Saima Shakil Malik ·
Nosheen Masood *Editors*

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This book is dedicated to the patients and their families past and future who have both encouraged and will one day benefit from the countless advancements in cancer genomics and to our own families for their ongoing patience and support for this book.

Foreword

In the past 20–30 years, we have witnessed the emergence of new areas with enormous contribution to the understanding of breast cancer biology. With the advancement in the field of steroid hormone nuclear receptors, responsible for controlling various genetic programs and their expression involved in cellular processes which are essential for normal and aberrant cell growth, plays a key role in the therapy and prevention of hormone-dependent cancers. The findings of the association between hereditary breast cancers and BRCAl and BRCA2 gene mutations, and the conception that they broadly represent a variety of mutations with varied degrees of penetrance within given families, have incorporated substantial understanding to the molecular and pathological basis of breast cancer. These advancements have been fastened with the development of recombinant DNA technology, being a revolutionary tool in probing the human genome. This explosion of knowledge on the genetic and hormonal basis of breast cancer with substantial advances in its early detection and therapeutic strategies have opened great hopes for the conquest of this disease. However, still the developed and developing countries both are facing the greatest burden of breast cancer mortality possibly due to changes in reproductive patterns and adopting westernized lifestyle.

The recognition that traditional developmental concepts need to provide the basic agenda for the elucidation of data generated by these modern techniques and novel therapeutic targets to explain the breast cancer etiology, pathogenesis, and progression has led Dr. Saima and her co-editor to design this book. This book has beautifully explained all the aspects of breast cancer from hormonal status to molecular profiling to diagnosis to therapeutic options to chemo-tolerance and the introduction to personalized medicine.

Towards the end, I would like to congratulate Dr. Saima and her co-editor for accomplishing this challenging task of writing a scientific book for pathologists, healthcare professionals, and breast cancer patients. Simplification of difficult terms without compromising readers' interest is extremely pleasant. I highly value Dr. Saima and Dr. Nosheen's immense efforts to turn this long awaiting book into reality!

The Foundation University Islamabad
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Mehreen Baig

Preface

The first part of the book explains about the foundations of breast cancer, briefly explains introduction to the disease, its epidemiology, risk factors, histopathological classification, subtypes depending on receptor status along with clinical and pathological staging. It also sheds light on relationship of breast cancer with other hormone-sensitive cancers. This knowledge is essential for determining the functional relevance of hormonal and genomic changes in cancer initiation and progression and for developing strategies for breast cancer prevention along with its relevance with other cancers. A chapter about cancer care and psychosocial needs would be valuable addition to this part. Breast cancer, with a complex landscape, requires discreate strategies to manage different molecular subtypes of this disease. Rapid advancements in the field of molecular biology have been mystifying for those involved in its study, detection, and management.

With the advent of next-generation sequencing, new insights have been provided in genomic and transcriptomic regulation. Alterations in the DNA structure/sequence have been correlated with varied disease outcomes and provide ways for novel therapeutic approaches. These advanced technologies have revealed the extensive contributions of epigenetic mechanisms such as histone modifications, non-coding RNA, and alternative splicing. All these changes together contribute to alterations in proteome with drastic consequences. Therefore, next part of the book covers the BRCA1-, BRCA2-associated breast carcinogenesis (screening, diagnosis, prevention, and limitations), early-stage progression of breast cancer (molecular classification, role of high and low penetrance genes in breast cancer), noninvasive biomarkers for early detection of breast cancer highlighting the mutational spectrum, and their role in response to particular drugs and adjustable dosage regimes. Breast cancer has variable disease heterogeneity therefore, discussing epigenetical involvement, role of fibrolytic mechanisms, microRNAs, non-coding RNAs and circulating tumor cells in breast cancer diagnosis, prognosis and treatment with specific influence on the concept of precision medicine would be helpful in understanding the complex and multifactorial disease etiology. The second part of the book also includes information about novel drug targets like PARP inhibitors in breast cancer leading towards personalized treatment with better survival rates, neoadjuvant, metastatic, and combination settings along with relationship to hormone receptor tumor types.

Breast cancer is caused by alteration in many types of proteins/hormones. There are many molecular subtypes of breast cancer and oncologists have come across resistance to therapies. Triple-negative breast cancer patients do not respond to many chemotherapeutic drugs and ultimately lead to death or recurrence. In the third part of the book, therapeutic options in BRCA1-linked breast cancer and systemic approaches, biomarkers for predicting drug response and disease severity, pros and cons of currently available drug regimens considering pharmacogenomics approach, transcriptional control leading to clinical outcomes in breast cancer cases, utility of personalized medicine in the treatment of different subtypes of breast cancer, association of molecular progression of breast cancer and personalized medicine in terms of clinical trials, chemo-tolerance of breast cancer and its management by personalized medicine, advances in breast cancer surgical pathology, and modern radiation therapy techniques and their toxicities for breast cancer. It also investigates the preclinical and clinical stage of nanostructures and nanomedicine for dealing with nanomedicine translation in breast cancer theranostics. It explains the discovery of new nanomedicines and their role in the early-stage breast cancer diagnosis and treatment. This part focuses on the design, characterization, and standardization of breast cancer nanomedicine and would be a great addition in this book. This book is an up-to-date collection of information with a novel idea of summarizing all the treatment options in one place. As therapeutic options are increasing day by day, there is always room for such books that gather all latest information from time to time. It is an ideal book for practicing clinical oncologists as well as trainees.

Overall, this book is a single-source collection providing complete insight into molecular and pathological basis of breast cancer using genomic, proteomic, computational, hormonal, and nanobiotechnological approaches.

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Rawalpindi, Pakistan Nosheen Masood

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Our special thanks go to Dr. Bhavik Sawhney (Associate Editor-Biomedicine Springer Nature), Selvakumar Rajendran (Production Editor), and whole production team for their countless support in the preparation of this book.

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About the Editors

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Nosheen Masood is currently working as an Assistant Professor at the Department of Biotechnology, Fatima Jinnah Women University, Rawalpindi, Pakistan. She completed her Ph.D. in Human Molecular Genetics at the Department of Biosciences at COMSATS University, Islamabad, Pakistan. Her main research interests include molecular biology and cancer genetics, focusing on epidemiological studies, risk assessment, genomic analysis, immunohistochemistry-based diagnostics, and protein–protein interactions. She has received various prestigious awards, notably, the Research Productivity Award 2014–2015 from the Pakistan Council of Science and Technology (PCST) category D, and Research Productivity Awards from COMSATS Institute of Information Technology for 6 consecutive years from 2010 to 2015. She has served as a reviewer for several international journals and has teaching experience in biotechnology, molecular biology, comparative genomics, environmental biology, regulation of gene expression, and molecular evolution. Dr. Masood has published several research articles in respected, peer-reviewed international journals and authored or co-authored various book chapters.

Part I

Overview of Breast Cancer

Epidemiology and Risk Factors of Breast Cancer

1

Tabassum Zafar, Ab Qayoom Naik, Manoj Kumar, and Vinoy K. Shrivastava

Abstract

The epithelial glandular tissue forms a major part of the breast cancer origin followed by lobular glandular tissue. The initial growth of the tumor is limited to the duct or lobule, without any symptoms and metastasis. The progression of the disease includes invasion of the neighboring breast tissue (invasive breast cancer), affecting nearby lymph nodes (regional metastasis), or spreads to other organs of the body (secondary metastasis or distant metastasis). The excessive metastasis because of tumor development leads to death of the patient. Breast cancer is considered the most common cancer with 2.3 million of women diagnosed with breast tumor and 685,000 deaths reported worldwide. During the last 5 years, 7,800,000 cases of breast tumor were reported globally. Breast cancer is neither an infectious nor transmissible illness. Other than gender (female) and age, breast tumor presents no manifestation of disease for almost 40 years of the age. Some of the significant contributing factors of the disease include harmful alcohol use, age, obesity, family history, exposure to harmful radiations, and reproductive factors. Besides, some factors are responsible for aggravating the incidence of breast cancer such as age at first menstrual period, use of tobacco, age at first pregnancy, and postmenopausal hormone therapy. The age at first menstrual period and age at first pregnancy, tobacco use, and postmenopausal hormone therapy are all variables that raise the risk of breast cancer.

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Keywords

Breast cancer · Epidemiology · Global distribution · Risk factors · Diagnosis

Abbreviations

25(OH)D	Vitamin D3 25-Hydroxyvitamin D
AFP	Alpha-fetoprotein
AICR	American Institute for Cancer Research
ATM	Ataxia telangiectasia mutated
BMI	Body mass index
BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
CDC	Centers for Disease Control and Prevention
CDH1	Cadherin 1
CHEK2	Checkpoint kinase 2
CK5/6	Cytokeratin 5/6
DCIS	Ductal carcinoma in situ
DES	Diethylstilbestrol
EDC	Endocrine-disrupting chemicals
EPIC	European Prospective Investigation into Cancer
ER	estrogen receptor
GLOBCON	Global Cancer Observatory
HCG	Human chorionic gonadotropin
HER2	Human epidermal growth factor receptor 2
HRT	Hormone replacement therapy
IARC	International Agency for Research on Cancer
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
NBN	Nibrin
NF1	Neurofibromin 1
NIEHS	National Institute of Environmental Health Sciences (NIEHS)
NTP	National Toxicology Program
PALB2	Partner and localizer of BRCA2, also known as PALB2 or FANCN
PR	Progesterone receptor
PTEN	Phosphatase and TENsin homolog deleted on chromosome 10
SIR	Standardized incidence ratio
TNBC	Triple-negative breast cancer
WCRFI	World Cancer Research Fund International

1.1 Introduction

The uncontrolled cellular growth of the breast tissue is referred to as breast cancer and is the most common female malignancy globally, with a high cure rate if treated at a non-malignant state. Depending on the type of breast cells, which turn into cancer, breast cancer is of different types and begins in different parts of the breast. All three parts of breast histoarchitecture including lobules (milk producing glands), ducts, and connective tissue (fibrous and fatty tissue). The ducts or lobules are the primary sites of breast cancer initiation. The metastasis affects other body parts through blood and lymph vessels (Fig. 1.1). The most common breast cancers include-

1.1.1 Invasive Ductal Carcinoma

The tumor development affecting other body parts and breast tissues other than ducts can metastasize to distant regions.

1.1.2 Invasive Lobular Carcinoma

Tumor cells from the lobules are potential tumor spreading cells that spread throughout the body, besides affecting neighboring breast tissues and cells. The less frequent breast cancers are Paget's disease, medullary mucinous, and inflammatory breast cancer.

Ductal carcinoma (DCIS) is a type of breast cancer in the early stages. The cancer cells have only spread to the duct lining and have not spread to other breast tissues. The characteristic symptoms of breast cancer vary from person to person, while some people have no disease symptoms. Some prominent warning signs of breast cancer include-

- Presence of new lump/outgrowth in the breast or armpit.
- Thickening/swelling of the part of the breast.
- Irritation/dimpling of breast skin.
- Redness or flaky skin in or around the nipple.
- Pulling in of the nipple/pain in the nipple area.
- Nipple discharge apart from breast milk.
- Change in the size/shape of the breast.
- Pain in any area of the breast.

There is no typical like the breast. The look and feel of the breasts may be affected by periods, having children, weight gain or weight loss, medications, and age. There are many conditions responsible for breast lumps, including cancer. However, besides cancer most of the lumps occur due to the conditions like fibrocystic breast condition, and cysts being the most common conditions. The fibrocystic state is

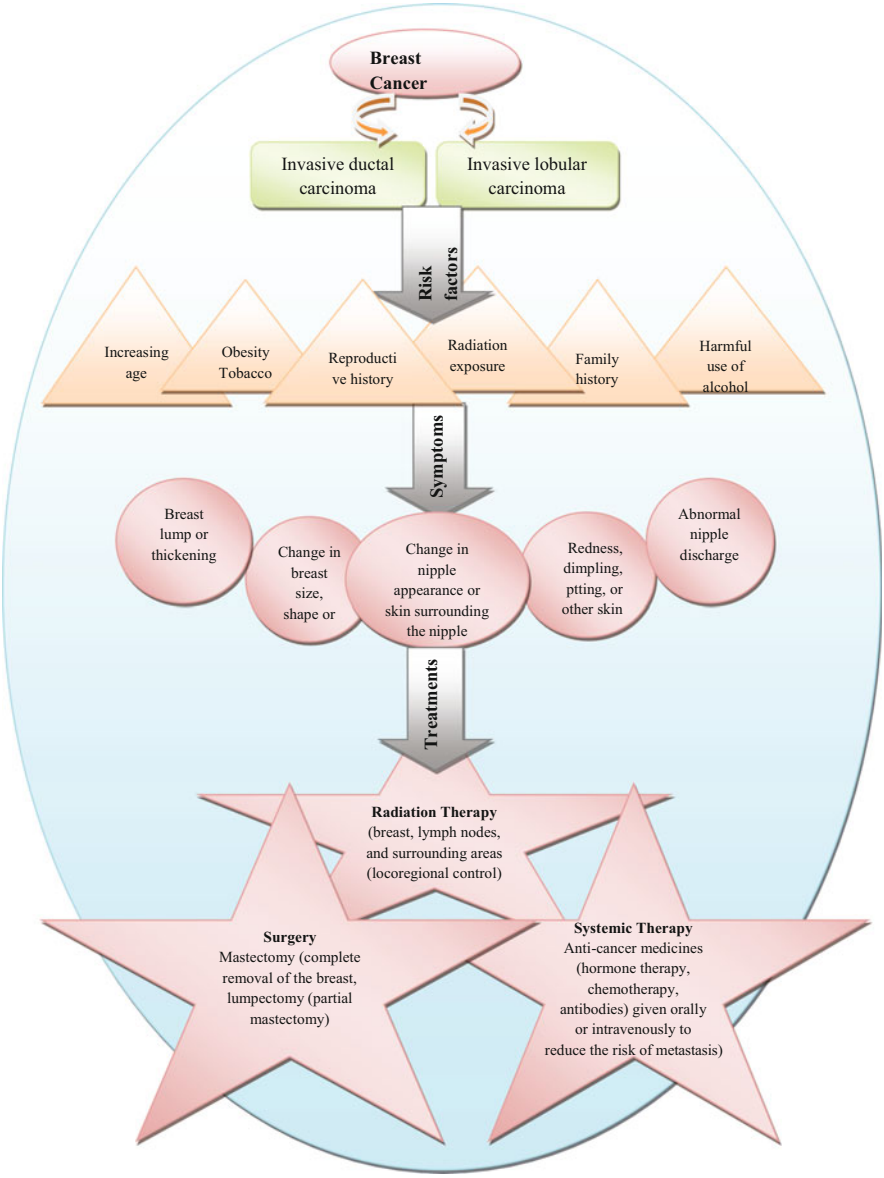

Fig. 1.1 Risk factors, symptoms, and preventation of breast cancer

responsible for non-malignant changes like formation of lump/outgrowth or tender, and sore breasts. On the other side, cysts are fluid-filled sacs that can develop in the breast (Division of Cancer Prevention and Control 2021).

1.2 Morphological and Molecular Classification of Breast Tumors

1.2.1 Morphological Classification

Traditional classification divides cancers into distinct categories based on their shape, which differ in behavior and prognosis. Breast tumors are classified morphologically according to their origin, the terminal ductal lobular units, and are divided into ductal and lobular neoplasia (Russo and Russo 1999). Besides, rare morphological types are tubuloductal, comedo, medullary, mucinous, and Paget types (Kumar et al. 1997). Treatment and prognosis mostly effected by morphological type. Breast tumors frequently have a variety of morphological forms, implying that a single genetic background can result in tumors of various morphologies. Non-specific ductal carcinoma and particular subtypes of invasive breast cancer are the current classifications for invasive breast cancer. Subtypes of breast cancer have distinct criteria; however, the non-specific type is a dumpster full of carcinomas that are not classified as specific subtypes. 60–75 percent of all breast cancers are non-specific invasive ductal carcinomas. The most prevalent kinds within this group are lobular, tubular, papillary, and mucinous tumors, which account for 20–25% of all tumors (Ellis et al. 2003; Weigelt and Reis-Filho 2009). Heterogeneity inside a single tumor (intratumorally) or between morphologically identical cancers of the same type (intertumoral) is now widely recognized. As a result, pathologists have developed new technologies that will allow doctors to monitor their patients better. The "histological grade," which is resolved by evaluating the stage of tumor differentiation (tubule production), nuclear pleomorphism, and proliferation (mitosis rate), is a necessary component of pathology reports.

1.2.2 Molecular Classification

Breast carcinoma is a collection of disorders with distinct clinical, histopathologic, and molecular characteristics. Breast cancer is a heterogeneous disease encompassing a broad range of cellular compositions, genetic changes, and clinical manifestations. Breast cancer molecular subgroups based on histological tumor grade and lymph node metastasis are powerful symptomatic and diagnostic indicators.

As a result, dividing breast cancer into relevant molecular subtypes is an essential part of treatment planning. Classical immunohistochemistry (IHC) markers including estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 play a crucial role in molecular subtyping (Viale 2012). The development of advanced methods including gene expression profiling (using complementary DNA microarrays) has therapeutic importance for molecular classification. Immunohistochemical study of tumors based on estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 is clinically practiced and is more accessible, and cost-effective having reliable results for molecular subtypes

(Goldhirsch et al. 2011; Kumar et al. 2013). Tumor immunohistochemistry-based molecular subtyping is now the gold standard for predicting tumor responsiveness to hormone therapy and successive trastuzumab therapy (Goldhirsch et al. 2011; Andre and Pusztai 2006). Newer classification approaches based on immunohistochemical, genetic, and molecular discoveries are also being developed (Kurian et al. 2010). Around 30 years ago, the advent of hormone (estrogen and progesterone) receptor markers signaled the start of molecular classification. Following the preceding breakthroughs, HER2/neu-based detection approaches were developed. Molecular subtyping based on tumor immunohistochemistry is now the gold standard for predicting tumor sensitivity to hormone therapy and successive trastuzumab therapy. Newer classification approaches based on immunohistochemical, genetic, and molecular discoveries are also being developed (Andre and Pusztai 2006; Kurian et al. 2010). Around 30 years ago, the availability of hormone receptor markers (Er and PR) signaled the start of molecular classification. Following the preceding breakthroughs, HER2/neu-based detection approaches were developed. Luminal A (ER+/PR+/HER2−/low Ki-67); Luminal B (ER+/PR+/HER2−/+/high Ki-67); HER2-overexpression (ER−/PR−/HER2+); and triple-negative breast cancers/TNBCs (ER−/PR−/HER2−) are the molecular subtypes of breast cancer according to the St. Gallen Consensus 2011. (Goldhirsch et al. 2011). TNBC, a basal-like subtype of breast cancer, was found to have basal marker (CK5/6) expression (Elesawy et al. 2014; Zhang et al. 2012).

1.3 Global Distribution and Epidemiology of Breast Cancer

The incidence of breast tumor occurrence has shown a steady increase during the last 50–70 years. Breast cancer is the most reported cancer type in females worldwide. Generally, developed countries have higher rates of breast cancer than developed countries due to certain lifestyle, and reproductive factors prevalent in developing countries. The increased incidence of breast cancer in developing countries is relatively exaggerated due to lower screening and incomplete reporting in such countries (Shulman et al. 2010). Breast cancer is the most prominent death cause in females living in developing countries. One of the most likely contributing factors leading to the global increased incidence in several reported cases is the Westernization of developing countries (GLOBOCAN 2012; Komen 2016). It is most frequently diagnosed in the age group of 55–64 years, and the median age at diagnosis is 61 years (Howlader et al. 2020). Less than 5% of breast cancer cases are reported in women below the age of 40, but the risk increases with age (National Cancer Research Institute 2021). However, the rate at which it increases decreases after menopause (Clavel-Chapelon and Gerber 2002). The female breast cancer is the main cause of death from cancer among women worldwide, accounting for one out of every ten new malignancies diagnosed each year. According to World Cancer Research Fund, there were more than two million breast cancer cases in 2018.

Factors responsible for premenopausal breast cancer include consumption of alcoholic beverages, a higher birth weight, and adult achieved height. According

to the Continuous Update Project Panel, the World Cancer Research Fund International (WCRFI) initiative, there is significant proof that robust physical exercise and higher body fatness prevent from premenopausal breast tumor. The panel found substantial evidence that alcoholic beverages, increased adulthood body corpulence, weight gain, and adult achieved height are all risk factors for postmenopausal breast cancer. Moreover, there is significant proof that increased body fatness and physical activity (particularly vigorous physical activity) in young adulthood protect against breast cancer occurrence during postmenopausal conditions. Likewise, there was substantial evidence that breastfeeding protects against breast cancer (unspecified menopausal status).

According to WHOs, International Agency for Research on Cancer, number of new breast cancer cases (of all ages and sexes) reported in 2020 were 2,261,419 which is 11.7% of all the cancer cases. The global mortality rate of breast cancer (of all ages and sexes) in 2020 was recorded at 6.9% of all the cancers, i.e., out of the 9,958,133 cancer mortalities of all types, 684,996 deaths were due to breast cancer. The worldwide and regional distribution of breast cancer incidence, mortality, and prevalence (both sexes) is shown below (World Health Organization (WHO) 2020).

1.4 Risk Factors Influencing Tumor Behavior

1.4.1 Genetic Factors

Heritable gene expression alterations that occur without changing the DNA sequence are known as epigenetic modifications. They have a pivotal involvement in the progression and prognosis of cancer. Accumulation of incorrect gene regulation leads to breast cancer. In addition to genetic factors, epigenetic mechanisms have a crucial role in breast tumor carcinogenesis. Changes or mutations in any of the genes alter cell growth, survival, and cellular function. Typographical errors in the DNA lead to wrong communication and direction, resulting in defective cell development (Table 1.1). These manual instruction errors result in the progression of disease that is beyond human control in natural settings.

1.4.1.1 BRCA

The inherited changes accumulate over a period, and are taken over by children from their parents. Inherited DNA changes are called germ-line alterations or mutations that cannot be stopped. Changes in BRCA1 and *BRCA2* are one of the leading causes of cancerous growth. *BRCA* genes are usually involved in DNA repair and cell damage and regulate the breast, ovarian, and other cells to grow normally (Shaik et al. 2021). Mutations that transfer from generation to generation make the individual prone to cancer progression. *BRCA1* and *BRCA2* mutations are widely validated cause of female cancers (10% of all breast cancers, or 1 out of every 10 cases). There are several genetic factors responsible for the incidence of breast cancer. Of the several genetic factors, BRCA1 and BRCA2 gene mutations inherited through autosomal dominant inheritance are responsible for nearly 40 percent inherited

Table 1.1 Genetic factors and their associated mutational disorders

Gene	In vivo function	Chromosome position	Level of associated risk	References
BRCA1	BReast CAncergene-1	17q21.31	45–87%	Thompson (2002); Antoniou and Easton (2006)
BRCA2	BReast CAncer gene-2	13q13.1	50–85%	Hoskins et al. (2008); Stratton and Rahman (2008); Antoniou and Easton (2006)
TP53	Tumor protein p53	17p13.1	20–40% & more	Børresen-Dale (2003); Garber et al. (1991); Birch et al. (2001)
CDH1	Cadherin-1	16q22.1	63–83%	Heitzer et al. (2013); Pharoah et al. (2001)
PTEN	Phosphatase and TENsin homolog deleted on chromosome 10	10q23.31	50–85%	Fusco et al. (2020); FitzGerald et al. (1998); Tan et al. (2012)
CHEK2	Checkpoint kinase 2	22q12.1	20–25%	Rainville et al. (2020)
PALB2	Partner and localizer of BRCA2	16p12.2	33–58%	Hu et al. (2020)
STK11	Serine/threonine kinase 11	19p13.3	32–54%	Angeli et al. (2020); Lim et al. (2004)
ATM	Ataxia telangiectasia mutated	11q22.3	20–60%	Foretová et al. (2019); Marabelli et al. (2016)
XRCC2	X-ray repair cross-complementing protein 1	7q36.1	ND	Kluźniak et al. (2019)
BRIP1	BRCA1 interacting protein 1	17q23.2	ND	Cantor and Guillemette (2011)

breast tumor cases (Cobain et al. 2016). According to a study, 55–65% BRCA1 mutation carriers along with 45% BRCA2 mutation carriers develop breast cancer around 70 years of age (Godet and Gilkes 2017). A prospective cohort study revealed that the likelihood of progressive breast cancer around 80 years was more than 70% in the BRCA1 carrier mutation (Kuchenbaecker et al. 2017). The onset and progression of breast cancer are also attributed to the changes in human interferon α-2b besides other risk factors (Ahmed et al. 2016; Yari et al. 2014).

1.4.1.2 PALB2

The PALB2 factor codes for a macromolecule that along with BRCA2 repair broken DNA and slow development. Another factor known as the PTEN factor is concerned with cell growth regulation. Cowden syndrome is associated with the development of benign (non-cancerous) and cancerous growth in the breast and a few other organs such as the GI tract, thyroid, uterus, and ovaries.

1.4.1.3 TP53

The *TP53* gene instructs the body for the synthesis of a protein that terminates tumor development. The inheritance of abnormal *TP53* gene causes Li-Fraumeni syndrome, a disorder related to the progression of soft-tissue cancers at an early age. Patients with this rare syndrome are likely to get encounter breast cancer, leukemia, brain tumors, and sarcomas.

1.4.1.4 ATM

The ATM sequence helps repair broken DNA. Ataxia-telangiectasia is a rare illness responsible for retarded brain development. Inheritable one abnormal ATM sequence has been connected to associate degree hyperbolic incidence of carcinoma and familial carcinoma. That's the result of the specific sequence inhibiting the cellular repairing of broken DNA.

1.4.1.5 CDH1

The CDH1 sequence forms a macromolecule that supports cells bind along to create tissue. Associate degree abnormal CDH1 sequence will increase the likelihood of a rare abdomen cancer at an associate degree young age.

1.4.1.6 CHEK2

The CHEK2 sequence directs for a macromolecule synthesis responsible for suppression of tumor development. Associate degree abnormal CHEK2 sequence will have a minimum of double the period risk of carcinoma. It may increase large intestine and glandular carcinoma risk.

1.4.1.7 STK11

The STK11 sequence promotes cell growth regulation. Associate degree abnormal STK11 sequence causes Peutz-Jeghers syndrome, a rare disorder within which individuals tend to develop a kind of polyp, referred to as a hamartomatous polyp, principally within the bowel, however conjointly within the abdomen and colon.

1.4.1.8 NF1

Autosomal dominant disease kind one caused due to NF1 mutation increases the chance of central system. An NF1 mutation causes a condition referred to as autosomal dominant disease kind one, which will increase the chance of central nervous system cancers and a specific kind of cancer affecting the abdominal wall or intestines, referred to as canal stromal tumors.

1.4.1.9 NBN

The production of a macromolecule, nibrin responsible for DNA damage in cells is controlled by the NBN sequence. Associate degree abnormal NBN sequence causes metropolis breakage syndrome, which ends up in slow growth in infancy and time of life.

1.4.2 Epigenetic Factors

Epigenetic changes are inherited changes in gene expression without any alteration to the DNA sequence. Epigenetic factors critically affect the cancer progression and prognosis (Karsli-Ceppioglu et al. 2014). This type of altered gene regulation also results in the progression of breast cancer. Methylation of DNA, histone modification, and chromatin structure are factors that affect the misreading of DNA sequences and their abrupt expression.

Methylation of the fifth positioned cytosines within DNA during post-replication modification is almost exclusively found to impact epigenetic modification (Bird 2002). Methylated cytosines are much more likely to be modified by endogenous and exogenous mutagenic factors (Pfeifer and Besaratinia 2009).

DNA packaging into chromatin is a highly sophisticated and dynamic process of forming protein–DNA complex, which further comprises the regulation of transcription. Any alteration between open (euchromatin) and closed (heterochromatin) chromatin close crosstalk by histone protein modification also contributes as an epigenetic factor to the chances of breast cancer.

Apart from genetic mutations, these epigenetic mechanisms are also an important influence of breast cancer. Research findings validate the role of aberrant epigenetic regulations in breast cancer occurrence. Identifying new epigenetic biomarkers and better understanding molecular mechanisms are potential future approaches towards breast cancer diagnosis and management (Nithya and ChandraSekar 2019; Madanikia et al. 2012).

1.4.3 Demographic Factors

Some demographic factors also play a vital role in breast cancer progression, including age, gender, and blood group. Breast cancer is a less common issue in men, while the disease is unique to women. Older males with endocrine disbalance radiation encounter, pedigree of breast cancer, mutation of BRCA2 generate some of the contributing factors to make them more susceptible to this disease (Giordano et al. 2002; Shaik et al. 2020; Abdelwahab Yousef 2017). Breast cancer incidents are more likely to occur in older age patients with a very high chances nearby menopause with a little decline later. Older age at the first full-term pregnancy increased the risk of breast cancer (Mahouri et al. 2007; Kim et al. 2015; Thakur et al. 2017).

1.4.4 Blood Group

Although no well-established correlation can be considered to discuss the possibility of breast malignancies in females of various blood groups, some studies are available to discuss the correlation between certain blood group type and breast cancer occurrence. Rhesus positive and blood group A bearing females are more likely to

develop breast cancer in comparison to the females bearing Rh −ve and AB blood (Saxena et al. 2015; Meo et al. 2017; Flavarjani et al. 2014).

1.4.5 Reproductive Factors

The relation between reproductive and endocrine dependent reasons of breast malignancies associated with the effect of female sex hormones rush during early adolescence exists which continue throughout the reproductively active period including several pregnancies and menopause (Thakur et al. 2017).

1.4.5.1 Menarche Age

Early menarche increases the probability of developing breast carcinoma two times (Laamiri et al. 2015). With a little exception, a large population study research that includes 11,000 women revealed that early menarche plays a vital role in expression of breast malignancy (Wu et al. 2006; Tamakoshi et al. 2005; Nguyen et al. 2016). However, durations of menstrual cycle and breast carcinoma are less likely to corelate (Fioretti et al. 1999). Menopause above 50 years was associated with a high occurrence rate of breast disease progression (Kim et al. 2015; Thakur et al. 2017).

1.4.5.2 Full-Term Pregnancy

Parous women are less likely to encounter with breast malignancy in comparison to women with high parity (Ma et al. 2010). A slight increase in breast cancer incidence has been associated with high age at initial complete term of pregnancy. According to some research, completion of early full-term pregnancy has an association with less vulnerability to breast histoarchitecture. Nulliparity is linked to an increased risk of breast cancer after the age of 40 years. Regardless of age at first birth, multiple full-term pregnancies lower the chance of breast cancer diagnosed after 40 years. On the other side, they may enhance the chances of breast carcinoma progression in females fewer than 40 years (Kelsey et al. 1993; Balekouzou et al. 2017). Besides, early age pregnancies also contribute to decreased prospects of breast tumor by up to 23% (Laamiri et al. 2015). The young age of first childbirth (around 26 years) has a positive correlation with lobular disease. The initial pregnancy during later years has a positive association of breast carcinoma progression (Williams et al. 2018). First full-term pregnancy at the age of 20 years and beyond does contribute at 40–50% of increased chances of breast tumor development (Clavel-Chapelon et al. 1995; Palmer et al. 2003; Bhadoria et al. 2013).

