substrates. As the number of ERK1/2 targets is easily in the hundreds (>600), suppression of MEK and ERK activities has profound effects on cell growth [81–86]. Activated ERK can also phosphorylate B-Raf, Raf-1, and MEK1, which alters their activity. Depending upon the site phosphorylated on Raf-1, ERK phosphorylation can either enhance or inhibit Raf-1 activity. In contrast, when B-Raf or MEK1 are phosphorylated by ERK, their activity decreases [87–90] (Fig. 4.1).

Activated ERK can translocate to the nucleus and phosphorylate additional transcription factors, such as Elk-1, CREB, Fos and globin transcription factor 1 (Gata-1) [85–92] that bind to promoters of many genes, including growth factor and cytokine genes which play important role in promoting growth and preventing apoptosis of multiple cell types. Aberrant regulation of this pathway can contribute to abnormal cellular proliferation and differentiation leading to cancer [93, 94].

Many of the effects of the Ras/Raf-1/MEK/ERK pathway on apoptosis are mediated by ERK phosphorylation of key apoptotic effector molecules (e.g., Bcl-2, Bad, Bim, CREB, Foxo, and Caspase-9 [95–99]. In addition, it also regulates the translation of weak mRNAs such as Mcl-1 involved in regulation of apoptosis. [97, 100–102]. Aberrant regulation of apoptosis is critically implicated in breast cancer and the activity of many key components in apoptotic cascades is sensitive to inhibitors that target this pathway [103].

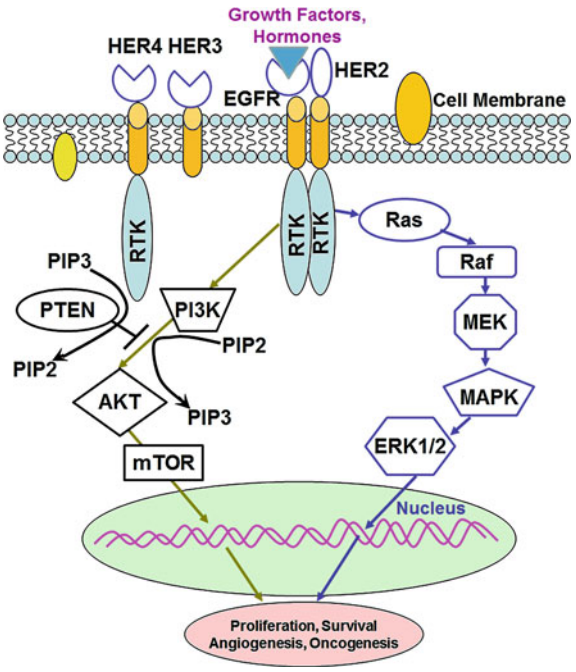

Fig. 4.1 Schematic of EGFR signaling pathway in breast cancer. Activation of EGFR initiates receptor dimerization and subsequently activates Ras/Raf/MEK/MAPK/ERK and PI3K/AKT pathways, two important survival pathways in breast cancer

The Ras/Raf/MEK/ERK pathway can also be activated by mutations/amplifications of upstream growth factors receptors. The aberrant overexpression or mutational activation of receptor tyrosine kinases (e.g., EGFR and HER2) can cause hyperactivation of Ras, which in turn, mobilizes the Raf/MEK/ERK cascades. In particular, the EGFR is overexpressed in 20–81 % of breast cancer [104, 105]. Overexpression and constitutive activation of EGFR in cancers often predicts poor prognosis. Recent reports have also suggested that EGFR levels may be elevated in the blood within 17 months prior to the diagnosis of breast cancer [105]. An important cause of sporadic breast cancer is overexpression of HER2, which occurs in approximately 30 % of breast cancer, leading to increased expression of Ras/Raf/MEK/ERK pathway [106]. Ras serves as a mediator between extracellular ligand binding and intracellular transduction of signals from the EGFR to the nucleus. HER2 causes transient activation of Ras, which in turn, associates with and activates multiple downstream effectors stimulating cytoplasmic signaling cascades that regulate cell proliferation, survival, and differentiation. In addition, a truncated constitutively-active EGF receptor, which lacks 267 amino acids in the receptor's extracellular domain, has been reported in breast cancer and the c-FMS/colony stimulating factor-1 receptor, which also signals through Ras, is expressed in around 15 % of breast cancers but not in normal breast tissue [107–109].

Another linkage between EGFR and Ras is mediated by the upregulation of expression of EGFR ligands by Ras signaling [79]. One important gene target of Ras activation involves transcriptional activation of the gene for transforming

growth factor alpha (TGFα), a ligand for the EGFR. Increased TGFα gene expression and secretion of TGFα and other EGFR ligands. (e.g., heparin-binding EGF, amphiregulin) has been observed in a wide variety of Ras- or Raf-transformed cell types [79]. The importance of this autocrine signaling loop for Ras transformation has been demonstrated by the ability of inhibitors of EGFR to block oncogenic Ras transformation. Additionally, the majority of Raf-induced changes in gene expression were found to be dependent on EGFR function. Hence, the EGFR can function both upstream, as well as downstream of Ras/MEK/ERK cascade [110–113].

The regularity with which this signaling cascade is activated in breast cancer suggests that it is critical in oncogenesis and makes it an appealing pathway for drug development. The considerable genetic and experimental observations provide strong support that inhibitors of the EGFR and Ras/MEK/ERK cascade will provide effective antineoplastic agents for the treatment of breast cancer. Many inhibitors of EGFR, Ras, Raf and MEK have been developed that target different components of ERK signaling, with some agents already approved as anticancer agents [79, 114, 115] (Fig. 4.1).

4.4 EGFR and PI3K/AKT Pathway in Breast Cancer

Another important signal transduction pathway regulated by EGFR is the PI3K/AKT pathways, which is reported to be one of the most commonly misregulated signaling pathways in many human cancers including breast cancer [4, 59, 75]. Since its discovery, PI3Ks has been found to play key roles in regulation of many cellular processes critical for cancer progression, including metabolism, cell survival, proliferation, differentiation and motility [116–121]. The primary biochemical function of PI3Ks is phosphorylation of the 3-hydroxyl group of phosphoinositides. PI3Ks are activated by RTKs and G protein coupled receptors (GPCRs), transducing signals from various growth factors and cytokines into intracellular messages by generating phospholipids. This leads to activation of AKT, the serine/threonine kinase and other downstream effector pathways [122].

PI3Ks, the family of lipid kinases, have been classified into three classes, based on their structural characteristics and substrate specificity [123, 124]. Of these, the most commonly studied are the class I enzymes that are activated directly by cell surface receptors.

4.4.1 Class I PI3Ks

Class I PI3Ks are further divided into class IA enzymes, activated by RTKs, GPCRs and certain oncogenes such as the small G protein Ras, and class IB enzymes, regulated exclusively by GPCRs.

Class IA PI3Ks, the most widely implicated class in human cancer, are heterodimers consisting of catalytic subunit (p110α, p110β, p110δ) and a regulatory subunit (p85α, p55α, p50α, p85β, p55γ). The regulatory subunit mediates receptor binding, activation, and localization of the enzyme. In humans, p85α (and its splicing variants p55α and p50α), p85β, and p55γ regulatory subunits, collectively called, p85 are encoded by the three genes, *PIK3R1*, *PIK3R2*, and *PIK3R3* respectively [125, 126].

Following activation by RTKs, the p85 subunit of PI3K interacts with tyrosine phosphate motifs on activated receptors directly (e.g. PDGFR) or to adaptor proteins associated with the receptors (e.g. insulin receptor substrate 1, IRS1) leading to recruitment of PI3K to the membrane. Binding removes the inhibitory effect of p85 on p110, resulting in full activation of PI3K and subsequently of multiple downstream signaling pathways regulating diverse cellular functions including cellular metabolism, proliferation, differentiation and survival.

Class IB PI3K is a heterodimer consisting of a catalytic subunit p110γ and a regulatory subunit p101. In addition, two new regulatory subunits, have been recently described which are p84 and p87PIKAP. p110γ is activated directly by GPCRs through interaction of its regulatory subunit with the Gβγ subunit of trimeric G proteins [124, 127].

4.4.2 Class II and III PI3Ks

Class II PI3Ks consist of a single catalytic subunit with three isoforms (PI3KC2α, PI3KC2β and PI3KC2γ) but no regulatory proteins. Class II PI3Ks are activated by RTKs, cytokine receptors, and integrins; however, little is known about the specific cellular functions of this family [125, 126].

Class III PI3K consists of a catalytic (Vps34) and regulatory (Vps15/p150) subunit. Vps34 is a nutrient-regulated lipid kinase which mediates signaling through mTOR (mammalian target of mTOR) and produces only PI(3)P which is an important regulator of membrane trafficking of proteins and vesicles [126].

The major function of the PI3K/AKT signal pathway is to promote growth factor-mediated cell growth, proliferation, migration and survival. Following activation of PI3Ks by a variety of stimulus through growth factor receptors such as HER2 and EGFR, the phosphatidylinositol bisphosphate (PIP2) is phosphorylated to phosphatidylinositol triphosphate (PIP3). PIP3 acts as a docking site for Akt, a serine/threonine kinase that is the central mediator of the PI3K pathway. Akt, also known as protein kinase B, is a phosphoinositide-dependent kinase and has three isoforms, AKT1, AKT2 and AKT3, encoded by the genes PKBα, PKBβ, and PKBγ, respectively, which are widely expressed in most human tissues [128–130]. AKT genes are amplified, or the protein is overexpressed, in a number of human cancers. AKT2 is genomically amplified in breast tumors and AKT3 is found to be overexpressed in estrogen receptor (ER) deficient breast cancer, suggesting that this enzyme may contribute to the more aggressive clinical

phenotype. All three isoforms comprise of an N terminal PH domain, a central serine/threonine catalytic domain, and a small C-terminal regulatory domain. The docking of the PH domain in the N-terminal region of AKT to PI(3,4,5)P3 on the membrane, results in a conformational change in AKT, exposing two critical amino acid residues for phosphorylation (T308 by PDK1 and S473 by PDK2). Both phosphorylation events are necessary for full activation of AKT [131, 132]. The primary source of PDK2 activity under most circumstances is mTORC2 (mTOR/rictor complex) [133]. mTOR, which belongs to a group of Ser/Thr protein kinases of the PI3K superfamily referred to as class IV PI3Ks, plays a pivotal role in the regulation of cell growth and proliferation by monitoring nutrient availability, cellular energy levels, oxygen levels, and mitogenic signals [134]. mTOR exists in two distinct complexes—mTORC1 and mTORC2. The mTORC1 complex is composed of the mTOR catalytic subunit, Raptor (regulatory associated protein of mTOR), PRAS40 (proline-rich AKT substrate 40 kDa) and the protein mLST8/GbL [134, 135]. mTORC2 is composed of mTOR, Rictor (rapamycin insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein 1) and mLST8/GbL [135]. When bound to Rictor in the mTORC2 complex, mTOR functions as PDK2 to phosphorylate AKT [133]. Once activated, AKT phosphorylates many other proteins, e.g. GSK3 (glycogen synthase kinase 3) and FOXOs (the forkhead family of transcription factors), thereby regulating a variety of cellular processes involved in protein synthesis, cell metabolism, proliferation, and survival [129, 136] (Fig. 4.1).

AKT can activate mTOR by phosphorylating both PRAS40 and TSC2 (tuberous sclerosis complex) to attenuate their inhibitory effects on mTORC1 [137–139]. The best-characterized downstream targets of mTORC1 are S6K1 (p70S6 kinase) and 4E-BP1 (4E-binding protein), both of which are critically involved in the regulation of protein synthesis [140]. Thus, activation of mTOR may provide tumor cells with a growth advantage by promoting protein synthesis.

In addition to the activating components of the pathway, another major mechanism of AKT activation is a loss of the function of novel tumor suppressor gene, phosphatase and tension homologue deleted on chromosome ten (PTEN) [99, 141–143] (Fig. 4.1).

PTEN has intrinsic lipid phosphatase activity and converts PI(3,4,5)P3 back to PI(4,5)P2. Thus the cellular level of PI(3,4,5)P3 is tightly regulated by the opposing activity of PTEN. Loss of PTEN therefore results in unrestrained signaling by the PI3K/AKT pathway resulting in cancer.

PI3K/AKT pathway has been shown to be aberrantly hyperactivated in breast cancer with high frequency. Activating mutations and deletions have been identified at multiple sites including at the level of RTK receptors, *PTEN*, AKT, and Ras. These mutations increase enzymatic function, enhance downstream signaling elements, and promote oncogenic transformation. Depending on the breast cancer subtype, about 20–25 % of breast tumors exhibit these mutations. For example, in hormone receptor–positive tumors, these mutations occur in > 30 % of cases and in $HER2^{+}$ disease, mutations are evident in about 25 % of

tumors, mutations in triple-negative breast cancer are less frequent [144]. Sporadic missense mutations in PTEN have been shown in 6 % of breast cancer.

Breast cancer cell lines with a constitutively activated PI3K/AKT pathway due to HER2 overexpression and/or loss of the PTEN suppressor gene has been shown to be resistant to HER2, EGFR targeted therapies and to endocrine therapy. The activation of AKT has been shown to be associated with a worse outcome among endocrine-treated breast cancer patients. In addition it has been revealed that breast cancer cell lines with activated AKT are especially sensitive to mTOR antagonism. Because the activation of PI3K/AKT pathway is strongly implicated in cancer development, this pathway currently attracts huge attention as a new target and inhibition of this pathway is now considered to be a promising strategy of developing effective therapies for breast cancer [145, 146].

4.5 Conclusion

In summary, EGFR signaling cascades have diverse cellular functions such as cell proliferation, survival, migration and differentiation and are frequently implicated in breast cancer. The study of EGFR family and its regulated pathways (Raf/Ras/MEK/ERK or PI3K/AKT) represent an exciting area of cancer research to promote our understanding of molecular changes that occur during breast cancer development. Manipulating the role of EGFR family of receptors in cell survival by targeting their expression levels, activity and nuclear translocation, has potential in the development of effective breast cancer treatment. However, the major obstacles in successful breast cancer treatment include concomitant aberrations and crosstalk between multiple signaling pathways in cancer cells which finally leads to the development of drug resistance. Therefore multiple targets need to be addressed for maximal clinical effects and to minimize development of drug resistance such as combination therapy involving use of EGFR inhibitors with inhibitors of various components of Ras/Raf/MEK/ERK, PI3K/AKT or other pathways. This approach holds great promise for more effective development of targeted anti-breast cancer therapy.

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Chapter 5
Breast Cancer Heterogeneity in Primary and Metastatic Disease

Jodi M. Saunus, Amy McCart-Reed, Majid Momeny, Margaret Cummings and Sunil R. Lakhani

Abstract The term 'breast cancer' describes a heterogeneous collection of neoplasms arising from the mammary epithelium. Tumors in different patients display diverse morphologies, molecular phenotypes, responses to therapy, probabilities of relapse and overall survival. Current histopathological classification systems aim to categorise tumors into subgroups to inform patient management decisions, but the diversity within subgroups is considerable. Molecular analyses such as gene expression profiling, and more recently, massively parallel sequencing technologies, have been employed to increase the degree of resolution in breast cancer taxonomies. It will take time for this information to be translated into the clinic. Sequencing projects have also been instrumental in revealing the true extent of intratumoral heterogeneity: three-dimensional variability in the genetic, phenotypic, cellular and microenvironmental constitution of individual tumors. This variability underlies clinical problems such as metastasis and drug resistance, and will present additional challenges as breast cancer diagnostics evolves to include higher resolution molecular analyses. Intratumoral heterogeneity will need to be carefully considered as we move towards more personalized models of breast cancer patient management.

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5.1 Introduction

Heterogeneity is defined as a lack of compositional uniformity. If we had a dollar for the number of times the phrase, or variations of "breast cancer is a very heterogeneous disease" was used to introduce publications in breast cancer research, we might *almost* have enough funding to understand the heterogeneity. Though now a cliché, the phrase is quite accurate, with at least 19 cancer types currently recognized by the World Health Organisation (WHO; Table 5.1) [1]. Breast cancer may be considered a collection of diseases arising from the mammary epithelium, with diverse risk factors, responses to therapy, probabilities of relapse and overall survival.

Histopathologists have observed diversity both between and within breast tumors for many years. Intertumoral heterogeneity is chiefly recognised as differences in the morphological, phenotypic and genomic features of different tumors. Critically, these features correlate with clinical behavior, and can be applied in prognostic and predictive settings. Intratumoral heterogeneity is observed as variations in morphology, phenotypic features and mutation spectra within the one tumor, and is largely responsible for clinical complications such as incomplete response to therapy, development of drug resistance and disease recurrence.

Categorizing tumors into broad diagnostic and prognostic groups is the current basis of personalized breast cancer patient management. The degree of resolution in this taxonomy continues to increase over time alongside technological advances in molecular profiling. There is conviction now embedded in clinical and research communities that we are not far from a *fully* personalized model of management, where genomic analysis will be routine in the diagnostic setting, offering precise and accurate molecular information to help guide patient management decisions [2, 3]. It is thought that the continual refinement of massively parallel sequencing (MPS) technologies will shorten the path to fully personalized medicine, however tumor heterogeneity will be one of the greatest challenges to manage in this endeavor [4].

This chapter will outline current breast cancer classification schemes, including the histopathological system used clinically, and molecular schemes that are revealing the biological basis of breast cancer heterogeneity, providing prognostic and predictive indicators and informing therapeutic development. We will also discuss intratumoral heterogeneity, including the major underlying sources of

Table 5.1 Key invasive epithelial malignancies of the breast. There are at least 19 histologically distinct breast cancer types, plus multiple morphological variants [1]

Histologic type		Prevalence
Invasive breast carcinoma		
1	Invasive breast cancer of no special type (NST), including: Pleomorphic carcinoma Carcinoma with osteoclast-like stromal giant cells Carcinoma with choriocarcinomatous features	60–75 %
2	Invasive lobular carcinoma, including: Classic, Solid, Alveolar, Pleomorphic, Tubulolobular and Mixed types	10–15 %
3	Tubular carcinoma	2 %
4	Cribriform carcinoma	0.3–0.8 %
5	Mucinous carcinoma	2 %
6	Carcinoma with medullary features, including: Medullary carcinoma Atypical medullary carcinoma Invasive carcinoma NST with medullary features	<1–2 %
7	Carcinoma with apocrine differentiation	4 %
8	Carcinoma with signet ring differentiation	rare
9	Invasive micropapillary carcinoma	<0.9–2 %
10	Metaplastic carcinoma NST, including: Low grade adenosquamous carcinoma Fibromatosis-like metaplastic carcinoma Squamous cell carcinoma Spindle cell carcinoma Metaplastic carcinoma with mesenchymal differentiation Mixed metaplastic carcinoma Myoepithelial carcinoma	1 %
11	Carcinoma with neuroendocrine features, including: Neuroendocrine tumor, well differentiated Neuroendocrine carcinoma, poorly differentiated Carcinoma with neuroendocrine differentiation	<1 %
12	Secretory carcinoma	<0.15 %
13	Invasive papillary carcinoma	rare
14	Acinic cell carcinoma	rare
15	Other rare types	<1–2 %
Epithelial-myoepithelial tumors		
16	Adenomyoepithelioma with carcinoma	rare
17	Adenoid cystic carcinoma	<0.1 %
Papillary lesions		
18	Encapsulated papillary carcinoma with invasion	<1 %
19	Solid papillary carcinoma—invasive	<1 %

For comparison between WHO prevalence data and other studies, see [73]

diversity and what we have learned from studying metastatic breast cancer from a range of sites in the body. Further, we will address genomic studies of matched primary and secondary tumors of other solid cancer types, introducing the key evidence that metastases are direct manifestations of clonal evolution.

5.2 Intertumoral Heterogeneity

Intertumoral heterogeneity results from patient-specific factors: the combined effects of genetic background, the presence of other risk factors (discussed in Chaps. 1 through 3), the tumor cell type-of-origin and any pre-determined differentiation programs, and the particular sequence of genetic alterations that occurs during progression. Pathologists began to highlight the morphological variability between breast cancers long ago, and over the last century or so have developed classification systems to provide prognostic and predictive information [1, 5, 6].

5.2.1 Types of Breast Cancer

5.2.1.1 Histological Subtypes

At the broadest level, breast carcinomas are divided into pre-invasive in situ lesions (ductal or lobular types) and invasive disease. The former are identified as malignant proliferation of cells confined within the basement membrane-bound structures of the breast, often detected by mammographic screening and generally associated with a favourable outcome. This chapter will focus on invasive disease, where tumor cells breach the basement membrane and invade the surrounding tissue (though in situ disease also comprises a heterogeneous group of lesions [7]). Invasive cancers are initially stratified according to architectural growth patterns into histological 'special types' with distinct morphology (25–30 % of cases, including 10–15 % lobular carcinomas), or the morphologically diverse invasive ductal carcinoma, no special type (IDC-NST; 60–75 % of cases) (Fig. 5.1 and Table 5.1) [5]. IDC-NST is an exclusion-based diagnosis, indicating there are no consistent and discriminating morphological features

In contrast to IDC-NST, special types are thought to represent relatively more homogeneous subgroups, with their distinctive appearances suggesting common underlying genetic alterations [8]. Indeed, several genotype-phenotype associations have been identified. For example, E-cadherin is inactivated or dysregulated in a large proportion of lobular carcinomas [9]; secretory carcinomas are associated with a t(12;15)(p. 13; q25) translocation and the resulting *ETV6-NTRK3* fusion gene [10]; and, like their relative in the salivary gland, adenoid cystic breast carcinomas consistently harbour the t(6;9)(q22–23; p23–24) translocation leading to *MYB-NFIB* gene fusion, and overexpression of the *MYB* oncogene [11].

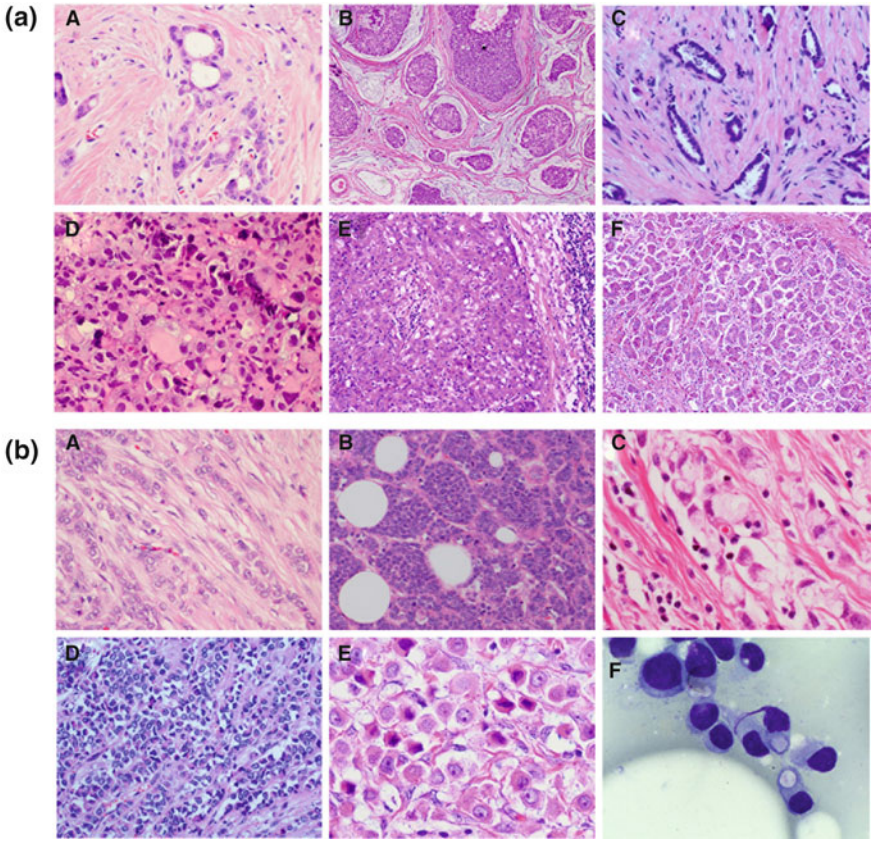


Fig. 5.1 Intertumoral heterogeneity shown by breast cancer histological types. **a**: (A) Invasive ductal carcinoma, NST. (B) Invasive mucinous carcinoma. (C) Tubular carcinoma. (D) Metaplastic carcinoma. (E) Medullary carcinoma. (F) Invasive micropapillary carcinoma. **b**: Morphological variation even within one histological type, invasive lobular carcinoma, shown histologically (A–E) and by fine needle aspiration (FNA) cytology (F). (A) Classic type. (B) Alveolar type. (C) Signet ring type. (D) Solid type. (E) Pleomorphic type. (F) Classic type

Furthermore, studying these groups has taught us that certain tumor-initiating cells may produce more homogeneous phenotypes; there is evidence that tumors with metaplastic features may derive from a CD10-positive (possibly myoepithelial) tumor-initiating cell [12].

Histotyping can provide prognostic information. For example, medullary carcinomas are usually associated with a good prognosis, but would be considered aggressive according to grade [5] (see below). However, the cases for which clinical behavior and treatment response cannot be robustly predicted based on histotype far outweigh these examples, and while typing may be biologically informative [13], its impact on clinical decision making is debatable [14]. There is fundamental variability in morphology (Fig. 5.1) and clinical behavior within

histological types. Addition of prognostic and predictive indicators controls for some of this diversity and allows stratification into clinically useful subgroups.

5.2.1.2 Prognostic and Predictive Subgroups

Apart from histological type, other clinico-pathologic factors are considered to provide greater prognostic accuracy and predict response to therapy. These include: (1) patient age and menopausal status, since tumors arising in younger, pre-menopausal women often show aggressive clinical behavior; (2) stage, determined by the size of the tumor, number of lymph nodes involved and the presence of metastases; and (3) histological grade, which refers to the degree of differentiation and reflects how closely the tumor resembles normal breast tissue. A grade of 1–3 is given based on the degree of nuclear pleomorphism, the percentage of tubule formation and the number of mitoses per ten high-power microscope fields [15]. The frequency of cells that express the proliferation marker Ki67 can be used to complement mitotic count, although it may not offer any advantage over mitotic score [16]. More than half a century since its introduction, histological grade remains one of the most powerful prognostic indicators in multivariate analysis [17, 18] and correlates with molecular features [19]. Grade is a key component of widely used prognostic algorithms such as the Nottingham and Kalmar Prognostic Indices [20, 21] and predictive algorithms used to guide the use of chemotherapy [22, 23].

Tumors are also routinely analysed using antibodies for oestrogen receptor (ER) and progesterone receptor (PR), giving an indication of the degree of hormone dependence of the tumor. PR is induced by oestrogen signalling (a surrogate for ER activity), and adds value to the power of ER for predicting response to therapy [24, 25]. ER/PR-positive tumors are good candidates for endocrine therapy (*e.g.* tamoxifen, aromatase inhibitors), tend to be lower grade and associated with better outcomes than ER/PR-negative cases. The third biomarker in routine clinical use is human epidermal growth factor receptor 2 (HER2) [26]. The gene encoding HER2 (*ERBB2*) is amplified and/or over-expressed in 15–20 % of invasive breast cancers, and correlates with prognosis [27–29] and sensitivity to Trastuzumab (Herceptin®) [30]. ER/PR and HER2 also define 'triple negative' breast cancer (TNBC), characterized by negativity for all three biomarkers. TNBC comprises 10–17 % of invasive cancers and has a higher risk of relapse within 1–3 years of diagnosis despite initial heightened sensitivity to chemotherapy, exemplifying the aggressive clinical behavior of these typically high-grade tumors [31, 32].

It is important to note that expression or amplification of ER/PR and HER2 is not always uniform, implying that not all tumor cells are dependent on their growth factor ligands. ER-positivity is currently defined by a diagnostic threshold of only 1 % [33], while 30 % of cells staining for HER2 is required before the intensity and cellular distribution of staining is considered. HER2 genetic heterogeneity has been reported to occur in ~10 % of HER2-positive cases [34–36]. Setting conservative cut-offs is done to ensure patients are eligible for therapy that

might have even marginal success, but in this context intratumor heterogeneity contributes to the clinical dilemmas of poor treatment response and drug resistance (*e.g.* HER2 heterogeneity is associated with shorter disease-free survival [34]). While diagnosing receptor negativity in TNBC is more straightforward, this group is still heterogeneous in other respects, comprising diverse histological types (*e.g.* IDC-NST, apocrine, and pleomorphic lobular), different molecular signatures (Sect. 5.2.2) and variable risks of relapse [31].

5.2.2 Molecular Heterogeneity at the Transcriptomic Level

The variation in morphology and clinical outcome observed across breast cancer subtypes implies that this is underpinned by molecular heterogeneity. For over a decade, there have been attempts to stratify breast cancers according to their molecular features to account for heterogeneity within the histopathological taxonomy. Historically, the field has been dominated by gene expression array approaches that generate averaged transcriptional 'signatures' from the sampled tissue. Signatures are biased towards transcripts expressed most differently from the mean, and/or towards high expression pattern frequencies amongst the cells sampled (e.g. a highly gene expressed in a proportion of the cells may be represented in the signature to a similar degree as a gene expressed at a moderate level in all cells).

Array profiling has been applied in three types of analysis: (1) class discovery: unsupervised identification of subgroups within heterogeneous cohorts based on gene expression profiles; (2) class comparison: supervised analysis to uncover molecular differences between predefined groups (e.g. lobular and ductal carcinomas [37, 38]); and (3) class prediction: an extension of class comparison that defines a molecular signature (single sample predictor, SSP) used to classify individual cases (*e.g.* risk of relapse in particular patient subsets [39–42]).

5.2.2.1 First Generation 'Intrinsic Subtypes' of Breast Cancer

The beginning of the breast cancer molecular subtyping era was marked by two of influential class discovery studies from Stanford University [43, 44]. The group initially stratified 38 invasive breast carcinomas into four groups by unsupervised hierarchical clustering of tumor gene expression profiles according to an 'intrinsic gene list' (genes that are most differentially expressed between tumor samples from different patients compared with duplicates from the same patients) [43]. The subtypes were named on the basis of phenotypic similarities to normal mammary epithelial compartments (luminal, basal-like and normal-like) or high expression of HER2 and associated genes (HER2+). Importantly, the highest order stratifier was ER status, a key prognostic and predictive indicator. Using a larger cohort, the group went on to show that luminal tumors could be segregated into at least two subgroups (A and B) with different clinical outcomes [44, 45] (summarized in

Table 5.2 Phenotypic features of key molecular breast cancer subtypes defined by gene expression profiling

		Typical histopathological features					
	Subtype	ER	AR	HER2[a]	Grade	Key phenotypic features	Differentiation state[b]
Intrinsic subtypes	Luminal A	+		−	1–2	Expression of ER-associated networks and low molecular weight cytokeratins (e.g. CK8/18)	Enriched with GEX signature of fully differentiated luminal epithelia
	Luminal B	±		∓	2–3	Expression of ER-associated networks as for luminal A, and also a proliferation GEX signature	Enriched with GEX signature of fully differentiated luminal epithelia
	HER2	∓		+	2–3	HER2 overexpressed and/or amplified, overexpression of genes at the 17q22.24 locus	Modest expression of mature luminal and luminal progenitor cell GEX signatures
	Basal-like	−		−	3	Often triple negative, high grade, frequent expression of myoepithelial markers (e.g. EGFR, CK5/6, CK14), *c-KIT* and *FOXC1*, frequent *TP53* mutations	Most similar to luminal epithelial progenitor cells
	Normal-like	∓		−	1	Expression of genes normally associated with adipose tissue and myoepithelia; low expression of luminal genes; clusters with fibroadenoma and normal breast samples	Enriched with MaSC and stromal GEX signatures
Newer subtypes	Claudin-low	−		−	3	Low expression of cell junction proteins, high expression of immune response and EMT GEX signatures	Primitive, highly enriched with stem cell and stromal GEX signatures
	Molecular apocrine	−	+	±	2–3	Frequent apocrine histologic features, activation of GEX networks associated with AR, calcium and ErbB signalling, lipid and fatty acid synthesis. HER2 overexpression not associated with amplification of genes at 17q22.24	Primitive, similarity to normal breast compartments not yet determined

[a] Presence of overexpression and/or gene amplification

[b] Differentiation state determined by comparison of tumor gene expression (GEX) profiles with those from FACS-sorted populations of normal mammary epithelia [80, 126]

Abbreviations: + positive; − negative; ± mostly positive; ∓ mostly negative; blank, not specifically determined; *AR* androgen receptor, *CK* cytokeratin, *EMT* epithelial-to-mesenchymal transition, *ER* oestrogen receptor, *ErbB* epidermal growth factor receptor tyrosine inase, *GEX* gene expression, *HER*2 human epidermal growth factor receptor 2, *IDC-NST* invasive ductal carcinoma of no special type, *MaSC* mammary stem cell

Table 5.2). The intrinsic subtypes have since been validated using more samples and newer, denser microarrays [45–48], and have also been correlated with survival [49, 50] and treatment response [51, 52], giving the classification scheme clinical context.

The ER-positive arm is characterized by high expression of genes associated with luminal differentiation, primarily ER signalling network genes (e.g. *ESR1*, *FOXA*1*, GATA*3) and the low molecular weight cytokeratins (*CK*8/18). Luminal A and B groups are distinguished by low and high expression of proliferation-associated gene networks [45]. Of all subtypes, luminal A has the best survival rates, followed by luminal B, consistent with their relative frequencies of high-grade tumors [43, 44, 48]. It has been postulated that failure to segregate weakly and strongly proliferative groups in the original study [43] was due to limited sample numbers and/or the use of paired 'before' and 'after' chemotherapy samples [44]. However, more recent meta-analysis suggests that expression of the proliferation signature in luminal tumors occurs in a continuum, and division into subgroups may be arbitrary [53].

The ER-negative arm is more transcriptionally heterogeneous, comprising at least three major subgroups: basal-like, HER2 and normal breast-like. The basal-like group is characterized by expression of high molecular weight cytokeratins (*CKs* 5, 6, 14), *c-KIT*, and *FOXC*1, frequent *TP*53 mutations, high proliferative activity and grade, and aggressive clinical behavior [32]. There is growing evidence implicating dysfunctional DNA repair in the phenotypic features and aetiologies of these tumors [54]. Sporadic basal-like tumors share phenotypic features with those from germline *BRCA*1 mutation carriers [55], and orthotopic xenografts of transformed epithelia derived from precancerous breast tissue of mutation carriers have basal-like features [56–58]. Surrogate IHC markers for this subtype have been developed [59], and evidence suggests ~75 % are ER-/HER2-negative plus EGFR- and/or CK5/6-positive [60]. Noteworthy, diagnostic assessment of these markers is not standardized, as the information does not alter patient management compared with a triple negative diagnosis [32, 61].

The basal-like group probably best exemplifies heterogeneity within the intrinsic subtype taxonomy. It is diverse in terms of histologic features, mutation profiles, response to chemotherapy, metastatic behavior, survival, and genomic landscape [44, 45, 49, 50, 62–68]. In cases defined using the IHC marker CK14 as a surrogate for the expression phenotype, there are at least two major prognostic groups: one associated with better survival than grade-matched non-basal cases, and the other succumbing to the first relapse within 1–3 years of diagnosis [68]. Clinical outcome has been associated with various parameters, including diffuse versus focal CK14 staining [68], the presence of lymphocytic infiltrate [69], and expression of B- and T cell, inflammation and angiogenesis signatures [70].

The HER2 signature is broadly defined by high expression of *HER*2 and other genes (*e.g. GRB*7) within the frequently amplified 17q22.24 locus. However, there is incomplete overlap between molecular and clinically-defined HER2 groups (~70–80 %), with a significant number of ER/HER2-positive tumors (which would be managed with Trastuzumab® and endocrine therapy) falling in the

luminal B cluster [47, 52], and some HER2-positive, ER-negative tumors in the basal-like cluster [47]. Also, up to one third of the HER2 intrinsic subgroup are not 'HER2′ by clinical criteria [47], suggesting the signature is not exclusively driven by *HER*2 amplification or overexpression. In a subsequent large cohort class prediction study, *HER*2-amplified tumors actually clustered into two molecular subgroups that were defined by ER and proliferative status [71]. These discrepancies underscore the heterogeneity that still exists within a subtype predicted to be the one of the most homogeneous, based on the notion that *HER*2 amplification is a common and dominant aetiological factor.

The normal-like subtype is characterized by high expression of genes associated with adipose tissue and other stromal cell types. This group has been contentious, with many studies failing to confirm its existence or suggesting it arises from contamination of tumor samples with breast stroma [47, 53, 72], but others support its designation as a distinct subtype [52, 71].

Despite intensive investment in the intrinsic subtype taxonomy, this approach was attached to limitations. The cohorts ($n = 38$ and 78) were small by today's standards, and the intrinsic gene lists may not have represented the full spectrum of tumor types or extent of heterogeneity in breast cancer. Indeed, comparison of special types with intrinsic subgroup-matched IDC-NST revealed critical differences [73]. For example, unlike basal-like, grade-matched IDC-NST, tumors with medullary and metaplastic features are usually associated with good prognosis [74] and poor chemotherapy response [75] respectively. In hindsight, it could be argued that intrinsic subtypes represent groups that are already (directly or indirectly) identifiable using standard diagnostic tests. They can replace histopathology (grade, ER and HER2) for predicting response to neo-adjuvant therapy, but add no benefit when these parameters are used [52]. Meta-analysis suggests the two 'bad-outcome' groups (basal-like and HER2) may ultimately act via proliferation [53], which is already assessed using histologic grade [19].

5.2.2.2 Emerging Molecular Subtypes

Subsequent research has attempted to address these limitations and reduce molecular heterogeneity in the taxonomy using larger sample cohorts with better clinical annotation, denser arrays and unified analytical approaches [45, 70, 71, 76–81]. ER expression, triple negative status and a proliferation signature appear to robustly discriminate clusters even in the larger studies. Predictably, the degree of sub-stratification increased. The biological and clinical significance of the many new subgroups identified is yet to be fully elucidated, although two were identified in independent studies [77–79, 81] (Table 5.2).

The claudin-low cluster was identified by comparing human and mouse mammary tumors [79], and is characterized by low expression of cell junction proteins (E-cadherin, *CLDN*3/4/7, *OCLN*), epithelial-mesenchymal transition and immune infiltrate-related networks [78]. Claudin-low tumors and cell lines are enriched with primitive, $CD44^+/CD24^-$, mesenchymal-like cells [78, 80], and comprise 7–14 % of

invasive cancers, mainly ER-negative IDC-NST that would otherwise be classified as HER2, basal- or normal-like [80]. A large proportion of cases with medullary and metaplastic features express the signature [78, 80]. Claudin-low tumors also have distinct clinical characteristics. Overall survival is better than that of the poor-prognosis groups but worse than that of Luminal A [49, 80]. Similar to basal-like tumors, they show metastatic proclivity for lung and brain [49], although their response to neoadjuvant chemotherapy is worse [80], and the claudin-low signature is enriched in residual cells sampled after chemo- or endocrine therapy [82].

The molecular apocrine (mApo) group was identified as a cluster of ER/PR-negative tumors with paradoxical expression of luminal genes [81]. The group was named on the basis of frequent apocrine histologic features (*e.g.* eosinophilic cytoplasm) [77]. A key distinguishing feature is expression of the androgen receptor (AR), which regulates ER-responsive genes [77, 81]. Notably, AR was a high-level stratifier in a large class discovery study where cases were preselected based on consistent subtype assignment with three clustering algorithms [71]. mApo tumors are relatively primitive [71], characterized by genes associated with AR, calcium and ErbB signalling, lipid and fatty acid synthesis [71, 77]. They comprise 8–14 % of invasive cancers, which would otherwise be classed as basal-like or HER2 [47, 71, 77]. The mApo phenotype is associated with early recurrence but good response to neoadjuvant therapy [71]. It has been suggested that AR blockade could be a specific therapeutic opportunity for some cases of TNBC, which currently lack specific therapeutic targets [83].

5.2.2.3 Clinical Translation of Breast Cancer Molecular Subtypes

A natural extension of class discovery has been the development of risk-stratification signatures. Various studies have associated particular signatures with clinical outcomes (*e.g.* the B cell:IL-8 metagene ratio [70]). However, many offer data that is of no significant utility beyond current practice standards. To progress from the realms of basic research into the clinic, prognostic and predictive signatures must: (1) show value independent of existing histopathological criteria in multivariate analysis; (2) robustly classify individual cases with high accuracy and precision; and (3) be standardisable, and practicable for introduction into a diagnostic laboratory. A handful of signatures satisfy these criteria to some extent, several have been commercialised, and a few are being assessed prospectively in ongoing clinical trials (Table 5.3).

From a clinical perspective, the most important contribution of molecular subtyping has been the recognition of the luminal A/B subdivision in ER-positive disease, which has informed the development of MammaPrint® [39, 84–87] and Onco*type* DX® [88–90]. These tests quantitatively assign the risk of recurrence in ER-positive, node-negative patients, and have implications for sparing a proportion of low-stage patients from receiving chemotherapy. However, relative to the amount of basic research in this area, progress translating array-based classifiers into the clinic has been disappointing, particularly for TNBC, a very broad

Table 5.3 Commercially available prognostic and predictive gene signatures for breast cancer management

Signature	Assay	Starting material	Indication	Prognostic value	Predictive value	Commercially available	FDA approved	Prospective trial	Ref
MammaPrint®	Microarray	FF	Age <61-yr, LN-neg, Stage I/II, Size ≤5cm (limited utility for ER-neg disease)	70-gene signature, predicts risk of metastasis in 5 year	Informs prescription of chemotherapy	Agendia	Y	MINDACT	[39, 84-87, 153]
Oncotype Dx®	qRT-PCR 21-gene pane; recurrence score (RS) 0-100	FFPE	ER-pos, LN-neg	RS associated with 10-yr distant recurrence. Included in ASCO guidelines	Predicts benefit of adding chemo-therapy to an endocrine therapy regime	Genomic Health	N	TAILORX	[88–90, 154]
Caris Target Now™	Assessment of predictive biomarkers using microarray combined with FISH and IHC	FP, FFPE	Patients with refractory metastatic disease	N	Highlights therapies more likely to provide benefit based on molecular profiling of biomarkers	Caris Life Sciences	N	N	[155, 156]
MapQuant Dx®	qRT-PCR, 8-gene genomic grade panel qRT-PCR	FFPE	All patients	Akin to histological grade	Not yet established	Ipsogen	N	N	[19, 157–159]
Breast Cancer Index℠	*HOXB13:IL17BR* and 5-gene molecular grade indices	FFPE	ER-pos LN-neg treated with surgery alone	Index associated with recurrence after adjuvant tamoxifen	Index may help to define benefit from endocrine therapy	bioThera-nostics	N	N	[160, 161]

For a comprehensive review of the strengths and limitations of these signatures, see [93]

Abbreviations and definitions: ASCO: American Society of Clinical Oncology; ER-pos/neg: oestrogen receptor positive or negative; FF: fresh frozen tissue; FP: fresh tissue with RNA preserved in RNA*later*® solution; FFPE: formalin-fixed, paraffin-embedded tissue; FISH: fluorescence *in situ* hybridisation; Genomic grade: transcriptional signature that correlates with histologic grade but is more strongly associated with relapse-free survival [19] and may help overcome inter-observer variability; *HOXB13*: Homeobox protein 13; IHC: immunohistochemistry; *IL17BR*: interleukin 17B receptor; LN-pos/neg: lymph node positive or negative; qRT-PCR: quantitative real-time polymerase chain reaction

prognostic group in great need of new pathological risk classifiers (e.g. TNBC patients are generally offered adjuvant chemotherapy, but many receive little benefit [91]). The reasons underlying this bottleneck are summarised below (and reviewed extensively in [92–95]).

First, array platforms, tumor cohorts and analysis methods differ between studies and the intrinsic gene lists are discordant, with molecular subtypes either unstable or different between studies (*e.g.* the normal-like cluster has been included in both ER-negative [43, 44, 48] and -positive [45, 47] arms). To some extent this has been unavoidable, due to technological advances. In order to utilise molecular subtyping in clinical diagnostics, there must be international consensus on the methodology. Second, there is an alarming lack of consistency between SSPs for classifying individual cases [96]. A SSP is based on similarity between the gene expression profiles of individual cases with the mean expression profiles of the possible molecular clusters (centroids). Apart from the basal-like category (which is not a clinically informative distinction), we are still not able to consistently classify individual tumors [96]. This is at least partly due to the derivation of centroids *en masse* from large cohorts. Samples that are not strongly aligned with the centroid are classified with lower precision. Finally, high cost and the requirement for fresh frozen tissue associated with gene expression profiling are practical hurdles that are difficult to overcome in a diagnostic setting.

5.2.3 The Next Generation of Breast Cancer Classification

Gene expression profiling has undoubtedly shifted the conceptual framework in which we consider breast cancer development and heterogeneity. However, interrogating molecular phenotypes using this approach has led to underestimation of the extent of diversity between and within (Sect. 5.3) breast cancers, and in terms of improving patient outcomes, progress has been incremental. Genomic analyses have begun to reveal the true extent of breast cancer heterogeneity, engendering a view that defining general prognostic and predictive indicators using gene expression profiling alone may no longer be justified [93, 94, 97].

Using a cohort of ~2,000 tumors, Curtis et al. recently took an innovative approach to breast cancer classification, integrating gene expression with gene copy number data, derived using expression and single nucleotide polymorphism (SNP) arrays respectively [98]. Expression data was used not to define expression modules or signatures, but to map the *cis*- and *trans*-acting copy number aberrations (CNAs) within the transcriptomic landscape (*cis/trans*: impacting expression of the altered region, or genes at distant loci respectively). Clustering based on *cis*-acting expression outlier genes identified ten 'integrative subgroups', which divided the intrinsic subtypes and were associated with distinct clinical outcomes. This approach has advantages over expression-based clustering alone. It is centred on identifying subgroups with shared driver mutations, discriminating these from non-pathogenic passenger changes. Also, SNP array data allows exclusion of

samples with low tumor cellularity, a robust and quantitative technology for which has been historically lacking. The significance of the integrative subgroups is not yet fully understood, but the field eagerly awaits further investigation, particularly the use of integrative clustering for class prediction.

We are in the midst of the massively parallel sequencing (MPS) revolution, which allows genome or transcriptome sequencing within a timeframe of several days per sample, at relatively low cost. MPS involves arraying short (*e.g.* 50–150 nt) fragments of DNA or RNA onto a solid surface (*e.g.* glass) and measuring the sequence of bases added to elongating complementary strands. The in situ nature of the technique allows millions of short fragments to be read in parallel; with each nucleotide position read hundreds of times. This redundancy creates huge depth of genome coverage that not only provides increased fidelity, but also allows the identification of rare somatic mutations present in a small proportion of the sample (*e.g.* present in a low percentage of cells and/or masked by high stromal content). In RNA sequencing, deep sampling of the transcriptome allows detection of all expressed sequences, including weakly expressed, non-coding, partially processed and unknown species, which could be below the background noise threshold in a microarray experiment [99].

MPS was initially used to study breast cancer progression in two landmark case studies, which brought new insights about breast cancer progression and clonality [100, 101]. Basal-like tumors are usually high-grade and recurrence occurs within 3 years [68]. Conversely, lobular tumors are typically lower grade, with metastatic progression over 10–20 years [9]. MPS analysis of a basal-like breast tumor and matched brain metastasis revealed only 3 somatic alterations were unique to the metastasis (private) [100], while comparison of a lobular cancer and its matching pleural effusion revealed 19 private somatic alterations ($\sim$60 % of non-synonymous changes in the metastasis) [101], suggesting the metastasis underwent a greater degree of genomic evolution prior to presentation, consistent with the longer latency period.

MPS analysis of treatment-naïve TNBCs [67] revealed a spectrum of altered pathways and clonal complexity (particularly in the basal-like subset). Although mutations in known TNBC drivers *TP*53, *PIK*3*CA* and *PTEN* were present at high clonal frequencies overall, in some cases their frequencies were incompatible with founder status. Disruption of pathways thought to be important in TNBC (*e.g.* ECM remodelling and cell motility) was highly variable in terms of clonal frequency, further highlighting heterogeneity in TNBC, and that features underlying or predicting clinical behavior may have to be determined on a case-by-case basis. The theme of enormous diversity was reiterated in MPS analysis of a cohort of 100 unselected tumors [102]. The study identified putative new cancer genes based on non-random clustering of somatic mutations in coding regions, and correlative observations (*e.g.* some were identified in genome-wide association studies).

Our understanding of the significance of breast cancer MPS data is still superficial; it will take some time (perhaps decades? [103]) for bioinformatics analysis pipelines to catch up to data generation. Developments eagerly awaited by the field include utilising MPS for class prediction, and integrating genomic and

transcriptomic data to identify recurrently altered, druggable pathways. Future clinical translation of this data ('medical genomics') is contingent on fully establishing the relationships between genetic alterations and clinical outcomes [104]. Although our newfound awareness of the extent of breast cancer heterogeneity is sobering, the potential for medical genomics remains a powerful driving force behind research and development in this area. This is exemplified by the highly innovative, randomised phase II 'IMPaCT' trial for metastatic pancreatic cancer, where genomic tumor profiling for selection of therapeutics with the greatest predicted efficacy is being combined with 'mouse avatars' (personalized tumorgrafts) to validate drug efficacy before clinical use [105].

5.3 Intratumoral Heterogeneity

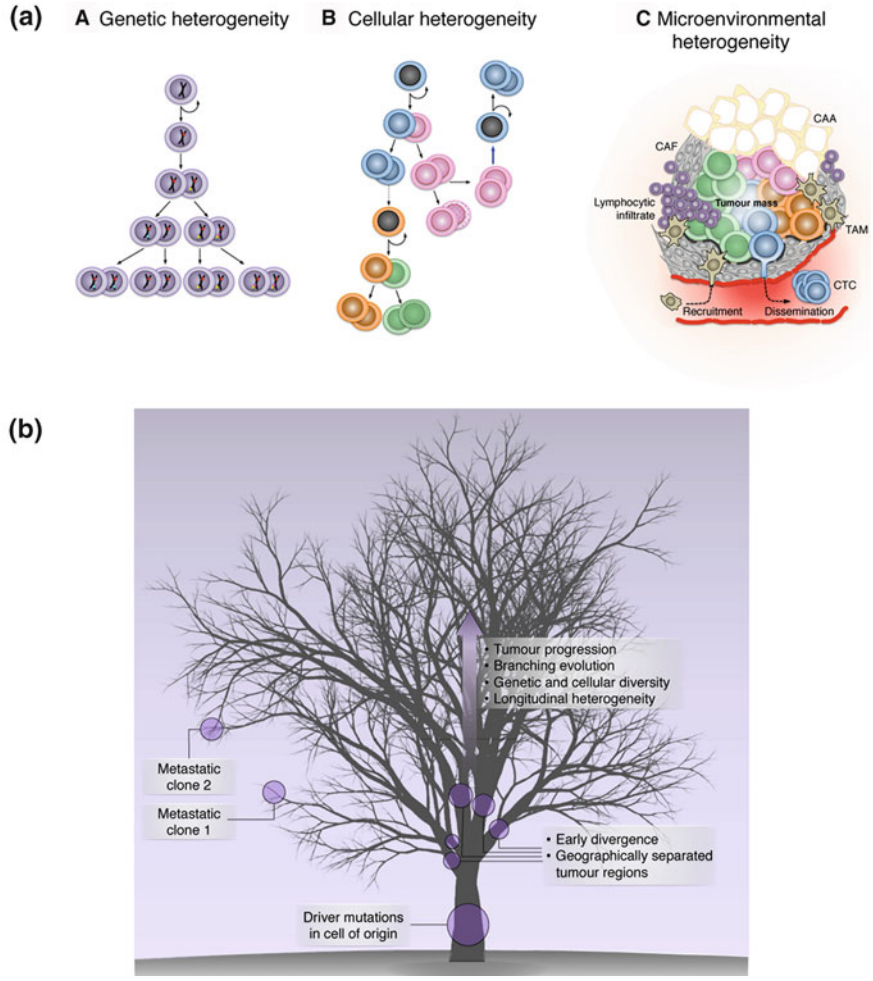
Most human tumors originate from a single cell that initially acquires a small set of somatic mutations sufficient to set the wheels of transformation in motion, yet the cells within a macroscopic tumor display heterogeneity at almost every level, implying a huge degree of genetic and phenotypic divergence during progression. In the diagnostic setting this presents as variation in morphologic and immunohistochemical features within a tumor.

Key molecular observations demonstrating intratumoral heterogeneity include that cells with *BRCA*1 and *BRCA*2 loss-of-heterozygosity occurred in spatially distinct clusters within precancerous breast tissues of mutation carriers [106]. Furthermore, MPS analysis has revealed breast cancers contain a huge spectrum of different alterations and mutation frequencies within each sample [67, 102]. These studies used DNA extracted from tissue sections, therefore probably underestimated heterogeneity, given analysis of other cancers shows genetically-distinct tumor cells can be separated by centimetres [106–108]. For pancreatic [107] and renal [108] cancers, reconstruction of phylogenetic trees using data from MPS analysis of topographically distinct samples demonstrated that mutation landscapes are both spatially and temporally heterogeneous.

Breast tumors also display functional heterogeneity, with cells displaying variability in critical features such as clonogenicity in vitro [109, 110], tumor repopulating capacity in vivo [111], sensitivity and resistance to chemotherapeutics [112] and metastatic potential [49].

5.3.1 Sources of Intratumoral Heterogeneity

Historically, the field has taken a gene-centric view to explaining intratumoral variability, where diversification is attributed to classic clonal evolution. A tumor cell 'clone' is a group of isogenic cells derived from a common ancestor. In this paradigm, the acquisition of a new, functional genetic alteration in a multipotent cell capable of self-renewal represents an evolutionary branching point, and the



initiation of a new subclone (Fig. 5.2A). Clonal diversity arises through reiterative rounds of clonal expansion, genetic and phenotypic diversification and natural selection [113, 114]. Addition of selective pressures (*e.g.* immune responses or chemotherapy) can destroy a large proportion of tumor cell clones, but can also facilitate the expansion of clones with inherent resistance. Clonal evolution dogma implies a gradualistic model of tumor progression, with steady progression through increasingly abnormal states. However, evidence from tumor and single-cell sequencing experiments suggests that clonal evolution can be determined by just a few major expansion events [115, 116]. There is now an appreciation that clonal evolution occurs through dynamic interplay between genetic and non-genetic factors, including adaptation within the tumor microenvironment [117, 118] (Sect. 5.3.1.3; Fig. 5.2).

◀ **Fig. 5.2** Mechanisms underlying intratumoral heterogeneity. **a**: Genetic, cellular and microenvironmental mechanisms cooperate to generate diversity. Cell molecular phenotypes and overall clonality are affected by genetic profile, any pre-programmed differentiation states as well as individual cells' responses to microenvironmental factors. (A) Classic clonal evolution involving stochastic acquisition of somatic mutations (coloured dots) and the generation of genetically diverse clones through cycles of cell division (*arrows*; *curved arrow* represents self-renewal). Addition of new selection pressure results in Darwinian selection of clones bearing advantageous genetic alterations. (B) Cell differentiation hierarchies are maintained within tumors. The differentiation states (different colours) of stem-like (*black nuclei*), committed progenitor and daughter cells contribute to phenotypic diversity (deterministic heterogeneity [117]). Phenotypic flux due to cell-specific biochemical processes (patterning in daughter cells) also contributes to phenotypic diversity (stochastic heterogeneity [117]). Daughter cells may acquire stem-cell activity through genetic alteration (*dashed arrow*) or de-differentiation (*blue arrow*), acquiring stem cell activity and initiating new clones. (C) Tumor cell phenotypes, clinical behavior and overall outcome are influenced by extrinsic factors, including stromal cell types (cancer-associated fibroblasts (CAF) and adipocytes (CAA)), the extracellular matrix protein milieu, recruitment of immune cell types (e.g. leukocytes and tumor-associated macrophages (TAM)) as well as humoral factors. There is further cross-talk between the tumor microenvironment and macroenvironmental factors like menopausal status and variations in body mass index. During progression there is active migration into local circulation, generating circulating tumor cells (CTCs), some of which may eventually form metastases. **b**: Branching model of intratumoral heterogeneity. Development of intratumoral diversity is analogous to the growth of a branching tree [152]. The trunk represents the founder mutations responsible for initially driving transformation, branch-points represent genetic and clonal divergence throughout progression and different branches represent genetically and/or phenotypically distinct subclones. The distance from the ground is proportional to the degree of divergence compared to the parent clone, and hierarchical relationships between subclones can be traced back to major branch points according to shared alterations

Intratumor heterogeneity can be interpreted within a framework that is based on three central ideas: (1) tumor cells can display inherent genetic instability, contributing to the activation of oncogenic pathways and inactivation of tumor suppressor genes; (2) intensive investigation of the cancer stem cell (CSC) concept has shown that tumors maintain cell division and differentiation hierarchies, which may be influenced by the phenotypic state of the founder cell; and (3) tumor cells respond to the local microenvironment and show dynamic phenotypic plasticity. Collectively these factors can achieve great diversification of tumor features over time (so-called, longitudinal heterogeneity), underpinning cancer progression.

5.3.1.1 Genetic Heterogeneity

Cancer initiation and progression are dependent on the sequential acquisition of mutations in cancer genes, and the consequential activation of oncogenic pathways and processes in order to attain the hallmarks of cancer [119]. This is catalysed by a degree of inherent genetic instability, which may be promoted by inherited susceptibility variants (e.g. *BRCA*1). The stochastic mutation process generates alterations that confer heritable selective advantages (drivers), and also thousands of mutations that do not immediately confer a selective advantage (passengers).

The presence of alterations affecting driver and drug resistance-associated genes has been shown to be heterogeneous in primary and metastatic cells from a range of human cancers [34, 67, 101, 108, 120, 121].

Driver alterations can be distinguished using algorithms that identify genes with high non-synonymous *vs* synonymous mutation rates, non-random clustering of mutations in coding regions and/or gene amplification combined with overexpression, which all imply positive evolutionary selection [122–124]. Prioritising oncogenic drivers helps to filter huge volumes of data and focus on alterations most likely to be functional and/or useful drug targets, but it is important to note that passenger mutations may have subtle effects on phenotypic state and plasticity, with significant roles in clonal evolution (Sect. 5.3.1.2) and that these algorithms are not infallible.

One of the long-term prospects for cancer MPS projects is the identification of recurrent changes with broad therapeutic potential [104]. An initial survey of 100 breast tumors showed that 7/40 predicted driver mutations and gene copy number alterations were present in >10 % cases, but most tumors were unique [102]. Cataloguing mutational landscapes is a necessary first step, but the diversity revealed thus far suggests thousands, rather than hundreds [104] of tumors may be required to find recurrent alterations with sufficient statistical power.

5.3.1.2 Cellular Heterogeneity

The differences between tumor cells are not solely due to genetic factors, but are the collective consequences of superimposing the mutational landscape over pre-programmed phenotypic determinants. According to the cancer stem cell (CSC) paradigm, tumor-propagating cells that possess or have acquired critical stem cell properties [125] are largely responsible for intratumoral heterogeneity, which is to some extent a blurred reflection of the differentiation states that exist in normal breast tissue [117].

Expression array studies have demonstrated that tumor signatures can be similar to particular differentiation states in the normal breast. The basal-like tumor profile resembles that of bipotent or luminal progenitor cells, whereas claudin-low tumors resemble mammary stem cells and luminal tumors are most similar to the differentiated normal luminal compartment [126]. Some have argued there could be a histogenic explanation for these similarities [127]. The significance of this is highlighted by the association between primitive, stem-like tumor phenotypes with poor outcomes in breast and other cancers [49, 80, 128], and mouse studies where the introduction of mutations into specific compartments generated tumors that phenocopied metaplastic carcinoma [12] and those arising in *BRCA*1 mutation carriers [129].

Whether a cell-of-origin can be reliably inferred from static phenotypic measurements is still being debated [130]. It is difficult to separate histogenic from adaptive factors by studying phenotypic endpoints. Tumors enriched with primitive, stem-like cells may be associated with metastasis and treatment resistance

because they have greater potential for generating clonal complexity and are good substrates for natural selection [117], and not necessarily because they possess equivalent normal stem cell functions like efficient drug efflux and slow cell cycling. Nevertheless, it is clear that tumor phenotypes are influenced by genetic and epigenetic pre-programming in CSCs, including the cell that sustained the founding oncogenic hits (cancer initiating cell) as well as those that arise through de-differentiation and phenotypic plasticity (cancer propagating cells) [125, 130] (Fig. 5.2B).

Intratumoral diversification is also achieved through phenotypic drift resulting from the dynamic biochemical processes occurring in each tumor cell (stochastic heterogeneity [117]) (Fig. 5.2B). This is reflected within the transcriptional 'noise' observed in gene expression profiling experiments, which some believe to be important in facilitating plasticity [131].

5.3.1.3 Microenvironmental Heterogeneity

Elements contributing to breast tumor stromal complexity include ECM, cancer-associated fibroblasts and adipocytes, tumor-associated macrophages and other leukocytes and endothelial cells [132] (Fig. 5.2C). Stromal features are strongly linked with breast cancer outcome [133], and a large effort has been directed at understanding the role of the tumor microenvironment in clinical behavior, including disease progression [134, 135]. There have been mixed reports on an association of lymphocytic infiltrate with outcome. Expression profiling and IHC have helped to discern different types of lymphocytes within lymphocytic infiltrate, and clarify cell type-specific effects. For example, Th1 and B cells are associated with good outcome in different contexts [70, 136], whilst regulatory T-cells are associated with progression and poor chemotherapy response [137]. Heterogeneity in tumor vasculature has important implications for drug delivery, exemplified in an elegant study using a circulation tracking dye together with radiolabeled lapatinib in experimental breast-brain metastases. The data showed that variation in blood brain barrier permeability was associated with variable distribution of lapatinib within the tumor [138], consistent with its limited efficacy in brain metastatic, HER2-positive breast cancer [139].

5.3.2 Heterogeneity in Metastatic Breast Cancer

Breast cancer is a systemic disease, with morbidity and mortality mainly due to development of distant metastases that lead to organ failure. That metastasis is a complication of end-stage disease historically led to an assumption that acquisition of metastasis-enabling changes and dissemination are universally late events in the course of disease. In this linear progression model, advanced clones would dominate both the primary tumor and its metastases. This idea was fuelled by gene

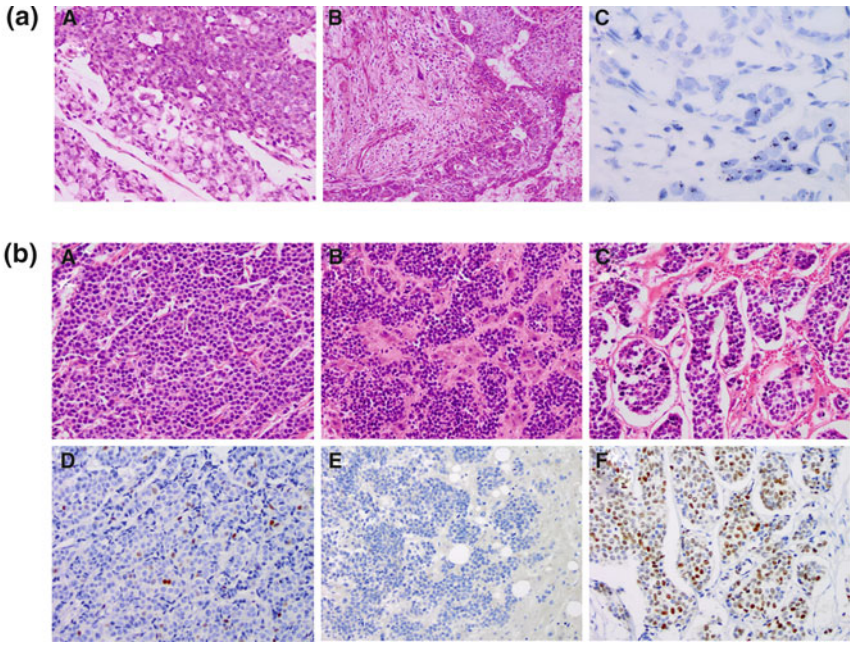


Fig. 5.3 Intratumoral heterogeneity. **a**: (A) at the epithelial level with an invasive ductal carcinoma, NST, mixed with an invasive mucinous carcinoma; (B) at both the epithelial and stromal level with both elements represented in a metaplastic carcinoma; (C) at the molecular level with some tumor cells showing HER2 amplification by SISH, while others remain diploid. **b**: Intratumoral heterogeneity between a primary breast carcinoma and its metastases with particular reference to expression of Progesterone receptor (PR) protein. (A) Primary breast carcinoma. (B) Liver metastasis. (C) Dural metastasis. (D) Breast primary with 1 % of cells positive for PR (E) Liver metastasis with no staining for PR. (F) Dural metastasis with all tumor cells positive for PR

expression array studies showing a high degree of similarity between primary tumors and matched metastases [140]. There are certainly examples of breast cancers that would fit with such a model [100], but there is also evidence suggesting that establishment of micrometastases can be an early event, even before diagnosis of the primary tumor [101, 141].

There is a body of evidence demonstrating heterogeneity between primary tumors and their metastases in breast cancer. Studying metastatic breast cancer from a range of sites in the body has shown that metastases may express clinically relevant molecular markers differently to their parent breast tumors [142–146] (Fig. 5.3). Furthermore, MPS studies have demonstrated parallel clonal evolution in primary tumors and metastases [100, 101, 141].

5.3.3 Clinical Implications of Intratumoral Heterogeneity

As described above, available data suggests that making decisions on the management of metastatic breast cancer based on primary tumor features is fraught with limitations. This is highlighted by HER2-positive breast cancer, where Herceptin therapy does not preclude later development of metastatic disease; in fact distant recurrence is common in this subtype [49] and relapse after therapy can be HER2-negative [147]. Furthermore, a prospective study indicated that biopsy of metastases altered the course of clinical management in one out of every seven metastatic breast cancer patients, based on re-evaluation of hormone receptors and HER2 alone [148]. Although it may not be possible to biopsy every recurrence, particularly if disease is widely disseminated, diagnosis might be more informative if the degree of heterogeneity within the primary tumor is considered [67, 117].

The potential consequences of basing management on information from limited tumor biopsies have been highlighted by heterogeneity in amplification and overexpression of HER2 [34], a key prognostic and predictive marker. Given that the suite of diagnostic tests used in clinical practice is still relatively low-resolution with conservative thresholds, in population terms tumor under-sampling is currently not a major dilemma. A future prospect is that personalized medicine will allow selection of rational combinations of targeted agents according to an individual tumor's mutation and phenotypic profile. Clearly the risk of under-sampling complexity would be amplified if higher resolution techniques were introduced. Tumor samples taken from different sites within the same renal cancer segregated differently according to gene expression signatures associated with good and poor outcome, demonstrating that tumor sampling could drastically affect the prognosis [108].

Relapse after chemotherapy is a major clinical complication of intratumoral heterogeneity. In order to be curative, drugs must be able to fully permeate a tumor at an efficacious dose, and eliminate all propagating cells. In many cases these requirements are not fulfilled due to heterogeneity in tumor microvasculature and/or because the tumor is primed for natural selection. MPS analysis has allowed tracing of relapsed tumor clones to the primary tumor, where they were present at much lower frequencies [108, 149]. Mutations may ablate the interaction between a compound and its target protein or pathway, or enable cells to bypass the target. Resistant phenotypes may also be generated through epigenetic mechanisms and phenotypic drift. In-depth discussion of this topic is outside the scope of this chapter, but the reader is directed to comprehensive reviews ([150, 151] and Chap. 19).

5.4 Concluding Remarks

Heterogeneity in breast cancer occurs at practically every level, and is apparent not only between tumors from different patients, but within individual tumors. Genetic and non-genetic mechanisms contribute to generating this diversity. Technological

improvements, including MPS techniques, have drastically improved the resolution at which heterogeneity can be assessed at the molecular level. There is expectation that applying MPS in oncology will inform the development of rational combinations of targeted therapies that minimise the occurrence of resistance and metastasis. A major barrier to this currently is our fragmented understanding of the associations between genetic alterations and clinical behavior. Data analysis, data management, drug design and functional validation studies are lagging behind rapid advancements in the physical technology, although this balance will change over time. Other barriers to MPS-based approaches becoming integral to routine diagnostics are economic and logistic in nature, including the same kinds of standardization issues that have applied to gene expression profiling (Sect. 5.2.2.3).

High-resolution molecular analysis of individual tumors is implicit in personalized therapy, addressing intertumoral heterogeneity. It is still unclear how we will manage the prodigious amount of intratumoral heterogeneity in breast cancer, but integrated, pathway-based analysis approaches may uncover more broadly applicable drug targets than those focussed on individual genetic alterations. Despite current limitations and barriers, the future looks bright and the realistic application of MPS technologies to personalized medicine is hotly anticipated.

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Chapter 6
Understanding Triple-Negative Breast Cancer

Ayca Gucalp and Tiffany A. Traina

Abstract It is estimated that over 200,000 new cases of invasive breast cancer will be diagnosed in the United States in 2012 and approximately 40,000 women will die from their disease. Triple-negative breast cancer (TNBC), which lacks expression of the estrogen receptor (ER) and progesterone receptor (PR), and does not overexpress the human epidermal growth factor receptor-2 (HER-2) represents approximately 15 % of all breast cancers and is often associated with a more aggressive underlying biology. Patients with TNBC more often experience rapid disease progression with poorer disease-related and overall survival in the first few years after diagnosis in comparison to their hormone-receptor positive counterparts. Furthermore, this subset of breast cancers has limited therapeutic options aside from traditional cytotoxic chemotherapy agents as they do not benefit from generally well-tolerated endocrine-targeted therapies and anti-HER2 drugs. In this chapter we will review the epidemiology, risk factors, prognosis and the varied molecular and clinicopathologic features that characterize TNBC. In addition this review summarizes the available data for the use of cytotoxic chemotherapy in the treatment of TNBC and explores the ongoing development of targeted therapeutic agents for the treatment of this subgroup of breast cancers.

Keywords Triple-negative breast cancer · Basal-like breast cancer · Molecular heterogeneity · Epidemiology · Prognosis · Chemotherapy · Targeted therapy · Platinum agents · Bevacizumab · Tyrosine kinase inhibitors (TKIs) · Poly (ADP-Ribose) polymerases (PARP) · Velaparib · Iniparib · Cetuximab · EGFR inhibitors

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6.1 Introduction

With the exception of non-melanomatous skin cancer, breast cancer is the most common cancer among women in the United States and remains the second most common cause of cancer-related death [1]. Estrogen receptor (ER) and progesterone receptor (PR)-negative breast cancer, which does not overexpress the human epidermal growth factor receptor-2 (HER-2), so-called triple negative breast cancer (TNBC), represents approximately 15 % of all breast cancers but accounts for a disproportionate number of breast cancer-related deaths each year [2, 3]. Patients with TNBC generally experience a more aggressive clinical course with increased risk of disease recurrence and poorer overall survival in the first few years after diagnosis [4]. Furthermore, while effective targeted therapies including drugs that target the ER (including the anti-estrogen drug tamoxifen, or aromatase inhibitors such as letrozole, anastrozole, or exemestane) and the HER2 protein (such as trastuzumab or lapatinib) greatly improve the outcomes of women with ER or HER2-positive breast cancer, there are few effective targeted therapies for TNBC [5–8]. Often, these tumors are treated with relatively non-specific and toxic chemotherapy. In this chapter we will review the epidemiology, risk factors, prognosis and the varied molecular and clinicopathologic features that characterize TNBC. In addition, this review summarizes the available data for the use of cytotoxic chemotherapy in the treatment of TNBC and explores the ongoing development of targeted therapeutic agents for the treatment of this subgroup of breast cancers.

6.2 Defining Triple-Negative Breast Cancer: Molecular and Histologic Features

Breast cancers represent a diverse group of tumors differentiated by risk factors, clinicopathological features, responses to therapy, patterns of recurrence, and clinical outcomes. Perou et al. [9], using complementary DNA microarrays, pioneered gene expression profiling techniques to identify 5 intrinsic subgroups of breast cancer which resembled normal breast cells, luminal epithelial cells (luminal A and luminal B), basal-like cells, and a HER2-amplified subtype. These distinct gene expression profiles have been validated in multiple independent data sets and correlate with disease progression and clinical outcomes [9–14].

Although approximately 85 % of all TNBC are categorized within the basal-like subgroup and significant overlap does exist between TNBC and basal-like breast cancers (BLBC) in terms of molecular, histopathologic, and clinical features, the two subgroups are not identical [15–18], and unlike other subtypes of breast cancer, TNBC is often defined largely by what it is not, and includes all breast tumors which do not express ER, PR, and HER-2. This definition, albeit somewhat simplistic, remains the primary method by which these tumors are

characterized in clinical practice in terms of both prognosis and in order to guide management. Our understanding of the underlying biologic heterogeneity of breast cancer in general, and TNBC as a subgroup, continues to evolve and not all TNBC are created equal. Hence, further subclassification is warranted. Recently, advances in molecular profiling have led to the classification of TNBC into multiple reproducible subgroups which demonstrate low expression of ER, PR, and HER2 and are distinguished by distinct gene signatures including basal-like, claudin-low, immunomodulatory, mesenchymal, mesenchymal stem-like, and molecular apocrine/ER (−) Class A/luminal androgen receptor subtype [19–21]. Although, the use of gene expression profiling is limited in clinical practice by the complexity and cost of testing, these innovations have allowed researchers to identify molecular targets which may have important implications for the development of novel therapeutic agents.

BLBC, as described by Perou et al., are characterized by low expression of ER/PR/HER2 and high levels of CK 5/6, CK 14, CK 17, p-cadherin, caveolin-1, carbonic anhydrase IX gene (CA IX), p63 (a member of the p53 family of transcription factors and a myoepithelial stem cell regulator), and epidermal growth factor receptor (EGFR or HER1) [22]. Multiple groups have proposed the development of a combination of immunohistochemical markers for routine clinical identification of BLBC primarily including ER, PR, HER2, basal cytokeratin markers (CK 5/6, CK 14, CK 17), EGFR and C-kit [18, 23, 24]. Using a four-marker combination including ER, HER2, EGFR, and CK 5/6, Nielsen and colleagues were able to correctly identify, with high specificity, tumors as "basal-like" among a panel of breast cancers pre-identified as BL by gene expression profiling [18]. Subsequent studies have shown that the presence of these immunohistochemical surrogate markers are associated with significantly poorer survival outcomes [18, 23–25].

Significant similarities have been described between BRCA-1 associated tumors and TNBC/BLBC in terms of their clinical, molecular and pathologic features. Interestingly, breast cancers associated with BRCA-1 germline mutations frequently lack expression of ER, PR, and HER2 and cluster closely with TNBC/BLBC on microarray sharing many common molecular features including expression of CK 5/6, 14, 17, p-cadherin, and EGFR [26–30]. Moreover, both subgroups are associated with altered BRCA function and genomic instability in addition to defective DNA damage repair theoretically rendering them more sensitive to certain therapies that induce DNA damage, as discussed later in this chapter.

A majority of tumors defined as triple-negative arise from the breast ducts and are associated with certain characteristic morphologic features including large tumor size, areas of central necrosis, pushing borders of invasion, lymphocytic invasion of the stroma, high nuclear and histologic grade, high mitotic index, and generally more aggressive histologic features [16, 30–34]. However, within the triple-negative subgroup there is a wide range of histologic presentations including less aggressive subtypes (medullary, secretory, and adenoid cystic tumors), that are generally associated with a more favorable prognosis, despite their classification within this subgroup [35–40].

6.3 Clinical Characteristics, Epidemiology and Risk Factors

Despite the diversity that exists within the triple-negative subgroup, multiple large population-based studies suggest that triple negative disease is associated with a distinct set of clinicopathologic features in addition to both modifiable and non-modifiable risk factors. Women with TNBC are on average significantly younger at the time of diagnosis [32, 41, 42]. In the United States, the prevalence of TNBC in comparison to other subtypes of breast cancer is lower, ranging from 15 to 20 % in the general population. However, the incidence and prevalence of triple negative disease varies by race and ethnicity and women in certain select populations such as African American or Hispanic descent have higher rates of TNBC in comparison to their Caucasian or Asian counterparts [31, 34, 42–46]. Moreover, women with triple-negative disease are more likely to have tumors associated with earlier age at menarche and at first pregnancy, increased parity, decreased breastfeeding, higher BMI, and lower socioeconomic status [32, 34, 42, 45, 47–53].

Higher rates of visceral versus osseus metastases have been observed in women with TNBC [44, 54]. In the largest multicenter retrospective to date, Lin and colleagues described the clinicopathologic features and sites of recurrences of greater than 12,500 women who were initially diagnosed with early stage breast cancer. Consistent with previously published findings, women with TNBC were more often of African American ethnicity, overweight, and younger with larger tumors and more advanced disease at the time of diagnosis. Data related to recurrence was available for 1,235 patients. Women with TNBC demonstrated a higher risk of developing visceral metastases when they initially recurred e.g., lung [Odds Ratio (OR) 2.27, 95 % confidence interval (CI) 1.50, 3.43; $p = 0.0001$] or brain (OR 5.32, 95 % CI 2.85, 9.91; $p < 0.0001$) and were less likely to develop osseus metastases (OR 0.23, 95 % CI 0.16, 0.33; $p < 0.0001$) in comparison to women with hormone receptor-positive disease [34].

Increased rates of central nervous system (CNS) metastases are well described in women with TNBC across multiple cohorts [55–57]. Women with TNBC often experience a shorter interval from the time of initial diagnosis and the development CNS disease as well as a shorter median survival once CNS involvement is documented. In a single-institution retrospective study of women with early stage (I–III) TNBC, Morris et al. reported a 29 % recurrence rate at a median follow-up of 5 years. In the women diagnosed with metastatic disease, 21 % had developed brain metastases. Among those individuals with CNS involvement, the median survival was 25 weeks [58]. Other studies have demonstrated comparable rates of survival after the development of CNS disease [56, 59].

6.4 Prognosis

For women diagnosed with TNBC, increased early recurrence rates and shorter periods of disease-free and overall survival have been described in multiple retrospective studies [9, 31, 32, 42, 60]. Additionally, patterns of recurrence have been shown to vary by breast cancer subgroup. One population-based study with a median follow-up of 8.1 years demonstrated that women diagnosed with triple negative disease experienced higher rates of both distant recurrence (hazard ratio, 2.6; 95 % confidence interval, 2.0–3.5; $p < 0.0001$) and death (hazard ratio, 3.2; 95 % confidence interval, 2.3–4.5; $p < 0.001$) within 5 years followed by a rapid decline in the subsequent years and limited recurrences after 8 years. This pattern of early recurrences followed by a rapid decline in the relapse rate among TNBC patients differed from other breast cancer subgroups that demonstrated a more constant rate of relapse during the follow up period [32]. Additionally, women with TNBC and BLBC experience shorter survival times once metastatic disease is diagnosed and overall worse outcomes in comparison to their hormone receptor-positive counterparts. Interestingly, retrospective analysis suggests that not all women with TNBC have similar survival outcomes and that triple negative tumors that do not express basal markers may be associated with improved prognosis [25].

6.5 Therapeutic Options

The mainstay of therapy for patients with TNBC in both the neoadjuvant and metastatic setting continues to be relatively non-specific cytotoxic chemotherapy. Treatment of this population remains clinically challenging, as these tumors are unresponsive to generally well-tolerated endocrine therapies and anti-HER2 agents. Furthermore, the lack of reliable prognostic features and predictors of response to therapy limits our ability to select for patients with more aggressive variants of this disease and tailor treatment regimens based on potential responsiveness to target-specific agents. In this section, we will review the current available treatment strategies for TNBC as well as those under development.

6.5.1 Chemotherapy

Numerous studies support the use of multi-agent chemotherapy regimens in the treatment of TNBC in the neoadjuvant setting [61]. Paradoxically, despite the poor prognosis associated with this subtype of breast cancer, these tumors generally are exquisitely chemosensitive albeit with shorter periods of durable response in comparison to other breast cancers. And although multiple trials have substantiated the efficacy of polychemotherapy and in particular the positive impact of

taxane-containing regimens on risk of recurrence, disease-free survival (DFS), and overall survival (OS) in TNBC [62–64] the superiority of one specific regimen over another remains an unanswered question.

6.5.1.1 Platinum Salts

It is in this setting that platinum-containing agents have become a particular focus of investigation for the treatment of BRCA-associated tumors and TNBC. In many ways, chemotherapy can be considered the original targeted agent. Due to their underlying mechanism of action, platinum compounds, which interfere with DNA function by producing interstrand DNA cross-links, may be of particular utility in the treatment of these two subsets of breast cancer. The BRCA1 gene is necessary to maintain DNA integrity and genomic stability and is integral in homologous recombination and repair of double-strand DNA breaks. In preclinical models, breast tumors deficient in this gene have demonstrated increased sensitivity to therapies that induce DNA damage such as alkylators and radiation [65–69]. It has been theorized that TNBC which are often characterized by altered BRCA function as well as impaired DNA damage repair may also demonstrate increased susceptibility to DNA damaging agents.

Antitumor activity in response to platinum agents has recently been described in BRCA1-associated breast cancers. In a retrospective analysis, Byrski et al. examined the use of preoperative chemotherapy in women with known BRCA mutations and the comparative rates of pathological complete response (pCR) achieved by regimen. Women treated with single-agent cisplatin achieved a pCR rate of 83 %, which was substantially higher than the rates seen with other regimens (cyclophosphamide/methotrexate/fluorouracil 7 %, doxorubicin/cyclophosphamide 22 %, cyclophosphamide/doxorubicin/fluorouracil 21 %, doxorubicin/paclitaxel 8 %). In contrast, Silver and colleagues evaluated the response to single-agent cisplatin in an unselected population of women with TNBC in a prospective preoperative trial. Eighteen of the 28 patients achieved a clinical response to cisplatin therapy defined as either partial response or complete response. Pathological complete response was documented in six of 28 patients (21 %) in response to neoadjuvant treatment. Notably the two BRCA germline mutation carriers enrolled on the trial were included among the 6 patients who achieved a pCR [70].

Platinum agents have also been examined for the treatment of TNBC in the metastatic setting. Recently at the 2011 annual meeting of the American Society of Clinical Oncology, Isakoff and colleagues presented data from a multicenter trial evaluating cisplatin or carboplatin in the 1st or 2nd line metastatic setting. Among the 86 women with TNBC treated the overall RR was 30.2 % (95 % CI 22.1 %–39.4) including 4 patients with radiographic complete responses (CR) and 22 with a partial response. The combined clinical benefit rate (CBR), defined as CR, partial response, or stable disease (SD) >6 months, was 34 %. Based on this series single-agent platinum demonstrated activity and was well tolerated in the metastatic setting [71].

Despite these promising findings, the addition of platinum compounds to preoperative or adjuvant regimens outside of a clinical trial remains non-standard. Additionally, the superiority of platinum-based chemotherapy over other agents in the metastatic setting has not been established and merits further evaluation. There are several trials currently underway in the both the preoperative/adjuvant (NCT00432172, NCT00861705) and metastatic setting (NCT0053272) that will provide additional information to help define how platinum agents should be utilized in this subpopulation.

6.5.2 Targeted Agents

To date there are no targeted agents FDA-approved specifically for the treatment of TNBC. The development of these agents in this setting has been difficult as testing for many potential targets is not standardized and remains variable across institutions subsequently influencing effective patient selection for clinical trials. In this section, we will review some of the targeted-agents currently under development for the treatment of TNBC.

6.5.2.1 Anti-Angiogenic Agents

Agents that target the angiogenesis pathway have been studied extensively for the treatment of breast cancer and in particular have been an attractive focus of investigation in the triple negative population which is characterized by high levels of VEGF expression and enhanced angiogenesis [72, 73].

Bevacizumab

Bevacizumab, a humanized monoclonal antibody to VEGF, is active in several solid tumors and is FDA approved for the treatment of certain types of colon, lung, kidney, and brain cancer [74]. Although the FDA recently reversed their accelerated approval of bevacizumab for the treatment of metastatic HER2-negative breast cancer in November 2011, the benefit of this agent for the treatment of TNBC continues to be an active area of investigation.

To date three randomized phase III trials of bevacizumab in combination with chemotherapy in the first-line metastatic setting have demonstrated activity with improvements in response rate and progression-free survival (PFS) across all breast cancer subsets. Unfortunately, all three of these studies failed to show an OS benefit for the addition of bevacizumab to a backbone of standard chemotherapy in this setting [75–77]. Notably, a metaanalysis conducted of all three studies identified improvements in both RR and PFS within the triple negative subset [78] (Table 6.1). Recognizing the inherent biases associated with retrospective

Table 6.1 Bevacizumab for the treatment of metastatic breast cancer (1st-line)

Trial	Intervention	Results/TNBC cohort
E2100	Paclitaxel ± bevacizumab	The objective response rate (36.9 vs 21.2 %, $p \leq 0.001$) and PFS (11.8 vs 5.9 months, $p \leq 0.001$) for the combination of bevacizumab and paclitaxel vs paclitaxel monotherapy
		Combination in TNBC: increase in median PFS from 4.7 to 10.2 months (hazard ratio (HR) = 0.45; 95 % CI, 0.33–0.61)
AVADO	Docetaxel ± bevacizumab (at 2 doses: 7.5 mg/kg or 15 mg/kg)	Median PFS for docetaxel monotherapy in comparison to the bevacizumab$_{7.5}$ and bevacizumab$_{15}$ groups was 8.0 vs 8.7 (HR 0.79 $p = 0.03$) and 8.8 (HR 0.72 $p = 0.001$) months respectively
		Combination in TNBC: Increase in median PFS from 6.0 to 8.1 months in the bevacizumab$_{15}$ arm (HR = 0.60, 95 % CI, 0.39–0.92)
RIBBON-1	Chemotherapy (anthracyclines/taxanes or capecitabine) ± bevacizumab	The combination of bevacizumab and capecitabine or an anthracycline/taxane resulted in significant improvement in PFS as compared to placebo 8.6 vs 5.7 months (HR 0.69, $p = 0.0002$) and 9.2 vs 8.0 months (HR 0.65 $p = 0.0001$), respectively
		Combination in TNBC: Increase in median PFS from 4.2 to 6.1 months (HR = 0.72, 95 % CI, 0.49–1.06) in the bevacizumab/capecitabine combination arm and from 8.2 to 14.5 months (HR = 0.78, 95 % CI, 0.53–1.15) in the bevacizumab/anthracycline/taxane combination arm

subgroup analyses, this comparison highlights the potential of bevacizumab for the treatment of a selected subpopulation of breast cancer patients.

More recently a pre-planned subgroup analysis of 159 patients with TNBC who were treated in the second-line metastatic setting with chemotherapy (paclitaxel, nab-paclitaxel, docetaxel, gemcitabine, capecitabine, or vinorelbine) ± bevacizumab (RIBBON-2) demonstrated that women with triple negative disease experienced a significant improvement in objective RR (41 vs 18, 95 % CI 7–39 %, $p = 0.0078$) and PFS (6.0 vs 2.7 months; HR 0.494, 95 % CI 0.33–0.74, $p = 0.0006$) when treated with the combination of bevacizumab and chemotherapy. Notably, these patients also demonstrated a trend towards an overall survival benefit as well on interim analysis (HR 0.624; $p = 0.0534$) [79, 80]. Similar results were seen in terms of RR, PFS, and median OS among the 585 TNBC patients treated with chemotherapy in conjunction with bevacizumab on the single-arm ATHENA study [81–83]. Multiple studies are currently underway in the neoadjuvant (NCT00861705, NCT01208480, NCT00777673), adjuvant

(NCT0052856), and metastatic settings (NCT00479674, NCT00733408, NCT0 1201265) to further explore whether this subpopulation of breast cancer patients has the potential to derive increased benefit from the addition of bevacizumab to standard chemotherapy.

Sunitinib and Sorafenib

In preclinical models of breast cancer, small molecule tyrosine kinase inhibitors (TKIs) such as sunitinib and sorafenib that target key steps in the angiogenesis-signaling pathway have been shown to have antiangiogenic and antitumor activity. Unfortunately, these agents have demonstrated limited activity as monotherapy for the treatment of metastatic breast cancer (MBC) [84–86]. Moreover, two large randomized phase III studies examining the addition of sunitinib to chemotherapy versus chemotherapy alone for the treatment of advanced disease (SUN1064: sunitinib plus docetaxel vs docetaxel; SUN1099: sunitinib plus capecitabine vs capecitabine) did not meet their primary endpoint of PFS [87, 88]. The RESILIENCE trial, a randomized phase III study comparing capecitabine in combination with sorafenib or with placebo was designed to provide definitive PFS data for the combination. This trial is currently open and enrolling patients (NCT01234337). Additionally, these agents are also being examined in the neoadjuvant setting in combination with platinum and taxane based chemotherapy (NCT00887575, NCT01194869).

6.5.2.2 Poly (ADP-Ribose) Polymerase (PARP) Inhibitors

In order to maintain genomic stability, multiple redundant repair mechanisms exist to correct both single-strand and double-stand DNA damage. Poly (ADP-Ribose) polymerases (PARP)-1/2 are part of a class of enzymes involved in single-strand DNA repair through the base excision repair pathway. In contrast, the BRCA gene encodes for proteins that are essential in homologous recombination and repair of double-strand DNA breaks. It is hypothesized that BRCA1-deficient tumors and TNBC, that often share a similar "BRCA-like" phenotype associated with impaired DNA repair, would be more susceptible to agents, such as PARP inhibitors that inhibit single-stand DNA repair and promote more error-prone DNA repair pathways.

Building on preclinical work demonstrating increased sensitivity of BRCA1-associated tumors to both DNA-damaging agents and PARP inhibition, multiple studies have evaluated the safety and efficacy of PARP inhibitors alone and in combination with chemotherapeutic agents such as cisplatin, carboplatin, or temozolomide. One such trial conducted by Tutt and colleague sequentially enrolled women to receive one of the following two doses of olaparib: cohort 1−400 mg twice daily (the phase 1 maximal tolerated dose) and cohort 2−100 mg twice daily (lower dose which was pharmacodynamically active and demonstrated

anti-tumor activity in the phase 1 setting). Two pre-planned analyses to assess differences in time to treatment withdrawal or dose escalation between the two arms were included in the trial design. Interim analyses favored the higher dose arm and patients in cohort 2 were offered the option of dose escalation irrespective of response to therapy. Objective responses [maximal dose cohort: 11 of 27 patients/RR 41 % (CI 25–59 %), low dose cohort: 6 of 27 patients/22 % (CI 11–41 %)] and prolongation of median PFS [maximal dose cohort: 5.7 months (CI 4.6–7.4), low dose cohort: 3.8 months (CI 4.6–7.4)] were demonstrated in both cohorts. Therapy was generally well tolerated with mostly grade 1–2 events [89]. This positive proof-of-concept trial strongly suggested the utility of PARP inhibition in this subset of tumors and highlighted the need for further trials to evaluate this approach.

Isakoff and colleagues evaluated the combination of another PARP inhibitor, velaparib (ABT-888), given with temozolomide in a phase II trial of women with MBC. Due to toxicity (grade 4 thrombocytopenia) the initial dose of velaparib was reduced (40 mg twice daily to 30 mg twice daily). The combination of velaparib and temozolomide demonstrated antitumor activity in MBC. However, that activity was limited to patients with known BRCA-mutations. An objective RR of 37.5 %, and a CBR (CBR = CR + partial response + SD > 16 weeks) of 62.5 % and was seen among the BRCA mutation carriers. Furthermore, median PFS of 5.5 vs 1.8 months was observed between the carriers and non-carriers ($p = 0.0042$) [90]. These findings support ongoing studies of this agent in the BRCA carrier population. However, the role of these agents in an unselected population remains an unanswered question requiring further investigation.

The efficacy of iniparib, a third agent in this class, was initially evaluated in a phase II trial of women with TNBC randomized to receive gemcitabine and carboplatin with or without the addition of iniparib. Treatment with the combination of chemotherapy and iniparib demonstrated a significant improvement in clinical benefit rate (CBR = CR + partial response + stable disease (SD) $\geq$ 6 months; 56 vs 34 %, $p = 0.01$), median PFS (5.9 vs 3.6 months HR 0.59, $p = 0.01$), and median OS (12.3 vs 7.7 months HR = 0.57, $p = 0.01$) when compared to chemotherapy alone [91]. However, a subsequent phase III trial of this agent failed to meet its co-primary endpoints of PFS and OS [92] raising many questions in terms of trial design, the choice of appropriate endpoints and whether the "BRCAness" associated with TNBC is an appropriate indicator of which non-BRCA mutation carriers are more likely to respond to PARP inhibition. Moreover, this study highlighted the need for further investigation aimed at better understanding the underlying mechanism of action of each of the agents in this class of drugs [93, 94]. Nevertheless, PARP inhibitors continue to be an area of active investigation in many tumor subtypes and currently at least 11 trials are actively accruing patients to evaluate the use of these agents for the treatment of early-stage and advanced breast cancers.

6.5.2.3 EGFR Inhibitors

EGFR (HER1) is commonly overexpressed in a majority of BLBC/TNBC (60–70 %) [18, 95–98]. In preclinical models of breast cancer, single agent activity of both erlotinib, a TKI targeting EGFR, and cetuximab, a monoclonal antibody to EGFR, were limited [99]. In contrast, when combined with chemotherapy EGFR inhibition demonstrated a signal of antitumor activity [100]. In the clinical setting, EGFR-targeted agents have been examined in the treatment of advanced breast cancer primarily in combination with chemotherapy with mixed results.

Cetuximab

Cetuximab has been studied in combination with various chemotherapeutic regimens in the metastatic setting. In a randomized phase II study of sequential cetuximab followed by carboplatin at the time of progression versus concurrent therapy in women with pre-treated TNBC, single agent cetuximab demonstrated limited activity prompting early closure of the sequential arm of the trial. In comparison, patients who received concurrent cetuximab/carboplatin experienced a response rate of 18 % and clinical benefit of 27 %, defined as partial response or SD $\geq$ 6 months. Despite these findings most patients on this study progressed rapidly with a median PFS of 2 months speaking to the underlying aggressive biology of TNBC [101].

A second study examining the addition of cetuximab (Day 1 then weekly thereafter) to the combination of irinotecan and carboplatin (Day 1, 8) versus chemotherapy alone reported an improvement in objective response rate (49 vs. 30 %) in subgroup analysis of women with TNBC. However, preliminary data from this trial failed to demonstrate improvement in either PFS or OS and increased toxicity prompted dose reductions in both arms. Of note patients who progressed on the chemotherapy alone arm were permitted to crossover and receive cetuximab at the time of progression. Consistent with previously reported studies single-agent cetuximab was minimally active following progression on irinotecan/carboplatin [102].

BALI-1, the largest study to date of cetuximab and chemotherapy (cisplatin) for the treatment of patients with TNBC was recently presented at the 35th annual congress of the European Society for Medical Oncology (ESMO). Among the 173 evaluable patients, who were randomized to receive either cisplatin or cisplatin and cetuximab, a modest improvement in PFS was reported in combination cetuximab/cisplatin arm, 3.7 vs. 1.5 months (HR 0.675 CI 0.470–0.969, $p = 0.032$). In addition a twofold increase was reported in ORR in the experimental arm 20 vs 10.3 % ($p = 0.11$). However, as the study failed to meet its primary endpoint of an overall RR exceeding 20 % among patients who received combination therapy these findings should be interpreted with caution [103]. This trial underscores the need for additional studies to evaluate the suitability of single

Table 6.2 Other potential therapeutic targets for the treatment of triple-negative breast cancer

Target	Agents	Rationale/select list of active trials
Androgen receptor (AR)	Bicalutamide, Enzalutamide (MDV3100), Abiraterone acetate	Preclinical data suggests a subset of hormone receptor-negative breast cancers that are AR + demonstrate AR-dependent tumor growth, which can be abrogated by exposure to AR-inhibitors such as flutamide [19] • Phase II: Bicalutamide for the treatment of androgen receptor positive [AR(+)], estrogen receptor negative, progesterone receptor negative [ER(−)/PR(−)] metastatic breast cancer patients (NCT00468715) • A Phase 1 open-label, dose escalation study evaluating the safety, tolerability, and pharmacokinetics of MDV3100 in patients with incurable breast cancer (NCT01597193) • Phase I/II open label study to evaluate the safety, endocrine effects and anti-tumor activity of abiraterone acetate (CB7630) in patients with estrogen (ER) or androgen receptor (AR) positive advanced or metastatic breast carcinoma (NCT00755885)
Src	Dasatinib	Preclinical studies in BLBC cell lines demonstrate response to Src-inhibition with dasatinib. ER/PR-negative breast cancer cell lines have shown increased sensitivity to combination therapy with dasatinib and chemotherapy [106, 107] • A Phase I–II study of dasatinib in combination with weekly paclitaxel for patients with metastatic breast carcinoma (NCT00820170) • Phase I/II trial of dasatinib plus ixabepilone in 2nd or 3rd line metastatic breast cancer
HSP90	PU-H71	In preclinical models of TNBC PU-H71 was shown to induce durable responses including tumor regression and complete response. The agent was non-toxic in murine models and did not result in the development of resistance over multiple exposures [108] • The first-in-human Phase I Trial of PU-H71 in patients with advanced malignancies (NCT01393509)

(continued)

Table 6.2 (continued)

Target	Agents	Rationale/select list of active trials
mTOR	Everolimus, Temsirolimus	In preclinical models of breast cancer upregulation of mTOR and aberrancies in the PI3 K/AKT signaling pathway increase sensitivity to mTOR inhibitors [109] • Temsirolimus plus neratinib for patients with metastatic HER2-amplified or triple negative breast cancer (NCT01111825) • A study of lapatinib in combination with everolimus in patients with advanced, triple negative breast cancer (NCT01272141) • Phase II trial of RAD001 plus carboplatin in patients with triple-negative metastatic breast cancer (NCT01127763) • A Phase II neo-adjuvant study of cisplatin, paclitaxel with or without RAD001 in patients with triple-negative locally advanced breast cancer (NCT00930930) • Phase I study of combined temsirolimus, erlotinib and cisplatin in advanced solid tumors (NCT00998036)
Human death receptor-5	Tigatuzumab	TRA-8 (an agonistic murine monoclonal antibody against human DR5) has been shown to induce apoptosis in triple-negative tumor cell lines and results in tumor regression in murine models of triple-negative disease both alone and in combination with chemotherapy [110, 111] • An open label, randomized, Phase II trial of abraxane, with or without tigatuzumab (a humanized monoclonal antibody targeting death receptor 5) in patients with metastatic, triple negative breast cancer

agent platinum therapy in this subgroup. Furthermore it remains unknown whether the use of agents, such as cetuximab, will be more effective in a selected population of patients who are known to express the target.

Erlotinib/Gefitinib

TKIs, which target EGFR, are also currently under investigation for the treatment of TNBC and have shown anti-tumor activity when combined with chemotherapy [104, 105]. However, further studies are necessary to better understand the role of small molecule EGFR inhibitors in combination with chemotherapy in the treatment of TNBC (NCT00491816).

6.5.2.4 Other Potential Targets for the Treatment of Triple-Negative Breast Cancer

The development of targeted agents for the treatment of TNBC is an area of active investigation. A number of other novel agents are currently undergoing evaluation in clinical trials (Table 6.2).

6.6 Conclusion

In summary, the triple-negative breast cancer subgroup is distinguished by its biologic heterogeneity, unique clinicopathologic features and risk factors, generally aggressive clinical behavior associated with the development of early metastases and distinct patterns of recurrence, lack of standardized treatments and poor prognosis compared to other breast cancer subtypes. Thus, the effective treatment of patients with TNBC remains a clinically challenging scenario with many unanswered questions. Research in this area is focused on evaluating the underlying biology of this subtype of tumors with the goal of developing improved therapeutic strategies for the management of this difficult to treat population.

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Chapter 7
The Biology of the Deadly Love Connection Between Obesity, Diabetes, and Breast Cancer

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Abstract Breast cancer is the most common malignant disease of women in the world and a leading cause of women's deaths. Many risk factors such as genetics, hormones, aging, and environment have been associated with breast cancer. Interestingly, a large number of epidemiological and clinical studies suggest that obesity and diabetes, especially type-2 diabetes, are associated with higher risk of breast cancer. Similarly, these chronic diseases, such as obesity, diabetes, and cancer, are also a major public health concern in the world. Fifty percent of the United States' population is overweight, thirty percent is obese, and ten percent has diabetes mellitus. Therefore, obesity and diabetes mellitus have been considered as potential risk factors for many cancers but this chapter is focused only on breast cancer. Although the mechanisms responsible for the development of these chronic diseases leading to the development of breast cancer are not fully understood, the biological importance of the activation of insulin, insulin like growth factor-1 (IGF-1) and its receptor (IGF-1R) signaling pathways in insulin-resistance mechanism and subsequent induction of compensatory hyperinsulinemia has been proposed. Therefore, targeting insulin/IGF-1 signaling with anti-diabetic drugs for lowering blood insulin levels and reversal of insulin-resistance could be a useful strategy for the prevention and/or treatment of breast cancer. Increased numbers of clinical studies have demonstrated that the

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administration of commonly used anti-diabetic drugs such as metformin decreases the risk of cancers, suggesting that these agents might be useful anti-tumor agents for the treatment of breast cancer. In this chapter, we will discuss the potential roles of anti-diabetic drug metformin as anti-tumor agents in the context of breast cancer, and will further discuss the potential roles of microRNAs (miRNAs) in the pathogenesis of obesity, diabetes, and breast cancer.

Keywords Obesity · Diabetes mellitus · Breast cancer · Metformin · MicroRNAs (miRNAs) · Body mass index (BMI) · Insulin resistance · Hyperinsulinemia · Insulin-like growth factor-1 (IGF-1) · Adipokines · Chronic inflammation · Oxidative stress · Sex hormones · Hypoxia

7.1 Introduction

As discussed in Chap. 1, breast cancer is the second most deadly malignancy for women with the highest number of newly diagnosed cases in 2012 [1]. Women between the ages of 20 and 59 are at the highest risk for death from breast cancer [1]. Breast cancer affects 121.2 per 100,000 people and its incidence rate is seen more in African Americans than in European Americans [1]. Survival rates have increased between 1987 and 2007 from 84 to 90 % [1], due to the early detection and effective treatment. Dietary pattern in breast cancer patients containing fruits, whole grains and fish, and vegetables but lacking animal fats and red meats has been positively associated with overall survival [2]. After the diagnosis of breast cancer, survival is dependent on appropriateness of treatment, as well as tumor characteristics [2]. Breast cancer has been considered as a disease of aging and is much more common in post-memopausal women than in pre-menopausal women. However, other factors such as genetics, hormones, and environments also contribute to the pathogenesis of breast cancer. A large number of studies have suggested that the most common metabolic disorders such as obesity and diabetes are highly associated with increased risk of breast cancer. However, the detailed molecular mechanisms by which these disorders contribute to the pathogenesis of breast cancer are not fully understood, and thus in this chapter we will summarize the state of our knowledge on the complexities of obesity, diabetes, and breast cancer.

7.2 Obesity and the Risk of the Development and Progression of Breast Cancer

Overweight and obese populations are widespread throughout the world. The World Health Organization (WHO) reported that, in 2008, more than 1.4 billion adults were overweight. Of these over 500 million adults were obese [3, 4]. Currently, 65 % of

the adult population in the United States are considered obese or overweight [5–7], therefore, obesity and overweight adults are a major public health concern. According to the Food and Agriculture Organization (FAO) and WHO, obesity and overweight conditions are diagnosed by the body mass index (BMI), which is measured by dividing kilograms of body weight into meters squared of height. The current categories of BMI are severely obese ($\geq$35.0), obese (30.0–34.9), overweight (25.0–29.9), normal weight (18.5–24.9), and underweight (<18.5) [6, 8, 9].

It is commonly accepted that obesity and being overweight increase the risks of hypertension, type-2 diabetes mellitus, and cardiovascular disease. Similarly, a large number of epidemiological and clinical studies have indicated that high BMI or obesity is positively associated with increased risk of several common cancers including breast cancer [10–19]. High BMI has been under recent study as a risk factor for breast cancer [11, 13, 20, 21]. A majority of emerging studies has shown that high BMI is associated with an increased risk of breast cancer or mortality from being diagnosed with breast cancer [21–26].

In 2004, 495,477 women were studied prospectively, and the results have demonstrated that obese women, with BMI $\geq$ 35.0, had the doubling death rate from breast cancer when compared to women in the lowest BMI category [27]. Multiple studies have shown that high BMI is positively associated with postmenopausal breast cancer, but not premenopausal [28, 29]. However, once breast cancer has developed, high BMI has adverse clinical consequences regardless of the person in either premenopausal or postmenopausal state [2, 22, 28]. These findings suggest that obesity plays an important role in the development and progression of breast cancer.

7.3 The Role of Obesity-Induced Insulin Resistance and Hyperinsulinemia in Breast Cancer

It is well known that obesity is related to aberrant features such as insulin resistance, hyperinsulinemia, glucose intolerance, and the subsequent development of type-2 diabetes mellitus [30–32]. A metabolic consequence of obesity, especially central adiposity, is the development of insulin resistance, which causes an elevation in the secretion of insulin. Increased levels of insulin production lead to compensatory hyperinsulinemia, which enhances insulin resistance [30, 32, 33]. The development of insulin resistance is one of the earliest negative effects of obesity, and is highly associated with the early alteration of glucose metabolism, chronic inflammation, oxidative stress, and deregulations of adipose hormone adiponectin and PPAR-γ, key mediators controlling adipogenesis [32, 34–36]. Moreover, the altered expression of inflammatory cytokines, adipose hormones such as adipokines, and PPAR-γ also leads to heighten the development of insulin resistance in obese subjects [37]. Consequently, insulin resistance and abnormal

glucose metabolism, even in the absence of diabetes, is associated with increased risk for the development of breast cancer [38–41].

It is known that insulin acts as a growth-promoting mitogen by binding to its receptor, leading to the activation of insulin signaling pathways. These growth promoting effects in the tissues have been shown by several in vitro studies [42]. High levels of insulin can enhance the synthesis of insulin-like growth factor-1 (IGF-1) and down-regulate IGF-1 binding proteins in the liver, leading to a higher bioavailability of free or active IGF-1, which activates its downstream signaling pathway through binding to its receptor (IGF-R). IGF-1 has been shown to be associated with cell proliferation and a higher risk of breast cancer [39, 43, 44]. Both insulin and IGF-1 can promote tumor growth by inhibiting apoptosis, stimulating cell proliferation and increasing angiogenesis [45–49]. Thus, one of the major biological contributions of obesity to the progression of breast cancer might be due to the development of insulin resistance, and its secondary impact on increased activity of IGF-1.

7.4 Regulation of Adipocyte-Producing Polypeptide Hormones in Obesity and Breast Cancer

Obesity, especially central adiposity, can impact the secretion and regulation of polypeptide hormones produced by adipocytes in adipose tissues. These adipocyte-produced hormones are known as adipokines, playing essential roles in the secretion, regulation and maintenance of normal metabolic and immune function [37, 50, 51]. So far, more than 50 different adipokines, for examples, adiponectin, leptin, resistin, and ghrelin, etc. have been identified in humans. Emerging evidence suggests that adipokines play a key role in the modulation of insulin sensitivity, glucose and lipid metabolisms, immune response, and angiogenesis [37].

Adiponectin and leptin are the most abundantly expressed adipokines by adipocytes in the body. Adiponectin is a polypeptide with 244 amino acids which is exclusively expressed and secreted by adipose tissue [37]. Adiponectin has been identified to increase insulin sensitivity, decrease insulin resistance, and reduce the risk of type-2 diabetes mellitus [52, 53], which suggests that it is potentially an endogenous negative mediator of diabetes. Contrary to other adipokines that are increased in obesity, the level of adiponectin is decreased in obesity and diabetes mellitus patients along with the subjects who are in the state of insulin resistance [54, 55]. Interestingly, it has been reported that reduction of adipose mass percentage and overall body weight leads to greater levels of adiponectin with a concomitant improvement in insulin sensitivity [52, 56, 57], which further suggest that low levels of adiponectin may lead to the development of insulin resistance in obesity, which is mechanistically associated with diabetes, resulting in the development and progression of cancers including breast cancer.

A number of clinical and experimental studies have demonstrated that adiponectin is similarly associated with the risk of cancers including breast cancer [58–63]. It is also noted that higher levels of adiponectin reduce the risk of cancer, while its low levels increase the risk of cancer [61]. Although the molecular mechanism by which how adiponectin protects against the development and progression of breast cancer is not clear; however, it is possible that in the pathogenesis of breast cancer adiponectin may be interconnected through several direct and indirect mechanisms. First, adiponectin can increase insulin sensitivity and minimize insulin resistance through tyrosine phosphorylation of insulin receptors in muscle tissue [64], leading to the down-regulation of insulin/IGF-1 signaling pathway. Secondly, adiponectin also functions as an anti-inflammatory cytokine, thus it can inhibit the expression of inflammatory cytokines, such as TNF-α and IL-6, and restrict the activation of NF-κB [65], which is likely to be associated with these chronic diseases. Therefore, the low levels of adiponectin promote the expression of inflammatory cytokines in obese subjects, which lead to subsequent development of diabetes and cancers. Moreover, several experimental studies have demonstrated that adiponectin could affect tumorigenesis more directly through the deregulation of the AMPK signaling pathway at a local tissue level [66, 67]. Adiponectin has also been shown to inhibit angiogenesis through the activation of PPAR-γ and cell proliferation by the induction of apoptosis in vivo through the activation of the caspase cascade [68], which could contribute to the anti-diabetic and anti-cancer effects of adiponectin. Together, these findings suggest that lower levels of adiponectin in obese subjects may be the risk factors in the pathogenesis of breast cancer mediated through complex deregulation of multiple signaling pathways.

Another adipokine leptin is expressed and secreted by adipocytes, which is positively associated with adiposity and insulin function [69]. By binding to its receptors in the hypothalamus, leptin activates cellular signals to suppress appetite and increasing energy expenditure. Insulin acts as a positive feedback on leptin gene expression and it has been shown that leptin and insulin levels are increased in obesity [69]. Interestingly, epidemiological and mouse model studies have demonstrated that leptin is positively associated with increased risk of cancers including breast cancer [38, 52, 63, 70–74]. It has also been shown that leptin functions as a mitogen for the growth and differentiation of a number of different cells including cancer cells [71, 75]. Several other experimental studies have shown that leptin exerts its anti-apoptotic and pro-angiogenic activities mediated through deregulation of vascular endothelial growth factor (VEGF). In addition, the binding of leptin to its receptor induces the signaling pathways of PI3K/Akt/MAP kinase and STAT, which are critical for cell survival, growth, proliferation and differentiation [69, 76]. Therefore, leptin may play a key role in the pathogenesis of breast cancer; however, further in-depth studies are warranted to fully understand the mechanistic role of leptin in obesity, diabetes and cancer.

7.5 The Role of Obesity-Associated Chronic Inflammation in the Pathogenesis of Breast Cancer

Chronic inflammation is well known to participate in the development and progression of tumors including breast cancer [14, 77–79]. It is known that obesity has been positively associated with chronic inflammation [80–82]. Obesity-associated chronic inflammation reflects one of the key features of the dysfunction of adipose tissue. Local inflammatory responses include macrophage infiltration and resistance to hormones such as insulin and leptin. Systemic inflammatory responses assessed by the increased production of inflammatory cytokines such as TNF-α, IL-1, IL-6, C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), fibrinogen and tissue-specific hormone activity through the activation of NF-κB and PPAR-γ signaling pathways, appears to be mechanistically linked with chronic inflammation and obesity [81, 83, 84]. The loss of body weight leads to a decrease in the levels of inflammatory markers whereas weight gain leads to an increase in these markers [85]. These findings suggest that body weight control may inhibit chronic inflammation, leading to a reduction in the risk for the pathogenesis of breast cancer.

Although the pathogenesis of obesity-related inflammation is not fully understood, sufficient evidence suggests that increased levels of inflammatory cytokines and proteins such as matrix metalloproteinases (MMPs), key mediators of cell invasion and metastasis, are associated with the high risk of cancers including breast cancer [77, 78, 86–91]. It is also known that insulin/IGF-1 signaling pathway plays an important role in the development and progression of breast cancer. Chronic inflammation also promotes the development of insulin resistance [92], which may eventually contribute to the increased risk of breast cancer as described earlier. Therefore, obesity-induced chronic inflammation appears to contribute to the development of breast cancer.

7.6 The Role of Obesity-Associated Oxidative Stress in Breast Cancer

Oxidative stress is a common phenomenon generated by an imbalance between the production of reactive oxygen species (ROS) and the detoxification of reactive intermediates in the body. The maintenance of oxygen homeostasis is important for normal health. The evidence suggests that obesity may cause systemic oxidative stress, if unresolved, leads to genomic DNA damage and inflammation, contributing to the development of tumors including breast cancer. Increased oxidative stress caused by chronic exposure to high levels of glucose and lipids, and decreased activities of antioxidants and relevant enzymes in the accumulated adipose tissue creates the dysfunctions of adipose tissues such as altered expression of adipokines and cytokines [93]. The data from several human and animal studies

have suggested that obesity is involved in lipid peroxidation, which is an aberrant interaction of lipids and oxygen [37]. It has been found that obese, non-diabetic subjects have elevated levels of lipid peroxidation [93]. Obese mouse models have shown similar increase in the lipid peroxidation along with a decrease in the activity of antioxidants [93]. These findings suggest that obesity could induce oxidative stress, which in turn, could induce the aberrant changes of a variety of biological processes such as genomic DNA damage, inflammation, apoptosis, and insulin resistance, contributing to the pathogenesis of cancer [94, 95]. ROS can also induce chronic inflammation by the activation of NF-κB, which has significant impact on the regulation of adiponectin in adipose tissue [37, 96, 97]. Therefore, obesity-induced oxidative stress may contribute to the increased risk of breast cancer; however, the detailed mechanism requires further investigation.

7.7 Altered Regulation of Sex Hormones in Obesity and Breast Cancer

Sex hormones are known to play important roles in the maintenance of homeostasis between cellular differentiation, proliferation and apoptosis. Deregulations of these hormones may promote cancer cell growth [11, 98]. The deregulation of sex hormones is also another key feature of obesity. Obesity induced high levels of insulin along with IGF-1 which can inhibit the synthesis of hormone binding globulin (SHBG) in the liver, the only source of SHBG synthesis [99]. SHBG is a major transporter protein for sex hormones such as testosterone and estradiol in the blood. Thus, a reduced level of SHBG results in an increase in the levels of unbound sex hormones, leading to the increased bioavailability of sex hormones to activate cellular signaling pathways [100]. Furthermore, adipose tissue is a major site for the synthesis of estrogens from androgenic precursors [101], which are known to play key roles in the pathogenesis of breast cancer.

A number of epidemiological and clinical studies have demonstrated that increased risk of various cancers including breast cancer associated with high BMI or obesity, especially central adiposity, is probably induced by the alterations in the levels of free sex hormones in the obese subjects [52, 102, 103]. For example, increased risk of postmenopausal breast cancer is associated with a low plasma level of SHBG, and an increased level of total and free androgens and estrogens [104]. These findings suggest that obesity-related alteration of sex hormone levels appear to contribute to the development and progression of breast cancer.

7.8 The Role of Adipose Tissue Hypoxia in Breast Cancer

Hypoxia is one of the fundamental features increasingly being recognized as important for the development and progression of solid tumors including breast cancer [105–108]. Hypoxia-inducible factor (HIF), a master transcription factor is known to control the expression of hypoxia responsive genes, which plays critical roles in increased cell proliferation, survival, angiogenesis and metastasis by the regulation of multiple cell signaling pathways, as reviewed elsewhere [109]. It has been demonstrated that tumor hypoxia and increased expression of HIF have been found to be associated with radio-chemotherapy resistance, cell invasion and metastasis, and poor outcome of patients diagnosed with solid tumors [108, 110, 111]. Hypoxia has been observed in the white adipose tissue in obese mice [112–114]. Obesity-associated hypoxia, especially in adipose tissue, is reported to participate in the development of insulin resistance, increased chronic inflammation, reduced excretion of adiponectin, and increased gene expression of leptin [113–115]. A number of experimental studies have suggested that hypoxia plays an important role in the maintenance of the phenotype and function of normal and malignant stem cells including breast cancer stem cells. Therefore, adipose tissue hypoxia might play a key role in increased risk of cancers including breast cancer.

Recently, it has been shown that HIF-1α increases the expression of leptin in colorectal and breast tumors [71, 73, 80]. High levels of insulin induce HIF-1 α-mediated expression of leptin in breast cancer cells, contributing to disease progression [38, 71]. Furthermore, obesity-induced hypoxia and HIF-1α increases the expression of MMPs and VEGF, suggesting that obesity-induced hypoxia and HIF-1α could be involved in the angiogenic and metastatic processes of tumors [115]. It is also known that hypoxia-induced of HIF proteins such as HIF-1α and 2α are involved in the regulation of stemness in a variety of cancers including breast cancer. These findings are consistent with clinical data showing that the levels of hypoxia-induced target VEGF, regulated mainly by HIF-1α and NF-κB, increases with high BMI [116]. Thus, obesity-induced hypoxia and HIF-1α may play a key role in the development and progression of breast cancer.

7.9 Diabetes Mellitus and the Risk of Breast Cancer

Diabetes mellitus is a common metabolic disorder with hyperglycemia, eventually impairing all systems in the body, and it is highly prevalent in the world. There are two types of diabetes mellitus, type-1 and type-2. In adults, type-2 diabetes mellitus accounts for 90–95 % of all diagnosed cases of diabetes mellitus. One of the main consequences attributed to diabetes mellitus is the impairment of multiple tissues and systems, including the cardiovascular and immune response systems. Moreover, the relationship between diabetes mellitus and the risk of tumorigenesis

has been investigated for more than a decade as briefly stated under obesity and the risk of breast cancer.

A large number of epidemiological and clinical studies have suggested that diabetes mellitus, particularly type-2 diabetes mellitus, is positively associated with the high risk of development of various common cancers including breast cancer [117–121]. In 2010, a meta-analysis of eight studies was conducted to examine the relationship between breast cancer and diabetes mellitus [122]. The results demonstrated a strong relationship between pre-existing diabetes mellitus and mortality from breast cancer. Three studies have clearly shown a positive association between pre-existing diabetes and the clinical state of breast cancer [123–125]. In one study, patients with breast cancer and diabetes were more likely to receive hormonal therapy and surgery [125]. Another study showed that patients with diabetes and breast cancer had an increased risk of being hospitalized for chemotherapy toxicity [124]. One recent clinical study confirms that diabetes is an independent predictor of low breast cancer-specific survival and overall survival [126]. These findings suggest that diabetes plays a role in the pathogenesis of breast cancer and perhaps the overall clinical outcome of patients diagnosed with breast cancer.

7.10 Diabetes Mellitus, Insulin/IGF-1R Signaling Pathways and the Risk of Breast Cancer

Current evidence suggests that diabetes mellitus is positively associated with increased risk of breast cancer; however, the exact molecular mechanism(s) has not been fully understood. Similar to the role of obesity in the development of breast cancer, insulin resistance and induced compensatory hyperinsulinemia are believed to be some of the most common underlying mechanisms that are causally association with diabetes mellitus and breast cancer.

It is known that insulin resistance and altered glucose metabolism are common pathological features for both obesity and type-2 diabetes mellitus. Moreover, diabetes mellitus has a more extensive degree of insulin resistance, hyperinsulinemia, and hyperglycemia, which may contribute to the development of breast cancer. Several clinical studies have demonstrated that high levels of insulin, increased fasting, and post-prandial blood glucose increase the risk of breast cancer [38, 41, 63, 127–130]. A high level of insulin stimulates the insulin/IGF-1R signaling pathway via insulin receptor substrates (IRS). In most cell types, these substrates mediate the PI3K/Akt/mTOR signaling pathway [131], which is known to play a pivotal role in the proliferation and survival of various cancers including breast cancer. Insulin use for controlling diabetes mellitus in humans has been shown to have an increased risk for the development of cancers including breast cancer [38, 72, 73, 132]. It is known that hyperinsulinemia increases IGF-1 activity by increasing its synthesis and inhibiting its binding protein synthesis. Thus, the

increased levels of insulin activity and consequently higher levels of free IGF-1 promote cell proliferation, inhibit apoptosis and enhance angiogenesis, all of which can contribute to the development and progression of breast cancer [38, 47, 71–73, 132, 133]. Insulin/IGF-1 could also up-regulate PI3K/Akt/mTOR signaling pathway by activation of IRS 1–4 [134], leading to the pathogenesis of cancers including breast cancer. Moreover, the activation of insulin/IGF-1 receptors by insulin can interplay positively with the hedgehog-signaling pathway, which regulates cancer cell growth by regulating cell proliferation and differentiation [135]. It is known that breast cancer cells express high levels of insulin, IGF-1R, and IRS-1,2 [136], suggesting that insulin/IGF-1R signaling pathway may play an important role in the pathogenesis of breast cancer. In contrast, the inhibition of IGF-1R has been shown to increase the sensitivity of colon cancer stem-like cells to chemotherapeutic drugs [137]. However, the cell signaling pathways regulated by insulin/IGF-1 receptors in breast cancer have not been fully elucidated, suggesting that further mechanistic studies are required to investigate the role of this signaling pathway in the pathogenesis of breast cancer.

7.11 The Role of Anti-diabetic Drug Metformin in Breast Cancer

It is known that anti-diabetic drugs are being routinely used to decrease blood insulin levels and increased insulin sensitivity in diabetes mellitus patients. Due to the relationship of diabetes mellitus with breast cancer, and existing knowledge on the roles of insulin/IGF-1R signaling pathways in the development and progression of breast cancer, anti-diabetic drugs have been widely investigated for their use in the prevention and/or treatment of cancers such as breast cancer for more than a decade. Currently, increased attentions have been paid to the anti-diabetic drug metformin for its benefit in the treatment of patients diagnosed with breast cancer.

Metformin, a biguanide class of oral hypoglycemic agents, is the most widely used anti-diabetic drug for the treatment of type-2 diabetes mellitus in the world. The primary systemic effect of metformin is to decrease the levels of blood glucose through the inhibition of hepatic gluconeogenesis and up-regulation of glucose uptake in peripheral tissues, including skeletal muscles and adipose tissue [138]. It also increases insulin sensitivity which results in decreased levels of insulin. Metformin is non-toxic and well tolerated. A large number of epidemiological studies have demonstrated that the administration of metformin in diabetes mellitus patients exhibits a protective effect by decreasing incidence of different tumors and improving prognosis of patients diagnosed with cancers [139–143]. Its protective roles in the pathogenesis of tumors prompted its investigation into its anti-tumor effect on site-specific tumors. Specifically, clinical studies in both diabetic and non-diabetic patients clearly suggest that metformin may decrease the risk of developing cancers including breast cancer. Furthermore, several

randomized trials have demonstrated its protective role when used as adjuvant therapy and for the prevention of breast and colorectal cancers [144–147].

For example, one recent study showed that breast cancer patients taking metformin had a pathologic complete response rate of 24 % as compared to 8 % of the patients who did not receive metformin [148]. Therefore, metformin appears to exert a protective role against the development and progression of breast cancer. In several other studies, metformin has shown promising results when treating breast cancer [149–151], suggesting its protective role in the pathogenesis of breast cancer.

A number of experimental studies have demonstrated that metformin can inhibit tumor growth in xenograft animal models [152–155]. The anti-tumor activity of metformin is probably related to its direct and indirect mechanisms although the detailed molecular mechanisms in support of these findings are not fully understood. It has been shown that increasing insulin sensitivity and decreasing insulin level by metformin could inhibit cancer cell growth by activation of AMP kinase (AMPK), which in turn inhibits mTOR signaling [156, 157]. Specifically, when the ratio of cellular AMP/ATP is increased, AMPK is activated, resulting in the down-regulation of the PI3K/Akt/mTOR signaling pathway by phosphorylation of mTOR. The mTOR is usually activated by mitogenic-responsive pathways such as Ras/ERK and PI3K/Akt as well as pathways that signal the availability of intracellular energy and nutrients such as glucose and amino acids. However, the inhibition of mTOR pathway by metformin through activation of AMPK leads to a rapid inhibition of cellular protein synthesis and growth [144–146]. Moreover, metformin can directly inhibit cancer cell growth and proliferation through the regulation of cyclin D1-mediated cell cycle, p53 expression and phosphorylation in various cancer cells including breast cancer [158–163]. Metformin has also been found to inhibit the production of inflammatory cytokines such as TNF-α and IL-6 as well as angiogenic cytokine VEGF, by inactivation of NF-κB and HIF-1α [164–166]. It has also been found that metformin could induce apoptosis and inhibit cell growth and proliferation in a variety of cancer cells by inhibition of insulin/IGF-1 pathway through the activation of AMPK [167, 168]. The data from in vitro *and* in vivo studies clearly suggest that metformin can block tumor growth by inactivation of breast cancer stem-like cells and epithelial-to-mesenchymal transition (EMT) phenotypic cells, and these cells are currently believed as one of the major causes of tumor recurrence and metastasis [150, 169]. Therefore, the rational application of metformin could have dual targeting for controlling the complexities of diabetes mellitus and breast cancer. Recently, we have demonstrated that metformin decreases cells growth, clonogenicity, cell migration, and the CSC self-renewal capacity, consistent with the inhibition of the expression of CSC surface markers CD44 and EpCAM, and up-regulation of anti-oncogenic miRNAs such as miR-101 in pancreatic cancer cells [170]. Other investigations continue to document similar conclusions on the biological activity of metformin against breast and other cancer cells. These findings suggest that metformin can have an anti-tumor effect

mediated through the regulation of multiple signaling pathways that are associated with tumor aggressiveness contributed by the presence and enrichment of CSCs.

7.12 The Regulatory Role of miRNAs in Obesity, Diabetes Mellitus and Breast Cancer

The microRNAs (miRNAs) are small non-coding RNAs with around 21–24 nucleotides long that act as regulators of genes at the post-transcriptional levels by binding to 3′-untranslated region of target genes, leading to the degradation of target mRNAs or inhibition of translation. Currently, miRNAs are believed to regulate the expression of most genes, and consequently they play critical roles in a wide range of biological processes such as cell differentiation, proliferation, death, metabolism and energy homeostasis [171, 172]. A large number of miRNAs have been reported to be associated with chronic diseases such as obesity, diabetes mellitus and cancers, and are considered to be mechanistically associated with these chronic diseases. However, their exact role in the pathogenesis of obesity, diabetes mellitus, and breast cancer are not fully understood.

A number of studies have shown that miR-21 is an oncogenic molecule and is up-regulated in various cancer cells [173–179]. Over-expression of miR-21 results in decreased expression of PTEN, a known tumor suppressor in cancer cells [173]. The miR-21 has also been reported to show anti-apoptotic, proliferative, invasive and angiogenic properties in cancer cells [179–181]. Additionally, it has been found that high level of glucose increases the expression of miR-21 and suppress PTEN, with a concomitant increase in the phosphorylation of Akt in renal cells [182]. An animal experimental study has shown that high-fat diet-induced obesity increases the expression of miR-21 in adipocytes of mice [183]. Moreover, decreased expression of miR-200 family, potential tumor suppressor molecules, has been observed in many cancer cells including breast cancer [179–181], suggesting that altered expression of these miRNAs may contribute to the invasiveness and metastatic characteristics of breast cancer. The possible roles of these two miRNAs in obesity and diabetes mellitus associated with the development of breast cancer require further studies.

Let-7 family, potential anti-tumor molecules, is abundantly expressed in pancreatic islet cells and considered to be important regulators of glucose metabolism [184–188]. Let-7b has been found to regulate the expression of PPAR-γ in adipocytes. In the pathogenesis of cancer, let-7 has been found to be down-regulated, thereby increasing the expression of Ras, c-Myc, Lin28 in malignant cells [175, 177, 179]. The down-regulation of let-7 family expression has been identified in breast cancer [189, 190]. The data showed that let-7 inhibits Lin28-mediated insulin-PI3K-mTOR signaling pathway; however, recent experimental studies have shown that over-expression of let-7 in mice results in impaired glucose tolerance and reduced glucose-induced pancreatic insulin secretion, consistent

with decreased fat mass and body weight. The knock-down of let-7 prevents impaired glucose tolerance in mice with high-fat diet-induced obesity, consistent with increased lean and muscle mass [187]. Therefore, further investigation is required to elucidate the direct mechanistic role of let-7 in the pathogenesis of obesity, diabetes mellitus and breast cancer.

Another class of miRNAs, miR-34a and miR-146a have been found to be highly expressed in obesity and diabetes mellitus [186, 191, 192], and over-expressions of miR-34a and miR-146a are known to cause alterations in glucose-stimulated insulin secretion and the induction of apoptosis in β-cells [186, 191]. In contrast, miR-34a has been shown to be induced by p53 and exhibit potent anti-proliferative and pro-apoptotic activities by targeting Notch-1 and CD44 in various cancer cells including breast cancer [175, 193]. Studies have also shown that miR-146a have angiogenic activity [186, 191], and altered expression of miR-34a and miR-146a are typically found in breast cancer cells [194–198] although the exact role of these miRNAs in obesity, diabetes mellitus, and breast cancer has not been fully investigated.

The increased expression of miR-143 has been found in obesity, diabetes mellitus, and breast cancer [185, 186, 197, 199]. It has been shown that one target gene of miR-143a is MAPK7, which is involved in the regulation of MAPK signaling pathway. While, over-expression of miR-143 is related to deregulation of PPAR-γ in adipose tissue of obese mice [185, 186, 197], the potential role of miR-143 in the development of breast cancer associated with obesity and diabetes mellitus is not clear, suggesting that further in-depth mechanistic studies are warranted for elucidating the possible role of miR-143 in the development of breast cancer in obese and diabetes mellitus patients.

The expression of miR-29 has been found to be increased in the insulin targeted tissues [184]. Over-expression of miR-29 inhibits insulin-stimulated glucose uptake, leading to the development of insulin resistance [184, 186, 191]. Several clinical and experimental studies have shown that the expression of miR-29 is up-regulated in obesity and diabetes mellitus animals and diabetes mellitus patients [184–186, 200]. Moreover, miR-29 was also found to be up-regulated in breast cancer cells [201, 202]. These findings suggest the potential role of miR-29 in obesity, diabetes mellitus, and breast cancer, and thus novel strategies by which specific miRNA could be down-regulated or up-regulated would become a novel approach for the treatment of chronic diseases including obesity, diabetes and breast cancer. Further studies have shown that miR-375 is highly expressed in β-cells in pancreatic islet. It down-regulates glucose-stimulated insulin secretion by controlling the expressions of 3′-phosphoinositide-dependent protein kinase-1 (PDK1), a key regulator of β-cell function, and myotrophin, a regulator of insulin secretion [186, 191]. An increased level of miR-375 has been found in obesity and type-2 diabetes mellitus patients [186, 203] where it represses insulin secretion. Conditional loss of miR-375 expression leads to an increase in insulin secretion [186, 203]; however, miR-375 knockout mice display marked hyperglycemia, suggesting that miR-375 is essential to regulate the insulin homeostasis [203]. Interestingly, the expression of miR-375 has been reported to be increased in

breast cancer cells [204, 205]. Increased expression of miR-375 results in the loss of cellular organization and acquisition of a hyperplastic phenotype [205]. These findings suggest a potential role of miR-375 in the pathogenesis of breast cancer associated with obesity and diabetes mellitus although mechanistic studies are lacking. The above sections underscore the importance of miRNA in the pathogenesis of chronic diseases; however, more in-depth mechanistic studies are warranted in order to fully appreciate the roles and therapeutic potential of miRNA targeting agents in the field of obesity, diabetes and cancer especially for the treatment of breast cancer.

7.13 Conclusions and Perspectives

A large number of epidemiological and clinical studies have provided solid evidence supporting a clear association between obesity and diabetes, which is also positively associated with increased risk of breast cancer, suggesting that both obesity and diabetes are additional risk factors for breast cancer. Insulin resistance and the induction of compensatory high levels of insulin, leading to the aberrant regulation of glucose and lipid metabolism, are common characteristics of obesity and type-2 diabetes mellitus, which appear to be the primary contributors to the pathogenesis of breast cancer mediated through deregulation of insulin/IGF-1R signaling pathways. Increased levels of insulin, fasting and post-prandial blood glucose have also been reported to be related to the high risk of breast cancer. Thus, targeting insulin/IGF-1 signaling by anti-diabetic drugs which will cause lowering of blood insulin level, could be useful for the prevention and/or treatment of breast cancer although further novel well thought-out clinical trial design is warranted. A large number of epidemiological and clinical studies have also demonstrated that the administration of anti-diabetic drug metformin, decreases the risk of cancers including breast cancer, suggesting that this drug could be useful anti-tumor agents for breast cancer especially because metformin could effectively eliminate cancer stem cells (CSCs) or EMT phenotypic cells which are believed to be the root cause of tumor maintenance, tumor recurrence and metastasis. Experimental studies have also shown that metformin can inhibit breast cancer cell growth by different mechanisms including alterations in the expression of genes mediated by the deregulation of miRNAs. Although the precise roles of miRNAs in the pathogenesis of obesity, diabetes mellitus and breast cancer are only beginning to be understood, selective up-regulation and down-regulation of important miRNAs such as miR-21, let-7, miR-29, miR-34a, miR-134, miR-146a, and miR-375 could become novel and newer strategies for the treatment of obesity, diabetes mellitus and breast cancer in the future (Fig. 7.1). Overall, the future looks brighter than ever before for exploring miRNA-targeting strategies by novel approaches for the management of chronic diseases such as obesity, diabetes and cancer especially in subjects who are at high risk for the development of breast cancer.

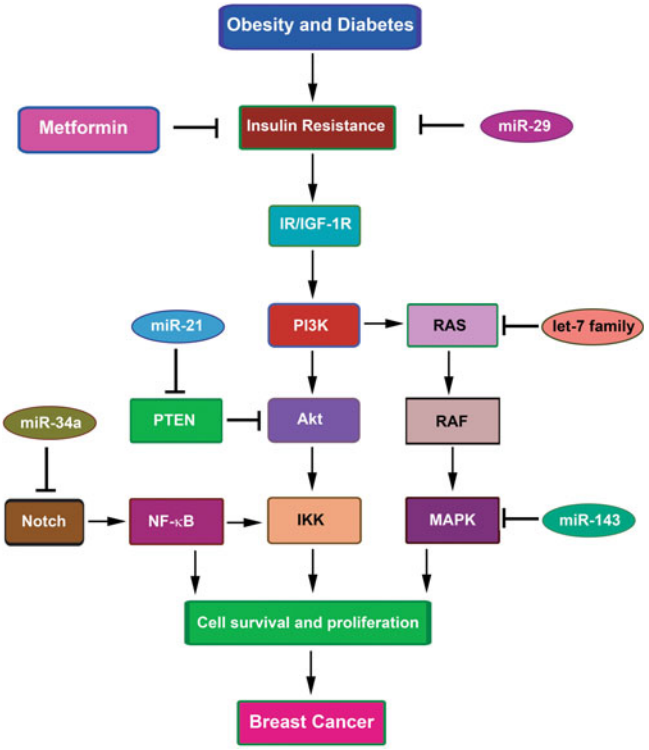

Fig. 7.1 The potential roles of miRNAs and metformin in obesity- and diabetes breast cancer (→: indicating an activation; ⊢: indicating an inhibition)

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Chapter 8
Progression of Early Breast Cancer to an Invasive Phenotype

Connor D. MacMillan, Ann F. Chambers and Alan B. Tuck

Abstract Histological and molecular evidence has led to a model of breast cancer progression in which cells from the terminal duct lobular unit give rise to atypical ductal hyperplasia or atypical lobular hyperplasia, which can progress to ductal carcinoma in situ or lobular carcinoma in situ, and eventually to invasive ductal carcinoma or invasive lobular carcinoma respectively. This review will present a histomorphological and epidemiological overview of the pre-invasive stages of breast cancer progression. As there is mounting evidence that these stages are likely rough phenotypes of underlying molecular changes, current knowledge regarding changes in genetic and epigenetic features of breast cancer progression will also be discussed. Microarray and CGH-based studies will be described, which suggest that low- and high-grade breast cancers can arise from normal terminal ducts through two distinct molecular pathways. Various in vitro and in vivo models used to study the cellular and molecular changes involved in early breast cancer progression will be presented. Lastly, the specific transition from pre-invasive to invasive breast cancer will be addressed, including possible molecular predictors of the invasive phenotype and a contemporary view highlighting the involvement of the tumor microenvironment during the transition to invasive disease.

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8.1 Introduction

Breast cancer continues to be a major health concern among women worldwide. In North America, there has been a decreasing trend in the mortality rate of breast cancer over the last several decades [1,2]. This is likely due to increased screening and improved diagnostic recognition of early curable stages. However, there will still be approximately 45,000 deaths due to metastatic breast cancer in North America in 2012 [1,2]. There continues to be a clinical need for molecular biomarkers that can predict which non-invasive breast cancers are likely to progress to malignancy. An important event in the progression of breast cancer is the transition from a pre-invasive lesion to an invasive phenotype. Upon diagnosis of an in situ lesion, 10–15 % of women develop subsequent invasive disease [3]; hence, there is a clinical problem of predicting which pre-invasive lesions are likely to progress to malignancy.

8.2 Histopathologic Description of Breast Cancer Progression

Evidence has led to a histological model of breast cancer progression in which cells from the terminal duct lobular unit give rise to atypical ductal hyperplasia (ADH) or atypical lobular hyperplasia (ALH), which can in turn give rise to ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), and eventually to invasive ductal carcinoma (IDC) or lobular carcinoma (ILC) respectively (Fig. 8.1) [4–9]. In this chapter, breast cancer progression will first be discussed from histomorphological and epidemiological perspectives, followed by molecular evidence to support the view of this progression model.

8.2.1 A Histopathological Overview of the Pre-invasive Stages of Breast Cancer

In order to provide a contemporary overview of the current molecular-based model of breast cancer progression, this section will build a conceptual framework of progression from normal breast tissue to the pre-invasive stages of breast cancer from a histomorphological perspective (Fig. 8.1).

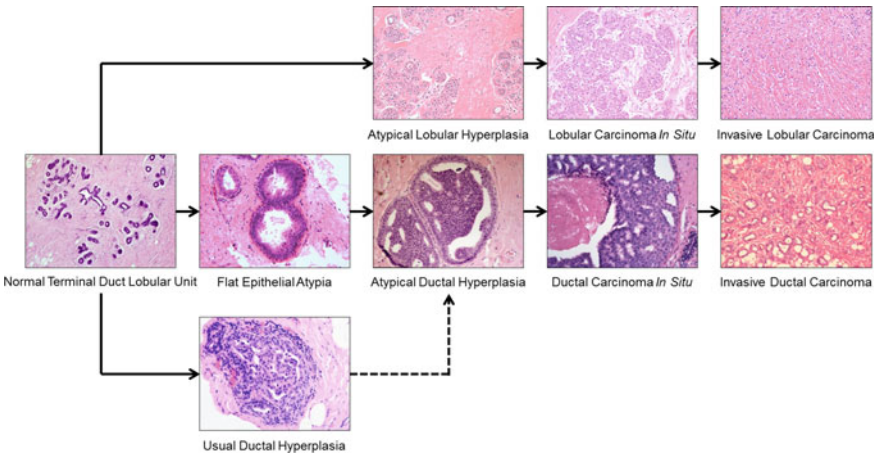

Fig. 8.1 Traditional linear model of breast cancer progression. Multiple lines of evidence (histomorphological, immunohistochemical, and molecular) support this model. Molecular alterations occurring in the normal terminal duct lobular unit (*TDLU*) can result in flat epithelial atypia (*FEA*). FEA may lead to additional changes that give rise to atypical ductal hyperplasia and ductal carcinoma in situ, upon which subsequent alterations in turn give rise to invasive ductal carcinoma (*middle*). Likewise, molecular alterations occurring in the normal TDLU result in atypical lobular hyperplasia, which can give rise to lobular carcinoma in situ, upon which subsequent alterations in turn give rise to invasive lobular carcinoma (*top*). There is some evidence that usual ductal hyperplasia may in some instances also be considered an early stage of breast cancer progression (*bottom*)

The human breast is composed of thousands of small glands lined by epithelial cells that produce milk. These glands are composed of a single terminal duct with multiple end acini (terminal ductules in the non-functioning state) and are referred to as the terminal duct lobular unit (TDLU). Once milk is secreted from cells of the TDLU, it is propagated outward through a series of interconnecting and increasingly larger ducts. The TDLU is composed of two cell layers: (a) an inner luminal epithelial layer composed of low columnar cells in the terminal duct and cuboidal cells in the acini/terminal ductules, and (b) an outer myoepithelial layer directly adjacent to the basement membrane. Pre-invasive epithelial lesions are characterized by a neoplastic epithelial cell proliferation, which remains confined to the ductal-lobular network and does not penetrate the basement membrane or invade into the surrounding stroma.

The two most common histologic types of invasive breast cancer are known as infiltrating ductal (also known as "no special type, NOS") and lobular carcinoma. These are matched by pre-invasive ductal and lobular neoplasias. Both types of breast cancers arise in the TDLU and the distinction between the two is based on morphological differences of the cells [10, 11]. Specifically, the lobular morphology consists of small, non-polarized cells that are discohesive, with vacuolated cytoplasm and a high nuclear to cytoplasmic ratio, resembling cuboidal cells of breast acini/terminal ductules. In contrast, the ductal morphology consists of larger, polarized cells in cohesive groups that resemble columnar cells of terminal

ducts. The pre-invasive lobular lesions include atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS). Pre-invasive ductal lesions include atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and possibly some columnar cell lesions, such as flat epithelial atypia (FEA). In addition, although more controversial, there is some evidence that usual ductal hyperplasia (UDH) and an entity known as "unfolded lobules" may in some instances also be considered early stages (non-obligate precursors) of breast cancer progression [8, 12].

FEA, ADH, and DCIS are considered non-obligate precursors of invasive ductal carcinoma (IDC). FEA is characterized by a proliferation and replacement of luminal cells of the TDLU by one or more layers of columnar epithelial cells that exhibit low-grade cytological atypia [13]. The cells of FEA may form either a single cell layer or multiple cell layers [14], such that FEA by present definition is comprised of both columnar cell change with atypia (1-2 cell layers) and columnar cell hyperplasia with atypia (multiple cell layers). Like FEA, ADH is also characterized by low-grade cytological atypia, but differs from FEA in that it exhibits architectural abnormalities such as solid patterns with even cell placement, punched-out secondary lumena, rigid bridging and cribriform or micropapillary morphologies. The differences between ADH and DCIS are based upon the degree of atypia and the extent of the atypical epithelial proliferation [15, 16]. DCIS is further classified based on cytomorphological (low, intermediate, or high nuclear grade) and architectural features, as well as the presence or absence of luminal necrosis, all of which have been associated with outcome. Comedo-type DCIS consists of cells that show a high degree of nuclear atypia and is associated with abundant central luminal necrosis. Comedo-type DCIS is generally more aggressive in terms of both risk for recurrence (with narrow margins of excision) and risk for associated invasion. Specific architectural types of DCIS also have different implications in terms of clinical behavior. For example, micropapillary type DCIS tends to be very extensive in the breast [17], whereas a centrally located papillary carcinoma in situ is more commonly a localized lesion with lower risk for recurrence upon complete excision [18]. Lastly, with the transition to invasive disease, important distinguishing factors between DCIS and IDC are the complete loss of the outer myoepithelial layer in the latter, with extension of neoplastic cells into the surrounding stromal compartment, beyond the basement membrane [19].

Lobular neoplasias form a spectrum of diseases and include ALH and LCIS, both of which are considered non-obligate precursors of invasive lobular carcinoma (ILC) [20, 21]. The main histological distinction between ALH and LCIS is based on the degree to which the TDLU is filled with neoplastic cells and the amount the lobular unit becomes distended as a result [4]. In ALH, the TDLU is colonized by a homogenous cell population of small, round, non-polarized, loosely cohesive cells that have a high nuclear to cytoplasmic ratio. The proliferation of ALH is limited (by definition involves less than 50 % of acini of a lobular unit) and leaves the acini/terminal ductules somewhat intact (lack distension/distortion). Conversely, cells of classical LCIS are the same cytomorphologically compared to ALH, but proliferation is extensive enough to completely fill and distend/distort the acini/terminal ductules of the TDLU. The loss of expression of membrane

E-cadherin is a hallmark feature of both ALH and LCIS [22]. Variants of LCIS have been described, including a pleomorphic variant, which consists of medium- to large-sized cells, with pleomorphic nuclei, and LCIS with central zonal ("comedo type") necrosis [23, 24].

8.3 Epidemiological Evidence of Breast Cancer Progression

Epidemiological studies have provided support for a linear model of breast cancer progression. Through long term cohort studies it has been shown that having a previous ADH or DCIS diagnosis greatly increases the risk of developing invasive mammary carcinoma, up to 4–5 times for ADH and up to 8–10 times for DCIS compared to the general population [4, 5, 25]. In addition, the relative risk positively correlates with grade, extent and presence/absence of zonal necrosis. Similarly, the risk of invasive disease in women diagnosed with LCIS (classic type) is estimated at 8–10 times greater than women in the general population [25]. The relative risk associated with a finding of FEA is not yet well-established, but studies to date suggest the risk for developing DCIS or invasive mammary carcinoma varies from a slightly increased risk to an increase in risk similar to ADH [13, 14, 26, 27]. Although epidemiologic data would suggest possible precursor status of usual ductal epithelial hyperplasia as well (1.5–2 fold increased risk for mammary carcinoma), molecular (loss of heterozygosity) studies indicate that this is likely a rare event [28].

8.4 Molecular Evidence of Breast Cancer Progression

The histological patterns observed during breast cancer progression are likely rough phenotypic indications of underlying molecular changes. There is interest in identifying the cellular and molecular events involved to determine which lesions are more likely to progress. An important barrier in understanding these changes has been the inability to accurately assess the molecular events as they relate to progression. Highly specific tissue-microdissection technologies and rapidly evolving high-throughput genomic and transcriptomic analyses have combined to identify a number of genomic and gene expression correlates between different stages of breast cancer.

8.4.1 Molecular Features of Ductal Carcinoma Progression

DCIS forms a spectrum of neoplastic lesions, with some behaving more aggressively than others. These different behaviors are to some degree associated with morphologic characteristics, as described above; however, it has been further

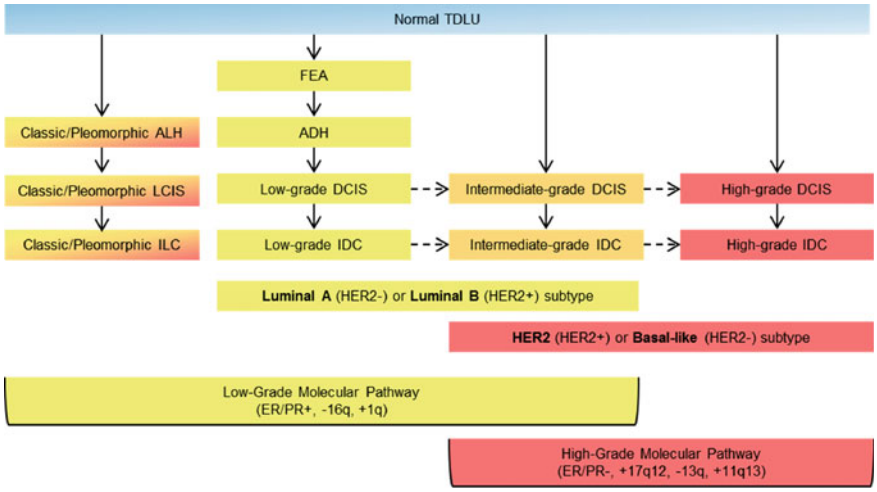

Fig. 8.2 A multistep model of human breast cancer progression based on immunohistochemical, genomic and gene-expression data. Molecular events that occur in the normal terminal duct lobular unit (*TDLU*) (*blue rectangle*) give rise to two distinct molecular pathways (low- and high-grade molecular pathways). Linear pathological progression occurs from normal TDLUs to invasive breast cancer (*solid arrows*) and intrastage progression occurs within ductal carcinoma in situ (*DCIS*) and invasive ductal carcinoma (*IDC*) (*dotted arrows*). The low-grade molecular pathway is characterized by loss of 16q, gain of 1q, and estrogen receptor (*ER*) and progesterone receptor (*PR*) positivity and is observed during pre-invasive and invasive stages of both ductal and lobular lesions (*yellow rectangles*). The high-grade molecular pathway is characterized by amplification of 17q12 and 11q13, loss of 13q, and ER/PR negativity (*red rectangles*). Pleomorphic lobular lesions [atypical lobular hyperplasia (*ALH*), lobular carcinoma in situ (*LCIS*), and invasive lobular carcinoma (*ILC*)] resemble high-grade tumors; however, immunohistochemical and genetic analyses support an association with the low-grade molecular pathway. The Luminal A and Luminal B subtypes of IDC constitute the majority of lesions in the low-grade molecular pathway. The human epidermal growth factor receptor 2 (*HER2*) and basal-like subtypes of IDC constitute the majority of the lesions in the high-grade molecular pathway. Abbreviations: ADH, atypical ductal hyperplasia; FEA, flat epithelial atypia [12]

revealed that different morphological subtypes of DCIS reflect distinct genomic alterations (Fig. 8.2). For example, comparative genomic hybridization (CGH)-based studies of DCIS revealed frequent loss of 16q in low- and intermediate-grade DCIS and gain of 1q and loss of 11q in intermediate-grade DCIS [29, 30]. Additionally, high-grade DCIS has been characterized by frequent loss of 8p, 11q, 13q, and 14q; gains of 1q, 5p, 8q, and 17q; and amplifications of 17q12 and 11q13 [29]. Further CGH analysis comparing DCIS and IDC revealed an almost identical pattern of genetic variations [29, 31] and there is also a correlation between copy number variations and progression [32]. Together, this data supports the view that DCIS is a direct precursor of IDC and that distinct genetic abnormalities are reflected by nuclear grade within the morphological spectrum of DCIS.

A number of loss of heterozygosity-based and CGH-based studies support the hypothesis that ADH is a precursor to low-grade DCIS. For example, LOH in

regions 16q and 17p in ADH is similar to the variations observed in low-grade DCIS [6, 33, 34]. Given that ADH and low-grade DCIS share many architectural and cytological features [15, 16], it makes sense that they share common chromosomal abnormalities. This supports the presumed sequence of progression of ADH to low-grade DCIS; however, the progression to high-grade DCIS is less clear. In terms of histological presentations and genetic aberrations, high-grade DCIS is more heterogeneous than low- and intermediate-grade DCIS. Despite the greater intricacy of the pattern of genetic aberrations found in high-grade DCIS (those with 17q12 amplifications), deletions of 16q are less frequent, suggesting that the majority of high-grade DCIS lesions arise de novo.

There is also molecular evidence suggesting that FEA is a precursor to ADH and/or low-grade DCIS. It has been shown that FEA has similar genetic alterations compared to ADH and both low-grade DCIS and low-grade invasive carcinoma [35]. There is an increase of loss of heterozygosity at chromosome 16q in FEA, low-grade DCIS, and low-grade IDC [33] and there are comparable chromosomal copy number gains and losses present in FEA, ADH, and low-grade DCIS [28]. A number of immunohistochemical approaches have also linked FEA, ADH, and low-grade DCIS. For example, the atypical/neoplastic cells of all three of these pre-invasive lesions show the same high-level expression of estrogen receptors, progesterone receptors and cytokeratin 19 [30, 36], an increase in expression of cyclin D1 [36], as well as identical negativity for cytokeratin 5/6 [30] and Human Epidermal Growth Factor Receptor 2 (HER2) [30, 37]. These data support the view that FEA may be a precursor to ADH and low-grade DCIS.