1.4.5.3 Abortion

Abortion is one of the reproductive factors of unclear etiological role in developing breast cancer. A higher incidence rate of abortion was associated with a high chance of breast cancer occurrence by one study, while another study could not find the same. However, a reanalysis of 53 epidemiological studies could not find any

association between naturally induced abortion with induction of breast carcinoma (Bhadoria et al. 2013).

1.4.5.4 Menstrual Cycle

Ovulatory menstrual cycle is one of the reproductive factors that show protection against breast malignancy (Balekouzou et al. 2017). The ovulatory menstrual cycle and some pregnancy characteristics, including initial pregnancy play a vital role in breast tumor progression. The mothers who deliver the first child (before 33 weeks) with early gestation are two folds more likely to encounter with breast carcinoma in later years. The results of a study revealed that multiple births increase the risk of developing breast cancer. According to the study, placental abruption is associated with an increased risk of developing breast cancer (Innes and Byers 2004). Several studies have also indicated the protective role of preeclampsia in breast carcinoma. Low estrogen, low insulin-like growth factor 1 (IGF-1) elevated IGF-1-binding protein, high human chorionic gonadotropin (HCG), and alpha-fetoprotein (AFP) have active effect on the disease occurrence (Vatten et al. 2002; Brasky et al. 2013).

1.4.6 Endocrine Factors

Tumor progression happens under the influence of various endocrine factors. Some of the factors are discussed here briefly.

1.4.6.1 Contraceptive Methods

Reports are available to discuss the role of the contraceptive pills in breast carcinoma enhancement (Marchbanks et al. 2002; Beaber et al. 2014; Kotsopoulos et al. 2014; Williams et al. 2018). However, McDonald and co-workers reported that contraceptive tablets had no association with an elevated risk of the breast tumor formation (Marchbanks et al. 2002). Medroxyprogesterone acetate usage is known to effect as an active enhancer of mammary gland carcinoma (Skegg et al. 1995). On the other hand, the withdrawal of endocrine contraceptives decreases the chances of breast cancer prospects; 5–10 years post withdrawal (Zolfaroli et al. 2018).

1.4.6.2 Ovulation-Stimulating Drugs

The prolonged use of ovulation-stimulating medications may enhance the possibility of breast carcinoma occurrence (Taheripanah et al. 2018). However, several studies state that ovulation-stimulating medications do not have any adverse effect on breast malignancy (Lerner-Geva et al. 2006; Brinton et al. 2004).

1.4.6.3 Postmenopausal Hormone Therapy

Hormone replacement therapy (HRP) has active association with progression of breast malignancy. High grade hormone replacement therapy (HRT) elevates the chances of breast tumor occurrence. The cessation of HRT by women who had earlier used the therapy likely have significantly reduced risk of breast cancer and breast cancer related mortality (Beral et al. 1997). Estrogen-progesterone

combination methods are more dangerous in comparison to the other available options. These findings are substantiated by a study that found that adding progesterone to HRT medication increases the risk of breast cancer considerably (Ross et al. 2000). The choice of progesterone use in HRT should evaluated before opting. The chances are low to progress breast carcinoma in estrogen–progesterone or estrogen–dydrogesterone therapies in comparison to the other available approaches (Fournier et al. 2007). The case-control studies revealed that the use of HRT in postmenopausal female and BRCA1 mutation carriers do not elevate the chances of breast carcinoma (Beral and Million Women Study Collaborators 2003).

1.4.7 Family History

Genetic predisposition contributes significantly to the progression of breast carcinoma. The fundamental structural and gene expression changes are considered to contribute the risk patterns in a varied approach. Approximately 20% of patients with mammary tumor family history reflect genetic predisposition, while 5% of the identified patients have a specific germ-line mutation identified in them (Easton et al. 1995; Thakur et al. 2017). The first case of familial breast cancer was described more than 100 years ago. Several other studies have attempted to define risk levels associated with varying degrees of positive family history. Systemic meta-analysis reveals the role of family history of breast cancer (Pharoah et al. 1997; Bravi et al. 2018; Ahern et al. 2017). Individuals with a first-degree relative who had developed breast tumor at 50 years of age or older had a relative risk of 1.8. At the same time, the relative risk was 3.3 for a first-degree relative who had developed breast cancer before the 50 years age compared to individuals with no family history of breast cancer. On the other hand, having a second-degree relative with breast cancer increased the relative risk by 1.5. The relative risk was 3.6 when two first-degree relatives (for example, mother and daughter) were impacted (Goldgar et al. 1996; Narod et al. 2014).

1.4.8 Breast Density and Lactation

Lactation and breast density those have antagonistic effects on disease incidence. Lactation prevents breast cancer and shows a protection of the breast tissue from any type of trouble, while breast density is an independent risk factor for breast cancer (Kim et al. 2015; Bravi et al. 2018; Nazari and Mukherjee 2018). The protective effect of lactation increases with the increment of lactation duration (Laamiri et al. 2015; Nazari and Mukherjee 2018). Lactation length of more than 13 months combined with two or more childbirth reduces the possibility of breast cancer to half (Jeong et al. 2017). Breastfeeding also have an active association with better prognosis, low reoccurrence rate, and better survival in breast cancer patients (Kwan et al. 2015). However, these connections are not so well established in few previous findings (Brinton et al. 1995; Michels et al. 1996; Socolov et al. 2015; Vierkant et al.

2017; Ahern et al. 2017). On the other hand, observations of a case-control study revealed that initiating estrogen and progesterone administration increases the breast density. They contribute to the increment of 3.4% in the breast malignancy with each 1% increase in mammary gland density (Byrne et al. 2017). A fivefold increase in the breast carcinoma occurrence in high breast density patients was predicted in various studies (Eriksson et al. 2017). Increment in breast density elevates ER-positive and ER-negative type invasive breast cancer in age dependent manner (Kerlikowske et al. 2017). Benign breast disorders are among the most prevalent risk contributors (Zendehdel et al. 2018; Román et al. 2017). According to the findings of case-control research, HRT and breast hyperplasia are linked to elevated occurrence of the breast tumor in females with benign breast disease. In postmenopausal women with benign breast disease, the risk of breast cancer diminishes (Arthur et al. 2017). On the other hand, the occurrence of benign breast disorders depends on factors like histological categorization of the disease and pedigree history (Hartmann et al. 2005).

1.4.9 Environmental Factors

1.4.9.1 Environmental Factors

Scientists determined that women living in places with greater levels of air pollution may have a high rate of breast tumor incidents, according to a National Institute of Environmental Health Sciences (NIEHS) report (Niehoff et al. 2020). Puberty may be accelerated or delayed because of exposure to common chemicals. Six chemicals that cause or may cause breast cancer have been identified by the National Toxicology Program (NTP) and the National Institute of Environmental Health Sciences (NIEHS). These are diethylstilbestrol (DES), a synthetic estrogen used to prevent miscarriages; steroidal estrogens used for menopausal therapy; X-ray and gamma radiation; alcoholic beverages; cigarette smoking; and ethylene oxide, a sterilizing agent (US Department of Health and Human Services 2016).

1.4.9.2 Radiation

The exposure of humans to ionizing radiations occurs due to natural, medical, and other artificial sources. Radiation is one of the external risk factors studied for cancer development, particularly regarding radiosensitive tissues and organs. Female breast tissue is susceptible to the carcinogenic effects of radiation exposure, particularly at a younger age. Some of the common reasons for radiation exposure in females include occupational, medical diagnostic procedures, and residence background radiation, but radiation effects from such sources have not been documented and are believed to be not a significant risk factor of breast cancer development. According to some radiobiological research, radiation-induced carcinogenesis may be produced by a specific event caused by radiation through recognition of the key target. Despite the low incidence of radiation-induced malignancies, ionizing radiation has been shown to have mutagenic and carcinogenic effects in vivo and in vitro (Zafar et al. 2016). Radiation-induced breast cancer is more common in female patients who have had irradiation for malignant or non-malignant disorders, including benign breast

diseases (Golubicic et al. 2008). The chance of getting breast cancer in women due to therapeutic radiation exposure related to past cancer treatment, screening for tuberculosis, or pneumonia surveillance is two to three times greater (John et al. 2007). Whileas, radiation exposure due to childhood cancer and whole lung irradiation treatment further increases the risk of development of breast cancer. As a result, researchers stress that the death rate linked with breast cancer is much greater in these cases (Moskowitz et al. 2014). People who have had high dose alkylator and anthracycline chemotherapy after surviving a sarcoma or leukemia are more likely to develop breast cancer at a younger age. This could be due to the high dose alkylator and anthracycline chemotherapy (Henderson et al. 2016).

1.4.9.3 Exposure to Various Chemicals

Girls that were exposed to high quantities of triclosan, which is found in some antimicrobial soaps, developed their breasts early (Wolff et al. 2016). Girls who were exposed to benzophenone 3, which is contained in sunscreens, developed their breasts later. Exposure to endocrine-disrupting chemicals (EDCs) throughout prenatal development, puberty, pregnancy, and menopausal transition increases breast cancer risk (Terry et al. 2019). In addition, about 300 chemicals found in personal care items, flame retardants, food processing, pesticides, and other applications were examined and found to influence hormones linked to an increased risk of breast cancer (Naik et al. 2021; Cardona and Rudel 2021).

1.4.9.4 Lifestyle Factors

Several risk factors that form part of our lifestyle have effect on the breast carcinogenesis responsible have been identified. Some of these risk factors are discussed here.

1.4.9.5 Body Mass Index (BMI)

BMI was not found to be a risk factor for breast cancer in premenopausal women, but it indirectly affects the overall performance of body in an adverse way (Zafar and Shrivastava 2017). Postmenopausal women, on the other hand, had a relative risk of breast cancer incidence in the highest versus lowest BMI quintile on the order of 1.1 in women aged 55–59, 1.18 in women aged 60–64, and 1.22 in women aged 65–69 years (Tretli 1989). Though modest, the highest BMI should be an essential factor for breast cancer risk because the fat cells are an important extragonadal source to affect estrogens in postmenopausal women (Verkasalo et al. 2001; Chen et al. 2016). Postmenopausal exposure to estrogens may increase the initiation and promotion time frame of breast cancer. Besides, high BMI has been associated with increased insulin and insulin-like growth factors which are known to have a high chance of breast carcinogenesis (Goodwin et al. 2002; Chen et al. 2016). High BMI and abdominal obesity results in hyperinsulinemia and contribute to the breast tumor progression (Stoll 1999). ($^{\#}$BMI calculated by Quetelet's index and subjects were divided into quintiles based on BMI).

1.4.9.6 Hyperglycemia

By interfering with molecular systems, diabetes and insulin-related problems raise the incidence of breast carcinoma (Wolf et al. 2005). Diabetes also has a strong link to the progression of breast tumor in obese women after the menopause (Tabassum et al. 2016). Women with glycemic alterations specifically type II diabetes had a 20% higher risk of breast tumor progression, according to a meta-analysis (Larsson et al. 2007). Postmenopausal women with high body mass index (<26 kg/m^2), abnormal blood glucose, insulin, or IGF-1 patterns are more likely to develop breast carcinoma. (Muti et al. 2002). According to the European Prospective Investigation into Cancer and Nutrition (EPIC), women with high range of insulin-like growth factor binding protein-3 (IGFBP-3) along with insulin-like growth factor (IGF-1) than controls had a considerably higher risk. This link is stronger in those whose malignancies are discovered after 50 years (Rinaldi et al. 2006). Controlling HbA1C below 7% can help breast tumor patients to get a better prognosis (Chang et al. 2018).

1.4.9.7 Alcohol Consumption

Alcohol consumption increases the risk of having breast carcinoma to the amount and frequency of alcohol intake. Several theories explain that alcohol consumption may increase the metabolism of carcinogens like acetaldehyde or diminish DNA repair effectiveness, or it could be related to a reduction in protective nutrients owing to alcohol consumption (Vogel 1998). However, the alcohol intake has a moderate incidence of breast cancer occurrence. Alcoholic drink consumption and hormone receptor-dependent breast cancer progression elaborate the role of timing of alcohol intake that may influence the risk of mammary gland tumor progression. Alcohol consumption during first full-term pregnancy results in a higher risk of developing breast cancer (Romieu et al. 2015). Some of the studies state that one drink or less per day (approx. 12 g alcohol) has no significant effect on the breast cancer development (Zhang et al. 1999; Manisto et al. 2000). Studies have also reported the effects of heavier to a very high dose of alcohol consumption and risk of breast cancer. However, the relative risk does not exceed 2. Whileas, no effect of alcohol type on relative risk was found (Wine vs. beer vs. distilled water) (Ellison et al. 2001). In 426 multigenerational breast cancer groups, the effects of alcohol usage and family were investigated (Vachon et al. 2001). According to the findings, daily alcohol consumption has been active associated with a highest relative risk of in first-degree relatives followed by second-degree relatives of the preexisting cancer patients, but least in unrelated females who married with relatives. Recent investigations, on the other hand, have not corroborated this (Ursin et al. 2002).

1.4.9.8 Smoking

Prenatal smoking and active smoking, including smoking after the menopause, have a high chance of breast carcinoma progression (Luo et al. 2011). Passive smoking is another active risk factor for breast tumor formation, and it increases the risk of ER+/PR+ double positive breast carcinogenesis the most (Tong et al. 2014).The duration and amount of smoke exposure (ten or more cigarettes per day) have a consistent

dose-response association between the number of smoking years before childbirth and the chance of disease occurrence (Bjerkaas et al. 2013).

1.4.9.9 Diet

Another important relative lifestyle factor for breast carcinoma progression is diet which has been addressed in several research studies. The studies have addressed the correlation among various factors such as quality, intake range, and type of nutritional diet with the rate of breast cancer incidence. Non-vegetarian diet was identified as a significant contributing factor for breast tumor progression (Thakur et al. 2017). A diet poor in polyunsaturated and saturated fatty acids is much essential than total fat consumption for the development of breast tumor (Jordan et al. 2013). On the other side, total meat and non-processed meat consumption enhance the chance of premenopausal breast tumor progression. Considering prospective European study on cancer and nutrition, saturated fat consumption is linked to an increased risk of breast cancer (Taylor et al. 2007; Sieri et al. 2008). An adolescent and early adulthood diet high in sugar-sweetened, artificial sweeteners, diet soda, soft drinks, refined flour, red meat, processed food are also worked as contributing factor. Absence of green vegetables, leafy plants, cruciferous vegetables, and coffee intake during at early age may contribute in the risk to develop breast cancer in childbearing years, without any correlation with postmenopausal disease progression (Harris et al. 2017).

1.4.9.10 Sleep Patterns

Diurnal rhythm is considered an active lifestyle factor that contributes to the progression of breast carcinoma. Longer sleep durations have a high chance of breast cancer occurrence than women who sleep for shorter periods of time (6–7 h). According to NTP's study of the effects of working at night, women who work night shifts for a long time alter their circadian rhythms are more likely to get breast cancer (National Toxicology Program 2021). Increasing sleep hours affects the occurrence of estrogen receptor-related breast cancer possibility. Sleep duration, on the other hand, was found to have no link to estrogen negative-receptor breast cancer (Lu et al. 2017). Insomnia was also linked to a high chance of getting breast carcinoma (Chiu et al. 2018). In cancer survivors, the amount of sleep they get has little bearing on their prognosis (Marinac et al. 2017). Varied sleep aspects, such as duration and quality, are also linked to a high risk of aggressive mammary cancers, which varies depending on the race of cancer survivors (Soucise et al. 2017).

1.4.9.11 Caffeine

There are conflicting reports about coffee consumption and mammary gland cancer progression. Several reports show no evidence of connection between caffeine consumption and breast carcinoma progression (Gierach et al. 2012; Boggs et al. 2010). In postmenopausal women, increasing daily coffee consumption was linked to a considerable reduction in ER-negative breast tumor (Li et al. 2011). The negative association between coffee intake and risk breast tumor (Oh et al. 2015) is also reported in literature, while another cohort study supports no relationship

between coffee intake and disease risk. However, a weak inverse association between coffee and postmenopausal breast cancer is reported by some researcher (Ganmaa et al. 2008) which defines the uncertainty of the condition.

1.4.9.12 Physical Activity

Physical activity has also been linked to reduction in breast tumor occurrence in postmenopausal women between the ages of 50–80 years (Mctiernan et al. 2003). Following a breast cancer diagnosis, physical activity may help to minimize the mortality rate from the disease. Several studies have proven that a routine moderate/brisk walk of approximately 3–5 h per week speed experiences the maximum benefits from exercise (Holick et al. 2008; Lee 2019).

1.4.9.13 Vitamin D

High vitamin D levels are related to the minor occurrence and better survival, improving treatment-specific outcomes, especially in postmenopausal patients (Hatse et al. 2012). Vitamin D deficiency contributes to progression of mammary neoplasms (Shekarriz-Foumani and Khodaie 2016). Vitamin D deficient females have a 27% increment in breast tumor progression risk (Park et al. 2015). High serum 25(OH)D levels and regular vitamin D supplement use decline the incident rate of postmenopausal breast carcinoma. These results may help establish clinical benchmarks for 25 (OH) D levels to support the hypothesis that vitamin D supplementation is helpful in breast cancer prevention (O'Brien et al. 2017).

1.4.9.14 Socioeconomic Status

In recent times, socioeconomic status contributes an positive influence for breast tumors. Females from high socioeconomic status are more likely to encounter with cancer risk due to high occurrence of associated risk factors including low physical activity, conception rate, and childbirth in later years and menopause. The higher prevalence of breast cancer is also affected by sedentary routine, obesity, and high fat intake (Orsini et al. 2016; Lundqvist et al. 2016; Thakur et al. 2017). These women, on the other hand, have much more support for breast cancer prevention, prognosis, diagnosis, and treatment well in comparison to the low socioeconomic females (Akinyemiju et al. 2015). The principal socioeconomic determinants of the breast carcinogenesis are education and work position (Fioretti et al. 1999; Balekouzou et al. 2017). Employed women are more likely to have a better access to medical care in combination with medical insurance. Although the incidence of breast cancer is higher in women of higher socioeconomic status, the rate of recurrence and mortality is high among low socioeconomic class (Gordon 2003; (Booth et al. 2010; Kuzhan and Adlı 2015).

1.5 Prevention Strategies and Treatments for Breast Cancer

There are numerous therapeutic options depending on the type of breast cancer and the duration of in vivo existence. According to the Centers for Disease Control and Prevention (CDC), females with breast malignancy frequently receive many treatments, including surgical options for the removal of cancerous tissue. Chemotherapy, which is the chemical-based cancer treatment approach, is a widely used successful therapy that involves the use of certain drugs to destroy or limit the cancer cells growth. The drugs can be taken orally or can be injected directly into the veins of the patients, or both. Hormonal therapy prevents cancer cells from gaining access to the endocrine secretions they require to thrive. Biological therapy uses the body's immune channel to aid in the fight against cancer cells or to reduce the adverse effects of other malignancy treatments. High-energy gamma rays or X-rays are also widely used in radiation treatment to kill cancer cells.

Prognosis and treatment of breast cancer in females with family history of breast cancer or not should be sufficiently aware about any possible underlying mutations (Melvin et al. 2016). Tamoxifen chemoprevention or increased breast tumor screening using magnetic resonance imaging is recommended for individuals with a pre-diagnosed family record of breast tumor (Metcalfe et al. 2009). Complementary and alternative medicinal treatments are also available to deal with early-stage breast cancers. Alternative medicine, naturopathy, and homeopathy work as an added therapeutic option in combination with the routine treatment. Meditation therapies, yoga sessions, and vitamin and mineral intake, coconut water and other liquid intake, and herbs consumption contribute to the better survival and recovery of patients. Several types of complementary and alternative medicine, on the other hand, have not been scientifically tested and may not be safe. The treatment strategies are more specific if the patient has any existing history of diabetic or similar metabolic issues. For the treatment of diabetic patients, metformin improves the overall and cancer-specific survivals (Xu et al. 2015; Tang et al. 2018). Affordable, point-of-care technologies and biosensor-based cancer care devices could be a new ray of hope in the field of cancer detection and management (Zafar 2019).

1.6 Conclusion

Despite the presence of many risk factors, most women do not develop breast cancer. It is advisable to identify the person-to-person chances of breast disease occurrence. The awareness about breast carcinoma screening and associated reduction strategies is highly advisable (Centre for Disease Control 2021). Breast carcinoma is one of frequent types of deadly cancer among females worldwide, with breast carcinoma mortality rates being greater in countries with less development. The incidence of breast cancer depends on caused by a variety of variables and various co-factors, including genetic factors, environmental factors, lifestyle, and much more. Being a woman and later years of age are two significant factors that increase your risk. The occurrence of such type of cancers is more likely to occur in postmenopausal age

group. In many cases, breast malignancy is reported in many patients without presence of any associated risk factor association. The awareness is advisable for all the female population to understand the presence of risk factor and the associated consequences to create a better knowledge about contributing risks and epidemic distribution of breast malignancy.

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2 Histopathological Characteristics: Breast Cancer Subtypes Depending on Receptor Status, Clinical and Pathological Staging of Breast Cancer

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Abstract

Breast cancer (BC) is a clinically heterogeneous disease affecting around 14% female population worldwide with unique histopathological and biological characteristics. Invasive carcinoma with no special type (NST) is common among histological type of cancer and about 25% breast cancer has been classified as histological *special type*. Nowadays, molecular classification of breast cancer is used for treatment. But molecular classification is also derived from NST and it has to be explored yet whether molecular classification is applicable to all histological subtypes. Therefore, in present chapter, we have reported the breast cancer classification by the detailed collection of data from different sources, i.e., PubMed, Web of Science, Science Direct, Scopus databases on a series of histological special type, i.e., invasive lobular carcinoma (ILC) and invasive ductal carcinoma (IDC) along with its subtypes: tubular, mucinous, neuroendocrine, apocrine (androgen-receptor positive and estrogen-receptor negative), micropapillary, adenoid, cystic, metaplastic, and medullary carcinoma. Understanding of present study focused on invasive carcinoma with histological types and subtypes with detail origin of heterogeneity may be helpful for the targeting novel therapeutic tools for breast cancer diagnosis and treatment.

Keywords

Breast cancer · Invasive ductal carcinoma · Invasive lobular carcinoma · Histopathological classification

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2.1 Introduction

Breast cancer (BC) is one of the major cancers among lung and colon cancer worldwide. In 2020, about 2.3 million BC cases have been diagnosed worldwide (Mubarik et al. 2019). BC is a heterogeneous disease and shows different phenotypes, molecular subtype, histological type, and treatment responses (Malhotra et al. 2010; Polyak 2011; Harbeck and Gnant 2017). Along with this, differences in histological type BC also showed a wide range of differences in clinical behavior (Simpson et al. 2005; Vargo-Gogola and Rosen 2007; Reis-Filho and Lakhani 2008; Weigelt and Reis-Filho 2009; Weigelt et al. 2010). Therefore, it is important to identify and define characteristics of breast carcinoma, which may be helpful for betterment and cure patient. Histopathological is an important aspect for the diagnosis, guidance for the management of patient, fatality prediction, and repetition of BC (Malik et al. 2019) by analyzing different parameter, including tumor nuclear grade, tumor histology, lymphatic & vascular invasion, and molecular study like progesterone receptor (PR), estrogen receptor (ER) status, epithelial growth factor, receptor, and expression of proliferation-related genes (Donegan 1997; Li et al. 2005; Tirada et al. 2018; Phung et al. 2019).

Thus, recent advances have increased the chances of molecular understanding heterogeneity of breast carcinoma. In this chapter, we will review recent clinical approach to classify BC, molecular based classification, and potential future for diagnosis and treatment.

2.2 Histological Classification of Breast Cancer

Breast cancer (BC) has classified into histological grade (Elston and Ellis 1991) and histological type (Ellis et al. 1992). Histological grade is an evaluation on the basis of tubule formation, nuclear pleomorphism, and proliferative activity of tumor (Elston and Ellis 1991). On the other hand, histological type of BC classification is based on the growth pattern of tumor. Study shows that pathologists have identified histological diversity on the basis of cytological and morphological histoarchitecture of tumor and linked with various clinical outcomes in breast adenocarcinomas. These patterns are known as "*histological type"* and include up to 25% among all BC (Ellis et al. 2003).

2.2.1 Invasive Breast Cancer

Invasive breast cancer is the most common type of breast cancer and develops among approximately 12% of women in her lifetime (Giordano and Gradishar 2017). Mostly BC starts to develop in the lobule and small sacs of milk producing glands or milk ducts, when infection of cancer spreads out from these cells to surrounding healthy tissue known as "*invasive cancer*." Present study focused on invasive carcinoma with histological types and subtypes (Fig. 2.1) of BC with

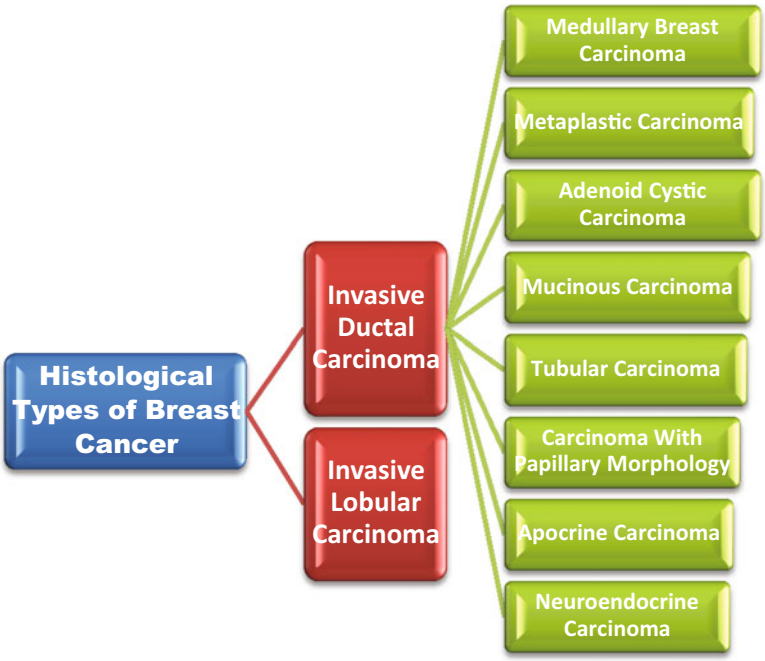

Fig. 2.1 Histological Classification of Breast Cancer

detailed origin of heterogeneity, which may be helpful for the targeting novel therapeutic tools for particular subgroups of BC patients.

2.2.1.1 Invasive Ductal Carcinoma

Invasive ductal carcinoma (IDC) is the most common pathological type accounting for 70–80% invasive carcinoma (Li et al. 2005). 50–80% of breast cancer included under the category of invasive ductal carcinomas not otherwise specified (IDC-NOS) or no special type (IDC-NST) (Ellis et al. 2003; Cserni 2020). According to American Society of Cancer, 80% BC are invasive cancer (American Cancer Society 2020). The term ductal carcinoma originated from the traditional concepts that such types of tumor developed from duct epithelium of mammary glands and ductal proliferation spread to the lymph nodes and other areas of the body (Ellis et al. 1992). IDC has been classified into subtypes on the basis of broad range of criteria, such as histoarchitecture (tubular, micropapillary, and papillary carcinoma), cell type (apocrine carcinoma), site of secretion, amount, and type (mucinous carcinoma) (Rosai 2011), and immunohistochemical study (neuroendocrine carcinoma) (Lakhani et al. 2012a). On the other hand, morphological variation, clinical behavior of IDC, including tumor shape size, and tumor cell and stroma proportion show different subtypes, such as medullary carcinoma, metaplastic carcinoma, and adenoid cystic carcinoma (Makki 2015).

2.2.1.1.1 Medullary Breast Carcinoma

Medullary carcinoma of breast (MCB) is an invasive rare type of breast cancer, with less than 5% rate of occurrence among all the breast cancer types, it was first introduced by Ridolfi and co-authors in 1977 (Ridolfi et al. 1977). Mostly MCB is unicentric but recently around 3–18% cases are found to associated with bilateral carcinoma (Foschini and Eusebi 2009). The WHO describes MCB as properly circumscribed, and invasive type of tumor which mainly consists of sheet of improper differentiated cells, lymphoplasmacytic infiltration with no gland-like formation (Kleer 2009).

Histological Organization of Medullary Breast Carcinoma

Based on Pederson et al. and Ridolfi's criteria, medullary carcinoma is categorized as atypical medullary carcinoma, typical medullary carcinoma, and non-medullary carcinoma. Typical medullary carcinoma shows better prognosis and survival rates (Jensen et al. 1997) and found to associate with most commonly with the BRCA-1linked mutations (Jacquemier et al. 2005). About 7.8–19% of MCB are among BRCA-1 linked cancers, while 2% are non-BRCA linked (Malyuchik and Kiyamova 2008).

According to WHO, histopathological characteristics of typical MCB consist of 5 criteria, i.e., histological non-invasive circumscription, lymphoplasmacytic infiltration, syncytial pattern of growth >75%, lack of glandular structure, and high-grade nuclei (Hanby et al. 2004). MCB histologically can display multinodular pattern with necrosis. Atypical tumor cells have many nucleoli and atypical mitoses. Tumor cells are arranged in the form of sheet without boundaries and smudged cell remains present. Atypical tumor cells which are multi-nucleated remain mixed with plasma cells and lymphocytes (Kleer 2009). Being negative for progesterone (PR), estrogen (ER) receptor and human epidermal growth factor receptor-2 almost 95% MCB are characterized as basal like breast cancer (Bertucci et al. 2006). On the other hand, some studies based on analysis of gene expression demonstrated that among the MCB around 10% are HER-2 positive and approx 40% are PR and ER positive tumor (Ridolfi et al. 1977).

2.2.1.1.2 Metaplastic Carcinoma

Metaplastic Breast Carcinoma (MBC) is an infrequent type of breast cancer reported with 0.2–5% occurrence of overall breast cancer and has recognized as distinct pathologic diagnosis by WHO in 2000 and due to its poor prognosis it contributes to the global mortality associated with breast cancer (Hanby et al. 2004). Metaplastic carcinoma term was first introduced by Huvos and colleagues in 1973 (Huvos Jr et al. 1973). MBC belongs to subcategory of basal like cancer which are marked generally as a triple negative cancer, i.e., lacking progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor-2 (HER-2) (Tray et al. 2019). MBC is characterized by high-grade tumor, aggressive, low axillary node penetration, negative hormone receptor, and large sized tumor due to which as compare to breast conservation treatment, mostly mastectomy and rarely chemotherapy is used (Pezzi et al. 2007). MBC is not responsive toward hormone

based treatment and thus poor clinical outcomes as compare to other breast cancers (Luini et al. 2007).