Much of the research on understanding the gene expression alterations that occur during the early pre-invasive stages of breast cancer have focused on the neoplastic epithelial cells of ADH and DCIS [38, 39]. For example, a patient-matched microdissection and microarray-based study showed that marked transcriptional alterations occur between normal TDLUs and ADH, which are sustained in DCIS and IDC [38]. However, in several studies, there were no major transcriptional profile changes between the pre-invasive and invasive stages [38–40]. This has led these authors to suggest that both pre-invasive and invasive stages of progression are clonal in origin and that genes expressed during ADH and DCIS may be responsible for progression. A number of studies have linked gene expression patterns during early stages of progression to the risk of developing IDC and metastasis [41–44]; however, there is a clinical need to further identify and characterize reliable markers of risk for progression.

Distinct differences in gene expression are also associated with grade [38, 45, 46]. For example, distinct gene expression patterns are present in low- and high-nuclear grade DCIS [38] similar to what is observed in IDC. Additionally, ADH and low--grade DCIS share gene expression patterns associated with ER expression, whereas high-grade DCIS has a gene expression pattern more associated with the cell-cycle and mitosis [38]. In a similar respect, gene expression analysis of intermediate-grade DCIS shows a combination of low- and high-grade characteristics [38, 46]. These gene expression analyses support the view that low- and high-grade breast cancers arise from normal TDLUs through distinct molecular pathways (Fig. 8.2). Defining

distinct molecular pathways and breast cancer subtypes (see Sect. 8.6.1) continues to be an evolving field as stratification of breast cancer into distinct subgroups and their molecular drivers involves an integrated view of the both the genome and transcriptome [47].

8.4.2 Molecular Features of Lobular Carcinoma Progression

CGH-based analyses of ALH and classic LCIS have revealed a similar pattern of chromosomal variation—loss of 16p, 16q, 17p, and 22q [48] in both. Further studies have identified a common loss of 16q in ALH, LCIS, and classic ILC [49, 50]. This supports the view that ALH and LCIS are closely related lesions and that all three (ALH, LCIS, and classic ILC) represent a progression continuum. Additionally, gene expression analysis of LCIS and classic ILC shows a pattern that is correlated with low-grade DCIS and IDC [50]. Taken together, these studies support a common—16q, low-grade molecular pathway that includes ALH, LCIS, and classic ILC, as well as FEA, ADH, low-grade DCIS and low-grade IDC.

A small subset of ILCs shows a more aggressive clinical course, and consists of neoplastic lobular cells with more marked nuclear atypia (pleomorphic ILC). These cancers share common genetic variations with classic ILC—e.g., loss of 16q and gain of 1q; as well as common features of high-grade IDC—e.g., amplification of 17q12 [51, 52]. However, a CGH-based study revealed that overall genetic variations of pleomorphic ILC are more closely correlated to those observed in classic ILC compared to IDC [52]. This suggests that pleomorphic ILC has a common molecular pathway of progression to that of classic ILC, that later accumulates alterations more characteristic of a high-grade lesion (Fig. 8.2). Similarly, there is CGH evidence that variant LCIS (pleomorphic LCIS, LCIS with necrosis) is of a common molecular background to classic LCIS (loss of 16q, gain of 1q), but that it is also associated with numerous further genetic aberrations that are more characteristic of a high-grade lesion [53].

8.5 Models and Methods Used to Study Breast Cancer Progression

In order to study the pre-invasive stages of breast cancer progression, several in vitro and in vivo models have been developed. Most take advantage of established human breast epithelial cell lines, which have been altered with activated oncogenes which drive production of these pre-invasive phenotypes [54–57]. In vivo models take advantage of the short time interval required for murine mammary progression and the high incidence of pre-malignant lesions in certain genetic backgrounds [58].

One such model system is the HMT-3522 series cell lines [54, 57], which consists of three cell lines derived from a single patient presenting with fibrocystic change. The HMT-3522/S1 cell line was produced during in vitro culture of the explant and was shown to be non-tumorigenic in a mouse xenograft model; whereas the HMT-3522/S2 cell line was established after an EGF-independent growth selection of the HMT-3522/S1 cell line and was shown to be tumorigenic. The third cell line, HMT-3522/T4-2, was derived from a HMT-3522/S2 tumor and is considered to be the most tumorigenic of the three cell lines. The HMT-3522 cell lines have undergone malignant transformation in vitro without being exposed to known carcinogenic agents and this transformation resembles some aspects of progression during pre-invasive breast disease [57]. Similarly, the MCF10AT cell lines represent a range of pre-invasive breast lesions [55, 56]. The MCF10A cells, also derived from a patient with fibrocystic change, are benign, immortalized breast epithelial cells. The MCF10AT cell line was derived from these cells by *ras* transformation. Subclones of the MCF10AT cells have generated a number of pre-invasive lesions including ADH and DCIS [55, 56]. Both the HMT-3522 and MCF10AT cell lines have proven useful; however, both model systems suffer from disadvantages. Both show mixed phenotypes and lack of stability of the phenotypes after culture. Additionally, the HMT-3522 cell lines lack a pre-DCIS stage, while the MCF10AT series is *ras*-transformation dependent, an uncommon event in spontaneous human breast cancers.

The 21T cell lines, derived from a single patient with metastatic breast cancer, represent a human breast cancer progression series [59, 60]. When grown in the mammary fat pad of nude mice, each cell line can reproduce a distinct stage of progression. For example, 21PT cells are non-tumorigenic and generate lesions of ADH, 21NT cells form lesions with the morphology of DCIS, and 21MT-1 cells generate IDC and are both tumorigenic and metastatic [60].

In vitro systems are very useful for high throughput studies. However, it has been shown that when grown in 2D in vitro culture, cell lines can have distinctly different morphology and genetic profiles compared to in vivo growth [61–66]. Also, important signals released by the extracellular matrix, which control normal homeostasis and tissue phenotypes, are lost when cells are cultured in 2D. When cells are cultured in a laminin-rich extracellular matrix, many of these signals remain intact [64]. By allowing cells to grow in a 3D conformation in contact with extracellular matrix proteins, certain characteristics of cell morphogenesis, proliferation, apoptosis and invasiveness may be studied in a highly controlled 3D environment. In fact, there have been many studies using 3D systems to examine molecular controls of morphogenesis in normal and neoplastic breast epithelial cells [65, 67–70]. There has been limited use of 3D in vitro systems to directly study progression through the pre-invasive to invasive stages of breast cancer; however, use of the HMT-3522 cell lines [71], the MCF10A-derived cell lines [72] and the 21T series cell lines [60] in 3D systems have proven useful in identifying potential regulators of progression.

In vivo breast cancer progression models have often made use of genetically engineered mice that have been designed to develop atypical lesions that mimic some pre-invasive lesions in humans [73]. In addition to genetic manipulation,

other murine models make use of viral, chemical or hormonal agents that induce pre-malignant lesions [58]. However, since these model systems are mouse-derived, they fail to mimic exactly human breast cancer progression, especially from a molecular perspective. Therefore, in order to study the molecular events underlying the pre-invasive stages of human breast cancer progression, researchers often make use of human cell lines in xenograft model systems. One such model system makes use of genetically engineered human breast organoids and activated human breast stromal cell xenografts. This approach has been useful in defining genetic events that are required to drive progression from pre-invasive stages to invasive carcinoma [74].

Breast cancer tissues are comprised of a complex mixture of healthy epithelial cells, invasive or in situ tumor cells, surrounding stroma, infiltrating immune cells, blood vessels, and capillaries. As a consequence, whole tissue lysates represent a variety of cell types, making analysis of tumor cell-specific signals very difficult. Laser capture microdissection technology has, however, proven useful in identifying different gene expression signatures of progression [29, 30, 32, 49–52, 75] that are representative of the different tissue components of a tumor or precursor lesion.

8.6 The Transition from Pre-invasive to Invasive Breast Cancer

One of the most important events in the progression of breast cancer is the transition from pre-invasive, in situ lesions, to an invasive phenotype, in which neoplastic cells of DCIS (or LCIS) gain the ability to break through the basement membrane and invade into the surrounding stromal tissue. First, to address the clinical problem of predicting which in situ lesions are likely to progress to malignancy, molecular markers of the invasive phenotype will be discussed. This will be followed by a discussion of the traditional epithelial centric view of progression, as well as a more contemporary view that includes involvement of the tumor microenvironment.

8.6.1 Molecular Predictors of the Invasive Phenotype

Microarray analysis has been used to identify gene expression patterns that are associated with clinical outcome of invasive breast cancers [41, 76–78]. These invasive breast cancers have been commonly categorized into four major subtypes: luminal A, luminal B, HER2 overexpressing/ER-, and basal-like. The basal-like subtype is typically ER-/PR- and HER2-, has high proliferation rates and is associated with a poor prognosis [77, 78]. There has been emerging refinement of these subtypes using paired DNA-RNA profiles that has revealed 10 novel subgroups based on clinical outcome [47].

Table 8.1 p16, COX-2, and Ki67 as molecular predictors of progression to an invasive phenotype

	p16	COX-2	Ki67	References
Normal stress-activation response	High	High	Low	[80]
Abnormal stress-activation response (poor prognosis DCIS)	High	High	High	[80, 81]
ADH prone to progression	No association	High	High	[82]

In women diagnosed with DCIS, 15–30 % will develop subsequent DCIS or IDC within 10 years after lumpectomy and radiation [3]. Of the 70–85 % that do not recur, it is likely that some are being overtreated. Conversely, since a majority of DCIS lesions are treated with lumpectomy (usually with accompanying radiation), some women are still prone to recurrence and/or subsequent invasive disease and require more aggressive treatment(mastectomies). Therefore, there is a clinical need for accurate markers that will predict if and when DCIS will progress to an invasive phenotype. Recently, expression profiling and immunohistochemical studies confirm the presence of molecular subtypes in DCIS [79–81] that parallel subtypes of invasive breast cancers, which may help to address this clinical problem. For example, it has been proposed that DCIS with high p16 and COX-2 expression in the absence of the cell proliferation marker Ki67 produces a normal stress-activation response that is protective against progression to an invasive phenotype [80]. In contrast, DCIS expressing high p16, high COX-2, and high Ki67 is interpreted as an abnormal response to cellular stress, and has been said to be associated with progression to a basal-like subtype of invasive breast cancer [80] (Table 8.1). In one study, DCIS with high p16, high COX-2, and high Ki67 was a better predictor for invasive breast cancer than nuclear grade [81]. In ADH, expression of p16, either alone or in combination with COX-2 and Ki67, was not found to be associated with progression to malignancy, although the combination of high COX-2 and Ki67 was found to convey stronger risk of breast cancer within 10 years [82] (Table 8.1). In DCIS at least, the expression signature of high p16, COX-2 and Ki67 may define a progression pathway of basal-like breast cancers to invasive disease, and could prove useful in the management of patients with high-grade DCIS. Identification of biomarkers indicating probability of progression to other subtypes of invasive cancer is ongoing and could further improve the clinical management of patients diagnosed with pre-invasive disease.

8.6.2 The Transition to the Invasive Phenotype: "Escape" versus "Release"

The transition from a pre-invasive to an invasive phenotype occurs when cells of DCIS (or LCIS) invade through the basement membrane and into the surrounding stromal tissue, thus representing a key event in the progression of breast cancer.

Work such as that described above has yielded a rudimentary understanding of the stage-specific molecular changes within the neoplastic epithelial cells themselves. However, there is evidence that the tumor microenvironment is important during progression and that molecular changes in non-neoplastic cells [39, 83, 84], in addition to neoplastic epithelial cells, have the potential to drive progression [72, 85–87]. For example, in a cell line model for DCIS, the transition from DCIS to IDC did not require additional molecular alterations within the neoplastic epithelial cells, but rather progression to IDC was promoted by fibroblasts and suppressed by myoepithelial cells that make up the stromal and periductal microenvironment of DCIS. Molecular profiling of isolated epithelial and myoepithelial cells identified a signaling interaction network involving transforming growth factor β (TGF-β), hedgehog, cell adhesion molecules and p63, which was required for the differentiation of myoepithelial cells. Elimination of this signalling network resulted in loss of the myoepithelial cells and progression to an invasive phenotype [72]. Similarly, the establishment of the self-sustaining TGF-β and stromal cell-derived factor 1 (SDF-1) autocrine-signaling loops in resident mammary myofibroblasts can give rise to carcinoma-associated myofibroblasts that promote progression to invasive mammary carcinoma [88]. In addition, carcinoma-associated fibroblasts may mediate tumor growth and angiogenesis through the secretion of SDF-1 by acting directly on neoplastic epithelial cells via the CXCR4 receptor and by recruiting endothelial progenitor cells respectively [85]. Additionally, tumor-associated macrophages can have progression-promoting effects through the secretion of immunosuppressive cytokines, the release of free radicals such as nitric oxide and hydrogen peroxide, and the secretion of angiogenic factors. It has been suggested that these signaling mechanisms may be useful as therapeutic targets to block the development of tumor-promoting stromal cells [89].

Studies such as these have changed our view of breast cancer progression as solely an epithelial/tumor cell-driven process. Two possible models of the DCIS-to-IDC transition ("escape" vs. "release") have been suggested [90]. The "escape" model proposes that genetic alterations accumulate in a subpopulation of neoplastic epithelial cells, which provides them with the ability to disrupt the myoepithelial layer and invade through the basement membrane into the surrounding stromal compartment. In contrast, the "release" model proposes that degradation of the basement membrane and subsequent invasion is due to alterations in the tumor microenvironment, particularly in the myoepithelial cells, myofibroblasts, fibroblasts, and tumor-infiltrating inflammatory cells. What is actually occurring is most likely a combination of both models whereby changes in neoplastic epithelial cells and non-neoplastic cells of the tumor microenvironment both contribute to the transition from pre-invasive to invasive disease.

8.7 Conclusion

Histological and molecular evidence has led to a model of breast cancer progression in which cells from the TDLU give rise to ADH or ALH, which can progress to DCIS or LCIS, and eventually to IDC or ILC respectively. Gene expression analyses suggest that low- and high-grade breast cancers can arise from normal TDLUs through two distinct gene expression pathways. The low-grade molecular pathway is characterized by loss of 16q, gain of 1q, and ER/PR positivity; whereas the high-grade molecular pathway is characterized by amplification of 17q12 and 11q13, loss of 13q, and ER/PR negativity. In addition, gene expression profiling has revealed distinct subtypes of invasive breast cancer based on clinical outcome. There is a clinical need to identify markers that will predict which pre-invasive lesions will progress, some of which may be unique to a particular subtype of IDC. Identification of such biomarkers is currently ongoing, which could improve the management of patients diagnosed with DCIS. It is important to bear in mind that the transition to invasive disease likely involves an interplay between the neoplastic cells themselves, as well as cells of the surrounding tumor microenvironment, such that both may be important in the future development of biomarkers and potential therapeutic targets.

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Chapter 9
Pre-Clinical Modeling of Breast Cancer: Which Model to Choose?

Claire Nash and Valerie Speirs

Abstract Breast cancer is a highly heterogeneous disease with several morphological and genetic sub-types identified in recent decades. The recognition that the breast microenvironment plays an active role in dictating mammary epithelial cell behavior calls for a need for models which better define the in vivo environment to use in breast research. However, given that breast cancer is so diverse one model is unlikely to recapitulate all aspects of breast cancer progression. Here we discuss the advantages and disadvantages of a variety of models available to researchers and outline their suitability to specific applications of breast cancer research.

Keywords Breast cancer · Two-dimensional (2D) in vitro models · Three-dimensional (3D) in vitro models · Extracellular matrix (ECM) · Tumorigenesis · Matrigel™ · Cell lines · Mouse · Xenografts · Syngeneic mouse model · Genetically Engineered Mice (GEM) · Tissue slice

Abbreviations

2D	Two dimensional
3D	Three dimensional
ECM	Extracellular matrix
GEM	Genetically engineered mice
MMTV-LTR	Mouse mammary tumour virus- long terminal repeat
WAP	Whey acidic protein

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9.1 Introduction

The start of the 21st Century heralded a new dawn for breast cancer research with the recognition through molecular profiling experiments that breast cancer heterogeneity observed morphologically was present at a genetic level [1, 2]. Recently this heterogeneity has been recognized further with breast cancer now classified into at least 10 molecular subtypes [3]. This poses a challenge when modeling breast cancer, with one single experimental system unlikely to be able to recapitulate this. As a result, various laboratory models are used to fully dissect the complex signaling pathways involved in breast cancer initiation and metastasis. One of the biggest challenges researchers face is finding a relevant model that allows manipulation of genes and proteins in a controlled laboratory environment while accurately reflecting the characteristic features of human breast tissue and tumors. Numerous models have been developed over the last few decades, which have been invaluable in revealing mechanisms of breast disease progression. These range from simple cell lines grown in two dimensions used to aid discovery of cell signaling pathways, to complex in vivo systems typically mouse, used for validation of drug response and metastasis studies. With the amount of different models available for breast cancer research increasing (each with their own advantages and disadvantages), choosing the right model to address specific biological questions becomes increasingly complex. The aim of this chapter is to review the different models available to scientists and to discuss how each model is suited and sometimes limited to specific aspects of breast cancer research.

9.2 Two-Dimensional (2D) in vitro Models

The simplest and most commonly used method of investigating breast cancer biology is the use of cell lines grown in vitro in 2D on tissue culture plastic. Several different cell lines representing the molecular subtypes of breast cancer defined by Perou et al. [1] are available, a selection of which are summarized in Table 9.1. Cell lines representative of pre-invasive ductal carcinoma in situ, including SUM225 [4] and MCF10DCIS.com [5], have enabled studies of early breast lesions. In addition, advances have been made in the isolation of breast cancer stem cells offering researchers good models to understand stem cell biology [Reviewed in 6]. It is yet to be established whether existing breast cancer cell lines can represent the further heterogeneity of breast cancer recently highlighted by Curtis et al. [3]. Nevertheless, cell lines have many advantages in that they are readily available, easy to propagate and are amenable to genetic manipulation. Further details on their biology and provenance can be found in some excellent reviews [Reviewed in 7–9]. However, it is increasingly acknowledged that a major problem with 2D culture is a loss of polarity and the lack of extracellular cues known to regulate breast tissue architecture in vivo [10]. While convenient, 2D

Table 9.1 Examples of breast cancer cell lines and their molecular classification

Cell line	Biomarker expression	Representative breast cancer sub-group
MCF-7	ER+, HER2−, ki67^{Lo}	Luminal A
ZR-75	ER+, HER2+, ki67^{Hi}	Luminal B
MDA-MB-468	ER−, PR−, HER2−, ki67^{Hi}	Basal
SKBR3	ER−, PR, HER2+, ki67^{Hi}	Her2
MDA-MB-231	ER−, PR−, HER2−, ki67^{Lo}, Claudin^{Lo}, E-Cadherin^{Lo}	Claudin-low

ER, estrogen receptor; *PR*, progesterone receptor; *Her*2, human epidermal growth factor receptor 2; *Lo*, low expression; *Hi*, high expression

culture cannot recapitulate in vivo conditions. Cells are highly sensitive to their environment and even defining the appropriate culture media to retain their in vivo phenotype remains a challenge. For this reason, more sophisticated systems are required to assess cell behavior that take into account the surrounding cell microenvironment.

9.3 Three-Dimensional (3D) in vitro Models

The development of 3D in vitro models has improved some of the limitations of 2D culture. Given that the extracellular matrix (ECM) which surrounds cells in vivo plays a crucial role in maintaining cell morphology and phenotype [Reviewed in 10], there has been a move towards developing methods of culturing cells in a 3D matrix. The basic premise of 3D culture is the incorporation of either single or multiple cell types into a matrix with a view to producing an environment reminiscent of the in vivo situation. There are several different natural and synthetic materials that can be used for this purpose. Synthetic matrices include polyethylene glycol, poly (lactic-co-glycolic) acid and modified hyaluronic acid. These are usually bioinert but can be engineered to include adhesion ligands and growth factors giving the advantage of complete control of matrix contents and physical properties with successful reproducibility [Reviewed in 11, 12].

Natural ECM materials isolated from murine sources such as the reconstituted basement membrane substance Matrigel^{TM} or Type 1 Collagen are preferred for 3D cultures. These materials provide a naturally occurring ECM which supports cell growth and under the right conditions can allow formation of duct-like structures [13]. Various techniques using natural ECM materials have been extensively reviewed [14–16]. Other bioinert materials such as agarose or a combination of this with either collagen or Matrigel^{TM} [17] have also been explored with similar results.

3D in vitro models have proved invaluable in elucidating many cell-extracellular matrix-signaling pathways. These have highlighted the mechanisms involved

in maintaining luminal cell polarity and non-tumorigenic acini architecture proving vital for cell invasion studies. Examples of key intercellular signaling proteins discovered to be important for this process include Desmosomal Cadherins and E-Cadherin and Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 [18]. In addition to this, the role of various cell surface molecules such as Tetraspanin CD151 [19] and α6, β1- and β4-Integrins [20] have shed light on the interactions of cells with their microenvironment. More recently, the role of collagen density and mechanical tension on cell morphology has also been revealed through use of 3D in vitro models [21, 22]. What's more, these interactions can be studied in conjunction with intracellular signalling pathways to better our understanding of the link between extracellular environment and cell transformation. Signaling molecules such as PI3K and PTEN have been studied in this manner [23]. 3D cultures also have the potential for genetic manipulation via RNAi techniques facilitating further study of complex signaling pathways.

The establishments of culture systems that resemble disease-free breast tissue architecture hold the key to investigating the subtle changes that occur during the early stages of tumorigenesis. Several examples of branching acini- and duct-like structures resembling in vivo breast tissue architecture have been achieved through use of 3D in vitro structures. Culture of MCF10A cells in 3D have yielded acini structures that are polarized and express several protein markers reminiscent of in vivo breast acini [24]. Successful culture of mouse mammary epithelial cells has even yielded polarized acini-like structures with evidence of milk protein expression [25]. Changes in polarization and disruption of these acini-like structures are factors that can be used to distinguish between non-transformed and transformed cells which are not apparent in 2D [26]. 3D cultures therefore hold the potential to facilitate cancer initiation studies which may have not been possible with 2D cultures.

Another benefit of these cultures is the ability to closely monitor the interactions between multiple cell types in a 3D setting. Examples include interactions between epithelial cells and fibroblasts [22, 27], epithelial cells with adipocytes and fibroblasts [28, 29], epithelial cells with vascular endothelial cells [30] and even the effects of different types of breast epithelial cells with fibroblasts [31]. This has led to a better understanding of how different breast cell types communicate with each other and investigating how this communication is altered in a tumor setting may give some more insight into how breast tumors are established and maintained.

In our lab, we have developed a model of non-tumorigenic breast using the HB2 epithelial cell line and myoepithelial cells isolated from breast reduction mammoplasty cultured together in 3D in the presence of collagen I. This model recapitulates the morphology and protein expression seen in vivo (Fig. 9.1). A similar example of a successful multicellular 3D model comes from a study whereby a tri-culture model of luminal and myoepithelial cells with fibroblasts shows distinct differences in structure formation and polarity in response to Hepatocyte Growth Factor and Matrix Metalloproteinase manipulation [32].

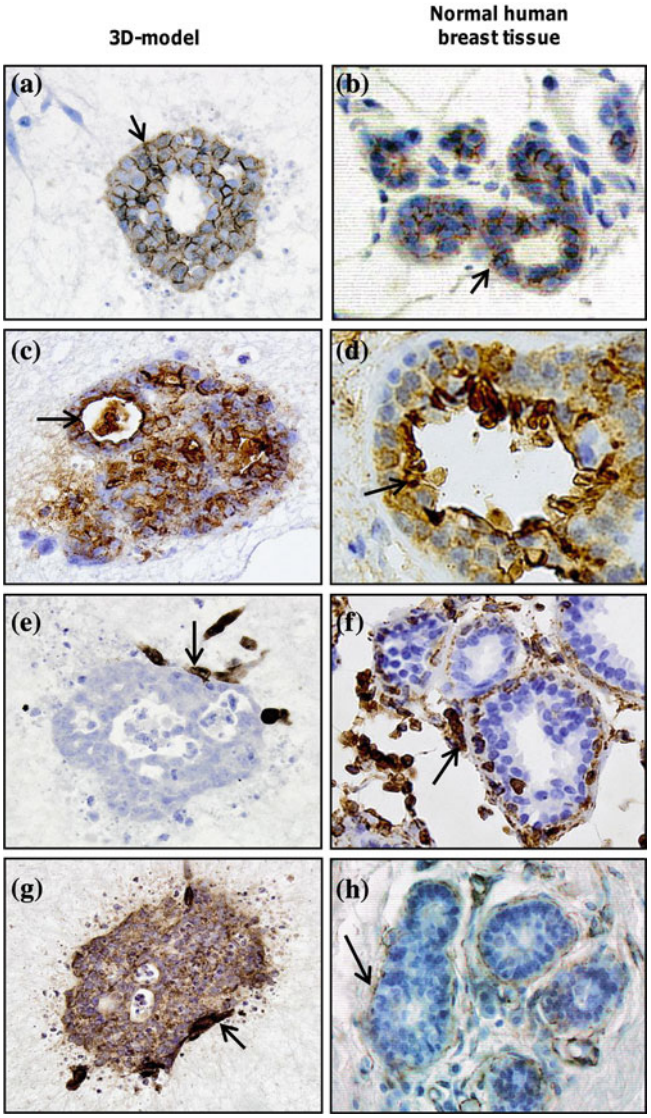

Fig. 9.1 Immunohistochemical characterization of a 3D in vitro model of non-tumorigenic breast against normal breast tissue. Immunohistochemical staining of 10 μm cross-sections of 3D cultures of Myo1089 myoepithelial cells and luminal HB2 cells embedded in rat tail collagen 1 (*left panels*) and 10 μm cross-sections of breast reduction mammoplasty tissue (*right panels*). In **a** E-Cadherin is limited to cell–cell junctions (*arrow*) between HB2 cells, recapitulated in **b** where E-Cadherin is situated at cell–cell junctions (*arrow*) between luminal epithelial cells at the lumen edge of breast acini. In **b** epithelial membrane antigen (*EMA*) is located at the apical membranes of HB2 cells (*arrow*) surrounding a lumen showing polarization of HB2 cells, also reflected in **d** where EMA is concentrated at the apical membranes of the luminal epithelium (*arrow*) surrounding the lumens of breast acini. Vimentin expression is limited to Myo 1089 cells (**e** *arrow*), which are distributed around the basal edge of the HB2 acini, also observed in (**f** *arrow*). Collagen 4 is distributed around the basal edge of HB2 acini structures (**g** *arrow*), also reflected in **h**, showing evidence of basement membrane production. All images 400x (Olympus BX51 microscope)

Potential disadvantages of 3D cultures include difficulty in tracking different cell populations in models consisting of multiple cell types. Labeling individual cell types with tracker dyes prior to incorporation into 3D culture allows cells to be tracked, but many of the cell tracker reagents available for this purpose are only stable for relatively short periods of time [31]. For longer-term experiments, this can be overcome by stable transduction with proteins such as Enhanced Green Fluorescent Protein. However, this aside, including just one or two cell types still does not account for influences imposed by adipose cells, immune infiltrates and cues from vascular endothelium which exists in vivo.

In addition, the matrix used for 3D cultures should be chosen with caution. For example, Matrigel™ is isolated from Engelbreth-Holm-Swarm mouse sarcoma and is rich in several key extracellular matrix proteins such as laminin, collagen 4, TGF-β, EGF, FGF and IGF and is a commonly used natural matrix [33]. However, levels of proteins such as collagen IV in Matrigel™ can differ in subunit composition to those seen in vivo [34] potentially increasing cell susceptibility to remodeling and proteolysis not commonly found in vivo. Matrigel™ can also provide transformed cells with additional survival and proliferative signals facilitating tumorigenesis [35].

An alternative in vitro approach developed more recently employs small fragments of breast tumors embedded in collagen 1 and grown in 3D [36]. This has allowed tamoxifen-sensitivity to be determined [37] suggesting that the model could prove valuable in assessing drug responses of individual patients. Allied to this is the tissue slice model [38], in which we have shown that 250 μm tissue slices can be maintained in a viable native state for up to 7 days post-surgery (Fig. 9.2). The ability to culture intact human tissue as outlined in these two models could provide an opportunity to validate new drugs on human tissue with fewer ethical implications which are associated with animal models. One could foresee the benefits of pre-testing patient samples with a range of drug therapies via ex vivo culture enabling a more accurate and individual treatment regime to be developed. Models such as these could improve current 3D models and start to bridge the gap between laboratory research and clinical practice. Nevertheless, the obvious limitation of all 3D in vitro models is the lack of a complex in vivo system complete with blood supply, immune infiltrates and regulation by hormonal cues. This limits these models to the study of cell interactions and signaling pathways. In order to study tumor progression and metastasis, animal models are required.

9.4 Animal Models

Several animal models have been used to study breast cancer. Tumor transplantation models involve implantation of either breast cancer cells or fragments of human or mouse tumors into immunocompromised or syngeneic mice, either subcutaneously or orthotopically. This provides tumors with a blood supply which

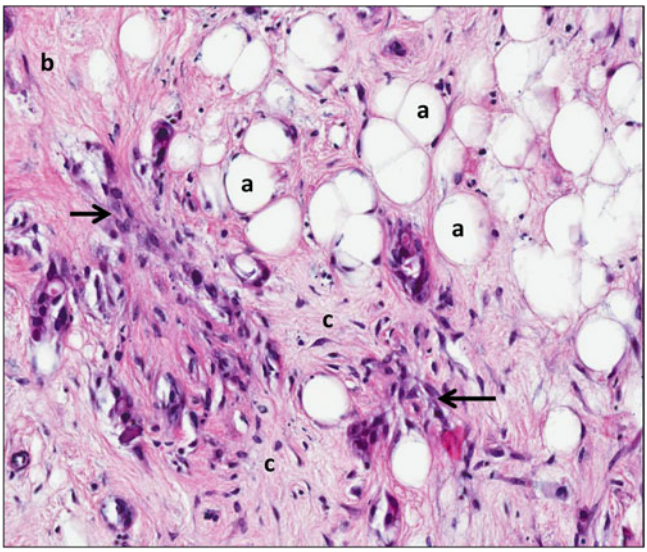

Fig. 9.2 Morphology of breast tumor tissue slice cultures after 7 days in vitro culture. Agarose embedded-250 μm slices of breast tumor were cultured in vitro for 7 days. Following formalin fixation and paraffin-embedding, Haematoxylin and Eosin staining of 5 μm sections demonstrated the preservation of original tumor morphology with intact adipocytes (**a**), tumor (**b**) and non-tumor associated stroma (**c**) plus tumor cells (*arrows*). Image was digitally scanned (Aperio). Original magnification x200

influences breast tumor progression and metastasis (Reviewed in 39) as well as adipose tissue which has the ability to regulate tumor cell behavior [40, 41].

One example of this model is xenograft, which involve implanting tumor cells (which may have been genetically manipulated) into the cleared mammary fat pad of immunocompromised mice. Xenograft models have already helped in the discovery of potential new drug therapies [42] and have contributed to the development of currently used drug therapies such as Herceptin™ [43]. Xenograft models also prove useful in mammary tissue development studies. The use of non-tumorigenic stem cells can reconstitute normal mammary tissue [44] and also offer an opportunity to study the role of cancer initiating stem cells.

A major caveat with xenograft models is that the mice used have to be immunocompromised in order to avoid human cell rejection. There is increasing evidence that immune and inflammatory responses influence neovascularization and accelerated tumor growth of breast cancers [45] as well as invasion and metastasis [46]. This problem can be addressed by using "humanized" mouse xenograft models which via transplant of human bone marrow haematopoietic stem cells reconstitute a human immune system in the xenograft mouse (Reviewed in 47). However, this procedure is technically complicated and reliant on the availability and successful expansion of haematopoietic stem cells.

Another weakness with xenograft is the physiological difference between mice and humans. Histologically, there are key differences between mouse mammary stroma and human mammary stroma [48] and differences in oncogenic cell signaling pathways between mouse and human mammary stromal cells has been highlighted [49]. Given that cells are highly sensitive to their environment, exposing human cells to a foreign environment could introduce growth factors and cell–matrix interactions that are absent in human breast tissue and thus cause changes in cell phenotype and behavior. In order to overcome this, xenograft have been developed to incorporate human stromal cells along with human breast tumor cells into the mouse mammary fat pad [50, 51], providing a more accurate mimic of the human mammary tumor environment.

A second tumor transplantation method is the syngeneic mouse model. This involves transplanting established tumor cells from one mouse into the cleared fat pad of mice from the same genetic strain. The advantage of this is that it avoids any host-versus-graft reactions and allows the role of the immune system on breast tumor progression to be investigated (Reviewed in 52). This is not possible in xenograft where mice are immunocompromised. Such models have permitted the study of how breast cells metastasize and home to specific organs [53].

A disadvantage of using syngeneic mice is that they are restricted to the study of the progression of mouse breast tumors. While this may give insight into the role of the immune system in breast cancer progression, one must always consider the physiological differences between mouse and human breast tumors and how these studies can relate to human in vivo systems.

Genetically Engineered Mice (GEM) models provide another type of in vivo model to study cancer. These involve the manipulation of critical oncogenic pathways via gain of function mutations of oncogenes or knocking out tumor suppressor genes. These are driven by tissue specific promoters, such as Mouse Mammary Tumour Virus Long Terminal Repeats (MMTV-LTR) and Whey Acidic Proteins (WAP). Many GEM models have been established that overexpress or knock out some of the most common oncogenes associated with breast tumor development. These include manipulation of Wnt-1 and Cyclin D1 [54], p53 [55] and Ras and c-myc proteins [56] to produce a variety of breast tumor phenotypes. GEM models have been engineered to overexpress the HER2/neu gene specifically in breast tumor tissue conserving the natural levels of expression in surrounding normal mammary tissue [57].

One of the biggest strengths of GEM is the ability to modify normal breast tissue to study causes of breast cancer initiation. Early breast lesions in humans are often hard to detect and inaccessible. GEM models allow the multiple developmental stages of breast cancer tumorigenesis to be studied from initiation to metastasis. In order to do this, genes need to be modified in a specific tissue at a specific time. Advances in inducible gene expression systems such as Tetracycline response systems allow genes to be switched on or off in a controlled manner [58]. Systems such as the Cre/LoxP recombinase also allow the ablation of genes inducibly [59]. Not only are these tools for cancer initiation and development studies, but they have also led the way for non-invasive in vivo imaging

technologies to be developed for effective tracking of tumor progression and metastasis [60, 61].

A major challenge in the use of GEM is the difference between mouse and human physiology. It has been proven that in most mouse tissues, telomerase remains active whereas in adult human tissues, telomerase is mainly inactive maintaining cellular senescence [62]. This causes mouse cells to be more susceptible to malignant transformation than human cells making it more challenging to assess the impact of genetic alterations. These species differences may account for the variation seen between human breast tumors and breast tumors produced from GEM mice. In a study carried out in 2000, a panel of breast pathologists compared GEM breast tumors with human breast tumors and found that many of the GEM models produced tumor phenotypes that were rare in human breast cancer [63]. More recently, differences between GEM breast tumors and human breast tumors are further being highlighted at a genomic level [64]. The failure of GEM tumors to recapitulate human breast tumors could also be due to the technique used to create them, as the choice of promoter used can affect the tumor types formed [65].

Because of they way they are created, GEM cannot yet mimic the genetic complexity that is seen within human mammary tumors as it is currently technically challenging to modify the expression of more than one gene at a time; while in human breast tumors, breast cancer results from a series of somatic genetic insults not a single event [66]. Since the way somatic mutations occur in breast cancer are not currently well understood and are highly heterogeneous between tumor sub-types, it seems GEM models are a long way from mimicking human breast tumor characteristics. Nevertheless, the homogenous tumors produced from these models will provide insight into the functions of single oncogenes or tumor suppressor genes.

In terms of drug development, GEM models have thus far been limited in modeling only estrogen-independent carcinomas [67]. This prevents the development of therapeutics using GEM for 60–70 % of breast cancer patients who have estrogen receptor positive tumors. Also, the difference in species means metabolism of cancer therapeutics [68] and molecular homology of protein targets [69] vary from mice to humans making the validation of drugs more challenging. What's more, the preparation of these models is both time-consuming (taking up to 6 months to produce a mouse line) and expensive, and with ethical considerations to be addressed, it is not always a convenient system to use in an academic environment.

9.5 Conclusion

Due to the complexity and heterogeneity of breast cancer there is no single model that can fully recapitulate all aspects of the breast microenvironment. The tools researchers can use are summarized in Fig. 9.3. Currently, there is no substitute for

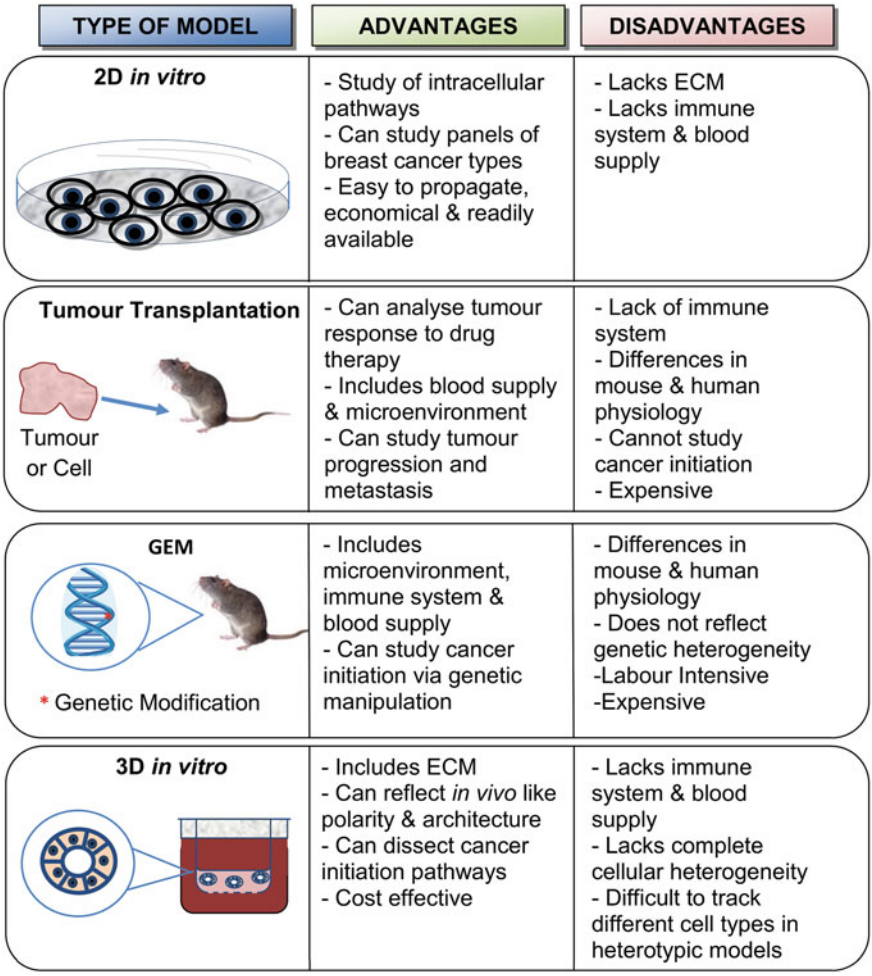

Fig. 9.3 Summary of pre-clinical models used in breast cancer research and their suitability for different applications

xenograft mouse models in the early development of drug therapies and this is also true for the study of breast cancer metastases. GEM models provide the opportunity to study multiple stages of breast carcinogenesis from cancer initiation to metastasis and the relevant pathways involved in these processes in vivo. However, due to the differences in mouse and human physiology, results must be interpreted with caution. While cell lines grown in 2D have contributed much to our understanding of breast cancer biology, it is now clear that 3D systems are more biologically relevant. However, there is still a need to refine current 3D in vitro models to encompass multiple components of the breast microenvironment. The development of 3D explant cultures and use of tissue slice models offer

a compromise between multicellular 3D models and animal models, offering a more realistic humanized model for scientists. Irrespective of the type of model, the comparison of these against human breast tissue specimens will remain the gold standard in assessing the validity and reliability of these models. The availability of good quality normal [70] and breast tumor [71] tissues from specialist breast tissue banks can facilitate this process.

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Chapter 10
Modeling Breast Cancer Progression in 4-D

Mansoureh Sameni, Stefanie R. Mullins, Kamiar Moin, Bonnie F. Sloane and Kingsley Osuala

Abstract Cell culture is among the most utilized techniques in biomedical research. The ability to grow mammalian cells in a dish has provided researchers with a tool to study the many mechanisms of biological function. The in vivo interactions in which cells participate, such as extracellular matrix adhesion, breakdown and deposition, along with cell to cell communications are key processes that need to be understood. Three-dimensional (3-D) cell culture has proved amenable to analysis of these interactions. Therefore, we have established a 3-D culture system, which utilizes co-culture of human breast epithelial cells with tumor-associated cells such as macrophages and/or fibroblasts. This unique 3-D model, which we have designated Mammary Architecture and Microenvironment Engineering or MAME, allows us to examine cellular function and mechanisms in a context more comparable to the in vivo microenvironment. In addition, we are able to visualize dynamic cellular processes such as proteolysis and invasion as they occur over time. In this manner we have taken cell cultures from 2-D to a multifaceted 4-D setting.

Keywords Modeling breast cancer · Tumor microenvironment · Hypoxia · Acidosis · Three-dimensional (3D) cell culture · Extracellular matrix · Mammary architecture and microenvironment engineering (MAME) · Live-cell imaging · Breast cancer · Malignant progression · In vitro models · Four-dimensional (4D) cell modeling

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10.1 Introduction

One hundred years since Carrel and Burrows' paper "On the permanent life of tissues outside of the organism", the science of cell culture has advanced to a level where we are engineering cell-type specific matrices and utilizing a wide-range of supplements to extend the survival of cells in vitro [1, 2]. Cell culture is a remarkable tool used to study drug toxicity, embryonic development, vaccine production, tissue grafting and organogenesis [3–6].

Although cell culture has facilitated the study of many biological processes such as the cell cycle, protein synthesis, cell motility, and apoptosis, we are still unable to replicate in vivo architecture in cell culture. Historically cell cultures have been of single cell types cultured independently of one another. We have disregarded the complex interactions that cells normally encounter in vivo. This issue has gained much attention over the past decade and the development of more complex culture systems is underway. Efforts to advance cell culture techniques are directed toward reproducing the in vivo microenvironment in an in vitro setting since it is how the cell functions in a tissue that we ultimately seek to understand.

10.2 Importance of the Tumor Microenvironment

A gap in our knowledge of breast cancer development and progression is our understanding of the contribution of the tumor microenvironment (TME). The TME is a dynamic locale, full of cytokines, growth factors, structural proteins, macrophages, lymphocytes and stromal cells. A number of studies have implicated the TME in tumor progression, indicating the necessity to study tumor cells in the context of their microenvironment [7–9]. The interactions between tumor cells and their surrounding TME come in many forms. Tumors begin to induce modifications of the TME at very early stages of tumor development. Two major alterations of the TME are hypoxia and acidosis. Hypoxia in the TME is a consequence of solid tumor growth. As a tumor increases in size, the core of the tumor becomes isolated from the oxygen rich vasculature at the tumor's periphery [10]. This often leads to phenotypic changes in cells at the core of the tumor as they modify glycolytic activity to tolerate the oxygen poor microenvironment [11]. These initial adaptations of tumor cells in response to hypoxic conditions are mainly physiological, such as disruption of Na^+ and K^+ exchange, ATP synthesis and glucose metabolism [12]. Acidosis in the TME has effects similar to hypoxia. Recent work suggests that tumor-mediated acidosis of the microenvironment directly promotes tumor progression, invasiveness, and therapeutic resistance [13–15].

For years the scientific community has commonly accepted that a cell's gene expression profile was mostly retained upon being removed from its in vivo microenvironment. We now know however that cells grown in vitro in monolayer

culture have a very different gene and protein expression profile than when grown in a state more like in vivo as on or within a 3-D matrix [16–18]. Others have shown that cell: extracellular matrix (ECM) interactions are pivotal for cell polarization and function [19–21].

Aside from the ECM of the tumor microenvironment, a number of cells can be found. These cells include adipocytes and stromal, myoepithelial and immune cells, all of which play an important role in the biochemical and biophysical properties of epithelial cells [16, 22]. One particular cell type found in the microenvironment of breast tissue is the myoepithelial cell. These smooth muscle actin expressing epithelial cells are contractile and distributed around the periphery of acini. They represent a fraction of the total cells present in breast tissue yet have a critical role in acinus formation and mammary gland morphogenesis [23]. In addition, myoepithelial cells are hypothesized to be tumor suppressors and have been shown to correlate with lower grade disease stages when present in tissue sections [24–27].

Immune cells such as dendritic cells, macrophages and lymphocytes are also important constituents of the TME and have been found to correlate with tumor progression and prognosis [28–30]. The presence of infiltrating dendritic cells is strongly associated with poor tumor prognosis, furthermore these cells appear to get reprogrammed at the tumor site and lose their antigen-presenting function [31, 32]. CD4+ lymphocytes have also been associated with having tumor promoting properties and this mechanism is mediated through the modulation of tumor-associated macrophages [33].

10.3 Cell Culture in Three Dimensions

We are working to recreate an in vitro TME that comprises the intricacy of the in vivo cell: cell and cell: microenvironment interactions. The use of 3-D culture systems will significantly aid in this effort as moving cell culture from a monolayer to a three-dimensional platform is an added level of complexity to cell culture techniques. Three-dimensional cell cultures unlike monolayer cultures allow cells to interface with one another in a 3-dimensional space. There are many established 3-D cell culture methods currently in use. These range from biological protein extract-derived matrices to synthetic scaffolds [34, 35]. Synthetic scaffolds are fabricated from silk, polymers or nanofibers and are primarily designed as housings for cell/tissue growth [36–38]. A major caveat of synthetic scaffolds is that they induce inflammatory and survival responses in cells [39–41]. Here we will primarily focus on the biological matrices and their relevance to the study of cellular function.

By growing a variety of cell types on reconstituted basement membrane (rBM), rBM has been found to be a suitable substrate for the recapitulation of normal tissue morphogenesis and organotypic functions [42–45]. For example, when human breast epithelial cells are grown on rBM they form spherical structures

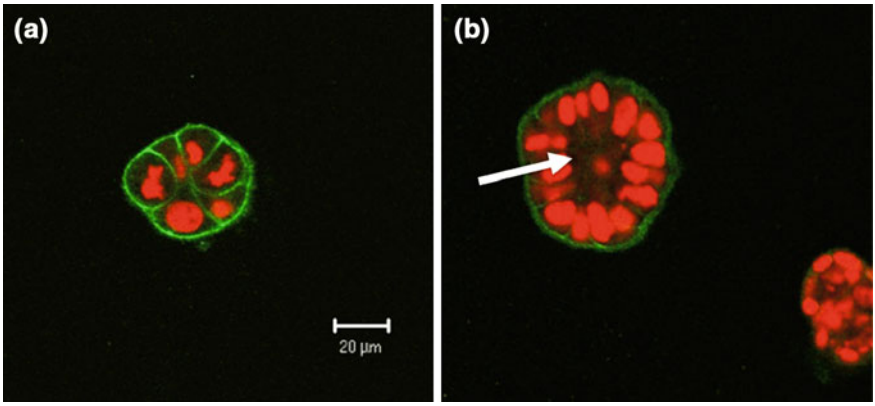

Fig. 10.1 MCF-10A are non-malignant breast epithelial cells that form acini with lumens (*arrow*) when grown in 3-D culture. To demonstrate polarity, cells were stained for β1 integrin (*green*) after 4 (**a**) and 12 (**b**) days in culture. Nuclei were stained with propidium iodide (*red*). Images represent a single confocal section taken at the equatorial plane of the structures. Scale bar, 20 µm

Table 10.1 3-D cell culture matrices utilized in breast cancer research

3-D culture matrix	Application	Single cell type	Co-culture
Reconstituted basement membrane (rBM) (+ or − % overlay)	Multilayer or mixed matrix	[17, 55, 56, 80]	[59, 80]
Collagen I + Collagen IV + rBM	Multilayer or mixed matrix	[56, 82]	[58, 59]
Collagen I or collagen I + rBM	Multilayer or mixed matrix	[22, 51, 53]	[22]

exhibiting appropriate cell polarity and acinar features reminiscent of those found in normal breast (Fig. 10.1) [45]. This has also been observed in 3-D culture of other cell types including: prostate, liver, skin and kidney [46–50].

The most widely used biological matrices are type I collagen, type IV collagen or reconstituted basement membrane derived from murine Engelbreth-Holm-Swarm Mouse Tumors, which is commercially produced and marketed as Matrigel™ and Cultrex® [17, 20, 51–57]. Some examples of currently used 3-D matrices are shown in Table 10.1. Although there are many substrates available for 3-D cell culture, a clear limitation is the functionality of a particular cell type on a given matrix. For example, when MCF-7 human breast cancer cells were embedded in type I collagen, they did not form epithelial acinar-like structures nor did they form lumens. In contrast, when these cells were grown on a Matrigel™ and collagen I mixture, distinct lumens were formed beginning at 2 weeks after seeding and remained throughout a six week culture period [22]. Because rBM is composed of laminins, collagen IV, integrins, entactins, and proteoglycans, it is ideal to utilize rBM substrates to study epithelial cell interactions as these proteins are key elements that the cells encounter in vivo since they reside on a basement membrane. Alternatively, when studying cell migration and tumor invasion, the use of collagen I is a suitable

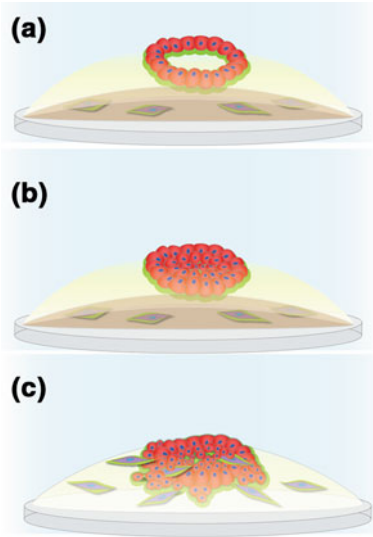

Fig. 10.2 Diagram of MAME (mammary architecture and microenvironment engineering) models. MAME tripartite: MCF-10A normal human breast epithelial cells (**a**) or MCF10.DCIS human ductal carcinoma in situ cells (**b**) are grown on top of a layer of reconstituted basement membrane (rBM) with an overlay of 2 % rBM. Human breast tumor-associated fibroblasts (TAFs) embedded in a lower layer of collagen I. MAME mixed: co-cultures in which epithelial cells and TAFs are plated as a mixture in rBM and a 2 % rBM overlay added (**c**). Dye-Quenched fluorescent substrates (DQ-collagens IV and I) are mixed with rBM and collagen I, respectively, with green representing the fluorescent cleavage products of these substrates (**a**–**c**)

substrate as collagen I is the major constituent of the ECM beyond the basement membrane.

We have developed a 3-D culture system to study the interaction of tumor cells with the ECM and neighboring cells. This model system has been termed MAME for mammary architecture and microenvironment engineering [58]. This 3-D culture system gives us the capability to co-culture the various cells that make up human breast tissue [59]. In Fig. 10.2a, we show a diagram of how human breast epithelial cells can be cultured in a 3-D matrix. We have developed two variations of the MAME culture model and termed them MAME tripartite and MAME mixed. In the tripartite model, we have incorporated a bottom layer of type I collagen containing embedded human breast fibroblasts. A second layer of rBM (Cultrex®) is placed on top of this, and a third layer of 2 % rBM in culture media is used as an overlay (Fig. 10.2a, b). In the mixed MAME model, we have combined human breast epithelial cells with breast fibroblasts and seeded them on top of rBM with the addition of the 2 % rBM overlay (Fig. 10.2c). This type of culture system allows for real-time evaluation of cell: cell and cell: ECM interactions [58, 60].

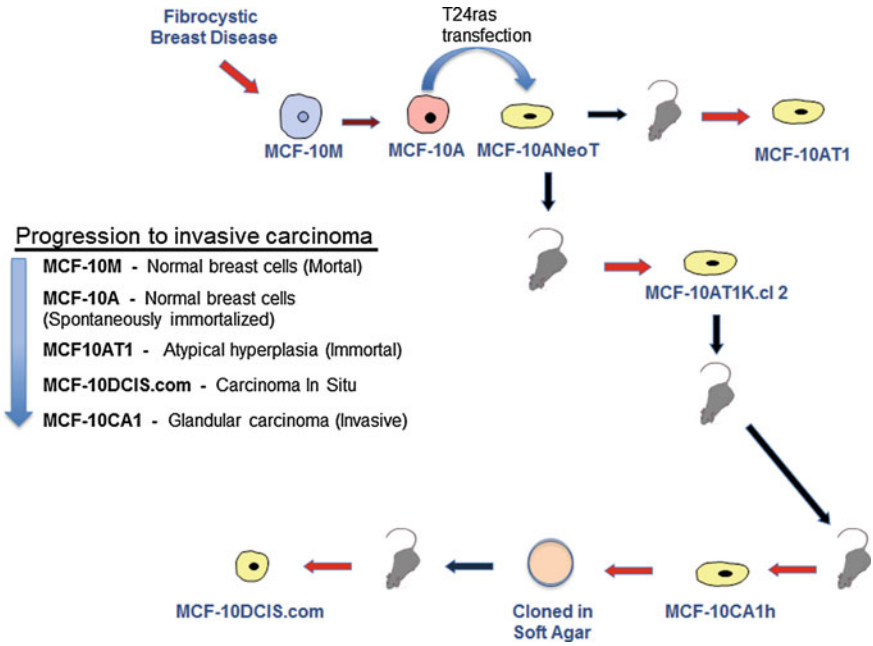

Fig. 10.3 Development of the MCF-10A breast cancer progression series. This diagram is a brief overview of the generation of isogenic variants derived from the parental MCF-10A cells [62, 63]

10.4 Modeling Breast Cancer Progression in 4D

Breast cancers are dynamic entities and the vast majority of our knowledge of breast cancer has come from culturing human breast and mouse mammary cancer cell lines. These studies have resulted in a better understanding of tumor biology; however, moving forward we must find novel ways to study tumor cell function as it pertains to interactions with the tumor microenvironment.

The generation of MCF10 cell lines that model the progression of breast cancer has been a catalyst for research on mechanisms that underlay progression [61–63]. This model system was originally derived from human reduction mammoplasty tissue and now consists of multiple variant cell lines (Fig. 10.3). One particular variant, the MCF10A, spontaneously immortalized in culture and was later transfected with the T24-ras oncogene to study tumor growth in vivo. These studies yielded in vivo tumor phenotypes that represent sequential stages of breast cancer as shown by histology [62, 64, 65]. The respective disease stages are also mirrored in 3-D cultures (Fig. 10.4). The isogenic origin and representation of premalignant breast cancer stages in the MCF10A cell lines provides a rare opportunity to study the properties that differentiate disease stages and progression.

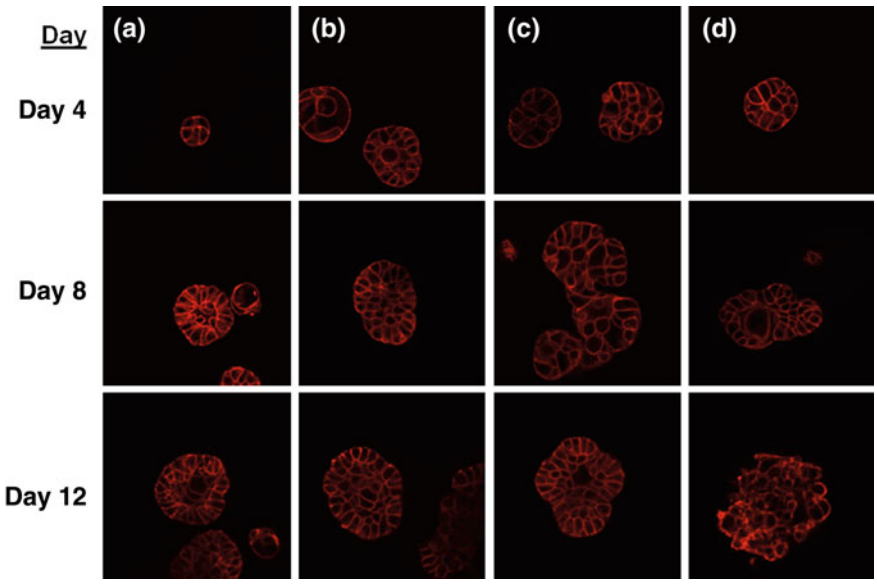

Fig. 10.4 MCF-10A variants in 3D culture replicate premalignant stages of progression to breast cancer. The MCF-10A cell line forms acinus-like structures with lumens over a 12-day period in 3-D culture (**a**). This phenotype is progressively lost in the MCF-10ANeoT (**b**), MCF-10AT1 (**c**), and MCF-10DCIS.com (**d**) cell lines, which form structures representing the premalignant stages of hyperplasia, atypical hyperplasia and ductal carcinoma in situ, respectively. Images of phalloidin staining (*red*) taken at 4, 8 or 12 days in 3-D cultures. Images represent a single confocal section taken at the equatorial plane of the structures. Magnification, 40X

Our 3-D culture system is also an ideal platform to study drug efficacy as it is being adapted for a microfluidics platform. Importantly, the study of drug efficacy in 3-D culture is proving to be invaluable as these studies show a reduced drug efficacy versus monolayer cell culture, which correlates with in vivo findings [66, 67]. Additionally, breast cancer cells in 3-D culture display a resistance to drug-induced apoptosis and mimic in vivo clonal dominance in contrast to the same cells in monolayer culture [68–71]. This difference between monolayer and 3-D cultures is due, in part, to the spherical structure of tumors growing in vivo or in 3-D [72–74].

The evident disparities between drug efficacy in monolayer cultures and in vivo emphasizes the necessity for an in vitro cell culture model that more closely resembles cellular architecture and interactions in vivo. Thus, the development of models like our MAME cocultures should increase the transferability of basic science to clinical application. The evaluation of breast cancer development in 3-D matrices has advanced our understanding of how cells alter their genomic and proteomic profiles based on their microenvironment [17, 21, 75, 76]. The next step is to characterize the functional properties of these cells over time in a 3-D culture system, i.e., in 4-D. Such a modality will facilitate the examination of cellular

functions like migration, invasion, proliferation, proteolysis, cell–cell interaction, spatial movement, cell orientation and morphogenesis. These types of analyses require state of the art imaging techniques that not only capture snapshots of live cells, but also image and record functional processes continuously in real-time.

Confocal imaging technologies allow for evaluation of biological cell processes over time, facilitating a better understanding of gene/protein expression and functionality [77–79]. The use of 4-D modeling is crucial in understanding and quantifying rates of changes in cellular proteolysis and responses to drug therapies, and less studied aspects like endothelial and lymphatic intravasation [80, 81].

10.5 Concluding Remarks

Modeling breast cancer in 4-D opens a new avenue for the evaluation of cell: cell and cell: ECM interactions. These types of functional studies raise new and exciting questions about the ever-evolving cancer cell. Our over-arching goal is to identify the functional changes that mediate progression to breast cancer so that we may identify druggable targets. However, in order to hit a moving target one can better define the target by visualizing it in 4-D.

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Chapter 11
Bone Metastasis of Breast Cancer

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Abstract Bone is the most common site of metastasis for breast cancer. Bone metastasis significantly affects both quality of life and survival of the breast cancer patient. Clinically, complications secondary to bone metastasis include pain, pathologic fractures, spinal cord compression, and hypercalcemia of malignancy. Because bone metastasis is extremely common in patients with metastatic breast cancer, clinical management of bone metastases is an important and challenging aspect of treatment in the metastatic setting. The skeleton is a metabolically active organ system that undergoes continuous remodeling throughout life. A delicate balance of the bone-forming osteoblasts and bone-resorbing osteoclasts in the dynamic microenvironment of the skeleton maintains normal bone remodeling and integrity. The presence of metastatic lesions in bone disrupts the normal bone microenvironment and upsets the fine balance between the key components. The changes in the bone microenvironment then create a vicious cycle that further promotes bone destruction and tumor progression. Various therapeutic options are available for bone metastases of breast cancer, and treatment can be tailored for each patient and, often requires multiple therapeutic interventions. Commonly used modalities include local therapies such as surgery, radiation therapy and

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radiofrequency ablation (RFA) together with systemic therapies such as endocrine therapy, chemotherapy, monoclonal antibody-based therapy, bone-enhancing therapy and radioisotope therapy. Despite the use of various therapeutic modalities, bone metastases eventually become resistant to therapy, and disease progresses. In this chapter, we describe the clinical picture and biological mechanism of bone metastases in breast cancer. We also discuss known risk factors as well as detection and assessment of bone metastases. We present therapeutic options for bone metastasis using a multidisciplinary approach. Further, we describe future directions for bone metastasis management, focusing on novel bone-specific targeted therapies.

Keywords Bone · Metastasis · Mechanism of bone metastases · Bone-targeted therapy · Therapy · Detection · Assessment · Resorption · Cytokine · Receptor activator of Nuclear Factor-κB ligand (RANKL) · Osteocystes · Denosumab · Integrin · Chemotherapy

11.1 Clinical Picture of Bone Metastasis in Breast Cancer

Bone metastasis develops in approximately 70 % of patients with advanced breast cancer and contributes to significant morbidity due to pain and skeletal related events (SREs) [1]. Among patients with bone metastases, two thirds will eventually develop skeletal related events [2]. Bone-only metastasis has been reported to develop in 17–37 % of women with metastatic disease [3–5]. SREs are often defined as a pathologic fracture, a requirement for surgical intervention and for palliative radiotherapy, hypercalcemia of malignancy, and spinal cord compression. Having pain alone, immobility and analgesic use do not define SREs. Table 11.1 summarizes the definition of skeletal-related events [2].

Bone metastasis not only adversely affects quality of life of the patient but also reduces overall survival. Sathiakumar et al. [6] studied 98,260 women with breast cancer who were U.S. Medicare beneficiaries between 1999 and 2005, among which 7,189 (7.3 %) had bone metastases either at the time of diagnosis or during the follow-up period. They found that the presence of bone metastases was strongly associated with a higher mortality rate among these women, and the association was stronger for bone metastasis complicated by SREs (HR of 1.5: 95 % CI 1.4–1.6). It is important to note, however, that several studies have shown that patients with bone-only metastatic disease tend to survive longer than those with visceral metastases, with median survival times of 26 months to 4.3 years for those with bone-only metastases whereas median survival was 13 to 18 months for visceral-only metastases [4, 5, 7].

Low-grade and ER-positive tumors are more likely to be associated with the development of bone metastases [8]. Colleoni et al. [9] found that ER-negative tumors had a higher early incidence of bone metastasis while ER-positive tumors

Table 11.1 Definition of skeletal related events [2]

Generally includes
Pathological vertebral fractures
Pathological non-vertebral fractures
Spinal cord compression
Surgery for bone complications
Radiotherapy for bone complications
Hypercalcemia
Does not include
Pain only
Immobility
Analgesic use
Non-hospital costs (physiotherapy)

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had a greater frequency of long-term incidence of bone recurrence, probably due to good recurrence control with endocrine therapy. Other factors associated with increased risk of bone metastasis include lymph node status at presentation of breast cancer (number of positive lymph nodes greater than 4), large tumor size (>2 cm), and younger age (<35 years) [9, 10]. Lousquy et al. reviewed 4,175 patients with non-metastatic disease and developed a nomogram to predict subsequent bone metastasis [10]. Furthermore, analyses of gene expression profiles have shown that a Src-response signature (SRS) in the primary tumor is more effective than ER status in predicting the likelihood of developing subsequent bone metastases [11].

In summary, the skeleton is the most common site of metastasis in breast cancer and it is important for clinicians to recognize the clinical problems associated with bone metastases in breast cancer. Also, in the future, it would be useful to develop reliable tools to predict who may be at higher risk for bone metastases so that both patients and clinicians have a more realistic understanding of the behavior of the disease.

11.2 Biological Mechanism of Bone Metastasis in Breast Cancer

In order to discuss therapeutic approaches to bone metastasis in breast cancer, it is important to review the biological mechanism of bone metastases. Remodeling occurs constantly in the healthy skeleton to regulate calcium homeostasis, to repair damage to the bone and to withstand new external stresses to the skeleton. In addition, remodeling is important to replace damaged and aging bone in order to preserve function of the skeletal system.

In adults, normal bone turnover mainly occurs through bone remodeling, which involves a well-coordinated activity of and interaction among osteocytes, osteoblasts, osteoclasts and chondrocytes. The basic multicellular unit (BMU), composed of osteoclasts and osteoblasts, is a temporary anatomic unit, which moves through the bone during remodeling. The leading group of osteoclasts in the BMU destroys the preexisting bone, a process called *resorption*, while the osteoblasts behind them rebuild and replace the matrix and minerals lost by resorption [12]. Recent studies suggest osteocytes, rather than osteoblasts, are the major source of cytokine receptor activator of Nuclear Factor-κB ligand (RANKL) and thus function as the chief driving component in bone remodeling [13]. RANKL is essential for differentiation and proper function of osteoclasts. Interestingly, the osteoclasts arising at different sites require different supporting cells. Although osteoblasts have long been recognized as the major source of RANKL, recent experimental data suggest hypertrophic chondrocytes are the major source of RANKL in endochondral bone formation whereas osteocytes are the major source of RANKL in cancellous bone remodeling [13]. Researchers speculate that osteocytes and hypertrophic chondrocytes, embedded within mineralized matrix, detect the need for bone resorption and send signals to stimulate osteoclast differentiation and activity [13].

Hormones, cytokines and growth factors modulate the proliferation of osteoclast and osteoblast progenitor cells, mainly through upregulating RANKL expression by osteocytes. Parathyroid hormone (PTH) promotes osteoclastogenesis by stimulation of RANKL in osteocytes [14]. When osteocytes undergo apoptosis, RANKL production increases from undetermined sources, promoting resorption of the bone [15]. Sex steroids suppress osteoclastogenesis, and loss of sex hormones may promote bone resorption by increasing osteocyte apoptosis [13].

When the normal balance among these key components is disrupted, it can result in bone destruction as observed in osteolytic metastases, which appear as "less dense than normal" areas on X-ray, or excessive bone deposition as observed in osteoblastic lesions, which appear "more dense than normal". In the healthy human, bone density declines after reaching a peak between age 25 and 30 [16]. In women, the bone loss accelerates after menopause around age 50, due to declining levels of estrogens, which have inhibitory effects on the bone-resorbing osteoclast [17]. Breast cancer survivors, after going through chemotherapy and adjuvant hormonal therapy including tamoxifen and aromatase inhibitors, are at an increased risk for low bone density and osteoporosis [18]. A well-balanced diet rich in calcium, adequate vitamin D levels, regular weight bearing exercise and, cessation of smoking are important to maintain bone health after breast cancer treatment [19, 20].

Once breast cancer cells metastasize to the bone, they disrupt the normal bone homeostasis and starts a vicious cycle. It is still unclear why certain types of breast cancer cells have a tendency to metastasize to bone. Stephen Paget (1889) postulated that the phenomenon of metastasis is not a random event but rather tumor cells growing selectively in the specific microenvironment of selected organs [21]. This model is named the "Seed and Soil" hypothesis. Multiple studies have demonstrated that neoplasms are biologically heterogeneous and that metastasis is an extremely

selective process, involving a series of alterations during the course of the disease [22]. The cancer cells that succeed in the multiple steps leading to metastasis, including invasion, embolization, survival in the circulation, arrest in a distant capillary bed, and extravasation into and multiplication within the organ parenchyma, can then establish metastatic lesions in the microenvironment that promote tumor-cell growth, survival, angiogenesis, invasion and metastasis [22]. The trabecular bone is highly vascular and appears to be the preferred site to which breast cancer to metastasize once breast cancer cells succeed in hematogenous spread [23].

Studies have shown that RANK is expressed on the surface of breast cancer cells and RANKL is overexpressed in bone [24]. Furthermore, CXCR4, a chemokine receptor, is highly expressed in breast cancer tissue and its ligand, CXCL12, is overexpressed in common metastatic sites in breast cancer, including bone marrow [25]. Cadherin-11 also promotes breast cancer cells to metastasize to bone [23]. These findings may explain the homing of breast cancer cells to the bone, in support of Paget's seed and soil hypothesis.

Bone metastases in breast cancer often have evidence of both osteolytic and osteoblastic features. Although osteolytic lesions usually predominate [26], 12–50 % of patients with bone metastases have predominantly osteoblastic disease [27]. Moreover, bone destruction in osteolytic lesions induces secondary new bone formation, leading to osteoblastic changes [28, 29], which may explain the presence of mixed lesions in bone metastases in breast cancer.

The process of metastatic lesion development in the bone is complex and involves various proteins and cytokines produced by metastatic breast cancer cells, which in turn stimulate the osteoblast to initiate a *vicious cycle*, leading to initiation of destructive bone lesions and tumor progression [30]. The initial step involves cancer cells in the bone, which produce several factors that promote differentiation of the osteoblast. These factors include parathyroid hormone-related peptide (PTHrP), interleukin-1 (IL-1), IL-6, IL-11, prostaglandin E2 (PGE2), tumor necrosis factor (TNF), and macrophage colony-stimulating factor (M-CSF) [31]. Furthermore, breast cancer cells produce Receptor Activator of Nuclear Factor-κB (RANK) and upregulate RANK ligand (RANKL) expression on the surface of the osteoblast [31]. RANKL then binds to RANK on the surfaces of monocytes, and under the stimulation of macrophage colony-stimulating factor (M-CSF), several monocytes fuse to form a multinucleated osteoclast [23]. RANKL also enhances the activity of preexisting osteoclasts by binding to RANK on their surface [31]. In turn, the osteoblast secretes osteoprotegerin (OPG), which competitively binds RANKL and suppresses the osteoclast activity [32]. PTHrP from cancer cells, however, suppresses the OPG activity [32]. Other factors that stimulate osteoclast differentiation include interleukin 6 (IL-6), IL-1, prostaglandins, and CSFs [23]. Once activated, the osteoclast reabsorbs bone by removing mineralized matrix as well as breaking up the organic bone [31]. Activated osteoclasts do so by first binding to the bone matrix via integrin proteins and then secreting acid and lysosomal enzymes to degrade bone [23].

The bone matrix stores several important growth factors including insulin-growth factor (IGF-1), transforming growth factor β (TGF-β), fibroblast growth factor

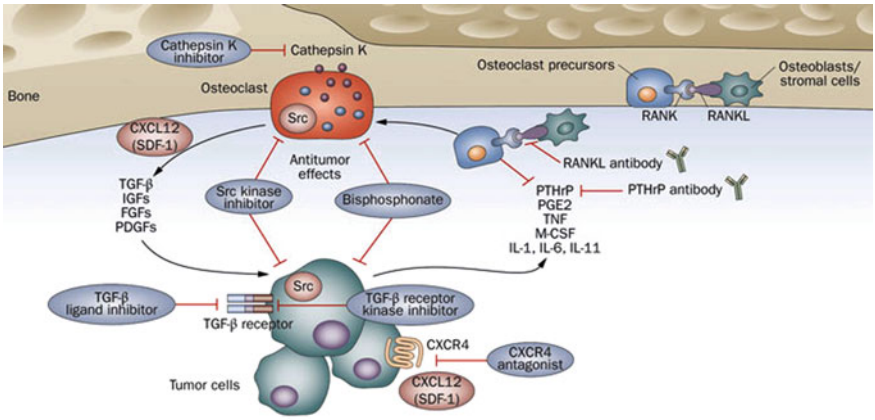

Fig. 11.1 A 'vicious cycle' accelerates both bone destruction and tumor growth as tumor cells secrete osteoclast-stimulating factors, and the bone marrow stromal cells secrete tumor growth factors. Various drugs targeting these factors, which include RANKL, Src kinase, cathepsin K, and TGF-β are under development. Abbreviations: *CXCL12* C-X-C motif chemokine 12, *CXCR4* C-X-C chemokine receptor type 4, *FGF* fibroblast growth factor, *IGF* insulin-like growth factor, *IL* interleukin, *M-CSF* macrophage colony-stimulating factor, *PDGF* platelet-derived growth factor, *PGE2* prostaglandin E2, *PTHrP* parathyroid hormone-related peptide, *RANK* receptor activator of nuclear factor κB, *RANKL* RANK ligand, *SDF-1* stromal cell-derived factor 1, *Src* proto-oncogene tyrosine-protein kinase, *TGF-β* transforming growth factor β, *TNF* tumor necrosis factor [97]. Future Directions of bone-targeted therapy for metastatic breast cancer. Nat Rev Clin Oncol. Doi: 10.1038/nrclinonc.2010.134

(FGFs), and platelet-derived growth factor (PDGF) as well as bone morphogenetic proteins (BMPs) [33]. These factors are released upon bone resorption. IGF-1 stimulates breast cancer cell growth and directs the cancer cells to migrate into bone by activating signaling molecules, such as PI-3 kinase, Akt, and NF-κB [23].

The enhanced bone resorption alters the calcium concentration in the affected bone, further weakening the bone. The various factors mentioned above as well as the environment high in calcium further enhance proliferation of the cancer cell as well as PTHrP secretion, which promotes the activity of the osteoclast [34]. The osteoclast activity can be measured by RANKL/OPG ratio, whose increased value indicates that the osteoclast activity is enhanced and the bone homeostasis balance has tipped toward the resorption side [31]. The interaction between tumor cells and other key components of the bone metastasis are shown in Fig. 11.1. The vicious cycle is summarized and divided into four major steps in Table 11.2 [31].

The Wnt signaling cascade, an important pathway in embryogenesis, promotes osteoblast differentiation and induces osteoblast activity [35]. Dickkopf-1 (DKK-1) is a gene in embryo development and is known to inhibit Wnt signaling [36], thus preventing osteoblast differentiation. Voorzanger-Rousselot et al. [36] showed DDK-1 was produced by osteolytic breast cancer cells and increased circulating levels were found in patients with breast cancer and bone metastases. In other words, DKK-1 blocks Wnt-signaling and, as a consequence, inhibits

Table 11.2 Four major steps of progression of lytic bone lesions in breast cancer [31]

Step 1: Breast cancer cells secrete parathyroid hormone-related peptide (PTHrP) and other factors, which stimulate the osteoblasts to produce RANKL
Step 2: RANKL stimulates the osteoclast, causing bone resorption
Step 3: Bone resorption stimulates production of growth factors, such as TGF-β which are released into the microenvironment
Step 4: Released growth factors promote cancer cell proliferation, which in turn further stimulates osteoclast activity

Created from text [31]

osteoblast differentiation [37]. Along with DDK-1, breast cancer cells secrete actin A (a member of TGF β superfamily) and noggin (bone morphogenetic protein [BMP] antagonist), all of which inhibit osteoblast differentiation [38], which favors osteoclastic activities and promotes osteolysis.

The mechanism of development of osteoblastic lesions is less well understood but accumulating evidence suggests it also is a complex mechanism involving various factors. Core binding factor alpha 1 (Cbfα1), also known as Runx-2, is a transcription factor linked to osteoblast differentiation [39]. Other factors which enhance the growth, differentiation and activity of the osteoblast include platelet derived growth factor (PDGF) [40], fibroblast growth factor (FGF) [41], TGF-beta [42], bone morphogenic proteins (BMPs) [43], and Endothelin-1 [44].

Endothelin-1 is known to mediate the development of osteoblastic metastases [44] by increasing osteoblast proliferation and activity through inhibition of expression of Dickkopf-1 (DKK-1) gene by marrow stroma cells [45]. As previously mentioned, DKK-1 blocks Wnt signaling and inhibits osteoblastic differentiation. When this inhibition is reversed, there will be more mature osteoblasts, favoring the development of osteoblastic lesions. Thus the mechanism of bone metastases appears to involve an intricate interplay between osteoblasts and osteoclasts as well as multiple factors in the bone microenvironment. Some breast cancer cell lines which cause osteoblastic metastases secrete endothelin-1, stimulating new bone formation [46].