Histological Organization of Metaplastic Breast Cancer

By the definition malignant and benign, both types of tumor in breast have mostly glandular epithelial origin, but sometimes change into non-glandular mesenchyme and this transformation is called as metaplasia (McKinnon and Xiao 2015).

Histologically, metaplastic carcinoma is poorly differentiated hetergenous tumor having ductal carcinoma cells mixed with squamous, spindle, chondroid and osseous elements. Along with this, all these cells may remain mixed with carcinoma type (Lee et al. 2012). On the basis of cells involved, metaplastic carcinomas can be tumor of low-grade like adenosquamous carcinoma of low-grade or spindle cell carcinoma of low-grade or it can be high grade which involves high-grade spindle cell or squamous cell carcinoma (Lee et al. 2012).

WHO classified MBC into two main types, i.e., epithelial type MBC and MBC of mixed type. The subtypes of epithelial type are adenosquamous carcinoma, squamous carcinoma, and adenocarcinoma along with differentiated spindle cell. The mixed type includes carcinoma with carcinosarcoma, chondroid metaplasia carcinoma, and carcinoma with osseous metaplasia (Hanby et al. 2004). Adenosquamous types demonstrate the presence of squamous cell undergoing differentiation, pavement like structure with keratin deposition. Along with this, it shows both glandular and squamous cell differentiation (Tan et al. 2015). Squamous cell infiltrating with keratin, eosinophilic cytoplasm, and polygonal cells presents squamous carcinoma, while adenocarcinoma displays spindle cell in the form of cohesive sheets (Hanby et al. 2004). Among the mixed type, carcinosarcoma demonstrates rare type of cancer which shows epithelial malignant cells infiltering carcinoma and sarcomatous elements (Mundhada et al. 2020). Metaplastic carcinoma with chondroid differentiation is rare among all subtypes of histological breast cancer showing infiltration of ductal carcinoma (Myint et al. 2015) with osseous metaplasia display carcinoma with sarcomatous stroma with osteoclastic cells and intervened with spindle cells (Lang et al. 2011). Tse et al. 2006 studied that MBC can be classified into three subtypes, i.e., monophasic carcinoma with spindle cell, biphasic epithelial & sarcomatoid carcinoma, and epithelial-only carcinoma. According to Wargotz and Norris classification, MBC has five subgroup of metaplastic along with osteoclastic giant cell, carcinosarcoma, squamous cell carcinoma, spindle cell carcinoma, and the matrix-producing carcinoma (Schwartz et al. 2013). Oberman has classified MBC into invasive ductal carcinoma along with morphology of extensive squamous metaplasia, spindle cell carcinoma, and invasive carcinoma along with pseudosarcomatous metaplasia (McKinnon and Xiao 2015). The heterogeneity is the basis of classification of MBC into various subtypes depending upon morphological and histological features encouraging finding new diagnostic and treatment strategies in future.

2.2.1.1.3 Adenoid Cystic Carcinoma

Adenoid cystic carcinoma (ACC) belongs to invasive type of carcinoma of breast and has been considered as a rare as well as special subtype of breast carcinoma (Lakhani et al. 2012b), resemble with ACC of salivary gland (Ro et al. 1987). It is less common type of breast carcinoma with the rate of occurrence less than 0.1% among all the main breast carcinoma (Sorlie et al. 2003). ACC of breast displays better prognosis and clinical study revealed the less changes of axillary node metastases (Glazebrook et al. 2010). As per clinical data available, the distribution of age for the occurrence of ACC of breast is 38–81 years with 60 years as a median age (Lakhani et al. 2012b). With the rare rate of occurrence in males, ACC of breast carcinoma is mostly found in females (Miliauskas and Leong 1991). ACC is a bilateral type of breast carcinoma which displays lesions in area below the areolar of the breast (Azzopardi et al. 1979). Mammographic study describes ACC as irregular tissue mass which is not symmetrical in densities; however, sonographic finding states it as hypoechoic, heterogenous mass (Glazebrook et al. 2010).

Histological Organization of Adenoid Cystic Carcinoma of Breast

The size of ACC ranges from 0.7 to 12 cm and the average size is approx 3 cm (Tavassoli 2009). ACC of breast is characterized by basal like tumor, lacking progesterone (PR) and estrogen (ER) receptor along with absence of human epidermal growth factor receptor-2 (Her-2) but it shows the presence of myoepithelial/basal like cell markers (Miyai et al. 2014). Alike ACC of salivary gland, ACC of breast shows the presence of mainly two types of basal cells—myoepithelial and luminal cell, arranged in different patterns like solid basaloid, cribriform, and tubular-trabecular (Lakhani et al. 2012b). The myoepithelial/basal cells display formation of pseudolamina, poor cytoplasm with oval shaped central nuclei and also exhibit immunoreactivity for myoepithelial markers, basal cytokeratin like CK 5,5/6,14 and 17 and for the factors like epidermal growth factor receptor (EGFR) and vimentin (Badve et al. 2011; Wetterskog et al. 2012; Reyes et al. 2013). On the other hand, luminal cells exhibit the presence of glandular space, eosinophil positive cytoplasm with round nucleus. It also shows the presence of epithelial membrane antigen, CD117 and cytokeratin 7,8, 8/18 (Badve et al. 2011; Wetterskog et al. 2012; Franzese et al. 2013). Many reports suggest that luminal and myoepithelial cells of ACC of breast also differ on the basis of protein expression, like epithelium differential and polarization related proteins are mostly exhibited by luminal cell; however, collagen type IV, fibronectin , laminin, and other basal lamina protein are associated with myo-epithelium-basal cell (Tavassoli and Norris 1986; Lamovec et al. 1989).

Some authors have classified ACC of breast carcinoma in three categories, including grade-I is characterized as a tumor devoid of solid component and with the presence of tubular-trabecular and cribriform pattern, grade-II ACC tumor exhibits $\leq$30% solid component, and grade-III represents with solid growth which should be greater than 30% (Ro et al. 1987). It is also reported that tumor of II and III grades are likely to become big in size, moreover they are also associated with the possibility of occurring again (Shin and Rosen 2002).

2.2.1.1.4 Mucinous Carcinoma

Mucinous carcinoma is a rare special type of BC representing 2% of invasive cancer of breast in elderly women usually at postmenopausal stage (Budzik et al. 2021). Mucinous carcinoma is also known as colloid carcinoma, mucous carcinoma, gelatinous carcinoma, and mucoid carcinoma (Rosai 2011). Furthermore, mucinous carcinoma is characterized by the secretion and presence of mucin protein in extracellular space (Tan et al. 2020).

Histological Organization of Mucinous Carcinoma

Tumor consists of small cluster of uniform epithelium cells containing mild nuclear floating in mucus. Tumor cells are arranged in cluster of small islands of 10–12 cells in uniform solid, acinar, or micropapillary structure in lakes of extracellular mucin (Ellis et al. 1992; Rosai 2011). The growing edge of these islands is embedded in loose fibrous stroma. Furthermore, WHO has classified mucinous carcinoma into two categories on the basis of cellular integrity, i.e., pure type mucinous carcinoma and mixed type mucinous carcinoma (Tan et al. 2020). Pure type of mucinous carcinoma contains tumor cells with extracellular and intracellular mucin over 90% tumor mass, but the mixed type of mucinous carcinoma is composed of both ductal and lobular infiltrating component with less than 90% mucin (Hanagiri et al. 2010). Along with this, on the basis, histoarchitecture pure type of mucinous carcinoma is classified into mucinous-A and mucinous-B. Mucinous-A is characterized by micropapillary, tubular, papillary, and cord-like growth pattern; however, mucinous-B consists of solid cluster of tumor forming cells floating in mucin (Chaudhry et al. 2019).

2.2.1.1.5 Tubular Carcinoma

Tubular carcinoma is a rare breast cancer subtype of IDC representing only 2% common in elderly women at age of 50 years and the site of pathogenesis is upper outer quadrant of breast with multifocality in 20% cases (Sullivan et al. 2005). In tubular carcinoma, tumor is made up of tube like structure with open lumina, surrounded by abundant stroma usually 1 cm in size (Goldstein et al. 2004).

Histological Organization of Tubular Carcinoma

Tubular carcinoma is characterized with open tubule consisting single layer of epithelium enclosed with clear lumen (Rosai 2011). Tubules are oval or rounded in shape arranged irregularly. Cells are moderate in size showing little nuclear pleomorphism with scanty mitotic structure (Limaiem and Mlika 2021). Study shows that 10–20% patients have multifocal tubular carcinoma and grow as a separate foci in one or multiple quadrants (Moinfar 2007). In the presence of invasive cancer and tubular cancer in different proportion in tumor known as tubulolobular carcinoma (Rosen 2009). Tubular carcinoma also present with flat epithelial atypia, less tubular neoplasia, and low-grade ductal carcinoma in situ (McDivitt et al. 1982; Fernández-Aguilar et al. 2005). Along with this, immunohistochemical study shows that tubular carcinoma is always positive for estrogen and progesterone receptors (Papadatos et al. 2001; Rakha et al. 2010).

2.2.1.1.6 Carcinoma with Papillary Morphology

Papillary carcinoma of breast represents 0.5% of invasive cancer of breast (Louwman et al. 2007). It is mostly seen in in-situ and invasive form in elderly the postmenopausal stage of female in elderly age and prevalence is reported in male as well (Pal et al. 2010). Breast papillary carcinoma is mainly intraductal lesion (Lakhani et al. 2012a). In the breast carcinoma with papillary pattern, malignant cells along with core of fibrovascular collectively form papillae like structure lacking myoepithelial component. The malignant cells display abnormal nucleus type (Moinfar 2007).

Histological Organization of Papillary Carcinoma

Papillary malignant neoplasm consists of papillary ductal carcinoma in situ (DCIS), intraductal papilloma lesion, solid, invasive, and encapsulated papillary carcinoma (Mulligan and O'Malley 2007; Collins and Schnitt 2008; Ueng et al. 2009). DCIS with papillary morphology exhibits core of fibrovascular surrounded by columnar monomorphic stratified epithelial cells which are neoplastic with micropapillary, cribriform, and solid proliferation (Hill and Yeh 2005; Collins et al. 2006). Intraductual papillomas are the type of benign mass of tumor caused by excessive proliferation of epithelial of duct in breast. In the central duct beneath the nipple mostly single intraduct papilloma are is found but multiple papilloma can be seen on peripheral side of ducts (Eiada et al. 2012). In some cases, it is palpable and shows discharge from nipple but mostly it is asymptomatic (Eiada et al. 2012). The large duct and terminal duct are in the location of intraductal papilloma with the feature exhibits core of fibrovascular component lined by myoepithelium and epithelium showing hyperplasia, abnormal proliferation, sclerosis, apocrine, and squamous metaplasia (Han et al. 2018).

Solid papillary breast carcinoma is characterized with circumscribed, thickly packed cells, extensile lumps of epithelial cells (Maluf and Koerner 1995; Nassar et al. 2006). The neoplasm is spindle shaped or oval in appearance, monotonous, abnormal low or intermedial nuclear grade and remains linked with extra and intracellular mucin. Sometimes due to the argyrophilic cells, it displays neuroendocrine characteristics. Furthermore, on the basis of presence of fibrovascular region categorize it as papillary carcinoma (Nassar et al. 2006; Moritani et al. 2007; Nicolas et al. 2007). Absence of myoepithelial cells supports solid papillary carcinoma which behaves as an invasive tumor instead of in situ intraductal lesions (Maluf and Koerner 1995; Nassar et al. 2006).

Invasive papillary carcinoma is among the rare type of carcinoma of breast and is characterized by well demarcated areas, oxyphilic positive cytoplasm, small calcium deposits, medial grade of histology, and interductal projection of papilla (Fisher et al. 1980).

Encapsulated papillary carcinoma is usually single structure, malignant proliferation with papillary projections, consisting cystic duct lined by fibrous thick capsule and devoid of myoepithelial cells. Lesions involve low to intermediate grade of nucleus (Collins et al. 2006). Some small proportion of encapsulated carcinoma are considered as invasive in nature due to infiltration from capsule to the surrounding

areas where it no more hold papillary property and stage of tumor are based on proportion of invasion (Mulligan and O'Malley 2007; Collins and Schnitt 2008).

2.2.1.1.7 Apocrine Carcinoma (Androgen-Receptor Positive and Estrogen-Receptor Negative)

Apocrine Carcinoma (AC) of breast is the rare type of invasive breast carcinoma with rate of occurrence $<1\%$ in female (Gogoi et al. 2012). On the basis of pattern of growth and prognosis, apocrine breast carcinoma is similar to invasive ductal carcinoma not otherwise specific (IDC-NOS), but difference can be seen in architecture of cell, hormones associated and clinical responses (Yerushalmi et al. 2009; Wader et al. 2013). Based on evidence, apocrine breast carcinoma expresses gross cystic disease protein fluid-15, i.e., GCDPF-15, androgen receptor and remains negative for estrogen and progesterone receptor, thus display the profile of ER−/PR−/AR+ (Durham and Fechner 2000; Tsutsumi 2012). Moreover apocrine breast carcinoma is often show over-expressed human epidermal growth factor receptor types2 (HER2) (Vranic et al. 2010); therefore, absence of HER2 receptor in apocrine carcinoma is considered as triple negative cancer of breast (Bosch et al. 2010).

Histological Organization of Apocrine Carcinoma

Benign apocrine lesions are different from apocrine carcinoma (Ng 2002). Apocrine differentiation occurs in a variety of breast lesions, both benign and malignant. Benign apocrine lesions represents low level of cellularity with cells properly display a pattern of flat regular type sheets the cells are full of cytoplasm and granules with round or oval nucleus monotonous with central nucleoli (Johnson and Kini 1989; Kulkarni 2012).

Apocrine breast carcinoma is well defined only when tumor is abundant (72%) with apocrine cell that exhibits granular and eosinophilic cytoplasm, from eccentric to centrally situated, round, vesicular and large nucleus with dominant nucleoli, ratio of nucleus-to-cytoplasmic should be $\geq$1:2 with demarcated cell boundary lining (Eusebi et al. 1986; Durham and Fechner 2000), also displaying the features of overlapped nucleus with pleomorphism (Johnson and Kini 1989; Kulkarni 2012).

2.2.1.1.8 Neuroendocrine Carcinoma

Neuroendocrine carcinoma is rare with 0.27–0.5% and unique subtypes of breast cancer representing only 2% of cases were first described by Feyrter and Hartmann in 1963 (Feyrter and Hartmann 1963). It is commonly diagnosed in elder women approximately 70 years old (Wang et al. 2014) and shows morphological as well as immunohistochemical similarity with neuroendocrine tumor of lungs and gastrointestinal tract and commonly in postmenopausal women (Fattaneh and Peter 2003). Neuroendocrine differentiation has been observed in 20% cases of mammary carcinoma, therefore it is very difficult to evaluate because the immunohistochemical biomarker has not been usually used for the diagnosis of BC (Inno et al. 2016). The most accepted theory for histogenesis of primary neuroendocrine breast cancer suggests that it is derived from the differentiation of neuroendocrine and epithelial neoplastic cells (Adams et al. 2014; Dalle et al. 2017). But, in another theory it has been hypothesized that neuroendocrine BC cancer originates from neural crest cells

and further migrates to mammary glands (Bussolati et al. 1985). Furthermore, BC cells have the potential to express benign neuroendocrine lesion and neuroendocrine biomarker, i.e., chromogranin, and synaptophysin, which have not been described in literature (Adams et al. 2014). Thus, in 2003 WHO recommends neuroendocrine carcinoma as special type of BC (Dalle et al. 2017). Neuroendocrine carcinoma results hypersecretion of calcitonin (Coombes et al. 1975), norepinephrine (Kaneko et al. 1978), and adrenocorticotropic hormone (Woodard et al. 1981).

Histological Organization of Neuroendocrine Carcinoma

Histoarchitecture of neuroendocrine carcinoma shows infiltrative growth pattern with solid aggregation of tumor cells in nest, alveolar, and trabeculae (Bussolati and Badve 2012). The tumor is 0.8–13.5 cm in size (Wei et al. 2010; Tang et al. 2011), and secretes soft and gelatinous mucin (Tang et al. 2011). The tumor cells are spindle, polygonal, and plasmacytoid in shape separated by fibrovascular septae with clear or vacuolated cytoplasm (Tang et al. 2011; Bussolati and Badve 2012). Nuclei are pleomorphic with irregular nuclear membrane, along with this salt and pepper chromatin are also visible in carcinoids of other sites (Tang et al. 2011). Apart from this, Wei et al. 2010 reported that neuroendocrine carcinoma are more ER and PR positive in comparison to IDC-NOS, therefore hormonal therapy could also be used for the treatment on the basis of receptor status (Angarita et al. 2013). Till date there is no specific guideline for staging, grading, and treatment of neuroendocrine carcinoma, thus, recommended that it should be treated like conventional BC (Angarita et al. 2013).

2.2.1.2 Invasive Lobular Carcinoma

Invasive lobular carcinoma (ILC) is the second common type of breast cancer (BC) after invasive ductal carcinoma (IDC) results 5–10% cancer (Li et al. 2003; Orvieto et al. 2008; Lee et al. 2010; Onitilo et al. 2013). ILC commonly diagnosed in postmenopausal age with ER/PR positive receptor (Mersin et al. 2003; Arpino et al. 2004) having distinctive metastatic pattern along with the involvement of gastrointestinal tract and peritoneal sites (Korhonen et al. 2013; Inoue et al. 2017; Mathew et al. 2017).

2.2.1.2.1 Histological Organization of Invasive Lobular Carcinoma.

Histoarchitecture of ILC represents the presence of uniform tumor cells present in stroma and enveloped by lobules (Rosai 2011). The growth of ILC tumor results disturbances in normal histoarchitecture having round nuclei, occasional cytoplasmic vacuole, thin cytoplasm, and concentric layer arrangement around normal ducts, this condition is known as "*targetoid appearance*" (Martinez and Azzopardi 1979; Reed et al. 2015). Apart from this, rare mitotic expression, hormonal receptor expression, and absence of immunohistochemical staining for E-cadherin are most important biomarker of pathogenesis of ILC (Arpino et al. 2004; Singhai et al. 2011). Classical ILC represents different histological variant, like pleomorphic lobular cancer shows growth pattern of classical ILC, but contains nuclear pleomorphism, marked cellular atypia, significant increase in mitotic range, and hormone receptor

negative expression (Jung et al. 2012; Ohashi et al. 2017). In solid and alveolar variant of ILC shows cells arranged in sheets, but diagnostic significance of these variants is not clear.

2.3 Conclusion

Breast cancer is the most common disease among cancer in women throughout the world and is the principal cause of mortality. Breast carcinoma is the complex heterogenous disease, but due to lack of sufficient data on its heterogeneity, it is difficult to understand. But histological types and subtypes of breast carcinoma with detail origin of heterogeneity may be helpful for the targeting novel therapeutic tools for particular subgroups of BC patients.

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Relationship of Breast Cancer with Other Hormone-Sensitive Cancers

3

Rahul Kumar Maurya, Alok Shiomurti Tripathi, Lucy Mohapatra, Shashank Soni, and Mohammad Yasir

Abstract

Women are diagnosed with breast cancer (BC) on a global basis every year, making it the most prevalent kind of cancer in women. A link between BC and various female hormones is significant because these hormones are used for a variety of purposes, including contraception and menopause treatment, by both reproductive and/or post-menopausal females. Hormonal changes that affect the mammary gland's development also appear to increase the incidences. Several studies have proposed that pregnancy and breastfeeding defend against future progress of BC in numerous women. Further, the chances decline with the cumulative overall extent of breastfeeding. Some epidemiological studies have proposed that there is a transient expansion in the relative risk of BC in females using oral contraceptives. The effects of the hormonal environment on the development, prognosis, and treatment of BC are covered in this chapter. The genetic basis for hormone-dependent tumors, relationships between breast and other hormonal cancer, and biomarker correlation that has recently been recognized are also covered in this chapter.

Keywords

Molecular biomarker · Breast cancer · Hormones · Menopause treatment · Estrogen receptor

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3.1 Introduction

Breast cancer is a complex illness with hereditary and environmental components. It is questionable whether or not cancer patients should get hormone replacement treatment. A link between BC and various female hormones is significant because these hormones are used for a variety of purposes, including contraception and menopause treatment, by both reproductive and/or post-menopausal females. Hormonal changes that affect the mammary gland's development also appear to increase the chances of disease. Estrogen and progestin hormone replacement therapy were used in a Women's Health Initiative research, after therapy the risk of BC was higher for these women than for those who got a placebo (Henderson and Feigelson 2000).

Several types of cancer are influenced by hormones, including hormone-sensitive or -dependent malignancies. Breast, endometrial lining, ovarian, prostate, testicular, thyroid, and osteosarcoma cancers are all caused by hormones in some way (Folkerd and Dowsett 2010). Breast cancer hormone levels may be effectively controlled with endocrine therapy since the hormones are influenced by tumor growth and regression. Cancers in the breast that respond to estrogen may be classified into two groups. These two female hormones encourage the growth of cancerous cells. It is thought that many types of BC have estrogen receptor sites on their cells, which helps the growth and spread of the cancerous cells as well as its kind.

Hormones play an important role in numerous processes in the body, including the development and functioning of certain cells and organs. Tumors are hormone-sensitive due to the presence of proteins on the surfaces of their cells referred to as receptors. Hormones attach to these receptors and alter the expression of certain genes, which stimulates cell growth when these receptors are active (Henry et al. 2020). It is believed that estrogen and progesterone are responsible for many of the physiological changes that occur throughout a woman's reproductive life and menopause. Breast cancer cells, as opposed to normal ones, have estrogen and progesterone receptors attached to them, and thus they need these hormones to thrive. BC cells, like normal ones, are fueled by estrogen and other associated hormones to develop (DeVita et al. 2018). Cancer's hormone receptor status may aid in determining how to treat it. Because hormone therapy alters the effect of estrogen (but not progesterone) on BC cells, the effectiveness of hormone therapy may differ depending on whether your tumor is estrogen receptor-positive or estrogen receptor-negative. The binding of hormones such as estrogen and progesterone to receptors helps to inhibit the development and spread of cancer. The effects of the hormonal environment on the development, prognosis, and treatment of BC are covered in this chapter.

Some gene-related pathways, such as PI3K, ACT, and mTOR, are activated in different malignant tumors and play an important role in cancer formation, progression, and treatment resistance. They are thought to be appealing and promising targets for cancer therapy, and numerous therapeutic compounds that target these pathways have been discovered (Atif et al. 2019). The genetic basis for hormone-dependent tumors, relationships between breast and other hormonal cancer, and

biomarker correlation that has recently been recognized are also covered in this chapter.

3.2 Role of Hormones in Breast Cancer

The hormones are considered as a chemical messenger and affect the cells and various tissues in the body reaching the target sites. Prevalence of BC observed to be higher with the exposure to estrogen and progesterone, released by the ovaries of premenopausal women. In women, BC is caused after puberty and its incidence increases rapidly with the growing age until menopause. Therefore, women with longer the normal ovarian function will be at higher prevalence of BC. Having more children may also be ineffectively protective, especially if they are born at a young age. BC is one of the common types of hormone-dependent (hormone-sensitive) cancers. The hormone-dependent cancer cells containing specific types of protein called hormone receptors (estrogen receptor or ERs and progesterone receptors or PRs). The estrogen receptors are abundantly located on breast cells. The estrogen evoked the proliferation of both normal and neoplastic breast epithelium (Fig. 3.1). These epithelial cells begin to proliferate during puberty and reach their peak between the ages of 12 and 18. Pregnancy is associated with proliferation at first, but then with marked differentiation of mammary epithelial cells, and there is some evidence that a first pregnancy increases and then decreases the risk of BC. The earlier a first pregnancy occurs, the less time there is for many undifferentiated susceptible stem cells to be present. As a result, the risk of developing BC is lower. Furthermore, early natural or artificial cessation of ovarian function is linked to

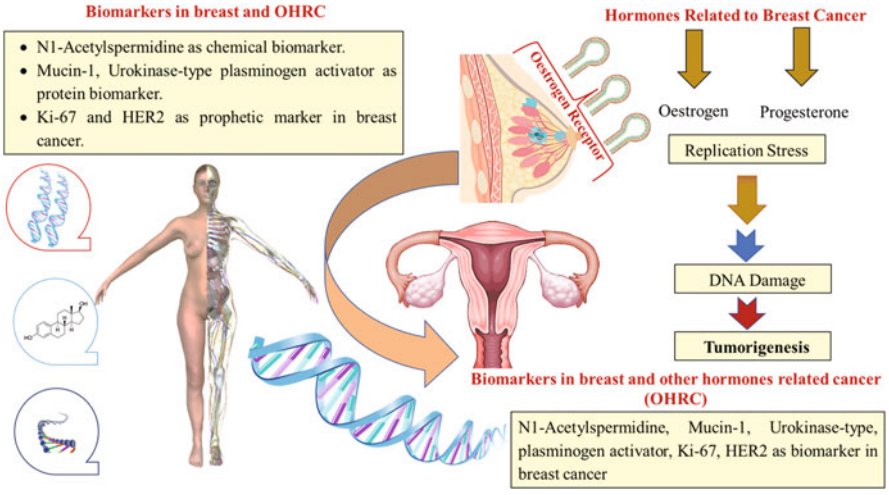

Fig. 3.1 Role of hormones in breast cancer, relation between breast cancer and other hormones related to cancer, biomarkers, and genetics behind hormonal breast cancer

breast involution which is characterized by a high degree of epithelial cell death and, as a result, a decrease in the number of basal and intermediate cells; this could explain the decline in the slope of the age-incidence curve after menopause, as well as the protective effect of early natural or artificial cessation of ovarian function. Progesterone also plays an important role in pregnancy and the regulation of the menstrual cycle. A thorough meta-analysis found that the time between menstrual cycle commencement and termination is strongly linked to incidences of BC. There is a 5% increase in lifetime risk of BC for every year younger a girl begins menstrual cycling. Similarly, for every year older at the time of menopause, the risk of BC rises by 3.5% (Hamajima et al. 2012).

3.2.1 Tumor-Promoting Effects of Estrogen

Nuclear steroid receptors control cell function when estrogen hormones are present. Internalization of hormones occurs when they attach to plasma membrane receptors, which results in hormone entry into cells. This is the typical paradigm for steroid hormone signaling. As a result, these complexes connect to DNA response elements, such as estrogen response sites, and alter target gene nuclear transcription, altering the biological response of cancer stem cells. One or both of these hormones may be found in BC cells. Breast cancer cells that are ER-positive have estrogen receptors. Breast cancer with progesterone receptors (PR) is referred to as PR-positive BC. The malignancy is designated ER/PR-negative if the cells lack any of these two receptors. ER and/or PR-positive BCs account for almost two-thirds of all BCs. Furthermore, a standard aspect of a BC diagnosis is a test for ER/PR in the tumor. Immunohistochemistry (IHC) is the most frequent approach for detecting ER/PR in a tumor. Endocrine treatment that blocks estrogen communication has been shown to be effective in treating breast cancer since it is a hormone-related malignancy. On the other hand, cancers linked to hormones are more likely to be resistant to hormone therapy. Endocrine tolerance may be explained by a variety of factors, including mutations in receptors and bridges in other communication networks. (Ellis et al. 2008; Li et al. 2013; Toy et al. 2017). The most important hormones that govern the stem cells of the human reproductive system are progesterone and estrogen. They have the potential to promote cancer stem cells (CSCs) growth and raise the risk of reproductive malignancies. As a result, estrogen and progesterone appear to be important regulators of CSCs quantity and function. Exploring the link between female hormones and CSCs is therefore critical in order to develop more cancer medicines that target CSCs (Joshi et al. 2010; Sun et al. 2017; Hilton et al. 2018).

3.2.2 Tumor-Promoting Effects of Progesterone

During adolescence, as well as during lactation and nursing, progesterone, an ovarian steroid hormone, is essential for proper breast development. The high-affinity progesterone receptors (PR), which include the traditional progesterone

receptor (PR)-A and -B isoforms, are found in numerous female reproductive tissues, including the uterus, ovary, and mammary gland (MG). Furthermore, PR play an important role in the growth and progression of BC (Giulianelli et al. 2021). A portion of PR-A and PR-B-expressing mammary epithelial cells (MECs) in the breast also expresses ERs, and estrogen is typically required to promote PR expression in these ER+ cells. As a result, separating the effects of progesterone and estrogen, which is also a potent breast mitogen, has proven difficult. In both normal and malignant breast cells, PR isoforms are severely understudied in comparison to ER (Trabert et al. 2020). In addition, progesterone/PR isoforms influence the creation of terminal end-buds (TEBs) or acini at the ductal ends that becomes milk-producing organelles in the lactating mammary glands. EGF and IGF-1, two additional necessary hormones, increase terminal end-bud proliferation during normal breast development and augment ductal expansion and side branching generated by estrogen plus progesterone (Ruan et al. 2005).

3.2.3 Melatonin and Hormone-Dependent Breast Cancer

The pineal gland's primary hormone, melatonin, is also known as N-acetyl-5-methoxytryptamine (N-AMPT) (a photoneuroendocrine transducer that turns light into humoral impulses). It is the suprachiasmatic nuclei of the hypothalamus that controls the generation of melatonin. It has been shown that melatonin is produced in a number of other tissues, including the digestive system (including the skin), bone marrow, retina, and lymphocytes (Stefulj et al. 2001). Melatonin synthesis begins with the melatonergic pathway's absorption of circulatory tryptophan (Trp). The kynurenine pathway has been associated with the development of BC. TNF and IFN are overexpressed in BC patients, as are pro-inflammatory cytokines such IL-1 and IL-6. cytokines that induce extrahepatic indoleamine 2,3-dioxygenase, which eliminates Trp from serotonin synthesis and favors the activation of the kynurenine pathway, increase the production of kynurenine and its acid, which are AhR ligands. On the other hand, stimulation of this receptor is very important in BC (Anderson 2019). When the aryl hydrocarbon receptor is activated in BC, it causes a rise in cytochrome P450(CYP)1b1 in the mitochondria, which then causes melatonin to be converted back to N-acetylserotonin (NAS). In contrast to melatonin, NAS activates the tyrosine receptor kinase B (TrkB) receptor. TrkB activation enhances the survival and migration of BC cells. Similarly, in mammary tumor cells, a drop in pineal hormone causes AhR actions.

Melatonin is a hormone that works in a variety of different ways. For example, the pineal gland secretes melatonin, which helps to regulate circadian and annual rhythms in response to light exposure. As a result of exposure to artificial light at night (ALAN), women who work night shifts have a higher risk of developing BC (Kelleher et al. 2014). These women's risk of BC related to nightwork would be lowered if they took a melatonin pill. However, using melatonin at the wrong time can have severe consequences, as morning doses can encourage tumor growth, afternoon injections have little impact, and evening injections have anti-proliferative

effects (Malhotra et al. 2004). Melatonin also modulates the immune system by acting as an immunostimulant on monocytes and lymphocytes via interleukins and other cytokines. As melatonin levels fall, the immune system is weakened, allowing tumors to spread unchecked. As a selective estrogen receptor modulator and estrogen enzyme regulator, melatonin may help to reduce the risk of BC. Antiestrogenic effects of melatonin can be attributed to its effects in a neuroendocrine-reproductive axis, where melatonin diminishes the production of ovarian estrogens and prolactin, which are important for both normal and tumor breast growth. A method is proposed by Borin et al. Melatonin suppresses the activity of Rho-associated protein kinase, which is connected to malignant transformation and spreading of breast cancer (Borin et al. 2016).

3.2.4 Hormonal Contraception and Breast Cancer

Breast cancer risk is believed to be impacted by contemporary societal conventions, such as breastfeeding, age at first live delivery, parity, diet, body fatness, physical activity, alcohol use, and plastic exposure. Breast tissue exposure to sex hormones throughout the course of a woman's lifetime is implicated in the majority of known risk factors for BC, including reproductive, menstrual, and nutritional variables. Hormonal pills, which are often used as contraceptives, need to be scrutinized more closely in this case since they may be a modifiable risk factor for BC. As a result of a growing trend in which women take oral contraceptives (OCs) earlier in life and for longer periods of time before having a child, and as a result of the fact that contemporary female lives expose them to more hormones than those of women in countries with naturally low fertility (Lovett et al. 2017). In postnatal mammary gland development, nuclear hormone receptors (HR) such as ER and PR play a crucial role in encouraging and coordinating. There are numerous developmental cycles for the mammary gland, including pregnancy, lactation, and involution. The mammary gland is a hormonally sensitive target tissue (Obr and Edwards 2012). For the alpha isoform of the estrogen receptor to function, it must be stimulated by growth hormones like fibroblast growth factor (FGF), epidermal growth factor (EGF), and others, such as estrogen (Arendt and Kuperwasser 2015). Seventy-five percent of BCs express the ER gene, which is important for the initiation and course of the illness. It is also important to note that progesterone-induced mammary gland growth takes place on two different levels. There are two treatments available, the first of which only targets PR-positive cells and is hence estrogen-dependent. In PR-negative cells, progesterone has a more enduring impact, acting as a regulator and affecting these cells through paracrine pathways (Obr and Edwards 2012). As molecular mediators, RANKL and its receptor, RANK, are critical in nuclear factor-kB signaling pathways. Menarche and parity age, two crucial reproductive events, have impact on lifetime BC risk, according to current epidemiological research. The finding that RANKL and its receptor RANK play a critical role in controlling progesterone's proliferation-inducing effects in the breast has speed up research into the hormone's carcinogenic mechanism. Cancer formation and progression

have been connected to proliferation bursts and RANKL expression. RANKL gene and protein expression levels have been found to correlate with circulating progesterone levels in clinical studies. As a result, RANKL expression was higher in both normal and cancerous breast tissue during the luteal phase, demonstrating that RANKL mediates progesterone action in both normal and cancerous tissue (Gonzalez-Suarez et al. 2010; Goss et al. 2011).

3.2.5 Menopause Hormone Therapy and Breast Cancer

Around 80% of women will experience menopausal symptoms. Therapy with menopausal hormones has been shown to be the most effective treatment to date. Menopausal hormone treatment (MHT) decreases the risk of BC in overweight women, although there is no indication that BMI in early adulthood (age 20) correlates with the age at which women first get pregnant. MHT usage increases the risk of BC not just in the general population, but also in people who have a greater risk due to a family history of cancer. The use of MHT has been discouraged for many women, especially those at increased risk of BC. Observational research on MHT and BC mortality has inconsistent findings. This discrepancy could not be explained by MHT subgroup analysis. Using estrogen alone or EPT has been shown to increase the chance of dying from BC in the Million Women Study (Beral et al. 2019). A possible explanation for the contradiction in these findings is that any causal influence of MHT on BC survival may provide such conflicting results since mortality represents the consequences of MHT on both prevalence and survivability (Beral et al. 2019). The benefits and risks of MHT were found to be complex, with lower rates of bone fracture, diabetes, and esophageal, gastroesophageal, and colorectal cancer being the most notable, while higher rates of stroke, venous thromboembolism, gallbladder disease, and breast and ovarian cancer were found to be more common. The recommendations state that MHT prescriptions must be based on an individual's risk-benefit analysis (NICE 2013; Marsden 2019).

3.3 Relation Between Breast Cancer and Other Hormones Related to Cancer

3.3.1 Different Hormones Sensitive Cancers

Hormones are important endogenous chemicals of our body involved in regulating numerous physiological functions. These chemicals consistently mark a whole lot of purposes concerning metabolism, menopause, and mood, particularly in females. However, they are also involved in supporting the progress of numerous cancers called hormone-sensitive or hormone-dependent cancers. All cancers are not fueled by hormones. The few types that can be considered as hormone-sensitive cancers are (Henderson et al. 1982):

1. Breast cancers, as some forms of such cancers, require hormones like estrogen and progesterone for their development.
2. Ovarian cancers, as such cancers are also greatly affected by estrogen.
3. Uterine cancers or endometrial cancers can be also fueled by estrogen and progesterone.
4. Prostate cancer, as hormone mainly testosterone plays a key role in the development and spread of this cancer.

In divergence to the extensively recognized models concerning chemicals (carcinogens) and viruses as predominant tumor promoters, the hormone-related cancers share a dissimilar mechanism of carcinogenesis. It has been proven that hormones, both endogenous and exogenous are capable of lashing cell proliferation, augment cell divisions and facilitate the chance for genetic mutations or random genetic errors (Henderson et al. 1988). The important difference amid this "cell proliferation" model related to the hormonal carcinogenesis equated with the chemical carcinogenesis model is that no précised promotor is required. As an alternative, errors in DNA replication during cell division stages can generate accidental genetic mutations upsurging malignant phenotype. To date, abundant evidence in the sustenance of the cell proliferation model of hormone-related cancers has continued to accrue (Henderson et al. 1988; Feigelson et al. 1996). The important contender genes associate with such hormone-related cancers include those involved in the endocrine pathway, DNA repair processes, tumor suppressor genes, and oncogenes (Sager 1989). For example, breast cancer gene 1 (BRCA1) and breast cancer gene 2 (BRCA2) are two such tumor suppressor genes (TSG) that have been found predominantly related to vulnerability to several cancers including breast, ovarian, etc. In addition to that, germline mutations in tumor protein 53 (TP53) are also related to augmented jeopardy of BC in numerous cases. The evidence of an effective anti-hormone treatment strategy in ending progression and in that way increasing the patient's life span strengthens the predominant etiological role of hormones in the development of several cancers.

3.3.2 Breast Cancer and Ovarian Cancer

The most common malignancy among women is breast cancer. But there are similarities between breast and ovarian cancer, such as the presence of comparable mutations (TSG and proto-oncogenes), alterations in hormone regulation, and the microenvironment, etc. (Kuchenbaecker et al. 2017). Briefly understanding the rapport prevailing amid these two forms of cancer, that extensively targets females will afford opportunities in the deterrence of metastasis and will thus allow the development of new ways to cure cancer. BRCA1 and BRCA2 gene abnormalities or hereditary breast or ovarian cancer have been related to an increased chance of developing cancer of the ovaries (Antoniou et al. 2003). The American Society of Clinical Oncology has recommended that women diagnosed with epithelial ovarian cancer receive genetic testing for hereditary variants of BRCA1, BRCA2, and other

susceptibility genes for ovarian cancer as well as clinical features of their disease and family history, thus supporting the connectivity existing between breast and ovarian cancer. People with hereditary mutations in the BRCA1 or BRCA2 genes (which increase a person's chance of acquiring breast or ovarian cancer) acquire these malignancies higher than the average persons. Breast cancer is caused by hereditary mutations or alterations in the BRCA1 or BRCA2 genes that are handed down in the family in roughly 3% of cases (7500 women per year) and ovarian cancer in 10% of cases (2000 women per year) (Brose et al. 2002). There is a greater chance of developing different cancers if the BRCA1 and BRCA2 genes are either deleted or duplicated. Even though some individuals do not have BRCA mutations, their malignancy has a behavior that resembles that of BRCA mutations and/or DNA repair system dysregulation. By analyzing variations in gene copy numbers and expression patterns, recent studies have sought to discover distinct kinds of mutations in these genes (Finch et al. 2002). Mutations in some DNA repair systems may thereby raise the risk of breast and ovarian cancer, as a result (Fig. 3.1).

Constitutional mutations refer to germline changes in the BRCA1 and BRCA2 genes that may be passed on to children. Complete gene deletions, massive insertions, duplicated sequences, frameshifts, and missense or nonsense mutations are all possible with these alterations. The frequency of these mutations, on the other hand, varies from one group to the next. Nearly 3500 mutations have been identified in these two genes, according to recent publications. For example, Persons of Ashkenazi Jewish descent are offered tests for all three mutations (BRCA1 and BRCA2) that can occur through knee-jerk full sequencing or deletion/duplication analysis (Kuchenbaecker et al. 2017).

In addition to that, the penetrance of mutations is also important for genomic rearrangements to develop into a detectable trait. These alleles are easier to detect than those with lower penetrability since they are always visible in a person who has them. However, there are many variances in low genetic risk alleles, and these low heterozygosity genes might increase the risk of cancer and its development (Tai et al. 2007).

For example, high penetrance alleles for breast/ovarian cancers are TP53 and Serine/threonine kinase 11 (STK11). The moderate penetrance alleles include Partner and Localizer of BRCA2 (PALB2), BRCA1 Interacting Helicase 1 (BRIP1), and S. cerevisiae (RAD51C) (Cavanagh and Rogers 2015). The KRAS-variant (a germline miRNA binding site disrupting variant) and Flap endonuclease 1 (FEN1) are the recently identified biomarkers for breast and ovarian cancer and can thus be used for early detection of these cancers (Nelson et al. 2013).

3.3.3 Breast Cancer and Endometrial Cancer

Endometrial cancer and BC are common malignancies found in women and because of estrogen dependence, a connotation is assumed to exist between these two forms of cancers affecting females. Endometrial cancer typically ensues after natural menopause and 60 being the middling age at diagnosis of this cancer type.

Endometrial cancer and BC share approximately similar reproductive and hormonal peril aspects, including nulliparity and acquaintance to unobstructed estrogen (Grady et al. 1995). Reports accumulated on double primary cancers in the same individual offer additional indication for an etiological connotation amid BC and endometrial cancer (Curtis et al. 1985; Storm et al. 1985). In addition to that, it appears probable that there are collective hereditary mechanisms involved in the etiology of some endometrial and BC cases. For example, Cowden syndrome and hereditary non-polyposis colorectal cancer (HNPCC) (Mutter et al. 2000). However, the familial association between breast and endometrial cancer is uncertain.

The BC treatment which is preferably been done with the drug named tamoxifen upsurges the jeopardy of emergent endometrial cancer. This menace essentially happens because of the mechanism of action of the drug, tamoxifen. Broadly, the drug acts contrary to the growth-promoting effects of the hormone estrogen particularly in breast tissue but acts like an estrogen in other tissues, including the bones and the uterus. In bones, it is beneficial as estrogen enhances bone density but increases the risk of cancer development and progress in the uterus (Chlebowski et al. 2015). Tamoxifen belongs to the drug class called selective estrogen response modifiers (SERMs) and has been found to upsurge the menace of endometrial hyperplasia. Though, hyperplasia itself is not cancer but can occasionally progress into cancer. In a case-control study, it was found that there was a 3.6% increase in the risk of endometrial cancer in women with BC treated with tamoxifen for at least 5 years compared to women without treatment. There was also an association between long-term use of tamoxifen and increased endometrial cancer risk (Odds ratio [OR] = 2.94, 95% CI 2.13–4.06, 3 years or longer; Odds ratio = 4.08, 95% CI 1.67–9.93) in women over 35 years of age compared to women 35 years of age and younger (3.5 ± 3.0 years) in BC patients using tamoxifen (Davies et al. 2011). Tamoxifen also somewhat upsurges the jeopardy of uterine sarcoma, cancer that starts in the muscle of the uterine wall. In a separate study of high-risk women with BC being prescribed tamoxifen as part of BC prevention, the median follow-up was 6.9 years and there were four sarcomas (1.7 for 100,000 patients per year) in the tamoxifen group and none in the placebo group. There were no differences in median age at the time of diagnosis or years of tamoxifen exposure (Sismondi et al. 1994). It can be concluded that a disease or state that upsurges the quantity of estrogen in our body can increase the risk of endometrial cancer. Females having BC/ovarian cancer might have augmented jeopardy of endometrial cancer too because the common dietary, hormone, and reproductive risk factors for breast and ovarian cancer likewise upsurge endometrial cancer risk.

3.4 Biomarkers in Breast and OHRC

When the body reacts to a cancerous tumor, tumor markers are generated by the cancer cells or created by the body itself. It is possible to monitor cancer progress, check for recurrence, or help screen for, diagnose, or stage cancer by testing these biomarkers in blood, urine, or other fluids. Biomarkers and tumor markers might be

proteins or DNA modifications, such as mutations and other abnormalities. Tumor tissue or bodily fluids may include a biomarker that aids in diagnostic and therapy options selection. When paired with clinical symptoms and imaging examinations, tumor marker data seldom serve as a diagnostic tool on their own, although they may provide insights.

3.4.1 N1-Acetylspermidine as a Chemical Biomarker

N1-acetylated spermidine and spermine derivatives were identified in high concentrations in mammary tumors, while these compounds were absent from normal breast tissue. There is evidence to suggest that the female sex hormone estrogen is involved in the development and course of the disease. In response to estrogen stimulation, ornithine decarboxylase (a polyamine biosynthesis enzyme) is expressed more than in the absence of estrogen stimulation (ODC). When compared to nearby normal tissues, tumor polyamine levels are higher in the former than in the latter. Research shows that polyamine catabolic enzymes spermidine/spermine N1-acetyltransferase (SSAT) and spermine oxidase (SMO) activation are crucial for the anti-proliferative and apoptotic actions of polyamine analogs and their combinations with chemotherapeutic drugs like 5-fluorouracil (5-FU) and paclitaxel. As a result, polyamine catabolic enzymes may serve as useful therapeutic targets and sensitive-marker indicators (Thomas and Thomas 2018).

The protein, known as SSAT-1, is widely distributed throughout mammalian tissues and may even be found in extremely low concentrations in the healthy cells of humans and animals. Toxic substances, hormones, medications, and growth factors are just a few of the things that might cause this (Matsui et al. 1981; Pegg et al. 1982; Seiler 1987). It has been shown that SSAT-1 is overexpressed in human prostate cancer, which helps to keep polyamine concentrations from becoming hazardous. Oncogenic polyamine synthesis increases polyamine and N1-acetylspermidine levels, indicating enhanced activity of SSAT-1. Tumor cells and tissue from both humans and animals have been shown to have higher levels of monoacetylated polyamines. As well as primary patient-derived tumor tissues, Maksymiuk et al. 2018 examined SSAT-1 expression levels in human normal and cancer cell lines. SSAT-1 expression levels were shown to be higher in some malignancies, and researchers believe that measuring SSAT-1 activity by measuring the excretion of N-acetylamantadine in urine might serve as a cancer diagnostic test in people (Maksymiuk et al. 2018).

3.4.2 Mucin-1, Urokinase-Type Plasminogen Activator as Protein Biomarker

Mucin 1 is a tumor-associated transmembrane glycoprotein that is the subject of much investigation (Jing et al. 2018). A glycoprotein found on the surface of epithelial cells in the pancreas, breast, lung, and gastrointestinal system, MUC1 is

typically expressed at low levels (Zhou et al. 2018). Since its upregulation may affect tumor cell invasion, proliferation, and survival by reducing cell–cell adhesion and cell–extracellular matrix adhesion, MUC1 has recently become a research topic in the diagnosis of cancers. Cancer development and invasiveness are controlled by MUC1, which has also been linked to EGFRs, β-catenin, and nuclear factor (NF)-κb signaling (Mansouri et al. 2016). Cancer angiogenesis and chemoresistance are both related with high levels of MUC1 expression (Pillai et al. 2015). Hence, MUC1 may have a role in carcinogenesis, progression, and dissemination and may be a prognostic factor for malignancies. Over 90 percent of breast tumors express MUC1, a surface protein of epithelial cells. There is still much to learn about the function of MUC1 in BC and the molecular pathways involved.

To put it another way, "Urokinase-type Plasminogen Activator" (uPA) is an essential serine protease that converts passive plasminogen into active plasmin, which is then implicated in a variety of metastatic cancer processes (Mahmood et al. 2018). For the first time, the ELISA method was used by Jänicke et al. to assess the quantity of uPA protein present in BC tissue (Janicke et al. 1989). Breast cancer patients with poor prognosis had an increased level of uPA antigen in their primary tissues, according to the study findings. Later, uPA biomarker testing was suggested for the evaluation of BC risk and the selection of suitable adjuvant chemotherapies for patients (Harris et al. 2007). There is still enzyme-linked immunosorbent assay-based techniques that are therapeutically useful for assessing BC outcomes and measuring uPA. As a result, the researcher has created uPA commercial ELISA kit products. FEMTELLE® is one of the tools used by uPA to see if chemotherapy is useful after initial BC surgery. (Mengele et al. 2010). ELISA-based tests have several drawbacks, like need of fresh specimens (Schmitt et al. 2008).

uPA mRNA expression has been measured by many organizations to detect malignancy (Spyratos et al. 2002; Lamy et al. 2007). Because mRNA can be extracted from formalin-fixed tissues, it has a significant advantage over ELISA-based tests. The influence of uPA on mRNA expression in cancer diagnosis has been studied although the results vary. Further investigation is necessary before a firm conclusion can be reached.

3.4.3 Ki-67 as a Prophetic Marker in Breast Cancer

The Ki-67 assay measures the rate at which BC cells divide and proliferate. Tissue samples are subjected to the examination, which utilizes an antibody known as MIB1. A sample of BC tissue may be tested for the Ki-67 marker to see how aggressive the tumor is likely to be (Mannell 2016). Tumor cells proliferate and divide more quickly when MIB1 is attached to more cells. The Ki-67 score may be useful in determining a patient's prognosis or recovery prospects. Cancers with greater concentrations of Ki-67 may have a worse prognosis than tumors with lower concentrations, according to research. (Zhu et al. 2020).

3.4.4 HER2 as a Prophetic Marker in Breast Cancer

HER2 is a fundamental biological marker for treatment that has been identified and established as a prognostic factor and a predictor of response. IHC and FISH, both well-established, quick, and inexpensive techniques for finding these markers, may be used to find them throughout the body. In conjunction with clinicopathological prognostic factors, these molecular markers predict cancer recurrence and progression the most accurately. Finally, molecular markers like hormone receptors and HER2 are critical for future medication development because of their therapeutic significance. The human HER2 gene (ERBB2 gene) produces the growth-promoting oncoprotein HER2, which is also known as tyrosine kinase, erbB2, CD340 (cluster of differentiation 34), ERBB2 (humans), Erbb2 (rats), NEU, HER2/neu, HER2, MLN 19, and TKR1. It is also known as the HER2/neu oncoprotein (Mohanty et al. 2020). Mammary cells include HER2 protein receptors, which assist and regulate the growth, division, and repair of mammary cells. However, the HER2 gene may malfunction and generate an excessive number of copies of itself, a condition known as HER2 gene amplification. This can lead to breast cancer. Then, because of the mammary cells making too many copies of the HER2 gene, the HER2 protein is overexpressed (Chen et al. 2021). This portends unrestrained growth, development, and multiplication of mammary cells. Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) make it simple to determine the presence of the HER2 gene and protein (Kalachand et al. 2017). Breast cancer is referred to be HER2 positive when the HER2 protein is overexpressed and the HER2 gene is amplified using FISH (Taneja et al. 2010). When the HER2 gene or the HER2 protein is not overexpressed in the tumor, it is known as HER2 negative BC. Indeed, over-expression of this oncogene contributes significantly to the development and progression of some aggressive forms of breast cancer (Burstein 2005).

3.5 Genetics Behind Hormonal Breast Cancer

Breast cancer prevalence is greater in first-degree relatives (McPherson 2000). Hereditary BC is approximately 10%, among all the BC and alteration (mutation) of the gene are directly involved in it (Apostolou and Fostira 2013). BRCA1 and BRCA2 are only two of the many genes now known to have a role in the development of BC. Furthermore, breast cancer rates in women are rising owing to an aging population. There are several causes of gene mutation in BC including hormones such as estrogen, which modulate these genes to alter the cellular process such as metabolism, apoptosis, cell cycle arrest, cellular proliferation, etc. (Apostolou and Fostira 2013).

3.5.1 BRCA1 and BRCA2 Gene Mutation

BRCA1 gene is localized to 17q21 in families, which is linked to the early onset of BC (Godet and Gilkes 2017). Mutations in the BRCA1 gene are related to age, as the risk of BC development is 200-fold higher in 40-year-old women who inherit a mutation in BRCA1 than the general population (Petrucelli et al. 1993). However, with the increase in age, the risk of the development of cancer reduces. This is evident that mutation in the BRCA1 gene promotes the cancer development tendency in hormone-responsive tissue such as prostate, ovary, and breast. Estrogen receptors are present in two isoforms in mammalian cells, i.e., ERα and Erβ, Erα regulates the function of mammary cells and thereby contributes to the development of the breast (Paterni et al. 2014). Estrogen receptor elements (E-ER) binds to ER, which stimulates cellular proliferation by upregulating the cell cycle regulating genes like Cyclin D1 and c-Myc and protein synthesis gene (Keyomarsi et al. 2011). This molecular upregulation in response to estrogen contributes to the development of the breast. Moreover, Erα also interacts with BRCA1, and the expression of Erα is inhibited with the inhibition of E-ER by BRCA1 (Gorski et al. 2009). This balance between BRCA1 and Erα disturbs due to mutation in BRCA1, which leads to inhibition of apoptosis and induction of proliferation of mammary cells.

Moreover, E-ER also regulates cellular metabolic function, as it promotes the Krebs cycling and glycolysis in presence of sufficient nutrients (Wang and Di 2014). This promotion of metabolic function such as glycolysis is achieved through upregulation of the PI3K-AKT signaling pathway, which causes mitotic division of cells and thereby induces cellular proliferation (Sever and Brugge 2015). However, cellular metabolism is negatively regulated by BRCA1, as it degrades the phosphorylated AKT and downregulates the expression of Igf-1 (Kang et al. 2012). Fatty acid synthesis is required for the growth of tumor cells and acetyl-CoA to malonyl-CoA contributes to the process of fatty acid synthesis. Acetyl-CoA carboxylase (ACC) was reported to stimulate the conversion of acetyl-CoA to malonyl-CoA, thus regulate the growth of tumor cells. However, BRCA1 is also known to directly inhibit the ACC and modulates the growth of these cells (Mashima et al. 2009). This evidence concretes the role of the BRCA1 gene in the regulation of tumor growth and mutation of it involved in the development of BC.

BRCA2 gene is the second most dominant BC susceptibility gene after BRCA1, which is involved in female BC. A study estimated more than 70% of lifetime risk of BC development for BRCA2 mutation carriers and BRCA2 gene mutation involved in approximately 2% of all types of BC. Moreover, recent evidence predicts that BRCA2 mutation is involved more in the development of BC in women in comparison to BRCA1. This projection of differences is due to the presence of BRCA2 mutation carriers in the aggressive tumor. Luminal B4 BC is an aggressive clinical behavior BC subtype, which occurs due to BRCA2 estrogen receptor (ER)-positive BCs in which recurrence score is higher than sporadic BCs (Li et al. 2016). The survival rate is higher in women with sporadic BC with ER-positive tumors than ER-negative tumors. Moreover, BRCA2 mutation-associated BC strongly expresses

ER and overexpresses HER2/neu protein of luminal B in contrast to BRCA1 (Fragomeni et al. 2018).

Risk of development of BC increases due to germline mutations in one allele of the BRCA2 tumor suppressor gene. BRCA2 gene is also involved in error-free DNA repair of double-strand breaks (DSBs) through homologous recombination (HR) and mutation in the gene disturbs the given mechanism of DNA repair, which leads to the development of BC (Joosse 2012). These statements suggest the role of BRCA2 gene mutation involved in aggressive BC by expressing ER.

3.5.2 High Menace Gene Mutations in PALB2, PTEN, and TP53 Genes

PALB2 is a localizer and partner of the BRCA2 gene, which is a moderately susceptible gene risk factor (Wu et al. 2020). PALB2 gene is involved in protein synthesis, which interacts with the protein synthesized by the BRCA2 gene. PALB2 is closely related to BRCA1 and BRCA2 genes to double-strand DNA repair, it functions as a tumor suppressor gene (Nepomuceno et al. 2017). Women with an abnormal PALB2 gene have a 14% of risk by 50 years of age for the development of BC, which enhances up to 35% in 70 years of age (De Angelis et al. 2021). A study reveals that BC risk for a PALB2 mutation carrier, even in the absence of a family history of BC (De Angelis et al. 2021).

PTEN is a multi-functional tumor suppressor gene identified on chromosomal band 10q23.3, it helps in the regulation of normal cell growth. PTEN gene product protects the domain of tyrosine phosphatase, which shares the homology with cytoskeleton proteins auxilin and tensin. Mutation in the PTEN gene consists of nonsense mutation and homozygous deletions. In more than 50% of cases of breast carcinomas, loss of heterozygosity affects 10q23.3, and germline mutation of PTEN is detected in autosomal dominant cancer included malignancy of breast (Chalhoub and Baker 2009). PTEN gene inactivation is involved in numerous cancers including BC.

PI3K signaling pathway is involved in more than 70% of cases of genetically abbreviated BC (Chalhoub and Baker 2009). Moreover, the molecular profile clarifies that ER-positive BC into an aggressive type of cancer, i.e., luminal B and resistance to hormonal therapy. ER reduced activity associated with estrogen-positive BC due to activation of PI3K pathway (Fu et al. 2013). PTEN gene downregulates the PI3K pathway and loss of it activates AKT/mTOR pathway which contributes to endocrine resistance therapy (Dong et al. 2021). A study on the mouse model justifies the dose-dependency of PTEN in the development of BC, as 20% downregulation of PTEN involves the high penetrance of BC.

TP53 gene is involved in the stoppage of growth cells by damaging DNA and it is frequently mutated in cancer (Mantovani et al. 2019). P53 is a transcription factor code with TP53, which involved in the repair of DNA, metabolism, apoptosis, cellular senescence, apoptosis, cell cycle arrest, and other process occurring after stress (Chen 2016). Moreover, mutated TP53 also involves in the cause of several

diseases such as sarcoma, brain tumor, leukemia, and BC. A study suggests that TP53 mutation is found in approximately 30% of cases of BC (Weng et al. 1999).

P53 has a complex signaling network that alters due to several modulations in intracellular and extracellular components and various components, which modulates P53 network need to regulate/ balance carefully, as it alters P53 function through which changes in the cellular response also occur (Rivlin et al. 2011). Erα is a modulator of the P53 signaling pathway. Mutation of the TP53 gene is highly evident in both ER-positive and ER-negative BC patients (Lu and Katzenellenbogen 2017). P53 forms a complex with Erα by direct interaction, which inhibits the function of the P53 gene. A study suggests that Erα binds directly to the P53 to accesses the p53 target gene promoters and function of P53 repressed by Erα, as it recruits HDAC1, MRT, and NCOR directly to modulate the interaction or complex formation between Erα-P53 (Konduri et al. 2010).

3.5.3 Moderate Menace Gene Mutations in ATM and cdh1genes

Ataxia-telangiectasia mutated (ATM) gene contributes to the DNA repair and is considered as on co-suppressor gene, which is located on chromosome 11q22.3 (Boultwood 2001). ATM gene belongs to family phosphatidylinositol 3-kinase-related protein kinases (PIKKs). ATM protein is involved in the activation of response to DNA damage pathway and participates in DNA repair (Boultwood 2001). Several cell processes such as telomere maintenance, oxidative stress, gene regulation, apoptosis, and cell cycle control are regulated by the ATM gene, and mutation in this gene is observed in many malignant conditions including BC. A report suggests that AT does not occur due to heterozygous mutation, however carrier of it enhances the risk of development of BC 2–3 folds (FitzGerald et al. 1997). Heterozygous mutation of the ATM gene is observed in nearly 40% of patients suffering from sporadic BC (Broeks et al. 2000). The risk of development of BC is strongly associated with the variant of ATM, as a high risk of development of BC is observed with the V2424G variant while the lower risk is associated with S707P, L546, and D1853V isoforms (Broeks et al. 2000).

Transcription of ATM is downregulated due to ER-α by the activation of miR-106a and miR-18a, which delays the DNA repair and induces cell cycle causing progression of the cell due to DNA damage and delayed response to it (Song et al. 2011). A report also suggests that BC treated with estrogen therapy sustained it without an increase in apoptosis and P53 activation due to failure of ATM activation. There are many genes such as ATM, CHK2, P53, BRCA1, and AKT that interact with ATM to influence the cell cycle process and lead to BC (Deng 2006). ATM activates and phosphorylates the checkpoint kinase CHK2, which controls cell cycle arrest (Hirao et al. 2002). Cell process changes lead to cancer formation when combined with an ATM gene mutation.

CDH1 gene encodes for E-cadherin/Cadherin 1 located on 16q22.1, which causes hereditary diffuse gastric cancer associated with germline mutation (Liu and Chu 2014). E-cadherin is a cellular adhesion protein that comes in a type of calcium-

dependent glycoprotein which involves cell–cell adhesion (Goud et al. 2020). There are several pathological features such as poor prognosis, lymph node metastasis, infiltrative growth, and poor differentiation associated with reduced expression of E-cadherin. Women having a family history of diffuse gastric cancer have a 50% risk of BC (Zhou et al. 2014). Alteration in the expression of E-cadherin was observed in several types of cancer including BC.

CDH1 germline mutation interacts mutually with the alteration of BRCA1/2 gene mutation, a mutation in the CDH1 gene commonly occurs extracellularly due to which alteration in the interaction between EGFR/E-cadherin occurs (Berx et al. 1998). This contributes to increased cellular mobility. A study also supports that EGFR inhibitor reduces the metastatic ability and cellular mobility (Luo et al. 2018).