As mentioned previously, PTHrP and TGF-β are important mediators in metastatic bone lesions and can be therapeutic targets. Other factors, which are also important in bone metastasis and thus can potentially become targets for therapy, include Matric metalloprotease (MMP), Cathepsin K, Proto-oncogene tyrosine-protein kinase (Src) and Chemokine receptors [31]. Important molecules and signaling pathways involved in bone metastasis are summarized by Theriault and Theriault as shown in Table 11.3 [23].

In summary, bone metastasis is a complicated biological phenomenon, involving multiple cellular and biochemical components interacting with each other. The complicated nature of bone metastasis makes it a challenge to develop targeted therapy and to completely stop or reverse the metastatic events. Regardless, multiple treatment modalities are currently available for combating bone metastasis in breast cancer. These are discussed next.

Table 11.3 Molecules and signaling pathways involved in bone metastasis of breast cancer [23]

Cytokines	Role	Result
Parathyroid hormone-related peptide (PTHrP)	Interacts with PTHR1 to cause expression of RANKL	Stimulates osteoclast-mediated bone resorption
Receptor activator of nuclear factor κB ligand (RANKL)	Binds to RANK receptor on precursor osteoclasts	Stimulates osteoclast development and activation, leading to bone resorption
Osteoprotegerin (OPG)	Acts as a decoy RANK receptor	Blocks RANK/RANKL interaction, inhibits osteoclast development
Insulin-like growth factor 1 (IGF-1)	Stimulates chemotaxis of cancer cells and directs migration	Causes proliferation of cancer cells in bone
Transforming growth factor beta (TGF-β)	Enhances production of PTHrP	Stimulates osteoclast-mediated bone resorption
Interleukin 6 (IL-6)	Induces osteoclastogenesis and suppresses osteblasts	Leads to bone resorption, decreased bone production
Interleukin 11 (IL-11)	Induces osteoclastogenesis and suppresses osteoblasts	Leads to bone resorption, decreased bone production
Prostaglandin E2	Increases expression of RANKL leading to enhanced osteoclast formation	Stimulates bone resorption
Macrophage colony-stimulating factor (M-CSF)	Induces osteoclastogenesis and suppresses osteoblasts	Leads to bone resorption
Tumor necrosis factor alpha (TNF-α)	Induces osteoclastogenesis and suppresses osteoblasts	Leads to bone resorption
Integrins	Allows cancer cells to arrest in target organs	Allows proliferation of cancer cells in bone
Cadherins	Unknown mechanism	Involved in migration and invasion
Osteopontin (OPN)/bone sialoprotein (BSP)	Stimulates osteoblast proliferation	Leads to bone resorption

11.3 Current Therapeutic Options

The multidisciplinary team, consisting of a medical oncologist, a diagnostic imaging physician, a radiation oncologist, and a surgeon, is often necessary for optimally treating patients with bone metastases. Currently, the mainstay of treatment for bone metastases includes external beam radiation therapy, systemic endocrine therapy, chemotherapy and supportive interventions including analgesics [47]. In addition, surgery can be utilized for patients with localized disease, with a single or few detectable metastatic lesions [48]. The treatment plan should be tailored for each patient, since the number, locations and biological features of tumors dictate the course of treatment most suitable for the patient. In most cases the goal is not curative but palliative. Surgery, radiation therapy, and radiofrequency ablation (RFA) are effective for pain control and for preventing pathological fractures [47].

A small percentage of stage IV disease (1–10 %) is potentially curable, especially when the metastasis is limited to an isolated loco-regional or distant site [49]. However, resection with curative intent for bone metastases has limited utility except for selected cases such as isolated spine or sternal legions [50, 51]. Surgical correction is useful in order to prevent impeding fractures in weight-bearing bones [52]. Surgery is especially indicated for locations such as the femur, humerus, pelvis, and vertebrae because pathological fractures at these sites may lead to significant disability. Several surgical techniques including plate osteosynthesis, nailing, and insertion of prosthesis are often employed for effective management [53]. External beam radiation is effective for alleviating pain and preventing fractures in weight-bearing bones and may be used in combination with surgical fixation [54]. For more emergent cases such as those involving spinal cord or cauda equina compression, high-dose corticosteroids in combination with external beam radiation or surgical decompression is needed to preserve neurologic function [55].

Once the patient develops bone metastasis, the disease is considered systemic and thus requires systemic treatments. Compared to patients with visceral metastases, those with bone only metastases have a more indolent course [4, 5, 56]. Thus, similarly to initial adjuvant systemic treatment, endocrine therapy is often selected as the first-line therapy for ER/PR receptor positive tumor subtypes [57]. HER2-directed therapies such as trastuzumab and lapatinib are indicated for tumor subtypes overexpressing HER2. Chemotherapy may be selected for patients with hormone receptor negative breast cancer and those with hormone receptor positive subtypes in which endocrine therapy has not been successful. However, similarly to metastases to other distant sites, the best treatment algorithms for bone metastases are difficult to determine at present time and it is not yet clear which treatment modality or combination of treatments is most effective in prolonging survival for patients with bone-only metastasis [58].

Among several molecularly targeted agents, osteoclast inhibitors such as bisphosphonates and denosumab target the osteolysis associated with bone lesions and deserve special attention here. Bisphosphonates reduce pain and incidences of SREs [59, 60]. Patients with stage IV disease confined to the skeleton at the time of diagnosis are most likely to develop SREs and may benefit the most from bisphosphonate treatment [61]. The mechanism of action of this class of drugs is inhibition of bone resorption by suppression of osteoclast activity. Early generation bisphosphonates (clodronate and etidronate) are taken up and metabolized by osteoclasts and induce apoptosis by their metabolites, cytotoxic ATP analogs. On the other hand, later-generation bisphosphonates (pamidronate, ibandronate and zoledronate) are internalized but not metabolized by osteoclasts. They inhibit the function of farnesyl diphosphonate (FPP) synthase, which is necessary for prenylation of GTPase such as Ras, Rho, and Rac, as part of post-translational modification. Without proper GTPase function, osteoclasts fail to form ruffled borders, which are necessary for adhesion to the bone surface [24].

Bisphosphonates reduce the SRE risk, delay the time to SREs, reduce bone pain and improve patients' quality of life [59]. Furthermore, bisphosphonates rapidly normalize calcium levels in tumor-induced hypercalcemia (TIH); therefore they

are the current standard of care in patients with TIH [62]. Bisphosphonates, however, do not appear to improve overall survival of breast cancer patients with bone metastases [63]. It is also important to note that bisphosphonates are associated with potentially serious side effects including renal failure, gastrointestinal side effects and osteonecrosis of the jaw, necessitating close monitoring during their use [64].

Denosumab is another effective osteoclast inhibitor, which is useful in management of bone metastasis in breast cancer. Denosumab has been approved by the U.S. Food and Drug Administration (FDA) for the prevention of SREs in patients with solid tumors, including breast cancer [64]. Denosumab is a monoclonal antibody, targeting the receptor activator of nuclear factor kappa B ligand (RANKL) [65]. As mentioned previously, upregulation of RANKL contributes to the vicious cycle of bone destruction in metastatic bone disease. Tumor cells in bone secrete cytokines, which in turn induce osteoblasts to secrete RANKL. RANKL then stimulates osteoclasts to resorb bone. Denosumab inhibits the function of RANKL, thus inhibiting bone destruction [65].

Denosumab has been shown to be superior to the bisphosphonate zoledronic acid in several trials involving patients with bone metastases. Compared to bisphosphonates, denosumab is more effective in reducing the risk of developing SREs as well as delaying the time to SREs in breast cancer [59]. Normalization of urine N-telopeptide levels, a marker of bone resorption, is observed more frequently in patients on denosumab compared to those on bisphosphonates [66]. Denosumab shows slightly lower treatment-related events or a frequency similar to bisphosphonates [59]. In a randomized, double-blinded study comparing denosumab with zoledronic acid in a total of 2,046 patients with bone metastases, rates of severe (defined as Common Terminology Criteria of Adverse Events grade ≥ 3) and serious adverse events (e.g. life threatening or requiring hospitalization) were similar between the treatment groups [67]. More cases of pyrexia, bone pain, arthralgia and renal failure were observed in the zoledronic acid group, while hypocalcaemia and toothache, not associated with osteonecrosis of the jaw (ONJ), were more frequent in the denosumab group [67]. Studies show denosumab has much less renal toxicity, and thus it may be beneficial for patients being treated with nephrotoxic compounds and for those with decreased creatinine clearance [65]. However, like bisphosphonates, denosumab does not make a significant difference in overall patient survival [59].

Another systemic treatment modality, which targets bone more specifically, are radioisotopes with affinity for bone. Isotopes such as strontium-89 and samarium-153, are given systemically but localize in sites of active bone turnover, treating all sites of bone metastases simultaneously [68]. These isotopes release beta-particles (electrons), which are cytotoxic to cancer cells in the metastatic bone lesions, providing effective pain relief with response rates ranging from 40 to 95 % [68]. Radium-223 is a promising, alpha particle emitting radioisotope, which is in a Phase II clinical trial for breast cancer with bone-dominant metastases no longer suitable for endocrine therapy. Radium-223 is further discussed in the new novel treatment section in this chapter.

Table 11.4 Current therapeutic options for bone metastatic breast cancer

Therapeutic options	Main indications
External beam radiation therapy	• Pain control • Prevention of pathological fractures
Surgery	• Curative intent for localized disease (rare) • Correction of pathological fractures • Prevention of pathological fractures
Systemic endocrine therapy	• Intent to control disease
Systemic chemotherapy	• Intent to control disease
Bone-targeted therapy (bisphosphonates, denosumab)	• Pain control • Reduction of SRE risks • Delaying time to SREs
Radioisotopes (Stribtuyn-89, samaruyn-153)	• Pain control
Supportive interventions (analgesics)	• Pain control

Although clinical management of bone metastases is challenging, multiple treatment modalities are currently available to alleviate pain and minimize the risk for SREs for breast cancer patients with bone metastases. The current therapeutic options for bone metastatic breast cancer are summarized in Table 11.4. These treatments, however, are only beneficial if given following good detection and under appropriate assessment of bone metastases. Further, bone specific therapies which specifically target the tumors need to be developed. In the next section, we discuss the current techniques used to detect bone metastases and to assess the response to treatment.

11.4 Detection and Assessments

To assess bone involvement from breast cancer, multiple imaging studies are currently available including plain x-ray films, bone scan (skeletal scintigraphy), computed tomography (CT) scan, magnetic resonance imaging (MRI), positron emission tomography (PET) scan and PET/CT. Studies indicate FDG-PET/CT has a very high sensitivity and specificity (PPV of 98 %, PNV of 91 %) when findings with both PET and CT are concordant [69, 70]. According to the National Comprehensive Cancer Network, use of these imaging studies for evaluation of patients with primary breast cancers is optional unless directed by symptoms or other abnormal laboratory results [71]. Excessive imaging by radiographs and CT is not only extremely expensive but also puts the patient at risk for unnecessary radiation exposure and/or invasive procedures undertaken because of false positive findings. When necessary, treatment response for bone metastases is assessed by a combination of methods, including imaging, blood analyses and symptomologies [72].

Three well-established organizations, namely Union International Against Cancer (UICC), World Health Organization (WHO) and MD Anderson Cancer

Center (MDA), have developed criteria to assess the bone response to treatment [72]. UICC recommends plain films only; WHO, plain films and bone scan; and MDA, plain films, bone scan, CT and MRI in order to classify the response into four distinctive types: complete response, partial response, no change or stable disease, and progressive disease [72]. On plain film, osteolytic lesions are recognized as a hole in the cortex while osteoblastic lesions appear dense and "whiter" than the surrounding bone [23]. The lesions in the cortex are best demonstrated on CT with bone windows, whereas trabecular lesions are best demonstrated on MRI [23]. Since CT and MRI are able to detect detailed anatomic changes, MDA criteria appear to be superior to UICC and WHO's to assess the response to treatment and to interpret the clinical behavior of bone metastasis [72].

At present, biochemical tests by blood analysis are not very specific for assessment of bone metastases. Bone resorption and osteoblastic markers may be, however, useful in identifying patients at increased risks of SREs as well as monitoring progression of the disease and evaluating treatment response [73]. Since the bone matrix contains type I collagen as its major organic component, byproducts of collagen breakdown are released during bone resorption [73]. These collagen-related biomarkers of bone resorption include: pyridinoline (PYD), deoxypyridinoline (DPD), urinary collagen cross-linked nitrogen-terminal N-telopeptide (NTX) and collagen I carboxyl-terminal C-telopeptide (CTX) [74]. These urinary waste products of the collagen crosslinks are very accurate predictors of the response to bisphosphonate therapy, compared to other markers such as bone alkaline phosphatase, urinary calcium and hydroxyproline [75]. Elevated levels of bone-specific alkaline phosphatase indicates the state of increased bone turnover and new bone formation, alerting the physician that new bone metastases may now be present or, if the presence of bone metastases is already known, that the disease is progressing [73].

As described in this section, various imaging studies and blood tests are available for detection and assessment of bone metastases. Since sophisticated imaging studies are expensive and false results can be problematic, surrogate biomarkers would be extremely useful. Thus, along with developing new therapeutic strategies, novel and effective biomarkers of bone disease and response are needed. Researchers continue to investigate potential prognostic markers such as circulating tumor cells (CTCs) and disseminated tumor cells in the bone marrow [97].

11.5 Resistant to Therapy and Future Directions for Bone Metastasis Management: Novel Treatments

As described previously, bone metastasis is a complex phenomenon, which involves multiple genes, signaling pathways, and cellular and biochemical components. Despite various types of treatment and a through multidisciplinary approach, the metastatic disease eventually becomes resistant to therapies and the disease progresses. The mechanism of resistance to bone-targeted therapies is not

well-understood. Some researchers postulate that cancer stem cells, which are insensitive to currently available therapies, are the culprit for therapy resistance in metastatic diseases including bone [76]. Epithelial-mesenchymal transition (EMT), which is viewed as the generation of cancer stem cell phenotypes, is another concept which might explain the mechanism of bone metastasis development and resistance to therapy [77].

Laboratory studies and clinical trials are being undertaken in order to develop new, more effective therapies. Recent advances in understanding the mechanism of bone metastases in breast cancer have led to several promising bone-specific, molecular targets under investigation, which target osteoclast activities, osteoblasts and the bone microenvironment favoring metastatic lesions [38]. The emerging therapeutic targets bring much hope and deserve special attention in this chapter. Recent development in new therapeutic targets and modalities for bone metastases in breast cancer is discussed here.

Integrin is a family of receptors expressed on the surface of osteoclasts and breast cancer cells. Among many integrins on the osteoclast surface, integrin $\alpha v\beta 3$ is important in tumor cell invasion and osteoclast-mediated bone resorption [78]. In animal models, integrin-$\alpha v\beta 3$-overexpressed breast cancer cells increases bone metastases as well as promotes both skeletal tumor burden and bone destruction [78]. Upon activation, integrin $\alpha v\beta 3$ stimulates an intracellular signalling complex consisting of c-Src and Syk (tyrosine kinases), which are required for proper functioning of the cytoskeleton in the osteoclast and for osteoclastogenesis. When integrin $\alpha v\beta 3$ is blocked, it leads to disorganized cytoskeleton in the osteoclast, creating decreased levels of bone resorption in vivo and in vitro [78]. Preclinical data with animal models show that integrin $\alpha v\beta 3$-targeting drugs, including S247, ATN-161, and cilengitide, effectively block osteolysis and inhibit tumor growth in bone metastases [79–81]. Some of the integrin antagonists, such as cilengitide, have entered clinical trials and more interesting data should come out in the near future.

c-Src is a proto-oncogene, encoding a non-receptor tyrosine kinase, which controls various signalling pathways in tumorigenesis. It is often overexpressed in human cancer cells and is activated by RANKL/RANK interaction and plays a central role in osteoclast function [38]. Mice injected with MDA-MB-231 cells and transinfected with wild type c-Src were observed to have more metastases [82]. Preclinical experiments show that osteoclasts in Src-null mice are incapable of resorbing bone, thus the animals are protected from osteolytic lesions [83]. Studies also showed that c-Src inhibitors such as CGP76030, effectively inhibit invasion, growth and bone metastases of MDA-MB-231 breast cancer in transinfected mice [82]. Furthermore, osteoclasts express high levels of Src, which is necessary for osteoclasts to migrate and form the ruffled border [84] to resorb bone. Src-targeting agents, including dasatinib, saracatinib, and bosutinib, are currently in clinical trials and preliminary data have shown these Src inhibitors have the potential to control bone metastases in breast cancer [85].

Cathepsin K is another key player in bone resorption. It is a lysosomal protease produced by osteoclasts and overexpressed in cancer cells which have metastasized to the bone. Cathepsin K becomes active in the acidic environment after

matrix dissolution, and it thereby breaks down the collagen matrix further [86], promoting bone resorption. Capthepsin K inhibitors, such as AFG-495, have been shown to reduce the formation and progression of bone metastases in mouse breast cancer models [87, 88]. Odanacatib (MK-0822), one of the most promising anti-cathepsin K drugs, has been shown to suppress bone resorption and increase type I collagen crosslinking [23, 89].

Activin A is a cytokine, which belongs to the TGF-β superfamily, and is involved in both normal remodeling of bone and pathological processes such as bone metastasis. In the setting of bone metastasis, it is produced by cancer cells and promotes osteolytic lesions by stimulating osteoclast differentiation and inhibiting osteoblast differentiation [90]. Serum levels of activin A are increased in breast cancer patients with bone metastasis [91, 92]. Interestingly, Activin A has dual effects depending on what type of tumor is in question. In breast cancer, actin A exerts growth inhibitory effects. In such a case, malignant cells are speculated to acquire resistance to Activin A in order to proliferate [90]. Because of the multiple effects of activin A on both normal and pathologic signaling pathways, it is a difficult target for which to develop a therapeutic agent. However, one of the Activin A receptors called Act RIIA (Activin type II receptor) has been studied as a potential target. Researchers have demonstrated that blocking this receptor stimulates bone formation and inhibits development of osteolytic lesions in murine models of breast cancer [93]. In addition, antibodies against Act RIIA have been demonstrated to reduce tumor burden and bone metastasis in a mouse model transplanted with breast cancer cell lines [94].

Another approach to lytic bone lesions may be the reversal of osteoblast inhibiters. As mentioned previously, if osteoblast function is suppressed, the effects of osteoclasts will be enhanced, favouring bone resorption and leading to lytic lesions. Dickkopf-1(DDK-1) inhibits the osteoblast promoting Wnt pathway, thus inhibiting osteoblast differentiation [38]. DDK-1 neutralizing antibodies decrease osteolysis as well as tumor growth in bone in a multiple myeloma model [95]. Although DDK-1 antibody has not been tested for breast cancer in clinical trials, data suggest DDK-1 is secreted by breast cancer cells that metastasize to bone [36]. Thus it warrants further investigation as a possible therapeutic target for breast cancer bone metastases [38].

Along the same line, interaction between endothelin-1 and its receptor can be a potential therapeutic target. Endothelin-1 is secreted by tumor cells and stimulates development of osteoblastic metastases via endothelin A receptor in vivo [46]. Endothelin A receptor blockage has been demonstrated to decrease bone metastases and tumor burden in a mouse model inoculated with ZR-75-1 cells, a breast cancer cell line which expresses ER/PR receptors [46]. Furthermore, endothelin-2 as well as endothelin-1 promotes breast cancer cell migration and invasion [96]. Thus, endothelin receptor antagonists could potentially be effective therapeutic targets for osteoblastic metastases in breast cancer [46].

It is clear that bone metastases are a complex phenomenon that involves multiple key components as well as alteration of the normal bone microenvironment. As stated in Paget's seed and soil hypothesis, tumor cells cannot survive

without an appropriate microenvironment in which to grow and proliferate. Thus, targeting the microenvironment and making the "soil" uninhabitable for cancer cells is another approach to control metastatic lesions. Among many targets, those that inspire researchers' interests are pathways involving peptides such as parathyroid hormone-related peptides (PTHrP), growth factors such as TGF-β, and chemokine receptors including CXCR4 [38, 97].

Parathyroid hormone-related peptide (PTHrP) is produced by both cancerous and normal cells. Almost all cancer cells metastasize to the bone secrete PTHrP, which supports osteoclastogenesis by upregulating RANKL in osteoblasts [97] and decreasing the RANKL antagonist called osteoprotegerin [98]. As a consequence, various growth factors such as TGFβ and minerals such as calcium are released during bone resorption, which initiates the vicious cycle [97]. A humanized monoclonal antibody against PTHrP was under development but the research has been suspended [74].

Transforming growth factor β (TGF-β) is known for having multiple effects on development and progression of bone metastasis. TGF-β regulates various cellular functions such as cell growth and differentiation, extra cellular matrix production, cell motility and immunosuppression [99]. Interestingly, TGF-β switches roles in cancer, exerting tumor suppressor effects in early stage and promoter effects as the tumor progresses [99]. As in activin A, a cytokine belonging to the TGF-β superfamily, the dual nature of TGF-β may pose a challenge upon developing therapies targeting TGF-β [97]. Several strategies targeting the TGF-β signaling system are under investigation, including monoclonal antibodies against TGF-β ligands, TGF receptor inhibitors, and antisense oligonucleotides, which inhibit TGF-β production. These have proven to be effective in preclinical studies and clinical trials [100].

Moreover, blocking TGF-β in ER negative breast cancer might prevent tumor cells from metastasizing [100]. In bone metastases, TGF- β inhibition interrupts the vicious cycle driven by TGF-β and other key components, halting tumor growth [100]. It is worth noting that serious side effects might arise from targeting TGF-β due to its pleotropic effects. Chronic inflammation and autoimmune reactions as well as the development of premalignant lesions may occur upon suppressing the immunosuppressive effect of TGF-β. Bone morphogenetic proteins (BMP) belong to a TGF-β superfamily with multiple effects on cellular differentiation, proliferation, and apoptosis as well as roles in bone repair and bone metastasis [101]. These also could be effective therapeutic targets for bone metastasis.

CXCR4 is a chemokine receptor that exclusively binds to stromal cell-derived factor 1 (known as SDF-1 or CXCL12) [97]. CXCR4 is highly expressed in breast cancer tissue and CXCL12 is overexpressed in common metastatic sites in breast cancer, such as bone marrow, lymph node, lung and liver [25]. In bone, CXCL12 is produced by multiple types of bone marrow cells, including osteoblasts [38]. Thus, the CXCR4/CXCL12 interaction is important in the homing of breast cancer cells to distant sites and is another attractive therapeutic target in bone metastasis [97]. Several CXCR4 antagonists such as CTCE-9908 have been tested in preclinical studies and have been demonstrated to reduce metastasis as well as primary tumor growth in animal breast cancer models [102–104]. Combination therapy with a

CXCR4 antagonist and chemotherapy agents is also of interest for many researchers. CTCE-9908, when used in combination with DC101 (the VEGFR2 blocking antibody) and docetaxel, demonstrated further inhibition of the primary tumor and lung metastases in a breast cancer model [103]. A phase I/II trial using CTCE-9908 as single-agent therapy for advanced solid cancers, including breast, showed good tolerability of CXCR4 antagonist and preliminary signs of efficacy [105]. Although CXCR4 agents seem promising for the treatment of bone metastases, the effect on normal bone turnover remains to be one major concern [24].

Radium-223 is a radioisotope, which emits alpha-particles, and is under investigation for management of bone metastases in breast cancer. It delivers an intense and highly localized radiation to the affected bone surface while delivering substantially less irradiation to healthy bone marrow, compared with standard bone-seeking beta-emitting radioisotopes [106]. This is due to alpha-particles having a lower penetration depth than beta-particles, thus sparing surrounding healthy bone and bone marrow tissues [107]. Radiotherapy with Radium-233 reduces pain secondary to bone metastases, decreases the incidence of SREs, and lowers bone-specific ALP concentrations [108]. Furthermore, data from phase III trial with Alpharadin (radium-223 chloride) have shown significant improvement in overall survival among patients with castration-resistant prostate cancer, with the treatment group having a median overall survival of 14 months compared with 11.2 months for the placebo group. The reduction in the risk of death was 59.5 % [107].

Alphardin has also shown promising preliminary results in an ongoing phase II trial in breast cancer. This trial recruited breast cancer patients with bone metastases no longer responsive to endocrine therapy. The preliminary results presented at San Antonio Breast Cancer Symposium in 2011 showed that Alpharadin was well-tolerated and reduced the levels of bone alkaline phosphatase as well as urine N-telopeptide, both of which are important bone turnover markers associated with bone metastases [109]. If radium-223 therapy shows clinical effectiveness against bone metastases in breast cancer, as observed in prostate cancer, it would be quite beneficial.

11.6 Conclusion

In summary, there are multiple potential therapeutic targets under investigation for bone metastases in breast cancer. Until definitive control of disease is possible, bone metastases in breast cancer remain difficult to cure and their resistance to pre-existing therapies continues to pose challenges. However, new discoveries elucidating the molecular mechanism of bone metastases and new emerging targets under study provide hope. Since bone metastasis in breast cancer is a complicated process, a multidisciplinary approach should continue to be employed in order to provide the best available care possible for breast cancer patients with bone metastases.

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Chapter 12
Cellular and Molecular Mechanisms Involved in Breaching of the Blood–Brain Barrier by Circulating Breast Cancer Cells

Hava Karsenty Avraham, Shuxian Jiang, Lili Wang, Yigong Fu and Shalom Avraham

Abstract Brain metastases are prevalent in lung, melanoma and breast cancers and are associated with high morbidity and mortality. Therefore, targeted treatments and preventative strategies of brain metastasis are needed. Brain metastases of breast cancer confer significant morbidity and appear to be increasing in incidence ($\sim$35 %) in subpopulations of metastatic breast cancer patients, particularly those with $Her2^+$ or "triple-negative" breast cancer (TNBC). Current therapy for brain metastases of breast cancer involves radiation, surgery and chemotherapy. Unfortunately, both disease progression in brain and treatments cause significant patient morbidity, including cognitive defects. The main question is how are circulating breast tumor cells (CBTCs) able to penetrate the blood–brain barrier (BBB) and gain access to the brain parenchyma, forming brain metastases. The BBB is a dynamic and highly selective barrier due to existence of tight junctions and adherens junctions between adjacent brain microvascular endothelial cells (BMECs). Although, the disruption of the BBB by brain metastases of human triple-negative and basal-type breast cancer was observed, very little is known on the cellular and molecular mechanisms involved in the process of CBTC infiltration through the BBB. This review focuses on the BBB and BMECs as well as several biological determinants by which breast tumor cells infiltrate the BBB and activate BMECs, resulting in co-option and colonization of tumor cells in brain.

Keywords Brain microvascular endothelial cells (BMECs) · Blood–brain barrier (BBB) · Breast cancer · Circulating breast tumor cells (CBTCs) · Metastasis · Colonization · "Triple-negative" breast cancer (TNBC) · Transendothelial electrical resistance (TEER) · Tight junction (TJ) protein complexes · Transmembrane

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proteins · Blood–tumor barrier (BTB) · V = vascular endothelial growth factor A (VEGF-A or VEGF) · Angiopoietins · Substance P (SP) · Integrins

Abbreviations

Ang	Angiopoietin
Ang-2	Angiopoietin-2
α-SMA	Alpha smooth muscle actin
BBB	Blood–brain barrier
BCM	Breast cancer metastasis
BCM/brain	Breast cancer metastasis in brain
BMECs	Brain microvascular endothelial cells
BTB	Blood–tumor barrier
CBTCs	Circulating breast tumor cells
CNS	Central nervous system
DMECs	Dermal microvascular endothelial cells
EC	Endothelial cells
ER^-	Estrogen receptor negative
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HUVECs	Human umbilical vein endothelial cells
HBMECs	Human brain microvascular endothelial cells
IHC	Immunohistochemistry
IF	Immunostaining
LCM	Laser capture microdissection
PR^-	Progesterone receptor negative
RT-PCR	Reverse transcription polymerase chain reaction
TJs	Tight junctions
TEER	Trans-endothelial electrical resistance
TNBCs	Triple negative and basal type breast cancer
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2
WB	Western blotting

12.1 Introduction

Brain metastasis is prevalent in lung, melanoma and breast cancers and is associated with high morbidity and mortality [1–4]. In the United States, more than 40 % of advanced cancer patients develop brain metastasis [5–7]. About 50 % of patients with lung cancer, 25 % of patients with breast cancer and 15 % of patients with melanoma cancer develop brain metastasis. Although the local control and therapy for metastasis to visceral organs have improved, the morbidity and

mortality due to late-diagnosed brain metastasis is expected to increase [1–3]. The median survival time for untreated patients is 1–2 months, and with conventional radiotherapy and chemotherapy, the survival time frame might be extended up to 6 months [1–3]. The resistance of tumor cells to chemotherapy in the brain parenchyma is due to the inability of circulating chemotherapeutic drugs to penetrate the blood–brain barrier (BB), thus preventing treatment of brain tumors.

Metastatic tumors are most common mass lesions in brain. To establish metastatic colony, tumor cells must: (1) grow within the primary site; (2) escape from the primary tumor; (3) penetrate circulating system either as single cells or small tumor embolism; (4) survive during circulation; (5) arrest in microvasculature of other organs; (6) extravasate into organ parenchyma; and (7) efficiently grow and invade tissue at secondary sites [5]. Brain metastases are often indicated by symptoms, such as seizures, loss of motor and sensory function and cognitive decline. These brain functions are confirmed by brain imaging lesions of several millimeters in size [1, 6]. Current treatments for brain metastases are palliative and centered on surgery and radiation therapy [1–4].

12.2 Breast Cancer Metastasis in Brain

Population-based statistics in the U.S. indicate that overall age-adjusted breast cancer mortality rates are higher among African American women (AA) than among Caucasian American women (CA), and the disparity is increasing [7–10]. There is a mortality disadvantage of between 1.5-fold and 2.2-fold that first appeared in the National Cancer Institute's Surveillance [11]. The triple negative breast cancer (TNBC) which accounts for 15–20 % of all breast cancer subtypes, have a proportionally larger number of breast cancer death, with high prevalence among young women and those of African descent [10] (discussed in Chap. 6). TNBC breast cancers are diagnosed at a younger age and have aggressive biologic behavior with the development of local–regional and distant metastasis within 5 years [12–16]. TNBC is associated with poor survival since recurrence and central nervous system (CNS) relapse is common. The high rate of CNS involvement is due to the lack of effective therapies in general for this aggressive subtype of breast cancer. Therefore, new treatment strategies are needed.

TNBC subtype is defined by the absence of ER and PR expression and HER2 amplification, underscoring the lack of understanding of key pathways driving TNBC [12]. Women with TNBC more often develop visceral metastases when compared to their hormone receptor-positive counterparts [12–21]. In a large multicenter study which included more than 2000 patients with TNBC, women with TNBC were more likely to develop lung or brain metastases as their first site of recurrence. Median survival for those with brain metastases was 6 months [17–23].

The poor survival outcomes seen with TNBC patients are in part due to a lack of therapeutic targets. A twofold higher incidence of TNBC in AA patients compared to their Caucasian counterparts was reported, regardless of age at

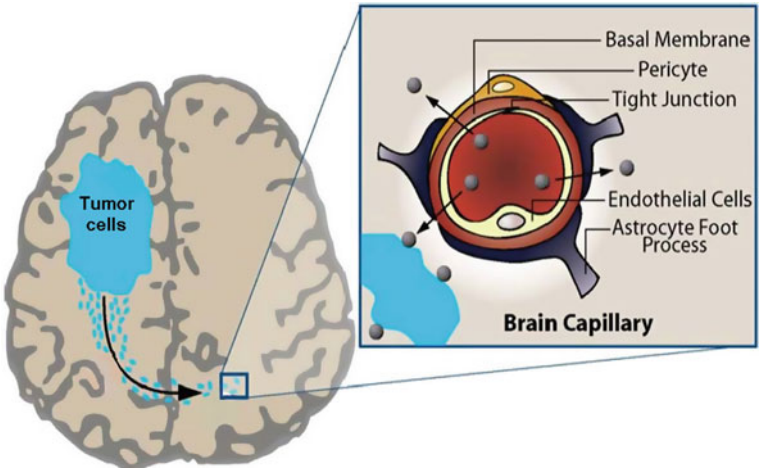

Fig. 12.1 The blood–brain barrier—structure. Rapid growth and highly invasive; the BBB limits access of the therapeutics (The figure was kindly provided by Dr. Miqin Zhang, Professor at University of Washington.)

diagnosis [10]. In AA patients under the age of 40, TNBC accounts for 50 % of all diagnosed breast cancer cases [10, 19–23]. Predominance of brain and lung metastasis in TNBC patients was also reported [18]. Since some of these patients with TNBC are more likely to develop distant metastasis early, we suggest that triple receptor status may be used as a prognostic marker for breast cancer patients that may develop brain metastasis.

12.3 The Blood–Brain Barrier and Brain Microvascular Endothelial Cells and Their Roles in Breast Tumor Cell Infiltration Across the BBB

The BBB is a dynamic and complex interface between the blood and the CNS [24, 25]. The BBB is critical for the maintenance of the homeostasis of the CNS and the regulation of the neural microenvironment [24, 25] (Fig. 12.1). This barrier is comprised mainly of BMECs, which exhibit many specialized properties, including a highly selective permeability and high transendothelial electrical resistance (TEER) (Fig. 12.2). BBB functions are mainly maintained by the tight junction (TJ) protein complexes between adjacent BMECs that include mainly claudins, occuldin, junctional adhesion molecules (JAMs) and the cytoplasmic zonula occludens-1 and -2 proteins (ZO-1 and ZO-2) (Fig. 12.3). Most forms of brain insults and tumor transmigration to the brain are associated with BBB disruption [26]. Disruption of the BBB by brain metastasis was observed in tumors of Triple-negative and basal type breast cancer [27].

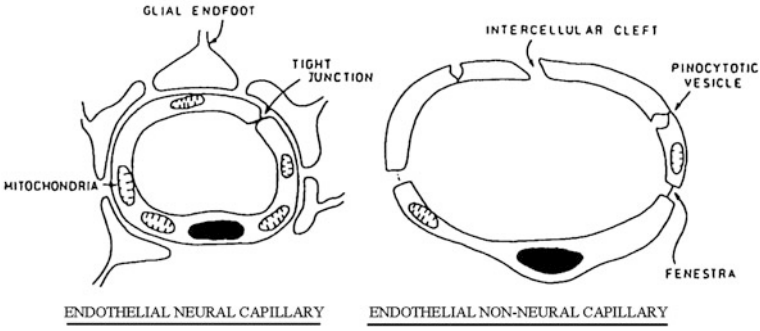

Fig. 12.2 Structure of the neural capillary endothelial cells VS non-neural capillary endothelial cells. (1) In CNS capillary endothelial cells joined by specialized "tight junctions". (2) Astrocytes involved in regulating ionic microenvironment (particularly Ca^{2+} and K^{+} ions). Functional differences between brain microvascular endothelial cells and peripheral endothelial cells: (a) Highly selective permeability to most substances. (b) Increased expression of transport and carrier proteins: receptor mediated endocytosis. (c) Limited paracellular and transcellular transport. For more details see references [24–26]

BMECs are the major cellular component of the BBB and line the cerebral capillaries unsheathed by astrocytic endfeet, which play an essential role in maintaining structure and function of the BBB [24, 25]. BMECs are distinguished from endothelial cells of other organs by several criteria including interendothelial TJs and paucity of pinocytic vesicles (Fig. 12.2). Examination of human brain tissue revealed decreased expression of TJs in BMECs in pathological conditions [26]. Recently the Hedgehog pathway was shown to promote BBB integrity and CNS immune quiescence [28]. In addition, pericytes are important in BBB formation and maintenance, vascular stability and angioarchitecture as well as regulation of capillary blood flow [29].

The genesis of brain metastasis is a multistep process that includes tumor cell dissemination and migration into the brain parenchyma followed by colonization of micrometastases and tumor growth [30–34]. The BBB, with its tight layer of BMECs and astrocyte foot processes, posses high selective permeability, which requires that the infiltration of CBTCs into the brain parenchyma to have highly specialized penetration functions, many of which remain to be characterized [35, 36]. CBTCs extravasate through the brain non-fenestrated capillaries, suggesting that brain metastases result from the ability of these cells to breach the BBB [4]. However, it is not known how CBTCs are able to infiltrate the BBB and gain access to the brain parenchyma to form brain metastases, and what are the dynamic interactions between CBTCs and BMECs within the brain microenvironment, which lead to tumor cell survival, co-option, colonization and growth in the brain.

The "seed and soil" hypothesis of metastasis indicates that successful outgrowth of metastatic tumors depends on permissible interactions between the metastatic cancer cells and the site-specific microenvironment in the host organs [2, 5, 37]. The brain's interaction with CBTCs largely stems from unique brain properties, which

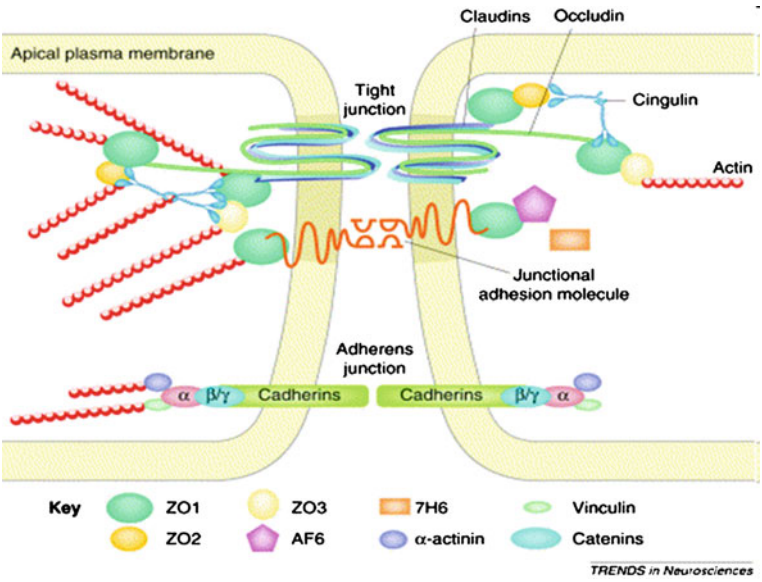

Fig. 12.3 BMEC—TJs. *Claudins*: (a) 22 kDa phosphoprotein, forms the "seal" of the BBB; (b) four transmembrane domains; (c) localized in TJ strands. *Junction adhesion molecules*: (a) 40 kDa, belong to immunoglobulin superfamily; (b) involved in cell-to-cell adhesion and monocyte transmigration through BBB; (c) regulates paracellular permeability and leukocyte migration with PECAM-1. *Occludin*: (a) 65 kDa regulatory protein; (b) its expression level correlates with permeability (TEER); (c) regulatory proteins: alters paracellular permeability. *Adherens junction*: (a) Complex between membrane protein cadherin and intermediary proteins called catenins; (b) E-Cadherin-catenin (alpha, beta) complexes are joined to actin cytoskeleton; (c) form adhesive contacts between cells; (d) assembled via homophilic interactions between extracellular domains of calcium ion dependent cadherins on surface of adjacent cells. *ZO-1, ZO-2*: (a) These scaffolding proteins are members of MAGUK family and function as adapters linking cytoplasmic and cell surface proteins to the cytoskeleton to regulate cell–cell adhesion, cell–cell communication and signal transduction; (b) maintenance of structural and functional integrity of endothelium; (c) crosslink transmembrane proteins. For more details see reference [64]

include the presence of blood–brain/tumor barriers, large nutrient supply and energy consumption and its status as a site of immune privilege. Metastatic cells take advantage of these properties for their successful growth in the brain. As such, the BBB plays a crucial role in brain metastasis. The initial arrest and extravasation of CBTCs, the recruitment of new vasculature and the effective delivery of chemotherapeutics are all influenced by the BBB permeability.

12.4 Tight Junction Structures and Their Regulation

TJ integrity determines the permeability of the BBB. TJs are complex structures found at the apical (luminal) region of intercellular junctions (Fig. 12.3) [24]. They appear as a continuous strand along the endothelial barrier tissues, forming

cell–cell junctional complex that contribute to the dynamic regulation of paracellular permeability across endothelial cellular sheets. TJs consist of various transmembrane and cytoplasmic proteins linked to the actin cytoskeleton, each with specific function in regulating cell-to-cell, assembly, adhesion and communication at the junctional complex. The transmembrane proteins form TJ strands that reach out and interact with signaling molecules and other proteins in the adjacent cells.

The paracellular route passes through intercellular and lateral spaces between adjacent endothelial cells and is mediated by the transmembrane proteins. The main transmembrane proteins include occludin, claudins, ZOs and JAMs [24, 25]:

(a) Claudins, considered as "sealers" of the BBB, act as the structural backbone of TJ and are the important determinant of TJ properties.
(b) Occlucin, an integral membrane protein localized at the TJ strand, also contributes to junction properties and regulates the exchange of small molecules between cells.
(c) Zonula occludens (ZO) function as scaffolding proteins. ZO-1 and ZO-2 are cytoplasmic proteins interact with the actin cytoskeleton, and act as cytoplasmic adaptors. The PSD95-DlgA-ZO-1 homology (PDZ) domains form a scaffold with other proteins or anchor transmembrane proteins to the cytoplasm via their specific C-terminus. ZO-1 and ZO-2 contain PDZ domains and form dimmers between claudin proteins and ZO-1 protein, which in turn interacts with JAM-1 protein [24, 25].
(d) The structure of JAM proteins are composed of a transmembrane domain and a C-terminal cytoplasmic domain. Studies have shown that inhibition of JAM-1 prevents reformation of TJ structure and stable TEER after experimental disruption of epithelial monolayers [24, 25].

TJs, along with adherens junctions (AJs) located at the basal region (see Fig. 12.3), are anchored to the perijunctional acto-myosin ring, formed by a strand of endothelial cells surrounded by actin and myosin II proteins at their apical region. The actin filaments interface with the TJs and regulate TJ structure and paracellular permeability.

12.4.1 Regulation

Signaling proteins, including protein kinase C (PKC), mitogen-activated protein kinases (MAPK), myosin light chain kinase (MLCK), and the Rho family of small GTPases, participate in regulation of the assembly, disassembly, and maintenance of TJ structure. These signaling pathways are activated to mediate interactions between transmembrane proteins and the actomyosin ring.

Myosin II regulatory light chain (MLC) phosphorylation is also implicated in the assembly and regulation of TJ. Inducing MLC kinases in fully differentiated monolayers led to a reduction in TEER and redistribution of ZO-1 and occludin

[24, 25]. The Rho family of small GTPases, RhoA, Rac and Cdc42, are implicated in the regulation of TJ structure and function and the perijunctional actomyosin ring [24, 25]. Downstream effectors of Rho, known as Rho kinases (ROCK), phosphorylate MLC and induce contraction of the actomyosin ring [24, 25]. Rho GTPase-mediated regulation of TJ is complex due to the multiple interactions between the different Rho proteins. For example, inactivation of Rho leads to redistribution of ZO-1 and occludin away from the cell membrane and reorganization of perijunctional F-actin, which leads to reduced TEER and increased paracellular flux. Increased activation of Rho, however, can also lead to increased TJ disassembly via contraction of the actomyosin ring induced by increased Rho/ROCK signaling and increased MLC phosphorylation [24, 25].

12.4.2 Mechanisms of BBB Breaching by CBTCs

The brain microenvironment may have a key role in the metastatic growth process and in resistance to antitumor therapies in brain [26, 38]. The function of the BBB in breast metastatic tumors and the role of the unique brain microenvironment provide a "sanctuary site" to tumor cells. Since the brain microenvironment regulates the establishment of brain metastasis, the cellular and molecular changes within the metastatic brain tumor vasculature and the microenvironment provide a targeting mechanism for therapeutic agents in brain.

It was shown that in numerous neurological diseases, including CNS inflammation in multiple sclerosis, experimental allergic encephalomyelitis (EAE), lymphocytic choriomeningitis virus infection, West Nile virus encephalitis and others, an increase in BBB permeability is detected by the leakage of markers from the circulation into the CNS tissues [39]. Further, studies in models of CNS autoimmunity and virus-induced neuroinflammation have provided evidence linking enhanced BBB permeability with the development of a CNS inflammatory response. Thus, the BBB plays an important role in protecting the CNS from immune-mediated pathology.

Factors known to experimentally disrupt the BBB include arachidonic acid and the eicosanoids, bradykinin, histamine and free radicals [39, 40]. These active compounds, released in pathological tissues may alter cytosolic calcium levels and induce second messenger systems, leading to alterations in BBB permeability. Extravasation of plasma proteins may occur via disrupted TJs, stimulation of fluid-phase vesicular transport or the formation of transcellular pores or channels [39, 40].

12.4.3 Blood–Tumor Barrier Heterogeneity

The disruption of the BBB by brain metastasis was observed with TNBC and basal type breast cancer(s) [27]. Examination of human brain tissues revealed decreased expression of TJs in BMECs in pathological conditions [25, 26]. To date, little is

known on the mechanism(s) by which CBTCs breach the BBB and infiltrate the brain to form micrometastases.

The role of angiogenesis in brain metastatic development is still unclear. Although several reports show co-option of existing vasculature by intravasated tumor cells, other reports support an involvement of vascular endothelial growth factor (VEGF)-induced angiogenesis [41]. Recent studies by Dr. Lockman group [36], showed no increase in vascular density in lesions, suggesting that angiogenesis may not be the cause of variability in blood–tumor barrier (BTB) integrity of the experimental brain metastases. On the basis of analysis of greater than 2000 metastases [36], statistically significant changes in BTB permeability were observed in ~89 % of 231-Br-Her2 and 96 % of 4T1-BR5 brain metastasis [36]. The BTB is variably compromised in most brain metastases greater than a minimal size (>0.1–0.2 mm^2), where as significant integrity changes were primarily associated with large lesions (>1–4 mm in diameter), in which different distances compromise oxygen and nutrient delivery and lead to angiogenesis [36]. With tumor cell delivery via the vasculature, two patterns of tumor growth, compact/solid and diffuse/infiltrative have been noted for brain metastases. Diffuse/infiltrative lesions may arise, in part, through perivascular tumor spread via blood vessel co-option. Although the BTB had observable permeability changes in most brain metastases [36], this is not an evidence of the absence of barrier function for brain metastasis. The leakiest of brain metastases (with ~33-fold increase), showed permeability that was still less than 12 % of that in peripheral breast tumor. Thus, BTB function is only partly compromised, retaining a significant ability to prevent changes in the BBB integrity.

BTB heterogeneity also determined drug efficacy and hereditary chemotherapeutic treatment of brain metastases for agents, such as paclitaxel and doxorubicin, which poorly penetrate the BBB [40]. Therefore, a new class of agents may be necessary for better chemotherapeutic activity to treat brain metastases. Such agents will need to be not only BBB permeable and active against metastatic breast cancer cells but also nontoxic to CNS constituents.

12.5 Biological Determinants in Breast Cancer Metastasis in Brain

Several genes were shown to facilitate the development of brain metastases and include the cyclo-oxygenase-2 (COX-2), the EGFR ligand HBEGF, and α2, 6-sialyltransferase ST6GALNAC5 [42]. COX-2 and EGFR ligands were examined for their roles as mediators of BBB transmigration using in vitro model of the BBB [43].

The vascular basement membrane serves as "soil" brain metastases [44]. In addition, reactive astrocytes were identified as the most active cells that immediately localizes to the individual invading tumor cell to brain following their extravasation across the BBB [42]. As detailed below, additional biological determinants that play important roles in breaching of the BBB by CBTCs are described below.

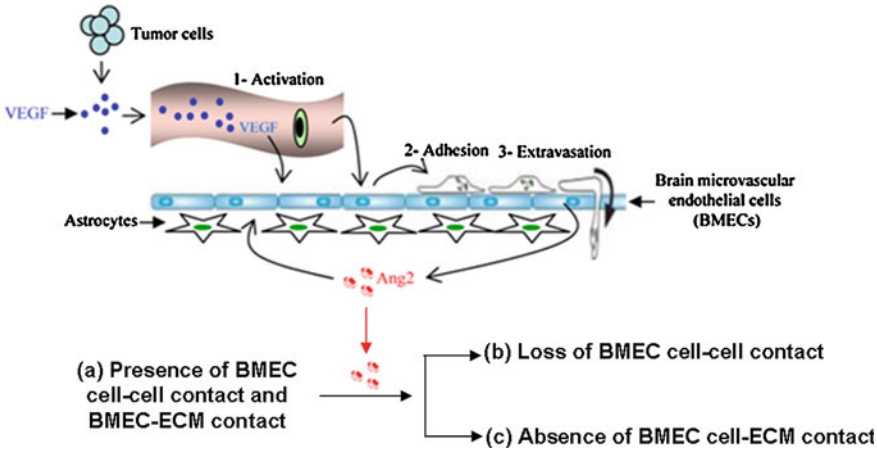

Fig. 12.4 Proposed effects of VEGF and Angiopoietin-2 (Ang2) on quiescent and activated brain microvascular endothelial cells (BMECs). Activated endothelium is a prerequisite step in endothelial sprouting induced by VEGF secreted from tumor cells. Quiescent BMECs (Panel *a*) are surrounded by perivascular support cells including pericytes, which secrete Ang1. Ang1 induces clustering at interendothelial cell–cell junctions, Tie2 activation and endothelial cell survival and stabilization. However, in activated BMEC by VEGF, BMECs secrete elevated levels of Ang2 which inhibit Ang1 binding to Tie2, resulting in decreased BMEC–cell–cell contacts (Panel *b*) and increased BMEC permeability as well as inducing Tie2 clustering at cell–ECM contacts (Panel *c*) [54, 55]

12.5.1 VEGF and Hypoxia

Marked neovascularization is a hallmark of many neoplasms in the nervous system. The tumor growth is dependent on the degree of tumor vascularity and the extent of peritumoral vasogenic edema [41, 45–47]. Several mechanisms have been implicated in angiogenesis, they include: (a) the sprouting of capillaries from preexisting blood vessels by endothelial cell proliferation; and (b) co-option of preexisting blood vessels by tumor cells, leading to expression of Ang-2 by those vessels' endothelial cells and tumor cell proliferation, followed later by involution of preexisting vessels in the core of the tumor, massive tumor cell apoptosis, organization of remaining tumor cells around areas of necrosis and tumor rescue at the margins by angiogenesis (Fig. 12.4).

The Ang-Tie system is crucial for the angiogenic switch in tumors and, together with VEGF, it promotes the initiation of angiogenesis and maturation of new vessels [45–51]. Resistance to anti-angiogenic therapy that target VEGF are attributed to the inherent heterogeneity of genetically unstable tumor cells, the presence of redundant angiogenic factors and the recruitment of hematopoietic cells and inflammatory cells into the tumor mass [52]. Therefore, therapeutic approaches that simultaneously target multiple angiogenic factors, inflammatory pathways and the metastasis process can translate into more clinically successful drugs.

Vascular endothelial growth factor A (VEGF-A or briefly termed VEGF) and its cognate receptors are central to the regulation of angiogenesis in both physiological and pathological states. VEGF was shown to promote neoangiogenesis in concert with Angiopoietin-2 (Ang2). The formation of new blood (angiogenesis) and lymphatic vessels is critical for normal development and is mediated mainly via the VEGF/VEGF-Receptors and by Angiopoietins/Tie receptors systems [46–48]. The endothelial cells lining both the blood and lymphatic vessels are critical in the pathogenesis of diseases with excess angiogenesis such as cancer [49, 50].

New blood vessel formation plays an important role in breast cancer growth and metastasis [49]. Tumor growth is preceded by the development of new blood vessels, which provide a pathway for metastasis and nutrients essential for growth. VEGF-A is a key angiogenic mediator that stimulates endothelial cell proliferation and regulates vascular permeability [41]. Highly proliferative tumors, such as TNBCs have enhanced angiogenesis that supports rapid growth and early metastasis and express high levels of VEGF [10, 12–23]. Thus, breast cancer patients that have tumor cells secreting high levels of VEGF may be of high risk to develop breast cancer metastasis to the brain, via modulation of the BBB (Fig. 12.5). VEGF also acts in concert with Ang2 to regulate blood vessel growth [47].

In cancer, local tumor hypoxia stimulates VEGF synthesis and VEGF levels are subsequently elevated in breast cancer and VEGF expression levels correlate with poor prognosis [47]. Blocking of the VEGF-VEGFR2 pathway is accepted as the first anti-angiogenic therapy. However, since tumors often develop resistance to this therapy, the development of new anti-angiogenic approaches is required for successful anti-angiogenic therapy. This can be achieved by better understanding of the receptors and pathways involved in BMEC remodeling in brain.

12.5.2 The Ang-2/Tie-2 Pathway in Breast Cancer Metastasis

Within the context of tumor biology, strong evidence supports a key contribution of angiopoietins, notably of Ang-2, to the control of tumor angiogenesis [45–51, 53–55]. Furthermore, overexpression of Ang-2 is associated with advanced disease and poor prognosis in several tumor entities [45]. Ang-2 was shown to stimulate breast cancer metastasis through the $\alpha v\beta 1$ integrin mediated pathway [47]. The angiopoietin (Ang)-Tie system is crucial for the angiogenic switch in tumors and together with VEGF-A (VEGF) promotes the initiation of angiogenesis and maturation of new vessels. The Ang-2/Tie-2 system is also involved in metastatic process, inflammation in pathways metastasis and lymphangiogenesis [45, 47]. Resistance to anti-angiogenic reagents that target VEGF are attributed to the inherent heterogeneity of genetically unstable tumor cells, the presence of redundant angiogenic factors and the recruitment of hematopoietic cells and inflammatory cells into the tumor mass [46–49]. Therefore, therapeutic approaches that simultaneously target multiple angiogenic factors, inflammatory pathways and the metastasis process can translate into more clinically successful drugs. In this

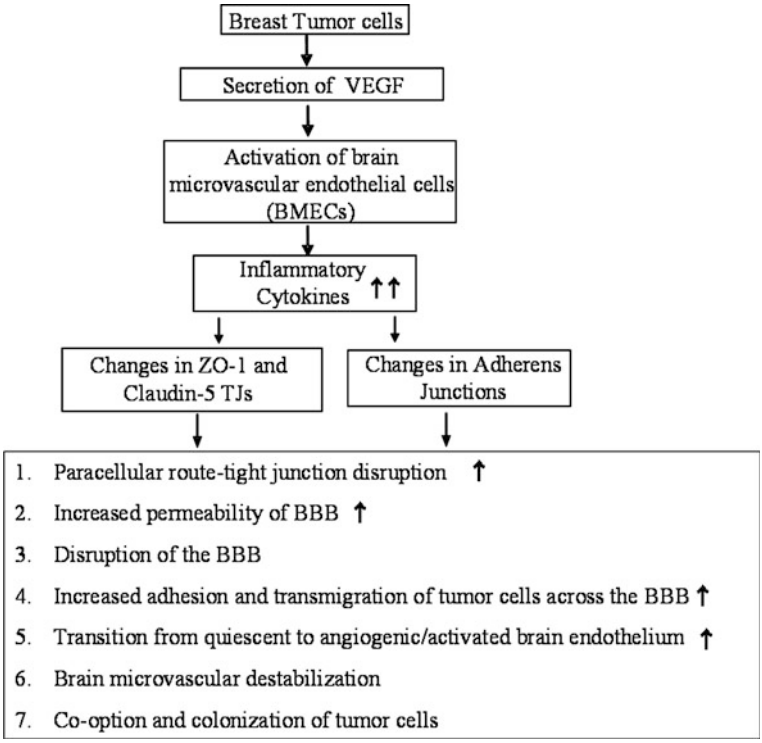

Fig. 12.5 Schematic diagram of the proposed mechanisms by which VEGF activates BMECs resulting in increase in the BBB permeability through paracellular routes and destabilization of BMECs

regard, the Ang-2/Tie-2 system may present a valuable therapeutic approach based on its effects for angiogenesis and vascular homeostasis, and it provides an important link between angiogenic and inflammatory pathways [46].

12.5.3 Substance P/NK-1R Axis in Breast Cancer Metastasis

Substance P (SP) is one of the most important neuropeptides that functions as a neuromodulator in the brain. SP, as well as its receptors NK-1 and NK-2, are expressed in breast cancer cells [52, 56–58]. SP is also an important mediator of neuroimmunomodulatory activity [52, 56–58] and was implicated in the neoangiogenesis connected with neurogenic inflammation. In breast cancer, the involvement of SP and its receptor in the acquisition of oncogenic properties and in facilitation of bone marrow metastasis has been described [52]. However, knowledge on the function of SP in breast cancer cell metastasis to the brain is still lacking.

12.5.4 Integrins

Another pathway for breast cancer metastasis to the brain is the high-affinity state of tumor cell adhesion receptor integrin $\alpha v\beta 3$ that critically promotes metastatic growth and recruitment of supporting blood vessels within the brain microenvironment [59–62]. Integrins are cell surface receptors composed of non-covalently linked α and β subunits that mediate cell–matrix and cell–cell interactions and transducer signals that have impacts on cell survival, proliferation, adhesion, migration, and invasion. Integrin $\alpha v\beta 3$ also plays a role on sprouting endothelial cells and contributes to angiogenesis [60, 61]. In several tumor types, including glioma, breast cancer, and melanoma, expression of $\alpha v\beta 3$ supports invasion and metastasis. Notably, these tumors either originate in the brain or frequently spread to the brain. Inhibition of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, which are preferentially expresses and activate angiogenic endothelial cells, induces tumor cell and endothelial cell apoptosis and impairs tumor angiogenesis [53–55, 62]. $\alpha v\beta 3/\alpha v\beta 5$ integrin signaling is mediated though interactions with an arginine-glycine-aspartic acid (RGD) peptide sequence found in matrix proteins such as vitronectin and can be abrogated by soluble function-blocking RGD peptides, such as cyclic RGDfV. Inhibitors of integrin $\alpha v\beta 3$ are undergoing clinical trials in cancer patients. The cilengitide (EMD 121974; Merck KGaA), an integrin $\alpha v\beta 3/\alpha v\beta 5$ function blocking RGDfV peptide, has shown so far encouraging activity in phase 1 and 2 trials against brain tumors in children and adult cancer patients [63].

12.6 Summary

Brain metastases are the most common malignant tumors of the central nervous system, out-numbering by ten times those that originate in the brain. Breast tumor cell metastasis to the brain is a complex process of a series of sequential steps. Initially, breast tumor cells have to detach from the primary tumor, migrate through the tissues and invade the lymphatic system or blood vessels. In the next step, circulating breast tumor cells adhere to microvascular endothelial cells and then extravasate by infiltrating the underlying basement membrane. Finally, the cells migrate to a suitable location where they form metastases. Understanding the molecular events by which breast tumor cells infiltrate the BBB and activate BMECs will provide better approach for designing new therapy for inhibition of brain tumor angiogenesis and brain metastasis of breast cancer. Specifically, current therapeutic targets for brain metastasis include inhibitors for COX-2, TNF-alpha, Ang-1/2, VEGF and VEGF receptors.

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Chapter 13
Resistance to Anthracyclines and Taxanes in Breast Cancer

Derek Edwardson, Simon Chewchuk and Amadeo M. Parissenti

Abstract Taxanes and anthracyclines are widely used in chemotherapy regimens for the treatment of invasive breast cancer. Whether used in the neoadjuvant or adjuvant settings, numerous clinical trials have validated their effectiveness in improving both progression-free and overall survival in breast cancer patients. However, while clinical response (decrease in tumor size by palpation) is common, for many patients this response is short-lived, after which tumors become refractory to treatment. In addition, some tumors exhibit innate (intrinsic) resistance to these regimens at the start of treatment. Consequently, the vast majority of patients do not exhibit either a pathologic complete response post-treatment or a survival benefit from chemotherapy. Numerous in vitro studies have identified potential mechanisms of action for the anthracyclines and taxanes and how tumors

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may evade the cytotoxic properties of these agents, but their clinical relevance remains questionable. In vivo studies of drug resistance are less subject to such criticisms, but false discovery rates can be high, in particular for genomic studies of biomarkers of drug response or resistance. Nevertheless, studies of drug response and resistance are now starting to provide useful tools to distinguish between responding and non-responding tumors and insight on how to best treat patients with tumors that are refractory to treatment.

Keywords Incidence rates · Adjuvant therapy · Estrogen Receptor (ER) positive cancers · Taxanes · Anthracyclines · In vitro studies · Drug transporters · Aldo–keto reductase enzymes (AKRs) · Microtubules · β-tubulin isotypes · Apoptosis · Chemotherapy resistance · Stromal cells · Tumor initiating cells (TICs) · Chloroquine

13.1 Introduction

Breast cancer is the most common form of cancer among women worldwide, with incidence frequencies continuing to rise [1]. This increasing incidence is generally attributed to prolonged life expectancy, urbanization and adoption of western lifestyles [1]. Global statistics as of 2004 from the World Health Organization (WHO) estimate that breast cancer comprises roughly 16 % of all female cancers worldwide [1]; of these, an estimated 519,000 women had succumbed to the disease in 2004 alone. The WHO estimates that a majority of these deaths occurred in developing countries, roughly 69 % [1]. While incidence rates vary greatly worldwide they have been recorded to be as high as 99.4 in 100,000 women in North America. Moderate incidence rates have been recorded in eastern Europe, southern Africa, eastern Asia and South America, with the lowest incidence rates occurring in most African countries [1, 2]. As in the case with incidence rates, survival rates also vary greatly worldwide, ranging from 80 % in high income nations to less than 40 % in low income nations. These discrepancies are mostly attributed to availability of early detection and treatment methods [1, 2].

Upon detection of disease that is contained within the breast, the primary treatment for breast cancer is typically surgical resection of the tumor with negative margins to prevent recurrence [3]. This is because many patients with early-stage disease respond well to this treatment method. If the disease is sufficiently advanced but within the axilla, many adjuvant treatments exist for breast cancer which include radiation therapy and a variety of chemotherapy regimens [3]. Adjuvant therapy is generally designed to treat micrometastatic disease or breast cancer cells that have escaped the primary tumor but not yet established identifiable metastases. Specific treatments differ depending on the nature of the tumor subtype [3]. Locally advanced and inflammatory breast cancers, however, do not respond well to primary surgical techniques and are therefore deemed

Table 13.1 Anthracycline and taxane containing regimens for the treatment of breast cancer

Treatment Regimen	Chemotherapy agents used	Dose	Frequency	Cycles	Reference
TAC	Taxotere (Docetaxel)	75 mg/m^2 IV	Every 21 days	6	[4, 5]
	Adriamycin (Doxorubicin)	50 mg/m^2 IV			
	Cyclophosphamide	500 mg/m^2 IV			
AC → T	Adriamycin	60 mg/m^2 IV	Every 21 days (14 days for dose dense)	4	[6, 7]
	Cyclophosphamide			4	
	Followed by		Every 21 days (14 days for dose dense)		
	Taxol (Paclitaxel)	600 mg/m^2 IV			
		175 mg/m^2 IV			
FEC 100	5-Fluorouracil	500 mg/m^2 IV	Every 21 days	6	[8]
	Epirubicin				
	Cyclophosphamide	100 mg/m^2 IV			
		500 mg/m^2 IV			
FAC	5-Fluorouracil	600 mg/m^2 IV	Every 21 days	4	[9, 10]
	Adriamycin	60 mg/m^2 IV			
	Cyclophosphamide	600 mg/m^2			
TC	Taxotere	75 mg/m^2 IV	Every 21 days	4	[11]
	Cyclophosphamide	600 mg/m^2 IV			
TCH	Taxotere	75 mg/m^2 IV	Every 21 days	6	[11]
	Carboplatin	AUC 6, IV			
	Trastuzumab (Herceptin)	4 mg/kg loading dose IV followed by 2 mg/kg/wk × 18 then q3wk × 12			

inoperable. Neoadjuvant chemotherapy regimens are thus used as the first treatment for these breast cancers and typically include the anthracyclines and taxanes. These regimens include but are not limited to: TAC [Taxotere (Docetaxel), Adriamycin (Doxorubicin), and Cyclophosphamide] [4, 5], AC → T (Adriamycin and Cyclophosphamide followed by Taxol) in both conventional and dose dense regimens [6, 7], FEC 100 (5-fluorouracil, Epirubicin, Cyclophosphamide) [8], FAC (5-fluorouracil, Adriamycin, Cyclophosphamide) [9, 10], TC (Taxotere, Cyclophosphamide) or TCH (Taxotere, Carboplatin, and Trastuzumab (Herceptin) for HER2-positive tumors [11] (see Table 13.1, adapted from WebMD http://emedicine.medscape.com/article/1946040-overview#aw2aab6b3). Each of these chemotherapy drugs serves a different function in treatment. The taxanes (paclitaxel and docetaxel) function as anti-microtubule agents disrupting the cell's ability to divide during mitosis [4, 5]. The anthracyclines (doxorubicin and epirubicin) function as DNA damaging antibiotics [6, 7]. Cyclophosphamide is an alkylating agent, adding alkyl groups to the guanine bases of DNA, and Trastuzumab

is a monoclonal antibody targeting and inhibiting the HER2 growth receptor present in some breast cancer types [9, 10]. Additionally, in early-stage breast cancer, adjuvant chemotherapy can play a critical role in the treatment of Estrogen Receptor (ER) positive cancers [12]. Adjuvant therapy in these cases involves the use of compounds that target the estrogen signaling pathway, either through interfering with estrogen synthesis (aromatase inhibitors (Letrozole) or through selective estrogen receptor modulators [SERMs (tamoxifen)] [13].

Even with such available treatments, disease progression typically occurs in advanced breast cancers, likely due to the presence or development of chemotherapy-resistant tumors [14]. Some patients possess tumors that exhibit innate resistance to chemotherapy and do not respond to initial treatment (often referred to as "primary chemotherapy"). These cancers are then typically treated with other chemotherapy drugs, if possible, or alternate treatments may become necessary, including surgery or radiation therapy [15]. Other patients have tumors that initially respond or show partial response to the therapy. In such cases, a fraction or the majority of the tumor cell population is killed [15, 16]. The remaining drug-resistant cells, however, survive and continue to replicate, resulting in disease progression. Here we will explore some of the mechanisms associated with resistance to taxanes and anthracyclines in the treatment of breast cancers as well as some of the current work being done to manage patients with drug resistant tumors.

13.2 Resistance to Anthracyclines and Anthracycline-Based Regimens In Vitro

Anthracyclines are believed to be cytotoxic to tumor cells through three mechanisms. First, they intercalate between strands of DNA or RNA molecules and interfere with normal synthesis of these macromolecules in rapidly dividing cells [17]. Second, they interfere with topoisomerase II, which is normally responsible for relaxing supercoiled DNA in order to facilitate DNA replication and transcription [18]. Finally, anthracylines cause cellular damage by facilitating the creation of iron-mediated oxygen free radicals [18].

Many of the biochemical and cellular mechanisms of anthracycline resistance that have been identified to date have been obtained from in vitro studies:

13.2.1 Drug Transporters and Anthracyclines

The innate or acquired overexpression of drug transporters has been proposed as a possible mechanism of resistance to anthracyclines in breast cancer and has been observed primarily in cells exposed to high concentrations of these agents [19–21]. The drug transporters are typically integral "ATP-binding cassette" (ABC) membrane proteins that actively transport anthracyclines and other chemotherapy

drugs from tumor cells in an ATP-dependent manner [19–21]. By exporting drugs from the cytoplasm into the extracellular space, tumor cells are protected from the damaging effects of the chemotherapy agents [19–21]. As revealed in a recent study [15], selection of tumor cells for survival in increasing concentrations of anthracyclines resulted in the acquisition of anthracycline resistance at a specific threshold dose. At or above this threshold dose, uptake of anthracyclines was substantially reduced. Co-incident with the acquisition of drug resistance and reduced drug uptake into tumor cells was the increased expression of various ABC transporters, specifically Abcb1, Abcc1, and Abcc2 [15]. The induced ABC transporter differed depending on the cell line examined: for example, epirubicin-resistant cells showed elevated levels of Abcb1 when the selection dose reached a 30 nM concentration, while doxorubicin-resistant cells only showed elevated levels of Abcc1 late during selection (100 nM doxorubicin) [15]. While the expression of these transporters correlated well with the reduced cellular uptake of drugs, their expression did not correlate well with drug sensitivity, suggesting that multiple factors were at play in the acquisition of drug resistance [15].

13.2.2 Alterations in Anthracycline Metabolism

An additional mechanism for anthracycline resistance appears to involve the ability of the liver and possibly breast tumor cells to convert chemotherapy agents into considerably less cytotoxic forms [22], thus protecting tumor cells from the killing action of these agents. One example of this is the overexpression of the aldo–keto reductase superfamily of enzymes (AKRs) [16]. The AKRs reduce ketones and aldehydes into secondary and primary alcohols [23] and their expression has been shown to be regulated by osmotic pressure, AP-1 transcription factors, and anthracycline-generated reactive oxygen species (ROS). The AKR1C family of enzymes has been shown to metabolize a variety of chemotherapy agents, including doxorubicin [23]. AKR1A1 and AKR1C2 have been shown to convert the anti-tumor agent doxorubicin into doxorubicinol, a significantly less toxic anthracycline [23]. In a similar fashion to AKRs, carbonyl reductases and quinone oxidoreductase-1 (NQO1) have been shown to metabolize doxorubicin into doxorubicinol [24]. The conversion of doxorubicin to doxorubicinol appears to result in altered localization of the drug to lysosomes [16], such that the drug no longer reaches its target in the nucleus.

13.2.3 Other Putative Anthracycline Resistance Mechanisms In Vitro

In addition to the expression of drug efflux pumps and drug metabolizing enzymes, other proteins have been implicated in anthracycline resistance in vitro, including the downregulation of topoisomerase II [25], changes in p53 function [26],

and reduced drug-induced apoptosis [27]. Gene expression profiling studies suggest that a variety of genes change expression as breast tumors acquire resistance to anthracyclines [27]. It remains unclear how many of these genes play a *bona fide* role in clinical resistance to anthracyclines and how many are "passenger" genes that change expression with the "drivers" of drug resistance.

13.3 Mechanisms of Resistance to Taxanes In Vitro

The taxanes block the growth of tumor cells by binding to microtubules and preventing their depolymerization, leading to mitotic catastrophe [28], multinucleation of cells, and the induction of apoptosis [29]. One such apoptosis-inducing agent upregulated by the taxanes is the cytokine TNFα [30]. Like other chemotherapy drugs, the efficacy of taxane treatment is limited by a tumors' inherent or acquired ability to resist their killing action. Taxane resistance can be the product of a variety of alterations in cell behavior [29]. A number of potential mechanisms of taxane resistance have been identified in vitro, including elevated expression of the ABC family of drug transporters, alterations in microtubule structure and stability, inhibition of apoptosis, as well as the activation of some survival pathways.

13.3.1 Drug Transporters and Taxanes

One of the most studied mechanisms of drug resistance is the overexpression of the ABC transporters [31]. ABC transporters are highly expressed in some tissues such as the intestinal epithelium and less differentiated cell types [32]. They are associated with the membrane and actively transport a variety of molecules out of the cell [31]. Among the ABC transporters is the permeability glycoprotein 1 (P-gp), also known as multi-drug resistance protein 1 (Mdr1) or Abcb1. It has been shown that P-gp contributes to taxane resistance in breast cancer cells in vitro, as its elevated expression correlates with low cytoplasmic concentration and decreased sensitivity to paclitaxel [33].

Breast tumor cell lines have been shown to develop P-gp mediated cross-resistance to drugs of the same class and in certain cases to drugs of different classes. Interestingly, breast adenocarcinoma cells selected for resistance to doxorubicin showed several thousand-fold cross-resistance to both docetaxel and paclitaxel [34]. An explanation for this may be that the anthracyclines first induce P-gp and that severe cross-resistance is observed because taxanes are a preferred substrate for P-gp than the anthracycline doxorubicin [35]. This observation may help to explain why patients were significantly less responsive to paclitaxel after late crossover from doxorubicin compared to treatment with doxorubicin after late

crossover from paclitaxel [36]. However, it appears unlikely that P-gp or other ABC drug efflux transporters play a prominent role in clinical resistance to taxanes in breast cancer patients (see 13.5).

13.3.2 Alterations in Microtubule Structure and Stability

Microtubules are dynamic polymers essential to the cell that can undergo elongation and shrinking with the ability to interact laterally with one another [29]. They are required for a variety of cellular processes including transportation of macromolecules and organelles, maintaining and changing structure of the cytoskeleton, and mitosis and cell division [37]. Microtubules are made up of α and β-tubulin subunits, which can be in either a polymerized or dimer form [29]. It is widely accepted that taxanes bind to β-tubulin in the polymerized form and increase polymer stability [29]. In the case of cell division, the increased stability of β-tubulin leads to cell cycle arrest in mitosis [29] and eventually cell death. The β-tubulins are comprised of a variety of isotypes which vary at their C-termini [38]. Molecular diversity among isotypes is accomplished by both the expression of distinct β-tubulin genes [38] and also post-translational modifications to β-tubulin gene products [39]. Expression of certain β-tubulin isotypes is tissue-specific, while other isotypes are constitutively expressed [29]. The functional specificity of different tubulin isotypes among tissues has yet to be determined [29].

Regardless of the β-tubulin isotype, polymers can form and the binding site for paclitaxel is only present in the case of the polymerized form of β-tubulin. Hence, it is suggested that selection for cancer cells in which the equilibrium between dimer and polymer has shifted toward dimer, could offer a survival advantage for a tumor that is treated with a microtubule-stabilizing agent such as paclitaxel [29].

Microtubule dynamics are also controlled by the differential expression of tubulin isotypes, mutations within tubulin genes, and also interactions with tubulin regulatory proteins. Tubulin regulatory proteins such as microtubule-associated proteins (MAPs) or stathmin interact with tubulin to promote polymerization or disassembly, respectively [29]. Increased stathmin mRNA levels have been measured in breast carcinoma tissue from patients with more aggressive disease [40]. It is also possible that post-translational modifications to tubulin such as phosphorylation, polyglutamylation, polyglycylation among others, may alter the binding of tubulin regulatory proteins, microtubule dynamics, and thus taxane efficacy [29].

13.3.2.1 Differential Expression of Specific β-Tubulin Isotypes

As mentioned, the tubulin isotype expressed in cells has an effect on the properties of polymer assembly and thus affects interactions with taxanes and microtubule dynamics. For example, microtubules assembled from βIII-tubulin are considerably

less sensitive to the suppressive effects of paclitaxel on their dynamics, than microtubules assembled from βII-tubulin [41]. This suggests that selective expression of certain β-tubulin isotypes may affect the cellular sensitivity to taxanes.

A number of in vitro studies suggest a relationship between βIII-tubulin isotype levels and taxane resistance. For example, one study examined tubulin isotypes and mutations in paclitaxel-resistant cells by combined isoelectric focusing and mass spectrometry, and found that class III β-tubulin expression did, in fact, correlate with resistance to paclitaxel [42]. Moreover, an association between class III β-tubulin expression and resistance to paclitaxel has been observed in a variety of human cancer cell lines of lung, ovarian, prostate and breast origin [43]. However, another study showed that βI, βII, and βIII-tubulin levels were decreased and βIV-tubulin levels increased when MDA-MB-231 cells were selected for taxane resistance [44]. Nevertheless, there is evidence of β-III tubulin's role in tumor resistance to taxanes in cancer patients (see 13.4.1), suggesting that differential expression of β-tubulin isotypes may be an important mechanism of taxane resistance in breast tumors.