3.5.4 Less Risk Gene Mutations in CHEK2, NBN, NF1, and STK11 Genes

There are several other genes, that are involved in the development of BC but the risk is associated with a lower side. However, still there is a need to discuss these genes and their role in BC.

Checkpoint kinase 2 (CHEK2) gene is located on chromosome 22q12.1, which is a mediator in DNA damage response, activates DNA repair, apoptosis, and cell cycle arrest (Nevanlinna and Bartek 2006). Kinase function is reported to be lost due to mutation of the CHEK2 gene in BC (Apostolou and Papasotiriou 2017). 1157T and 1000delC CHEK2 germline variants mutations found in patients suffer from BC, risk of BC enhances two-fold in women with 1000delC variant, which enhances to 10 folds in men (Nevanlinna and Bartek 2006). BC associated with CHEK2 gene mutation are ER-positive, a report suggests that in women 72% of cancer-associated ER-positive and postulates that tamoxifen could be used for the intervention in BC associated with CHEK2 mutation as these cancers are ER-positive (Shiovitz and Korde 2015).

NBN gene is also known as NBS1, which is an encoding of nibrin. NBN gene provides the protein named nibrin, which has a role in several cellular functions including response to DNA damage (Desjardins et al. 2009). NBN interacts with RAD50 and MRE11A proteins to guide them to work together to prepare the broken strand of DNA (Zhang et al. 2006). Heterozygous NBN status is associated with several types of cancer including BC and the risk of it enhances 2-3-fold in the carrier of a deleterious mutation in the NBN gene (Seemanova et al. 2007).

NF1 is a gene located at chromosome 17 involved in the synthesis of neurofibromin protein which regulates cell growth (Shen et al. 1996). Mutation in the NF1 gene causes the development of CNS cancer due to the loss of neurofibromin. NF1 gene mutation also enhances the risk of development of several other types of cancer including BC, which is especially before the age of 50 in women (Sharif et al. 2007). NF1 mutation leads to loss of neurofibromin which promotes tumorigenesis by dysregulating the RAS/MAPK signaling pathway (Shaikh et al. 2019). A preclinical study was evident that NF1 mutation or deletion

is involved in dysregulation of ER and RAS signaling and suggests that NF1 could be the therapeutic target for endocrine resistance BC (Dischinger et al. 2018).

STK11 gene is involved in the synthesis of an enzyme known as serine/threonine kinase 11, which is a tumor suppressor enzyme as it regulates the growth of cells (Granado-Martínez et al. 2020). Mutation in the STK11 gene develops a rare disorder Peutz-Jeghers syndrome. Population suffering from Peutz-Jeghers syndrome are at higher risk of development of ovarian, lung, and BC. Women suffering from Peutz-Jeghers syndrome are at a 45% risk of development of cancer throughout the life (Alkaf et al. 2017). Erα signaling is activated by STK11, as it acts as a coactivator of it and enhanced expression of it promotes the risk of development of BC. SKT11 phosphorylate Akt leads to suppression of apoptosis of tumor cell and thus STK11 acts as oncogenic gene (Lima et al. 2019).

3.6 Conclusion

This chapter finds that choosing a promising approach may help cure BC. Because the global incidence of BC is rising, it is vital to identify the influence of modifiable risk variables so that future primary preventative interventions can be better guided. High levels of estrogen and progesterone generated by premenopausal women's ovaries are linked to an increased risk of BC. Since contradicting findings from epidemiological research appeared, the link between hormone tablets and the risk of BC has been a widely disputed topic. BCs growing complexity has raised questions regarding the risk accumulates in any of its numerous histological forms, or women at greater risk react more selectively to oral contraceptives. It may be possible to provide entirely "personalised" treatment by supporting "prevention" tactics with the full "participation" of women who are empowered to make healthy lifestyle choices based on research concentrating on "precise" biological pathways.

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Cancer Care and Psychosocial Needs

4

Saima K. Beigh and Hira Gul

Abstract

When an individual is diagnosed with cancer, s/he is embarking on a long journey that affects physical health, emotional well-being, and relationships with family, friends, and others. Cancer patients are at risk for long-term physical impairment, disability, and inability to perform everyday tasks, as well as psychological and social issues that may arise as a result of the diagnosis and its sequelae. Many cancer survivors claim that their caregivers are unaware of their psychosocial needs and often fail to identify and properly treat depression and other stress symptoms. From the communication of the diagnosis to the management of the end-of-life process, psychological problems play a significant role in oncology settings. When the patient is diagnosed with cancer, approximately 30% of patients experience psychological distress or other serious mental health issues. We are going to explain the type of caring for a cancer patient entailed on a personal, relational, and socio-cultural level. The chapter will cover a wide range of topics, including the importance of personality and the psychological distress that cancer patients can encounter, as well as the caregiver's critical position and the significance of socio-cultural influences.

Keywords

Cancer care · Survivors · Physical health · Psychological problems · Social impairments

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4.1 Introduction

Cancer has been there since the beginning of time with findings indicating that tumors were present in animals as well as plants (Hajdu 2011). The first medical document describing cancer was Egyptian Edwin Smith Papyrus, wherein breast cancer was described as a grave disease with no treatment (Breasted 1930). Later, Hippocrates and Galen coined the term cancer and tumor, respectively, but they could not do much as they lacked sophisticated pieces of equipment (Brannon et al. 2013).

Humans are made up of cells and these cells normally grow, multiply, and die eventually being replaced by new cells. Sometimes, these cells divide abnormally, forming tumors and invading nearby organs via blood or lymph which is referred to as metastasis (Chambers et al. 2002). There are many types of cancers but all of them have one thing in common, i.e., the presence of neoplastic tissue cells (Fidler 2003; Gupta and Massagué 2006; Nguyen et al. 2009).

Neoplastic cells are characterized by abnormal growth that deprives the host organ of nutrients. These cells can be benign or malignant, benign tumors grow slowly and do not spread to other body parts, whereas malignant ones spread to other areas (Brannon et al. 2013; Hanahan and Weinberg 2000; Steeg 2006).

4.1.1 Death and Survival Rates

The global cancer burden is expected to rise 47% in 2040 (Sung et al. 2021). In 2020, the estimated incidence of cancer patients in India was 679,421 (94.1 per 100,000) for males and 712,758 (103.6 per 100,000) for females. One in 68 men (lung cancer), one in 29 women (breast cancer), and one in nine Indians (0–74 years of age) will develop cancer during their lifetime (Mathur et al. 2020). India has a high burden of tobacco-related cancers in men and cervical cancers in women, which are related to low socio-economic status (Mint et al. 2020).

Even globally, cancer accounted for ten million deaths in 2020, with the most common cause of death being lung cancer (1.80 million), colon and rectum (935,000), liver (830,000), stomach (769,000), and breast cancer (685,000) (Piñeros et al. 2021).

4.1.2 Risk Factors

Cancer often results from certain behaviors like cigarette smoking, alcohol abuse. However, some factors are largely beyond anyone's control which include inherent and environmental risk factors (Stine et al. 2018; Wu et al. 2018; Yari et al. 2018; Xu et al. 2019; Machlowska et al. 2020).

4.1.2.1 Inherent Risk Factors

Inherent risk factors include ethnicity, gender, age, and family background (Taitt 2018; Guerrero et al. 2018; Gupta et al. 2019). Although these hazards are beyond one's control, persons with cancer risk factors can minimize their risk by changing their lifestyles, such as diet, exercise, and stopping smoking. Much of the research about family history of cancer has focused on breast cancer.

Breast cancer is hereditary disease (Colditz et al. 1993). Age is also a factor in breast cancer as women who are diagnosed with breast cancer are often women who have reached or passed menopause (Ban and Godellas 2014). Breast cancer gene 1 and 2 (BRCA) are protective factors against breast cancer as they provide a protective protein (Paull et al. 2001) and they have been implicated in the case of pancreatic cancer in both men and women (Lynch and Smith 2005).

Ethnicity refers to the different cultural identities, socio-economic, religious beliefs, language, and diet (Wiencke 2004). These variables contribute to cancers, their diagnosis, treatment, and attitude towards diseases (Brannon et al. 2013). For cancers, European Americans, African Americans have 40% to 50% more incidence of cancers than Asian Americans, Hispanic Americans, and Native Americans which can be attributed to diet and practices (Nguyen et al. 2009). Age is one of the strongest risk factors for cancer, there is a steep increase in cancer mortality with progressing age in men and women but men are more at risk (Brannon et al. 2013).

4.1.2.2 Environmental Factors

Environmental factors contribute to the development of cancer and sometimes increase the vulnerability to develop cancer, these things include asbestos, pesticides or living near a nuclear facility (Boffetta 2004; Siemiatycki et al. 2004). Prolonged exposure to asbestos, diesel exhaust, welding fumes can increase the susceptibility of lung cancer to 9% compared to people who are not exposed to such environments (Gustavsson et al. 2000; Yano et al. 2001). Nuclear plants also contribute to the development of rectum, colon, lung, and testicular cancers in the workers (Ashmore and Sewell 1998; Sont et al. 2001).

4.1.2.3 Behavioral Risk Factors

Behavioral risk factors are those that you or your doctor can change, treat, or modify. Behavioral risk factors for cancer include diet smoking and alcohol abuse, exposure to ultraviolet light, physical inactivity, and sexual behavior (Brannon). Lung cancer is often prevalent in men and women who smoke, in the United States alone 660,000 people were diagnosed with tobacco-related cancers and 343,000 people succumbed to these cancers from 2009 to 2013 (Henley et al. 2016; Freedman et al. 2016). An unhealthy diet is a major risk factor for cancer. Foods that are adulterated or contain chemicals and preservatives are often carcinogenic and contribute to the cancers of the stomach, colon, kidneys, thyroid, and esophagus (Murtaugh et al. 2004).

Exposure to ultraviolet light has been recognized as a major cause of skin cancer. Melanoma is prevalent in light-skinned people who have been exposed to the sun (Holick 2004).

Sexual behavior is also one of the factors responsible for cancers, Kaposi's sarcoma and non- Hodgkin's lymphoma are malignancies caused by AIDS, and often cancers of the prostate, cervix, vagina, and ovary are because of unsafe sexual practices (Henke-Gendo and Schulz 2004; Rosenblatt and Hart 2000).

4.2 Living with Cancer

Each year millions of people around the globe receive diagnosis and treatment of cancer which is often accompanied by feelings of anger as well as anxiety largely because the treatment is accompanied by some unpleasant effects like hair loss, weight loss, and often disfiguring wherein surgeries are involved. The three most common methods of treatment are surgery, radiation, and chemotherapy Singer et al. (2010).

Surgery is done when cancer has not metastasized and the surgeon comes to a conclusion that the procedure will be successful. These surgeries involve the removal of some parts of the body and sometimes leave men and women disfigured which affects their body image and self-esteem. Radiation and chemotherapy have side effects like nausea, hair loss, fatigue and anxiety, and sometimes burns.

4.2.1 Adjusting to Diagnosis and Treatment

Receiving the diagnosis of cancer is a very stressful period accompanied by bouts of anxiety and reservations about treatment. People who receive the diagnosis of cancer often suffer from depression, and chronic depression often speeds up the progression of cancer (Spiegel and Milstien 2003).

People who take up the fighting spirit often have an advantage over people who do not fight back (Dobson and Bell 2005). Older women who receive breast cancer diagnosis are better adjusted and fight off cancer well than young women (Helgeson et al. 2004) (Fig. 4.1).

4.3 Cancer Induced Stressor

4.3.1 Psychosocial Stressors

Physical as well as psychological limitations may lead towards serious social issues, such as the inability to work or perform other socially acceptable duties. Cancerous patients have been found to have symptoms that shows (post-traumatic stress disorder) PTSD and (post-traumatic stress symptoms) PTSS (Kangas et al. 2002; Bruce et al. 2006). According to the research of APADMD (American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders), suffering a lethal disease or witnessing it in someone close to you can be a qualifying event for PTSD. Even individuals who do not acquire clinical syndromes may experience severe distress due to anxieties, fears, and other forms of psychological stress. Guilt,

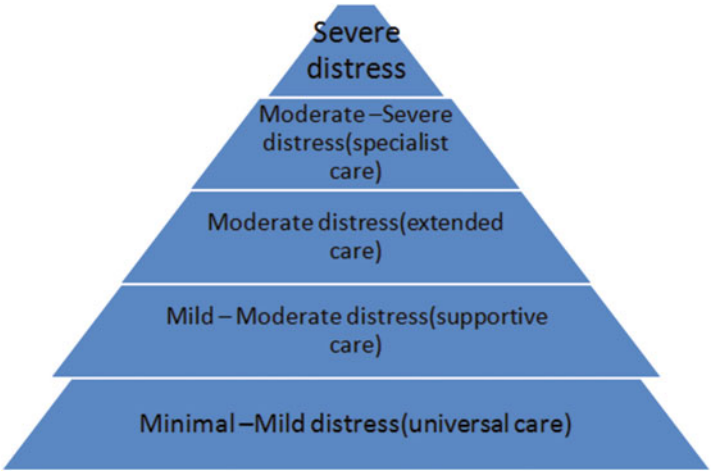

Fig. 4.1 Pyramid showing the psychosocial needs for cancerous patients

feelings of loss of control, rage, grief, perplexity, and dread are all common side effects of chronic illness (Charmaz 2000; Stanton et al. 2001). Challenges that are all common among cancer patients are shifts in mood, fear of rejection, body image problems, and anxiety attacks (Kornblith 1998). As a result of this stress, people's self-esteem suffers. Patients may also experience generalized anxiety, fear for the future, inability to make plans, uncertainty, and a sense of vulnerability, as well as other concerns, such as the possibility of second cancer, changes in sexual function and reproductive ability, and changes in one's role within the family and other relationships. Furthermore, cancer patients may have spiritual and existential concerns related to their faith, their perception of god, and the likelihood and meaning of death. Some cancer survivors express sentiments of rage, alienation, and helplessness. When a family member is diagnosed with a life-threatening illness, there is a dread of losing the loved one and worry about the agony he or she may undergo Mitchell et al. (2011).

4.3.2 Consequences of Stressors

Some stresses (discussed in the previous sections) occur as a side effect of cancer, whereas other stresses occur before diagnosis and are imposed by the healthcare system. Although these issues may not affect any individual treated for cancer, those who do require the information and proper skills to operate and treat them best. Health conditions may suffer with the unavailability of these resources and thus patient suffers.

Even if patients have the knowledge, skills, and information, they need to cope with their condition, a lack of logistics and material resources, such as transportation, medical equipment, and supplies, can hinder them from doing so.

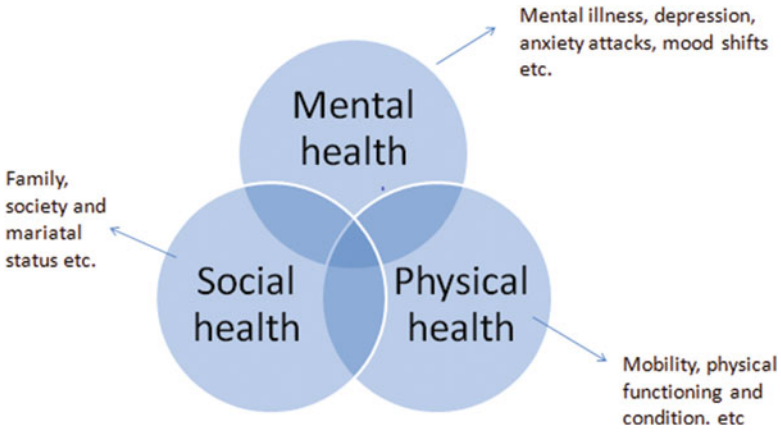

Fig. 4.2 Issues faced by cancer survivors

High medical costs combined with joblessness, work reductions, and unemployment, as well as corresponding decline in salary (expenditure), can make getting the necessary resources difficult for cancer treatment. Many of these resources can be provided or secured by relatives, family members, friends, and other informal sources of support system (Eakin and Strycker 2001), although such type of sources are occasionally unavailable or sometimes overwhelmed by patients' treatment requirements (Fig. 4.2).

4.4 Psychosocial Aspects of Cancer

4.4.1 Social Support

The social support of family members, close friends, co-workers, relatives, and neighbors is very helpful for cancer patient (Thoits 1985). It is the emotional and material resources that are provided to an individual through interpersonal communication (Moak and Agrawal 2010).

Patients and families dealing with cancer diagnoses are likely to suffer a range of emotions and stress. Every cancer patient has fears of mortality, interruption of life goals, changes in imagination and self-confidence, changes in social roles, lifestyle, and financial concerns. Such variables would have a distinct impact on each cancer patient Koehly et al. (2008).

While social relationships are a primary source of beneficial disease adjustment, improper social relationships can have negative impacts on disease adjustment. It is important to remember that social support with facilitation in the process of cognitive and stressful events helps patients adjust to their circumstances; nevertheless, if social reactions are negative and anti-disease, the process will be slowed (Baider and Surbone 2014).

Social support is associated with better treatment results for different types of chronic illnesses, including breast cancer, it also helps in alleviating the stress that comes with a cancer diagnosis along with improving emotional well-being. The most important type of support for a cancer patient is informational support, cancer patients should be provided with information about their diagnosis, prognosis, and possible side effects of the treatment (Hagedoorn et al. 2000).

4.4.2 Communication

Communication is a crucial clinical skill in oncology, yet few physicians or specialist cancer nurses have undergone formal training in this area. Patients and their families may be distressed by insufficient communication since they often require far more information than is often delivered. Many patients leave consultations with questions about their diagnosis and prognosis, the meaning and necessity of additional diagnostic testing, about therapeutic process, and the management plan of treatment.

Furthermore, issues with communication may obstruct patient enrollment for clinical tests, thus leading towards delaying the introduction of effective new cancer therapies into clinics. Confusion and a loss of trust among the team might result from a lack of good communication across specialists and departments. Oncologists themselves admit that a lack of communication and management skills training is a major contributor to their own stress, job satisfaction, and emotional fatigue Chaturvedi et al. (2014).

4.4.3 Management of Psychosocial Aspects

Anxiety and worry around a cancer diagnosis can cause major disruption in practically anyone's life. A cancer diagnosis might put one's general feeling of security and order in life at jeopardy. Despite the fact that the vast majority of malignancies are curable, many people still have deep-seated anxieties that cancer would cause them pain, suffering, and death Vehling et al. (2012).

A cancer diagnosis brings with it a slew of challenges, including coping with disease-related medical symptoms and treatment, as well as dealing with financial concerns, understanding the illness's existential dimension, and the search for a soothing philosophical, spiritual, or religious belief structure or ideals that give life and death meaning.

Whether or not a person expects the diagnosis, bewilderment, numbness, and worry are common reactions. For many people, receiving a cancer diagnosis is connected with a spike in negative mood and discomfort. Waves of powerful emotions, akin to a grieving reaction, are typical, as are intervals of tranquilly. Most people are able to formulate a plan of action after the early days after obtaining the diagnosis, a practical plan of action.

Professionals in the healthcare field must keep in mind that no matter how good they are at what they do patients may still be upset, no matter how caring and skillful the person who delivers the bad news is it will elicit strong emotional responses (Shell and Kirsch 2001).

4.4.4 Psychotherapy with Cancer Patients

The individual may be aware of changes in his or her body that could signal cancer prior to the diagnosis (e.g., a lump, abnormal bleeding). Most people experience a sense of hyperalertness as a result of this awareness, which eventually leads to action. The speed with which this process takes place is determined by a number of factors, including personal or family history of cancer.

A cancer diagnosis may prompt some people to seek medical help right away. Others' experiences may lead them to avoid seeking medical help because they are afraid of what the symptoms could indicate. People are more likely to seek medical help if they are experiencing pain or discomfort as a result of their symptoms.

Other factors, such as discomfort around healthcare providers, financial considerations, fear of being dependent, and the fear of disfigurement may lead towards delays. Fear of cancer therapy could play a role to a person's acknowledgement of symptoms Koehly et al. (2008).

Family members who share the same ideals may unintentionally encourage the same delaying behaviors as the patient. A delay could also be caused by a lack of awareness about the symptoms.

In the United States, clinicians have told patients directly about their cancer diagnoses due to adherence to the ethical ideal of autonomy. The principle of autonomy states that each person has the right to choose his or her own path of action based on a self-made plan (Beauchamp and Childress 2001). In the sphere of healthcare, this means that everyone has the right to know about and participate in all medical choices. A physician's obligation, according to the recorded original 1847 Code of Ethics of the (AMA) American Medical Association (quoted in Katz, 1984) is to avoid all those things that could cause depression to a patient.

Families may still request that patients not be informed of their diagnoses. This puts healthcare providers in an ethical bind when it comes to getting informed consent for treatment from their patients. Having to use phrases like "growth" for cancer or "special medicine" for chemotherapy makes it more difficult to provide care to these individuals.

Dunn et al. (1993) found that not disclosing the diagnosis leads to patients suspecting it and assuming that the disease is so bad that even doctors and nurses refuse to acknowledge it. The avoidance of the term "cancer" promotes the anxiety associated with it (Holland 2002).

When cancer strikes a person's life, it affects family members and close friends as well. Cancer affects the multiple aspects of patients such as mental, emotional, physical, and spiritual well-being of patients and their families, according to research (Northouse et al. 2005). It shows a significant crisis for patients and their relatives

(Glajchen 2004). Psychotherapy can help an individual in dealing with these issues and transient with knowledge and proper communication.

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Part II

Cell and Molecular Biology of Breast Cancer (Diagnosis and Prognosis)

Implications of BRCA1, BRCA2 Gene in Overall Development and Prognosis of Breast Cancer

5

Sheikh Mansoor, Usma Manzoor, Aabid Mustafa Koul, Shahid M. Baba, Ina Amin, Iqra Anwar, Qurat ul Aein, and Arshad A. Pandith

Abstract

Thousands of genes are there but BRCA1 and 2 are the most talked about and cited with reference to many common malignancies and in particular breast cancer. These genes have a pivotal role to repair DNA but their mutations either heritable or somatic enhance sensitivity to carcinogenic agents that damage DNA. Being highly penetrant, BRCA1/2 furnishes access to various DNA damage pathways and aberrant cell cycle surge. Subsequent to the mutations in both genes, the impacted pathways augment the sensitivity to ionizing radiation coupled with condensed competence of the individual to restore the insult caused to DNA that consequently raise the vulnerability to breast carcinogenesis. Especially acquaintance with the prototype of inheritance of disease with reference to BRCA gene mutation manifests its distinctive feature to identify families that harbor the mutation that facilitate their therapeutic preventative measures to guard against the disease. Classification and revelation of BRCA mutation-affected patients manipulate the options for treatment and impact the outcome of breast cancer survival, and in addition could be a vital tool to identify their kins that harbor the inherited mutated gene. Therefore, these mutations highly implicated in breast cancer are actionable in the era of personalized medicine to treat the

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patients that prolong their survival outcome and free the patients from disease. Thus, it is imperative to develop and opt for the screening methods best available to identify the BRCA 1/2 mutations.

Keywords

BRCA1/2 · Mutation · Breast cancer · Modalities · Diagnosis · Treatments · Management

5.1 Introduction

Breast cancer is the commonest malignancies affecting women globally, with around 570,000 deaths in 2015. Every year, about 1.5 million women worldwide that amount for 25% of all cancer patients are diagnosed with breast cancer (Stewart and Wild 2014; Basse and Arock 2015). This metastatic disease may spread to distant organs such as the bone, liver, lung, and brain, which explains why it is incurable. Early diagnosis of the disease has been associated with favorable prognosis and high survival rate. (DeSantis et al. 2016). The majority of breast cancer instances are found in women and the number is 100 times greater than in males (Siegel et al. 2017). In America, although the prevalence of breast cancer is increasing every year, the fatality rate is decreasing owing to extensive early screening for the disease and better treatment modalities. In recent years, biomedical therapeutics have been created and have proven to be useful for breast cancer management. Breast cancers often begin with ductal hyperproliferation and progress to benign or metastatic tumors after being continually stimulated by numerous carcinogenic elements. Stromal effects and macrophages that comprise tumor microenvironments play critical roles in the initiation and progression of breast cancer. Also, differences in DNA methylation patterns between normal and tumor-associated microenvironments have substantiated that epigenetic changes in the tumor microenvironment can promote carcinogenesis (Polyak 2007; Basse and Arock 2015).

5.1.1 Genes Related to Breast Cancer

Mutations and aberrant amplification of oncogenes play important roles in genesis and development of tumor. Risk of breast cancer has been linked to germline pathogenic mutations in cancer predisposition genes included in hereditary cancer multigene testing panels (Stewart and Wild 2014; Basse and Arock 2015; DeSantis et al. 2016; Siegel et al. 2017). Detection of pathogenic variants in predisposition genes has benefited carriers of BRCA1 and BRCA2 pathogenic variants by improving access to risk-reducing prophylactic surgery and targeted therapies, as well as access to enhanced mammography and MRI based screening among carriers of

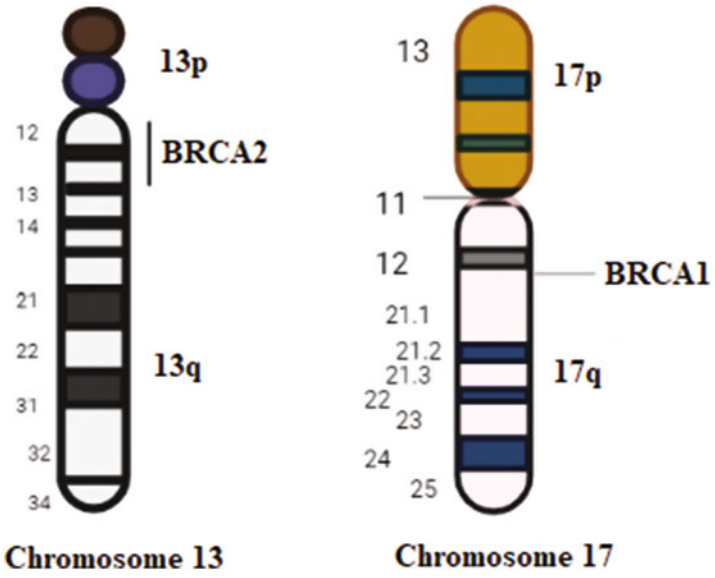

Fig. 5.1 Chromosomal location of BRCA1 and BRCA2

pathogenic variants in several established genes that predispose to breast cancer (Drukteinis et al. 2013; Majeed et al. 2014).

Breast cancer related genes 1 and 2 (BRCA1 and BRCA2) found on chromosomes 17q21 and 13q12, respectively, are the two well-known anti-oncogenes that influence breast cancer risk (Fig. 5.1).

Both of these genes code for tumor suppressor proteins. If a person inherits deleterious mutations in either the BRCA1 or BRCA2 genes, their chance of developing breast cancer is considerably enhanced. Even when the second allele is normal, BRCA1/2 mutations are inherited in an autosomal dominant manner. Loss of BRCA1 results in genetic instability, cell cycle checkpoint dysregulation, aberrant centrosome duplication, and finally death. Expression of BRCA1 has been shown to be suppressed by "pocket proteins" such as p130, p107, and the retinoblastoma protein in an E2F-dependent way. Also, BRCA1 regulates its expression by forming a loop between its promoter, introns, and terminator regions which interacts with its own promoter (Deng 2006; Dine and Deng 2013; Tan-Wong et al. 2008; Hegan et al. 2010). Breast cancers linked with BRCA2 are more generally high-grade, invasive ductal carcinomas with a luminal phenotype (Bane et al. 2007). Normally, BRCA2 protein modulates recombinational repair in DNA double-strand breaks by interacting with RAD51 and DMC1 (Sanchez et al. 2017; Martinez et al. 2016). Signalling pathway involving BRCA1 or BRCA2 in breast is shown in Fig. 5.2.

BRCA1/2 mutations cause approximately 20–25% of hereditary and 5–10% of all breast cancers (Balmana et al. 2011; Paluch-Shimon et al. 2016). Chen's meta-analysis revealed 57% and 49% risk of breast cancer in women over 70 years of age with BRCA1 or BRCA2 mutations (Chen and Parmigiani et al. 2007). The

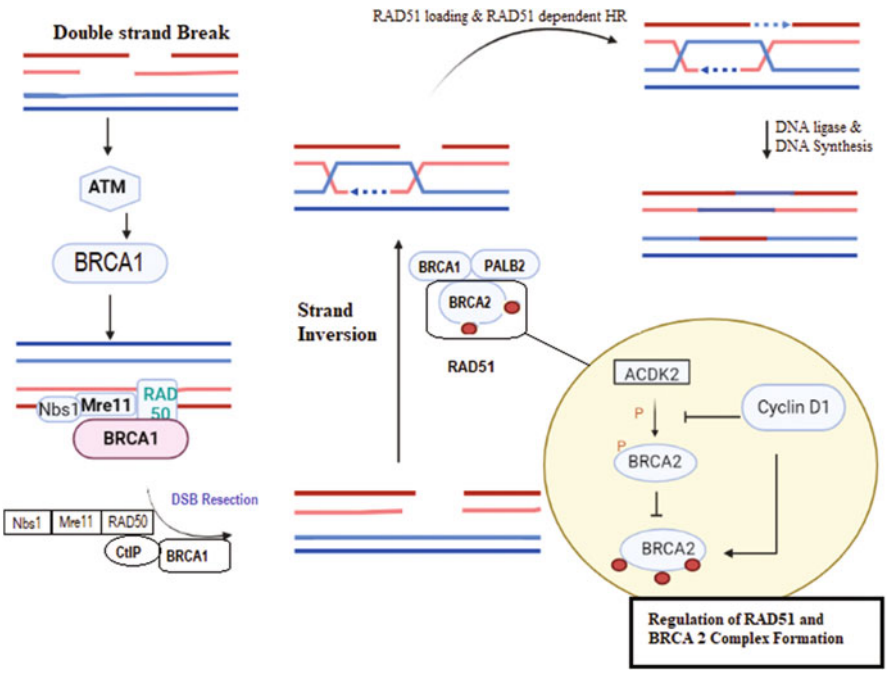

Fig. 5.2 BRCA1 and BRCA2 signalling pathway involving DNA repair system and different complexes, i.e., Mre11-RAD50-Nbs1 (MRN) complex, BRCA1–abraxas–RAP80 complex, BRCA1–CtIP and RAD50 complex, and BRCA1–PALB2–BRCA2 complex that is dependent on the phosphorylation of S988 on BRCA1 by CHK2

National Comprehensive Cancer Network has also proposed a risk stratification in the selection of unaffected and afflicted women for testing. 15 In contrast, the American Society of Breast Surgeons has advised germline genetic testing in all women with breast cancer to rule out hereditary cancers. Separately, mass screening for BRCA1 and BRCA2 mutations has been advocated in all women over the age of 30. 16 However, large-scale community-based studies providing estimates of the proportion of pathogenic mutations in predisposition genes are sparse.