13.3.2.2 Point Mutations in Tubulin

The binding of taxanes to β-tubulin subunits in microtubules can also be affected by mutations in genes coding for either β- or α-tubulin [45]. These mutations can affect the sensitivity of cells to taxanes by causing a change in microtubule dynamics. It has been observed that cells with specific βI-tubulin mutations become resistant to paclitaxel in vitro [46], and that some paclitaxel-resistant cell lines depend on paclitaxel for survival [29]. A potential explanation for this is that certain tubulin mutations shift the equilibrium in favor of the dimer form, such that cells harboring these mutations become hypersensitive to drugs that bind the dimer form of tubulin such as colchicine and vinblastine [47]. In some cases, the equilibrium is shifted to such an extent that the resulting lack of polymer stability compromises the cell's basic functions and thus paclitaxel's polymer stabilizing effects shift the polymer-dimer equilibrium in a more favorable direction, promoting survival [29].

Another form of taxane resistance can occur from a mutation in either α- or β-tubulin that alters the drug-binding site on β-tubulin polymers, such that it has less affinity for taxanes. In vitro reports of point mutations associated with taxane resistance in breast cancer cells have been reported, but in other studies, including clinical ones, no association between point mutations in tubulin genes and taxane resistance has been observed. For example, no mutations in β-tubulin genes were found when β-tubulin sequence information was compared between two docetaxel-resistant variants of the MDA-MB-231 and MCF-7 human breast adenocarcinoma cell lines and their drug-sensitive parental cell lines [44]. A clinical study in 2003 also revealed that mutations in the class I β-tubulin gene did not

predict response to paclitaxel in breast cancer patients [48]. Thus, despite in vitro reports showing an association between β-tubulin mutations and taxane resistance, this association is not observed in breast cancer patients treated with taxanes.

13.3.3 Inhibition of Apoptosis

The arrest in mitosis caused by taxane-binding to microtubules appears to promote the induction of apoptosis. The trigger for apoptosis is governed by the effects of taxanes on key apoptotic regulatory proteins. For example, it is believed that taxanes induce hyperphosphorylation of Bcl-2 and Bcl-x_L, which subsequently blocks their ability to bind to and antagonize the apoptosis-inducers Bax and Bak [49, 51]. Bax and Bak are then free to dimerize and cause pore formation within the mitochondrial membrane, thus mediating apoptosis by the intrinsic apoptotic pathway [49–51]. Taxanes also can cause Bax upregulation to promote apoptosis [49–51]. It has also been suggested that paclitaxel can directly bind and sequester Bcl-2, a microtubule-independent mechanism of cell death [52].

The function of Bcl-2 is often regulated post-translationally by a variety of growth factor and cytokine signaling pathways [53]. These pathways can drive Bcl-2 upregulation and induce paclitaxel resistance [54]. For example, exposure to estrogen in estrogen responsive breast adenocarcinoma cells (MCF-7) is associated with an increase in Bcl-2 levels and resistance to paclitaxel-induced apoptosis [55]. Interestingly, one study found that induced recombinant ERα expression in ER-negative breast cancer cells caused resistance to paclitaxel by inhibiting apoptosis, while blocking ERα receptor activity in ER-positive breast cancer cells caused sensitization to paclitaxel [56]. There is also clinical evidence that patients with ER-positive breast tumors are less responsive to paclitaxel than patients with ER-negative tumors [57–59].

Breast cancer cells selected for resistance to escalating doses of docetaxel were shown to have alterations in TNF signaling pathways. Specifically, the TNFRI receptor, which promotes cellular apoptosis, became downregulated upon resistance to docetaxel [30]. This downregulation of TNFRI lead to increased activation of the transcription factor NF-κB, which promotes expression of anti-apoptotic survival genes such as c-FLIP [60] XIAP, and Bcl-$_{XL}$, which are known to cause chemotherapy resistance [61, 62].

13.3.4 Activation of Survival Pathways

A cell's tendency to live or die is determined by the net balance of opposing death and survival pathways. Induction of survival pathways in breast cancer cells is often associated with resistance to taxanes. Taxane-resistant breast adenocarcinoma cells have been observed to possess an amplified positive-feedback loop

involving the TNF-dependent activation of NF-κB, which promotes expression of pro-survival genes. This involves the expression and secretion of cytokines, which complete the loop by way of autocrine or paracrine signaling [30]. Increased nuclear staining of NF-κB in tumors (indicative of activated NF-κB) has been shown to be associated with resistance to chemotherapy treatment with anthracycline- or taxane-containing regimens in breast cancer patients [63]. Nevertheless, the true clinical relevance of such pathways in taxane resistance in breast cancer can only be determined through repeated clinical investigation.

13.4 Mechanisms of Resistance to Anthracyclines and Taxanes In Vivo

While providing significant insight into potential mechanisms of taxane or anthracycline resistance, the majority of the above in vitro studies fail to address important characteristics of human tumors that can impact on drug response and resistance. Such characteristics include their three-dimensional nature, the vasculature that provides nutrients and oxygen, and a complex tumor microenvironment comprised of surrounding stromal tissue, the extracellular matrix, and cells recruited by tumors (endothelial cells, fibroblasts, inflammatory cells of the immune system and pericytes). It is likely that some of these characteristics can account for the lack of relevance of some in vitro drug resistance mechanisms in clinical studies. This tumor microenvironment creates the potential for cells within a tumor to be deprived of oxygen and nutrients, evade drug exposure, and exhibit a reduced proliferation rate, all of which could present a barrier to taxane or anthracycline cytotoxicity.

13.4.1 Changes in Tubulin Isoform Expression

As mentioned previously, in vitro studies have shown that there may be a correlation between expression levels of specific tubulin isoforms and taxane resistance in breast cancer cells [43, 64]. Clinical studies appear to support such a view, as one study showed that breast cancer patients with high levels of class I and class III β-tubulin transcripts are less likely to respond to docetaxel than patients with the following levels of tubulin transcripts: class I-low/class III-low, class I-high/class III low or class I-low/class III high [65]. Also supporting this study, high tumor levels of tubulin β-I and β-III transcripts were found to correlate with clinical resistance to paclitaxel in advanced breast cancer [66]. While these reports are compelling, further studies are required to assess whether tumor levels of tubulin β-I and β-III transcripts can serve as an effective biomarker of taxane resistance in multiple cohorts of breast cancer patients.

13.4.2 Interactions with Stromal Cells

Interactions between epithelial and stromal tissue play an important role in the function of healthy mammary glands [67] and mediate suppression of transformation to preneoplastic phenotypes [68]. It has been suggested that cancer could be a physiological response to an abnormal stromal environment in some cases [69], as reviewed by Barcellos-Hoff and Medina [70]. In addition, stromal tissue can affect chemotherapy response through its tumor-supporting behavior [71].

Human cells communicate by secreting cytokines, chemokines, and growth factors that convey signals to nearby cells or travel through the bloodstream and affect more distal tissues. Activation of the innate immune response originates from the site of infection or inflammation, whereby signals are made available to components of the immune system, including monocytes, via the bloodstream. In breast cancer, signals originating from tumor or nearby stromal cells can strongly affect the host (patient) and may affect tumor response to chemotherapy. Accumulation of tumor-associated macrophages has been associated with poor prognosis in breast carcinoma, as they are suggested to exhibit a tumor-supporting phenotype in some cases, which can include secretion of cytokines that promote proliferation, angiogenesis, and metastasis [71].

It has recently been demonstrated that stromal gene expression can be an important factor in the clinical outcome of breast cancer patients treated with adjuvant chemotherapy [72]. In this study, tumor stromal samples were classified as being from a patient with good, poor or bad outcome after assessment of clinical status post-treatment. Stromal overexpression of a specific set of immune-related genes, including T cell and natural killer cell markers, typical of a $T_{H^{-1}}$ type immune response, was correlated with a good clinical outcome in patients [72]. On the other hand, stroma from individuals in the poor-outcome group showed markers of hypoxia and angiogenesis, along with a decrease in chemokines that stimulate natural killer cell migration and mediate pro-survival signals in T-lymphocytes [72, 73]. In another clinical study, mesenchymal/stromal gene expression signatures were shown to be useful in predicting resistance to neoadjuvant chemotherapy in breast cancer [74].

13.4.3 Nutrient Deprivation, Hypoxia, and Acidity

Tumors are generally less vascularized than healthy tissue. As cells within a tumor reside farther from blood vessels, the level of nutrients falls and tumor cells in these areas tend to have decreased proliferation rates [75]. It is suggested that since most anticancer drugs including taxanes and anthracyclines tend to be most toxic to rapidly dividing cells, slowly proliferating cells tend to be more drug-resistant [76]. As nutrient levels are lower at distances further from vessels, so are pH and levels of molecular oxygen [77]. Such hypoxic regions typically have increased

expression of P-gp [78], which as mentioned, can cause taxane or anthracycline efflux from tumor cells. It has been suggested that anthracyclines may rely on superoxide formation as a means of cytotoxicity [79] and thus tumor cells in hypoxic regions may be less likely to suffer an attack of this nature [77].

Low pH in the tumor microenvironment is typical, as cancers often rely more heavily on glycolysis than normal tissues [80, 81] and slower clearance of breakdown products [82–84]. This can influence the cytotoxicity of anticancer drugs like doxorubicin, which are weakly basic. Protonation of such weak bases in acid environments could then result in decrease cellular drug uptake [84, 85].

13.4.4 Drug Penetration in Tumors

Both taxanes and anthracyclines are administered intravenously and must cross capillary vessel walls to reach cancer cells. For cells in the interior of tumors, this requires extensive diffusion through multiple layers of tumor cells (referred to as "packing density") [76]. By visualizing the location of doxorubicin through its natural fluorescence, it has been shown that high concentrations of doxorubicin are found within and around blood vessels, but concentrations of doxorubicin are considerably lower as the distance from the nearest blood vessel increases [86]. It is suggested that the inability of both doxorubicin and epirubicin to penetrate deep into tumors may be the result of its sequestration in perinuclear endosomes and other organelles at the tumor surface or nearby host tissue [87].

13.4.5 Role of Tumor Initiating Cells in Anthracycline and Taxane Resistance

Solid tumors are generally heterogeneous, a product of their relatively high genetic instability. This results in tumors containing cells with a diversity of phenotypes, including rare cells exhibiting stem cell characteristics (quiescence, pluripotency, increased capacity for DNA repair) and both ABC transporter expression or dependence on surrounding stromal cells for survival [32]. Such "stem cells" within tumors are referred to as tumor initiating cells (TICs)—due to their ability to initiate tumor formation when injected into mice. Such cells may have significant relevance in taxane and anthracycline resistance in patients with breast cancer.

TICs have been identified in a variety of cancers including multiple myelomas [88], leukemias [89, 90], colorectal [91], prostate [92], and hepatocellular carcinomas [93]. Breast cancer TICs are defined by specific cell surface markers ($CD44^+/CD24^-/ALDH1^+$). Additionally, in many cases, breast cancer TICs have been shown to be dependent on developmental signaling pathways [94], particularly the Notch, WNT and Hedgehog pathways [94]. Since TICs tend to possess properties similar to less differentiated cells, they may possess the ability

to adapt to the adverse conditions caused by chemotherapy treatment [94, 95]. In addition, since TICs are relatively quiescent, they are less sensitive to chemotherapy agents targeting rapidly dividing cells, such as the taxanes or anthracyclines. They also may overexpress ABC drug transporters, which are known to play a role in resistance to both taxanes and anthracyclines, as mentioned in previous sections [94, 95]. Nevertheless, there has been controversy about the cell surface markers that define breast TICs and which stem cell markers are correlated with chemoresistance [95, 96]. "Basal-like" breast cancers are associated with poor patient prognosis and have many of the properties of TICs [97], but such cancers remain some of the most chemoresponsive tumors [98]. Moreover, while clear subtypes of breast cancer have been identified through gene profiling studies [99] and while these subtypes differ in response to adjuvant chemotherapy [100], there are currently no pre-treatment genetic or protein biomarkers that can definitively distinguish between tumors that are responsive to anthracycline or taxane-chemotherapy regimens and those that are not [101].

13.5 Management of Breast Cancer Patients with Drug-Resistant Tumors

Even if the appropriate biomarkers can be found to identify chemoresistant tumors, the challenge of how to manage patients with such tumors remains. Typically upon failure to respond to chemotherapy with anthracyclines and taxanes, treatment moves to other chemotherapy drugs in the adjuvant setting or to surgery and/or radiation therapy in the neoadjuvant setting. Strategies used to treat drug-resistant breast cancer involve the employment of drugs with mechanisms of action distinct from taxanes and anthracyclines, including capecitabine [102], navelbine, gemcitabine [103], and carboplatin.

Currently there has been little success in restoring drug sensitivity to patients whose tumors have acquired resistance to anthracyclines and taxanes [3]. With increased knowledge of clinically relevant drug resistance mechanisms, it may be possible to interfere with these mechanisms to restore chemosensitivity. An early example of attempts to re-establish drug sensitivity by interfering with a drug resistance mechanism involves the employment of P-gp inhibitors in patients with chemotherapy-resistant tumors [104]. Two such inhibitors, Verapamil and Tariquidar, were found to restore sensitivity to doxorubicin in drug-resistant cells [104], but had little effect on restoring clinical response to anthracycline- or taxane-containing chemotherapy regimens [105].

Another possible mechanism to restore sensitivity to chemotherapy regimens in breast cancer patients may involve the use of chloroquine. Chloroquine (Resochin) was originally developed as a drug to prevent malarial infections in humans [106]. Its use has been expanded to include treatment for autoimmune disorders such as rheumatoid arthritis and recently as a radiosensitizing or chemosensitizing agent in

cancer and HIV chemotherapy [107–110]. In the case of cancer treatment, chloroquine is thought to act by inhibiting autophagic survival while activating apoptotic pathways [109, 110]. This occurs because chloroquine preferentially accumulates in lysosomes of the cells where the pH of the lysosomes traps the chloroquine [107]. Additionally, chloroquine permeablizes the lysosomes allowing for the release of lysosomal enzymes into the cytosol [107]. Thus, chloroquine may sensitize tumor cells to radiotherapy or chemotherapy by interfering with autophagic survival pathways induced upon exposure to chemotherapy agents [107]. Several clinical trials are currently under way to assess the efficacy of chloroquine as a possible tool to restore sensitivity to chemotherapy agents, such as the anthracyclines and taxanes. Research is also being performed on other autophagy inhibitors as sensitizing agents for chemo-resistant tumors.

Given that patient tumors vary in response to chemotherapy agents (both prior to and after previous rounds of chemotherapy), an additional approach to manage breast cancer patients would be to accurately assess tumor response to chemotherapy early in treatment, such that patients with non-responding tumors could be quickly switched to other downstream regimens such as surgery, radiation therapy, or other chemotherapy drugs. A recent study revealed that locally advanced breast cancer patients exhibiting a pathologic complete response to epirubicin/docetaxel chemotherapy post-treatment exhibited significant reductions in RNA integrity during chemotherapy [111]. This "response biomarker" may be of particular value in patient management, if tumor response can be determined after one or two cycles of chemotherapy. The true value of this biomarker will only be determined through additional studies involving the assessment of multiple cohorts of breast cancer patients at various cycles during chemotherapy treatment.

13.6 Concluding Remarks

Anthracyclines and taxanes are powerful chemotherapy drugs used in the treatment of breast cancer, in particular for those patients that achieve a pathologic complete response to treatment with these agents. However, the majority of patients exhibit innate or acquired resistance to anthracycline- or taxane-containing regimens. While much has been learned from in vitro and in vivo studies on resistance to anthracyclines and taxanes in breast tumor cells, it appears likely that breast tumors evade the action of these agents through multiple mechanisms. Moreover, these mechanisms likely vary among patients and among the cell population within a given tumor. This makes it difficult to predict chemotherapy response and to identify a single small molecule that will block innate or acquired drug resistance. Nevertheless, significant advancements have been made in understanding the molecular diversity of breast cancers and their differential sensitivity to anthracyclines and taxanes. These tools are helping guide the oncologist in assessing a particular patient's risk of treatment failure. In addition to such predictive biomarkers, the development of response biomarkers may help

confirm drug resistance early in treatment, such that non-responding patients can be moved more rapidly to alternate and potentially more beneficial treatments. The development of agents to prevent or combat resistance to anthracyclines and taxanes in select or multiple cohorts of breast cancer patients would help further improve the therapeutic benefit to patients with breast cancer. Given that the majority of patients do not receive a survival benefit from adjuvant or neoadjuvant chemotherapy with anthracycline and taxanes [112, 113], there is still significant and challenging work to be done.

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Chapter 14
Understanding Tamoxifen Resistance of Breast Cancer Based on Integrative Bioinformatics Approaches

Y. Dai and L. Huang

Abstract Global gene expression profiles on tumors are not only useful in developing prognosis signatures but also rich resource for the elucidation of underlying mechanisms related to poor clinical outcome and drug resistance. In this chapter we present a panel of bioinformatics strategies to derive biological insights based on gene expression profiles on estrogen receptor positive breast tumors that were collected prior to adjuvant tamoxifen treatment. The analyses reveal that the tamoxifen resistant tumors are highly proliferative and display a distinctive expression profile for genes related to inflammation and angiogenesis compared to tamoxifen sensitive tumors. The bioinformatics analysis also identifies a set of small molecules that may reverse the tamoxifen resistance in breast tumor.

Keywords Tamoxifen resistance · Tamoxifen sensitive tumors (TamS) · Tamoxifen resistant (TamR) tumors · Gene expression · Transcription factors (TFs) · Gene set enrichment analysis (GSEA) · Co-expression network analysis · Intrinsic cell proliferation · Angiogenesis · Inflammatory defense · Resensitize · Small molecule

14.1 Introduction

Several prognosis gene signatures for breast cancer have been developed from various studies and methods based on global gene expression profiles of breast

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tumors during the last decade [1–6]. These findings based on unsupervised learning approaches have provided new classification of breast cancer and identified the association of a proliferative gene signature to distant metastasis and relapse [7]. Gene signatures have also been derived based on supervised learning methods. That is, using the information of the clinical endpoints, such as metastases or recurrence of cancer, to retrospectively classify patients into groups based upon which gene signatures were obtained [8–16]. For example, several studies have identified different gene signatures using gene expression profiles from tamoxifen resistant and sensitive breast tumors.

Gene signatures are useful in stratifying patients with poor prognosis [17, 18]. However, they display little overlap, presenting challenge to further advancing our understanding of breast cancer [19]. Bioinformatics approaches to the analysis of entire gene expression profiles offer a better opportunity in providing not only the genome-level understanding of the disease but also in generating new hypotheses to investigate specific mechanism such as signaling pathways that govern the processes of formation, maintenance and expansion of tumor [20, 21]. For example, a gene set linked to the growth factor signaling was found significantly enriched in the Luminal B tumors based on the gene set enrichment analysis (GSEA) [22]. In another study, multiple pathways were identified by mapping gene sets derived from from estrogen receptor (ER) positive (ER+) and ER negative (ER−) tumors to Gene Ontology (GO) Biological Process (BP) terms, and among them pathways related to apoptosis, cell division and G-protein coupled receptor signal transduction are predictive for ER+ and ER− tumors in an independent set [23]. Elucidating mechanisms involved in the disease could lead to new therapeutic strategies to specific molecular phenotypes.

In the remainder of this chapter, we focus on gene expression profiles of tumors collected from patients before receiving tamoxifen adjuvant therapy. Tamoxifen is used as an adjuvant treatment to prevent breast cancer recurrence and as a therapy to ER+ patients for reduction of tumor recurrence and improvement of overall survival. However, about 50 % of patients with metastatic disease do not respond to the first-line treatment with tamoxifen, and many who receive it as adjuvant therapy experience relapse despite an initial response. The mechanisms responsible for these treatment failures remain unclear [24]. The gene signatures derived from multiple gene expression profiles on tamoxifen sensitive (TamS) and tamoxifen resistant (TamR) tumors are useful for the prediction of clinical outcome. However, understanding of the tamoxifen resistant is challenged not only by the lack of overlap among the gene signatures, but also by the lack of commonly differentially expressed genes identified from different gene expression profiling studies [25]. Apparently, the gene-based analysis would be unreliable. Instead, the approaches based on gene sets or pathways are effective for uncovering the common biological themes represented by distinct gene sets.

Using three publically available gene expression data, we demonstrate the utility of integrative bioinformatics strategies for in silico discovery of relevant biological pathways and potential transcription factors that may regulate the differential gene expression between TamR and TamS tumors. We further show that

Table 14.1 Summary of the three data sets from tamoxifen resistant and sensitive breast tumors used in this study

		Number of tumors	
Data source	Platform	Tamoxifen resistant	Tamoxifen sensitive
GSE6532	Affymetrix HG-U133A	85	91
GSE9195	Affymetrix HG-U133 Plus 2.0	26	138
GSE9893	Qiagen-Operon Oligo Set 2.1.3	49	98

the co-expression network analysis is capable in identifying a set of genes, which are differentially connected with other genes in the TamR and TamS co-expression networks. Finally, we describe how to use the gene expression profiles to identify small molecules that may have potential to resensitize tamoxifen resistance.

14.2 Identify Common Biological Pathways and Transcription Factors Related to TamR Through Gene Set Enrichment Analysis

The three datasets were selected from several microarray gene expression studies of breast cancer published previously [14, 15]. The raw data were downloaded from the Gene Expression Omnibus [26] (accession numbers GSE6532, GSE9195 and GSE9893) (Table 14.1). The differential expression analysis for each data set based on common protocols of preprocessing and statistical analysis has led to the identification of three different sets of differentially expressed (DE) genes between TamR and TamS tumors [25]. Only four genes are common in the three DE gene sets (Fig. 14.1). They are chemokine C-X3-C motif receptor 1 (CX3CR1), which is under-expressed in tamoxifen resistant tumors; cyclin E2 (CCNE2), kinesin family member 4A (KIF4A) and non-SMC condensin I complex subunit G (NCAPG), which are over-expressed in TamR tumors.

In order to search for common biological processes implied by the gene expression profiles of TamR and TamS tumors, the functional enrichment analysis tool, DAVID [27], was applied to the individual DE gene sets. This analysis identified a set of enriched GO BP terms for each over-expressed and under-expressed gene set in TamR tumors. Taking the overlap from the sets of the enriched GO BP terms, a list of commonly enriched GO BP terms in the over-expressed genes in TamR tumors was obtained (Table 14.2). Specifically, these terms are mainly associated with cell-cycle and DNA replication, suggesting an elevated cell proliferative capacity in TamR tumors compared to TamS tumors. Similarly, four pathways, DNA replication, G1_to_S cell cycle, cell cycle, and purine metabolism, were found to be commonly enriched by all three over-expressed genes sets in TamR tumors. This result further indicated that TamR

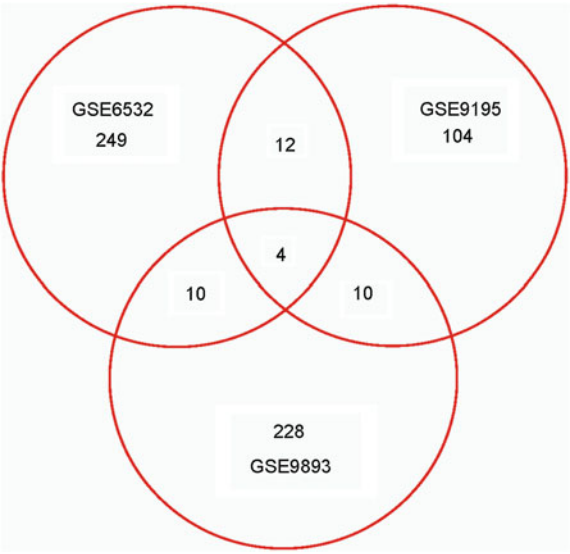

Fig. 14.1 The Venn diagram of the three differentially expressed genes sets identified from the three microarray gene expression studies. Gene expression analysis was performed using packages in Bioconductor [12]. The preprocessing protocols and differential gene expression analysis can be found in [25]

tumors likely have an intrinsic highly proliferative profile, which is shared by the Luminal B subtype tumors [25].

To predict the upstream transcription factors (TFs) that maybe responsible for the gene expression profiles, the GSEA was employed [28]. The GSEA can identify TFs whose encoding genes are not differentially expressed, but their transcriptional regulatory activities are indirectly observed in the change of expression levels of their target genes. Using the TF target gene sets that are available from the Molecular Signature Database (MSigDB) [29], the TFs whose predicted target gene sets are commonly enriched in three microarray datasets were searched. Four TFs, TFDP1, TFDP2, E2F1, and E2F4, were identified for the TamR tumors [25]. TFDP1 and TFDP2 are transcriptional coactivators that can stimulate E2F-dependent transcription of a number of genes whose products are involved in control of cell-cycle progression from G1 to S phase, DNA replication, and p53-dependent/independent apoptosis [30]. It was shown recently that ERα-dependent E2F transcription could mediate resistance to estrogen deprivation in human breast cancer [31]. Taken together, the findings from the enrichment analyses of functional annotation, canonical pathways, and TF target gene sets, are linked to the concept that the elevation of cell proliferation pathways is a hallmark of tamoxifen resistant breast tumors.

Table 14.2 Commonly enriched GO Biological Process terms in the three over-expressed gene sets in tamoxifen resistant tumors

GO Accession number	Synonyms
GO:0000087	M phase of mitotic cell cycle
GO:0000278	Mitotic cell cycle
GO:0000279	M phase
GO:0000280	Nuclear division
GO:0006259	DNA metabolic process
GO:0006260	DNA replication
GO:0007049	Cell cycle
GO:0007067	Mitosis
GO:0007346	Regulation of mitotic cell cycle
GO:0010564	Regulation of cell cycle process
GO:0022402	Cell cycle process
GO:0022403	Cell cycle phase
GO:0048285	Organelle fission
GO:0051301	Cell division
GO:0051726	Regulation of cell cycle

For each microarray dataset, over-expressed and under-expressed genes in tamoxifen resistant compared to sensitive tumors were tested for enrichment of functional annotation categories (GO Biological Process) using tools in the Database for Annotation, Visualization and Integrated Discovery (DAVID) (v6.7) [27]. The *P* values for the functional annotation enrichment were corrected by the Benjamini–Hochberg method for multiple testing and the threshold for significance was set at 0.1 for the adjusted P-values [25]

14.3 Uncover Novel Gene Modules Related to TamR Through Co-expression Network Analysis

The gene co-expression network analysis is a systems biology method for describing correlation patterns at the genome scale. Particularly, the weighted correlation network analysis (WGCNA) can detect clusters (aka modules) of highly co-expressed genes, and organize modules into a hierarchical structure [32, 33] based on a topological similarity measure defined for a pair of genes. The identified modules in various applications have been shown to be highly biologically relevant [34, 35]. The WGCNA can also detect highly connected hub genes within each module to facilitate the understanding of underlying mechanisms.

To reveal distinct patterns on how genes are co-expressed in TamR and TamS tumors, the WGCNA was applied to the gene expression profiles of the TamR and TamS tumors (GSE6532) separately. Figure 14.2 shows the hierarchical clustering of modules in the co-expression networks corresponding to TamR and TamS tumors, respectively. Genes in modules of the same color in the two networks overlap significantly. Based on the significantly enriched GO BP terms in each cluster, it can be seen that the WGCNA approach identified highly biologically relevant co-expressed genes that are common in both TamR and TamS tumors. It also revealed a marked distinction in clustering structures between the two

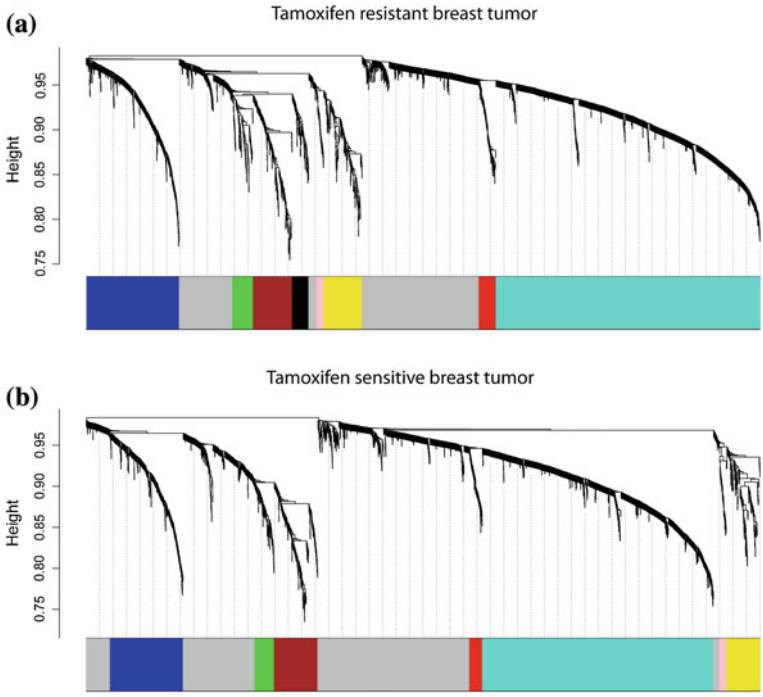

Fig. 14.2 The hierarchical clustering of genes generated from the WGCNA for TamR and TamS tumors. The WGCNA [32] was applied to the gene expression profiles of TamR and TamS tumors (GSE6532) separately. The weighted correlation coefficient matrix of gene expression was first constructed using the power function with $\beta = 7$ [32]. Panel A and B show the dendrograms generated by the average linkage hierarchical clustering of 3,064 genes with largest variance using topological overlap for TamR and TamS profiles, separately. The horizontal colored bars under each dendrogram directly correspond to the module (color) designation for the clusters of co-expressed genes. Modules with significant overlap between two co-expression networks have the same color. *Black* module is unique to the co-expression network derived from the tamoxifen resistant tumors. The GO BP terms were identified by the DAVID (FDR < 0.05). Major terms for each module are: *blue*: GO:0008270—zinc ion binding and GO:0045449—regulation of transcription; *Green* GO:0001568—blood vessel development and GO:0001944—vasculature development; *Brown* GO:0030199—collagen fibril organization and GO:0001568—blood vessel development; *black* GO:0001568—blood vessel development and GO:0001525—angiogenesis; *pink* GO:0006955—immune response; *yellow* GO:0042110—T cell activation, GO:0006954—inflammatory response and GO:0050778—positive regulation of immune response; *red* GO:0007049—cell cycle; *Turquoise* GO:0065003—macromolecular complex assembly and GO:0006091—generation of precursor metabolites and energy

co-expression networks: the pink and yellow modules are grouped with different modules in the two clustering structures. These two modules are clustered closely with the brown and green modules in the TamR network; while in the TamS network, they are relocated next to the turquoise module. The two modules represent many genes related to immune and inflammatory response, suggesting a different level of inflammatory activity in TamR tumors in comparison to TamS

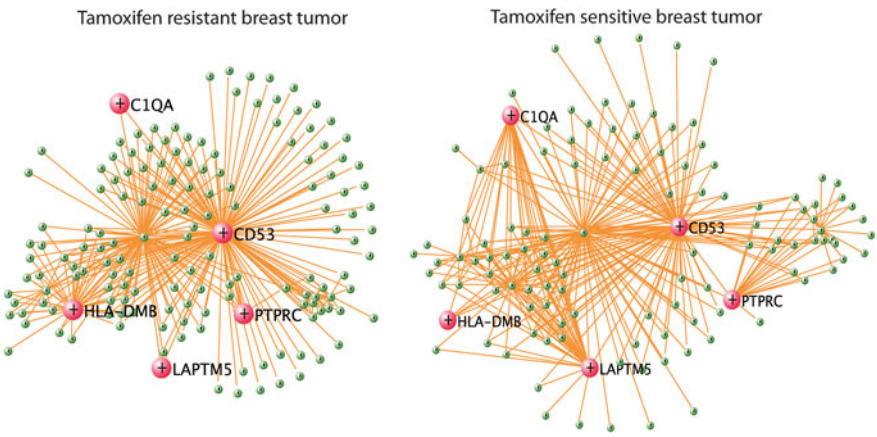

Fig. 14.3 The VisANT plots of the connections among the most highly connected genes within yellow module in the co-expression networks of tamoxifen resistant and sensitive breast tumors. The connections among the most highly connected genes were defined by the topological overlap (TO) measure [32] within yellow module in the co-expression networks of tamoxifen resistant (TamR) and sensitive (TamS) breast tumors. Only the strongest within-module gene–gene interactions based on the dynamically determined thresholds of TO are shown (connections of yellow module for TamR and TamS are 318 and 332 with TO value greater than 0.31 and 0.29, respectively). Nodes in pink represent genes with significant change in numbers of connections from TamR to TamS networks (CD53: 133 → 89; HLA-DMB: 40 → 5; LAPTM5: 3 → 36; C1QA: 2 → 24; and PTPRC: 6 → 20)

tumors. In addition, this activity also seems to be highly correlated to angiogenesis in TamR tumors, as two modules are grouped with the modules (black, brown and green), which represent activities related to angiogenesis and extracellular matrix. Further examination of the co-expression pattern in the yellow module revealed that five hub genes were distinctively co-expressed with other genes within the same module in TamR and TamS tumors respectively (Fig. 14.3). These genes are CD53, HLA-DMB, LAPTM5, C1QA and PTPRC. Our finding provided the evidence that the tumor microenvironment may be different between TamR and TamS tumors. This result is intrigue, as previous studies have indicated that inflammation may promote aggressive breast cancers [36, 37], and a quantitative difference in genes corresponding to immune functions and extracellular matrix components between ER+ and ER− breast cancer [38] and a predominant immune component in breast cancers based on DNA methylation profiling [39] have been reported.

Table 14.3 Common top ranked compounds with expression profiles opposite to those of the TamR tumors

Compound	Cell Line	Rank		
		GSE6532	GSE9195	GSE9893
Trichostatin A	MCF7	12	3	3
LY-294002	MCF7	3	4	5
Resveratrol	MCF7	9	13	143
Trifluoperazine	MCF7	24	14	11
Thioridazine[a]	PC3	29	6	6
DL-thiorphan	MCF7	32	73	92
Harmine	MCF7	26	31	67
0297417-0002B	MCF7	38	20	21
Chrysin	MCF7	51	35	116
Trimethylcolchicinic acid	MCF7	62	68	89
Galantamine	MCF7	92	46	77

Entrez gene identifiers of the three DE gene sets were first mapped to Affymetrix HG-U133A probe sets using Affymetrix Human Genome U133A set annotation data implemented in package hgu133a.db in Bioconductor. All mapped probe sets in each individual microarray dataset were then submitted to the Connectivity Map website [49]. A connectivity score based on the Kolmogorov–Smirnov statistic was calculated to estimate the enrichment of both over- and under-expressed query genes in a Connectivity Map instance as described [40]. Instances were ranked in ascending order of connectivity scores. Permutation tests were performed to estimate the significance of the instance sets ranked by the connectivity scores. Compounds were selected from top ranked instance sets with negative connectivity scores at P-value <0.05 for each individual inquiry gene list [25]

[a] The compound, thioridazine, was identified as it induced a gene expression profile that is opposite to that observed between tamoxifen resistant and sensitive breast cancers. It has been validated for its ability to reduce the number of viable cells in a dose-dependent fashion in MCF-7 cells that developed spontaneous resistance to tamoxifen [25]

14.4 Predict Small Molecules that May Resensitize TamR Using the Connectivity Map Analysis

Gene expression profiles on TamR and TamS tumors can be used to predict small molecules that may alter the gene expression profiles, and potentially resensitize tamoxifen resistance in TamR tumors. The Connectivity Map (C-Map) is a tool to connect gene expression to small molecules [40, 41]. The C-Map (build 02) contains 6,100 such gene expression profiles on 5-cultured human cell lines treated with 1,309 compounds. The C-Map facilitates the screening of compounds by comparing a ranked list of genes based on the association to a disease phenotype with the expression profiles from the several types of cell lines treated with compounds. A small molecule is given to a negative score if it produces a gene expression pattern that is opposite to that observed between TamR and TamS tumors. The molecules with negative scores are considered to have potential to reverse the tumor expression pattern if the tumors were treated with them.

The C-Map analysis for the three DE gene datasets has generated a ranking list of compounds based on the negative connectivity scores for compound/cell combinations [25]. A partial list of the top ranked compounds is provided in Table 14.3. Among the top-ranked compounds, three drugs (trifluoperazine, thioridazine, and prochlorperazine) belonging to the same structural family of phenothiazine compounds were identified. These drugs were originally designed as anti-malarial drugs but have been shown to act as anti-histamines, anti-emetics, suppressants of psychotic symptoms, and anti-cholinergics. The three compounds have been validated for their ability to reduce the number of viable cells in a dose-dependent fashion in MCF-7 cells that developed spontaneous resistance to tamoxifen [25]. It was also validated that the expression level of CCNE2 was reduced after the treatment in the same resistance cells. The CCNE2, one of the four DE genes that are common in the three DE gene sets [25], was over-expressed in the TamR tumors. Interestingly, another antipsychotic drug (chlorpromazine), identified from our analysis, has also been shown to reduce the cell growth and metabolic activity in TamS and TamR human breast cancer cells [42]. These results suggest that the bioinformatics analysis could lead to the identification of a novel usage of therapeutic drugs that have the potential to inhibit proliferation of both tamoxifen-sensitive and tamoxifen-resistant breast tumors.

14.5 Conclusions

We have demonstrated the utility of a variety of bioinformatics approaches (GSEA, C-Map, and WGCNA) in analysis of gene expression profiles from the breast tumors that are retrospective classified as tamoxifen sensitive and resistant. The extension of analysis to the entire expression profiles has revealed an intrinsic highly proliferative profile and possible relevance to inflammation in tamoxifen resistant tumor. The analysis also linked a set of small molecules that may have potential to reverse the tamoxifen resistance in breast tumors. However, the understanding of underlying mechanisms and identification of drug target cannot be achieved with the analysis of gene expression alone. The integration of various data from genetic, genomic and proteomic studies is indispensable. For example, the ability and mechanism of microRNA-221/222 in resensitizing tamoxifen resistance in tamoxifen resistant breast tumor cells have been discovered [43–46]. Molecular pathways and networks regulated by copy number aberrations in molecular phenotypes of breast cancers have been identified by an integrative genomic and transcriptomic analysis [47]. There is no doubt that integrative bioformatics approaches will continue their roles in facilitating systems level understanding of breast cancer and in generating new hypotheses for interrogation of the disease and drug resistance mechanisms [48].

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Chapter 15
Current Understanding of Drug Resistance Mechanisms and Therapeutic Targets in HER2 Overexpressing Breast Cancers

Aamir Ahmad and Fazlul H. Sarkar

Abstract A subset of breast cancers is marked by overexpression of HER2 receptor and activated HER2-mediated signaling. Targeting HER2 offers a unique therapeutic approach for the treatment of such breast cancers. Trastuzumab, lapatinib, and, more recently, pertuzumab, have been approved by FDA to treat HER2 overexpressing breast cancers. Although the drugs effectively target HER2 leading to a favorable clinical response in patients initially, a majority of patients turn refractory to HER2 targeted drugs as early as within a year of administration. Trastuzumab, being the first HER2 targeted drug, has been investigated in detail in relation to acquired resistance, and emerging reports are evident for such drug resistance in lapatinib treated HER2 overexpressing breast cancers as well. This chapter takes a look at the progress of treatment options in HER2 overexpressing breast cancers with focus on mechanisms that are believed to be responsible for drug resistance. We also discuss the current strategies being investigated to overcome drug resistance.

Keywords Drug resistance · HER2 · Trastuzumab · Lapatinib · Pertuzumab · Steric hindrance · De-regulated intracellular signaling · Cell cycle regulators · Combinational therapies · Mitogen-activated protein kinase-interacting kinase (MNK) · Reversing trastuzumab resistance · MicroRNAs (miRNAs)

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15.1 Drug Resistance: The Problem

Drug-resistance remains a major clinical problem that hinders the successful management of breast cancer patients. A number of targeted therapies are available for breast cancer subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR) and overexpression of HER2. While some cancers do not respond to the therapy at all, right from the beginning (the phenomenon being called de novo drug resistance), many breast cancers initially respond to the targeted therapy but develop resistance with the passage of time and continued administration of therapeutic agent, and this is called acquired drug resistance. De novo drug resistance is by itself challenging but acquired drug resistance is clinically a much bigger problem. Breast cancers with acquired drug resistance are known to be far more aggressive and linked to poor prognosis and overall poor survival.

15.2 HER2

HER2 is encoded by *ERBB2*, an oncogene located at the long arm of human chromosome 17(17q21-q22). It was first characterized, named and reported by Coussens et al. in 1985 [1]. It was named HER2 because of its close resemblance to epidermal growth factor receptor (EGFR) which is also known as HER1. 'Neu' in HER2/neu refers to its origin from a rodent glioblastoma cell line, a type of neural tumor. While reporting HER2, Coussens et al. found it to be similar to *neu* oncogene that they commented on the possibility of having identified the human counterpart of rat *neu* oncogene. HER2 is also called ErbB-2 because of its similarity to *ERBB* (avian erythroblastosis oncogene B), an oncogene that codes for EGFR. Additionally, HER2 is also known as p185 [2] and CD340.

The EGFR/ErbB family of receptors comprises of four plasma membrane-bound receptor tyrosine kinases—EGFR/HER1, HER2, HER3 and HER4. All of these receptors contain an extracellular ligand binding domain, a transmembrane domain and an intracellular domain that interacts with multiple intracellular signaling molecules. HER2 is unique among the family members and is also called 'orphan receptor' because it has no known ligand. All the other EGFR/ErbB receptors have known ligands and form either homodimers or heterodimers when bound to ligands. HER2 heterodimerizes with all of the three receptors and is known to be the preferred dimerization partner of the other EGFR/ErbB receptors [3, 4]. Dimerization leads to autophosphorylation of tyrosine residues within the cytoplasmic domain of these receptors and such activated receptors then modulate a number of signaling pathways that affect the cell growth.

15.3 HER2 in Breast Cancer

It is believed that HER2 is overexpressed or amplified in 20-30 % invasive breast cancers. These HER2 over-expressing breast cancers are invariably linked to worse prognosis and poor survival [5]. HER2 is expressed not only in breast tissue but in many others, such as ovaries, lungs, liver, kidneys as well as central nervous system where it is necessary for normal development and growth [6]. In normal tissues its expression is low but in breast cancer cells its expression is so high that there can be up to two million receptors on a single cell [5–7].

15.4 HER2 Targeting Drugs

In the breast cancer cells that are marked by overexpression of HER2, targeting HER2 via targeted therapies is the preferred therapeutic regimen. With better understanding of HER2-mediated cellular signaling and resulting cell growth, a number of HER2-targeting drugs have been approved for clinical use. This section describes some of these drugs.

15.4.1 Trastuzumab

Marketed as 'herceptin', trastuzumab is a monoclonal antibody that is very effective in HER2-overexpressing breast cancers. Trastuzumab has unique place in cancer research as this was the first monoclonal antibody approved for use against a solid tumor. It was approved by FDA in the year 1998. The antibody was originally developed in mice but was subsequently developed as a humanized antibody. The greatest efficacy of trastuzumab is seen only in HER2-over-expressing breast cancers. In a phase III trial [8], the efficacy and safety of trastuzumab was tested in women with metastatic breast cancer that overexpressed HER2, by randomly assigning 234 patients to receive standard chemotherapy alone and 235 patients to receive standard chemotherapy plus trastuzumab. It was concluded that trastuzumab increases the clinical benefit of first-line chemotherapy in HER2-overexpressing metastatic breast cancers. This was based on the observations that addition of trastuzumab to chemotherapy resulted in a longer time to disease progression, a higher rate of objective response, a longer duration of response, a lower rate of death at 1 year, longer survival and a 20 percent reduction in the risk of death. However, the biggest adverse event noted in this trial was cardiac dysfunction, which was observed in up to 27 % of patients administered trastuzumab in combination with other drugs.

Trastuzumab remains a standard of care for the treatment of HER2 over-expressing breast cancers in adjuvant as well as in metastatic settings although

there are concerns about the high costs associated with trastuzumab treatment, with costs of the treatment ranging from $50,000 to $100,000. In addition to being used as a single agent, trastuzumab is also administered in combination with other drugs, as discussed in individual reports latter in this chapter. The therapeutic action of trastuzumab is not clearly understood but is believed to involve several mechanisms—antibody-dependent cell mediated cytotoxicity, inhibition of dimerization of HER2 with other family receptors, inhibition of downstream intracellular signaling pathways, induction of apoptosis, induction of cell cycle arrest, modulation of cell cycle pathways as well as inhibition of angiogenesis [9].

15.4.2 Lapatinib

Similar to trastuzumab, lapatinib (or lapatinib ditosylate/Tykerb) is also a drug that targets HER2; however, it is not an antibody. Chemically, lapatinib is an oral small molecule derivative of 4-anilinoquinazoline [10]. Also, it does not target HER2 alone but is rather known to inhibit the tyrosine kinase activity of HER2 as well as EGFR. It was approved by FDA in 2007 for clinical use with capecitabine in combination therapy for breast cancer patients. In 2010, it was approved for the treatment of postmenopausal women with hormone receptor positive, HER2 overexpressing metastatic breast cancers. The mode of action of lapatinib involves targeting of C-terminus tyrosine kinase domain of target HER2/EGFR where it binds to ATP binding site, resulting in the inhibition of phosphorylation and subsequent activation of downstream intracellular signaling pathways. Lapatinib has shown promise against trastuzumab-resistant cells [11, 12], which is of interest to clinicians dealing with the drug resistance associated with trastuzumab.

15.4.3 Pertuzumab

Pertuzumab, marketed as ‘Perjeta’, is also a monoclonal antibody, similar to trastuzumab. It is the latest drug to be approved for the treatment of HER2 positive metastatic breast cancer and received its approval from FDA on June 8, 2012. This drug is the first of its class in a series of drugs called ‘HER dimerization inhibitors’. As mentioned above, HER2 is the preferred binding partner for dimerization for all the EGFR/ErbB receptor family members. By binding to and inhibiting HER2, pertuzumab inhibits the potential dimerization of HER2 with all other family receptors, resulting in the inhibition of tumor growth [13].

15.4.4 Other HER2 Targeting Drugs

In addition to the drugs mentioned above that have been approved by FDA for use in clinics against HER2 overexpressing breast cancers, a few others are also making their way through the preclinical and clinical studies [14, 15]. Some examples are Afatinib, Neratinib and NeuVax. Afatinib and neratinib inhibit both EGFR and HER2, and thus are similar to lapatinib. NeuVax is a peptide-based immunotherapy that harnesses patient's own immune system and directs immune T cells to target and destroy HER2 expressing cancer cells.

15.5 Drug Resistance Mechanisms in HER2 Overexpressing Breast Cancers

Although the targeted therapies offer improved clinical outcome, they suffer from acquired drug resistance. The resistance against trastuzumab, the prototype HER2-targeting drug, is both 'inherent' as well as 'developed' [7]. First of all, less than 35 % of all HER2 overexpressing breast cancer patients ever respond to trastuzumab [7, 16]. Secondly, of the trastuzumab responding breast cancers, as many as 70 % develop resistance, leading to progressively aggressive and metastatic disease within a year [17]. It is estimated that approximately 5000 HER2-positive breast cancer patients succumb to this disease every year in the US alone [9].

A number of molecular/biochemical mechanisms have been proposed for the observed trastuzumab resistance [9, 18]. These include changes in the HER2 receptor expression, increased expression of other HER family receptors which compensate for HER2 inhibition, steric effects which disable binding of trastuzumab to HER2, constitutive activation of downstream PI3K/Akt pathway which no longer needs message from HER2 activation and inhibition of cell cycle inhibitory p27.

15.5.1 Trastuzumab Resistance Pathways

15.5.1.1 Increased Expression of Alternate HER Family Receptors

With the inhibition of HER2, breast cancer cells switch to other HER family receptors for sustained growth and proliferation. For example, there is evidence to suggest increased expression of both EGFR (HER1) as well as HER3 to compensate for the blocked HER2 activity, leading to resistance against trastuzumab [19]. Further, trastuzumab is highly specific for HER2, which means it does not effectively inhibit any other family members, thus providing an easy escape route for cancer cells. Elevated HER3 signaling has been proposed to be a mechanism of

trastuzumab resistance [7], a mechanism, which needs more mechanistic details and direct clinical evidence. A further complex crosstalk between signaling pathways, leading to trastuzumab resistance, has also been reported and this involves a crosstalk between HER2, HER3 and insulin-like growth factor-I receptor (IGF-IR) pathways [20]. Interactions between the three signaling pathways were observed exclusively in trastuzumab-resistant cells, suggesting a crosstalk that might be important for the progression to trastuzumab resistant phenotype as well as sustenance of trastuzumab resistant phenotypic cells. Furthermore, down-regulation of HER3 or IGF-IR resulted in an efficient induction of p27. Since p27 is an inhibitor of cell cycle, an increase in p27 levels resulted in re-sensitization of trastuzumab-resistant cells to trastuzumab. Another complex crosstalk influencing trastuzumab resistance is the one that involves EGFR, IGF1R, PTEN, PI3K, Akt and mTOR (mammalian target of rapamycin) signaling [21].

15.5.1.2 Steric Hindrance of HER2-Trastuzumab Interaction

HER2 receptor is known to undergo proteolysis where it can mutate, leading to a receptor without the extracellular domain [7, 22–24]. Since trastuzumab works via binding to the extracellular domain of the receptor, absence of this domain is likely to result in resistance to drug action. Further, the mutated HER2 is linked to constitutively-activated intracellular signaling, resulting in highly aggressive disease. It may be important to point out that such mutated HER2 is largely known to develop randomly, as opposed to in response to exposure to trastuzumab. It is not known if trastuzumab treatment can lead to HER2 mutation and, if so, what are the mechanisms involved.

A direct steric hindrance of binding of trastuzumab to HER2 is afforded by membrane-associated glycoproteins studies [25, 26]. This largely involves overexpression of mucin-4, a highly O-glycosylated membrane protein. Interestingly, mucin-4 is itself known to be associated with poor prognosis of multiple cancers, and is therefore an attractive target for therapy not only for multiple human cancers but specifically for trastuzumab resistant breast cancers. Again, the mechanism by which HER2 overexpressing breast cancer cells increase the expression of mucin-4, leading to trastuzumab resistance, is poorly understood.

15.5.1.3 De-regulated Intracellular Signaling Pathways

As briefly mentioned above, constitutively activated PI3K/Akt signaling provides resistance to HER2 inhibition by trastuzumab. Activated HER2 signaling is known to activate PI3 K/Akt signaling and, therefore, a constitutively activated signaling of this pathway redundates its dependency on activation of HER2 receptor. An important factor contributing to this is the loss of inhibitory PTEN. For instance, it has been reported that up to 36 % HER2-expressing stage IV breast cancers are marked by the loss of PTEN [7, 27]. As expected, the patients with

PTEN-deficient cancers had significantly lowered response to trastuzumab. Additionally, a quarter of trastuzumab refractory breast cancers were observed to harbor PI3K activating mutations [28], an observation that provided direct connection between trastuzumab resistance and constitutively activated PI3K/Akt signaling. The PI3K activating mutations, namely E545 K and H1047R, were found to be directly correlated with increased resistance to trastuzumab.

Src signaling has recently been proposed as a candidate of interest with respect to both de novo as well as acquired resistance to trastuzumab [29]. This study demonstrated reversal of trastuzumab resistance, via targeted inhibition of src, through effective blockage of Akt. Another pathway that has been implicated in trastuzumab resistance is the IGF-1R signaling pathway [19, 26, 30]. Activation of this pathway also leads to the activation PI3K/Akt. Other de-regulated cellular signaling implicated in trastuzumab resistance is the increased activity of rac1 [31] and met [32]. The study on met receptor in trastuzumab resistance showed frequent expression of met in HER2 overexpressing breast cancer cells. Interestingly, HER2 over-expressing cells tend to up-regulate met expression in response to treatment with trastuzumab, possibly as a means to overcome the proliferation inhibition that they are subjected to, post trastuzumab-treatment. Inhibition of met led to re-sensitization of trastuzumab-resistant cells to trastuzumab.

15.5.1.4 Altered Cell Cycle Regulation

Increased expression of HER2 provides a proliferative advantage leading to uncontrolled cell division and growth. Resistance to trastuzumab involves modulations in cell cycle regulatory proteins. Cyclin D1 and the inhibitory p27 in particular, have been documented for their role in trastuzumab resistance. A number of reports have indicated the role of these cell cycle regulators, leading to re-sensitization of trastuzumab resistant cells to trastuzumab treatment. For example, trastuzumab induces the expression of p27 [33], and induction of p27 leads to the inhibition of cyclin-CDK complex, resulting in cell cycle arrest. It is, therefore, logical to observe down-regulation of p27 in trastuzumab-resistant breast cancer cells and, indeed, this was observed in a cell line model when trastuzumab-resistant variant of HER2 over-expressing breast cancer cell line, SKBR3, was observed to exhibit significantly reduced p27 [34]. A more recent report has further elucidated the mechanism of p27 down-regulation where it has been shown that a serine/threonine phosphatase, PPM1H, is mechanistically involved in trastuzumab resistance [35]. PPM1H dephosphorylates p27 and since phosphorylation is a signal for proteasomal degradation, a dephosphorylated p27 is saved from degradation. This means that PPM1H is a positive modulator of p27 and, therefore, low expression of PPM1H translates into lower expression of p27, all of which is directly connected to trastuzumab resistance.

Trastuzumab also functions via down-regulation of cyclins [36], in addition to induction of p27 discussed above. Both of these cellular events—induced p27 and decreased cyclins—lead to an effective cell cycle arrest. PD 0332991, a selective

inhibitor of CDK4 and CDK6, was reported to increase the efficacy of trastuzumab in HER2 over-expressing cells [37]. In this study, analysis of 47 human breast cancer cell lines revealed that Rb phosphorylation is blocked only in drug-sensitive cells as opposed to drug-resistant cells. Since Rb phosphorylation is a measure of CDK activity, an effective inhibition of CDKs by trastuzumab in HER2 over-expressing trastuzumab-sensitive cells resulted in reduced Rb phosphorylation. The resistant cells became refractory to CDK inhibitory action, suggesting that CDK inhibition can potentially benefit trastuzumab-resistant breast cancers [38]. As a direct clinical data supportive of a significance of cell cycle regulatory proteins in trastuzumab resistance, it has been reported that the prognostic importance of HER2 is significantly better for patients whose tumors overexpress cyclin D1 [39]. In patients with overexpression of cyclin D1, HER2 overexpression strongly correlated with increased risk of recurrence and mortality.

15.5.2 Lapatinib Resistance Pathways

With the FDA approval of lapatinib for treatment of HER2 overexpressing breast cancers, this drug has been used in the clinical setting. However, similar to resistance against trastuzumab, as discussed in the above section, the resistance against lapatinib has also been documented. In this context, increased expression of another receptor tyrosine kinase, AXL, has been proposed to lead to lapatinib resistance [40]. In this report, in addition to lapatinib resistance, cells were also found to be resistant to trastuzumab. Inhibition of AXL restored sensitivity to not only lapatinib, but also trastuzumab. Suggestive of the activation of alternative signaling pathways, there is evidence in support of activation of ER signaling in lapatinib resistant cells [41, 42]. This is indicative of a switch from dependence on HER2 signaling to dependence on ER signaling, in the case of targeted therapy against HER2. Using a panel of breast cancer cell lines, it has recently been demonstrated that sustained inhibition of HER2 signaling by use of trastuzumab and lapatinib results in functioning of ER signaling as the key survival pathway [43]. This advocates additional targeting of ER signaling in drug resistant HER2 overexpressing breast cancers.

15.6 Strategies to Overcome Drug Resistance in HER2 Overexpressing Cells

As discussed in the section above, HER2-targeted therapies suffer from the development of drug resistance, which results in metastatic and aggressive disease. It is therefore important to develop novel strategies to overcome the problems of drug resistance in HER2 overexpressing breast cancers. A number of such

strategies have been proposed [44, 45] and we will discuss some of them in this section.

15.6.1 Targeting HER2 Extracellular Domain

Pertuzumab, the most recently approved drug against HER2, has been advocated for its use in trastuzumab resistant breast cancers [41]. Although it is similar to trastuzumab in being a monoclonal antibody that targets extracellular domain of HER2, it differs from trastuzumab in the epitope that it recognizes on the extracellular domain. Further, being an inhibitor of HER dimerization, pertuzumab is able to inhibit homodimerization as well as heterodimerization of HER-family receptors. This ensures a very efficient inhibition of pan-HER family of receptors. As discussed above, signaling through alternate HER family receptors is also an important mechanism by which trastuzumab-treated breast cancers develop resistance. Simultaneous inhibition of multiple HER receptors ensure a more efficient reversal of trastuzumab resistance.

15.6.2 Combinational Therapies

Since drug resistance against trastuzumab involves activation of alternate signaling pathways, it is logical to include inhibitors of such alternate pathways in an effort to reverse trastuzumab resistance. The same holds true for lapatinib as well. It has recently been reported that a combination of trastuzumab and lapatinib results in a significantly increased pathological response rate [46]. Multiple studies/trials have also reported a synergy between blockage of HER2 and ER/PR pathways leading to increased progression-free survival [41]. In view of the reported inhibitory effect of trastuzumab on VEGF-directed angiogenesis, the synergy between trastuzumab and angiogenesis inhibitors is also the subject of many investigations [9].

Based on the observation that Notch-1 oncogene (discussed in Chapter 17 in this book) is activated in trastuzumab resistant breast cancers, the use of Notch inhibitor, γ-secretase, in combination with trastuzumab, has been suggested in trastuzumab-sensitive as well as trastuzumab resistant breast cancers [47]. In preclinical models, simultaneous inhibition of mTOR signaling (discussed in the next sub-section) and extracellular signal-regulated kinase (ERK) has also shown promise against trastuzumab and lapatinib resistance [48]. Combination of Akt inhibitor triciribine with HER2 antibody has been reported to inhibit tumor growth in trastuzumab-resistant breast tumor mouse models via inhibition of intracellular signaling pathways in addition to increased T cell infiltration in the tumor microenvironment [49].

15.6.3 Targeting Downstream Intracellular Pathways

Constitutively activated PI3K/Akt pathway has been implicated in multiple studies that focused on trastuzumab resistance (please see the section on mechanisms of drug resistance above). It is, therefore, intuitive to inhibit PI3K/Akt pathway in an effort to reverse trastuzumab resistance. Towards this end, use of an investigational drug NVP-BEZ235, an inhibitor of PI3K, has been found to be effective in reversing trastuzumab resistance in breast cancer cells that harbor PI3K activating mutations [50]. A similar efficacy of this drug has also been reported against lapatinib resistant breast cancers [40, 41], suggesting a general usefulness of such approach in reversing drug resistance phenotype in HER2 overexpressing breast cancers. This drug, NVP-BEZ235, is also an inhibitor of mTOR, a molecule that closely associates with PI3K and Akt. A modulatory effect of this drug on drug resistance underlines the importance of PI3K/Akt/mTOR pathway in trastuzumab/lapatinib resistant mechanisms in HER2 overexpressing cells. mTOR inhibitors, by themselves, have also been reported to be effective in sensitizing trastuzumab resistant cells to trastuzumab [41, 51, 52].

Recently, the involvement of $\beta 1$ integrin in mediating an alternate survival mechanism for development of trastuzumab as well as lapatinib resistance has been reported, and thus inhibition of $\beta 1$ integrin and its downstream signaling can be a strategy to overcome trastuzumab and/or lapatinib resistance [53]. Consistent with the observation that rac1 is implicated in trastuzumab resistance [31], its inactivation has been linked to reversal of trastuzumab resistance in HER2 overexpressing breast cancer cells SKBR3 [54].

15.6.4 Other Approaches

In addition to all the above-described strategies that are currently under investigations for possible roles in reversing resistance in HER2 overexpressing breast cancers, there are a few more which need to be mentioned. One of these is the approach that targets heat shock protein 90 (Hsp90) [55] eliciting favorable response in HER2 positive metastatic breast cancers when used in combination with trastuzumab. Another proposed approach is the inhibition of anti-apoptotic protein Bcl-2 [56]. This approach is based on the observation that breast cancers cells BT474 show an increased expression of Bcl-2 when they acquire trastuzumab resistance. Targeted inhibition of Bcl-2 by ABT-737 leads to improved sensitivity to trastuzumab in these resistant cells. It has also been proposed that inhibition of fatty acid synthase can potentially reverse the resistance to trastuzumab and lapatinib [57]. Recently, it has been shown that inhibition of mitogen-activated protein kinase-interacting kinase (MNK) family member MNK1 [58] and calpain4 [59] can re-sensitize resistant cells to trastuzumab.

In recent years, microRNAs (miRNAs) have made a big impact on cancer research [60, 61] and it is now increasingly being realized that modulation of specific miRNAs can influence the sensitivity of refractory cancers to specific therapeutic drugs [62, 63]. In one of the earliest report connecting miRNA with trastuzumab resistance, it has been reported that miR-210 levels are significantly higher in trastuzumab resistant cells and this raises the possibility of monitoring circulating miR-201 levels for sensitivity to trastuzumab [64]. Trastuzumab has also been shown to up-regulate miR-26a and miR-30b leading to drug resistance [65], and thus targeting of these miRNAs has the potential of reversing trastuzumab resistance.

15.7 Conclusions and Perspectives

A number of HER2 targeted therapies are now available for clinical management of HER2 overexpressing breast cancers. These therapies, however, suffer from de novo as well as acquired drug resistance. While a number of factors have been implicated in the development of drug resistance phenotype, the knowledge on the subject is far from being complete. As a means to overcome drug resistance in these HER2 overexpressing breast cancers, use of inhibitors of several proposed signaling molecules/factors has been advocated. A common practice is the combination of HER2 targeting therapy, such as trastuzumab, with the proposed inhibitor. While the individual studies report some benefit, there are always concerns about the increased toxicity in such combinational treatments. The treatment options for HER2 overexpressing breast cancer patients, particularly long term treatments in view of acquired resistance, are limited. The most recently approved drug, pertuzumab, offers a new perspective by being an inhibitor of HER dimerization and needs to be investigated in detail. A number of drugs are in various stages of trials and emerging reports have indicated a role of miRNAs in trastuzumab resistance. All these developments mark an exciting phase in the research area and we wait eagerly for targeted therapies in the near future that not only target HER2 overexpressing breast cancers effectively but are also tolerated well and do not result in rapid development of acquired resistance.

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Chapter 16
Platinum and Ruthenium Complexes for the Therapy of Breast Cancer Diseases

Bernhard Biersack and Rainer Schobert

Abstract Breast cancer is still the leading cause of cancer deaths among women worldwide, and new therapies for the treatment of this dangerous disease are desperately sought for. Complexes of metals such as platinum and ruthenium have been frequently found efficacious against breast tumors, in particular highly aggressive multidrug resistant and triple-negative subtypes. Numerous platinum and ruthenium complexes with enhanced selectivity for breast cancer and with reduced side effects have been developed recently. This chapter is intended to give an insight into the latest developments in the field of platinum and ruthenium based drugs against breast cancer. Chemical formulae and a brief description of the manifold biological activities of some important such compounds are provided which might be of interest to inorganic chemists, medicinal chemists, biologists, and clinicians alike.

Keywords Platinum complexes · Ruthenium complexes · Triple-negative breast cancers (TNBC) · Estrogen receptors (ER) · Tamoxifen · Organometallic compounds · Anticancer agents · Breast cancer · Drug resistance · DNA binding · Tumor targeting · Cytotoxic activity

16.1 Introduction

The platinum complex cisplatin holds a salient position in the chemotherapy of various solid tumors. Meanwhile, further platinum complexes like carboplatin and

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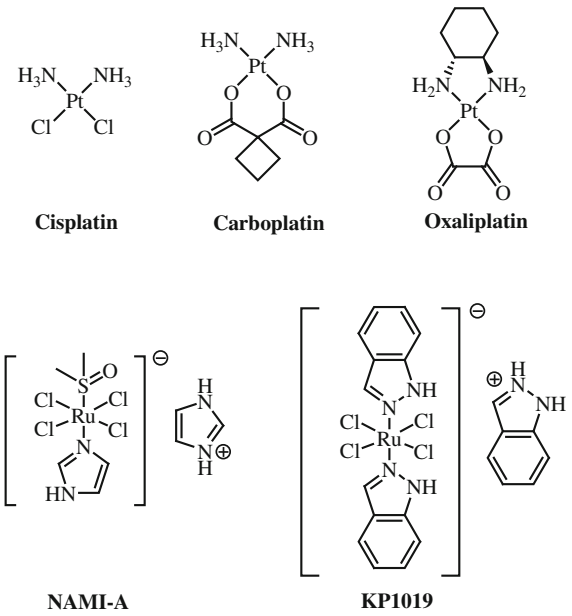


Fig. 16.1 Anticancer active platinum and ruthenium complexes

oxaliplatin featuring reduced side effects and a lack in cross resistance with cisplatin are applied for the therapy of cancers (Fig. 16.1) [1]. It is commonly accepted that these platinum complexes exert their biological activity via the damage of DNA [2]. However, in the treatment of breast cancer they play but a limited role. One of the few applications of platinum drugs in the clinical therapy of breast cancer is that combination regimen of docetaxel, carboplatin, and trastuzumab, which proved to be a real alternative to anthracycline, taxane, and trastuzumab based treatments of HER2-positive breast cancer [3]. Lately, a more mechanistically oriented interest emerged in the effect of platinum complexes against triple-negative breast cancers (TNBC), which generally have a poor prognosis and which lack EGF, estrogen, and progesterone receptors [4]. In Western societies TNBC comprise 15–20 % of all breast cancers [5]. BRCA1 mutation is common among TNBCs leading to genomic instability and affecting DNA repair [6]. Hence, DNA damaging agents such as platinum complexes are a promising compound class for the treatment of TNBC [7].

The success of platinum complexes has also intensified research efforts to develop and exploit related anticancer drugs based on metals other than platinum, e.g., ruthenium which is only a knight's move away from platinum in the periodic system of elements. Several ruthenium complexes have since been found which add significantly and complementarily to the spectrum of antitumoral effects of platinum complexes. Some ruthenium complexes (e.g., NAMI-A, KP1019, Fig. 16.1) have already entered clinical trials [8]. In the following, an overview is presented of platinum and ruthenium complexes at an advanced stage of medicinal evaluation or clinical trials for the treatment of breast cancer with an emphasis on those types associated with a poor prognosis.

16.2 Platinum Complexes

Cisplatin, carboplatin, and oxaliplatin are approved for the therapy of a wide range of solid tumors. Cisplatin was the first platinum complex to be clinically employed in 1978, and it still is the gold standard for the treatment of testicular cancers achieving cure rates beyond 90 % [9, 10]. A renowned patient who got completely cured by cisplatin from testicular cancer despite already suffering from metastases is cyclist Lance Armstrong who later on even won the Tour de France seven times. Meanwhile, carboplatin has replaced cisplatin in some cases (e.g., ovarian cancer) due to its lower toxicity and other practical advantages. Oxaliplatin was found to overcome cisplatin resistance in colon cancers and is currently applied together with folinate and 5-fluorouracil (FOLFOX) for the treatment of colon cancer diseases. Yet, as already mentioned above, Pt complexes play only a minor role, so far, in the treatment of breast cancer diseases until now.

16.2.1 Targeted Pt Conjugates Connected to Estrogens

Estrogens play a crucial role in the growth of the majority of breast cancers. In addition, these steroids may act as DNA targeting devices due to their affinity for estrogen receptors (ER), which upon binding of an estrogen ligand migrate to specific ER binding sites on the DNA. Similar effects should be initiated by selective estrogen receptor modulators (SERM) such as tamoxifen, which is commonly applied for the treatment and prevention of breast cancer. A Franco-Canadian group (Bérubé et al.) has prepared a series of potent platinum (II) complex conjugates **1a–b** with tamoxifen-like triphenylethylene derivatives (Fig. 16.2). The complexes **1a** and **1b** were more active in cells of ER negative MDA-MB-231 breast carcinoma (IC_{50} (**1a**) = 1.6 μM, IC_{50} (**1b**) = 1.3 μM) than in ER-positive MCF-7 breast cancer cells (IC_{50} (**1a**) = 4.3 μM, IC_{50} (**1b**) = 4.9 μM) [11]. ER binding assays revealed that these complexes did not bind to ER though they were distinctly cytotoxic also against ER-positive MCF-7 cells. The high lipophilicity of these complexes appears to play an important role for their in vitro cytotoxic activity. Similar results have been reported by Schobert and co-workers, who changed the diamine ligand fragment and used 6-amino-methylnicotinate instead of en as a diamine ligand [12]. Their *para*-methoxy substituted triphenylethenyl conjugate **1c** (Fig. 16.2) selectively led to a complete growth inhibition of MDA-MB-231 cells at 10 μM, while cells of MCF-7 (ER^+) breast carcinoma and its mutant form MCF-7 (ER^-) were not noticeable affected by this compound. This pattern of activity is possibly due to certain regulative proteins, different in MDA-MB-231 and MCF-7 cells, being the actual targets rather than the estrogen receptor or DNA. Bérubé and co-workers likewise prepared a very active estradiol conjugate **2** [13]. Again, its

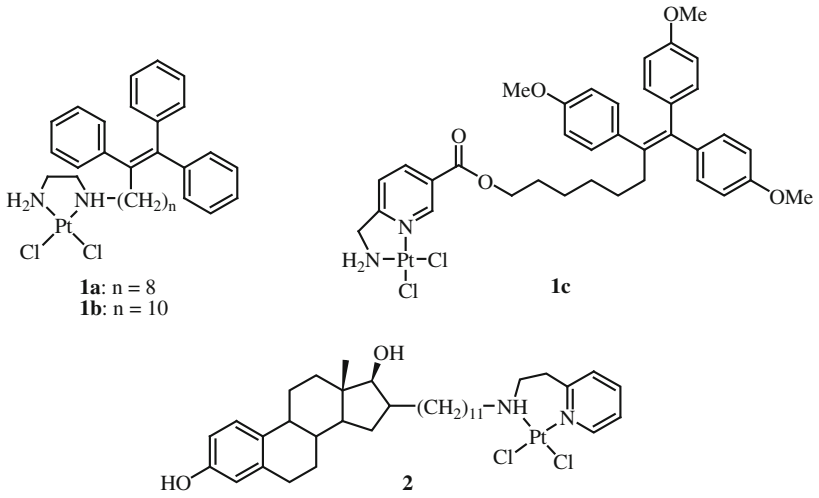


Fig. 16.2 Triphenylethylene and estradiol conjugates of Pt(II) with significant activity against MDA-MB-231 cells

selectivity was unincisive, however, its cytotoxic activity reached nanomolar ranges both in MCF-7 and MDA-MB-231 breast cancer cells ($IC_{50} = 0.5$ μM).

Essigmann and Croy designed an estradiol platinum (II) complex conjugate **3** connected via a non-hydrolysable linker and which allowed the juxtaposition of the estrogen receptor with DNA damage sites in previous works of the authors (Fig. 16.3). This complex **3** showed significant ER binding affinity (relative binding affinity RBA compared with estradiol = 28 %), which was retained even after binding to DNA. It was also more efficacious against ER-positive MCF-7 cells when compared with ER-negative MDA-MB-231 cells [14].

Lippard and co-workers found out that cisplatin adducts are shielded from nucleotide excision repair (NER) by the protein HMG1 (high-mobility-group 1) which is activated by estrogens [15]. Hence, this group pursued an approach towards DNA-damaging estrogen-platinum(IV) conjugates [16]. They prepared bis-17*β*-estradiol-*cis*-diammine-dichlorido-platinum(IV) conjugates **4a–e** with one to five methylene spacer groups between the 17-O-atom of the steroid and the O-donor atom at the Pt(IV) center (Fig. 16.3). In the hypoxic environment of a solid tumor Pt(IV) complexes **4a–e** are supposed to be reduced to Pt(II) complexes and to release one equivalent of DNA-damaging cisplatin and two equivalents of a linker modified estrogen which was hoped to up-regulate HMG1 expression after enzymatic hydrolysis. The cytotoxicity (IC_{50}) of the complexes **4a–e** in MCF-7 (ER^+) and HCC-1937 (ER^-) breast cancer cells was in the range of 2.1–5.5 μM.

Schobert and co-workers have developed an estradiol platinum(II) complex conjugate **5** (Fig. 16.3), which bound both to the ER and to the sex hormone

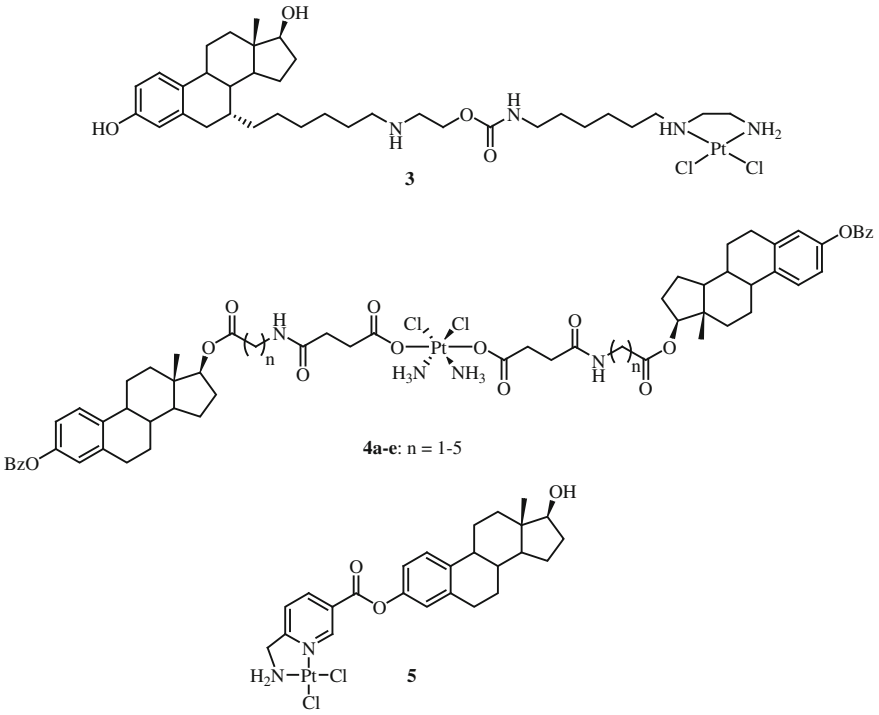


Fig. 16.3 Estradiol complex conjugates of Pt(II) and Pt(IV) with significant selectivity for hormone dependent breast cancers

binding globulin (SHBG) [17]. SHBG acts as a carrier for estrogens in blood and also interacts with plasma membranes of cells stimulating intracellular signalling pathways [18]. In addition, complex **5** elicited distinct growth retardation of MCF-7 (ER^+) breast cancer cells although it did not bind significantly to isolated DNA.

Gust and co-workers investigated a series of [1,2-bis(4-fluorophenyl)-ethylenediamine][dicarboxylato]platinum(II) complexes [19]. The most potent fluoro-derivatives **6** of this series reached the activity of cisplatin and surpassed carboplatin in MCF-7 breast cancer cells by far. The same group also developed additional Pt(II) complexes containing similar 2,6-difluoro-3-hydroxyphenyl fragments for the endocrine therapy of breast cancer. While complex **7a** and its racemic diastereomer **7b** revealed high activity in a hormone-sensitive MXT-M-3,2 breast cancer mouse model, in vitro tests using this cell line gave only moderate results [20]. The high in vivo activity of these complexes might originate from a significant reduction of the estrogen level in the animals since these complexes interfere with ovarian steroid biosynthesis (Fig. 16.4).

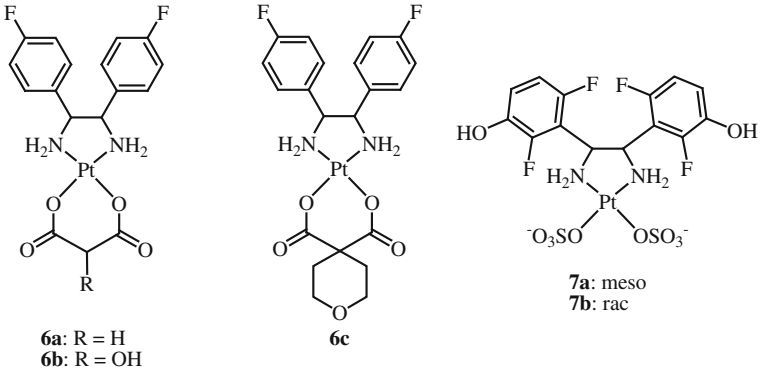


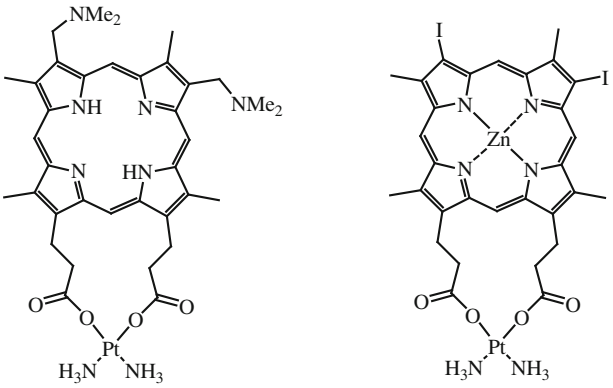
Fig. 16.4 Fluorinated Pt(II) complexes for the endocrine therapy of breast cancer

16.2.2 Further Platinum Complex Conjugates and Complexes with Functional Ligands

The team of Henri Brunner has been investigating since 1994 a series of porphyrin dicarboxylate complexes of Pt(II). Porphyrins are well known photosensitizers which could add significantly to the DNA damaging effects of Pt(II) complexes. In addition, porphyrins are enriched in tumors which need these molecules to grow and expand. Complex **8a** (Fig. 16.5) represents one of the first porphyrin Pt complexes with significant activity (38 % of control cell proliferation, 10 μM) against MDA-MB-231 breast cancer cells after irradiation, a result which exceeds that by cisplatin (44 %) [21]. Based on these findings for **8a,** Brunner and co-workers then designed complexes with improved cytotoxic activity against MDA-MB-231 cells, e.g., complex **8b** (Fig. 16.5), which bears a iodo-porphyrin zinc ligand and which was distinctly more cytotoxic than cisplatin [22].

Padhye, Sinn and co-workers conceived a testosterone thiosemicarbazone ligand which was subsequently coordinated to $PtCl_2$ fragment giving the dichloridoplatinum complex **9**. This complex was more cytotoxic against MCF-7 breast cancer cells at low concentration (0.3 mg/mL) than cisplatin [23].

Zoldakova, Biersack, and Schobert have prepared and studied Pt(II) complex conjugates with vascular-disrupting chalcones such as **10**. In collaboration with breast cancer researchers from the Karmanos Cancer Institute Detroit (Ahmad, Padhye, and Sarkar) and DNA biochemists from the Czech Republic (Brabec et al.), they carried out detailed studies of DNA interaction and in vivo mode of action. Complex **10** surpassed the activity of the free chalcone precursor and also of cisplatin with an excellent IC_{50} value of 80 nM (after 72 h) in MDA-MB-231 cells. On a molecular basis, this is due to its more potent DNA damaging property both in terms of the number of DNA lesions which complex **10** causes and their amenability to repair [24]. Hence, the combination of a potent vascular disrupting agent (VDA) with a reactive Pt complex appears to be very promising for the treatment of TNBC.



8a **8b**

Fig. 16.5 Porphyrin Pt(II) complex conjugates for the phototherapy of breast cancer

Trávnícek and coworkers came up with interesting Pt(II) complexes of the CDK inhibitor bohemine. The complexes **11a** ($IC_{50} = 3.3$ μM) and **11b** ($IC_{50} = 4.9$ μM) showed improved activity against MCF-7 breast cancer cells when compared with cisplatin ($IC_{50} = 10.9$ μM) [25] (Fig. 16.6).

A planar heterocyclic nitrogen donor ligand able to intercalate into DNA has been used by Italian scientists [26]. The cationic complex **12** containing a 2,9-dimethyl-1,10-phenanthroline and 1-methylcytosine (Fig. 16.7) showed high water solubility and pronounced cytotoxicity in MCF-7 and MDA breast cancer cells in the nanomolar range (IC_{50} ca. 0.2 μM). In addition, complex **12** overcame several cisplatin resistance mechanisms.

Similarly, Aldrich-Wright and co-workers prepared complexes **13a** and **13b** bearing 5-methyl-1,10-phenanthroline or 5,6-dimethyl-1,10-phenanthroline ligands aside an (1*S*,2*S*)-diaminocyclohexane ligand (Fig. 16.7). These complexes were strongly active against MCF-7 breast carcinoma with IC_{50} values of 35 and 28 nM, respectively [27]. In addition, they were also active in cancer cells resistant to cisplatin and were taken up by cancer cells more readily and to a larger extend than cisplatin.

16.2.3 Miscellaneous Platinum Complexes

Spanish scientists (Navarro-Ranninger, Alonso et al.) designed platinum(II) complexes with high activity against MDA-MB-468 breast cancer cells. The acetate-bridged cyclometalated organoplatinum(II) complex **14** (Fig. 16.8) exhibited an ID_{50} value of 2.0 μM against these breast cancer cells (cisplatin: 3.3 μM), and led to a drastic modification of plasmid DNA [28]. Fifteen years later another Spanish group (Ruiz et al.) introduced another organoplatinum(II)

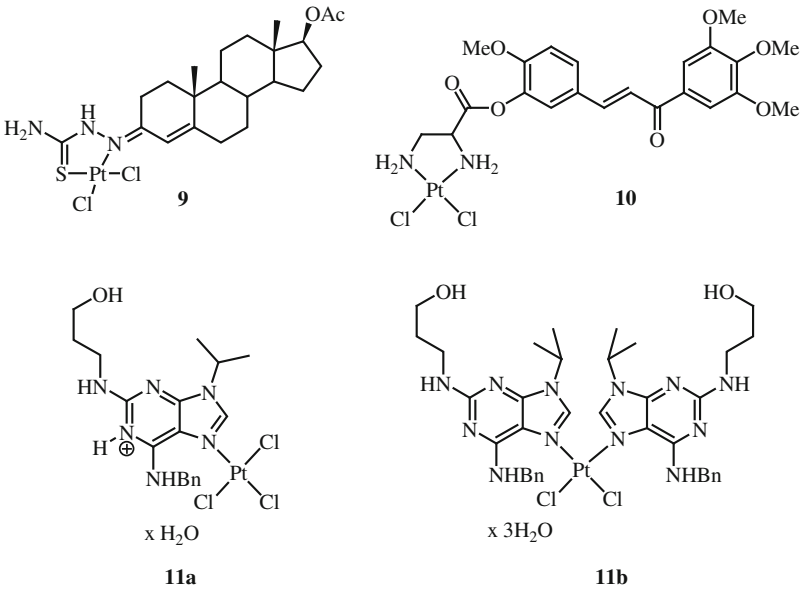
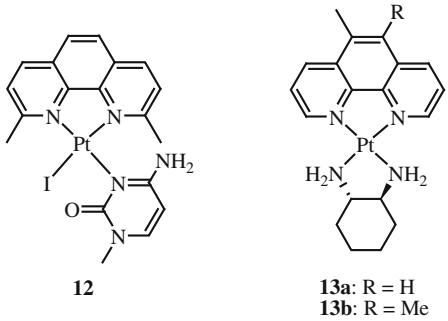


Fig. 16.6 Pt(II) complexes with androgen (**9**), VDA (**10**), or cytokinin (**11a–b**) moieties

Fig. 16.7 Pt(II) complexes with DNA intercalating 1, 10-phenanthroline ligands



complex **15a** featuring an N,C-chelating 2-(dimethylaminomethyl)phenyl ligand (dmba) and the hypoxanthine analogue 4,7-dihydro-5-methyl-7-oxo[1,2,4]triazolo[1,5-a]pyrimidine (HmtpO), as well as a related complex **15b**, *cis*-[Pt$(C_6F_5)_2$(HmtpO)$_2$] (Fig. 16.8). Both complexes showed distinct activity against the cisplatin-resistant breast cancer cell line T47D (IC_{50} = 4.6 μM) [29]. They caused significant DNA distortion of plasmidic DNA as to electrophoretic mobility shift assays (EMSA) and AFM experiments. A joint cooperation between the Karmanos Cancer Institute in Detroit/Michigan (Ping Dou et al.) and the Shiraz University (Hemmateenejad et al.) in Iran yielded an N,C-chelate (deprotonated 2-phenylpyridine) Pt(II) complex **16** (Fig. 16.8) bearing a bis(diphenylphosphino)methane (dppm) ligand. Complex **16** showed no toxicity but distinct growth inhibition of MDA-MB-231 xenografts in mice which was associated with proteasome

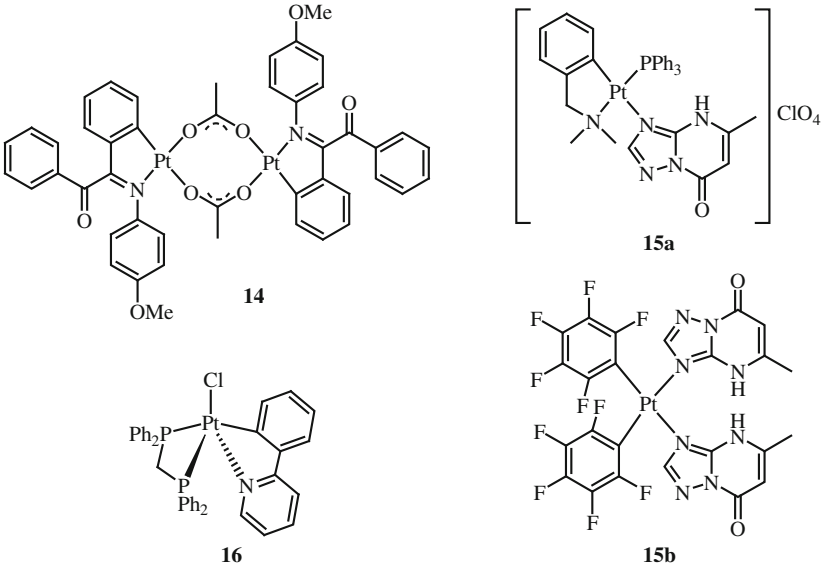


Fig. 16.8 Organometallic Pt(II) complexes with activity against breast cancer

inhibition, apoptosis induction, and DNA binding [30]. Thus complex **16** is a promising multimodal drug candidate.

Reedijk and co-workers have developed the dinuclear platinum(II) complex **17** (Fig. 16.9) of 5,7-dimethyl-1,2,4-triazolo[1,5-a]pyrimidine (dmtp), which revealed distinct and selective cytotoxic in vitro activity ($IC_{50} = 2.3$ μM) against T47D breast cancer cells (cisplatin: $IC_{50} = 10.7$ μM) [31]. Messori, Navarro-Ranninger and co-workers recently found out that a simple "rule-breaking" iodidoplatinum(II) complex (**18**) exhibits pronounced biological activity. Its antiproliferative activity in T47D breast cancer cells ($IC_{50} = 2.6$ μM) was superior to cisplatin ($IC_{50} = 15$ μM), although it showed a lower DNA distorting effect on plasmid DNA suggesting an alternative mode of action (Fig. 16.9) [32].

Bertani and co-workers conceived the *trans*-platinum(II) complex **19** (Fig. 16.9) featuring amidine and cyclohexylamine ligands with improved activity compared with cisplatin against MCF-7 breast cancer cells (IC_{50} ca 2.5 μM) [33]. Complex **19** also overcame cisplatin and doxorubicin resistance and caused DNA damage leading to apoptotic cell death. First in vivo assays using lung cancer xenografts (C57BL mice) were successful and revealed distinct tumor growth inhibition at non-toxic doses. The simpler *trans*-dichloridoplatinum(II) complex **20** (Fig. 16.9) bearing methylamine and dimethylamine ligands in *trans* position was likewise proven to be very active (and more active than cisplatin) against MCF-7 cells ($IC_{50} = 1.4$ μM) [34].

Dyson and co-workers designed *trans*-platinum(IV) complexes with functionalized aromatic carboxylate ligands. Complex **21** (Fig. 16.10) showed excellent in vitro activity against MCF-7 breast cancer ($IC_{50} = 0.77$ μM), which was in line

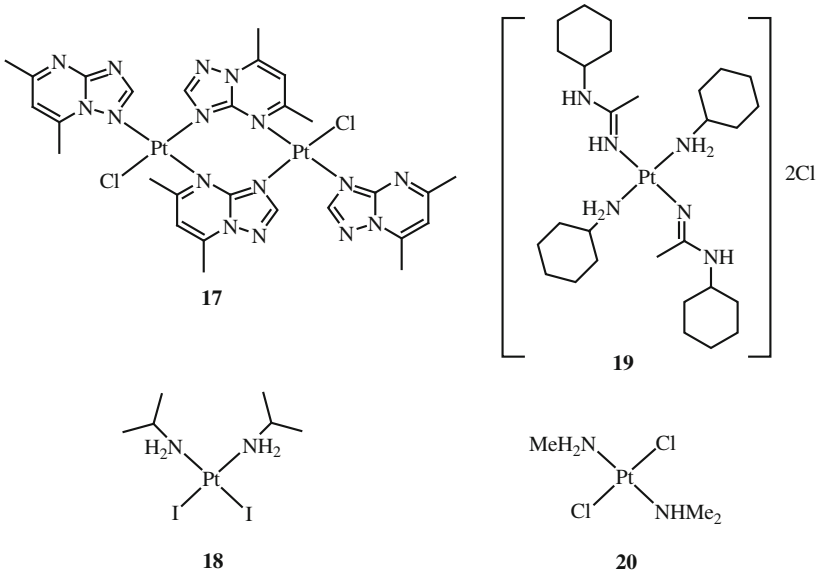
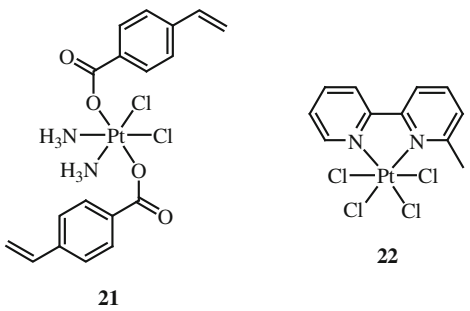


Fig. 16.9 Unusual Pt(II) complexes with potential against breast cancer

Fig. 16.10 Pt(IV) complexes with distinct activity against breast cancer



with a significantly enhanced uptake of this lipophilic complex [35]. Recently, an Iranian group (Ostad et al.) has reported Pt(IV) complex **22** (Fig. 16.10) to have market and selective activity against T47D breast cancer cells ($IC_{50} = 1.7$ μM), while non-malignant mouse fibroblasts responded less well by a factor of 38 [36]. This is an encouragingly broad therapeutic window.

16.3 Ruthenium Complexes

Ruthenium based drugs possess certain advantages over platinum complexes. They are known to be less toxic than their platinum congeners since they are able to mimic iron in protein binding sites, e.g., in transferrin [37]. Tumor cells have a

strong demand for iron and so overexpress shuttle and transport proteins in order to safeguard a sufficient iron uptake and supply. Another characteristic of many strategies employing ruthenium coordination complexes is the concept of "activation by reduction" according to which non-toxic Ru(III) complexes are supposed to reach hypoxic regions of solid tumors where they subsequently get activated by reduction to the bioactive Ru(II) complexes proper [37]. Recently, certain ruthenium(II) arene complexes have gained interest due to their promising antitumor activities [38].

16.3.1 Ruthenate Complexes

Alessio and Sava have developed Na[*trans*-$RuCl_4$(DMSO)Im], a ruthenate(III) complex with antimetastatic activity [39]. This complex reduced tumor growth and increased survival time of MCa mammary carcinoma bearing mice. In addition, this complex reduced metastasis when administered at low doses. Its close imidazolium congener NAMI-A (Fig. 16.1) also reduced lung metastasis and in a phase I clinical trial led to stable disease in a lung cancer patient for 21 weeks [40]. In this study, NAMI-A was safely administered i.v. at doses of 300 mg/m^2/day for five days.

Keppler and co-workers have designed another ruthenate complex, *trans*-indazolium [tetrachlorobisindazoleruthenate(III)] (KP1019, Fig. 16.1) which targets individual tumor cells as well as metastasis of solid tumors. This compound gets activated by reduction to Ru(II) species and it is selectively transported into the tumor cells when bound to transferrin. Inhibition of tumor colony formation by freshly explanted breast tumors was achieved in seven out of ten specimens by long-term exposure to 100 μg/mL of KP1019 [41]. KP1019 has already successfully undergone a phase I clinical trial with patients suffering from colon adenocarcinoma and endometrial carcinoma where it was well tolerated at doses ranging from 25 to 600 mg twice weekly for three weeks leading to a stable disease for ten weeks in several patients [42]. KP1019 reduced the weight both of the primary tumor and of lung metastases in mice bearing MCa mammary carcinoma. Since the number of metastases remained unaffected, direct cytotoxic effects appear to be the reason for the reduction of the metastasis mass [43]. KP1019 was also tested for its activity against tumors with intrinsic and acquired forms of multidrug resistance. The exposure of various tumor cells to KP1019 for as long a period as two years diminished its activity by a factor of merely two. Thus, the probability to elicit drug resistance during therapy with KP1019 is rather low. In contrast, multidrug resistant breast cancer cells expressing the BCRP transporter (MCF-7/bcrp and MDA-MB-231/bcrp) were extremely sensitive to KP1019 (collateral sensitivity) [44].

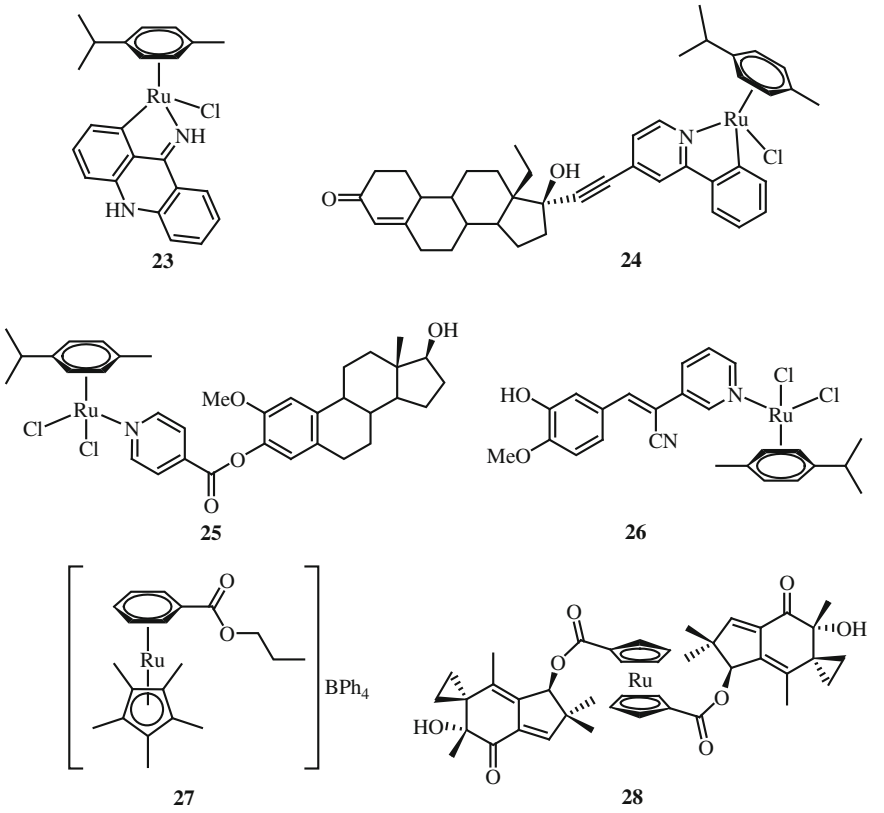


Fig. 16.11 (Arene)Ru complexes with activity against breast cancer

16.3.2 Ruthenium Arene Complexes

Ruiz and co-workers have devised a new (η^6-*p*-cymene)ruthenium complex **23** (Fig. 16.11) with 9-aminoacridine as an N,C-chelate ligand. Complex **23** revealed an activity in breast cancers two to three times that of cisplatin ($IC_{50} = 10$ μM, T47D; $IC_{50} = 6.8$ μM, MCF-7) [45]. Its interaction with plasmid DNA was evaluated by EMSA and was found to be less pronounced than that of cisplatin. The same group also designed an (η^6-*p*-cymene)ruthenium conjugate **24** containing a lipophilic levo-norgestrel group (Fig. 16.11). Complex **24** had an eightfold higher activity against T47D breast cancer cells ($IC_{50} = 7.4$ μM) compared with cisplatin ($IC_{50} = 60$ μM) [46]. A strong distorting interaction with plasmid DNA was found for **24** as to EMSA.

Schobert and co-workers disclosed new (*p*-cymene)Ru(II) complexes conjugated to various isonicotinate linked steroids with activity against MCF-7/Topo cancer cells (e.g., **25**: $IC_{50} = 6.5$ μM) [47]. In contrast to its platinum predecessor **5** this Ru(II) conjugate did not bind to the ER, but to the sex hormone binding

globulin (SHBG, $IC_{50} = 219$ nM). The addition of SHBG to MTT assays even lowered the cytotoxicity of complex **25** (Fig. 16.11) probably be sequestration and reduction/retardation of its cellular uptake. A ruthenation of cellular DNA as well as of salmon sperm DNA was observed, however, without any influence on the topology of the DNA according to EMSA. Biersack et al. have investigated (arene)Ru(II) complex conjugates of epidermal growth factor receptor binding tyrphostins [48]. Conjugate **26** (Fig. 16.11) was especially active against multidrug resistant EGRF(+) MCF-7/Topo breast cancer cells ($IC_{50} = 0.2$ μM) when compared with the metal free ligand ($IC_{50} = 1.5$ μM). Again, ruthenation of DNA occurred without causing much DNA distortion.

Williams, Loughrey, and co-workers have prepared cytotoxic Ru(II) arene Cp* sandwich complexes [49]. Complex **27** (Fig. 16.11) revealed good activities both in hormone dependent (MCF-7, $IC_{50} = 2.33$ μM) and in hormone independent breast cancer cells (MDA-MB-231, $IC_{50} = 3.36$ μM) compared with non-malignant NFF cells ($IC_{50} = 10.6$ μM). The ruthenocene conjugate **28** of the fungal toxin illudin M (Fig. 16.11) has been developed by Schobert and co-workers and it displayed selective activity against the resistant breast cancers MCF-7/Topo ($IC_{50} = 0.57$ μM) and MDA-MB-231 ($IC_{50} = 0.8$ μM) [50]. It proved a strong inducer of apoptosis in MDA-MB-231 cells, and inhibition of JNK did not reduce its cytotoxic activity.

16.4 Conclusions

Platinum complexes are an important compound class with potential to overcome resistance of triple-negative breast cancers. There are several new platinum complexes with improved activity against breast cancer cells compared with cisplatin, which might add significantly to the therapy of incurable breast tumors in the near future. The combination of platinum with protein binders (e.g., oncoproteins, tubulin) appears especially promising. Some of the newly discovered platinum complexes also target DNA by binding to estrogen receptors, however, it is important to ensure that the cytotoxic activity of the platinum fragment overrides any proliferation promoting responses induced by the estrogen. Ruthenium complexes are convincing due to their impressive selectivity and unique modes of action, e.g., anti-metastasis activity. Their selectivity is based on binding to transferrin, but there are also examples which use estrogen derived compounds and EGFR inhibitors as selectivity enhancing ligands. In addition, ruthenium fragments were used to potentiate the selectivity of generally toxic agents (e.g., illudin M). Despite the recent efforts in this field of bioinorganic chemistry, the search for new fine-tuned platinum and ruthenium complexes is ongoing, either by formation of conjugate ligand systems or by applying new ligand systems with novel intrinsic properties.

Acknowledgments Own work referenced in this review was supported by grants from the Deutsche Forschungsgemeinschaft (Scho 402/8).

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Chapter 17
Development of Notch Pathway Inhibitors for Cancer Therapy

Ingrid Espinoza and Lucio Miele

Abstract Notch signaling is an evolutionarily conserved cell-signaling pathway involved in cell fate during development, stem cell renewal and differentiation in postnatal tissues. Roles for Notch in carcinogenesis, in the biology of cancer stem cells and tumor angiogenesis have been reported. These features identify Notch as a potential therapeutic target in oncology. A series of pre-clinical studies using primarily small molecule inhibitors of γ-secretase have demonstrated anti-tumor effects. Phase I trials have identified a reasonable safety profile for these agents, especially with intermittent administration. Mechanism-based combinations specific for individual indications are being investigated. Several other classes of Notch inhibitors are being developed. In this review, we describe the basics of Notch signaling, the role of Notch in normal and cancer stem cells; finally we describe opportunity and challenges in the development of Notch inhibitors as novel targeted agents for cancer patients.

Keywords Notch signaling · Notch receptors · Notch inhibitors · Ubiquitination · Drosophilia · Gycosylation · Gamma secretase inhibitors (GSI) · Breast cancer · Stem cells · Carcinogenesis · Neutralizing notch antibodies · Decoys · Blocking peptides · Natural dietary supplements

Abbreviations

N^{IC}	Notch intracellular domain
N^{EC}	Notch extracellular domain
N^{TM}	Notch transmembrane domain
EGF	Epidermal growth factor

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NEDD4	Neural precursor cell expressed developmentally down-regulated 4
HES1-5	Hairy/enhancer of split family 1-5
GSK3b	Glycogen synthase kinase 3 beta
T-ALL	T cell acute lymphoblastic leukemia
GSI	Gamma secretase inhibitors
DLL 1, 3, 4	Delta-like 1, 3, 4
OFUT1	O-Fucosyltransferase 1
MAML1	Mastermind-like 1
PS1	Presenilin 1
CSC	Cancer stem cells

17.1 Introduction

17.1.1 Notch Receptors

The Notch pathway is one of the fundamental signaling pathways used in developmental processes. It is involved in both cell type specification and organogenesis [1, 2]. The name originated because partial loss of Notch function causes notches at the end of Drosophila wing blades [3–5]. Drosophila Notch was cloned in the mid-1980s by two groups, Artavanis-Tsakonas [6] and Young [7] and encodes a single-pass transmembrane receptor [8]. Typically, Notch is expressed in a cell and its ligands are expressed in neighboring cells. Upon interaction between Notch and a ligand, a canonical signaling pathway is triggered that has been the subject of intense studies over the past 25 years Notch signaling is evolutionarily conserved from sea urchins to humans. Mature Notch proteins are non-covalent heterodimers [9]. The extracellular subunit (N^{EC}) of Notch possesses multiple Ca^{2+} binding epidermal growth factor-like repeats (EGF-like) that are required for ligand interaction [10], followed by a negative regulatory region (NRR) which is composed of three cysteine-rich Lin12/Notch repeats (LN) each containing a Ca^{2+} binding site [11, 12] and a C-terminal hydrophobic region. The C-terminal hydrophobic region of N^{EC} together with the N-terminal region of the transmembrane subunit (N^{TM}) forms the heterodimerization domain (HD) [13]. The LN repeats are not required for the interaction between the subunits but stabilize it by preventing ligand-independent cleavage by metalloproteases [13]. As the name suggests, the NRR holds the mature Notch heterodimer in an auto-inhibited state. The transmembrane subunit includes a short extracellular region containing a pair of conserved cysteines [7, 14, 15] thought to participate in heterodimerization [16]. This is followed by a Type I transmembrane region and an intracellular region that contains a RBP-jk association module (RAM) that interacts with its transcriptional coactivator RBP-Jk or CSL (CBF-1/Suppressor of Hairless/LAG1) [17]. Seven ankyrin (ANK) repeats [18] that interact with CSL and other transcriptional regulators [19], two nuclear-localization signals (NLSs) [14], a transactivation

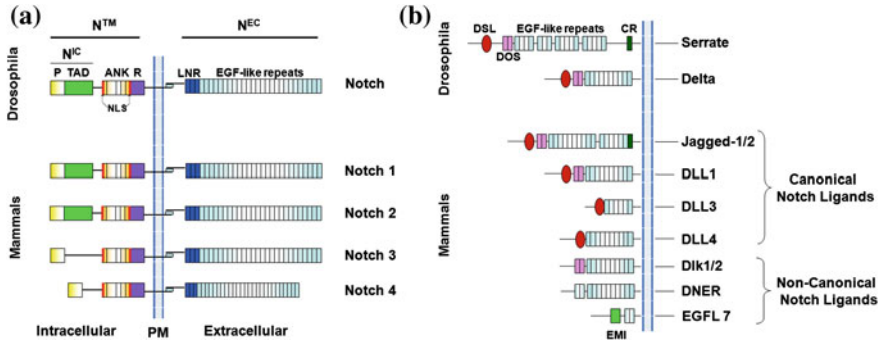

Fig. 17.1 Notch structure and ligands

domain (TAD) [20] which ends in a polyglutamine stretch (OPA) [20, 21] and a C-terminal PEST sequence (a region rich in Proline, Glutamic acid, Serine, and Threonine) that is phosphorylated and ubiquitinated and is involved in receptor turnover [22].

While Drosophila has only one Notch gene, the mammalian Notch family consists of four members (Notch1, 2, 3, and 4) that are approximately 60 % homologous to each other and to Drosophila Notch [23, 24]. Although the overall structures of Notch receptors are similar, they show significant differences. The Notch1 and Notch2 receptors contain 36 EGF repeats [16, 25] in their extracellular domains, whereas Notch3 has 34 repeats [26] and Notch4 has 29 [27]. Three of four Notch receptors contain a loosely defined evolutionarily divergent Transcriptional Activation Domain (TAD) [28]. Notch1 and Notch2 contain a strong and weak TAD, respectively [20], Notch3 has a potent but specific TAD best suited to the activation of the *hes5* promoter [29]. In contrast, Notch4 does not contain a TAD (Fig. 17.1a). These structural differences may offer clues to the functional divergence among mammalian Notch paralogs.

Drosophila has 2 canonical ligands, Delta and Serrate. Mammals express five canonical Notch ligands: three are homologous to Delta and are named Delta-like-1,-3 and -4 (DLL1, DLL3 and DLL4) and two are homologous to Serrate and are named Jagged1 and Jagged2 [30–34]. These ligands are Type I single-pass transmembrane proteins with an extracellular region consisting of an N-terminal region, a cysteine-rich DSL (an acronym for Delta, Serrate and LAG-2) motif and varying number of EGF-like repeats similar to the Notch proteins [28]. The N-terminal region, the DSL domain and the first two EGF-like repeats are necessary for interaction with EGF repeats 11 and 12 of the Notch receptors [35, 36]. The Jagged family contains double the number of EGF-like repeats compared to DLL ligands and an additional cysteine-rich sequence (CR) downstream of the EGF-like repeats [8]. The intracellular regions of DSL ligands are not conserved, but some contain multiple lysine residues and a C-terminal PDZL (PSD-95/Dlg/ZO-1 ligand) motif. These are thought to be required for the poorly understood ligand signaling activity and interactions with the cytoskeleton, respectively [37]. Notch signaling can also

be activated by "non-canonical" ligands other than Delta/Jagged, such as F3/contactin [38], DLK1 & 2, DNER, EGFL7 [39, 40] (Fig. 17.1b).

The structural variability observed in mammals for the four Notch proteins and their differential contex-dependent functions open the possibility of specific targeting with monoclonal antibodies (mAbs) against the least conserved regions of the proteins.

17.1.2 Notch Signaling Pathway

Most of our information on the canonical Notch signaling pathway is derived from studies on Drosophila Notch and its mammalian orthologue Notch1. The Notch precursor protein is produced as a single-chain transmembrane protein in the endoplasmic reticulum where it interacts with O-fucosyltransferase 1 (OFUT1 in Drosophila, POFUT1 in mammals) [41]. It is then transported to the Golgi where it is cleaved by a Furin-like convertase at site 1 (S1) [42, 43] and glycosylated by OFUT [44–46] and Fringe family N-acetylglucosaminidyl transferases [47]. Cleaved, glycosylated Notch is transported to the cell surface as a mature heterodimer.

Notch signaling is initiated by a Notch receptor-ligand interaction between two neighboring cells, which induces two successive proteolytic cleavages within the N^{TM} subunit that are required to release the intracellular fragment of Notch (N^{IC}) from the membrane [15]. The interaction between Notch and its ligand DSL generates an activating *trans* interaction on neighboring cells. In contrast, inhibitory *cis* interactions between receptor and ligand in the same cell suppress Notch signaling [48–51]. More recent work indicates that this *cis* interaction between Notch and DSL is bidirectional: Notch inhibits its ligand (preventing it from activating Notch receptors in *trans*), and the ligand inhibits Notch (preventing it from being activated by ligands acting in *trans*) [52–54]. Ubiquitin ligases Mindbomb [55] or Neuralized [56–59] interact with the ligand intracellular domain to promote its ubiquitination and internalization. Internalization and recycling to the plasma membrane may be required for ligand activity [60–62]. After receptor-ligand interaction, a ligand-N^{EC} complex is transendocytosed to the ligand-expressing cell [63]. This endocytic process may be required to generate sufficient mechanical force to disrupt the hydrophobic interactions between the N-terminal portion of N^{TM} and the C-terminal portion of N^{EC} [64]. At least for some Notch paralogs (e.g., Drosophila Notch, mammalian Notch1), Ca^{2+} chelation with EDTA disrupts the tertiary structure of N^{EC} to a sufficient extent to cause subunit dissociation and receptor activation [9]. Subunit dissociation exposes a cleavage site (S2) on N^{TM} on the extracellular side of the membrane for A Disintegrin And Metalloprotease 10 (ADAM10) or ADAM17 [15, 65]. ADAM10 or ADAM17 cleave the receptor at the S2 site. Ligand-dependent Notch activation is thought to prefer ADAM10, while ADAM17 is involved in ligand-independent activation [66]. ADAM cleavage leaves a short extracellular truncation fragment,

and a clipped transmembrane spanning region called NEXT (Notch Extracellular Truncation) which serves as a substrate for the final proteolytic cleavages [67]. The latter occur at site 3 (S3) [68] and site 4 (S4) within the transmembrane domain and are mediated by the γ-secretase activity of a multi-protein complex consisting of four subunits, presenilin 1 or 2 (the catalytic subunit) [69], nicastrin (which maintains complex stability and regulates intracellular protein trafficking) [70], APH1 (anterior pharynx-defective 1; required for the proteolytic activity) [71] and PEN2 (presenilin enhancer 2; stabilizes the complex after presenilin proteolysis has generated the activated N-terminal and C-terminal fragments) [72, 73]. γ–secretase-mediated cleavage releases the N^{IC} [74]. γ-secretase cleavage can occur at the cell surface or in an endosomal compartments, but cleavage at the membrane is thought to produce a more stable form of N^{IC} [28, 75]. Monoubiquitination of Notch has been proposed to occur after S2 cleavage by ADAM and to be necessary for endocytosis and subsequent γ-secretase cleavage in the endosomes [76]. Aquaporin family member Big brain (BIB) has been suggested to mediate the release of endosomally generated N^{IC} from endosomes [28, 77]. It is important to point out that the details of this process are not as clearly understood for mammalian Notch paralogs other than Notch1. Following γ-secretase cleavage, N^{IC} translocates to the nucleus where it binds to its downstream transcription factor CSL and drives canonical Notch-mediated gene transcription [78]. CSL is initially thought to be bound to target DNA in a repressive complex that contains histone deacetylases [79, 80], co-repressors SMRT (silencing mediator for retinoid and thyroid receptor)/N-CoR (nuclear receptor co-repressor) [81], CIR (CSL interacting repressor) [82] and SHARP (SMRT/HDAC-1-associated repressor protein)/MINT/SPEN [83, 84]. N^{IC} competes with the co-repressor complex to bind to CSL and interacts first through its RAM domain [19]. The ANK domain then associates with CSL to recruit the coactivator Mastermind-like1 (MAML1, one of three mammalian MAML homologues of Drosophila Mastermind or MAM) [85, 86]. The Notch-CSL-MAML1 ternary complex in turn recruits other coactivators like histone acetyltransferases CBP/p300 [87, 88] or PCAF/GCN5 [89], which convert CSL from a transcriptional repressor to a transcriptional activator. Crystallographic data have shown that the ankyrin domain of N^{IC} and the N-and C-terminals of the Rel homology domain of CSL form a complex with the long, kinked N-terminal helix MAML1 [19]. In this complex, the relatively unstructured N-terminal region of the ANK domain, which includes the RAM sequence, folds to form the N-terminal ANK repeat, creating a 7-repeat domain. CSL-binding sites on some Notch promoters exist in pairs in a head-to-head arrangement and could recruit dimeric Notch transcription complexes [90], which could increase the strength of the Notch signal. The end result of canonical Notch activation is transcriptional de-repression of a group of genes, many of which are themselves, transcription factors or transcriptional repressors. This generates a cascade of gene regulatory events that can modulate virtually every aspect of cell fate decisions depending on cellular context. Recent ChIP-Seq data [91–93] have started to shed light on factors contributing to “cellular context”, at least in T- and B- lineage cells. In T-ALL (T-lymphoblastic leukemia) cells, ETS and RUNX family factors

are frequently bound to chromatin close to CSL, and appear to cooperate with Notch/CSL, consistent with their known roles in T cell development and Notch signaling. CREB also appears to cooperate with Notch/CSL at low affinity CSL sites. Zinc finger protein ZNF143 may control the accessibility of CSL to Notch/CSL complexes. ZNF143 sites were associated with a prevalence of repressive chromatin marks, as were CSL-only sites that contained CSL but not Notch. In proliferating lymphoblastoid cells (LCLs) expressing EBNA2, Wang et al. [92] found that EBNA2 and CSL bind predominantly at nonpromoter sites. EBF, ETS, RUNX, PU.1, and NF-κB (RELA) sites were found within 500 bp of CSL sites. This correlated strongly with actual occupancy data for these transcription factors. Thus, the choice of genomic CSL sites at which Notch activates transcription may depend, among other factors, on the presence of additional transcriptional regulators that can cooperate with or antagonize the Notch-CSL transcriptional complexes. Different cells or different cellular states may have a variety of Notch target sites based on similar mechanisms. "Classical" Notch target genes include among others nuclear basic helix-loop-helix proteins (bHLH) of the Hairy/Enhancer of Split family (HES1-5) [94, 95], the Hairy-related family (HRT) [96], and the Hairy/Enhancer of Split-related with YRPW motif (HEY) families [97]. These negatively modulate the expression of genes such as the Achaete-Scute family that induce neuronal differentiation. N^{IC} is also thought to upregulate Deltex [98], several members of the NF-kB family, at least in bone marrow hematopoietic cells, [99, 100], the PPAR family [101, 102], as well as cell cycle regulators p21WAF1-CIP1 [103], cyclin D1 [104], and c-Myc [105].

Non-canonical pathways activating Notch signaling have been described. These pathways have been characterized as signals that respond to Notch independently of CSL complex (Type I), signals that activate Notch independently of S3 cleavage (Type II), or signals that activate CSL-dependent genes without Notch cleavage and N^{IC} release (Type III) [106]. Non-canonical Notch signaling pathways may be important in maintenance of hematopoietic progenitors, and in the regulation of immune response [107]. Among suggested mechanism of non-canonical Notch signaling are interactions of Notch with non-CSL transcription factors, such as β-catenin [108], HIF-1a (hypoxia-inducible factor-1a) [109], NF-κB (Guan, Wang, see Osipo and Miele for review), the estrogen receptor ERα [110] and others. Additionally, there is evidence that Notch activation results in activation of the PI3 K-AKT-mTOR pathway in many different cell types [111–128]. At least in some cases, these effects have been shown to be mediated by cytoplasmic N^{IC} [129]. Physical interaction of $Notch1^{IC}$ with PI3 K p85 has been described in T cells [111, 117]. Finally, we and others [110, 130–134] have described physical interactions between $Notch1^{IC}$ and the IKK signalosome or nuclear IKKα. These effects are suggested to mediate Notch-induced activation of NF-κB [133] and ERα [110]. $Notch3^{IC}$ has also been shown to bind IKKα homodimers, resulting in activation of the NF-κB alternative pathway [135]. Conversely, nuclear IKKα has been shown to activate Notch-dependent transcription in colon cancer cells [131].

Notch signaling is regulated at several levels by different types of post-transcriptional modifications. Glycosylation of Notch receptors by Fringe enzymes

(*N*-acetylglucosaminidyltransferases) affects binding affinities between ligands and specific EGF-repeats [136]. Fringe glycosyl transferases initiate elongation of O-linked fucose residues on specific EGF-like repeats of Notch receptors [137–139]. This modification prevents Notch activation by Jagged ligands, but not by Delta-like ligands [140]. In Drosophila, a recently identified glycosyltransferase, RUMI, also modifies Notch by adding O-glucose to serine residues on particular Notch consensus sequences [141] but the importance of this modification in mammals remains to be demonstrated. In mammals three Fringe genes are known, *Lunatic Fringe* (*Lfng*), *Manic Fringe* (*Mfng*), and *Radical Fringe* (*Rfng*) [142]. Reduced Lfng expression has been recently demonstrated in basal-like triple-negative breast cancer (TNBC). Importantly, targeted deletion of Lfng in the mouse mammary gland induces TNBC-like mammary cancers with high expression of cleaved Notch receptors. In this model, Lnfg blocked the mammary stem cells proliferation [143].

Another post-transcriptional modification of Notch is ubiquitination. Monoubiquitination has been proposed to result in Notch activation [76]. Conversely, polyubiquitination can lead to downregulation of Notch signaling. The Ring Finger E3 ubiquitin ligase Deltex along with β-arrestin/Kurtz [144], E3 ubiquitin ligases Itch/AIP4 (Atrophin-1 interacting protein 4) [145, 146], NEDD4 (neural precursor cell expressed developmentally down-regulated 4) [147] and Cbl (Casitas B-lineage lymphoma) [148] can poly-ubiquitinate Notch in the cytoplasm and direct Notch receptor endocytosis towards lysosomal degradation or toward recycling to the plasma membrane [149]. Endocytosis can sort Notch to either activation (see above) or degradation pathways. Numb is a cytoplasmic negative regulator of Notch. Numb, in cooperation with the AP2 (adaptor protein-2) component α-adaptin promotes Notch endocytosis [150, 151] followed by proteasome-mediated degradation [152]. Prolyl isomerase Pin-1 can modify N^{IC}, increasing its intracellular half-life [153]. Pin-1 in turn is regulated by mixed lineage kinases (MLK), potentially placing this pathway upstream of Notch [154].

In the nucleus, N^{IC} can be phosphorylated by kinases like GSK-3β [155] or CDK8 [156]. Several E3 ubiquitin ligases including Fbw7/Sel-10 [157], Itch [145], c-Cbl [148], and Deltex [144] can ubiquitinate active Notch and target it to the proteasome for degradation. Sel-10-mediated degradation extinguishes the Notch signal fairly rapidly as the Notch coactivator MAML1 itself recruits CDK8, which phosphorylates the PEST region, inducing Sel-10-mediated ubiquitination and proteasome-mediated degradation of N^{IC} [156]. Acetylation is another post-translational modification that can control the stability of N^{IC} ([158, 159]. SIRT1 deacetylase has been reported to regulate endothelial Notch signaling [160]. Numerous other oncogenic pathways crosstalk with Notch. Thus, Notch1 is required for the transforming activity of H-Ras [161] and TGF-α [162]. Notch activates the PI3 K-AKT pathway [111] while the AKT pathway upregulates Notch1 in response to VEGF (vascular endothelial growth factor) [163]. Both the AKT and ERK pathways cooperate with Notch4 in transforming breast epithelial cells [164]. Glycogen synthase kinase 3 beta (GSK3β), which is negatively regulated by AKT, decreases the half-life of Notch [155]. Our group has demonstrated

that in ERα-positive breast cancer cells estrogen causes accumulation of inactive Notch1 and inhibits Notch signaling while estrogen deprivation reactivates Notch signaling [165]. We also reported that HER2/neu overexpression inhibits Notch signaling while downregulation of HER2/neu or inhibition of its signaling caused reactivation of Notch signaling [166]. Recently, the Clementz et al. demonstrated that Notch1 and Notch4 are transcriptional targets of PEA3 [167], a transcription factor whose expression has been associated with malignant phenotype [168, 169] and with HER2/neu expression in breast carcinoma [170], and predicted worse overall survival in this malignancy [171]. Targeting PEA3 may indirectly inhibit Notch pathways, and provide a new therapeutic strategy for triple-negative and possibly other breast cancer subtypes [167].

In summary, we can conclude that Notch is the nexus of a unique and versatile signaling network that regulates and is regulated by a variety of cellular mechanisms highly dependent on cellular context. Thus, therapeutic targeting of the Notch pathway presents both promise and challenges. Successful development of Notch-targeting agents will require a mechanistic understanding of the role of Notch in specific diseases, and ideally, mechanism-based combination regimens (Fig. 17.2).

17.2 Targeting Notch Signaling

17.2.1 Notch Signaling and Cancer

Notch was first identified as an oncogene in T-cell acute lymphoblastic leukemia (T-ALL) in which a t(7;9) chromosomal translocation fuses the N-terminal region of the T-cell receptor beta (TCRβ) to the C-terminus of Notch1 [172]. This leads to expression of a truncated Notch1 protein lacking the extracellular subunit and hence constitutively active [173]. It was later discovered that over 50 % of T-ALL have a variety of mutations that activate Notch1 [174]. These mutations are concentrated in the heterodimerization region, leading to destabilization of the interaction between the two subunits, and/or in the C-terminal PEST region and prolongation of the intracellular half-life of Notch. Further, loss of the E3 ubiquitin ligase Fbw27/Sel-10, or mutations that target the Fbw7-binding pocket can cause Notch pathway activation in T-ALL [175]. The intracellular forms of all four Notch proteins are potentially oncogenic and capable of transforming normal cells [24, 176–178].

Deregulated expression of Notch proteins, ligands, and targets has been described in a multitude of solid tumors, including cervical [179], head and neck [180], endometrial [181], renal [182], lung [183], pancreatic [162], ovarian [184], prostate [185], esophageal [186], oral [187], hepatocellular [188], and gastric [189] carcinomas, osteosarcoma, mesothelioma [190], melanoma [191], gliomas [192], medulloblastomas [193]. Dysregulation of Notch signaling has been reported in

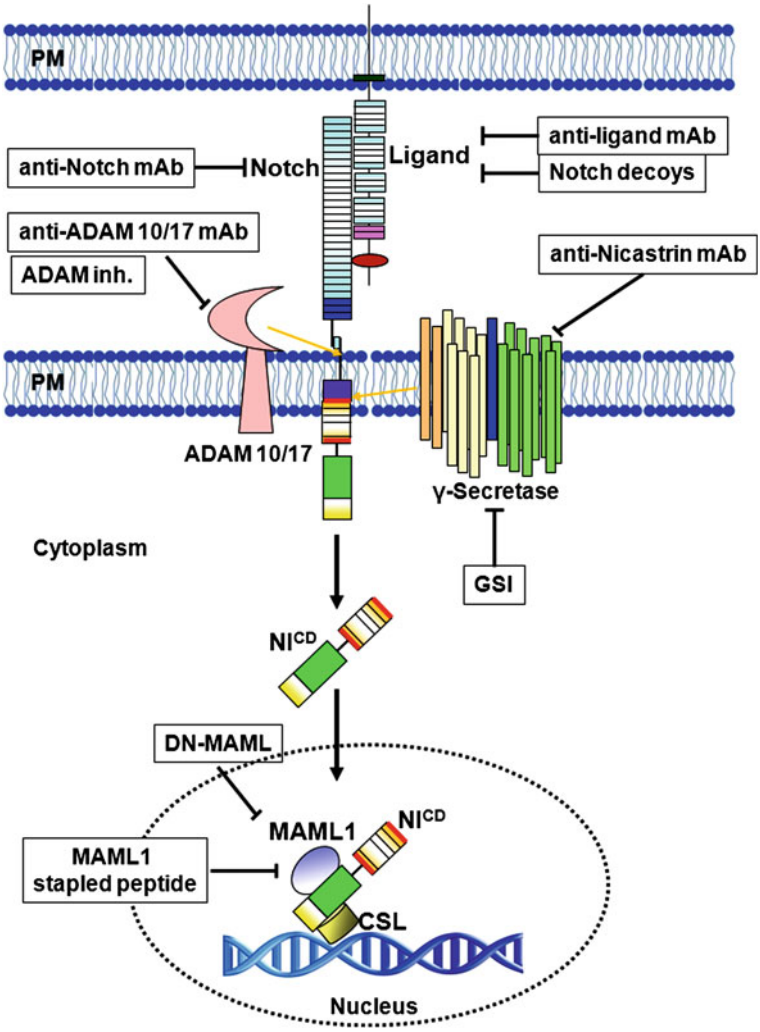

Fig. 17.2 Notch signaling pathway

some hematological malignancies other than T-ALL. These include Hodgkin lymphomas, anaplastic large-cell non-Hodgkin lymphomas [194], some acute myeloid leukemias (AML) [195], B cell chronic lymphoid leukemias (B-CLL) [196], multiple myeloma (MM) [197, 198]. For a recent review, see Pancewicz et al. [199].

A large set of studies has addressed the role of Notch in breast cancer. The first indication of a link between Notch signaling and breast cancer came from a study characterizing a frequent insertion site of the mouse mammary tumor virus (MMTV) in mice [200] which resulted in the overexpression of truncated Notch4

proteins. These truncated forms of Notch4 contained the transmembrane and intracellular domains, and similar to the truncated Notch1 subsequently discovered in T-ALL, they were constitutively active and caused spontaneous mammary tumors. This was confirmed when truncated Notch4, expressed in transgenic mice under the control of either the MMTV long terminal repeat or the whey acidic protein promoter [201, 202] led to mammary carcinogenesis. Besides Notch4, there is evidence that constitutive activation of Notch1 and Notch3 [203, 204] in mouse models [178, 205, 206] causes mammary tumors. Conversely, Notch2 has been associated with better prognosis in breast cancer [207]. In vitro, it can cause apoptosis of MDA-MB231 cells [208]. Notch2 may function as a Notch1 antagonist due to its lower transcriptional activity [209].

The first evidence of a role for Notch1 in human breast cancer came from a study showing increased expression of Notch1 protein in four breast tumors that overexpressed H-Ras [161]. Subsequently, two large studies showed that the loss of Numb, a negative regulator of the Notch pathway [210] and high-level co-expression of Jagged-1 and Notch1 mRNA [211, 212] in breast carcinoma samples correlated with poor prognosis. This was independently confirmed when accumulation of the intracellular domain of Notch1 and loss of Numb was observed by Stylianou et al. [213]. Immunohistochemical studies reported that high-level expression of Notch4 correlates with proliferative marker Ki67, while expression of Notch1 correlates with node status [214]. Recently, chromosomal rearrangements leading to the formation of Notch1 and Notch2 fusion transcripts have been described in breast cancer [215]. Fusion proteins behave as constitutively active Notch mutants. Tumors carrying such mutations may be sensitive to Notch inhibition.

Mechanistically, Notch may contribute to carcinogenesis by inhibiting differentiation, inhibiting apoptosis or promoting proliferation. The intracellular forms of Notch induce transformation when it is expressed with oncoproteins that disable the G1-S checkpoint, such as adenovirus E1A, human papillomavirus E6 and E7, Ras, myc, or SV40 large T-antigen. Depending on context, Notch also can activate the expression of several oncogenic pathways via direct or indirect induction of cyclins D1 [216] and D3 [217], cyclin A [218], SKP2 [219], c-Myc [220–222] or via activation of PI3 K-AKT-mTOR [111, 128], NF-kB [128, 134, 135, 223–227] and NF-kB2 [228], β-catenin [128, 229–232], signal transducers and activators of transcription-3 (STAT3) [233–235]. Notch can also co-operate with oncogenic pathways such as Wnt [236–238] or Her2/Neu [239]. Recent evidence suggests that Notch1 can induce expression of multidrug resistance transporter MRP1 (ABCC1) in breast cancer cells [91].

In addition to its cell-autonomous effects on oncogenic pathways, there is strong evidence for a role of Notch in tumor-stroma interactions. Notch signaling can mediate bidirectional tumor–stroma interactions and tumor–endothelium interactions [240]. For example, myeloma cells overexpress Jagged-2, activating Notch in stromal cells, which in turn produce IL-6, a growth factor for myeloma cells [197]. Conversely, stromal cells express Jagged-1, activating Notch in myeloma cells [198]. Head and neck squamous cell carcinomas overexpress

Jagged-1, which activates Notch in endothelial cells, promoting angiogenesis [241]. Productive tumor angiogenesis requires cooperation between VEGF and Notch signaling in the endothelium. Both DLL4 and Jagged-1 ligands participate in this process, with complementary roles [240]. Another poorly understood facet of the role of Notch in tumor microenvironment is the well-documented role of Notch signaling in a variety of immune system cells that can affect tumor growth through inflammation, angiogenesis and cytokines [reviewed in [240].

In contrast to its oncogenic role in numerous tissues, Notch has a tumor suppressor effect in the epidermis. Notch1 induces differentiation in murine [103] and human [102] keratinocytes. This has been confirmed by tissue-specific ablation of Notch1 in conditional knockout mouse models [242]. The mechanism for the tumor suppressor activity of Notch1 is still unclear. Cell-autonomous effects have been described, such as induction of p21 [103], calcineurin [243] and IRF6 [244]. Additionally, Notch signaling is essential for epidermal differentiation/barrier formation as Notch1 KO skin loses barrier integrity leading to inflammation and production of cytokines such as TSLP-1 [245]. Chronic inflammation and cytokine production in turn can lead to keratinocyte transformation, as well as distant effects such as B-lymphocyte proliferative disorder [246] or myeloproliferative syndrome [245]. Recently, Notch1 inactivating mutations have been described in a subset of oropharyngeal squamous carcinomas, suggesting that Notch1 may have a direct or indirect tumor-suppressor role in some of these tumors [247, 248]. The role of the other 3 Notch paralogs was not investigated. Conversely, increased expression of Notch1 and Jagged-1 has been reported by other groups to be associated with poor prognostic characteristics in Asian head and neck squamous carcinomas [249–252]. This suggests molecular heterogeneity in these tumors. Whether this correlates with HPV status is currently unclear.

17.2.2 Notch Signaling and Cancer Stem Cells

In recent years, Notch activity has been reported in cancer stem-like cells (CSC) [253]. Notch activity has been implicated in the maintenance of this "cancer stem cell" phenotype in breast cancer [254–258], embryonal brain tumors [259], glioma [260, 261], hepatocellular carcinoma [262] and pancreatic carcinoma [263].

CSC are thought to constitute a small subset of cancer cells with stem-like phenotype that are a reservoir of self-sustaining cells with the ability to self-renew, presumably leading to recurrence. The stem-like phenotype is also characterized by enhanced resistance to chemo- and radio-therapy [253] and [264]. Recently, we demonstrated that breast CSCs of different subtypes and in secondary mammospheres from clinical specimens show higher levels of Notch activity compared with the majority of the tumor cells. Notch inhibition by γ-secretase inhibitors (GSIs) inhibited sphere formation, proliferation and anchorage independent growth in soft agar [265]. This data supports a crucial role for Notch signaling in maintenance of breast cancer stem-like cells and suggest that Notch inhibition may

have clinical benefits in targeting them. Indeed, recent evidence in a Her2/Neu positive xenograft model [167] indicates that GSIs used in combination with Herceptin do not increase the effects of Herceptin on tumor volume, but completely abrogate tumor recurrence. This strongly suggests an anti-CSC effect.

17.3 Notch Inhibitors

Based in our current understanding of the Notch signaling pathway, we can identify several steps that can potentially be targeted to inhibit Notch signaling: (1) expression of ligands, (2) ligand ubiquitination and trans-endocytosis, (3) expression of Notch receptors, (4) ligand-receptor binding, (5) heterodimer dissociation during Notch activation, (6) ADAM-mediated cleavage of Notch, (7) subsequent ubiquitination and endocytosis of the γ-secretase substrate, (8) γ-secretase-mediated cleavage of Notch, (9) assembly of the coactivator complex with Notch and CSL, (10) heterodimerization of Notch transcriptional complexes, (11) Notch post-translational modifications and (12) expression of Notch targets. In the next section we will describe currently available Notch inhibitors and their development (Table 17.1).

17.3.1 Neutralizing Notch Antibodies

Blocking monoclonal antibodies (mAb) directed against Notch 1, 2 and 3 are under study. Two classes of blocking anti-Notch antibodies had been developed. One is directed to the extracellular negative regulator region (NRR) of Notch, blocking the conformational change that allows the ADAM protease cleavage [266]. A second class consists of a ligand-competitors directed against the EGF-repeat region of Notch receptors, blocking the ligand binding domain (LBD) [266]. Both NRR- and LBD-Notch antibodies induce a strong and specific downregulation of Notch1 signaling, but LBD required higher antibody concentrations to exert the inhibitory effects [266]. Interesting, Notch 1 NRR (NRR1) antibodies are also capable to bind and inhibit Notch1 carrying the "class I" NRR mutations (single amino acid substitutions or short insertions or deletions in the NRR domain of Notch 1 that cause increase Notch1 activity) in T-ALL cells [266]. Specific NRR antibodies such as anti-Notch1 (NRR1), Notch 2 (NRR2) and anti-Notch 3 (NRR3) antibodies that bind to the extracellular binding domain of Notch had been developed and they are in preclinical or in in vitro studies [266–268]. NRR1 also showed anti-angiogenic effects, inhibited blood circulation to the tumor and dramatically inhibited tumor growth [266–268]. Based in the success of in vitro and preclinical studies using blocking Notch antibodies, a dose escalating Phase I clinical trial as single agent has been opened using a humanized mAb that blocks Notch 2 and Notch 3 signaling, OMP-59R5. This clinical trial is directed to

Table 17.1 Notch inhibitors and their current development stage

Agent	Notch pathway target	Compound	Condition	Development phase
Neutralizing antibodies	Interference with ligand-induced Notch subunit separation and Notch ligands Specific for Notch 1, 2, 3; DLL1, 4	OMP-59R5 anti-Notch2/3 mAb (OncoMed Pharmaceuticals)	Solid tumors	Phase I NCT01277146
		NRR1 anti-Notch1 mAb (Genentech and exelixis; Merck)	Breast cancer Colon Cancer Anaplastic carcinoma T cell leukemia T-ALL cell line [1–3]	Preclinical and In vitro studies
		NRR2 anti-Notch2 mAb (Genentech and Exelixis)	Breast cancer Colon Cancer Anaplastic carcinoma HEK293T cell line [3]	Preclinical studies
		NRR3 anti-Notch3 mAb (Genentech)	HEK293T cell line [1]	In vitro studies
		OMP-21M18 anti-DLL4 mAb (OncoMed Pharmaceuticals)	Colorectal cancer Small cell lung cancer Pancreatic cancer Solid tumors	Phase I NCT01189929 NCT01189942 NCT01189968 NCT00744562
		DLL1-Fc and JAG1-Fc Anti-Delta-like1 and Jagged 1 Fc chimeric mAbs	Autoimmune encephalomyelitis [4, 5]	In vitro studies
		A5622A Anti-nicastrin mAb	T cell leukemia T-ALL tumor [6]	T cell leukemia T-ALL tumor [6]

(continued)

Table 17.1 (continued)

Agent	Notch pathway target	Compound	Condition	Development phase
Decoys	Interference with ligand-receptor interaction	Soluble forms of Notch1, Dll1 and Jagged 1	Endothelial cells [7–9]	Preclinical studies
γ-Secretase Inhibitor (GSI)	Notch 1, 2, 3, 4; Notch ligands	RO4929097 (Roche)	Breast cancer	Phase I
			Brain tumors	NCT01088763
			Colorectal cancer	NCT01198535
			Melanoma	NCT01149356
			Solid tumors	NCT01141569
			T cell leukemia	NCT01196416
				NCT01218620
				NCT01217411
				NCT01270438
				NCT01238133
				NCT01208441
		MRK-003 (Merck)	Breast cancer	Preclinical
			T cell leukemia	studies
			[10, 11]	
		MRK-0752 (Merck)	Breast cancer	Phase I
			Brain tumors	NCT00756717
			Neoplasms	NCT00803894
			Pancreatic cancer	NCT01295632
			T cell leukemia	NCT01098344
				NCT00645333
				NCT01243762
				NCT00572182
				NCT00106145
				NCT00100152

(continued)

Table 17.1 (continued)

Agent	Notch pathway target	Compound	Condition	Development phase
		PF-03084014 (Pfizer)	Neoplasms Solid tumors Lymphoid leukemia T cell leukemia	Phase I NCT00878189
		MRK-0752 (Merck)	Breast cancer Brain tumors Neoplasms Pancreatic cancer T cell leukemia	Phase I NCT00756717 NCT00803894 NCT01295632 NCT01098344 NCT00645333 NCT01243762 NCT00572182 NCT00106145 NCT00100152
		PF-03084014 (Pfizer)	Neoplasms Solid tumors Lymphoid leukemia T cell leukemia	Phase I NCT00878189
Blocking peptide	Interference with Notch nuclear co-activator MAML1	MAM peptide antagonist SAHM1 (Aileron Therapeutics)	T cell leukemia T-ALL tumors [12, 13]	Preclinical studies
Natural compounds	Downregulation of Notch activity and Notch pathway	Genistein Sulforaphane Quercetin Curcumin Resveratrol	Pancreatic cancer Prostate cancer Thyroid cancer Carcinoid T-ALL cells Glioblastoma cells Oral cancer cells [14–22]	Preclinical and In vitro studies

metastatic or relapsed patients who have received prior treatment with standard chemotherapeutic drugs. The estimated date of conclusion for this trial is July 2012. Some mAbs specific for the negative regulatory region of Notch3 have been shown to inhibit ligand-induced Notch activation by stabilizing the autoinhibited conformation of the receptor and preventing dissociation of the heterodimer [267].

Blocking antibodies against Notch ligands are under development. Anti-Dll4 mAb [269] and soluble Dll4-Fc fusion proteins [270, 271] that bind Notch receptors and prevent their activation by endogenous Dll4 have been generated. These antibodies inhibited Notch signaling in endothelial cells, caused disorganized angiogenesis and inhibited tumor growth [269]. They are therefore being developed as anticancer treatments [272, 273]. Recent studies using the humanized anti-Dll4 mAb OMP-21M18 that blocks the interaction with Notch1 and Notch4, showed an anti-tumor activity in patient-derived xenografts independent of any effect on angiogenesis [274]. Clinical trials using the OMP-21M18 antibody were designed for treatments of patients with solid tumors as colorectal cancer, pancreatic cancer, and small cell cancer. Currently, four active clinical trials using OMP-21M18 are ongoing using it as a single agent (NCT00744562) or in combination with chemotherapeutic drugs (NCT01189968, NCT01189942, NCT01189929) in different solid carcinomas. The mAb approach has the advantage of potentially exquisite specificity, with the disadvantages of mAbs including limited biodistribution and prolonged half-life. Specificity may decrease toxicity in cases where a specific Notch signaling protein is pathogenetically involved. On the other hand, when multiple Notch paralogs are involved, targeting of individual receptors may not be the most effective approach.

Recently, a novel mAb against the extracellular domain of nicastrin, A5226A, has been generated. This antibody recognizes the full glycosylated mature nicastrin in the active γ-secretase complex on the cell surface, and inhibits the γ-secretase activity by competing with the substrate binding in vitro. The A5226A antibody abolished the γ-secretase activity-dependent growth of T-ALL cell lines and tumor growth of a T-ALL xenografts mouse model [275]. Such a mAb would ideally cause γ-secretase inhibition (and potentially pan-Notch inhibition) without the potential off-target effects of small molecules.

17.3.2 Decoys

Decoys are soluble forms of the extracellular domain of Notch receptors or Notch ligands. Soluble decoys compete with their endogenous cell surface-bound counterparts and abrogate Notch signaling due to the lack of a transmembrane region necessary for receptor activation. A Notch1 decoy that acts as a ligand-dependent Notch antagonist blocks Notch signaling in endothelial cells, and affects tumor neoangiogenesis and growth. It also reduced Notch1 activity and interfered with Dll1, Dll4 and Jagged1 activities, making it a pan-ligand inhibitor [276]. Soluble forms of the DSL type ligands Dll1 [277] and Jagged1 [278] have also

been successfully used to inhibit Notch signaling. The presence of endogenous soluble Notch ligands has been reported as a result of endogenous metalloproteases activity [279–281]. Thus, there is evidence to support the use of soluble Notch ligands as a therapeutic tool. The extracellular domain of Dll1 binds to the EGF-like repeats of Notch and it can exist in a membrane-tethered and in a soluble form [282]. Another no-canonical Notch ligand is EGF-like domain 7 (EGFL7), a secreted angiogenic factor expressed in endothelial cells. Its bind to the extracellular domain of the four Notch receptors and inhibits Notch activation induced by Jagged. EGFL7 inhibits neural stem cells renewal [39] and inhibits Notch activity in post-natal retina and in primary endothelial cells [283]. These results suggest that EGFL7 could be used as a Jagged antagonist in cancer cells. The potential efficacy of decoys will depend in large part on their pharmacokinetics and biodistribution. A decoy that achieves better biodistribution than mAbs inside solid tumors may be an attractive therapeutic candidate.

17.3.3 γ-Secretase Inhibitors (GSI)

The activation of Notch depends largely on γ-secretase activity [284]. Thus, γ-secretase is a promising target for Notch inhibition. Non-selective GSIs, often referred to as "Notch inhibitors" in oncology are widely assumed to be equivalent in terms of biological activity and to have cytostatic or cytotoxic activities in cancer cells. Gamma-secretase inhibitors are under clinical trials for T-ALL and several solid tumors [285–289]. Several classes of GSIs have been developed. Most of them are directed to bind and block the catalytic activity of presenilin. The dipeptide inhibitor, z-Ile-Leu-CHO (GSI-I) was showed to have Notch1-dependent anti-neoplastic activity in Ras-transformed fibroblasts [161] and induced apoptosis in melanoma xenografts [290]. A similar tripeptide inhibited the proliferation of MDA-MB231 cells and tumor growth in MDA-MB231 xenografts. It also inhibited the growth of ERα^+ T47D:A18 cells and had a synergistic inhibitory effect in combination with Tamoxifen on ERα^+ xenografts [165]. These peptides, however, are not candidate human drugs due to poor pharmacokinetics and off-target effects. GSI Compound E inhibited growth and induced apoptosis by increasing the G0/G1 fraction and decreasing the S-phase fraction in T-ALL cell lines [291]. LY411,575, a GSI that binds to presenilin 1 (PS1) has been widely used in Alzheimer's disease, where it reduced the accumulation of amiloid-β peptide [292, 293]. In the HER2$^+$ breast cancer cell line BT474, LY 411,575 treatment increased apoptosis and re-sensitized resistant HER2$^+$ cells to trastuzumab [166]. GSI MRK-003 had good preclinical activity in breast cancer and T-ALL [286, 289]. This compound is more effective than LY411,575 in human mammospheres [264] and it completely abrogates recurrence in HER2$^+$ xenografts [288] in combination with trastuzumab. A similar compound, MK-0752, also binds to PS1. It is currently in several Phase I clinical trial for pediatric and adult oncology treatment [288, 294–296]. We have completed a pilot clinical trial with MK0752

in combination with endocrine therapy in the preclinical setting [297]. This combination was safe and well tolerated, and, importantly, showed molecular evidence of anti-proliferative and pro-apoptotic effects in tumor tissue. GSI RO4929097 appears to differ from other GSIs in that it induces a less transformed, compacted and slower-growing tumor phenotype without appreciable pro-apoptotic effects [298]. Currently it is in several Phase I clinical trials for treatment of solid tumors and T-ALL. Whether this is due to selectivity for specific Notch paralogs is unclear. GSI PF-03084014 has shown an effect in tumor growth and inducing apoptosis in several tumors [287] and it is currently in Phase I clinical trials for T-ALL and solid tumors. In vivo, most GSIs show evidence of anti-angiogenic effects in addition to direct effects on tumor cells. This is most likely due to inhibition of the Notch-VEGF cross-talk essential for angiogenesis (see above). The relative importance of anti-angiogenic versus direct anti-tumor effects in the in vivo mechanism of action of GSIs is still unclear, and may depend on tumor model and class of GSIs. Interestingly, pro-angiogenic cytokines IL6 and IL8 have been reported to cause resistance to GSI RO4929097 [299]. The possible role of Notch inhibition in other tumor-stroma components, including T cells, macrophages, tumor-associated fibroblasts and others is poorly understood.

In summary, it is safe to say that GSIs have shown anti-tumor effects in numerous preclinical models. Anti-angiogenesis and anti-CSC effects are likely to contribute to their mechanism of action in vivo. Due to the broad spectrum of substrates of γ-secretase, GSIs are likely to have multiple off-target effects in vivo. Their toxicity, however, appears to be almost exclusively Notch-mediated. The most serious adverse effect is diarrhea, caused by goblet cell metaplasia of the small intestine, which in turn is due to Notch inhibition in intestinal epithelial stem cells. This effect can be dose-limiting and in many cases it requires intermittent administration. The relative lack of specificity of GSIs is not necessarily a therapeutic problem, and may even be an advantage provided that mechanistically relevant pharmacodynamic biomarkers are identified. However, successful development of these agents will require evidence of target inhibition in tumor tissue to guide dose escalation. Molecular biomarkers indicative of Notch inhibition may differ in different tumors and the classical Notch targets (e.g., HES1) may not be the best biomarkers. Whenever possible, neo-adjuvant clinical trials guided by strong preclinical evidence may be the best approach to development.

17.3.4 Blocking Peptides

Numerous studies on Notch signaling have demonstrated that the activation of Notch and its nuclear access are required to maintain tumor cell growth and survival. Thus, blocking the transcriptional nuclear complex formed by Notch, CSL and coactivators may be another possible therapeutic tool. In 2003 the first dominant negative peptide derived from MAML1 was developed, this peptide forms a transcriptionally inert complex with Notch1 and CSL. It has been shown to

inhibit the growth of transformed T-ALL cell lines [291]. Six year later, a new synthetic, cell-permeable, stabilized α-helical, hydrocarbon-stapled peptide derived from MAML1 was generated (SAHM1) [300]. Stapled peptides are a new generation of drugs consisting in peptides outfitted with chemical braces or "staples" [300]. SAHM1 peptide showed a direct binding to pre-assembled Notch1–CSL complexes and competitive inhibition of the MAML1 co-activator binding. In addition, SAHM1 induced a direct transcriptional repression that resulted in anti-proliferative effects on T-ALL cell lines. SAHM1 treatment also showed an inhibition of leukemic progression through inhibition of Notch signaling in a murine model of T-ALL [300].

The use of stabilized, cell-permeable peptides to interfere with protein complex formation possesses several attractive features; these molecules have relatively small size, they have a high structural compatibility with target proteins, and have the ability to disrupt protein–protein interfaces. Pharmacokinetics will dictate to what extent these molecules can be used therapeutically in humans.

17.3.5 Natural Compounds

Natural dietary supplements have received much attention, primarily because epidemiological studies have shown that the consumption of fruits, soybean and vegetables is associated with reduced risk of several types of cancers [301–303]. Such compounds have notoriously pleiotropic activities, but in many cases their biological effects are very promising. As a result, many groups have focused on elucidating molecular mechanisms and identifying the targets of action of these natural products. Several dietary derived compounds target Notch signaling. Isoflavone genistein, found in soy products, inhibits Notch signaling, decreases cell proliferation and induces apoptosis in pancreatic cancer cells via downregulation of NF-κB activity [304]. In prostate cancer cells, genistein reduces cell viability and induces apoptosis through downregulation of Notch1, AKT and FoxM1 [305]. Sulforaphane, a natural compound derived from cruciferous vegetables such as broccoli, inhibits breast CSCs growth in vitro and in vivo through down-regulation of the Notch and Wnt/beta-catenin pathways, and inhibits growth of CSC-xenografts derived from prostate and pancreatic tumors [306]. Quercetin is a major polyphenol and flavonoid commonly found in many fruits and vegetables. It has been reported that quercetin decreases the levels of Notch1 protein and its active fragment in a leukemia cell line with constitutive Notch1 activation [307] and has a synergistic effect with GSI on Notch1 activity [308]. Quercetin also targets CSCs and the epithelial-mesenchimal transition (EMT) phenotype of pancreatic cancer cells [309]. Curcumin is an active compound found in Curcuma longa, which is widely used as a flavoring agent in food (e.g., turmeric). It has been shown to have antitumor activity. Curcumin downregulates Notch1 and induces apoptosis through inactivation of NF-κB in pancreatic cancer cells [310] and in oral cancer cells [187]. Resveratrol, a polyphenolic compound found in grapes,

red wine, purple grape juice, peanuts, and some berries, induces apoptosis in part by inhibiting Notch and PI3 K/AKT in T-ALL cells [311] and in glioblastoma cells [312]. Recently, it has been reported that resveratrol can also activates Notch2 as a mechanism of induction of apoptosis in medullary thyroid cancer [313] and in carcinoid, a neuroendocrine tumor [314]. Notch2 may function as a Notch1 antagonist due to its lower transcriptional activity.

Considering the relatively non-toxic effects of natural products, the idea of such compounds inhibiting Notch in tumor cells is potentially attractive. Chronic, partial Notch inhibition by natural products may contribute to chemopreventive activity. Therapeutic uses in established cancers are likely to require combinations with conventional chemotherapeutic agents.

17.4 Conclusions

In this brief commentary, we attempted to summarize the role of Notch proteins in cancer, with emphasis on breast cancer, and current Notch-targeting therapeutic tools. Deregulation of Notch proteins has been associated with specific pathologies including cancer development and progression, including the self-propagation of cancer stem cells. These and other features of Notch signaling, identify Notch as a candidate diagnostic and prognostic biomarker, and an attractive target for cancer therapy. Currently, most Notch-directed therapies involve the use of GSIs, but a variety of biopharmaceuticals and natural products deserve further investigation. As is the case for most developmental pathway inhibitors, the development of Notch inhibitors will need to be guided by biology. Biomarkers indicative of Notch activity (and of it inhibition by investigational drugs) will have to be identified and validated in each indication. Additionally, mechanism-based combinations will play a key role. We have demonstrated that combinations with endocrine therapy and trastuzumab can have remarkable therapeutic activity compared to single agent treatment. Importantly, in the case of Her2-positive breast cancer the effect of Notch inhibition was to prevent recurrence rather than to decrease tumor volume [289]. This implies that tumor volume may not be the most informative endpoint in clinical trials of Notch-targeting agents. Recurrence-free survival and/or good surrogate endpoints predictive of survival (e.g., circulating tumor cells, mammosphere-forming cells) are likely to be more informative. These challenges do not diminish the tremendous therapeutic opportunity offered by a pathway that is essential for CSC maintenance, angiogenesis and in many cases proliferation and survival of cancer cells.

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Chapter 18
Systems Biology Approaches in Breast Cancer Studies

Zhiwei Wang, Shavali Shaik, Hiroyuki Inuzuka and Wenyi Wei

Abstract Breast cancer is the second most common malignancy for women. Currently, only several prognostic and predictive factors are used clinically for managing breast cancer patients. Recently, systems biology approaches based on high-throughput technologies such as DNA microarrays, mass spectrometry-based proteomics and metabolomics have began to be used to investigate the expression of a wide range of genes and proteins in the dissected breast tumors. Moreover, these expression signatures have been found to provide potential and independent prognostic information in patients diagnosed with breast cancer. Furthermore, these molecular signatures could not only help to identify new therapeutic targets, but also allow physicians to design more effective and targeted therapeutic strategies for achieving better treatment outcomes of breast cancer patients.