In the present scenario, molecular testing for BRCA 1/2 can be achieved through sequencing platforms by high throughput technology coupled with convention robust techniques and both pathogenic BRCA1/2 mutations recognized as germline and somatic should irrefutably be established in the tumor. This is important as the tumor phenotype varies as per the specific germline or sporadic mutation that influences the treatment and management of breast cancer. This chapter appraises the current modalities related to BRCA 1/2 associated breast carcinogenesis that focuses on screening, diagnosis, and prevention of the disease.

5.2 Importance of Detection of BRCA Mutations

Hereditary breast cancer comprise around 5–10% of total breast cancer cases. Germ line mutations in BRCA gene are thought to be linked with hereditary breast cancer, which affect 2.0–4.7% of breast cancer patients (Malone et al. 2006). The BRCA1 and BRCA2 genes code for various tumor suppressor proteins that are vital for DNA repair and genomic stability. Mutations in BRCA1 and BRCA2 are known to increase the risk of breast and ovarian cancer in women. In the coding and non-coding areas of these two genes, over 2000 distinct mutations have been discovered. The most prevalent mutation forms include small insertion/deletion frame-shift, non-synonymous truncation, and splice-site changes which result in truncated non-functional proteins of BRCA (Walsh et al. 2006). BRCA1/2 mutations are predicted to affect 1% of the general population (BRCA1: 0.04–0.24%; BRCA2: 0.14–0.4%) (Malone et al. 2006; Whittemore et al. 2004). According to rough estimates, around 5×10^5–1×10^6 people in the United States of America carry BRCA1/2 mutation. The presence of a BRCA mutation has associated with an increased risk of getting breast cancer in particular. According to estimates, at the age of 70 years, carriers of BRCA1 mutation have 46–65% risk of breast cancer, whereas BRCA2 mutation carriers face a 43–45% lifetime risk (Chen et al. 2006; Antoniou et al. 2003).

To manage higher risk of breast cancer in healthy BRCA mutation carriers, numerous strategies have been proposed. Mutations in BRCA gene may allow carriers to tailor their cancer prevention tactics, like employing breast MRI instead of mammography, preventive mastectomy, salpingo-oophorectomy, or chemoprevention (Meijers-Heijboer et al. 2001; Scheuer et al. 2002; King et al. 2001). As a result, early diagnosis of a BRCA mutation allows for appropriate screening or therapeutic procedures to be implemented. Therefore, keeping in view the diagnostic importance of these two gene mutations, the genetic testing methods for them must be precise and comprehensive, since it will have a direct impact on the decision making of carriers or their family members.

Identifying BRCA gene mutation carriers among patients of breast cancer can have a big impact on their treatment options. Mutation in BRCA gene may be suspected due to demographics, tumor biology, or family history of the patient. Breast cancer in numerous relatives in more than one generation, at an early age, bilateral breast cancer, male breast cancer, or linked cancers are all personal or family history qualities that suggest hereditary breast cancer (Metcalfe et al. 2008). When considering family history and carrier probability, the new UK National Institute for Health and Care Excellence recommendations advocate using an acknowledged calculation method (Gronwald et al. 2007). The National Comprehensive Cancer Network and the American College of Radiology now recommend an annual mammography and MRI screening for breast cancer starting at 25–30 years of age or 10 years prior to the age of the earliest 1° relative diagnosed with breast cancer (Laitman et al. 2019; Metcalfe et al. 2008). Screening, however, does not prevent breast cancer; rather, it facilitates early detection and targeted therapies to minimize mortality (Walsh et al. 2006; Thompson and Easton 2004).

Therapeutic agents like tamoxifen and raloxifene are shown to reduce breast cancer incidence in postmenopausal women (Smith et al. 1996; Moisan et al. 2006). However, due to its side-effect profile, hormone chemoprevention has a poor acceptance rate in premenopausal women (Zhang et al. 2010). Therefore, prophylactic risk-lowering surgery is becoming more popular. After childbearing, all BRCA mutation carrying females should have a risk-reducing bilateral salpingo-oophorectomy (RRSO). It can almost completely eliminate the ovarian cancer risk and reduce breast cancer risk by half (Mazoyer 2005; Schouten et al. 2002). In women with a BRCA gene mutation, after a mean follow-up of 4.5–8.5 years, prophylactic bilateral risk-reducing mastectomy (RRM) has proven beneficial in reducing the occurrence of breast cancer up to 2% (White et al. 2004) and 1% (Morozova and Marra 2008; Mardis et al. 2013; Mardis 2008). Also, the rate of complications associated with RRM have been found to be very scarce (Morozova and Marra 2008). In terms of therapeutics, BRCA mutation carriers who develop breast cancer have a plethora of extra therapeutic options. The type of therapeutic modality in these patients is determined by the risk of surgical morbidity, impact of adjuvant therapy, and the effectiveness of future breast screening. Studies have demonstrated comparable long-term survival rates for early breast cancer patients managed with breast-conserving treatment (BCT) or mastectomy (Walsh et al. 2010; Feliubadalo et al. 2013). However, many patients opt for contralateral prophylactic mastectomy (CPM) keeping in view higher risk of ipsilateral breast tumor recurrence (IBTR) and contralateral breast cancer (CBC). Therefore, in BRCA gene mutation carriers, the prophylactic role of RRSO or the use of adjuvant radiation, chemotherapy, and hormonal therapy necessitates extra considerations. Screening after BCT is also distinct. Keeping this in view, patients with hereditary breast cancer particularly the BRCA mutation carriers that constitute only a small fraction of the total breast cancer cases need to be considered and investigated extensively to provide high-quality diagnosis and treatment choices to improve their outcomes.

5.3 Pathology of *BRCA1* and *BRCA2* Associated Breast Cancers

BRCA1 and *BRCA2* are tumor suppressor genes that repair DNA double-strand breaks, control cell cycle, and regulate transcription, all of which contribute to genome integrity (Yoshida and Miki 2004). A significant lifetime risk has been associated with breast and ovarian cancers due to alterations in these genes. Carriers of these two gene mutation have a 45–80% likelihood of acquiring breast cancer (King et al. 2003; Antoniou et al. 2003). Clinical data such as age at diagnosis, ethnicity, and most importantly family history have a significant role in evaluating the likelihood of *BRCA1* or *BRCA2* germline mutation. Indeed, a strong family history of breast and ovarian malignancies is the major risk factor for acquiring this condition. Several studies have examined clinical characteristics and pathological features of *BRCA1* and *BRCA2* positive breast cancer (Veronesi et al. 2005; Musolino et al. 2007; Atchley et al. 2008; Tung et al. 2010). The outcomes of these investigations revealed that *BRCA1* and *BRCA2* positive cancers had distinct

morphological and immunohistochemical features. Breast cancers associated with *BRCA1* and *BRCA2* vary on morphological as well as molecular levels from a pathological standpoint. *BRCA1*-associated tumors differ from *BRCA2*-associated tumors in terms of younger age at diagnosis, higher prevalence of interval cancers, lower proportion of ductal carcinoma in situ (DCIS), and an unfavorable tumor size at diagnosis (Rijnsburger et al. 2010). At the morphological level, *BRCA1*-associated breast carcinomas typically present as high-grade invasive ductal carcinomas with minimum to no tubule or glandular development, pleomorphic nuclei (substantial variation in shape and size), vesicular chromatin, large nucleoli, and strong mitotic activity. A "medullary" look has also been observed, with sheet-like tumor cell growth pushing boundaries, abundant peri- and intra-tumoral lymphocytes, and necrosis (Hodgson and Turashvili 2020). On the other hand, *BRCA2*-associated tumors are much more like sporadic malignancies (Lakhani et al. 2005; Lakhani et al. 2002), often moderately or poorly differentiated carcinomas (grade II and III) due to relatively less tubule formation, increased nuclear pleiomorphism, and enhanced mitotic rates (Agnarsson et al. 1998; Lakhani et al. 1998; Palacios et al. 2003). The imaging features of breast tumors may reflect these changes in natural history based on *BRCA* mutation type. So far, number of studies have examined breast malignancies in carriers of *BRCA1* and *BRCA2* mutation (Schrading and Kuhl 2008; Noh et al. 2013; Gilbert et al. 2009); however, these studies analyzed a small group of patients and moreover, the correlations between pathologic results and imaging features including molecular subtype are yet unknown. According to new research, there is a marked difference in the imaging properties of *BRCA1*-and *BRCA2*-associated breast cancers, which are coherent with differences in clinicopathologic aspects between the two tumor types (Rijnsburger et al. 2010). With each imaging modality, *BRCA1*-associated breast tumors appear to have benign morphologic characteristics, but more aggressive pathologic traits, like in case of triple-negative phenotype. In contrast to random breast cancers, *BRCA*-associated breast tumors have distinct morphologic characteristics on imaging. According to Kuhl et al. 2000, 23–38% of hereditary breast cancers, particularly of *BRCA1*-associated type that resemble fibroadenoma or cysts, have benign morphologic characteristics. When compared to *BRCA2* lesions, *BRCA1* lesions more commonly appear as round or oval shaped with a confined border on mammography (Ha et al. 2017).

Breast cancers in high hereditary risk patients have a higher nuclear and tumor grade, and morphologic presentations on imaging modalities in these individuals are linked. Because of their rapid growth, high-grade cancer appears on mammography as distinct masses, but intermediate and low-grade tumors induce a desmoplastic response and generally appear as a speculation (Blaichman et al. 2012; Lamb et al. 2000). Also evident from the findings of Ha et al. 2017, that *BRCA1* mutant group appears to have higher nuclear and histologic grades. The triple-negative subtype was also connected to *BRCA1* malignancies, whereas the luminal B subtype was linked to *BRCA2* cancers, which is in accordance with Larsen et al. 2013 findings. The triple-negative phenotype has been linked to higher nuclear grade, worse histologic grade, early metastasis, younger age of onset, and shorter life expectancy (Atchley et al. 2008; Dent et al. 2007; Rakha et al. 2008). Furthermore, on MRI,

these tumor types generally have a smooth margin and rim enhancement (Uematsu et al. 2009).

Patients with *BRCA1* mutation associated breast tumors unlikely have microcalcifications compared to *BRCA2* mutation carriers, who in addition to an increased proportion of calcification-related DCIS have a higher mammographic sensitivity (Rijnsburger et al. 2010).

Schrading and Kuhl (Schrading and Kuhl 2008) described one such cohort of *BRCA1*-associated invasive breast cancers in which none had calcification or an intraductal location, both of which are known to reduce mammographic sensitivity. *BRCA1* mutation carriers have also been shown to have metaplastic carcinomas (Breuer et al. 2007; Zhang et al. 2015), whereas *BRCA2*-associated tumors are strikingly comparable to sporadic "luminal-type" tumors (Perou et al. 2000). This category includes luminal A (most prevalent intrinsic molecular subtype of breast cancer) and is characterized by varying expression of genes found in the luminal breast epithelium as well as those linked to the estrogen receptor (ER) (Perou et al. 2000). Morphologically, tumors with *BRCA2* mutations are mostly invasive ductal carcinoma of no special type of variable grade and appear having no explicit morphology, however, lobular carcinomas have been associated with *BRCA2* mutations (Mavaddat et al. 2012).

BRCA2-associated cancers are generally immunohistochemically positive for PR, ER, and low molecular weight keratins but lack *HER2* protein overexpression (Lakhani et al. 2002), whereas according to other histopathological characteristics, *BRCA1* tumors are mostly poorly differentiated (grade 3), having a high mitotic count with high frequency of necrotic areas (Van der Groep et al. 2008). Tubule formation is reduced, but there is a greater degree of pleomorphism, all of which point to a more aggressive phenotype (Armes et al. 1998; Honrado et al. 2005; Lakhani et al. 1998; Marcus et al. 1996). Additionally, tumors are frequently well-defined, with a high incidence of lymphoplasmocytic infiltration and lymphovascular invasion (Heerma van Voss et al. 2010).

5.3.1 BRCA-1 and BRCA-2 Probability Models

The probability of harboring a genetic mutation varies greatly from woman to woman and is dependent on family history of breast cancer and similar malignancies. In such respect, risk-prediction algorithms which completely represent our understanding of the nature of the inheritance process can make a significant contribution to a woman's decision. A precise assessment of the likelihood that a woman possesses a mutation is a critical step in counselling a woman confronting these dilemmas. Furthermore, once the test/s are completed, the crucial factor for decisions is the prior probability of mutation, assuming the test findings. Other models have focused on the probability of breast cancer in women with family history of this cancer. Despite valuable, none of these prediction models explicitly discusses the likelihood that the woman contains *BRCA*1/*BRCA*2 mutation, as is required in genetic testing and counselling decisions (Parmigiani et al. 1998). Since no risk

evaluation model is suitable for each and every patient, doctors usually select a model over the other for certain patient population. In a medical scenario, a patient might be given a number of risk estimations based on the models that are thought to be relevant for her situation. Also by offering the spectrum, the patient will be able to recognize that risk estimate is an approximate way. Screening and preventive measures can then be customized individually based on an assessment of the risk figures presented.

5.3.2 BOADICEA Model

BOADICEA (Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm) model was built in 2002 by Antoniou et al. to estimate the likelihood of *BRCA*1/*BRCA*2 mutation, thus predicting risk of breast and ovarian cancers (Antoniou et al. 2005). The model was constructed by the use of intricate separation analysis to predict probability of breast and ovarian cancer by using two data sets based on number of breast cancer cases and multiple families in a population. The model considers familial breast cancer that which have mutations in both *BRCA*1 and *BRCA*2 genes and a polygenic component. In addition, the model provides the possibilities of genetic modification that may alter the penetration of both *BRCA*1 and *BRCA*2 mutations. Polygenic factors are attributed for the remaining non-*BRCA* cancer clusters in families. The model, developed in 2005, was shown to appropriately determine the carrier probability. A web-based software interface is being developed by the researchers that will allow clinicians to enter pedigree data and analyze probability of the entered data. To measure the risk, might also use the already published tables (Antoniou et al. 2005). For clinicians, using many *BRCA* probability models is laborious, thus it may well be preferable to select one or two models which suits best the particular patient (Culver et al. 2007).

5.3.3 BRCAPRO Model

The *BRCA*PRO (*BRCA* Probability) model (Berry et al. 1997; Parmigiani et al. 1998) assesses breast and ovarian cancer risk depending on the chance that a person inherits mutation in *BRCA*1/2 gene. This tool utilizes Mendelian genetics as well as Bayesian analysis to determine the mutation probability in *BRCA* gene and from such probability determine the risk of breast and ovarian cancer based on a patient's current age, history of cancer, and familial history of breast and ovarian cancer in 1° and 2° relatives. This model specifically evaluates the likelihood of a mutation in either, both or neither of the genes (Berry et al. 2002). This model takes into account every family member (up to 2° relatives), their ovarian and breast cancer history, bilateral breast cancer and whether the familial background is Ashkenazi. Cancer risk estimations are solely applicable to unaffected persons; however, probabilities of mutation may be computed for impacted as well as non-impacted individuals. The model also considers the family's mutation status. *BRCA*PRO models software

output is simple to read; nevertheless, to achieve the precise mutation probability, pedigree must be included, that may be time-consuming. Further disadvantage *BRCA*PRO model is that the penetrance data utilized to develop this particular model was primarily from the Caucasian race, hence its application may be confined. In 2002, validation experiments were carried out by correlating the predicted *BRCA* mutation calculated using *BRCA*PRO with the traditional genetic findings. According to these investigations, *BRCA*PRO provides a reliable calculation of the likelihood of a mutation and so is a valuable tool in the counselling approach (Berry et al. 2002). Because the *BRCA*PRO model is based on the Bayesian analysis which takes family size into account, so this model is frequently effective if a family is notably big or small. Furthermore, *BRCA*PRO is beneficial if the results of genetic screening conducted in the family are negative but calculating the frequency of a mutation in other close relatives is necessary. Moreover, this model is perhaps one of the only validated model that takes bilateral breast cancer into consideration (Culver et al. 2007).

5.3.4 Couch Model

The Couch model is a logistic regression model meant for predicting the risk of *BRCA*1 mutation in a particular family (Couch et al. 1997). To predict the mutation frequency, the model uses individual as well as family history of ovarian and breast cancer in 1° and 2° relatives. Couch model also takes into account the population of Ashkenazi Jewish descent. The mutation probability is calculated using the median age at initiation of breast cancer within family, however for ovarian cancer it is not calculated. The offered risk represents likelihood of having a *BRCA*1 mutation within a family and pertains to almost any ovarian and/or breast cancer affected and/or diagnosed family members. The mutation probability is nearly half in the unaffected 1° relatives of ovarian and/or breast cancer patients when compared to the nearest family members. For analogy, if a family is having 10% likelihood of a Couch mutation, then the affected members daughter of the same family has 5% chance of getting a *BRCA*1 mutation, since the probability of receiving the mutant allele via her mother is 50% (Couch et al. 1997). The limitations of Couch model should be taken into consideration when used in clinical context. This model excludes additional malignancies related with the *BRCA* genes, male breast cancer as well as female bilateral breast cancer. The model is even more restrictive because it exclusively predicts *BRCA*1 mutation type, and the sample population was mostly Caucasian women. The model could be amended in a clinical setting to incorporate *BRCA*2 mutation (Shih et al. 2002). The basic Couch model, dubbed as "Penn II," has been improved and modified to predict *BRCA*1/*BRCA*2 mutations as well as consider relevant cancer histories of an individual or their family. The model considers three generations of families with a history of ovarian / breast cancer along with other *BRCA*-related malignancies such as pancreatic cancer, prostate, and male breast cancer. Since the model predicts the probability of cancer in families with several affected members, it must not be utilized in a family with only a single

instance of breast or ovarian cancer. Also, the projections of this model should be changed to accommodate *BRCA*2 probability, in families with multiple affected members (Culver et al. 2007).

5.3.5 Manchester Model

The Manchester Model uses statistical approach which calculates whether a family has at least 10% likelihood of having a *BRCA*1/*BRCA*2 mutation (Evans et al. 2004) or has a 20% collaborative risk (Evans et al. 2005). At the city of Manchester, in England this model was developed, thus its name. The creators of this model devised a statistical score system based on a specific demography to assess if a family might be carrying a harmful mutation in *BRCA*1/*BRCA*2 genes. The statistical scoring method in this model covers cancers like female breast cancer, male breast cancer, ovarian, prostate, and pancreatic cancer. Each cancer diagnosed is assigned points between 1 and 8 on the basis of its type and the age of onset. Greater scores are assigned for younger onset ages, and the time of diagnosis is incorporated into the score calculation for breast cancer cases. A comparison is established among ovarian, pancreatic, and male breast cancers diagnosed prior or beyond 60 years of age. *BRCA*1 and *BRCA*2 scores are calculated separately and a cumulative score of 10 for one lineage within a family corresponds to10% chance of a mutant gene. Employing this approach, firstly, gene with highest score may be evaluated; if there is no detectable mutation in that gene, scores for the other gene may be revised thereafter. For a family with higher cumulative scores, testing is warranted with firstly *BRCA*1 testing, followed by *BRCA*2 testing. This model has a significant benefit in that the scoring method is simple to apply in the clinical scenario. Furthermore, validation analyses of this model performed in comparison to *BRCA*PRO, Couch, and Myriad models authenticated Manchester model outperforming other models in distinguishing families exhibiting a mutation risk of 10% (Evans et al. 2004). However, this model does not support the use of mutation analysis in a solitary case of ovarian or breast cancer at any age. One key shortcoming of this approach is it does not evaluate the actual chance of a mutation, rather it determines whether or not a family satisfies the 10% or 20% probability criterion. This model might not be quite as effective in a clinical setup that performs *BRCA* testing using a separate probability threshold or does not utilize any precise numerical probability threshold. Furthermore, this model is not intended for usage among Ashkenazi Jewish population. When a 10% mutation probability is utilized as a criterion for delivering genetic testing in a scenario with resource constraints, the Manchester model becomes even so important and useful (Culver et al. 2007).

5.3.6 Myriad Model

Based on methods originally published by Frank et al., Myriad Genetic Laboratories provided mutation prevalence tables to offer conveniently available risk assessments

for discovering a *BRCA* mutation (Frank et al. 2002). This model estimates risk by using *BRCA* testing results from persons with an individual or family history of ovarian or breast cancer. Myriad issued an updated data on mutation prevalence on the internet in March 2006, based on patient's clinical test findings having complete gene sequencing profiles as well as those tested for three founder mutations among Ashkenazi Jews. The most significant benefit of utilizing Myriad tables is that the risk predictions are predicated on a larger clinical samples which are also classified on Ashkenazi Jewish ancestry vs non-Ashkenazi Jewish ancestry. Furthermore, the tables are simple to use which are often revised. When presenting mutant risk predictions in the clinical context, the limitations and benefits of the Myriad model should be addressed. The relative risks reported in these tables, nonetheless, do not consider the factors such as the exact age at breast cancer onset, number of kins affected, bilateral breast cancer, kins who are not affected and other *BRCA*-related malignancies. Also, the model shows no difference among first 1° and 2° relatives or among affected paternal and maternal relatives. Additionally, these risk predictions are solely based on the information regarding individual/family history furnished on patient detail forms filled by the doctor, which is susceptible to mistakes and exclusions by medical professionals. In short, the model offers risk predictions ahead of *BRCA* testing, but it may underestimate or exaggerate the risk of identifying a mutation in *BRCA* gene in specific families and therefore must be used cautiously. This model is suitable both for solitary patients and families, calculating probability ratios quickly and is particularly valuable in the clinical set up (Culver et al. 2007).

5.3.7 Tyrer-Cuzick Model

This model is a risk assessment approach (Tyrer et al. 2004) that computes the chance of a mutation in *BRCA*1 or *BRCA*2 genes as well as the chance of a weak penetrance mutation in other genes and individual risk variables. For a single normal female, Bayes' theorem is employed to utilize family history in order to calculate the probability of a *BRCA* gene mutation. After that, the related risk of breast cancer is computed and updated to indicate the comparative risk linked with the female's individual risk variables like age, age at perimenopause or menopause, pregnancy, age upon first livebirth, history of atypical hyperplasia/in situ lobular carcinoma, body mass index and height. The model also has been integrated into a software that generates extremely easy to use outputs regarding the possibility of the patient having breast cancer as well as possessing a *BRCA* gene mutation. This model takes into account 1° and 2° relatives who have ovarian and breast cancer as well as their ages of cancer onset. The model does not calculate the mutation probability for an unaffected individuals, who are typically not the best option for commencing diagnosis within a family, also this model solely applies to unaffected females. This model has the advantage of providing both breast cancer risk estimation and *BRCA* gene mutation probability, however, it is often more insightful to investigate an affected family member initially, so a probability prediction for that participant may well be required, which this model does not calculate (Culver et al. 2007). Currently

there are a plethora of models available to evaluate the individuals likelihood of carrying a *BRCA*1/2 genes mutation which have been published in peer-reviewed journals (Apicella et al. 2003; Antoniou et al. 2004; Antoniou et al. 2005). A number of these algorithms are also capable of predicting specific cancer risks. Several of such models had been created as early as 1997, shortly after clinical genetic testing of *BRCA* became available. Numerous tables, algorithms, and complex web-based applications have also been built over the last decades to determine the prior possibility of *BRCA*1/2 gene mutations (Claus et al. 1994; Parmigiani et al. 1998; Antoniou et al. 2004; Tyrer et al. 2004). The efficacy of such models has been mostly examined in female cohorts of varied ethnic origins (Nanda et al. 2005; Parmigiani et al. 2007; Antoniou et al. 2008; Kwong et al. 2012a, b; Fischer et al. 2013; Moghadasi et al. 2018). From 2015, two novel approaches to classify *BRCA*1/2 genetic variants are in practice; one established on the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology (AMP) criterions and rules (Richards et al. 2015), and other built on a Bayesian structure proposed by the International Agency for Research on Cancer (IARC) suggestions (Greenblatt et al. 2008; Plon et al. 2008). Lately, the ClinGen Sequence Variant Interpretation Working Group revealed that the ACMG/AMP benchmarks of 2015 are attuned with Bayesian statistical reasoning (Tavtigian et al. 2018). Several repositories for *BRCA*1 or *BRCA*2 genetic variations have been generated so far (Béroud et al. 2016; Cline et al. 2018; Parsons et al. 2019). The majority of the data, nevertheless, is focused on European populaces. It is a fact that cancer risk and genetic diversity vary among different populations (Rebbeck et al. 2018; Ledford 2019; Bhaskaran et al. 2021), hence research on non-European groups must be prioritized (Park et al. 2021).

5.4 Implication of BRCA1/2 in Hereditarily Onset of Breast Cancer

Breast cancer is a hereditary disorder with a very high level of penetration (Sadia et al. 2021; Shaw et al. 1996). About 10% cases of breast cancer are hereditary. Heterozygous germinal mutations of either maternal or paternal origin are found in cancer sensitivity genes (Sadia et al. 2021). Age and ethnicity are the most determining factors for hereditary cancer (Sadia et al. 2021). After the discovery of BRCA1 (Li et al. 2019; Sandoval et al. 2021) and BRCA2 (Felix et al. 2014) in the 90s, the genetic and molecular basis of hereditary risk of breast cancer began to be uncovered and currently these two genes are mainly associated with hereditary breast cancer (Silva 2019; Lalloo and Evans 2012). Mutations in BRCA1/2 genes are thought to enhance the breast cancer risk by 82% (Sadia et al. 2021; Shiovitz and Korde 2015).

BRCA1 or BRCA2 gene mutation can have either maternal or paternal origin. A person possessing mutation in one of these genes has a 50% probability of passing down the mutation to its sibling (Petrucelli et al. 2016). Even if a carrier inherits deleterious mutation in BRCA1/2 from one parent, normally, then he/she would

have inherited a normal copy of the gene from the other parent. However, in certain cells, the inherited normal copy might be lost or changed during lifetime. Such a transformation is called somatic alteration. Without functioning BRCA1 or BRCA2 proteins, cells become cancerous (BRCA gene mutations: cancer risk and genetic testing 2021). Bi-allelic mutations in the BRCA1 gene generally prove fatal during embryonic development, whereas same mutations in the BRCA2 gene can cause Fanconi anemia type D1, which is associated with an increased juvenile cancer risk (Sadia et al. 2021; Moatter et al. 2011). De novo (or "new") variation is a variation that is not inherited from either parent (BRCA gene mutations: cancer risk and genetic testing 2021). BRCA1 and BRCA2 genes often have 4153delA, 185delAG, 5382insC, 3819del5, 4075delGT, and 580del4 mutations, respectively (Wang et al. 2012). In consanguineous populations, the founder mutations are predicted (Sadia et al. 2021; Rashid et al. 2017; Torres et al. 2017).

Three variants in BRCA1/2 gene, viz. BRCA1 c.68-69delAG (BIC: 185delAG), BRCA1 c.5266dupC (BIC: 5382insC), and BRCA2 c.5946delT (BIC: 6174delT) collectively account for around 99% of the pathogenic variants among people of Ashkenazian Jewish descent (Petrucelli et al. 2016). In Europe's ancestral populations, the frequency of CHEK2 founder mutations has been found to higher (c.1100delC, c.470T>C) (Sandoval et al. 2021; Felix et al. 2014; Silva 2019). Founder mutations also occur in other groups, such as Norwegians, Netherlands, and Islanders (Nelson et al. 2013a, b).

Over the past 10 years, many genes have been genetically discovered for the inheritance of breast cancer. Among them, most widely studied are ATM (Ataxia Telangiectasia-Mutated) (Robson et al. 2015), BRCA1 (Breast cancer Genes 1), BRCA2 (Breast Cancer Genes 2), BRIP1 (Parts and Localizers of BRCA 2), PTEN (Phosphatase and Tensin homologue), TP53 (Tumor Protein p53) (Meric-Bernstam-F, 2018) RAD51C (RAD51 Paralog C),CHEK2 (Checkpoint kinase 2) (Liang et al. 2018), and PALB2 (Partner and localizer of BRCA2) (Silva 2019). The MUTYH gene, which has previously been found to be related with male breast cancer (Rizzolo et al. 2018), has also been hypothesized by researchers to be implicated in female breast cancer (Tadashi 2020). Around 5% of western population is affected by heritable mutations in cancer-susceptibility gene with BRCA1 being the most prevalent mutation (Katarzyna et al. 2020; Tung et al. 2015). In the general public the prevalence of BRCA1/2 is around 0.2%–0.3% (or about 1 in 400). In Ashkenazi Jews, the rate of mutation might be greater (Katarzyna et al. 2020; Zhu et al. 2016) with approximately 2.0% of individuals carrying a deleterious mutation in one of the two genes (BRCA gene mutations: cancer risk and genetic testing 2021). Interestingly, among Asians, the rate of BRCA1/2 mutation is lower than in whites (Katarzyna et al. 2020; Zhang et al. 2012). However, in Asians, BRCA2 mutations are more frequent than BRCA1(Sadia et al. 2021; Kim and Choi 2013). Two oncogenes, BRAC1/2, also known as caretaker genes repair and maintain genomic integrity through homologous recombinations (Silva 2019). These genes translate to proteins that help in repairing damaged DNA, cell cycle control, and gene transcription regulation and apoptosis (Silva 2019). BRCA1 gene codes for BRAC1 protein which interacts with tumor suppressors proteins, DNA damage sensitive proteins,

and cell signalling proteins form a multiprotein complex known as BRCA1-associated genome surveillance complex (BASC) (25). These proteins also interact with histones and RNA polymerase in order to promote transcription and its regulation (Lalloo and Evans 2012), cell cycle progression, and ubiquitination (Robson et al. 2015). There are two main domains of BRCA1 protein. The DNA repair function is related to BRCA1 C terminus domain (BRCT) and the ubiquitination function is related to the zinc finger domain (Meric-Bernstam et al. 2018). BRCA2 forms a complex with RAD51 and PALB2 in order to identify homology regions in DNA (Silva 2019; O'Donovan and Livingston 2010). Proteins encoded by BRCA2 help to repair breaks in DNA strands and also maintain genomic stability. It plays a pivotal role in meiosis. Serious growth problems and infertility have been observed in mice that have BRCA2 mutations (Shaw and Cantley 2006). The structures of BRCA1 and BRCA2 are distinct, yet they have linked roles and are related to DNA repair functions (Silva 2019).

BRCA1-associated breast tumors are generally smaller in size, more often high-grade, poorly differentiated infiltrating ductal carcinomas with increased cytokeratin 5/6, cyclin E, and p53 expression and are mostly triple negative (Katarzyna et al. 2020). Patients with BRCA1 associated breast cancer are usually younger than those with mutation in the BRCA2 gene (Katarzyna et al. 2020). Also, an increased risk of serous adenocarcinomas has been associated with females having germline BRCA1/2 pathogenic mutations (Petrucelli et al. 2016; McLaughlin et al. 2013). Considering relative chances of having breast cancer, men in their third and fourth decade of their life have a greater risk which then declines with increasing age. Men with BRCA2 pathogenic mutations exhibited greater relative and cumulative risks than males carrying pathogenic variants of BRCA1 gene (Tai et al. 2007). Comparatively, 87% chance of developing cancer has been reported in females carrying BRCA1/2 pathogenic mutations against only 20% in men. Numerous studies have found an incidence of about 5% of germline BRCA1/2 mutation in unscreened breast cancer patients of any ethnicity (Chen et al. 2020; Sun et al. 2017; Wen et al. 2018; Li et al. 2019). Germline mutations in BRCA1/2 confer a higher risk of acquiring ovarian and breast cancer. The cumulative incidence of female breast cancer has been reported to be around 71.4–87% and 77–88%, respectively, in BRCA1 and BRCA2 mutation carriers by the age of 70–80 years (Torres et al. 2017; Kim and Choi 2013; Antoniou et al. 2005).

The BRCA1 mutation increases ovarian cancer risk by 59–65%, whereas the BRCA2 mutation increases the risk by 34.5–37% (Katarzyna et al. 2020; Van der Kolk et al. 2010). Chen et al., observed that 5.53% of patients carried a germline BRCA1/2 mutation out of which 2.10% had BRCA1 and 3.44% had BRCA2 mutations (Chen et al. 2020). Further, Aejaz et al., reported that 13% of younger patients and 5.8% elderly patients carried BRCA1/2 gene mutation. Also, 3% of younger and 2% of elderly patients carried TP53 mutation (Sadia et al. 2021). Sandoval R et.al observed that 61% of breast cancer patients harbored pathogenic variant in high penetrance gene compared to 15.2% in moderate penetrance gene. They also reported that 89.7% of breast cancer patients had at least one 1° or 2° family member affected by cancer and 64% of patients with breast cancer had

affected family members. Mutations in BRCA1/2 gene have also been found to increase the risk of numerous other malignancies (Katarzyna 2020). Fallopian tube cancer (Finch et al. 2006) and primary peritoneal cancer are two cancers that develop in women. Breast cancer and prostate cancer (Nyberg et al. 2020) are more common in men with BRCA2 mutations. Further, a higher risk of pancreatic cancer has been reported in both men and women who have mutations in BRCA1/2 genes (Hu et al. 2018). Also, a prevalence rate ranging from 10 to 20% for germline BRCA1/2 mutation has been reported in patients with triple-negative breast cancer, whereas about 80% of patients with breast cancer have germline mutations in the PTEN gene (Silva 2019).

For high-risk patients, breast cancer screening guidelines have been designed. BRCA1/2 are the most often examined genes in clinical practice, especially for patients with triple-negative breast cancer diagnosed at a young age or who have a strong family history of ovarian, breast, or other malignancies (Chen et al. 2020). Patients with breast cancer and their families can be screened for BRCA1/2, PTEN and TP53 mutations, as well as get preventative treatment including chemoprevention and prophylactic surgery. People with specific hereditary cancer predisposition diseases, such as Cowden syndrome, Peutz-Jeghers syndrome, Li-Fraumeni syndrome, or Fanconi anemia, should be assessed for breast cancer risk (BRCA gene mutations: cancer risk and genetic testing 2021). With the increased use of next-generation sequencing, there has been 26% increase in reports of hereditary ovarian and/or breast malignancies among Caucasians (Sadia et al. 2021; Antoniou and Easton 2006). Genetic counselling is important for educating and providing support to families who are at high risk which can help detect early stage breast cancer (Silva 2019).

5.5 Advantages and Disadvantages of Screening and Genetic Testing

Cancer genetic testing searches for particular hereditary alterations (mutations) in a person's genes that are linked to a high-to-moderately elevated cancer risk. Patients with a personal or family history of increased risk of a certain hereditary cancer type may benefit from single/limited gene screening. BRCA1/2 mutations, which account for 15% of ovarian cancer cases and 5–10% of total breast cancer cases have a prevalence of around 1 in 300–500 females (Anglian Breast Cancer Study Group. 2000; Antoniou et al. 2002). Women with mutations of clinical significant in the BRCA1/2 genes have higher risk of breast cancer (Chen et al. 2006). As a direct result of a hereditary defect in the genes, almost 10% of breast and ovarian malignancies are caused (Economopoulou et al. 2015). Hereditary breast cancer (HBC) has historically been linked to pathogenic variants in the BRCA1/2 genes; however, there is growing evidence that additional genes (such as ATM, CHEK2, and PALB2) have an important role in inherited breast cancer risk in the post-genomic era (Peshkin and Isaacs 2020; Buys et al. 2017). Women who are carriers of a non-functional copy of BRCA1/2 have a considerably increased lifetime breast

cancer risk, particularly when they are young. Screening for hereditary component of breast and ovarian cancer allows for better medical management of high-risk cases. Significantly, screening may also identify women from "high-risk" families who did not inherit cancer predisposition, enabling patients to escape needless medical intervention. Through genetic testing and counselling, possible complexity of the findings, such as the possibility of discovering variants of uncertain clinical significance (VUSs), as well as the consequences of the results for the family are explored (Scott et al. 2019; Daly et al. 2017). If an individual with mutated BRCA1/2 gene has a family member with negative results, the genetic counsellor may be able to say with greater certainty that she has relatively low risk of ovarian or breast cancer in the overall population. If the test results are positive, actions can be taken to reduce the risk of these cancers, or to try to identify these malignancies early if they do occur.

5.5.1 Advantages of Screening and Genetic Testing

- Genetic testing allows patients to get preventive therapy with selective estrogen receptor modulators (SERMs) such as tamoxifen, raloxifene, and aromatase inhibitors (Ais) like Aromasin, which may lower the developing breast cancer risk (Thorat and Balasubramanian 2020; Nelson et al. 2019a, b), or oral contraceptives, which may lower ovarian cancer risk.
- Other benefit is the possibility of doing prophylactic bilateral salpingo-oophorectomy (PBSO) which is surgical removal of both ovaries before cancer develops. PBSO results in a 96% and 50% reduction in incidences of ovarian and breast cancer irrespectively (Xiao et al. 2019; Kotsopoulos et al. 2016). Bilateral risk-reducing mastectomy (RRM) is another effective strategy of lowering the risk of breast cancer especially in women with hereditary breast cancer (Giannakeas and Narod 2018; Flippo-Morton et al. 2016).
- An individual can have more regular clinical checkups and breast screenings, every 6 months instead of once a year in addition to digital mammography and/or MRI. Mammography and MRI lower the incidence of interval cancers in women who are at high-risk and are commonly used in females who opt for monitoring over risk-reducing surgery (Pilewskie et al. 2019).
- Following genetic testing, patients can make lifestyle adjustments to help lower their cancer risk.
- In case the patient develops malignancy, the doctor may be able to make treatment decisions based on the patient's genetic information.
- An individual can participate in studies that may aid in the prevention or cure of breast or ovarian cancer.
- Knowing that one possesses a defective gene related to breast cancer risk may motivate the subject and their family members to adopt lifestyle and family planning adjustments, as well as other considerations, that may help decrease cancer risk.

Men who tests positive for abnormal BRCA1/2 gene mutations are at higher-than-average risk for prostate cancer. They undergo screenings like annual digital rectal examinations and prostate specific antigen (PSA) blood tests. Though the risk of breast cancer is low in males, but it is higher than those males who do not have abnormal gene.

5.5.2 Disadvantages of Screening and Genetic Testing

Genetic testing also has limitations and possible drawbacks, including the following:

- It is not yet apparent what might or might not be done once a person receives the findings of a genetic test. Using drugs such as tamoxifen, raloxifene, Aromasin for breast cancer can sometimes result in other pathologies. Tamoxifen is usually associated with enhanced thromboembolic and endometrial cancer risk whileas raloxifene is associated with leg cramps and fever events (Nelson et al. 2013a, b, 2019a, b).
- Surgical interventions like mastectomy and oophorectomy or salpingo-oophorectomy are associated with a range of post-surgical complications like infection, pain, bleeding, swelling, numbness, tingling, breast hardness, organizing hematoma, breathing problems, thrombosis, pulmonary embolism, and failed reconstruction (Nelson et al. 2019a, b; Alamouti et al., 2015; Nurudeen et al., 2017; Borreani et al., 2014).
- Consistent observation including frequent examinations and screening may not always result in the early detection of ovarian and/or breast cancer. Even intensive screening including MRI for breast cancer and transvaginal ultrasound for ovarian cancer demonstrated high rates of false-positives (Le-Petross et al., 2011; US Preventive Services Task Force et al., 2019).
- An abnormal test result might cause anxiety, depression, or rage in some female patients (Lieberman et al., 2017; Low et al., 2008). Although the result does not imply that a patient will certainly acquire breast cancer, many females with a defective gene think they will.
- Depending on the genetic information, individuals may encounter discrimination when applying for life insurance or employment. Genetic testing might not be able to address all of the queries.

5.6 Future Perspective

Detection of BRCA gene mutations in breast cancer patients impacts management and outcome in addition to its significance for their kins. Possibly deleterious mutations of BRCA1/2 are not only related with increased breast cancer risk but influences ovarian, peritoneal, and fallopian tube cancer. Therefore, knowing for germline BRCA1/2 mutations has a well-known prognostic role in breast cancer risk consideration. Of late, evidence show BRCA1/2 mutational status as clinically

applicable in the choice of therapy for breast cancer patients. It is thus highly proposed to offer BRCA detection to breast cancer patients even though that are classified as high-risk group. No doubt if every breast cancer patient is accessible for BRCA testing, the possibility for breast cancer management and treatment linked with such confirmation can be made better even further than current testing norms suggest. We believe that health professionals dealing with breast cancer patients necessitate to be conscious of recommendation testing potential for healthy individuals with breast cancer in the family, and the rationale behind the low compliance should be taken into consideration. Wholesome approach for better plan and policy to improve both diagnostic and prognostic BRCA gene confirmation testing will help to categorize further actionable mutation positive individuals preceding to breast cancer development. Thus, proper knowledge to know the pattern of these BRCA mutations either sporadic and germline abets in recognition of breast cancer affected individuals and their families, who can then be imparted proper counselling for their prospective health management.

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Early-Stage Progression of Breast Cancer

6

Hira Gul, Iqra, and Nosheen Masood

Abstract

Breast cancer consists of group of molecularly heterogenous disease in which breast cells start growing in a prolific way. Globally, breast cancer is one of the most common cancers and is the main cause of cancer deaths in women. As compared to developing countries, developed countries showed higher prevalence rate of cancerous cells of breast among women. There are five different stages of breast cancer. The proliferation of breast cells can start in different areas of breast, e.g., ducts, lobules, and tissues lie in between them. Stage zero breast cancer is non-invasive ductal carcinoma in situ DCIS, whereas stage 1 through stage 4 is called invasive breast cancer. The commonly known categories of invasive type breast cancer are IDC (Invasive ductal carcinoma) and ILC (Invasive lobular carcinoma). Early-stage breast cancer has considerably high potential to cure. Sensitivity of the chemotherapy is affected due to the cancer characteristics and by molecular classification. Molecular classification of breast cancer based on estrogen receptor negative group such as HER2 enriched, normal breast, and basal shaped is more sensitive to chemotherapy as compared to estrogen receptor positive group having luminal type disease. Etiology of breast cancer involves both genetic and non-genetic factors. Genetic counseling of the patient must consider both profiles of family history along with mutation location inside the body. Susceptibility genes associated with breast cancer etiology and prognosis can be classified as low penetrance genes and high penetrance genes. Mutations and polymorphisms in low penetrance genes are frequent in population and are linked with low risk as compared to high penetrance genes mutations that

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are linked with high risk. Mutations involved in breast cancer are identified by clinical genetic testing that provides survival benefits to the patients by focusing on early screening and prevention strategies. A wide range of cancer cases are reported because of the mutations in any of the high penetrance breast cancer genes including PTEN, STK11, BRCA1, TP53, BRAC2, CDH1. Different studies have reported that few number of low penetrance genes have been used as an advance genetic testing methods. Association of the low penetrance genes such as BARD, BRIP, MLH, MSH2 & 6, PMS2, NBN, RAD51C & 1D with breast cancer is still a question of debate.

Keywords

Breast cancer · Non-invasive ductal carcinoma in situ · Invasive breast cancer · Invasive ductal carcinoma (IDC) · Invasive lobular carcinoma (ILC) · Breast cancer genes

6.1 Early-Stage Progression of Breast Cancer

Worldwide, breast cancer (BC) is the most commonly occurring cancer, and it poses crucial health challenges on a global scale. Breast cancer consists of group of molecularly heterogenous disease in which breast cells start growing in a prolific way (Guedj et al. 2012). There are different kinds of breast cancer according to the breast cells. The proliferation of breast cells can start in different breast areas such as lobules, ducts, or the tissue in between them (Shi et al. 2017).

From stage 0 to stage 4, there are five stages of breast cancer. Stage zero breast cancer is called DCIS (non-invasive ductal carcinoma in situ), while breast cancer from stage 1 through 4 is called invasive breast cancer. (Invasive ductal carcinoma) IDC and (Invasive lobular carcinoma) ILC are most common classes of breast cancer (Balakrishnan et al. 2011) (Fig. 6.1).

6.2 Epigenetics in Breast Cancer Progression

Both epigenetic and genetic changes are important in breast cancer progression. Epigenetic is defined as heritable changes in expression of gene without any specific type of change in sequence of DNA. Remodeling of nucleosomes, modifications of histone, and DNA methylation are major epigenetic changes that are seen in breast cancer progression. Many different genes that are involved in cell metastasis, invasion, proliferation, and anti-apoptosis may undergo epigenetic changes in breast cancer (Basse and Arock 2015).

Epigenetic changes are not only limited to posttranslational modifications of histones and CpG islands but it also includes miRNAs (microRNA). Hypermethylation of different microRNA such as mir152, mir91, mir663, mir148, and mir124a3 is found in breast cancer patients. Erosion of telomere and aneuploidy

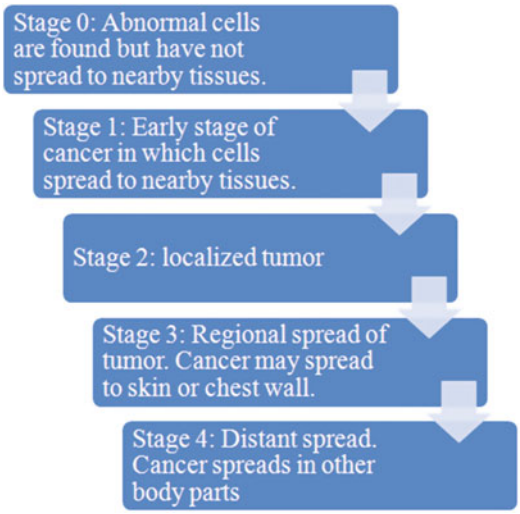

Fig. 6.1 Chart showing the detailed description of stages of cancer

(genetic instability) are additional epigenetic changes that are associated with epigenetic changes (Lustberg and Ramaswamy 2011).

6.3 Molecular Classification of Breast Cancer

Four classes of breast cancer have been recognized.

1. Luminal like
2. Basal
3. Normal breast
4. Positive HER2

Luminal subtype is derived from ERPT (Estrogen receptor positive tumors), while basal-like normal breast-like and HER2-enriched subtypes are derived from ER negative cancers (Simpson et al. 2005). The best prognosis is carried by the luminal A, whereas the worst prognosis is carried by HER2-enriched and the basal-like despite improved response to chemotherapeutic treatment.

6.4 ERPT Positive Group: Luminal Type

Luminal-like type is divided into two subtypes:

1. **Luminal (A)** consists of ERPT (Estrogen receptor positive tumors) of low histologic grade (de Ronde et al. 2010).

2. **Luminal (B)** consists of ERPT (Estrogen receptor positive tumors) of high histological group and displays high expression levels of PRG (Proliferation related genes) (Smid et al. 2008).

The division of luminal like type in two categories shows high level of prognostic signification. A type luminal tumor is associated with good prognosis, whereas a B type luminal tumor carries aggressive behavior than A type luminal tumor (Shi et al. 2017).

These two subtypes discriminate highly from each other. The other area of discrimination between these two subtypes (Luminal A and B) is in their response to chemotherapeutic treatment (Sørlie et al. 2001). Although generally it seems bad for both A and B luminal subclasses, pathologic as well as clinical effect to chemotherapeutic treatment is reported higher in B type luminal cancers (Foulkes et al. 2010). The separations of luminal subtypes tumors are primarily dependent on the expression level of genes related to proliferation.

6.5 ERNT Negative Group

ER negative type group consists of the following subtypes:

1. HER2 enriched
2. Normal breast
3. Basal shaped

6.5.1 HER2 Enriched Subclass

The gene of HER2 over expressed in about 15% of invasive like breast cancer. Compared to ER-positive cancers, HER2 tumors have shown a worse outcome (Parker et al. 2009). There are total 33 HER2 tumors, by analysis of gene expression 64% shows HER2 enriched type, and about 6% were group as basal-like tumor. Furthermore, 9% of negative HER2 tumors were grouped as HER2-enriched.

6.5.2 Breast Like

The scientific importance of tumors that seems breast like remains unclear and is yet in research to be fully characterized (Tang et al. 2018). There are many researches that illustrate that mostly normal like breast tumors constitute tissue procurement object, samples with a high stromal and normal like breast epithelial cells content (Perou et al. 2000).

6.5.3 Basal Like

Basal-like cancers consist of tumors of heterogeneous group, prevalent in adult women (Wirapati et al. 2008). In correspondence to HER2 and ER-positive tumors, the molecular nature of basal type tumors is more hostile; however, special cytological breast tumor types show a phenotype of basal-like cells such as ACC (Adenoid Cystic Carcinomas) and SC (Secretory Carcinomas), which shows remarkably indolent (inactive) clinical course (Reis-Filho et al. 2005). Basal-like cancers show bad prognosis despite chemotherapy sensitivity has a higher tendency for visceral metastasis. High gene expression levels of (EGFR) epidermal growth factor receptor are reported in more than 60% of basal-like tumors, and TP53 gene mutations are seen in more than 90% of cases (Weigelt et al. 2010) (Fig. 6.2).

6.6 Progression of Breast Cancer and Role of Cell Specific Polarity Proteins

Polarity of cells plays a very prime role in maintaining integrity of tissues and development of cells. Loss of cell polarity leads towards cancer progression. For maintaining cell type and tissue structure, three conserved complexes of polarity proteins play a vital role. Par, Scribble, and Crumbs are the polarity proteins complexes (Ellenbroek et al. 2012). Par3 and Par 6 belong to Par complex. Scribble complex consists of lgl1/2 and scribble, whereas crumbs polarity protein complex consists of crb3, PATj, and pals1 proteins (Fig. 6.3).

6.7 Role of High and Low Penetrance Genes

Breast tumor etiology involves both genetic and non-genetic factors. Approximately 15% of the breast cancer patients show positive family history signifying the major risk for mutation carriers. Its risk depends upon the genes involved as well as

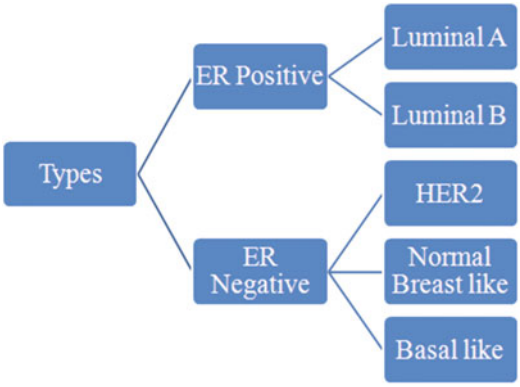

Fig. 6.2 Flowchart showing the types of breast cancer

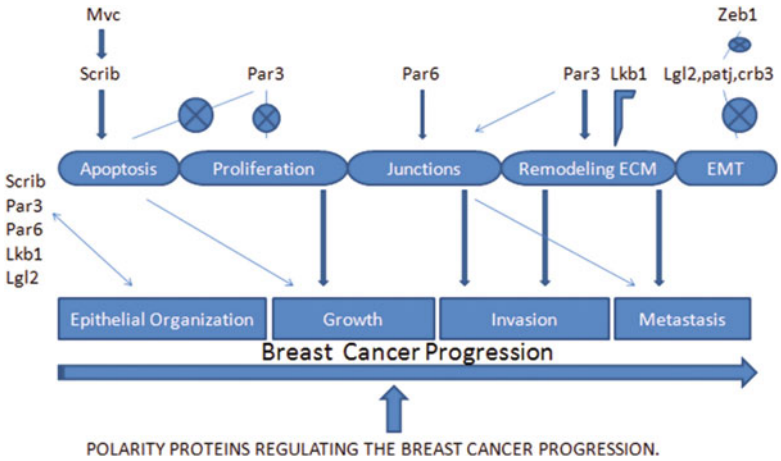

Fig. 6.3 Diagram showing the breast cancer progression regulated by polarity proteins complexes. Different cellular processes such as remodeling of extracellular matrix, proliferation, transition of epithelial mesenchymal cells, and apoptosis. Arrow of Lkb1 shows that it is not well known that remodeling of ECM will affect metastasis or invasion of cells that will further lead towards breast cancer progression (Rejon and McCaffrey 2015)

location of mutation present (Kuchenbaecker et al. 2017). Therefore, genetic counseling must consider both the family history profiles along with mutation location inside body. Susceptibility genes associated with breast cancer etiology and prognosis can be classified as (LPG) low penetrance genes and (HPG) high penetrance genes (Kang and Choi 2021).

High penetrance genes mutations are not very common in population but associated with very high risk (relative risk of these genes between carriers and non-carriers is 5 to >20). Low penetrance genes mutations and polymorphisms are very common and are linked with low risk as compared to high penetrance genes mutations. Mutations involved in breast cancer are identified by clinical genetic testing providing survival benefits to the patients by early screening and prevention strategies. Identification of all variants including rare and common that could be associated with predisposition of breast cancer can be done through next generation sequencing (Hamdi et al. 2020).

6.8 High Penetrance Breast Cancer Genes

If an individual becomes the victim of breast cancer disease at young age or belonged to the family where other cases pre-exist, then this infers that those individuals should be candidates for screening for mutations. Despite advanced medical research, patients with suggestive personal or positive family history possess less than 30% of the identified gene mutation. A wide range of cases are caused because of the mutations in any of the high penetrance breast cancer genes including

PTEN, STK11, BRCA1, TP53, BRAC2, CDH1, and different strategies and practices help in the management of these cases.

6.9 BRCA1 and BRCA2

Due to the BRCA1 and BRCA2 genes mutations, the most of the cases are of hereditary breast cancer. Both these genes are tumor suppressors and encode proteins which play a role in homologous recombination repair. Variants which are pathogenic in both BRCA1 and BRCA2 affect one in 400 individuals in general population. They exhibit autosomal dominant pattern of inheritance and possess life time accumulative breast cancer risk of 69% and 72% for BRCA2 and BRCA1, respectively (Pouptsis et al. 2020) (Fig. 6.4).

6.9.1 PALB2

The PALB2 is BRCA2 binding protein and regulates it by localizing and stabilizing within vital nuclear structures. This PALB2 was categorized as high-risk breast cancer gene having 95% CI = 5.1–11.1 and OR = 7.4 (Ellsworth et al. 2019). Likewise, in European-Caucasian cohort, about 46% of tumors in breast cancer cases possess PALB2 mutations (Slavin et al. 2017).

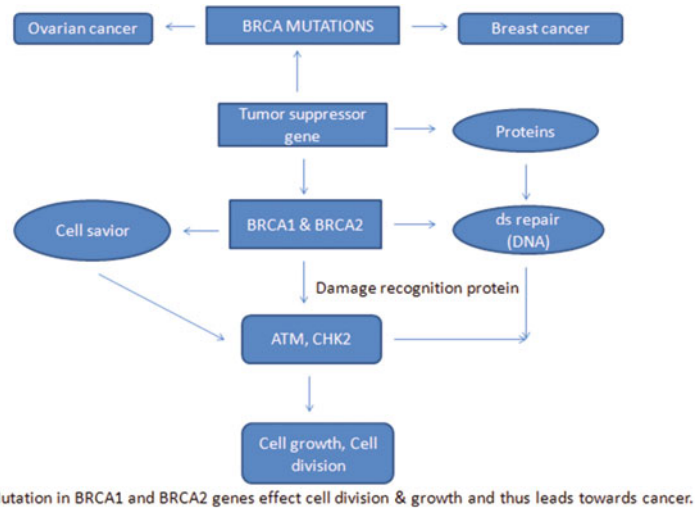

Fig. 6.4 Diagram explaining the mode of action of BRCA1 and BRCA2 genes

6.9.2 TP53

TP53 is among one of the most frequently mutated gene and is mutant in 30% of all types of breast cancer. In breast cancer management, the role of this gene is not clear yet. Depending upon the treatments given, clinical outcomes of mutant p53 can be beneficial or harmful. This is possibly due to the diverse activities of mutant p53 as a result of various treatments and each one has its own survival time (Shahbandi et al. 2020).

6.9.3 PTEN

The gene phosphatase and tensin homolog (PTEN) deleted from chromosome 10 is a tumor suppressor. It is negative regulator of PI3K/AKT signaling pathway, playing a role in survival of cell, apoptosis, and proliferation. Inactivation of PTEN was related to tumorigenesis of various human cancers together with breast cancer (Zhang and Yu 2010). Frequency of variants of PTEN has not been yet explored fully. In the literature, it has stated that low expression or deletions of PTEN is found in 63% and 4% of breast cancer individuals. Various groups studied PTEN expression patterns and their association with clinicopathological characteristics along with clinical outcomes. However, results were inconsistent (Li et al. 2017).

6.9.4 STK11

The STK11 (serine/threonine protein kinase 11) is a gene in breast cancer which is highly penetrant and regulates the energy metabolism and polarity of cell. Mutation present in STK11 causes (PJS) Peutz-Jeghers syndrome with elevated risk for several cancers together with breast cancer (lifetime risk from 24 to 54%) (Rousset-Jablonski and Gompel 2017).

6.9.5 CDH1

Cadherin 1 gene (CDH1) basically encodes an adhesion molecule that is involved in repairing the morphology of epithelial cell. CDH1 germ line mutation associated with high risk of hereditary diffuse gastric cancer, malignancy predisposition syndrome related with high lifetime risk of breast cancer, mostly invasive lobular carcinoma (ILCA)(Vargas et al. 2011). Invasive lobular carcinomas (ILCA) are mostly estrogen positive, but link between CDH1 and TNBC mutations is unclear. Similarly in CDH1, mutations in family were not very common (0.0–0.3%) in TNBC (Buys et al. 2017).

6.10 Low Penetrance Breast Cancer Genes

Different studies reported that few number of low penetrance gene have been used as advance genetic testing methods. Although these may play a role as risk factor in a polygenic fashion, this is probably significant to minority of cases and their finding should not be mostly from daily practice. Mutation testing is required for high index of suspicion, and next generation sequencing improves the finding of genes and medical administration of all cases. Many case studies reported that there is no genetic susceptibility identified and lifetime breast cancer risk calculated by standard tools. These are low penetrating genes BARD, BRIP, MLH, MSH2 & 6, PMS2, NBN, and RAD51C & D; there association with breast cancer is still a question of debate.

Two of the five paralogs of RAD51 are RAD51C and RAD51D genes and their associations with other repairing genes of DNA on Ds (double-stranded breaks) are due to homologous recombination. Yang et al. (2020) explain in their research study that comparable breast cancer risk for a patient with mutation in RAD51C gene is 1.99 probably.

The BARD1 gene is a protein and function as a tumor suppressor. Its mutations affect the splicing sites in this gene. In 2015, Tung et al. reported that mutation in BARD (1) gene shows no prominent risk for breast tumor (Tung et al. 2016). While Kurian et al. (2017) showed that BARD1 comparison to breast tumor is about 1.94.

The (BARD1): (BRCA1 Associated Ring Domain 1 gene) binds with a binding partner gene BRCA1 (Weber-Lassalle et al. 2018). Other researches studies have discussed that the linkage of mutations in BRIP1 possesses clinically and scientifically relevant risk for TNBC with OR greater than 2 (Shimelis et al. 2018; Hu et al. 2020) (Fig. 6.5).

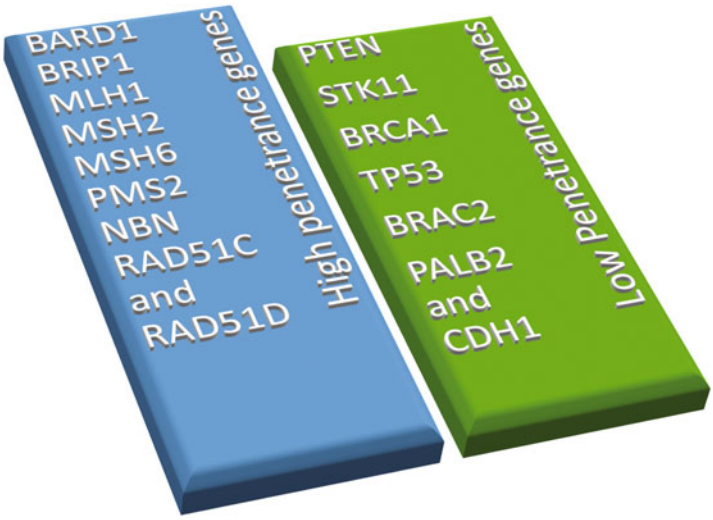

Fig. 6.5 Illustration of Low & High penetrance genes in breast cancer

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Noninvasive Biomarkers: Emerging Trends in Early Detection of Breast Cancer

7

Amisha Patel and Sejal Shah

Abstract

Breast cancer (BC) is one of the leading mortality among women throughout the globe. Screening of breast cancer and early detection aids better prognosis and successful therapeutic upshots. Currently, the mammography is the only method used for the diagnosis as the gold standard offers effective revenue, limits to its effectiveness, accuracy, overdiagnosis and its inability to detect small-scaled cancers, mainly in female with high density breast tissues, remains a foremost problem in breast oncology. Hence, it is an unmet clinical need to recognize convenient noninvasive biomarkers from an easily approachable source that could overcome the shortcomings of mammography. Promising biomarkers using epigenetic approach just like miRNAs, circulating tumor cells (CTCs), circulating cell-free DNAs or RNAs, lipids, proteins, volatile organic compounds as well as biomarkers from tears, nipple aspirate fluid (NAF), sweat, and urine have shown great potential to detect BC at pre-invasive stages. In addition, there is a superior requirement of mutational signature for individualized treatments; the high-penetrance molecular biomarkers including *TP53, BRCA1, BRCA2 are* risk alleles for the breast cancer progression. The current chapter would focus on the recent discoveries aimed to harnessing novel molecular biomarkers for the early detection of BC biomarkers as well as the therapeutic implications that need to be overcome.