Keywords DNA microarray · Mass spectrometry-based proteomics · Metabolomics · Factors for increased breast cancer incidence · Estrogen receptor (ER) · Progesterone receptor (PR) · Human epidermal growth factor receptor 2 (Her2) · Triple-negative breast cancers (TNBC) · Systems biology · Systems biology approaches · Tissue microarray · MicroRNA microarray · Gene annotation tool to help explain relationships (GATHER) · Dissociable antibody microarray (DAMA) · cDNA-mediated annealing selection extension and ligation (DASL)

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18.1 Introduction

Breast cancer is the second most common form of human cancer just behind lung cancer. According to the American Cancer Society estimation, 229,060 American women will be expected to develop breast cancer, and 39,920 will unfortunately die from it in 2012 [1]. More than 1.3 million women worldwide will be diagnosed with breast cancer each year. Although death rates have been decreasing since 1990 due to early detection through screening and treatment advances, nearly half-a-million females die from this disease each year around the world [1].

It is worthy to mention that the causes of breast cancer development remain largely elusive. However, many factors have been found to be associated with increased incidence of breast cancer, such as aging, race, family history, smoking, drinking, diet, and lifestyle [2]. For example, a woman who has a first-degree relative (mother, sister, daughter) with breast cancer will have a higher risk of developing breast cancer [3]. To this end, approximately 15 % of women with a family member diagnosed with breast cancer will get this deadly disease in their life time. More importantly, about 5–10 % of breast cancers can be linked to gene mutations, including BRCA1 and BRCA2 genes [4]. Women with BRCA1 and BRCA2 gene mutations have 80 % risk of developing breast cancer in their lifetime, and it often occurs at a relatively younger age [5]. Moreover, it has been shown that many genes and cell signaling pathways play critical roles in the development and progression of breast cancer [6–8].

Breast cancer has been characterized by the expression of various hormones and growth factor receptors including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2) [9]. Approximately 70 % of patients with breast cancer have higher expression of ER and PR, while 20 % of breast cancers have elevated Her2 expression [10]. The remaining 10 % of breast cancer cases are defined as triple-negative type of breast cancers (TNBC) that are negative for ER, PR, and Her2 expressions. Patients with TNBC tend to have a poor prognosis [11]. Currently, clinical treatments for breast cancer include locoregional treatment with surgery and radiation, plus systemic treatment with chemotherapy, hormonal therapy, and biologic therapies [12]. Although these treatments have increased 2 % survival rate every year, a significant number of breast cancer patients die from it, indicating that understanding the molecular mechanisms underlying the development of breast cancer is urgently needed to design the novel targeted therapeutic strategy for achieving better treatment outcomes.

18.2 Systems Biology Approaches

Systems biology is mainly an attempt to construct models of the behavior of complete biological systems [13]. To achieve this goal, it will use multiple research fields involved in mathematics, physics, engineering, and computer

science in addition to the biological sciences [14]. In other words, systems biology depends on the computational technology and numerical techniques to simulate biological networks, leading to a potentially better understanding of the complicated system processes, mechanisms, and principles [14]. It has been documented that systems biology is a novel approach to get the wealth of information much more efficiently than the conventional biological study approaches regarding to cellular mechanisms. For example, systems biology has been used to determine the relationships and interactions between different parts of a biological system such as gene and protein networks [15]. Specifically, systems biologist typically used high-throughput techniques attached to computerized data mining to quantify differences in the genome, proteome, and metabolome after stimulus from cellular outsides [15]. To this end, microarrays measure the miRNA changes in the genome, and mass spectrometry determines changes at the protein and metabolite levels. Mass spectrometry also studies protein modifications and identifies specific protein/protein interactions [16].

Systems biology methods have been used to define the molecular mechanism underlying tumor development and progression in recent years [17]. The advances and accessible in genomics, proteomics, and metabolomics high-throughput experiments promote the current oncology research rapidly evolves from the conventional "one gene one lab" mode to the systems biology era [18]. It is clear that the number of published papers based on microarray data has increased 15 folds within 10 years. It is known that the development of cancer requires many different pathways to regulate cell growth, cell apoptosis, and cell cycle. This integrated nature of cancer pathways results in difficulty to get the better treatment if we only target specific pathway components [19]. Therefore, getting the comprehensive models of cancer-related cellular signaling pathways in tumorigenesis will help us tremendously to design the novel strategy for targeting multiple key pathway components [19]. To accomplish this goal, systems biology coupled with new analysis software tools could generate the complete picture of many cancer cell-signaling pathways.

18.3 Systems Biology Approaches for Breast Cancer

Over the past decade, systems biology approaches such as various genomics-based techniques including DNA microarrays, mass spectrometry-based proteomics and metabolomics, miRNA microarrays have been widely used to define the molecular characterization of breast tumors in the genome (DNA), transcriptome (mRNA), proteome (proteins), or metabolome (metabolites) [14]. Moreover, these systems biology methods have been applied to detect the prognosis and the prediction of outcome and treatment in breast cancer [20]. Furthermore, proteomics-based techniques have been used to discover the biomarkers of early diagnosis, prognosis and prediction of outcome response to breast cancer therapies [21]. In the following

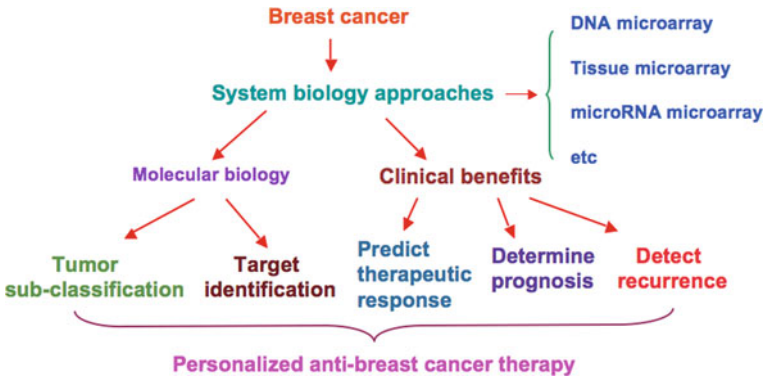

Fig. 18.1 The roles of systems biology approaches for breast cancer in personalized medicine. Systems biology approaches such as DNA microarray, tissue microarray, microRNA microarray could be applied to various areas in the clinical management including breast cancer screening, diagnosis, detecting recurrence, and predicting therapeutic response

paragraphs, we will discuss the several systems biology approaches that have been reported to identify the characterization of breast cancers and have potential clinical applications for breast cancer diagnosis and prognosis (Fig. 18.1).

18.3.1 DNA Microarray

Recently, microarray-based gene expression profiling of human breast cancer has been studied [22]. The advantage of the microarray technology is to measure the gene expression of thousands of genes at one time within a single experiment, and get the gene expression pattern among different genes in or between various breast tumor samples [22]. A number of different DNA microarray platforms have been generated to assess gene expression signatures [23]. However, the common methods are cDNA and oligonucleotide-based microarrays. Specifically, RNA extracted from breast tumors is converted into either cDNA or cRNA, followed by hybridization to the surface of the DNA microarray and subsequent detection of their interactions with probe-targets [24].

Perou and colleagues reported their groundbreaking work that characterizes the gene expression variations between sporadic breast tumor samples [25]. In this study, this group used complementary DNA microarrays representing 8,102 human genes to define the gene expression patterns in a set of 65 human breast tumor samples from 42 different individuals [25]. Based on "an intrinsic gene set" of 476 cDNAs and hormone receptor as well as Her2 status, they classified breast tumors into four major subtypes: (1) a "normal" epithelial group; (2) a "luminal cell-like" group expressing ER; (3) a Her2-positive subset; (4) a "basal cell-like" group lacking ER expression, but expressing Interrin 4, Laminin, Cytokeratins 5 and 17. Moreover, in the following study, the same group identified 456 genes that

could segregate the breast tumors into six defined subgroups: (1) normal breast-like; (2) ER-positive, luminal subtype A; (3) ER-positive, luminal subtype B; (4) ER-positive, luminal subtype C; (5) ER-negative, Her2-positive; (6) Basal-like, ER-negative, PR-negative, Her2-negative [26]. More importantly, these subtypes were found to significantly correlate with overall survival [26].

Another study used DNA microarray analysis on primary breast tumors of 117 young patients and applied supervised classification to identify a gene expression pattern that predicts a short interval to distant metastases in young patients with lymph node negative [27]. The poor prognosis signature includes multiple genes involved in cell cycle, invasion, angiogenesis and metastasis [27]. Moreover, the same group classified a series of 295 consecutive patients with primary breast tumors that have a gene expression signature correlated with poor or good prognosis [28]. They found that among the 295 patients, the 10-year survival rates were 55 % for 180 patients with a poor prognosis signature, while 10-year survival rates were up to 95 % for 115 patients with a good prognosis signature [28]. These studies indicated that the gene expression profile could be a powerful predictor of the outcome of breast cancer in young patients.

As a further support role of genome-wide measures of gene expression, Wang et al. identified a 76-gene signature consisting of 60 genes for patients with ER-positive and 16 genes for ER-negative patients [29]. This gene profile was correlated with distant metastases within 5 years in lymph-node-negative breast cancer patients without receiving adjuvant systemic treatment. The gene expression signature provides a powerful tool for detection of patients whether they will have distant recurrence later. It will also help physicians to design the less aggressive therapeutic treatment, but not adjuvant systemic therapy, for patients with a favorable prognosis [29]. Consistent with this notion, Pawitan et al. reported that a subset of 64 genes were found to spare the breast cancer patients from adjuvant therapy with good and poor outcomes [30]. In line with these findings, multiple studies from different groups using whole genome approaches also identified prognostic gene sets that mediate metastasis to distant organs [31–34]. Taken together, DNA microarray could be a useful method for diagnostic tests, identifying prognostic factors and predictors of treatment response.

18.3.2 Tissue Microarray

It is accepted that DNA microarray experiments are currently relatively expensive. In addition, the large volumes of data from DNA microarray require validation. To overcome this pitfall, the use of tissue microarray has become a common methodology to identify and validate breast tumor biomarkers [35]. It is known that tissue microarray is a collection of tissue specimens arranged on a glass slide, subsequent probing with different specific antibodies, leading to the simultaneous investigation of biomarkers in many tissue specimens [35]. So far, the most commonly used assay on tissue microarray is immunohistochemistry (IHC).

Recently, it has been reported that in situ hybridization (ISH) for DNA and RNA has been performed based on tissue microarray. Interestingly, tissue microarray has also been used for protein blotting and infrared spectroscopy [36, 37].

The first application of tissue microarray was towards the identification of six genes including ERBB2, CCND1, MYC, MYBL2 in addition to p53 and ER expression in breast cancer for defining new subgroup of tumors [35]. This new technology, validated by Camp and colleagues, confirmed that IHC based on tissue microarray was equivalent to whole tissue sections in breast cancer [38]. Moreover, Callagy et al. detected the ability of tissue microarray to sub-classify breast cancer using formalin-fixed paraffin-embedded tumor archives [39]. This group found that the pattern of expression of 13 different protein biomarkers could be used to divide breast cancer into two main groups correlating with tumor grade and nodal status [39].

Tissue microarray has increasingly been used to validate the data from DNA microarray-based gene expression profiling studies. For example, one study led by Dr. Gilks used tissue microarray to validate 31 potential biomarkers based on DNA microarray profile in 438 invasive breast carcinoma cases with 15 years of follow-up. They demonstrated that 17 of 31 markers showed prognostic significance, suggesting that multiple markers validated by tissue microarray could be prognostic indicators in breast cancer [40]. Similarly, another study also used tissue microarray to evaluate 25 biomarkers in 1,076 invasive breast cancer cases [41]. These biomarkers are mainly based on DNA microarray profiles and well-characterized commercially available molecules related to epithelial cell lineage, differentiation, hormone and growth factor receptors in breast cancer [41]. They identified six main clusters by these markers, demonstrating that breast tumors could be classified into biologically and clinically distinct sub-groups by the tissue microarray analysis [41].

Recently, tissue microarray has also been used to evaluate whether the previously identified biomarkers have biological and therapeutic significance in breast cancer. For example, cyclooxygenase-2 (COX-2) was highly expressed in 41 % of cases in 200 breast carcinomas by tissue microarray [42]. Wulfing et al. also detected the expression of histone deacetylases (HDAC-1) in 200 breast tumor samples by tissue microarray [42]. Their finding suggests that HDAC-1 expression could be a potential marker of prolonged disease-free survival and tumor aggressiveness [42]. Rakha et al. used tissue microarray and examined the several mucins in 1447 cases of breast cancer with a long-term follow-up [43]. Their results showed that MUC1 and MUC3 are potential prognostic indicators, and MUC1 has the strongest relationship with patient outcome [43]. A study by Li and colleagues identified that glioma-associated oncogene homolog 1 (GLI1) and forkhead box C2 (FOXC2) are associated with the basal-like breast cancer phenotype and with a poor rate of disease-free survival using tissue microarray in breast cancer [44]. In addition, phospho-signal transducer and activator of transcription 3 (STAT3) has been discovered as an important independent prognostic marker in node-positive breast cancer patients by tissue microarray technology [45]. More recently, multiple markers identified by tissue microarray including CD151 [46], protease-activated receptor 1 (PAR1) [47], glucose transporter 1 (Glut-1) [48], claudin-7 [49],

ABCG2 [50], and fibroblast growth factor receptor 3 (FGFR3) [51] have been reported to have important biological functions in breast cancer. These studies suggest that tissue microarray is a useful tool to discover novel biomarkers.

18.3.3 MicroRNA Microarray

Recently, microRNA microarray has been employed to detect short non-coding RNAs. It is well known that microRNAs play critical roles in tumorigenesis through post-transcriptional regulation of the gene expression. Specifically, alterations in microRNA expression have been found to correlate with tumor growth, metastasis and poor prognosis in breast cancer [52]. Moreover, some microRNAs are associated with molecular subtypes of breast cancer such as HER2, ER and PR expression levels. In addition, some microRNAs are correlated with breast cancer clinicopathological factors including tumor stages, invasion, and tumor metastasis. Interestingly, some microRNAs have tumor suppressor functions, which are frequently downregulated in breast cancer, while some microRNAs that have high expression play oncogenic roles in breast carcinomas [53].

The microRNA microarray has been recently used to understand the mechanisms by which breast cancer arises and develops. For example, Ota et al. performed microRNA microarray analysis to compare microRNA levels in bone marrow and investigate whether microRNA could serve as a prognostic marker for cancer recurrence in breast cancer [54]. They found that microRNA-21 and microRNA-181a levels were highly expressed in the recurrent breast cancer cases. Moreover, the expression of microRNA-21 and microRNA-181a was significantly associated with shortened disease-free survival and overall survival, respectively [54], suggesting that these two microRNAs could be prognostic markers for breast cancer. Furthermore, one study by Zhang et al. explored differential expression of microRNAs by microRNA microarray between breast cancer cells and mammary epithelial cells [55]. They reported that 113 microRNAs were up-regulated whereas 60 were down-regulated in breast cancer cells. Strikingly, microRNA-18a and microRNA-195 were highly expressed in breast cancer cells [55]. Tanic et al. used microRNA microarray and mRNA microarray and demonstrated that microRNA-146a, microRNA-99b, and microRNA-205 bind and regulate TRAF2 gene expression and NF-κB activity in breast cancer [56].

Recent multiple studies have shown that microRNA microarray analysis could be used to identify the specific microRNAs that are related to drug resistant in breast cancer. For example, Kastl et al. found that more than 200 microRNAs are altered in docetaxel-resistant cells [57]. Specifically, increased expression of microRNA-34a and microRNA-141 as well as decreased expression of microRNA-7, microRNA-16, microRNA-30a, microRNA-125a-5p and microRNA-126 are associated with docetaxel resistance in breast cancer [57]. Smeets and co-workers revealed 10 novel microRNAs suppressing lymph node invasion and one microRNA promoting lymph node invasion by microRNA microarray and gene expression profiling,

suggesting that deregulation of the microRNAs could be potentially responsible for lymph node invasion [58]. Notably, one study led by Caldas performed systematic comparisons of microarray profiling, real-time PCR, and next-generation sequencing technologies for identification of differential microRNA expressions [59]. They validated the 89 microRNAs from microRNA microarray by real-time RT-PCR and found the inconsistent results between these two methods, suggesting that microRNA microarray is currently not a "gold standard" assay, and further thorough studies are needed to improve this approach, or to synergize with other conventional approaches for more accurate predication/diagnosis [59].

18.3.4 Other Systems Biology Approaches

Recently, multiple new systems biology approaches have been established. One group led by Tsunoda developed an antibody proteomics system that facilitates the screening of biomarker proteins from many breast cancer patients by rapid preparation of cross-reacting antibodies using a phage antibody library technology [60]. Using this new technique, they validated that Eph receptor A10, TRAIL-R2 and cytokeratin 8 could be promising breast tumor biomarkers for drug development [60]. Additionally, DASL (cDNA-mediated annealing, selection, extension and ligation) has been developed as a high-throughput gene expression profiling system to generate reproducible data from degraded RNAs such as those extracted from breast formalin-fixed paraffin-embedded (FFPE) tumor samples [61, 62]. Additionally, Song et al. developed a dissociable antibody microarray (DAMA) staining technology that combines the protein microarrays with immunostaining methods [63]. This technique can detect the expression and subcellular location of hundreds of proteins in cultured cells and tissue samples at the same time. DAMA has been used to identify potential biomarkers for breast cancer. This group examined the expression profiles of 312 proteins, and identified 10 proteins that could be potential biomarkers for the diagnosis of breast cancer [63]. To understand the full meaning of the biology captured in molecular profiles, Chang et al. developed a new systems approach known as gene annotation tool to help explain relationships (GATHER) to integrate various forms of available data to elucidate molecular signatures from high-through post-genomic assays [64]. Due to the space limitation, many other systems biology approaches currently used or developed for breast cancer studies were not included for further discussion.

18.4 Conclusion

Systems biology approaches have impacts on understanding mechanisms of breast cancer over the last decade. DNA microarray and tissue microarray have provided the insights into identification of biomarkers for breast cancer. The microRNA microarray analysis has also been used to identify the specific microRNAs that are

related to development and progression of breast cancer. Moreover, several new techniques such as DASL, DAMA, and GATHER have been established to explore the molecular signatures in breast cancer. It is important to note that combination of different assays might be a better way to reveal the biomarkers in breast cancer. For example, DNA microarray-based gene expression profiling often need to be validated by tissue microarray. Lastly, we believe that more technologies will be developed and applied to breast cancer research in the near future, leading to important impact on translational breast cancer research.

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Chapter 19
Epigenetic Factors in Breast Cancer Progression

Samriddhi Shukla and Syed Musthapa Meeran

Abstract Breast carcinogenesis involves genetic and epigenetic mechanisms for its initiation and progression. These mechanisms include genetically driven mutational changes in the tumor suppressor genes or proto-oncogenes, and epigenetic modifications leading to transcriptional up- or down-regulation of key regulatory genes involved in breast cancer progression. While, the participation of the genetic constituents in the carcinogenesis process has been known for decades, the knowledge of involvement of epigenetic machinery in tumor initiation and promotion is comparatively newer, furthermore less explored. The major epigenetic modifications including DNA methylation, histone modifications and miRNA-mediated transcriptional silencing are crucial processes involved in the initiation and progression of breast carcinogenesis. This chapter describes the major epigenetic modifications involved in breast carcinogenesis and use of various epigenetic modulators in preclinical as well as in clinical trials against breast carcinogenesis.

Keywords Breast cancer · Epigenetics · DNA methylation · Histone modifications · miRNA · Breast carcinogenesis · Hypermethylation · Hypomethylation · Methylation-sensitive transcription factors · Histone deacetylases (HDACs) · Steroid hormone receptors · Selective estrogen receptor modulators (SERMs) · Selective estrogen receptor down-regulators (SERDs) · Aromatase · Breast cancer susceptibility gene 1 (*BRCA1*)

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19.1 Introduction

Carcinogenesis is a multi-step process which starts with tumor initiation and then advances with tumor progression, subsequently metastasizing to the distant body parts. The role of de novo as well as inherited mutations in carcinogenesis is well established. Mutational changes drive to cellular transformations by two important mechanisms, through inactivation of tumor suppressor genes and through conversion of proto-oncogenes into oncogenes. In addition to these genomic changes, there are many different epigenetic modifications which have the capability to regulate the process of gene expression without directly altering the DNA sequences. These epigenetic alterations include methylation of CpG islands in the promoter regions of genes, modifications of the histone tails leading to changes in chromatin structure as well as microRNAs (miRNAs), the short regulatory RNAs (20–30 nucleotides in length) which undergo sequence-specific binding to the 3' untranslated region (3'UTR) of their target genes and lead to their degradation or inhibition of translation.

The epigenetic machinery gets significantly altered in the process of breast carcinogenesis leading to drastically changed genetic expression profiles in cancerous cells. The altered expression patterns facilitate these cells to achieve selective advantage over the non-cancerous cells. Nearly all the cellular pathways for example those involved in cell growth, proliferation and survival are affected by these epigenetic alterations.

In this chapter on epigenetic factors in breast cancer progression, we are focusing on the basics of epigenetics, the major types of epigenetic modifications such as DNA methylation, histone modifications, and miRNA silencing in breast cancer progression along with the epigenetic targeting strategies against prevention and therapeutics of breast carcinogenesis.

19.2 Epigenetics of Breast Cancer

Breast cancer is the most frequently diagnosed cancer in females and the leading cause of cancer death among women globally. Although acquired genetic alterations in the important tumor suppressor genes such as *BRCA1* or proto-oncogenes such as *c-Myc* are considered as the driving forces for breast cancer initiation, epigenetic malfunction has also been proven to contribute equally in the process of breast carcinogenesis. Epigenetics is defined as the study of heritable changes in gene expression which occur without alterations in the underlying DNA sequences. Epigenetic mechanisms constitute a regulatory layer which coordinates all the crucial biological processes and alterations in the dynamics of these critical players is associated with a variety of human cancers, including breast cancer.

DNA methylation, histone modification and miRNA silencing are the three major epigenetic players of transcriptional regulation of gene expression. The first

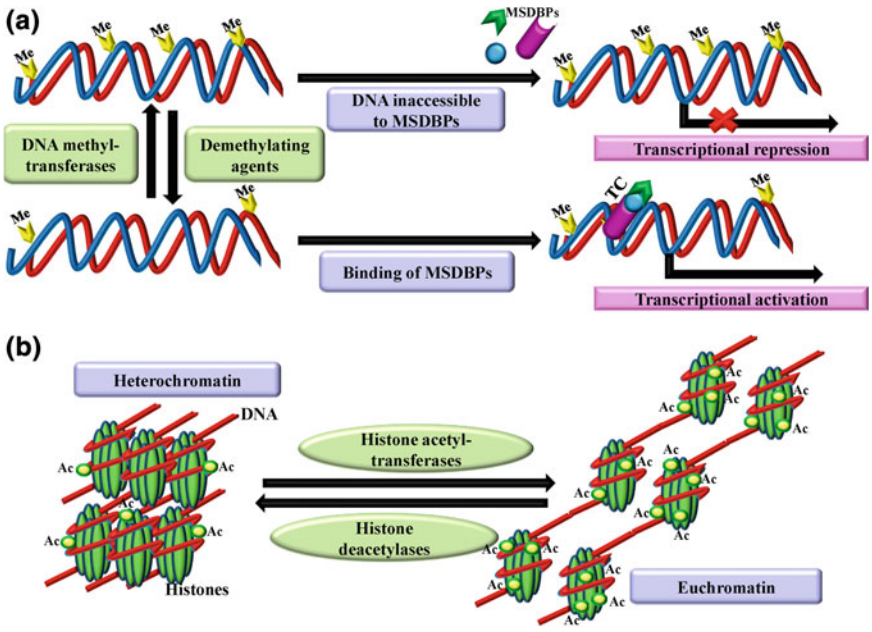

Fig. 19.1 Role of DNA methylation and Histone acetylation in transcriptional regulation. Schematic diagram illustrating the transcriptional changes associated with DNA and histone modifications. (**a**) The DNA methyltransferases add methyl groups to the cytosine residues of the CpG dinucleotides in the gene promoters which render the MSDBPs unable to bind to the promoter and transcriptional inactivation of genes takes place. The demethylating agents remove the methyl groups from the cytosine residues leading to binding of transcriptional complexes (TC) and the gene becomes transcriptionally active. (**b**) The histone acetyltransferases transfer the acetyl moieties to the histone tail and lead to unwinding of the DNA resulting in the transcriptional activation of the genes. The histone deacetylases counterbalance the process by removing the acetyl groups and lead to conversion of chromatin from an active (euchromatin) to inactive form (heterochromatin). Abbreviations: *Me* methyl groups; *Ac* acetyl groups; *MSDBPs* methylation sensitive DNA binding proteins; *TC* transcriptional complex

key player in epigenetic regulation machinery, DNA methylation, is the process of covalent addition of a methyl group to the fifth carbon of the cytosine nucleotide in the CpG dinucleotide sequences. The methylation is catalyzed by a group of enzymes known as the DNA methyltransferases (DNMTs) and the methyl group is donated by S-adenosyl methionine (SAM). The process of DNA methylation is involved in many important biological phenomenon such as genomic imprinting, X-chromosome inactivation as well as developmental processes. The genes with lower promoter methylation status are in general transcriptionally active, for example the housekeeping genes have lower level of promoter methylation, while those with higher promoter methylation are in general transcriptionally repressed as illustrated in Fig. 19.1a. In breast cancer, the aberrant methylation of numerous genes results in altered level of their expression. The retinoblastoma (Rb) gene was the first gene reported to be hypermethylated in cancer [1].

Histone tail modifications are the second most important players in transcriptional regulation. Histones are the basic proteins which constitute the nucleosome core around which DNA is tightly packaged to form chromatin. The amino-terminal tails of these histones protrude out from the nucleosomal core and are the subject of numerous bio-chemical modifications. Lysine residues are the most often modified amino acid residues with a variety of modifications including acetylation, methylation, ubiquitination and sumoylation. Other histone modifications include methylation and deimination of arginine, phosphorylation of serine or threonine, proline isomerization and ADP-ribosylation of glutamic acid residues. The modes of alteration in transcriptional regulation are specific to the type and position of the modified amino acid residues.

The various types of histone modifications are regulated by many different histone modifying enzymes including histone acetyltransferases (HATs) and histone deacetylases (HDACs), involved in lysine acetylation and deacetylation, respectively; histone lysine methyltransferases (KMTs) and histone lysine demethylases (KDMs) implicated in lysine methylation and demethylation, respectively; E3 ubiquitin ligase, ubiquitin protease and nuclear deubiquitinase involved in ubiquitination; E1 activating and E2 conjugating enzymes involved in histone lysine sumoylation; histone arginine methyltransferase and peptidyl arginine deiminase 4 involved in arginine methylation and deimination; different kinases and phosphatases which contribute to serine or threonine phosphorylation and dephosphorylation; proline peptidyl isomerase, an important enzyme for proline isomerization and poly (ADP-ribose) polymerase leading to ADP-ribosylation of glutamic acid. These enzymes write the histone code of cells predicting the expression profiles of genes. These modifications lead to either eu- or hetero-chromatin conformations and facilitate the accessibility or inaccessibilty of DNA to the transcription complexes. Fig. 19.1b shows the role of histone acetylation in transcriptional regulation.

Micro-RNAs (miRNAs) are small, non-coding, phylogenetically conserved RNAs which are regulatory in function. The third most important key players in post transcriptional regulation, miRNAs bind to 3'-untranslated region (3'UTR) of their target mRNA transcripts by perfect complementarity and lead to their degradation. They can function as tumor suppressors when they target proto-oncogenic transcripts or as oncogenes, targeting tumor suppressor gene transcripts. These regulatory RNAs are also involved in promoting epithelial to mesenchymal transition (EMT) and malignancy. Global down-regulation of miRNA expression is a very frequent event in breast carcinogenesis. Fig. 19.2 illustrates miRNA mediated silencing in breast carcinogenesis.

19.2.1 DNA Methylation in Breast Cancer

Approximately, more than half of the genes in the human genome have CpG islands in their promoters. Both normal cellular development and disease processes are regulated by patterns of methylation of CpG islands. Promoter methylation has dual

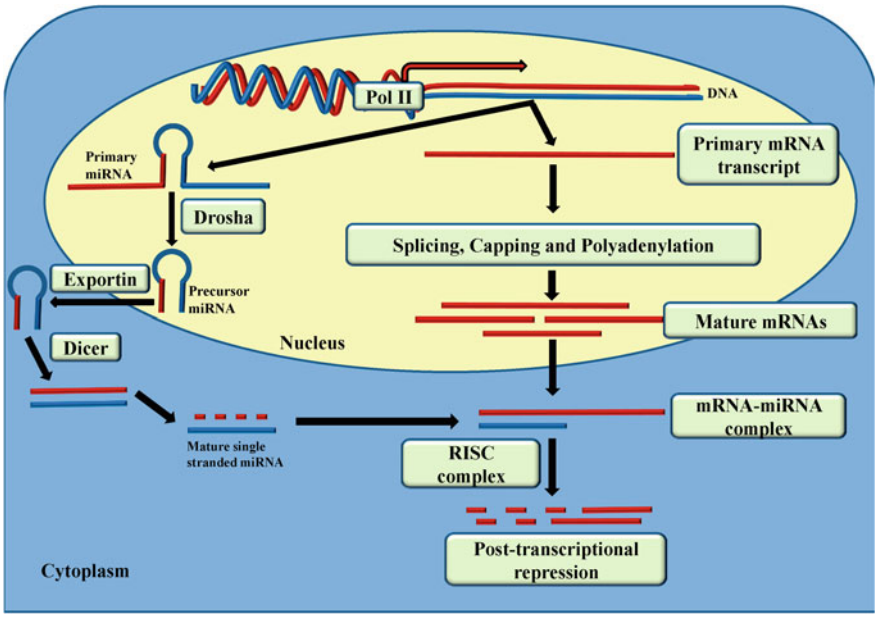

Fig. 19.2 miRNA-mediated transcriptional silencing. The process of gene transcription involves the synthesis of primary RNA transcripts which undergo post-transcriptional mRNA processing involving splicing, capping and polyadenylation. The mature mRNAs then enter the cytoplasm. The miRNA are synthesized as the primary miRNA transcripts which are cleaved by the nuclear enzyme drosha to form precursor miRNAs. These precursor miRNAs containing hair-pin loop then enter the cytoplasm, their entry being facilitated by a nuclear membrane protein exportin. In the cytoplasm, dicer cleaves the hair-pin loop leading to the formation of mature miRNA, which binds to the mRNAs containing region of sequence complementarity. The RNA-induced silencing complex (RISC) cleaves the double stranded mRNA-miRNA complex leading to post-transcriptional repression

roles in terms of transcriptional inactivation and chromatin remodelling. The methylation profiles of the breast cancer tissue and corresponding normal tissues are quite different. The local hypermethylation of CpG islands in the tumor suppressor gene promoters and global hypomethylation of the genome is a commonly observed paradox in case of all cancer types including breast cancer. The DNA methyltransferases (DNMTs) are the group of enzymes actively involved in cytosine methylation in the CpG islands. These enzymes are of two types depending on their target DNA. The DNMT1 is the maintenance methyltransferase which methylates the daughter DNA strands based on the pattern of methylation in parental strands. The other two DNA methyltransferases, DNMT3A and DNMT3B are de novo DNA methyltransferases which by themselves set up the patterns of methylation early in the development.

19.2.1.1 Hypermethylation in Breast Cancer

The active promoters are less methylated at their CpG islands and are considered open to the transcription factors and other transcriptional co-activators. The breast cancer cells achieve substantially altered methylation profiles in the process of carcinogenesis. Analysis of DNA methylation has been advanced to an enormous degree by genome-wide high throughput sequencing approaches, which allow quantitative and cost-effective analysis of the target genome for methylation studies. A genome-wide high-throughput methylation enriched immunoprecipitated-DNA (MeDIP-DNA) sequencing based study confirmed a global promoter hypomethylation at the genomic level and a cell-type specific regulation of gene methylation at the gene level in eight different breast cancer cell lines [2]. The major genes which are reported to be hypermethylated in breast carcinogenesis are regulatory genes coding for hormonal receptors (*ERα, ERβ, PR*), genes regulating cell cycle progression ($p16^{INK4A}$, *CCND2,* $p57^{KIP2}$), growth inhibitory genes (*TGFβ, RASSF1A, SOCS1, RARβ, SYK, HIN-1, NES1*), genes involved in DNA repair mechanisms (*BRCA1, MGMT, GSTP1* etc.), pro-apoptotic genes (*DAPK, FHIT, Twist, HOXA5, TMS1, GPC3*), genes regulating angiogenesis (*MASPIN, THBS1*), genes involved in cell to cell and cell to matrix interactions and cytoskeletal changes (*E-Cadherin, CDH13, APC, prostasin, TIMP-3, BCSG1*) [3, 4].

$p16^{INK4A}$ hypermethylation has been clearly associated with the immortalization potential of the normal human mammary epithelial cells (HMECs). The HMEC cells having hypermethylation at $p16^{INK4A}$ promoter have demonstrated to bypass senescence barrier known as stasis which is mediated by Rb and another p53 involving telomere-length-dependent pathway. When these cells overcome another barrier known as agonescence or crisis, they undergo immortalization. The hypermethylation profiles of these immortalized HMECs for a cluster of 30 genes known as 'cancer proliferation cluster' were found to be remarkably similar to the methylation profiles of the non-invasive breast cancers [5]. The methylation status of $p16^{INK4A}$ was also found to be significantly associated with frequencies of promoter methylation of *BRCA1, BRCA2, ERα,* and *RARβ2*. Thus, the hypermethylation of $p16^{INK4A}$ gene is indicative of global pattern of epigenetic modifications at the time of early breast carcinogenesis [6]. The methylation profiling of women at high breast cancer risk without having *BRCA1* and *BRCA2* mutations have demonstrated higher frequencies of promoter methylation of the several tumor suppressor genes such as *RARβ, ER,* $p16^{INK4A}$, *BRCA1, PR-A, PR-B, RASSF1A, HIN-1* and *CRBP1* in periareolar fine-needle aspirate samples [7].

Hypermethylation of promoters in *RARβ2, APC* and *RASSF1A* have been considered as early events in breast carcinogenesis. More specifically, *RASSF1A* is the marker for breast cancer as well as benign breast susceptibility, while *RARβ2* methylation is indicative of personal history of breast cancer. *APC* promoter hypermethylation correlated inversely with the parity of women. Parity has been associated with the decreased risk of breast cancer [8, 9].

The incidence of hypermethylation at certain key breast cancer regulatory gene promoters is an early biomarker to detect breast carcinogenesis. This

hypermethylation can also be used as a marker of pre-invasive and invasive breast cancer [4, 10]. In contrast, a genome-wide analysis showed that the presence of a breast CpG island methylator phenotype (B-CIMP), having higher proportion of genes hypermethylated, can predict the possibility of metastasis or survival, where higher methylation was associated with lower risk of breast cancer metastasis [11]. Quantitative multiplex methylation specific PCR (QM-MSP) is a high-throughput, very sensitive DNA methylation detection method which is used to quantitate cumulative gene promoter hypermethylation in samples with very limited content of DNA.

Promoter methylation of genes encoding for the regulatory miRNAs is another mechanism involved in deregulation of cellular activities observed in case of breast carcinogenesis. The level of miRNA expression is the measure of aggressiveness of the breast tumor. Hypermethylation of genes for tumor suppressor microRNAs such as *let-7* family*, miR-206, miR-17-5p, miR-125a, miR-125b, miR-200, miR-34,* and *miR-31*, have been shown to be a common event in breast tumors [12, 13]. Another human micro RNA, miR-335 involved in tumor recurrence in breast cancer, was reported to be inactivated through dual mechanisms, deletion and promoter hypermethylation [14].

19.2.1.2 Hypomethylation in Breast Cancer

In normal cells, hypermethylation of repetitive DNA sequence elements is a universal regulatory mechanism leading to silencing of these elements to circumvent the chromosomal rearrangements such as translocations and gene disruptions through insertions of reactivated transposable elements [15, 16]. Cancerous cells have globally hypomethylated genome which leads to chromosomal aberrations, loss of genomic imprinting, activation of repetitive elements and chromosomal instability due to increased mutational events [17]. Promoter hypomethylation of numerous proto-oncogenes leading to uncontrolled proliferation and metastasis (genes for *synuclein γ* and *urokinase*) and development of drug resistance (genes encoding *N-cadherin*, *ID4*, *β-catenin*, *annexin A4* and *WNT11*) is the other major epigenetic mechanism of breast carcinogenesis [17]. Even the early cancerous lesions display widespread global hypomethylation patterns and patterns of hypomethylation of CpG sequences in satellite DNA, suggesting it to be an early event in breast carcinogenesis [18]. Analysis of three repetitive DNA elements, long interspersed repetitive DNA elements (*LINE1*), short interspersed repetitive DNA elements (*Alu*) and satellite DNA elements (*Sat2*) lead to the conclusion that their methylation level is significantly lower to those of adjacent normal breast tissue [19]. *BRCA1* mutations have been proved to be associated with aberrant regulation of DNMTs leading to global hypomethylation and increased expression of various proto-oncogenes such as *c-Fos, Ha-Ras* and *c-Myc* [20].

19.2.1.3 Methylation-Sensitive Transcription Factors in Breast Cancer

The mechanism of transcriptional repression by hypermethylation of CpG islands in gene promoters may function in two different ways. The first mechanism may be by creating hindrance in binding of methylation-sensitive transcription factors to the gene promoters leading to transcriptional repression [21]. The other mechanism may be by recruiting the methylation-dependent transcriptional repressors to the hypermethylated CpG islands, which subsequently recruit the HDACs. The HDACs in turn deacetylate the histone proteins leading to conversion of euchromatin into heterochromatin. The members of a family of nuclear proteins, methyl-CpG-binding domain proteins (MBDs) which comprises of MBD1, MBD2, MBD3, MBD4 and MeCP2 are capable of binding specifically to methylated DNA (with the exception of MBD3). These nuclear proteins are involved in DNA methylation mediated transcriptional repression. MBD3 lacks a functional methylated DNA binding domain but is an integral subunit of the histone deacetylase Mi_2-NuRD complex recruited by MBD2 [22, 23]. The MBD2 expression is found to be involved in repression of tumor-promoter gene human telomerase reverse transcriptase (*hTERT)* in breast as well as in other cancer cell lines [24]. MIRA-assisted microarray profiling of global DNA methylation patterns in ductal carcinoma led to the conclusion that hypermethylation of the transcription factors encoded by homeobox genes is also a very common event leading to their silencing which suggests a critical role of homeobox gene methylation in breast carcinogenesis [25].

19.2.2 Histone Modifications in Breast Cancer

Among all the earlier discussed histone modifications, histone acetylation and methylation are relatively stable and are considered to be potential marks of the epigenetic modification carried over through multiple cell division cycles. The histone modifications have the tendency to open-up the chromatin or leading to its compaction, giving rise to 'open' or 'closed' conformations of chromatin. The chromatin in an open organization makes the DNA thread approachable to transcription factors and co-activators and such chromatin is transcriptionally active, called as euchromatin. The compacted state of chromatin is transcriptionally inactive and is known as heterochromatin. In general, histone acetylation at lysines (symbolized by K) H3K5ac, H3K8ac, H3K9ac, H3K12ac, H3K18ac and H4K16ac as well as H3K4me and arginine dimethylation H4R3me2 are considered as the markers of euchromatin [26–29]. The heterochromatin histone markers include mono (me)-, di (me2)-, or tri (me3)-methylated histone H3 at lysine 9 (H3K9me, H3K9me2 and H3K9me3), H3K27 (H3K27me, H3K27me2 and H3K27me3), and H4K20 (H4K20me, H4K20me2 and H4K20me3) which initiate and maintain the heterochromatin state [17, 26, 30, 31]. Mutations of p300 and its co-activator *CBP* lead to aberrant histone acetylation patterns in many cancer types including breast

cancer [32]. Aberrant expression of other histone methyltransferases may also be implicated in breast cancer. The HDACs counterbalance the actions of HATs by deacetylating the lysine moieties leading to the compaction of chromatin. These enzymes are divided into four types based on their homologies to the yeast counterparts, cellular localization and acetylation activities.

Class I HDACs include HDACs-1, 2, 3, and 8, class II HDACs have two subclasses: class IIA having HDACs-4, 5, 7 and 9; class IIB including HDACs-6 and 10. The class III HDACs include seven HDACs namely sirtuin 1-7 and class IV HDAC has a single member HDAC11. Class I HDACs (except for HDAC8) have been found to be up-regulated in breast cancer. A tumor suppressor gene, *ARH1*, is repressed through multiple histone deacetylases in breast cancer [33]. The inhibition of class II HDACs have effects on cell cycle progression, apoptosis, gene expression and estrogen receptor signalling in breast tumor cells [34]. Overexpression of HDACs-1, 6 and 8 is implicated in breast cancer invasion and also in the expression of matrix metalloproteinase-9 (MMP-9) [35]. The regulation of transcription by methylation of lysine moieties is dependent of location of that particular lysine and the degree of methylation. Histone methyltransferases (HMTs) are the enzymes responsible for this histone methylation process. Over expression of EZH2, a type of HMT has been associated with the breast cancer invasion and progression [36]. This protein works in association with HDACs and KMTs leading to transcriptional repression of associated genes [37]. Mono-, di- and tri-methylations on the histone H3K27 are the histone modifications which generally induce transcriptional repression and thus are regulatory in controlling gene expression patterns. Arginine methylation at the histone H3R2 by the enzyme PRMT6 antagonizes the binding of histone remodelling enzymes and transcriptional co-activators and thus leads to the transcriptional repression [38]. The tissue microarray of 880 breast tumor cases and normal breast tissues have shown a higher levels of acetylation at H3K18 while lower H4K16 acetylation in the breast tumors. Assessment of the relationship between histone modifications and patient outcome showed that, in the whole cohort, low-level detection of histone modifications was associated with adverse patient outcome. The histone modifications H3K4me2, H4K16ac and H4K20me3 in normal breast acini were reported to be higher than corresponding cancerous tissues. Longer disease free survival was reported in women with high levels of histone modifications such as H3K18ac, H4R3me2 and H3K9ac. In poorer prognostic subtypes of breast cancers, including basal carcinomas and HER2-positive tumors, moderate to low levels of lysine acetylation (H3K9ac, H3K18ac and H4K12ac), lysine methylation (H3K4me2 and H4K20me3), and arginine methylation (H4R3me2) were observed, which indicates the involvement of these histone markers in repression of transcription of the tumor suppressor genes [39].

19.2.3 miRNA Silencing in Breast Cancer

In 2005, altered miRNA expression in case of human breast cancer was first reported [40]. miRNAs interact with a variety of genes involved in different cellular pathways such as genes involved in cell division and cell cycle regulation. The silencing of miRNAs may take place either through hypermethylation of promoter sequences of genes coding for them, or through copy number variations as demonstrated by a study that 73 % of miRNA encoding genes are present in the chromosomal regions which are either frequently deleted or amplified in breast cancer [41]. Significant changes in the level of expression of the enzymes involved in miRNA processing (Dicer and AGO1) have been observed during breast carcinogenesis. The alternative mechanism of miRNA deregulation may be the alterations in the miRNA processing machinery [42].

Aberrant expression of miRNAs in human breast cancers is a frequent biological event [43]. miR-21 is an oncogenic miRNA which targets multiple tumor suppressor genes involved in p53 suppression pathway. This miRNA also promotes breast cancer invasion and metastasis [44]. miR-27a is a breast cancer oncogenic miRNA which down regulates expression of cell cycle inhibitors, leading to unregulated cell proliferation [45]. Up-regulation of miR-10b has been proven to be involved in breast cancer invasion and metastasis by targeting the *HOXD10* mRNA [46]. Another group of researchers proved that miR-373 and miR-520c help in cancer cell migration and invasion, working as metastasis-promoting miRNAs through a CD44 suppression mechanism [47]. Conversely, some miRNAs (miR-335, miR-126, and miR-206) have been found to be human breast cancer metastasis-suppressor miRNAs. miR-335 functions in metastasis repression by targeting mRNA transcripts of a transcription factor SOX4 [48].

Five known groups of miRNAs are found to be the direct regulators of cell cycle progression. Let-7 is the most well studied family of miRNAs, which is involved in the targeting of *Ras, HMGA2,* and *caspase-3* genes [49–51]. Many important cell cycle regulatory genes are repressed by let-7 such as *cyclin D1, cyclin D3, cyclin A, CDK4*, *CCNA2, CDC25A, CDK6* and *CDK8* [50, 52].

19.3 Epigenetic Targets and Therapeutic Strategies in Breast Cancer Treatment

19.3.1 Role of DNA Methyltransferases in Breast Cancer

Epigenetic targeting of DNMTs to reverse the down-regulation of methylation-silenced tumor suppressor genes is a promising approach for breast cancer prevention. The *DNMT1* was found to be over-expressed in pre-invasive breast tumors when compared with the normal breast tissue [53]. DNMT1 inhibition by ASO98 in MDA-MB-231 cells lead to re-expression of estrogen receptor α *(ERα)*

mRNA in these cells with other methylation silenced genes such as *PGR, RARα* and *cyclinD1* [54]. Inhibition of DNMT1 has also been shown to inhibit anchorage-independent growth and tendency to invade, but on the contrary combined inhibition of DNMTs leads to activation of pro-metastatic genes leading to increased invasiveness [55]. Over-expression of DNMT3B was found to be a frequent event in breast carcinogenesis [56]. Inhibition of these enzymes to reactivate methylation silenced tumor suppressor genes as well as to inhibit the tumor promoter genes is currently the main therapeutic approach against breast cancer.

19.3.1.1 Nucleoside Analogue DNMT Inhibitors

A well known DNMT inhibitor, 5-azacytidine (AZC) is a ribonucleoside analogue and binds to RNA and DNA. It acts by inhibiting mRNA translation and by inhibiting DNA methylation by trapping DNMTs. At higher concentrations, this drug forms high levels of enzyme–DNA adducts. Another nucleoside analogue, 5-aza-2'-deoxycytidine, is a deoxyribonucleotide analogue which does not bind to RNA. This nucleoside analogue inhibits DNA methylation by the same mechanism as that of 5-azacytidine. Both these compounds are FDA approved for the treatment of myelodysplastic syndrome. 5-fluoro-2'-deoxycytidine is also a deoxyribonucleotide analogue of 5-azacytidine which induces DNA hypomethylation and cellular differentiation. Zebularine is another deoxyribonucleotide analogue, functioning in the similar way as other deoxyribonucleotide analogues [57].

19.3.1.2 Non-Nucleoside Analogue DNMT Inhibitors

Epigallocatechin-3-gallate (EGCG) is a major and most effective constituent of green tea polyphenols and is involved in direct inhibition of DNMT by forming hydrogen bonds to hinder the entry of cytosine into its active site [58]. It also leads to decrease in the level of available S-adenosyl-L-methionine (SAM) and an increase in S-adenosyl-L-homocysteine (SAH) and homocysteine levels, thereby providing evidence of an indirect inhibition of DNA methylation [59]. MG98 is an anti-sense oligonucleotide which highly specifically targets the 3'UTR of *DNMT1* causing decreased DNA methylation [60]. Hydralazine is a compound which binds to the DNMTs in a similar manner as of 2'-deoxycytidine, 5-azacytidine, and 5-aza-2'-deoxycytidine as predicted by molecular docking experiments [61]. Procainamide is a non-nucleoside analogue which reduces affinity of DNMT1 for both DNA and S-adenosyl-methionine (SAM) causing a decrease in DNA methylation [62]. The structures of some of the nucleoside and non-nucleoside analogues having DNMT inhibitory activity are given in Fig. 19.3.

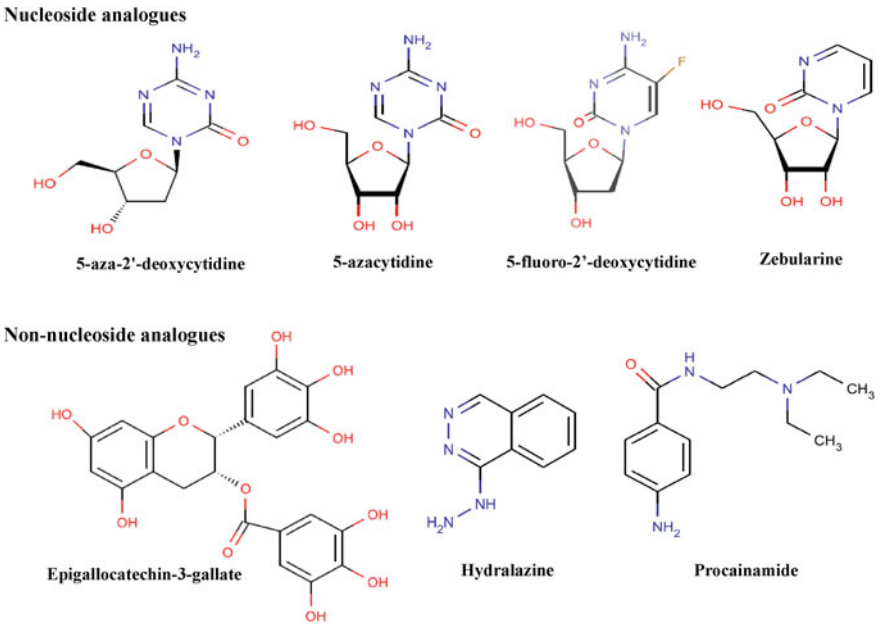

Fig. 19.3 Different nucleoside analogue and non-nucleoside analogue DNMT inhibitors

19.3.2 Role of Histone Deacetylases in Breast Cancer

Aberrant expression of histone deacetylases (HDACs) is observed in the process of breast carcinogenesis leading to the silencing of different types of tumor suppressor genes such as the genes involved in cell cycle regulation and apoptosis. The histone deacetylases, HDAC1 and HDAC6 were found to be over-expressed in breast tumor tissues [63, 64]. Inhibition of HDACs may lead to activation of silenced genes through two important mechanisms. The HDAC inhibitors can either help in the opening up of the chromatin by causing the accumulation of hyper-acetylated histones H3 and H4 or they can alter the nuclear DNMT dynamics to prevent the hypermethylation of the tumor suppressor genes. Cellular inhibition of *SIRT1* using RNAi constructs in breast cancer MDA-MB-231 and MCF-7 cell lines had revealed the re-expression of tumor suppressor genes despite full retention of hyper-methylation of the promoters [65]. HDACs inhibition was reported to cause degradation of DNMT1 protein through an ubiquitin-dependent proteasomal degradation mechanism [66]. The following types of histone deacetylase inhibitors have been tested for breast cancer treatment and some of them have proved to be successful in reactivation of the silenced tumor suppressor genes and suppression of proto-oncogenes in breast cancer.

19.3.2.1 Short Chain Fatty Acids

Butyrate is a short chain fatty acid which causes hyper-acetylation of H3 and H4. Valproic acid (VPA) inhibits HDAC activity and relieves HDAC-dependent transcriptional repression. It has an added benefit of anti-tumor effects on both estrogen-sensitive and estrogen-insensitive breast cancer cells [67, 68]. Valproic acid in combination with 5-flurouracil is undergoing phase II clinical trials.

19.3.2.2 Hydroxamic Acids

Trichostatin A (TSA) is a HDAC inhibitor which acts by enhancing acetylation as well as the stability of the ERα and p300 protein. TSA synergizes with the demethylating agent 5-aza-2'-deoxycytidine in the re-expression of ER-α gene in breast cancer cells [69]. Suberoylanilide hydroxamic acid (SAHA) is another hydroxamic acid which inhibits both the class I and class II HDAC enzymes and found to be effective in breast cancer patients with amplification of Her2/neu. This is an FDA approved drug for the treatment of cutaneous T cell lymphoma patients.

19.3.2.3 Cyclic Tetrapeptides

Trapoxin (TPX) binds covalently to the histone deacetylases through the epoxide moiety and thus inhibits histone deacetylation irreversibly. TPX is an inhibitor of the cell cycle in eukaryotes and it also reverses the morphological changes in transformed cells [70]. Depsipeptide (FK228, FR901228), a bicyclic bacterial product, is a pro-drug which is activated by the action of glutathione and yields two free sulfhydryl groups which are capable of chelating the zinc in the HDAC active site. This compound exhibits a stronger direct inhibition of class I HDACs in comparison with class II HDACs [71, 72]. Apicidin [cyclo(N–O-methyl-l-tryptophanyl-l-isoleucinyl-d-pipecolinyl-l-2-amino-8-oxodecanoyl)] has been shown to increases the levels of acetylated histone H3 and H4 through inhibition of histone deacetylases in H-Ras-transformed human breast epithelial (MCF10A-Ras) and non-transformed mammary epithelial (MCF10A) cell lines [73].

19.3.2.4 Benzamides

Entinostat (MS-27-275), an active benzamide derivative, causes hyper-acetylation of nuclear histones H3 and H4 by selectively inhibiting class I HDAC enzymes. This drug also sensitizes TRAIL-resistant breast cancerous cells to radiation treatment [74]. CI-994 (N-acetyl-dinaline), a novel oral histone deacetylase inhibitor, is another substituted benzamide derivative which leads to accumulation of acetylated histones possibly in an indirect manner [75].

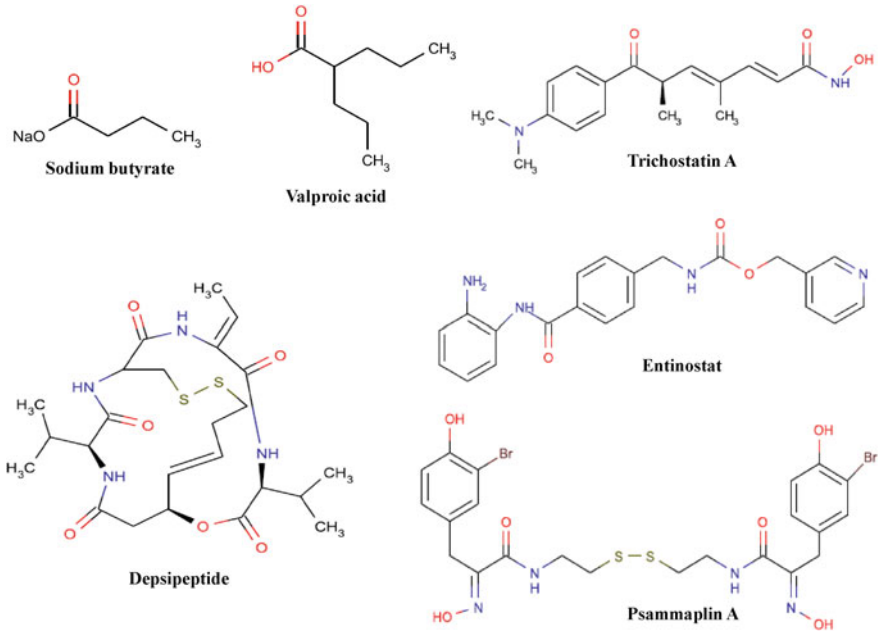

Fig. 19.4 Different types of HDAC inhibitors in breast cancer treatment

19.3.2.5 Psammaplins

Psammaplins are marine metabolites which were previously considered to be having dual DNMT and HDAC inhibition activity. Recent studies demonstrated that psammaplin A can efficiently inhibit bacterial DNA methyltransferase, but not the human DNMT enzyme. But these compounds can efficiently inhibit histone deacetylation activity in breast (MCF7), lung (A549), and normal human lung fibroblast (WI-38) cell lines [76]. Figure 19.4 depicts structures of some of the compounds with HDAC inhibitory activity.

19.3.3 Role of Steroid Hormone Receptors in Breast Cancer

The two estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ) are the nuclear hormone receptors. In unstimulated state, the estrogen receptors (ER) reside in cytoplasm bound to molecular chaperones. There are two modes of estrogen receptor activation, ligand-dependent, where ER is activated by estrogen binding and ligand-independent, which is the alternative mode of activation through growth factor signalling by phosphorylation of the receptor. In a classical ER response mechanism, after dimerization, ER binds to the estrogen response elements (EREs) present in the promoter regions of certain genes and mediates cellular proliferation as depicted in Fig. 19.5.

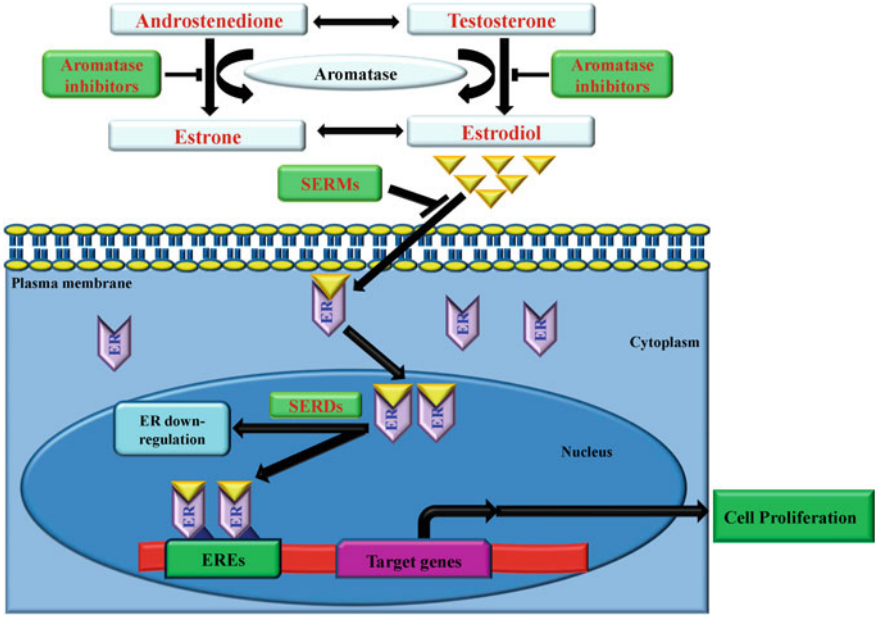

Fig. 19.5 Classical estrogen response pathway of breast cancer proliferation. Estrogens (estrone and estradiol) are synthesized by the enzyme aromatase from the precursors, androstenedione and testosterone. The steroid hormone estrogen binds with the estogen receptors (ER) present in the cytoplasm and this estrogen-bound ER then enters the nucleus. After dimerization, the complex binds to the promoter of the genes containing estrogen responsive element (ERE) and leads to their transcriptional activation ultimately leading to cellular proliferation. The selective estrogen receptor modulators (SERMs) compete with the binding of estrogen to the ER while the selective estrogen receptor down regulators (SERDs) function in the down regulation of ERs

ER has also been shown to regulate transcription of various key regulatory genes by non-classical pathway through binding with the non-estrogen responsive element sites such as activator protein (AP1) and specificity protein 1 (SP1) on the promoter regions of other transcription factors such as Jun and Fos. In such cases, ER functions as a co-regulatory protein (Fig. 19.6). Another non-genomic function of ER has been shown to be the activation of other growth factor receptors by some still unknown mechanism. Due to these three different modes of actions, ER is proven to be important for breast cancer progression and thus targeting of this gene may prove fruitful in the breast cancer therapy [77].

Expression of progesterone receptor (PR) is dependent on the expression of *ERα* in breast cancer cells. Previously, the gene coding for PR was known to be involved in inhibiting the proliferation of the uterine endometrium. Due to this inhibitory role, this gene was thought to be of lesser importance in breast carcinogenesis. However, later studies proved that progesterone helps in proliferation of breast cells in a mechanism independent of estrogen, and thus it is associated with breast cancer risk. Progesterone primarily induces cellular proliferation in a paracrine fashion [78]. Paracrine progesterone secretion-induced development of

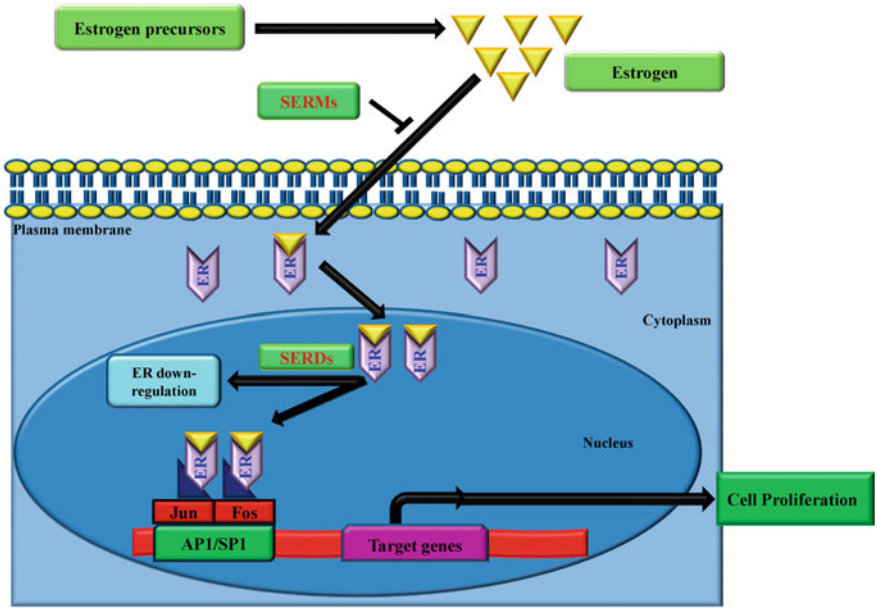

Fig. 19.6 Non-classical estrogen response pathway of breast cancer proliferation. The steroid hormone estrogen binds with the estrogen receptors (ER) present in the cytoplasm and this estrogen-bound ER then enters the nucleus. After dimerization, estrogen-bound ER promotes transcriptional activation by binding to the non-estrogen responsive element sites such as AP1 and SP1 on the promoters of other transcriptional factors such as *Jun* and *Fos*. Thus, estrogen may also function as a transcriptional co-activator leading to the transcriptional activation of genes associated with cellular proliferation. The selective estrogen receptor modulators (SERMs) compete with the binding of estrogen to the ER, while the selective estrogen receptor down regulators (SERDs) function in the down regulation of ERs

alveoli is mediated by receptor activator for nuclear factor-κB ligand (*RANKL*)-mediated downstream signalling in PR-negative breast cancer cells. Progesterone receptor also affects proliferation through *CCND1*-dependent mechanism in PR-positive breast cancer cells in autonomous manner [79]. This dual role of progesterone in cell proliferation in both the *PR*-positive and *PR*-negative cancer types makes it a strong candidate for epigenetic targeting. The treatment of DNMT and HDAC inhibitor combination (5-azacytidine and Trichostatin A) lead to re-expression of *PR* gene in *PR*- negative breast cancer cell lines [80]. Inhibition of either the estrogen receptors or alternative inhibition of estrogen synthesis is an important strategy for treatment of hormonal receptor positive breast cancer.

19.3.3.1 Selective Estrogen Receptor Modulators

These are small synthetic molecules which compete with estrogen for binding to the ER and can exert different effects on the estrogen target genes. The typical example of this category is tamoxifen, which functions by acting as a competitive

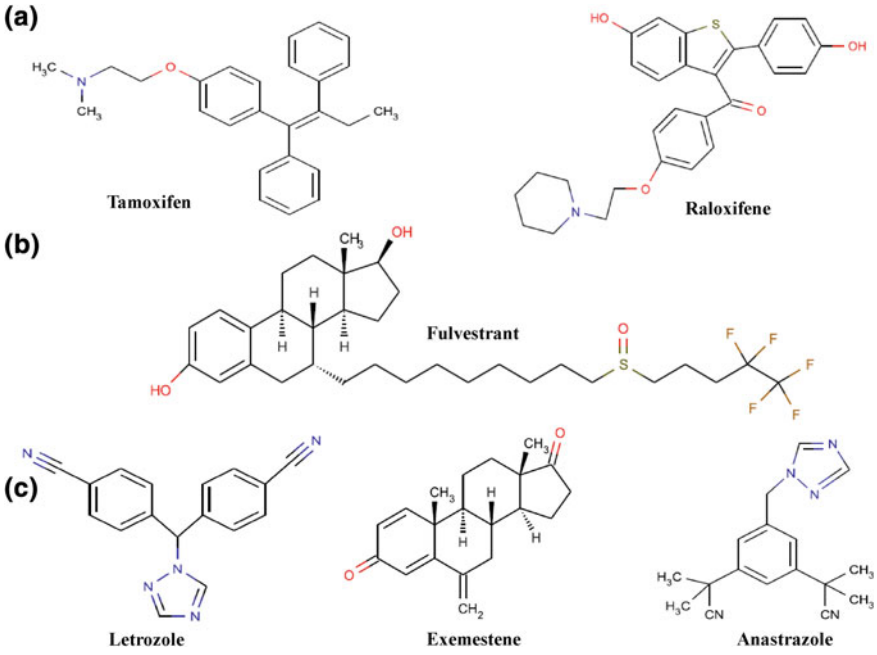

Fig. 19.7 Different types of estrogen receptormodulators, downregulators and aromatase inhibitors in breast cancer treatment

inhibitor of estrogen to show agonistic or antagonistic effects on the estrogen-related transcriptional patterns in breast cancer cells to inhibit cancer cell proliferation. The tamoxifen bound ER can dimerize and bind to the DNA, but the downstream effects are different. For example, the receptor bound balance of co-activators and co-repressors differs for estrogen-bound and tamoxifen-bound estrogen receptors. This results in different effects on different target genes. Tamoxifen-bound ER may block AF-2 mediated gene transcription but AF-1 mediated transcription remains unaffected [81]. Later on, to improve the agonist/antagonist profile, tamoxifen like triphenyethylene SERMs were developed. Toremiphene, a chlorinated analogue of tamoxifen, has no significant difference in efficacy or binding affinity to the former [82]. Droloxifene (3-hydroxytamoxifen) was shown to have a 10-fold higher binding affinity for ER relative to tamoxifen [83]. Idoxifene was another tamoxifen analogue which was later on found inefficacious. The fixed ring selective estrogen receptor modulators were developed as the second or third generation of SERMs. This class of SERMs include raloxifene, arzoxifene, acolbifene, ERA-923 etc. They also function in similar way to tamoxifen but have comparatively lesser side effects. Fig. 19.7a shows the structures of some of the available SERMs.

19.3.3.2 Selective Estrogen Receptor Down-Regulators

These pure anti-estrogens induce destabilization and degradation of estrogen receptor and have no agonistic activity. They function by sterically hindering the dimerization of the ER leading to increased ER turnover and disruption of nuclear delocalization leading to lesser ER molecules in cells [84]. The typical example of this class is the steroid fulvestrant, which has been approved for the treatment of metastatic breast cancer (Fig. 19.7b). Fulvestrant-mediated destabilization of the ERs is dependent on pattern of ubiquitination of the protein [85]. Other examples of SERDs include IC182780 and ZK-703. Use of SERDs has been proved to be the most effective in the treatment of *ER*-positive tamoxifen-resistant breast cancers.

19.3.4 Role of Aromatase in Breast Cancer

Aromatase is an enzyme involved in synthesis of estrogens, estrone and estradiol from androgenic substrates androstenedione and testosterone respectively [86]. The enzyme is encoded by a *CYP19A1* gene and the process of transcription of this gene is regulated by tissue specific promoters. Majority of breast cancer tumors show higher levels of aromatase activity than corresponding non-malignant mammary tissues. Inhibition of aromatase is a well-known therapeutic strategy to reduce estrogen expression for the treatment of estrogen receptor-positive breast cancer in post-menopausal women. But, nonspecific aromatase inhibitors (AIs) lead to reduction of aromatase activity in other tissues such as bone, brain and adipose tissue. Due to this non-specificity, they are associated with undesirable side effects such as bone loss and abnormal lipid metabolism. Breast tumor-specific inhibition of aromatase can prove to be a far better approach in this regard.

19.3.4.1 Aromatase Inhibitors

There are two types of aromatase inhibitors including irreversible steroidal activators and reversible non-steroidal imidazole-based inhibitors. Steroidal agents are irreversible inhibitors of aromatase, which inhibit aromatase by competing with the physiological aromatase substrates (androstenedione and testosterone). The non-steroidal inhibitors reversibly interact with the cytochrome P450 moiety of the enzyme and thereby inhibit enzymatic activity. Examples of this type of aromatase inhibitors are letrozole, anastrozole, exemestane [87]. The structures of some of the aromatase inhibitors are given in Fig. 19.7c.

The first generation aromatase inhibitor AG3 which was used for breast cancer treatment for more than one decade, showed potential side effects. It was then withdrawn from the market. Then, the second generation steroidal aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA, formestane) had a limitation of pharmacological availability. The third generation aromatase inhibitors are being

used as adjuvant therapy in place of tamoxifen, the ER inhibitor in the treatment of post menopausal breast cancer, because they are well-tolerated and are more effective. Exemestane (6-methylenandrosta-l,4-diene-3,17-dione) is a orally administered third generation steroidal aromatase inhibitor available in the market [88].

19.3.5 Role of Breast Cancer Susceptibility Genes in Breast Cancer

Although mutations of the breast cancer susceptibility gene 1 (*BRCA1*) are well known to be involved in a majority of breast cancers, the methylation status of the proximal portion of *BRCA1* promoter is also highly influential in deciding the *BRCA1* expression level in the breast cancer cells. Hypermethylation of the *BRCA1* promoter significantly increases in the breast cancer cells in comparison to the normal tissues [89]. *BRCA1* product, a multifunctional protein is involved in DNA repair, cell cycle check point control and chromatin remodelling as well as protein ubiquitination. It also interacts with a variety of proteins involved in epigenetic modifications such as histone deacetylases, and different components of chromatin remodelling complexes. Hypermethylation of *BRCA1* promoter may also function as the second hit to silence the expression of this gene in a subset of *BRCA1* mutation carrier women [90]. Higher levels of *BRCA1* promoter methylation were also correlated with advanced breast cancer stages and increased mortality [91].

Mutations in the other breast cancer susceptibility gene *BRCA2* are also implicated in the development of some sporadic breast cancers. This gene shows transcriptional activation either through the presence of HAT activity or through its association with transcriptional activator p300/CBP-associated factor (P/CAF). This gene was found to be over-expressed in breast cancer [92]. *BRCA2* was found to be hypermethylated in approximately 70 % of the patients [93]. The breast cancer patients defective in *BRCA1* and *BRCA2* genes depend on an alternate DNA repair process mediated by poly (ADP-ribose) polymerase 1 (PARP1). Thus, tumors deficient in *BRCA1* gene can be well targeted by PARP1 inhibitors leading to lack of DNA repair and enhanced cell death. The examples of PARP1 inhibitors include nicotinamide analogs (first generation), benzamide analogs (second generation) and 3-aminobenzamide analogs (third generation *PARP1* inhibitors). AG014699, veliparib (ABT-888), olaparib and iniparib are the *PARP1* inhibitors which have been undergoing the different stages of clinical trials [94].

19.4 Conclusion

The process of breast carcinogenesis is well-orchestrated by genetic and epigenetic mechanisms and therapeutic strategies targeting both these processes at the same time may prove to be a better approach for effective breast cancer treatment. The epigenetic mechanisms mainly DNA methylation and histone modification have been shown to play key regulatory role in breast cancer initiation and progression. Silencing of important tumor suppressor genes and activation of proto-oncogenes into oncogenes by epigenetic modifications facilitates the mammary cells to acquire transformed phenotype. Methylation status of frequently methylated genes such as *RASSF1* and *BRCA1* in breast cells has proven to be important early biomarker to detect breast cancer susceptibility. Further, genome-wide methylation profiling would add an extra mile to identify other important early biomarkers to detect the risk of developing breast cancers in highly susceptible women. Targeting one or more epigenetic modifications such as DNA methylation, histone modifications and miRNA-mediated silencing is currently growing therapeutic approach against breast carcinogenesis. Many different types of anti-cancerous compounds with epigenetic mechanism of action are being used in breast cancer therapy and a variety of new compounds are also being investigated in clinical trials. However, detailed understanding of these epigenetic modifications with respect to their part in breast carcinogenesis might lead to the significant advancement in the prevention and treatment strategies against breast cancer.

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Chapter 20
Breast Cancer Stem Cells and miRNAs

Saurabh Singh

Abstract Breast cancer remains one of the leading causes of cancer-related deaths in women. Following the initial diagnosis and treatment, a significant number of patients suffer eventual relapse characterized by chemoresistant form of the disease and poor prognosis. For this reason, there is an urgent need to discover new disease targets for successful therapy outcomes. Breast cancer stem cells (bCSCs) are a niche population that is chemoresistant, possess self-renewal capacity and contribute to malignant disease and poor clinical outcomes. In humans, bCSCs express increased levels of ALDH and cancer stem-cell marker CD44. Several studies have linked these cells to advanced breast cancer. miRNAs are small non coding RNA molecules that control gene activity via post-transcriptional regulation. There is evidence that miRNAs are involved in survival and in maintaining self-renewal capacity and chemoresistant potential of bCSCs. Thus, it may be possible to devise novel and highly effective therapy regimens that rely on identifying specific miRNAs and targeting them to prevent chemoresistance and relapse. While treatment strategies relying on replacement of antitumor miRNAs or inhibition of oncogenic miRNAs are still in their infancy, there is increasing excitement toward this RNAi approach to treat breast cancer. Many groups have started combining anti-miRNA molecular drugs with chemotherapy drugs to prevent chemoresistance. Technical and experimental strategies and advances reported here will improve the clinical outcomes for breast cancer patients.

Keywords Chemoresistance · Cancer stem cells (CSCs) · Breast cancer stem cells (BCSCs) · microRNAs (miRNAs) · Epithelial to mesenchymal transformation (EMT) · Mammospheres · Metastasis · Antisense oligonucleotides

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(ASO) · Anti miRNA oligoneucleotides (AMOs) · Locked nucleic acids (LNAs) · Antagomirs · Small-molecule drugs

20.1 Introduction

Breast cancer is the leading malignancy and second leading cause of cancer-related death in women globally. In the United States, nearly 12 % of all women are likely to be diagnosed with breast cancer during their lifetime. This translates to roughly 230,480 new cases of invasive and 57,650 new cases of non-invasive breast cancer in 2011 [1, 2]. Currently, breast cancer therapy usually involves mastectomy (removal of the entire breast) or a lumpectomy (conservative surgery to remove only the tumor and some surrounding tissue). This is usually followed by adjuvant therapy that may involve chemotherapy, radiation therapy, hormonal therapy, or a combination of these treatments. For tumors that are inoperable, neoadjuvant therapy to shrink the tumor is often prescribed prior to surgery.

Following therapy, nearly 40 % of all patients will experience a relapse and around 70 % of this patient population will be diagnosed with metastatic disease [3, 4]. While localized disease is largely curable, relapsed or metastatic disease carries a dismal prognosis. Following treatment, the overall survival rate for women diagnosed with localized, early stage breast cancer is around 98 %. For women diagnosed with late stage cancer with metastasis, the survival rate drops to around 23 % [5].

Chemoresistance is a major factor responsible for poor prognosis, morbidity, and mortality in breast cancer patients [6]. It stands to reason that any successful treatment of advanced breast cancer should focus on overcoming chemoresistance. As chemotherapeutic drugs act by a variety of mechanisms, there are multiple and complex mechanisms involved in mediating chemoresistance in breast cancer. These include ABC-transporters [7–9], increased DNA repair and dysregulation of the apoptotic and proliferation machinery [4], increased cytoplasmic inactivation of drug metabolites and overexpression of oncogenes or downregulation of tumor suppressor genes and epigenetic alterations [3, 10, 11]. Despite these advances, our understanding of chemoresistance especially regarding breast cancer is incomplete which makes the task of finding new therapies even more challenging.

Most solid tumors are heterogeneous and are composed of bulk cancer cells and a small population of pluripotent cells that are chemoresistant, capable of self-renewal, and can maintain and regenerate the tumor [12, 13]. There is increasing evidence that chemoresistance in breast carcinomas is mediated by these tumor initiating cells (TICs) or cancer stem cells (CSCs) which leads to poor prognosis [4, 11]. Essentially, the inherent drug resistance and survivability of CSCs can predispose to failure of therapy, relapse and metastasis during conventional therapy. Since most cancer related deaths occur due to metastatic tumor growth, new therapeutic strategies that selectively ablate this cellular subset are being investigated to improve prognosis for patients with advanced breast cancer.

In addition to CSCs, development of chemoresistance is also linked to dysregulation of non-coding small RNA molecules, the microRNAs (miRNAs) [6–8, 10, 14]. Apart from being posttranscriptional regulators of many biological processes in eukaryotic cells, miRNAs are also implicated in etiology of various cancers and can function either as oncogenes (oncomirs) or tumor suppressors [15]. More importantly, miRNAs can regulate self-renewal and survival of various CSCs, including that of breast cancer stem cells (bCSCs) [14, 16–18]. Thus, miRNAs have rapidly emerged as promising diagnostic and prognostic markers and targets for the development of novel anticancer therapeutics. miRNA-based cancer therapy aims to either replace tumor suppressor miRNAs or inhibit oncomirs using a variety of approaches [9]. This chapter focuses on the role of breast cancer stem cells (bCSCs) and miRNAs in chemoresistance and failure of therapy. Clinical implications of targeting bCSCs and/or miRNAs are also discussed.

20.2 Breast Cancer Stem Cells (bCSCs)

Cancer stem cells (CSCs) are a niche population amongst the bulk or non-cancer stem cells in solid tumors. They are highly tumorigenic and like normal stem cells, can differentiate into non-stem cell progeny [19]. Most CSCs possess cell surface markers such as CD44, CD24, CD133, epithelial-specific antigen (ESA) and aldehyde dehydrogenase-1 (ALDH1) which are of use to researchers for isolating, identifying and characterizing CSCs. Breast cancer stem cells (bCSCs) or mammary gland tumorigenic CSCs were first identified and characterized in 2003 by Al-Hajj et al. by flow sorting a subpopulation from clinical breast cancer samples. It was found that these cells expressed high levels of cell surface markers, CD44 and epithelial surface antigen (ESA) and low levels of CD24. These cells could initiate tumors in immunodeficient mice at numbers as low as 200. In contrast, nearly 0.5×10^6 unsorted cells were required to induce similar tumors. Further, xenografts tumors had both bulk and CSC populations which mirrored the phenotypic heterogeneity of the original tumors [20]. While they share some gene expression markers with embryonic stem cells, bCSCs express specific intracellular and membrane makers such as CD44 and ALDH. bCSCs are resistant to radiation and chemotherapy, are more invasive in vitro and more metastatic in vivo and may contribute to clinical metastases. There is a need to devise strategies to target and ablate these populations to prevent relapse and improve outcomes in clinical setting.

20.2.1 Origin of bCSCs

There are currently two theories outlining the origin of bCSCs. The first postulates that dysregulation of normal stem cell pathways, particularly those involved in self-renewal and differentiation, generates cancer cells capable of self-renewal and

differentiation. As stem cells are relatively quiescent and long-lived, there is an increased chance of mutations that yield CSCs [21, 22]. Accordingly; CSCs can regenerate tumors by asymmetric division. This results in two progenies; one is a stem cell, which allows self-renewal, and another cell, which is the precursor for 'bulk' cancer cell incapable of self-renewal. Unlike the bulk cancer cells, CSCs share many features with normal cancer cells including their capacity for plasticity and increased expression of stem cell markers such as SOX2, OCT 3/4 and Nestin. In breast cancer patients, increased expression of these stem cell markers was statistically correlated with increased disease progression, invasion, metastasis and decreased survival [15, 21, 23–25] suggesting their potential use as markers of clinical outcome. The second theory of CSCs origin focusses on the role of cancer cells that have assumed a mesenchymal phenotype from an earlier epithelial one; a phenomenon termed as epithelial to mesenchymal transformation (EMT). It is known that post-transformation, not only are cancer cells more motile and invasive, they are also resistant to the various cytotoxic drugs, express mesenchymal markers and have self-renewal properties [24, 26–28]. These properties of EMT cells closely mirror those of CSCs [25], an observation validated by experimental evidence indicating that CD44 (high)/CD24 (low) cells from normal and neoplastic clinical breast tissues express genes associated with cells that have undergone EMT. For example, cells with CD44 (high)+/CD24(low)− phenotype also had higher expression of mesenchymal markers N-cadherin, Vimentin, Fibronectin and reduced expression of epithelial marker E-cadherin [26]. Taken together, it may be stated that EMT can promote the generation of cancer stem cells from more differentiated cancer cells. This theory of CSC biogenesis from EMT cells is bolstered by involvement of the canonical and noncanonical Wnt pathways and the TGFβ pathway, which can induce mesenchymal transformation of human mammary epithelial cells (HMECs) and maintain their CSC-nature [27].

20.2.2 Characterization of Breast Cancer Stem Cells

In recent years enrichment and isolation techniques based on the enzymatic activity of ALDH1 has allowed the isolation of bCSCs and normal mammary stem cells [29]. In addition, ALDH1 is a diagnostic marker and a therapeutic target of BRCA1 related breast cancer and its increased expression is an indicator of poor prognosis in clinical setting [30–32]. Ginestier et al. demonstrated that only ALDH1 expressing human breast cancer cells could form tumors in mice. They further demonstrated that $CD44^{+}/CD24^{-/low}$ cells which expressed ALDH 1 were highly tumorigenic and only 20 of these could generate tumors [30], suggesting that these are the most relevant markers for highly tumorigenic bCSCS. Breast cancer stem cells, normal or cancerous, possess the ability to form non-adherent spheroidal colonies termed as mammospheres [33]. When these colonies are initiated using $CD44^{+}/CD24^{-/low}$ or ALDH1 expressing breast cancer cells, significantly enriched bCSCs capable of self-renewal can be harvested [30, 32–34]. bCSCs with $CD44^{+}$/

$CD24^{-/low}$ phenotype also express OCT3/4, a key marker of all stem cells, and overexpress genes associated with Hedgehog pathway known to be involved in maintaining a variety of stem cells [34, 35]. Other signaling pathways common to most CSCs include Wnt, Notch, p53, PI3K and HIF [36, 37]. Several of these are involved in modulating key features of bCSCs such as chemoresistance, metastasis and invasiveness and have been discussed in this chapter.

20.2.3 Resistance to Chemotherapy and Metastatic Potential

Breast cancer stem cells can be enriched by chemotherapy and radiotherapy in mouse and in vitro models as evinced by an increase in cancer cells with $CD44^{+}/CD24^{-/low}$ phenotype which form mammospheres in vitro [14]. Tanei et al. examined clinical samples from patients previously treated with paclitaxel and epirubicin and found increased percentage of $ALDH1^{+}$ cells [38]. Epirubicin also increased the number of cells with $CD44^{+}/CD24^{-/low}$ phenotype in experimental mouse xenograft models [14]. In mice with human breast tumor burden, radiotherapy significantly enhanced the $CD44^{+}/CD24^{-/low}$ cells significantly [39, 40].

At the molecular level, bCSC resistance to drugs or radiation is due to the alteration of signaling pathways which are responsible for self-renewal and cellular fate. These include Notch, Wnt, Hedgehog, and/or HER-2. The Hedgehog pathway maintains the characteristics of various cancer stem cells [41–43]. Notch-1 overexpression in bCSCs endows chemoresistance [44] and resistance to radiation therapy [40]. As Notch signaling can upregulate the antiapoptotic gene BIRC5 and induce expression of cell proliferation factor cyclin D1 [45], this may allow bCSCs to avoid cell death resulting from drug and/or radiotherapy and contribute to genetic instability [34, 40, 44]. Notch1 signaling is important for establishment of breast cancer and requires cyclin D1 since it is responsible for self-renewal of normal and cancer stem cells and is indispensable for Notch1-induced mammary tumorigenesis [46, 47]. Since cyclin D1 is also a downstream target of Wnt, Stat3, β-catenin, and NF-κB signaling, it acts as a common regulatory node that ensures survival of stem cells [48].

Since bCSCs can survive the initial onslaught of chemo/radiation therapy and regenerate tumors, they are actually responsible for the relapse and reestablishment of aggressive and chemoresistant disease. Interestingly, it is now known that bCSCs are not inherently resistant to therapy and specific molecular cues and pathways are responsible, perhaps in response to the environmental conditions. bCSC population within a triple (estrogen receptor (ER), progesterone receptor (PR) and HER-2) negative cell line are susceptible to radiotherapy, supporting the hypothesis that bCSCs are not resistance *per-se* and that underlying molecular mechanisms regulate the resistant phenotype [39]. Elucidation of the molecular mechanisms which ensure bCSCs survival can identify novel targets for improved breast cancer therapy.

Metastasis is a complex and well-tuned multistep process that involves cancer cells escaping from the primary tumor site utilizing the circulatory system to implant at distant sites. According to the cancer stem cell hypothesis, only CSCs are

tumorigenic and sustain tumor growth and thus, bCSCs will play a major role in metastasis [49]. Breast cancer stem cells are motile, invasive and their gene signature panels include metastasis markers such as ALDH1 and CD44. Recent work by Liu et al. has shown that $CD44^{+}/CD24^{-/low}$ cells isolated from human breast cancer samples when injected into the mammary fat pad were able to produce metastatic nodules in lungs and these cells retained tumor-inducing potential through successive passages [46]. Another set of studies by Charafe-Jauffret et al. demonstrated that upon their injection into cardiac region, only ALDH expressing breast cancer cells generated distant metastases possessing the heterogeneity of the parent tumor [32]. Together, these results support the idea that the metastatic population of breast cancer cells is contained within the bCSCs. The molecular mechanisms and regulatory factors that control bCSCs are discussed below.

20.3 Micro RNAs and Breast Cancer

Micro RNAs (miRNAs) are endogenous, noncoding RNA molecules about 22 nucleotide (nt) long that can negatively modulate post-transcriptional expression of genes by binding to their complementary sequence in the 3′ untranslated (UTR) region of mRNA targets [50]. It is believed that almost 1200 miRNAs regulate expression of almost a third of the vertebrate genome [51]. Mature miRNAs exist as hairpin structures in the cytoplasm after undergoing two processing steps. In the nucleus, miRNAs are transcribed by RNA polymerase II into precursor units termed the pri-miRNAs [52]. These transcripts which can be several kilobases in length, are processed by Drosha, an RNase III enzyme, into 70 nt long stem-loop structures [52, 53]. Processed pre-miRNAs are then actively exported out of the nucleus by the GTP-driven exportin 5 transporter to the cytoplasm for further processing by the Dicer-TRBP (tar binding protein) microprocessor complex [54–56]. This causes the release of a double-stranded RNA duplex composed of the mature miRNA bound to its complementary strand. The mature miRNA strand is separated from its complement due to differences in thermodynamic stability at the 5′ end and is loaded into a RISC where it has the capacity to regulate target genes. The bound mRNAs are either targeted for destruction by RISC resulting in a decreased number of transcripts or remain untranslated resulting in a decrease in the proteins they encode. In animals, miRNAs mediate RNAi via a translational inhibition mechanism involving partial base pairing (imperfect complementarity) and this recognition requires nucleotides 2–7 in the 5′ end of the miRNA (seed region) to be perfectly complementary to the target mRNA [50, 57, 58].

miRNAs are involved in a number of cellular processes including development, differentiation, proliferation, apoptosis, and stress response [59]. They can also regulate gene networks or pathways controlling biological functions, thus playing an important role in development and differentiation. Since they function by binding to complementary sites on target mRNAs to induce cleavage or repression of productive translation, identification of miRNA targets is the key to analyzing their function. miRNA expression profiling has been used to demonstrate

characteristic miRNA signatures in tumor tissue [60–62] suggesting that miRNAs have the potential to be used as diagnostic and prognostic tools [63, 64].

20.3.1 miRNA Involvement in Breast Tumorigenesis and Progression

Several lines of evidence point to the role played by miRNAs in the etiology of breast cancer. Key miRNAs, including let-7, miR-34, miR-125, miR-200 family, miR-205, miR-21, miR-10 and the miR-17-92 cluster, involved in breast and other cancer cell proliferation, apoptosis, cancer stem cell expansion, and tumorigenesis have been reviewed elsewhere [65, 66]. Initially, it was believed that breast cancer genesis and progression involved global downregulation of miRNA expression. However, since miRNAs can be tumor suppressors or oncomirs, each stage of the disease is like to have a distinct expression profile [67]. One of the first studies to explore miRNA involvement in breast cancer was carried out by Iorio et al. who utilized gene expression microarrays and northern blot analysis to identify 29 dysregulated miRNAs in 76 human breast cancer tissues and 14 breast cancer cell lines. They established that miRNAs such as mir-125b, mir-145, mir-21, and mir-155 as being significantly altered and concluded that miRNA expression profiling could not only distinguish between normal and cancerous breast tissue but also delineate the specific breast cancer pathology, such as tumor staging, invasiveness or cellular proliferation [68]. On similar lines, Rothe et al. conducted a global microarray based miRNA expression profiling in systemically untreated breast cancer patients who already had the mRNA profiles available [63]. By comparing gene signatures it was observed that tumor response such as estrogen receptor status, tumor grade, and gene expression grade index, had a direct association with unique miRNA expression profiles. miRNAs such as miR-210 were associated with poor clinical outcome in a some patients [63]. Enerly et al. investigated the relationship between miRNA and mRNA expression in primary breast carcinomas and its clinical relevance [64]. The authors identified significant differential expression of miRNAs between molecular intensive subtypes and between samples with different levels of proliferation. Similar studies have also been reported for other tumors [69, 70]. These studies point to the correlation between miRNA expression profile with breast tumor formation and progression.

20.3.2 miRNAs, Breast Cancer Stem Cells and Chemoresistance

miRNA involvement in tumor formation by CSCs is well known. A recent report by Hwang-Verslues et al. has shown that miR-495 expression was significantly increased in breast cancer cells capable of initiating tumorigenesis (i.e. possessing

the CD44(+)/CD24(−/low) phenotype), suggesting the role of this miRNA in maintaining CSCs. Further, when miR-495 mimics were transfected into breast cancer cells prior to their injection in mice, colony formation was seen in vitro and tumor formation occurred in mice. miR-495 mediated cell invasion was correlated to a simultaneous decrease in epithelial marker E-cadherin expression, suggesting that miRNAs potentially regulate cancer cell stemness and EMT-transition of breast cancer cells [71]. Let-7 is a miRNA expressed in differentiated cells while its expression in embryonic stem cells (ES cells) is negligible, a pattern that was mirrored in breast cancer stem cells [14]. In this study, Yu et al. experimentally enhanced breast CSCs population by transfecting undifferentiated cells with let-7, resulting in these cells being able to form mammospheres in vitro, forming tumors upon injection into mice [14]. A very recent study by the same group has established the significance of miR30 in maintaining bCSCs. Since mir-30 expression is decreased in bCSCs, expression of its target genes Ubc9 (ubiquitin-conjugating enzyme 9) and ITGB3 (integrin beta3) is enhanced. The ability of bCSCs for pluripotency and self-renewal is lost upon restoration of miR-30 leading to cell death. When miR-30 expression in differentiated breast cancer cells was experimentally downregulated, surface markers for CSCs were observed, indicating de-differentiation of these cells to an earlier stem cell like phenotype [72]. In another study, Shimono et al. identified 37 differentially expressed miRNAs upon comparing expression profiles of human bCSCs and bulk/non CSC breast cancer cells [73]. Their major observation was the downregulation of three distinct clusters, miR-200c-141, miR-200b-200a-429, and miR-183-96-182 in bCSCs and normal mammary stem/progenitor cells. miR-200 family members suppress EMT by targeting ZEB1 and SIP1 transcripts in various cancers [74]. Breast cancer stem cells which underwent miR200c replacement were unable to form colonies in cell cultures and tumors in vivo [73] reaffirming the central role of miRNAs in regulating self-renewal capacity in both normal and cancer stem cells. Another negative regulator of bCSCs is miR-34c. CSCs, which were transfected with miR-34c mimic lost ability for self-renewal, and demonstrated inhibition of EMT and invasiveness via silencing of the Notch4 gene [75]. Finally, bCSCs have reduced miR-128 expression which is an indicator of chemoresistance and poor survival [76]. Constitutive expression of miR-128 restored chemosensitivity of bCSCs to doxorubicin, resulting in apoptosis and DNA damage [76].

20.4 Future Implications and Therapeutic Strategies

Chemoresistance and metastasis in breast cancer is a complex phenomenon due to the broad nature of escape routes utilized by tumors. For example, intrinsic resistance may be modulated by existing gene mutations, miRNA alterations, epigenetic alterations, establishment of a resistance cancer stem cell population, reemergence of efflux mechanisms, and the enhancement of DNA repair mechanisms [4, 7, 8, 11, 77–82]. Since CSCs, which are involved in cancer initiation and

maintenance, are under the control of various miRNA-driven pathways [83, 84], it stands to reason that miRNA determinants of bCSC maintenance are also mirrored in the dysregulated patterns of miRNAs in breast tumors [28]. Recent findings suggest an interrelationship between EMT phenotype, CSCs, chemoresistance and miRNAs [16]. miRNAs via their regulation of EMT also impart increased chemoresistance and self-renewal capacity to cancer cells, turning them into CSCs. Therefore, targeting of miRNAs—the common regulator of EMT and cell stemness—can enhance the clinicians' armamentarium for treating chemoresistant and metastatic breast cancer [26, 85, 86]. Down-regulating or re-expressing (miRNA-replacement) specific miRNAs could be a new strategy for overcoming chemoresistance and eliminating metastasis in patients with breast cancer. At present, a number of RNA-inhibition strategies are in use in preclinical setting and only a few of these have made it to clinical trials or to the market. Some of these include antisense oligonucleotides (ASO), anti-miRNA agents such as locked nucleic acids (LNAs) and antagomirs [87, 88]. There is also sustained interest in naturally occurring polyphenolics and small molecules for targeting miRNAs. Some of these strategies are detailed below.

20.4.1 Antisense Oligoneucleotides

Antisense oligoneucleotides are single-stranded, DNA-like molecule 17–22 nt in length, chemically modified to increase stability and are principally designed to complementarily bind to a specific messenger RNA (mRNA) transcript. This results in a duplex formation which triggers RNAse-H mediated cleavage of the transcript and inhibition of gene expression. Using this approach, Yu et al. delivered an antisense oligoneucleotide (ASO) against anticancer miR-30 and showed enhanced metastasis in NOD/SCID mice bearing human mammary tumor xenografts [72]. Presently, a number of ASOs such as Genesense (Genta Inc., targets BCL-2, Phase III), AP 120009 (Antisense Pharma, targets TGFβ, Phase II), LY2181308 (Eli Lilly, targets survivin, Phase II for leukemia, lung and prostate cancer), Custirsen (OncogeneX, targets clusterin, phase III in combination with docetaxel, for advanced prostate cancer) are in various stages of clinical trials for treating a wide variety of cancers.

20.4.2 Anti miRNA Oligoneucleotides, Locked Nucleic Acids and Antagomirs

Anti miRNA oligoneucleotides are structurally and functionally similar to ASOs and are directed against miRNAs. Like ASOs, they can be chemically modified to prevent nuclease mediated degradation and improve their performance and

potency. To act effectively, anti miRNA oligoneucleotides (AMOs) need to bind with high affinity to the 'seed region' spanning from bases 2–8 from the 5′ region of target miRNA [89]. Several preclinical studies have demonstrated the effectiveness of this approach in miRNA inhibition and loss-of-function suggesting the potential for AMO-based therapeutics. Using this method, Esau and colleagues inhibited miR-122 in normal mice by intraperitoneally (i.p.) injecting an unconjugated modified anti-microRNA antisense oligodeoxyribonucleotide (AMO) resulting in enhanced expression of miR-122 target genes and a decrease in plasma cholesterol [90]. Systemic delivery of chemically modified anti-miR-132 effectively reduced tumor burden and angiogenesis in mouse bearing breast cancer xenografts [91]. LNAs are a class of bicyclic RNA analogues capable of inhibiting individual miRNAs or entire miRNA families with minimal side-effects. Due to the locked ribose moiety, a significant increase in the hybridization properties is observed [92]. Very recently, phase II clinical trials of Miravirsen (Santaris Pharma), an LNA-modified ASO against miR-122, have shown promising results as monotherapy in hepatitis C patients (http://clinicaltrials.gov/ct2/show/NCT01200420?term=miravirsen&rank=1). Of late, cholesterol-linked, single strand RNA molecules called antagonimirs have been utilized to study the gene regulatory effects of miRNAs. These are 21–23 nts in length and are complementary to the mature miRNA sequence. Successful use of cholesterol-linked antagonimirs enables stable delivery in vivo and has enabled research into functions and effectiveness of miRNA in rodent models [93–95]. Recently, systemic delivery of miR-10b antogomir to mice orthotopically-implanted with human breast cancer tissue reduced expression of miR-10b and its target gene Hoxd 10 and prevented breast cancer cell metastasis in a sequence-specific manner [96].

20.4.3 Naturally Occurring Phytochemicals and Small Molecule Drugs

While the abovementioned molecular drug strategies have typically yielded good animal data, there are significant challenges associated with their use. For example, nucleic acid drugs after their in vivo delivery are susceptible to nuclease-mediated degradation, subjected to binding with serum proteins and removed from circulation, and may go off-target causing systemic toxicity [89]. For this reason, recent efforts have also focused on the development and use of small-molecule drugs capable of targeting and modulating expression of specific miRNAs. The secondary structure of miRNAs, including the stem-loops of miRNA precursors and bulges in mature miRNAs, makes them druggable and allows targeting by small molecules. Most of these drugs are small synthetic organic molecules around 800 Da and can bind directly to miRNAs thereby inhibiting their biological function. They have good solubility and pharmacokinetic and pharmacodynamics profiles, making them ideal candidates for targeting specific miRNAs. For these reasons, their cost is likely to be significantly low compared to molecular drugs.

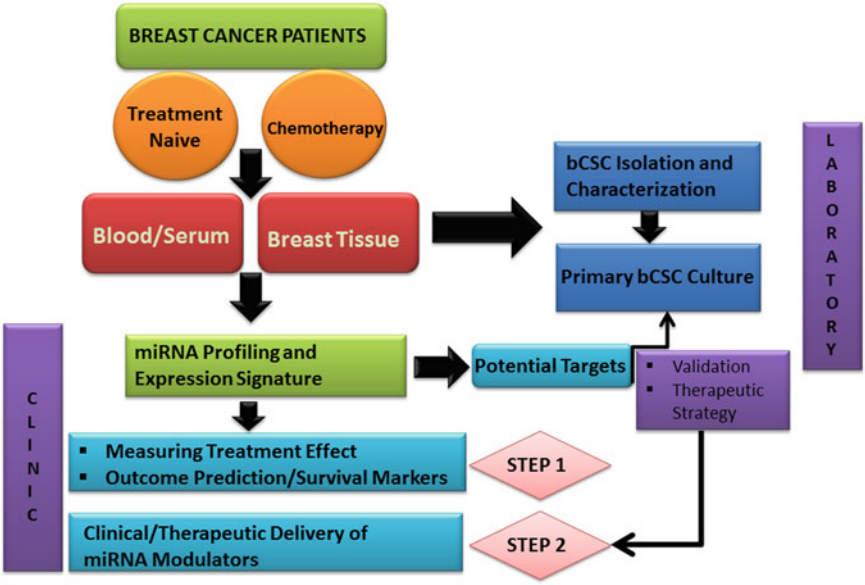

Fig. 20.1 Retrosepective/prospective therapeutic and experimental strategy for treating advanced breast cancer by targeting patient specific miRNAs and bCSCs. Proposed strategy for clinical and laboratory applications of miRNA-based therapy. Core biopsy specimens from breast tumor and serum samples isolated from patients untreated ('Treatment Näive') and those subjected to neoadjuvant and adjuvant therapies ('Chemotherapy') will be utilized for creating patient, stage and treatment specific miRNA expression signatures. One set of samples will be used for isolating and establishing cultures of bCSCs. miRNA signatures from patients subjected to chemotherapy will create a baseline for treatment-effected sampling. Samples from na patients will create markers for predictive outcomes, chemotherapy response, as well as patient survival. In parallel, miRNA expression signatures from patients undergoing neoadjuvant chemotherapy will allow side-by-side comparison of pre and post-treatment miRNA expression profiles for validating chemotherapy effectiveness. Isolated bCSC cultures will allow in vitro testing of new strategies such as miRNA replacement, miRNA sponges and small molecule drugs for restoring chemosensitivity to drug-resistant cancer cells by targeting miRNA dysregulation. Data obtained from these studies can be used for subsequent clinical applications

Recent work in this field has been carried out by Dieter's lab. Using in silico tools and structure–activity functional studies, they successfully screened a small molecule library and identified diazobenzene and its derivatives as effective inhibitors of miR-21 formation [97]. In a subsequent study, Dieter and colleagues also demonstrated the effectiveness of a small molecule drug targeting anti-miR-122 in animal model of hepatocellular carcinoma [98]. Enoxacin (Penetrex), a fluoroquinolone antibacterial drug, has been shown to increase expression of anticancer miRNAs such as let-7a, let-7b, 143 and 205 by directly binding to the miRNA biosynthesis protein TAR RNA-binding protein 2 (TRBP) [97, 99, 100]. Successful establishment of this strategy is likely to revolutionize disease treatment since unlike traditional small molecule drugs that target specific proteins

and effector molecules, miRNA-targeting agents can be cheaply produced and effectively delivered in vivo with few of the problems associated with miRNA mimics, ASO and related therapeutic nucleic acids.

Accumulating evidence further suggests some easily available, natural, non-toxic and well-studied compounds (e.g., curcumin, indole-3-carbinol, epigallocathechin gallate (EGCG) and isoflavone) can regulate or target miRNAs, CSCs and EMT-phenotype in various cancers making them likely and less-toxic candidates for treating breast cancer [101–104]. Sarkar and colleagues have demonstrated the feasibility of commonly used anti-diabetic drug metformin in reducing CSC population in pancreatic cancer. Metformin treatment not only reduced the expression of CSC markers such as CD44, EpCAM, EZH2, Notch-1, Nanog and Oct4 but also restored expression of miRNAs such as let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c which are usually lost in advanced pancreatic cancer [105]. This suggests that CSC and miRNA axis in breast cancer can be targeted using similar strategy. Metformin treatment eas found to restore sensitivity to trastuzumab in mouse models of breast cancer by preferentially killing tumorigeneneic CD44(high)/CD24(low) cells [106]. The fact that post-menopausal diabetic women taking metformin had reduced incidence of breast cancer [107] and metformin reduces the stemness of breast cancer stem cells by targeting OCT4 [22] further validates this approach.

Translation of the abovementioned strategies from bench-to-bedside while seemingly feasible still requires a significant investment in efforts. However, with the availability of new experimental and in silico tools, a critical threshold in therapy targeting specific miRNAs or miRNA-clusters has been crossed and it is a matter of time when such molecular drugs reach the market in an affordable fashion. Further, combination of anti-miRNA molecular drugs with existing chemotherapy can potentiate the drug regimen and prevent chemoresistance. This will have a significant improvement in the quality of life of breast cancer patients globally. Figure 20.1 is a summation of some of the current and potential therapeutic strategies targeting miRNAs and CSCs for treating breast cancer.

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Chapter 21
Breast Cancer Stem Cells: Responsible for Therapeutic Resistance and Relapse?

Hasan Korkaya and Fayaz Malik

Abstract Since the "war on cancer" was waged more than 30 years ago, the fact remains that the metastatic breast cancer is still incurable and patients will ultimately die from this disease [1]. American Cancer Society has estimated that in the year 2012, there will be about 229,060 new cases of breast cancer and an estimated 39,920 new deaths caused by breast cancer in the United States alone [2]. Majority of breast cancer-related deaths are primarily due to metastatic disease which display poor prognosis with an estimated 5-year survival of $\sim$20 %. Furthermore, therapeutic resistance and relapse are strongly associated with metastatic disease in breast cancer patients [1]. Despite the fact that the heterogeneity of tumor cells had been widely acknowledged, it has not been validated until the 1990s due to lack of markers and techniques. D. Bonnet and J. Dick were the first to describe the hierarchical organization of acute myeloid leukemia (AML) and the existence of cancer stem cells (CSC). This was quickly followed by the identification of CSCs from number of malignancies enabling us to better characterize these cells in mouse models and preclinical settings. These ongoing functional studies suggested that CSCs may explain the failure to treat advance metastatic tumors. Thus the "seed and soil" hypothesis proposed by Stephen Paget more than 120 years ago may re-framed in a modern context explaining the ability of subset of tumor cells "seed" or "CSCs" to disseminate and metastasize to secondary organs where nutrient-rich microenvironment "soil" stimulates the secondary tumor growth by enhancing CSC self-renewal.

Keywords Cancer stem cells (CSCs) · Biomarkers · Aldehyde dehydrogenase (ALDH) · Trastuzumab resistance · Breast cancer stem cell self-renewal ·

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21.1 Introduction

More than 150 years ago, German scientist Julius Conheim proposed "cancer stem cell" hypothesis due to similarities between embryonic and neoplastic developments. It was only recently John Dick and his colleagues isolated such cells from acute myeloid leukemias (AML) where a small subset of CD34+/CD38- cells that comprised less than 0.01 % of tumor cells could transform human AML into NOD/SCID mice while the remaining cells lacking this phenotype failed to do so. To date, CSCs were identified from majority of human malignancies including brain, breast, colon, prostate and pancreas etc. These ongoing studies have given considerable support to the CSC model explaining the failure of conventional therapeutics. According to the CSC hypothesis, tumors are organized in a hierarchical structure whereby self-renewing CSCs drive tumorigenesis while differentiated cells form bulk of the tumor [3]. The CSC model fundamentally differs from the traditional or "stochastic" model of carcinogenesis in which any cell may have equal malignant potential. Based on the stochastic model, most therapeutic strategies have been selected for their ability to cause tumor shrinkage by targeting rapidly cycling cells. For breast cancer, however, tumor regression does not necessarily correlate with patient survival. In contrast, the CSC model suggests that tumor initiating cells comprise only a small fraction of tumors and thus alterations in this population may not be reflected by changes in tumor volume. While the existence of CSCs in multiple human tumors has been firmly established, the functional and clinical significance of these cells are currently under extensive investigation [4]. As illustrated in Fig. 21.1, CSCs may also exist in two different forms representing quiescent and proliferating CSCs, which may explain the different outcomes in different breast tumor subtypes.

The development of mammary gland at puberty and cycles of lactation and involution at each pregnancy provided circumstantial evidence that this organ is maintained by self-renewing stem cells. To provide experimental support, Kordon and Smith demonstrated the existence of mouse mammary epithelial cells with the ability to repopulate mouse mammary gland through a serial transplantation of retrovirally tagged epithelial cells [5]. With the advancement of techniques and development of cell surface markers, Jane Visvader and colleagues demonstrated that a single mouse mammary stem cell with Lin-CD29hiCD24+ phenotype was able to reconstitute a complete functional mammary gland in a recipient mouse in a serial transplantation assays [6]. Furthermore, these multipotent self-renewing cells were expanded in mouse mammary tissue during the pregnancy. Together these suggest that the stem cells in mammary gland may have indefinite number of self-renewal potential. Although the experimental evidence is currently lacking, it

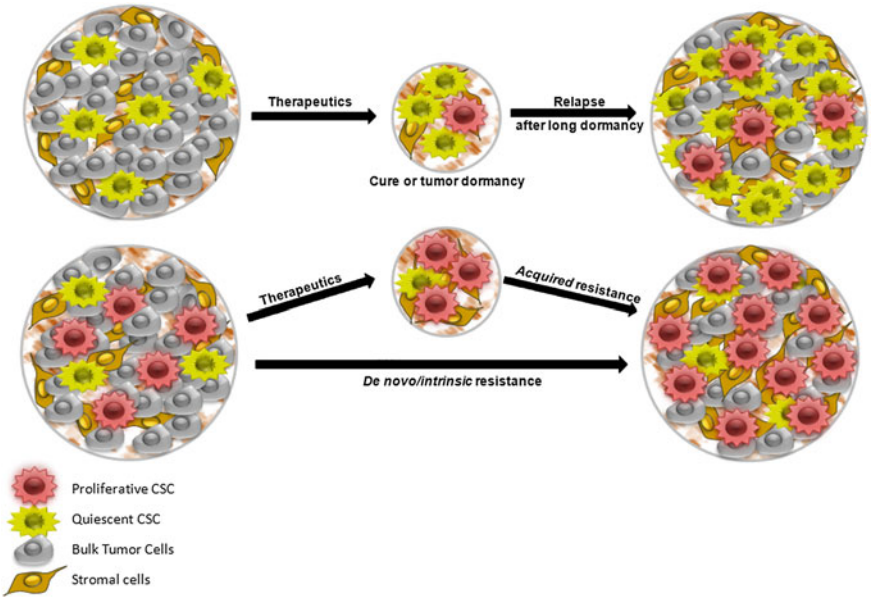

Fig. 21.1 The breast cancer stem cells may exist in two different forms representing quiescent and proliferating CSCs in different breast cancer subtypes. In luminal breast tumor subtypes, activation of the quiescent CSCs takes longer time and thus resistance or relapse develops after many years. However, highly proliferative CSC in basal/claudin low tumors either show de novo resistance or develop resistance within a very short time

has been speculated that mammary stem cells as compared to differentiated cells may have the potential to accumulate number of genetic mutations resulting in malignant transformation. However, the breast CSCs may not necessarily be derived from normal mammary stem cells, thus it is also believed that the differentiated cells may acquire capabilities of stem cells such as self-renewal and lineage differentiation upon activation of self-renewal pathways. Therefore the term CSC is a functional definition based on the similarities between self-renewing tumor cells and normal mammary stem cells.

21.2 Identification of Breast Cancer Stem Cells

Embryonic and tissue specific stem cells are characterized by two distinct properties; (i) self-renewal, infinite number of cell division while maintaining the undifferentiated state, and (ii) multipotency, differentiation or the ability to generate distinct cell types that make up the organ. Tissue specific stem cells are distinguished from embryonic stem cells in that their differentiation is primarily limited to cell types of a particular organ. Although trans-differentiation of tissue

specific stem cells has been reported, this plasticity is often attributed to fusion of stem cells of different origins [7–9]. The mammary tissue is organized in cellular hierarchy, where stem cells are able to generate all progeny, committed able to generate all progeny, committed progenitors and terminally differentiated cells with specialized functions such as the production of milk by alveolar cells. In fact these early studies on normal organ development and tumors suggested that the tumors are indeed organ-like structures resembling to their normal counterparts in that they are both comprised of heterogeneous cell population.

The development of biomarkers to identify CSCs as well as validation of in vitro and mouse models, has facilitated the isolation and characterization of these cells from both murine and human tumors. Michael Clarke, Max Wicha, and their colleagues previously described a subpopulation in breast cancer that displayed stem cell properties and was characterized by expression of the cell surface markers ESA (EpCam) and CD44 in the absence of CD24 expression [10]. These cells have been called "breast cancer stem cells" (BCSCs). As few as 200 EpCam + CD44 +/CD24-Lin$^-$ cells were able to generate tumors in immunocompromised NOD/SCID mice, whereas 100-fold more cells without these markers isolated from the same tumors were non-tumorigenic[10]. Furthermore, the tumor-initiating populations regenerated tumors that recapitulated the heterogeneity of the initial tumor [10].

In addition to CD44 +/CD24- phenotype, a number of other markers and means have been identified in recent years. The "mammosphere" assay was developed to enrich normal and malignant stem cells in suspension culture [11, 12]. This in vitro assay has been widely utilized to investigate various aspects of CSCs and to screen CSC-targeting drugs [13–15]. Based on the Hoechst 33342 dye efflux a "side population" (SP) of cells with enriched stem cell activities was first demonstrated in bone marrow cells [16]. This SP method was also utilized to isolate and characterize CSCs in a number of solid malignancies [17–19].

Expression level or the activity of aldehyde dehydrogenase (ALDH) as assessed by immunohistochemistry (IHC) and by the Aldefluor assays respectively has also been established as means of identifying and enriching normal and malignant mammary stem cell populations [20]. Interestingly, these markers identify overlapping but not identical cell populations [13, 20]. Furthermore, these markers can be utilized to isolate CSC populations from established breast cancer cell lines as well as primary tumor xenografts [20, 21]. The identification of ALDH-A1 as the primary ALDH isoform expressed in stem cells has allowed us to utilize IHC to identify these cells in situ in formalin-fixed paraffin embedded tissue sections [22]. The proportion of ALDH-positive cells is increased in HER2-amplified breast cancer and triple negative breast cancers (TNBC) and ALDH1 expression is associated with poor patient outcome. The CD44 +/CD24- phenotype has been linked to the TNBC subtype, a 10-year lower median age, and unfavorable prognosis in BC patients [23].

Another method of enriching normal breast stem cells or CSCs is the use of PKH26, a fluorescent marker that utilize the functional characteristics of the cells by labeling only quiescent cells while it fades away every division in the

Table 21.1 Cancer stem cell markers in breast cancer

Marker	Isolation technique	Phenotype and properties	Reference
$CD44^+/CD24^-$	Flow cytometry	Stem cell, self renewal	[10]
$ALDH^+$	Flow cytometry	Stem cell, self renewal	[22]
Hoechst 33342^-	Flow cytometry	Stem cell, self renewal	[17–19]
$PKH26^-$	Flow cytometry	Stem cell, self renewal	[24]
Tumorshpere/ Mammosphere Assay	Suspension culture in a serum free media	Stem cell, self renewal	[11, 12]
$CD49f^-/EpCAM^+$	Flow cytometry	Differentiated cells, luminal cancers	[25]
$CD49f^+/EpCAM^+$	Flow cytometry	Luminal progenitors	[25]
$CD49f^+/EpCAM^-$	Flow cytometry	Stem cell, basal or claudin-low tumors	[25]

proliferating cells. Thus, this PKH26 dye has facilitated the isolation of quiescent cells with CSC activity and their subsequent characterization in in vitro and mouse xenografts [24]. Furthermore the PKH26-positive cells formed both basal and luminal cells and were able to re-generate mammary gland while PKH26-negative cells failed to do so, in the cleared fat pads of immune-compromised mice Table 21.1 [24].

21.3 Functional Characterization of Breast Cancer Stem Cells

Following the identification of these breast CSCs in mouse models and primary xenografts, functional characterization of these cells demonstrated that the breast CSCs have far greater invasive and metastatic potential than differentiated cells which comprise the tumor bulk. Furthermore, there is increasing evidence that by virtue of their relative resistance to radiation and chemotherapy, these cells contribute to relapse following therapy [26, 27]. There appear to be multiple mechanisms that account for this resistance: radiation resistance may be mediated by decreased level of oxidants [28] or by increased efficiency of DNA repair [29] in these cells. Furthermore, most chemotherapeutic agents preferentially target and kill rapidly dividing cells, whereas many CSCs are able to persist in a quiescent non-cycling state [26, 28]. Other mechanisms of chemotherapy resistance include increased expression of multi-drug resistance transporters [30, 31] and overexpression of anti-apoptotic proteins [32]. In fact, ALDH, the marker used to isolate these cells, is able to metabolize and inactivate cyclophosphamide [33], an agent frequently used to treat BC.

In recent years, developments of the molecularly targeted therapies provided a breakthrough in cancer treatment extending the overall survival of the patients. However a resistance to molecularly targeted therapies also develops by activation

of alternative pathways. Among these, the HER2-amplified tumors are found in 20–30 % of breast cancer patients and strongly associated with aggressive metastatic disease that is resistant to trastuzumab [34–36]. Following the identification of HER2 gene, a humanized anti-HER2 monoclonal antibody, trastuzumab [Herceptin] was developed to target these types of breast tumors [36]. Despite the clinical benefits of trastuzumab in improving progression free and overall survival, a substantial fraction of patients treated in the adjuvant setting still relapse, one-third of patients with advanced disease fail to respond and half of the initial responders demonstrate disease progression within 1 year [37].

Although a number of mechanisms mediating "de novo" or "*acquired*" trastuzumab resistance have been proposed, the most common molecular alteration associated with this resistance is inactivation of the tumor suppressor PTEN, found in over 40 % of HER2-positive breast cancers [38]. The PTEN activity mediates trastuzumab resistance via activation of the downstream signaling molecule Akt, bypassing the requirement for HER2 activation [39]. In addition, we and others have shown that both HER2 and PTEN are important regulators of subpopulations of breast cancer cells which display stem cell properties [13, 40]. Our recent studies demonstrated that PTEN deletion in HER2 overexpressing breast cancer cells activates an IL-6 mediated inflammatory feedback loop which may provide resistance by regulating breast CSCs. This feedback loop expands the cancer stem cell population displaying an EMT phenotype through both autocrine and paracrine mechanisms which provide trastuzumab resistance [41]. As illustrated in Fig. 21.2, PTEN deletion in HER2 amplified breast cancer cells results in activation of NF-κB mediated IL-6 production and establishment of the positive feedback loop, which provide resistance by driving the EMT phenotype and increased stemnes. In addition, our studies demonstrate that interfering with this feedback loop utilizing an IL-6 receptor (IL-6R) antibody reduces the cancer stem cell population overcoming resistance and inhibiting tumor growth and metastasis [41].

Interleukins 6 (IL-6) and 8 (IL-8) have been demonstrated to regulate the breast cancer stem cell self-renewal [42, 43]. Although these cytokines are regulated by multiple factors, HER2 overexpression in breast cancer stem cells has been shown to increase IL-6 production [44]. The IL-6 links inflammation to malignant transformation by activating the NF-lB pathway, which, in turn, drives constitutive IL-6 production generating a positive feedback loop. In addition, IL-6 is able to induce epithelial-mesenchymal transition (EMT), which has been implicated in generation of stem cell phenotype [15, 42, 45]. The clinical relevance of these studies is demonstrated by the strong association between serum IL-6 levels and poor clinical outcomes in breast cancer patients [46, 47].

The metastasis which accounts more than 90 % of cancer related deaths is a complex process requiring not only tumor cells to acquire EMT phenotype but also depend on the tumor microenvironment. Considerable pre-clinical and clinical evidence now suggest that factors in tumor microenvironment may induce CSCs with MET phenotype and subsequent metastasis [48, 49]. For example, Wnt/beta-catenin signaling has been shown to regulate normal and malignant intestinal stem cells [50]. Vermeulen et al. reported that despite the APC mutation resulting from

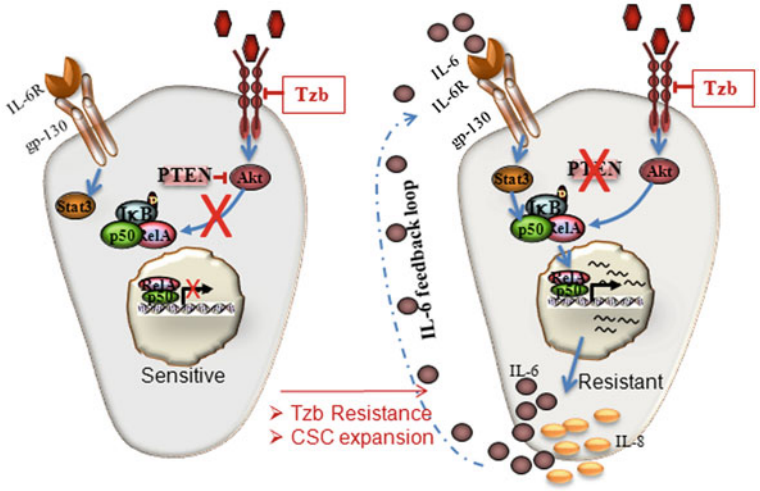

Fig. 21.2 Development of Herceptin resistance in Her2 amplified breast tumors. Deletion of PTEN in HER2 amplified breast tumors results in activation of IL-6 feedback loop that is mediated by AKt/Stat3/NF-κB pathway. Activation of this alternative pathway overrides HER2 requirements leading to Herceptin resistance

Wnt/beta-catenin activation, most colorectal tumors show cellular heterogeneity when beta-catenin nuclear localization is analyzed [51]. Moreover, increased Wnt activity was observed in cells located around the invasive edges of tumors where stromal myofibroblast-secreted hepatocyte growth factors (HGF) activate Wnt signaling through a paracrine mechanism [51, 52]. The HGF has been show to induce EMT phenotype in breast cancer cells [53]. Accumulating evidence also points to a link between inflammatory states and cancer development. In clinical studies, elevated biomarkers of inflammation are associated with reduced survival among the breast cancer patients [54]. The development of chronic inflammation has been shown to result in production of cytokines IL-1β, IL-6 and IL-8 by a variety of inflammatory cells, which are recruited to tumor microenvironment [55–58]. Interestingly, the genetic polymorphism in these genes encoding cytokines predispose affected individuals to cancer [59]. Furthermore the Stat3/NF-κB pathway plays a critical role in inducing and maintaining a pro-tumorigenic inflammatory microenvironment [60–62], while IL-6 mediated NF-κB signaling may influence tumor growth by stimulating the CSC self-renewal [42, 63–65]. In addition to regulating CSCs, cytokines also induce EMT phenotype, which may represent the aggressive tumor phenotype due to self-renewal capacity [15, 42, 45].

More recent studies demonstrated that the CSCs have greater potential to invade and metastasize to secondary organs. Advances in optical imaging and reporter constructs enabled researchers to demonstrate the direct role of breast CSCs in tumor progression and metastasis in live animals. Liu et al. utilized such technique to determine spontaneous metastasis of CD44+ breast cancer cells in

mouse xenografts [66]. Together these studies provide experimental support for the existence of CSCs and their clinical relevance in tumor progression.

21.4 The CSC Targeted Therapies and Their Clinical Implications

In the last 30 years, we have made limited progress in the treatment of certain types of cancers, primarily childhood cancers. However, our failure to treat advanced metastatic cancers suggested that an alternative approach is needed. In addition to de novo drug resistance, the recurrence of tumors after initial regression by conventional therapies is very frequently observed which suggest inadequacies of current therapies. In addition, the need to design molecularly targeted therapeutics for tumors based on their molecular diversity has long being recognized. Most recent studies demonstrating the role of CSCs in therapeutic resistance and metastasis suggest that one potential reason may be the failure of current therapies to target CSCs. Therefore the design and development of alternative therapeutics may require targeting and elimination of CSCs. Although such phase I clinical trials testing the efficacy of CSC-targeting compounds are currently under investigation, preclinical studies provided early indication that inhibiting CSC-specific pathways may target these cells in mouse models and xenografts. In this regard, the developmental or stem cell specific pathways such as Notch, Hedghog (Hh), Wnt and NF-κB have received great deal of attention.

Of these, Notch activation is activated in number of malignancies and inhibiting Notch may target CSCs in those tumors leading to better outcome. HER2 over-expressing breast cancer cells show increased Notch activation and these cells are characterized by their CSC phenotype [67, 68]. Thus inhibition of γ-secretase, which cleaves and activates Notch can reduce the breast cancer growth [69].

Cyclopamine is a natural steroidal alkaloid that specifically inhibits Hh pathway by directly binding and neutralizing the Smo receptor [70]. Recent studies using Cyclopamine inhibits cell lines and tumor growth in a number of malignancies including breast, prostate, medulloblastoma, small cell lung cancer, pancreas and glioma [71–74]. Small molecule inhibitor of Hh has been shown to treat one patient with refractory medulloblastoma to multiple treatments [75], suggesting that this developmental pathway may be driving the therapeutic resistance.

The canonical Wnt pathway is essential for embryonic development, mice deficient in any of the Wnt pathway components such as Wnt3, LRP5/6 or β-catenin fail to develop primitive streak and lack mesoderm [76, 77]. In addition, MMTV/Wnt transgenic mice show expansion of mammary stem/progenitor cell population expressing keratin 6 and Sca-1 stem cell markers [78]. Several ongoing studies utilizing the Wnt specific inhibitors are aimed at directly targeting the Wnt/β-catenin complex. A high-throughput screen identified a number of compounds that inhibit TCF/β-catenin transcription complex in a reporter assays [79]. These studies may have potential implications for the treatment of Wnt driven tumors.

We have previously demonstrated in mouse xenotransplantation assays that Akt mediated Wnt signaling regulates normal and malignant breast stem cells [13]. Inhibition of Akt pathway substantially reduces breast CSCs in mouse xenografts suggesting that agents that inhibit this pathway are able to effectively target tumorigenic breast cancer cells [13]. Interestingly, deletion of PTEN in HER2 amplified breast tumors provides resistance to HER2 targeting agents by activating this Akt/Wnt pathway.

NF-κB has also been implicated in the regulation of mouse mammary stem cells [80]. Elevated levels of progesterone during pregnancy induce the production of RANKL by differentiated breast epithelial cells. RANKL in turn stimulates breast stem cell self-renewal via activation of NF-κB pathway in these cells [81, 82]. Therefore the NF-κB pathway also represents an attractive target. Promising preclinical studies and early stage clinical trials using NF-κB inhibitor parthenolide support the testing of this drug in future trials [83].

Together these studies will determine whether the targeting of these CSC specific pathways alone or in combination improves efficiency of anticancer therapies.

21.5 Conclusion

There is accumulating evidence that human cancers including breast are driven by a subset of cells (CSC) which retain/acquired properties of their normal stem cell counterparts. Like normal stem cells, CSCs show ability to self-renew and lineage specific differentiation in serial transplantation assays. In recent years, considerable progress has been made in the breast CSC research leading to alternative strategies and novel targets. These alternative therapeutics may have the potential to benefit patients in future clinical studies.

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Chapter 22
MicroRNAs in Breast Cancer Research: Progress and Promise

Alia Ahmed, Ashhar S. Ali, Shadan Ali, Aamir Ahmad, Philip A. Philip and Fazlul H. Sarkar

Abstract Over the years, remarkable progress has been made in regards to our understanding of breast cancer biology and consequently the development of novel treatments. One idea that has proven to be immensely valuable is the use of microRNAs (miRNAs) in cancer diagnosis, prognosis, and even for treatment. The miRNAs are short RNA molecules that are able to post-transcriptionally regulate the expression of genes at multiple levels. Past and current research has continued to classify miRNAs as either highly or rarely expressed in cancer cells in relation to their normal non-cancerous counterparts. This classification is also used to organize the various miRNAs as either tumor suppressing or oncogenic. For example, aberrant expression of certain miRNAs is widely accepted to signify different stages of cancer. This chapter summarizes our current understanding of the role of miRNAs in cancer, while enlightening the readers with the role of specific miRNAs in breast cancer development and progression, and their exploitation for designing innovative therapeutic strategies.

Keywords miRNA · Metastasis · Tumor suppressor · Oncogene · Therapies · Let-7 family · Natural agents · Triple negative breast cancer cells · Chemotherapy · Radiation · miRNA-200 family · Treatments

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22.1 Introduction

22.1.1 What are MicroRNAs?

MicroRNAs (miRNAs) are small, non-coding RNA's that aid in the regulation of gene expression during translation [1]. As they are gene regulators, they play important roles in a variety of biological processes within the human body. Such processes are in the areas of development, proliferation, differentiation, apoptosis, survival and stress response [2, 3]. Furthermore, miRNAs are practical molecules to work with not only because of their size, but because they are "naturally abundant and evolutionarily conserved" [3]. It remains unclear how exactly miRNAs function, which is still a mystery that is being intensely investigated at the present time. In the subsection below we are summarizing what is generically known about the manner in which miRNAs function while extensive and more in-depth research are being conducted by many laboratories. This chapter will focus on providing readers with a general knowledge on the outcome of breast cancer research in relation to miRNAs by examining their tumor suppressive and oncogenic ability.

22.1.2 How miRNAs are Synthesized (Biogenesis and Processing)

The miRNAs are primarily found in the introns of different protein coding genes [4]. These miRNA genes are transcribed as long primary transcripts, called pri-miRNAs, by RNA Polymerase II [1, 2, 4]. Subsequent processing of the miRNA gene takes place in two different locations, firstly in the nucleus, and then in the cytoplasm. The pri-miRNAs made in the nucleus are characterized by common eukaryotic messenger RNA (mRNA) components, such as the 5′ cap and 3′ polyA tail. In addition, unique to the miRNA are hairpin structures that are first cleaved by RNAse III Drosha. Drosha cleaves the pri-miRNA to produce "a stem-loop precursor molecule," now called pre-miRNA [1]. Pre-miRNA is generally 70 nucleotides long, significantly shorter than the original pri-miRNA. Transportation of the pre-miRNA molecules from the nucleus to the cytoplasm occurs, as the next processing enzyme, RNAse III Dicer, is confined to the cytoplasm [4]. However, transportation of the pre-miRNAs requires Exportin-5 and Ran-GTP, which carry the pre-miRNA molecules out of the nucleus and into the cytoplasm, where the mechanism of miRNA processing is completed. RNAse III Dicer then cleaves away any remaining loops on the pre-miRNA, leaving only a double stranded portion approximately 22 nucleotides in length, called miRNA/miRNA* [2]. Finally, the mature miRNAs are incorporated into the miRISC complex (miRNA-containing RNA-induced silencing complex). In this complex, one of the strands of the double stranded miRNA/miRNA* disappears (most likely degraded),

while the other remains as a template strand to continue as a mature miRNA [4]. The mature miRNA acts as a guide of the RISC complex to search for the target mRNA. Together, the mature miRNA and the target mRNA are able to post-transcriptionally regulate gene expression [2] where a single miRNA could regulate hundreds of genes although, such regulation and specificity is context dependent [2].

22.2 The Role of miRNAs in Cancer

For years, researchers have been searching for a potential link between a variety of molecules and cancer. Recently, miRNAs were found to be involved in multiple pathways that are intricately linked with the development and progression of cancer. Most importantly, miRNAs play a pronounced role in cancer invasion and metastasis [5]. Generally speaking, accumulating knowledge dictates that miRNAs can be divided into two main classes: tumor suppressors and oncogenes. Such classes are also determined by the amount of expression of the miRNA as compared to normal non-cancerous cells. For example, as cancer develops, the expression of certain regions of the chromosome that contain miRNAs could repress the expression of tumor suppressors possibly by increasing their levels [2]. The tumor suppressor gene would then be silenced as a result of increased expression of miRNAs. In contrast, other miRNAs that tend to suppress oncogenes are typically located on more fragile areas of the genome. Thus, unfortunate occurrences including mutations or deletions of that area in the genome are more likely, leading to decreased expression of such miRNAs and consequently increasing the expression of their target oncogenes [2]. Although, the regulation and homeostasis of the expression of genes regulated by miRNAs sounds simplistic, the biological interplay of mRNA and miRNA in the development of cancer is rather very complex. This complexity is further exacerbated due to context-dependent regulations within the tumor microenvironment, suggesting that there are many layers of regulations prior to the development of cancer and progression to metastatic disease.

Emerging evidence suggest the advantage of the deregulation of miRNAs toward cancer therapies together with chemotherapy or radiation. The miRNAs have the ability to adapt tumor cells that are generally resistant to therapeutic drugs to alter their receptivity towards the drug [5]. Additionally, novel studies have illustrated that natural agents can play a role in the deregulation in the expression of miRNAs, allowing conventional therapies to be more effective through regulation of cancer cell growth and inducing cell death [6]. Furthermore, profiling of the many different miRNAs is extremely important for understanding the biological complexity of cancer and their resistant behavior to conventional therapeutics. Classification of miRNAs can allow them to be useful and important biomarkers beyond just cancer detection, but also for diagnosis, prognosis, and treatment of cancer [7]. Emerging concepts are being investigated for assessing

the value of miRNAs in predicting therapeutic outcome in cancer patients especially for targeted agents, and such knowledge would likely be beneficial in designing personalized therapy in the near future.

22.2.1 Honing in: The Role of miRNAs in Breast Cancer

The variety of implications of miRNAs in cancer mentioned in the previous section apply to breast cancer as well; however, this subsection will further elaborate upon the role of miRNAs specifically in breast cancer cells. Multiple receptors within the body affect the regulation of breast cancer. Three main types of receptors are known, the estrogen receptor (ER), the progesterone receptor (PR), and the Her-2 receptor. Estrogen is a hormone found in both men and women that is known for its regulatory role in cell growth and differentiation [8]. It is able to express its function using two different hormone-dependent transcription regulators, ER-alpha and ER-beta [8]. ER-beta is often nonexistent in breast cancer cells [9]. This receptor functions by binding to certain sites on a gene and inhibiting the synthesis of pri-miRNAs, which would be beneficial only for miRNAs that are up-regulated in breast cancer cells [10]. Furthermore, emerging studies have exemplified that ER-beta can inhibit proliferation, invasion, and tumor formation in breast cancer cells [11, 12]. Thus, it is widely accepted that the presence of ER-beta indicates a less aggressive cancerous phenotype [13]. However, ER-alpha is frequently found to have an effect upon cancer cells, and research is being conducted on whether or not miRNAs and ER-alpha have any biological relationship. It is being speculated whether miRNAs could potentially regulate epithelial-to-mesenchymal transition (EMT) based on their interactions with ER-alpha [14]. Some findings have suggested that between ER-alpha-positive and ER-alpha-negative breast cancers, certain miRNAs are differentially expressed [14]; however, the biological significance of such findings awaits further in-depth investigations.

Much more research has been conducted with regard to the ER than PR; however some studies have shown a correlation between miRNAs and PR. While many correlations have been made, presently, only a few studies have attempted to determine whether there is a miRNA that directly targets PR; or if there is then what is the principal connection between the two [15]. Furthermore, the Her-2-receptor has also been significantly less studied in comparison to the ER receptor in the context of miRNAs. However, triple negative (ER, PR, and Her-2) breast cancer cells are commonly investigated in the context of the expression or lack of expression of certain miRNAs [16]. It is our contention that the regulatory role of miRNAs in breast cancer in the context of breast cancer sub-types is still in its infancy, and thus further research opportunity exists as an open area waiting to be exploited.

22.3 Key miRNAs Pertinent to Breast Cancer

This chapter will evaluate the role of specific miRNAs in breast cancer. The miRNAs will be broadly classified under two separate categories: miRNAs accepted to act as tumor suppressors and miRNAs that are accepted to be oncogenes. Table 22.1 provides a brief introductory outline for the miRNAs that will be discussed.

22.3.1 Tumor-Suppressing miRNA in Breast Cancer Cells

22.3.1.1 The Let-7 Family

The let-7 (lethal-7) family consists of 12 miRNAs, each with related target genes and functions [17]. It is one of the earliest discovered miRNAs and has been studied extensively. It is widely accepted that in breast cancer cells, let-7 miRNAs commonly function as tumor suppressors; thus they play a critical role in tumorigenesis. In the early stages of breast cancer, epithelial cells are dominant. Such cells exhibit a high expression of let-7 [18]. In spite of this, as breast cancer progresses and EMT occurs, cells begin to lose their ability to express let-7 [19]. In accordance with this, several studies have attempted to alter the amounts of let-7 in breast cancer cells and have found that proliferation, self-renewal and metastasis were affected [20]. Furthermore, one study indicated that by creating anti-sense oligonucleotides of let-7 and introducing them into specific cells in vitro, self renewal of non-metastatic cells was achieved [20].

A correlation between let-7 and the estrogen receptor has also been proposed. It was shown that the function of ER-alpha was decreased in certain type of breast cancer cell line when let-7 expression was increased [17]. Consequently, cell proliferation was restrained [17]. Recent investigations have also proposed that experimentally increasing the levels of let-7 in cancer cells could potentially aid in the advancement of certain therapies [21].

22.3.1.2 miRNA-200 Family

The large miRNA-200 family is organized into two clusters. The first includes miR-200a, miR-200b, and miR-429, and these are found on chromosome 1 in humans and chromosome 4 in mice. The other cluster is made up of miR-200c and miR-141, and is located on chromosome 12 in humans and 6 in mice. Members of the miRNA family, listed above, target a common subset of genes, implying their redundant function [22]. The family is generally regarded to be anti-metastasis and associated with poorly invasive phenotype. Among the most prominent processes involved is that of the acquisition of EMT phenotype mediated through targeting of specific molecular markers.

Table 22.1 Regulation of microRNAs in breast cancer and their targets

miRNA	Regulation	Function	Targets	References
Let- 7	Down-regulated	Tumor suppressor; metastasis suppressor	RAS, HMGA2, ER-alpha	[17, 20, 45–48]
miRNA-200	Down-regulated	Tumor suppressor; metastasis suppressor	Cadherin, vimentin, ZEB1, ZEB2	[17, 22, 23]
miRNA-125	Down-regulated	Tumor suppressor; metastasis suppressor	BMPR1B, BRMS1	[26, 28]
miRNA-205	Down-regulated	Tumor suppressor; metastasis suppressor	ZEB1, ZEB2, HER3	[23, 25]
miRNA-31	Down-regulated	Tumor suppressor; metastasis suppressor	RhoA, WAVE3	[30, 31]
miRNA-17/20	Down-regulated	Tumor suppressor	Cyclin D1	[32, 33]
miRNA-10b	Up-regulated	Metastasis promoter	HOXD10, RhoC, RhoA	[34, 36]
miRNA-155	Up-regulated	Oncogene	FOXO3a, socs1	[38, 39]
miRNA-210	Up-regulated	Metastasis promoter	Hypoxic cells, MNT	[40, 41]
miRNA-9	Up-regulated	Metastasis promoter	MYC	[43]
miRNA-373	Up-regulated	Oncogene; metastasis promoter	LATS2, CD44	[45, 46]

The EMT markers that are targeted by miR-200 family include cadherin, the marker for epithelial phenotype, and vimentin, ZEB1 and ZEB2, which are markers for mesenchymal phenotype [17]. All members of the miRNA-200 family are significantly down-regulated in cells having EMT phenotype [23], and re-expression of this family was sufficient to block the acquisition of EMT phenotype. Furthermore, ectopic expression of miR-200 family resulted in the reversal of EMT resulting in the mesenchymal-to-epithelial (MET) phenotype [17].

Our current understanding, based on consensus of cancer researchers and numerous studies, is that the expression of miR-200 family is negatively correlated with migration, invasion, and metastasis of breast cancer cells. Although one recent study [24] seemed to challenge this notion, the majority of researchers presently agree that the expression status of miR-200s correlate with a non-invasive or poorly invasive phenotype and that the loss of expression of miR-200 family is associated with cancer aggressiveness [17].

22.3.1.3 miRNA-125

There are two types of miRNA-125: miRNA-125a and miRNA-125b. Both can be classified as miRNAs with tumor-suppressing ability. Evidence in support of this phenomenon came from the observation that both are down-regulated in breast tumors [25]. In addition, certain breast cancer cells with high amounts of miRNA-125 displayed decreased ability of migration and invasion, or in other words, decreased ability to metastasize [26]. Breast Cancer Metastasis Suppressor 1 (BRMS1) was shown to up-regulate the anti-metastatic miRNAs such as miRNA-125, while at the same time down-regulate the metastatic miRNAs [26]. Thus, BRMS1 plays a keys role in the regulation of miRNA-125 and its tumor suppressing abilities.

Moreover, a few studies have indicated a correlation between single nucleotide polymorphisms (SNPs) and breast cancer [18]. Two such SNPs were found in both miRNA-125a and miRNA-125b. In regards to miRNA-125a, the polymorphism in the mature miRNA resulted in impaired maturation and function, thus elevating patient risk towards breast cancer [27]. On a similar note, a higher risk of breast cancer is possible when the target site in the 3′ un-translated region (UTR) of BMPR1B is mutated, as this is suggested to impair the function of the miRNA [28].

22.3.1.4 miRNA-205

The miRNA-205 is also widely known as a tumor suppressing miRNA. Multiple targets of miRNA-205 have been studied and these targets are affected by the presence or absence of the miRNA. The miRNA-205 is generally expressed in the myo-epithelial layer of breast cancer cells [29]. When EMT takes place, the expression of miRNA-205 is significantly down regulated, and in some cases, non-existent [29]. Targets of miRNA-205 include ZEB1, ZEB2, and HER3 [23, 25].

ZEB1 and ZEB2 are both transcriptional repressors that regulate the expression of E-cadherin [23]. E-cadherin is commonly referred to as a marker of epithelial cells, and thus cells with E-cadherin expression may be regarded as less invasive tumor cells. Therefore with, the loss of miRNA-205, such transcriptional repressors will no longer be targeted and will no longer perform their function of repressing transcription and indirectly repressing E-cadherin expression, indicating a more aggressive cancerous phenotype. The HER3 receptor is also a target of miRNA-205. When activated, the receptor inhibits the activation of the PI3K/Akt survival pathway [23]. Certain investigations have portrayed that the forced re-introduction of miRNA-205 in mesenchymal breast cancer cells led to the acquisition of MET phenotype [23, 25, 29]. Collectively, the loss of expression of miR-205 is associated with aggressive cancerous phenotype, and thus strategies for the re-expression of miR-205 could be a useful therapeutic strategy for breast cancer.

22.3.1.5 miRNA-31

Similar to the miRNAs mentioned above, studies have shown that higher levels of miRNA-31 expression in breast cancer cells confer less aggressive and less metastatic cancerous phenotype. This miRNA was able to decrease metastasis by targeting different genes that promote metastasis, such as RhoA and WAVE3 [30]. An inverse relationship between miRNA-31 expression and the expression of WAVE3 and RhoA exists [31]. The relationship is such that when miRNA-31 expression is decreased; WAVE3 and RhoA expression is increased, also increasing the progression of breast cancer associated with the acquisition of mesenchymal phenotype. Along the same lines, re-expression of WAVE3 or RhoA reversed the suppression of cancer cells from metastasizing by miRNA-31 [30, 31]. Therefore, targeted re-expression of miRNA-31 by novel strategies could also be useful for the treatments of breast cancer, and the expression status of miR-31 may also function as a molecular tool for patients' prognosis.

22.3.1.6 miRNA-17/20 Cluster

The findings showing that the expression of miRNA-17/20 cluster are absent in breast tumors suggests that the miRNA-17/20 cluster also played a significant role in the development of breast cancer. A couple of findings reported that Cyclin D1 and miRNA-17/20 cluster regulate each other, orchestrating the "regulatory feedback loop," where Cyclin D1 was found to initiate the expression of miRNA-17/20 cluster, while at the same time miRNA-17/20 inhibits Cyclin D1 [32, 33]. This is important because Cyclin D1 is known to regulate multiple genes, and amplifies the metastatic processes including the acquisition of mesenchymal phenotype. Therefore, miRNA-17/20 can potentially have a central therapeutic role in regards to breast cancer especially strategies that will allow for the re-expression of miRNA-17/20.

22.3.2 Oncogenic miRNA in Breast Cancer Cells

22.3.2.1 miRNA-10b

In contrast to the miRNAs discussed above, miRNA-10b has been found to promote metastasis of breast cancer cells. Rather than disappearing in breast tumors, it is highly prevalent and seems to have a broader effect on many features of breast cancer aggressiveness. Twist, a specific transcription factor, stimulates miRNA-10b production, and a direct correlation has been found with increased expression of miRNA-10b and the invasive and metastatic behavior of breast cancer [34–36]. Once Twist induced miRNA-10b, it goes on to suppress homeobox D10 (HOXD10), a specific messenger RNA encoding homeobox [34]. Suppression of HOXD10 causes an increase in the expression of RhoC and RhoA, which are generally categorized as pro-metastatic genes [34, 36]. However, certain studies have also shown that miRNA-10b can be downregulated by CCN5: Cysteine-rich 61-connective tissue growth factor nephroblastoma-overexpressed 5 [35]. CCN5 is regulated by Twist; thus the expression of Twist must be inhibited in order for CCN5 to be stimulated, also allowing miRNA-10b to be inhibited and breast tumor metastasis to be suppressed [35]. Furthermore, a popular therapeutic option that has been widely studied and discussed is producing anti-miRNAs for miRNAs such as miRNA-10b that amplify oncogenesis. One specific study conducted at the University of California demonstrated that an anti-miRNA-10b inhibitor positively modulated the expression of HOXD10, therefore decreasing the expression of RhoC/RhoA, allowing tumor cell invasion to slow down [34]. Despite these results, there are some studies showing no significant correlation between miRNA-10b and breast cancer metastasis [37]. Hence, further research must be done to support or repute the popular belief that miRNA-10b is a metastatic miRNA.

22.3.2.2 miRNA-155

The miRNA-155 is an additional miRNA upregulated in breast cancer cells. While it is not primarily a metastatic miRNA, it still appears to be important. The expression of miRNA-155 is inversely correlated with the expression of FOXO3a and the suppressor of cytokine signaling 1 gene, also called socs1 [38, 49]. It was found that increased expression of miRNA-155 directly correlated with decreased expression of FOXO3a in multiple breast cancer cell lines [49]. However, one study has shown that a modified version of FOXO3a, one without its 3′-UTR in the coding sequence, was able resist the negative effects of miRNA-155, such as cell survival and resistance to chemotherapy, suggesting novel and targeted therapeutic strategy could be devised using miRNA-155 and such a strategy could be incorporated with existing therapeutics [49]. Additionally, miRNA-155 is thought to stimulate oncogenesis by acting as a negative regulator

of socs1 [37]. Similar to FOXO3a, a mutation in the 3′-UTR of socs1 was identified to reduce the expression of miRNA-155 in breast tumors [37]. Multiple other studies have also found that variations in the 3′-UTR generally cause a decrease in the number of miRNA binding sites, subsequently repressing the harmful effects of miRNAs in such cells. Thus, this may also be a possible approach by which one could regulate different miRNAs such as miRNA-155 in order to find a more effective remedial approach to breast cancer therapy.

22.3.2.3 miRNA-210

The expression of miRNA-210 has been noticeably up regulated in breast tumors while promoting metastasis [50]. Specifically, miRNA-210 was elevated in cells existing in a hypoxic environment, an environment containing a relatively low concentration of oxygen [50]. Hypoxic breast cancer cells stimulated the production of miRNA-210 under the transcriptional control of the hypoxia-inducible factor (HIF) pathway [51]. Similar to miRNA-155, miRNA-210 binds to the 3′-UTR of MNT, an MYC antagonist that is a direct target of miRNA-210 [40]. Thus, if MNT is mutated then the progression of the cell cycle is affected where the ability of miRNA-210 to overcome hypoxia-induced cell cycle arrest is abrogated, thus inducing the survival of existing breast cancer cells and eventually leads to metastasis [40]. Therefore, miRNA-210 targeted therapeutic approach could be useful in breast tumors that are highly aggressive and associated with hypoxia [42].

22.3.2.4 miRNA-9

Another miRNA that is activated by MYC due to binding at the 3′-UTR is miRNA-9, and it was also found to be highly expressed in various breast cancer cell lines [43]. The miRNA-9 is able to down-regulate the expression of E-cadherin by targeting CDH1, which is highly significant because E-cadherin is referred to as an epithelial marker. Thus down-regulation of its expression indicates a more invasive and metastatic behavior [43, 44]. Furthermore, certain studies have illustrated that miRNA-9 was able to positively modulate the expression of vimentin in certain cell lines, but not all, suggesting that in certain cells within a particular environment, miRNA-9 has the ability to promote EMT, instigating more aggressive behavior of breast cancer [43].

22.3.2.5 miRNA-373

The elevated expression of miRNA-373 in breast cancer cells also promotes oncogenesis by stimulating tumor invasion and metastasis. Originally, all that was known about miRNA-373 was that it is able to form tumorigenic cells from regular

epithelial cells [45], and further experimental proof came from studies showing that miRNA-373 could directly targets the LATS2 tumor suppressor gene in addition to CD44, a metastasis suppressor [45, 46]. Inhibition of the expression of LATS2 suppresses the p53 pathway and ultimately leads to tumorigenesis [45]. Down-regulation of CD44 on the other hand leads to breast cancer metastasis [46]. These results collectively suggest the oncogenic role of miRNA-373 in breast cancer; however, further studies are warranted to prove whether targeting miRNA-373 could be a useful strategy for designing novel therapies for breast cancer.

22.4 Treatments, Clinical Applications, and the Future with miRNAs in Breast Cancer

There are multiple reasons for in-depth continued investigations on miRNAs. Although significant progress has been made to-date, the therapeutic implications of miRNAs intrinsically associated with the biology of breast cancer remains to be uncertain. First and foremost, miRNAs play significant roles as diagnostic and prognostic markers of various cancers, including breast cancer. However, there are some contradictory findings as to the relevance of the expression of miRNAs in different types of cancers, and occasionally in the same type of cancers that, challenges the paradigm of the generic classification of each miRNA as tumor suppressing or tumor promoting. Overall, the metastatic progression of breast cancer can be determined by the aberrant expression of certain miRNAs, allowing for a more accurate diagnosis and prognosis of the cancer.

Another important advancement in breast cancer treatments involving miRNAs is the use of antagomirs. Antagomirs are able to knock-down the function of targeted miRNAs by competitively inhibiting their function [6], and although this approach is a sound experimental approach much remains to be done for appreciating the value of miRNA targeted therapy for human breast cancer. This is an exciting area of research especially because anti-miRNAs would be able to bring the cancer cells back to its normal phenotype. Conversely, re-expression of lost miRNAs in breast cancer could also be useful for the treatment of breast cancer and such novel strategies would be the welcome news to millions of women who are inflicted with this deadly disease.

Recent studies have demonstrated the effectiveness of the use of natural agents like curcumin and isoflavone as adjuncts to chemotherapeutics [47] especially because such natural agents are able to modulate miRNA expression in cancer cells by allowing them to become more sensitized to the conventional chemotherapeutics. Furthermore, chemotherapy or other targeted therapies can be improved by deregulating the miRNAs that are involved in specific pathways, like apoptotic or proliferative pathways [2]. While such methods are possible, in order to successfully employ the use of miRNA targeted agents in cancer therapy, the stability of such strategies must first be perfected. The antagomirs and anti-miRNAs are not always

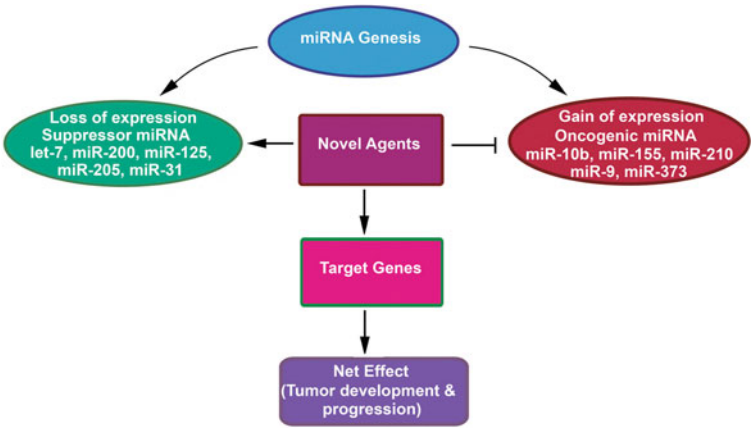

Fig. 22.1 miRNAs and the treatment pathway

stable, and their stability must be optimized in order to prove their therapeutic potential. Moreover, studies are being conducted in order to ensure effective transfer of miRNAs into cancer cells, but the molecular biology of the role of miRNAs within the tumor microenvironment still require in-depth investigations. However, the future appears to be brighter than ever before in understanding the role of specific miRNAs and their targets in a given tumor system especially in different sub-types of breast cancer, which will allow for the development of novel strategies either to activate or inactivate the expression of specific miRNAs for breast cancer therapy. Figure 22.1 provides a brief glimpse into the mechanism of the pathways between potential treatments and the discussed miRNAs.

22.5 Conclusion

Breast cancer remains to be one of the leading causes of morbidity and mortality in women in the United States and in the world. The Last decades have witnessed significant progress in our understanding of the development and progression of breast cancer together with advanced molecular understanding of breast cancer aggressiveness. With this deeper understanding, we have been able to identify innovative mechanisms, and further assisted in the developed of targeted agents in the fight against breast cancer; whereas the field of miRNA research and miRNA targeted therapeutic development is still in its infancy. Empowered with the knowledge from the discovery and identification of specific miRNAs present in breast cancer, and their role in the up-regulation or down-regulation of their specific target genes as shown in Fig. 22.1, will allow for the development of strategies for the use of miRNA in the diagnosis, predicting prognosis and assessing therapeutic

outcome. Moreover, this knowledge will allow for developing miRNA targeted therapeutic strategies for pre-clinical in vitro and in vivo animal studies, and the positive outcome of such studies will lead to the translation of such an approach in the clinical setting for ultimate testing. In conclusion, the field of miRNA research is very fertile and the seed has already been planted, and we are only waiting to harness the fruits of such research in order to eradicate breast cancer.

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Chapter 23
Erratum to: Bone Metastasis of Breast Cancer

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