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Keywords

Breast cancer · Early diagnosis · Noninvasive biomarkers · Mutational signature · Detection

7.1 Introduction

Breast cancer (BC) is the one of the most diagnosed sarcomas with 684,996 deaths and 2,261,419 new cases among women in 2020 (Sung et al. 2021). It is metastatic cancer and predominantly transfers to other organs including liver, lungs, brain, and bone which resulted in incurability (DeSantis et al. 2016). The majority of people detected with breast cancer have no known origin. However, potential threat elements have been identified for developing breast cancer together with family history of BC, female gender, age group, early menstruation, late menopause, late age at first childbirth, therapeutic chest radiation, benign tumor in breast and genetic alteration such as BRCA1 and BRCA2 (Leaf et al. 2021). Screening of breast cancer and diagnosis at an earlier stage play a vital role in successful therapeutic upshots and prognosis (Lehtimäki et al. 2011). Mammography is the benchmark for screening and diagnosis of breast carcinoma. However, it has prominent limitations such as radiation, limited sensitivity, over diagnosis as well as it cannot detect all breast cancers especially women with dense breast (Drukteinis et al. 2013). Petite studies have been observed that the overdiagnosis through mammogram may actually contribute adverse effects ranging from 0 to 40–50% and X-ray used for mammography can lead to the onset of breast cancer (Heinävaara et al. 2014; Jacklyn et al. 2018). Moreover, digital breast tomosynthesis is the incremental improvement in mammography which reduces the false positive rates of BC screening (Friedewald et al. 2014). Surgical biopsy is the traditional technique to diagnose cancers which stands invasive practice, but it is not necessary when tumors are benign. Hence, significant efforts require much attention for the identification and development of noninvasive and reliable biomarkers.

Biomarkers are the molecular signatures or molecules secreted by tumor cells or by normal tissue as its reaction or biological markers which can be visualized using various tools such as imaging technology and molecular technology (Yeen et al. 2018). It offers a dynamic and potent approach to evaluate the biological state of disease which can predict tumor behavior, progress of disease, and treatment response (Hinestrosa et al. 2007; Giridhar and Liu 2019). Breast cancer associated tumor suppressor genes *BRCA1* and *BRCA2* are well recognized in heritable BC. Approximately, 20–25% hereditary BC and 5–10% all BC caused by *BRCA1/2* mutation (Paluch-Shimon et al. 2016). There are a number of mutagens and carcinogens responsible for onset of cancer, even HPV is one of the most responsible factors involved in the development of various cancers (Bhavika et al. 2018; Patel et al. 2021b).

Previous study reported that noninvasive biomarkers including CTCs, circulating mRNAs and miRNAs, circulating extracellular vesicles (EVs), circulating

carcinoma antigens (CAs), circulating cell-free DNA (cfDNA), nipple aspirate fluid (NAF), volatile organic compounds (VOCs), tears, sweat, and urine hold significant potential to early detection of breast cancer and it enables real-time monitoring of ongoing fluctuations in the tumor (Alimirzaie et al. 2019).

7.2 Types of Noninvasive Biomarkers

The application of noninvasive molecular signature has become an indispensable component in clinical practice. It needs to develop noninvasive technologies for cancer diagnosis as it has many advantages with accuracy during in vivo analysis (Nisar et al. 2020). Noninvasive biomarkers used in cancer detection are mainly classified in blood-based and non-blood-based biomarkers (Fig. 7.1).

7.2.1 Blood-Based Biomarker

Blood-based biomarkers are enabled for monitoring treatment response or guidance of better treatment (Lewandowska 2014). One of the noninvasive, cost effective, and convenient method for BC detection that relay on liquid biopsy approach. To upgrade the clinical utility, circulating biomarkers should be incorporated into evaluating new therapies for diagnosis and prognosis of cancer patients.

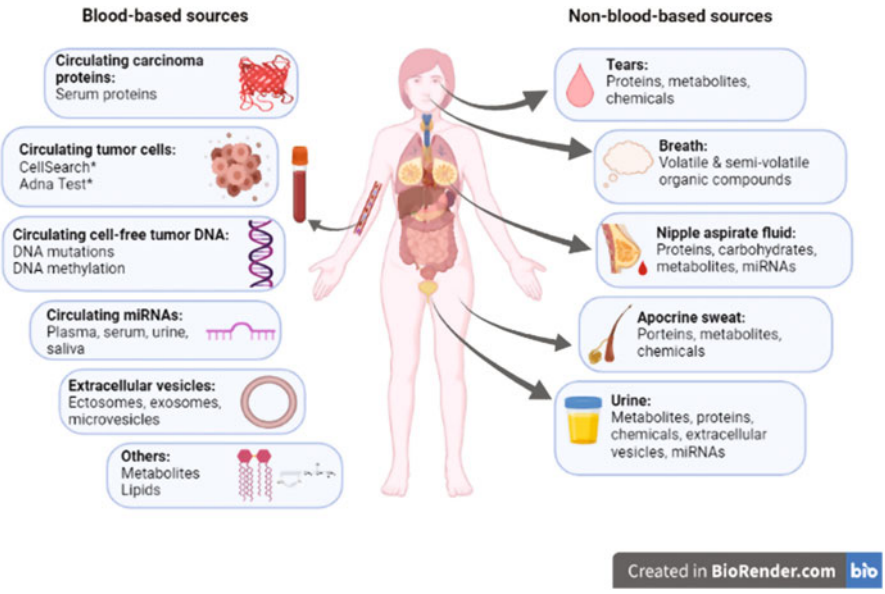

Fig. 7.1 Noninvasive biomarker detection sources for breast cancer

7.2.1.1 Circulating Carcinoma Proteins

Proteomics has opened the door to understanding the dynamic picture of all expressed proteins and it can provide a functional landscape of proteins within the cancer cells (Tyers and Mann 2003; Ősz et al. 2021). Proteins are involved in proliferation, migration, oncogenic signaling, and angiogenesis and found as biomarkers as well for breast cancer diagnosis (Winkler et al. 2020). Several identical diagnostic methods for proteomic analysis involving immunohistochemistry (IHC), enzyme-linked immunosorbent assays (ELISA), and reverse-phase protein array (RPPA) are based upon the principle of antibody–antigen interaction and analytical methods such as flow cytometry and mass spectrometry (MS)-based technologies (Manuscript 2014).

IHC is the traditional touchstone for the clinical evaluation of protein expression including Estrogen Receptor 1 (ESR1), Progesterone Receptor (PGR), and Human Epidermal Growth Factor Receptor 2 (HER2) markers in patients with breast cancer. Patients diagnosed with ESR1 and PGR positive have better survival than negative one. These receptors are widely used to determine patients for endocrine therapy. Overexpression of HER2 related to poor prognosis during chemotherapy. Combination of anti-HER2 therapies with chemotherapy in patients with HER2 positive have led to exceptional survival rate. HER2 has better prognosis than HER2 negative patients (Ősz et al. 2021). Petite study reported that jury of trefoil factors (TFF1, TFF2, and TFF3) expressed differentially in each protein of BC patient's serum and it has remarkable discriminative feature; however, it is the promising biomarker for screening of breast cancer (Ishibashi et al. 2017).

7.2.1.2 Circulating Tumor Cells

Thomas Ashworth discovered the first breast CTCs in 1869 through an autopsy examination of cancer patients, and the isolation method for CTC was described in the late nineteenth century. CTCs are rare in the early stage of BC, for non-metastatic, the ratio would be 1 cell/10 mL which may not be the practical choice to link with most fluorescence-activated cell-sorting techniques. There are several CTC analysis methods developed over the decades based on physical appearance, ELISA or immunofluorescence, immunomagnetic separation, and RT-PCR (reverse transcription polymerase chain reaction) assay. For BC, AdnaTest® (AdnaGen AG, Langenhagen, Germany) and CellSearch® (Janssen Diagnostics, Raritan, NJ, USA) were recommended (Andreopoulou et al. 2012). As per initial theoretical concern, CTCs served as a prognostic biomarker for patients with metastatic breast cancer (Pang et al. 2021). But CTCs are not continuously shed in circulation due to their short circulating half-life in bloodstream, lower sensitivity and reproducibility (Thery et al. 2019). CellSearch® is the CTC test system recently approved by the US FAD for monitoring circulating cancer cells in the blood metastatic breast cancer patients (Habli et al. 2020).

7.2.1.3 Circulating Cell-Free Tumor DNA

In 1948, Mendel and Metais identified ctDNA in the bloodstream of both cancer patients and healthy individuals. The discovery of improved techniques described in

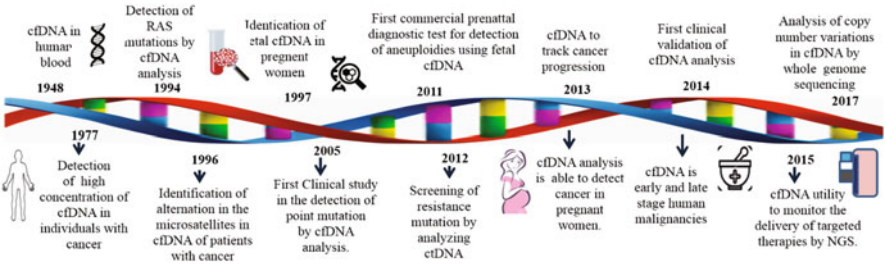

Fig. 7.2 Timeline of improved discoveries in ctDNA

Fig. 7.2. Several studies reported that the concentration of ctDNA significantly improved in cancer patients compared with healthy individuals. In 1963, *Stroun* et al. established the specific fraction of circulating DNA originating from tumor cells which identify decreased strand stability. This discovery has emerged recently as the new hallmark for cfDNA biomarkers in precision medicine. Several studies suggested that methylation prestige of different genes including RASSFIA, APC, MGMT, ITIH5, BRCA1, GSTP1, and FOXA1 spotted in ctDNA could be used for early detection of BC. ctDNA is still investigational, though it is the spearhead in the field of biomedical research as it opens the door to mirror the tumor's lifespan (Panagopoulou et al. 2021).

7.2.1.4 Circulating miRNAs

MicroRNAs are very short sequences that do not code RNA, average having 22 nucleotides which prevent messenger RNA (mRNA) translation silencing or mRNA degradation and regulate different gene expression covering various biological processes. Initially it was reported in 1993 by Lee Rosalind in *Caenorhabditis elegans* (Lee et al. 2007). Body fluid components, whole blood, plasma, serum, urine as well as nipple aspirate fluid. miRNAs play a key role in development of cell, tumor progression and apoptosis. Earlier studies reported >2000 miRNAs have been identified to be responsible for many chronic diseases as well cancer. First oncogenic miRNA found to be overexpressed in metastatic BC. In addition, they are stable as well as easy to collect, however they gained tremendous attraction as noninvasive biomarkers but challenges are still raised due to smaller size and lower abundance of miRNAs. To overcome these challenges several techniques have been established such as next generation sequencing (NGS), various PCR techniques including digital droplet PCR (ddPCR) and quantitative reverse transcription polymerase chain reaction (qRT-PCR), NanoString nCounter® and microarray assay. Among various detection methods qRT-PCR has been widely used to address the technical improvement as it is extremely sensitive and cost effective for miRNA detection and quantitation (Fig. 7.3). Abundant research of miRNAs has highlighted the concrete efficacy of miRNAs as biomarkers in BC

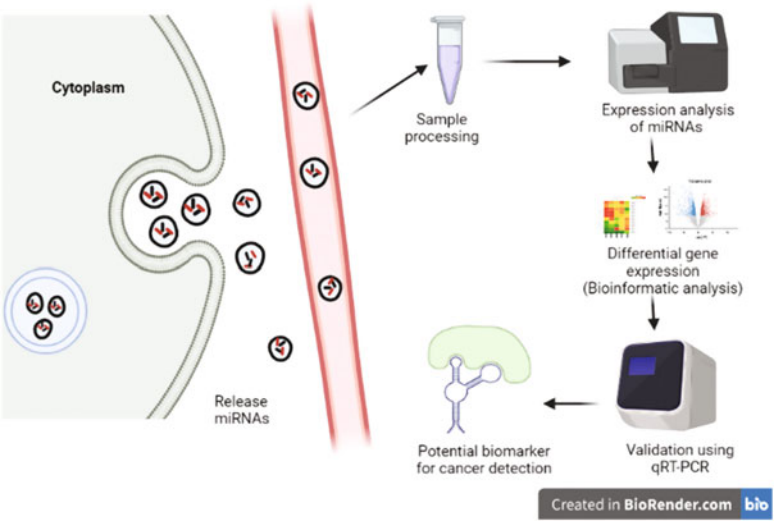

Fig. 7.3 Circulating miRNAs as biomarker in cancer diagnosis

(Table 7.1). Most promising microRNAs observed are miR-145, miR-21 and miR-221 in patients with BC (Aggarwal et al. 2020).

7.2.1.5 Extracellular Vesicles

Cells secrete extracellular vesicles (EVs) into an extracellular environment and are composite of proteins, RNAs, DNAs, and lipids. They are differentiated by origin, biogenesis, composition of membrane as well as physical properties such as size (Vader et al. 2014). They might be an important biomarker as they carry treasured information of cancer origin and the state of the tumor. It is found that there is a significantly higher number of EVs found in cancer patients (Sadovska et al. 2015). EVs have been classified based on various features including origin (protostomes & oncosomes), biological function (tolerosomes & vexosomes), and biogenesis (exosomes, ectosomes, & apoptotic bodies). Exosomes involved in tumor growth promotion, angiogenesis, and metastasis. Fibronectin (FN) existing on the surface of the EVs was selected for biomarker detection as the amount of FN significantly elevated ($p < 0.0001$) at all stages of breast cancer and reduced after the treatment (Moon et al. 2016a). Although, many vital aspects of EVs such as functions, biogenesis and release need to be investigated.

7.2.1.6 Other Blood-Based Biomarkers

Recent investigation has increased attention towards genetic biomarker arising somatic mutations. TP53, PIK3CA, and GATA3 are prime driver alterations in cancer (Shah 2011; Shah et al. 2015, 2018). Deregulation of PTEN gene is the predictor of trastuzumab resistance, PI3K inhibitor might overcome this (Shah et al. 2017).

Table 7.1 List of molecular biomarkers for BC identification

Blood-based biomarkers for breast cancer				
Type	Year	Biomarkers	Methodology	References
Circulating carcinoma proteins	2019	TNF-α, HER2, AFP, CA19–9	ELISA	Henderson et al. (2019)
	2017	PTN	ELISA	Jiang et al. (2016)
	2017	TFF1, TFF2 and TFF3	ELISA	Ishibashi et al. (2017)
	2016	HE4	ELISA & qRT-PCR	Lu et al. (2017)
	2016	ApoC-1	SELDI-TOF-MS & MALDI-TOF/TOF MS	Song et al. (2016)
Circulating tumor cells	2012	CTCs (gene expression of GA733–2, MUC-1, HER2)	CellSearch system and AdnaTest	Andreopoulou et al. (2012)
Circulating cell-free tumor DNA	2021	MGMT, GSTP1, APC, RASSFIA, FOXA1	Machine learning approach	Panagopoulou et al. (2021)
	2014	PIK3CA, E545K and H1047R mutations	ddPCR	Beaver et al. (2014)
	2019	DELF1	–	Cristiano et al. 2019
CirculatingmicroRNAs	2019	miR-21	qRT-PCR	Abdulhussain et al. (2019)
	2019	Five-miRNA board, miR-1246,miR-1307-3p, miR-4634, miR-6861-5p, andmiR-6876-5p	Microarray analysis	Shimomura et al. (2016)
	2016	miR-182	qRT-PCR	Mihelich et al. (2016)
	2019	miR-90b, miR-130a, miR-200b, miR-452	Meta-analysis	Expressions et al. (2019)
Extracellular vesicles	2016	Fibronectin	ELISA	Moon et al. (2016b)
Metabolites	2021	Ethyl (R)-3-hydroxyhexanoate, caprylic acid	LC-QTOF-MS	Wei et al. (2021)
	2021	Five unknown metabolites C9H16O3S, 278.1552@9.641, C26H43CIN4S3, C26H51N5O4, C23H30N2S	LC-MS	Jové et al. (2017)

(continued)

Table 7.1 (continued)

Non-blood-based biomarkers for breast cancer				
Tears	2009	Proteomic profile of tear	SELDI-TOF-MS	Lebrecht et al. (2009)
	2012	Quantification of tear protein level.	MALDI-TOF-TOF	Böhm et al. (2012)
Breath	2003	VOC markers of oxidative stress	GC-MS	Phillips et al. (2003)
	2014	BreathLink™ system	Gas chromatography	Phillips et al. (2014)
	2018	Two commercial electronic noses	GC-MS	Herman-Saffar et al. (2018)
NAF	2006	NAF protein profile	ICAT labeling, SDSPAGE, LC-MS.	Pawlik et al. (2006)
	2012	Deglycase DJ-1 protein	ELISA	Oda et al. (2012)
	2005	TF & its receptor Tn	ELISA	Kumar et al. (2005)
	2010	TF, Tn, & age information	Direct immunoassay	Deutscher et al. (2010))
Apocrine sweat	2019	20 aspirants of sweat markers	–	([CSL STYLE ERROR: Reference with no printed form.])
Urine	2019	miRNA-21	RT-PCR	Ando et al. (2019)
		MMP-1	Western blotting	
	2020	Four combine candidates of urinary miRNAs	qRT-PCR	Hirschfeld et al. (2020)

Apart from these, lipidomics is the emerging field of biomedical research providing a snapshot of lipid metabolism in the cell. Previous study (Chen et al. 2016) discovered 15 lipid species panels from plasma which need to be validated the first stage of BC. Whereas, additional study described detection of fatty acid from serum able to identify early stage BC (Zhang et al. 2014). In recent years, metabolomics is the alternative approach to characterize small volatile organic compounds involved in cancer progression. Our earlier study (Patel et al. 2021a) reported propionic acid as a potential diagnostic biomarker for oral cancer. There are several analytical techniques including NMR (nuclear magnetic resonance), mass spectroscopy-based methods such as gas chromatography (GC), liquid chromatography (LC), and ultra-performance liquid chromatography (HPLC) coupled with MS used to quantification of unknown compound from biological samples. Earlier study reported ethyl (R)-3- hydroxyhexanoate and caprylic acid as diagnostic biomarker for breast cancer with ~0.80 univariate area under the receiver operating

characteristic curve (AUROC) which is used to evaluate specificity and sensitivity of aspirant biomarker (Wei et al. 2021). Remarkably, Jové et al. (2017) identified five unknown metabolites such as C9H16O3S, 278.1552@9.641, C26H43ClN4S3, C26H51N5O4, and C23H30N2S as well as four known metabolites including taurine, caproic acid, linoleic acid, and stearamide in BC patients with high specificity and sensitivity. Many scientific literatures suggested different metabolites found to be present in BC, hence it needs to be validated in a large cohort.

7.2.2 Non-blood-Based Biomarkers

Unlike peripheral blood, other body fluids are also investigated for the diagnosis of cancer diseases.

7.2.2.1 Tears

Tears are three-layered, transparent fluid containing various biomolecules including proteins, lipids, metabolites, and electrolytes. These biomolecules reflect corneal biochemistry as well as physiology as they are constantly exchanged with ocular surface epithelial cells. In addition, tears produced by filtration of blood plasma which circulates around different organs within the body. Thus, it is believed that tears are an abundant source of clinical information. Number of studies reported tears as biomarkers for various diseases including cancer, diabetes mellitus, cystic fibrosis, Alzheimer's disease, etc. (Barmada and Shippy 2020).

Different types of mammaglobin and lacryoglobin have been identified as biomarkers for the development of metastatic BC. Mammaglobin-A expressed in the mammary gland, whereas Mammaglobin-B can be found in breast, uterus, tears, kidney, etc. Recently, a tear protein based biomarker kit has been developed by one of the kit designer companies, Ascendant Dx. (Morton et al. 2020). Tears over the blood as a source of biomarker have merits as it is noninvasive method, easy sample collection, no protein filtration required. The main demerit of the tears as a source of biomarker is that it is very difficult to get higher concentration of some of the molecules compared to blood. Hence, a reliable diagnostic kit is the need of the hour in the recent scenario. Though different biosensors based on Raman spectroscopy have been designed with high specificity and sensitivity (Kim et al. 2020).

7.2.2.2 Breath

Breathomics is an emerging technology to analyze various diseases which investigating volatile organic compounds (VOCs) released through metabolic activity caused by pathological disorders. The resulting metabolites appear to have a potential to detect breast cancer. Exhaled breath analysis increases an interest in disease diagnosis mainly because of their noninvasive and rapid diagnostic nature. However, still it is extremely limited in clinical practice due to lack of validation and proper certification (Yang et al. 2021). Petite study reported 94.1% sensitivity and 79.8% specificity for VOC markers of oxidative stress in the breath which differentiate BC patients from normal individuals (Phillips et al. 2003).

Moreover, Menssana Research Inc. has developed a noninvasive breath system called BreathLink™. It is a rapid point-of-care device for breath collection and analyzing the VOCs for breast cancer detection. One study presented abnormalities in mammograms (Phillips et al. 2014). Breath testing could reduce the use of mammograms for BC detection but the limitation is the accuracy of breast test results affected by several factors including method of breath collection and analysis, physiological condition of patients, and test environment as well. Hence, more attention will be needed to standardize the procedure for breath testing to detect breast cancer (Hanna et al. 2019).

7.2.2.3 Nipple Aspirate Fluid

Nipple aspirate fluid (NAF), a fluid is secreted by breast epithelial duct cells in non-lactating healthy women. It can be collected by various methods such as breast massage, nipple aspiration, and by using milk-expressing pumps. The intensity of color and the specific biomarker present in NAF could be used to detect breast cancer. It is considered to be a mirror of the cellular fluctuation of breast microenvironment as it comprises high concentration of proteins, hormones, carbohydrates, and metabolites including organic acids, fatty acids, and amino acids. It is the enriched source of predictive biomarkers for precancerous and cancerous transformation as well as considerably less invasive, hence it may provide faster and cheaper tumor assessing aspects. Thus, it has the potential to reduce the risk of complications while assessing breast disease (George et al. 2021). Both Thomsen–Friedenreich (TF) antigen and its biosynthetic precursor, Tn antigen, are found on epithelial cell surface proteins and lipids. They are also detected in primary BC tissue (Kumar et al. 2005). Moreover, miRNA expression profiling from NAF also provides a big shot for diagnosis of breast cancer. Significant upregulation of microRNA-3646 and microRNA-4484 as well as the downregulation of microRNA-4732-5p was found in NAFs of BC patients compared with the patients having benign tumor. Despite the analysis, the NAF collection is simple, faster, and reliable (Zhang et al. 2015). Hence, it may facilitate breast cancer detection through noninvasive approach and it may provide prognostic biomarkers for early detection of breast cancer.

7.2.2.4 Apocrine Sweat

Apocrine sweat produced by apocrine sweat glands, a coiled tubular gland found in epidermis. It plays a critical role in thermoregulation and maintains core body temperature by evaporation. Moreover, apocrine sweat gland is located all over the body surface and secretes oily substances containing steroids, proteins, lipids, and small metabolites including amino acids, carboxylic acids, xenobiotics, and antimicrobial peptides through hair canals. This biological matrix is involved in communicating the health status of an individual; hence, it can be useful for diagnostic purposes. The traditional advantage of sweat analysis is noninvasive collection, but the metabolome analysis from sweat is still limited as it has some disadvantages in terms of variety of metabolites and difficulty in reproducibility of sweat. A study on metabolome analysis for breast cancer using sweat has identified 20 sweat biomarkers with 72% specificity and 97% sensitivity (Kr et al. 2019). Sweat

biomarkers can be used for secondary confirmatory tests to locate tumors in the body or can be used as a prescreen for mammograms. It is believed that apocrine sweat analysis will lead to a totally new approach for cancer screening (Oaks 2003).

7.2.2.5 Urine

Urine is considered to be an ideal bio-fluid for the detection of biomarkers as it allows for ease and noninvasive collection (Gasparri et al. 2017). Several studies investigated urinary metabolites from breast cancer patients using various technologies such as gas chromatography-mass spectrometry (GC-MS) (Herman-Saffar et al. 2018), nuclear magnetic resonance (NMR) spectroscopy (Zahran 2021) as well as capillary electrophoresis coupled to mass spectrometry (Kim et al. 2010). Combine urinary metabolites (1-MA, 1-MG, and 8-OHdG) with CA15–3 discriminates BC from the non-cancerous group with sensitivity range from 80.15 to 91.5%, specificity from 83.2 to 95.2% as well as AUC range from 0.82 to 0.95 (Zahran 2021). Exosomal microRNAs can be detectable from urine analysis. The petite study found significantly altered miRNAs including miR-21, miR-125b, miR-155, and miR-451 from breast cancer patients (Erbes et al. 2015). These results propose noninvasive innovative biomarkers for the detection of breast cancer. However, as a urinary biomarker still in the discovery phase, it needs to be validated in a large cohort.

7.3 Biomarkers Involved in Cancer Progression/Proliferation

Cancer is a multifactorial disease. Several miRNAs and proteins elucidated to regulate the cancer cell proliferation. Circulating RNAs (circRNAs), miRNAs, and proteins circulating in peripheral blood which may be possible biomarkers for BC progression.

7.3.1 circRNAs

circRNAs are a multifunctional component of liquid biopsy as it is involved in many physiological operations. Overexpression of circ_MYO9B (Wang et al. 2018), circ_0084927 (Gong et al. 2021), and circ_ABCB10 (Liang et al. 2017) knockdown significantly suppressed cell proliferation, apoptosis, and BC invasion by sponging miR-4316, miR-142-3p, and miR-1271, respectively. Moreover, a petite study reported that circRNA_000911 was highly expressed than adjacent normal tissue and could be involved in cell proliferation as well as cell invasion for the onset of BC. miR449 provoked cirRNA_000911 to regulate cell proliferation through Notch1 and NF-kB signaling pathways. However, the network of circRNA_000911/miR449a/Notch1/NF-kB may bring a new road map for the development of therapeutic strategy for BC.

7.3.2 miRNAs

Overexpression of miRNAs in BC cells downregulates the expression of programmed cell death protein (Abdulhussain et al. 2019). Earlier study discovered that miR-26a & miR-26b downregulate the expression of ST8 alphaN-acetyl-neuraminide alpha-2,8-sialyltransferase 4 and inhabit the BC progression. Hence, miR26a/26b could be a possible marker for BC progression (Ma et al. 2016). Most recent study reported that expression of miR-383-5p was downregulated, whereas PD-L1 was upregulated in BC tissues. Transfected miR-383-5p inhibits the PI3K/AKT/mTor pathway to suppress the PD-L1 expression (Azarbarzin et al. 2021). Excitingly, many miRNAs including miR-205, miR-206, miR-124, let-7 (lethal-7 family) were identified as tumor suppressor miRNAs (Liang et al. 2019). Each finding needs to be certified through larger-scale studies.

7.4 Biomarkers Involved in Cancer Metastasis

BC is a metastatic disorder that also refers to advanced breast cancer that can invade other parts of the body such as lungs, liver, brain, and bones. Different organ metastasis resulted in different outcomes, hence organotropism metastasis has been well-studied while translating research to clinical practice (Studies et al. 2017).

7.4.1 miRNAs

Almost all metastatic breast cancer resulted in mortality which underlined development of precision medicine-based prognosis approach of BC. The participation of miRNAs in cancer metastasis was examined by Ma and colleagues who reported that overexpressed miR-10b was the first miRNA to be found in breast cancer metastasis. It is proven that RhoC is a downstream effector of miR-10b which encourages cell migration and invasion (Ma et al. 2007). Bit later, Tavazzoie and other members of Joan Messague group exposed that miR-335 targets the extracellular components such as the transcription factor SOX4 as well as tenascin C and suppresses the cancer migration and metastasis (Huang et al. 2008). Another study revealed the involvement of miR-373, miR-126, and miR-520c in the promotion of cancer metastasis (Negrini and Calin 2008). These discoveries strengthen the outcomes of BC research and may be an effective strategy against metastatic breast carcinoma.

7.4.2 Chemokines

In the 1980s, chemokines were found to be small, secreted proteins aid leukocyte migration and positioning. It plays a vital role in epithelial–mesenchymal transition (EMT). The chemokine superfamily has been categorized in four subfamilies including 27 CC–, 17 CXC-, 2 XC-, and 1 CX3C-chemokines. It modulates tumor

growth, proliferation, and migration, so it can be targeted for novel therapeutic strategies for cancer metastasis. The tumor promoting CCL5 is primarily found in tumor epithelial duct cells, but rarely found in normal breast cells. Moreover, multiple cytokines such as CCL2, CCL4, and CXCL8 were highly expressed in cancer tissue compared to normal adjacent tissues, whereas the ligand CXCL12 also revealed the mRNA peak expression in the secondary site of metastatic BC. CXCR4 is the receptor for chemokine CXCL12, a strategic mediator of metastatic breast malignancy. In addition, level of CXCR4 regulated by the hypoxia induced Hif-1α pathway. Hence, targeting chemokine networks could reduce the tumor cell growth and proliferation and it can be a promising target for hindering metastatic breast cancer (Ali et al. 2007).

7.5 Biomarkers Involved in Cancer Drug Resistance

7.5.1 circRNA

circRNAs are not only a transcription factor but also act as microRNA sponges and regulate gene expressions. Petite study (Sang et al. 2019) reported that hsa_circ_0025202 expression improved the sensitivity of tamoxifen (TAM) and inhibit breast cancer cell progression by regulating the miR = 182-5p/FOXO3a axis, whereas another study (Liang et al. 2019) found that hsa_circBMPR2 suppressed cell proliferation, invasion, and TAM resistance by sponging mir-553 to increase expression of USP4in breast cancer. Moreover, petite study (Ma et al. 2019) proved that circAMOTL1 (circular RNA angiomotin-like 1) knockdown reduces the function of paclitaxel by AKT pathway regulation which induces paclitaxel resistance in BC cells. Hence, circRNAs can be a novel target for achieving improved therapeutic strategy for BC patients.

7.5.2 miRNA

Mahor challenge for the number of miRNAs emerged as drug resistance for BC. The overexpression of miR-34a induces the MCF-7/ADR sensitivity to doxorubicin resistance by targeting NOTCH1 pathway as miR-34a found to be downregulated in MCF-7/ADR cells (Li et al. 2012; Park et al. 2014). Another study (Zhou et al. 2010) observed that microRNA-125b, 221, 222, and 923 were overexpressed in paclitaxel resistant breast tumor cells which indicate it might be possible biomarkers for paclitaxel resistant breast cancer. A systematic review and meta-analysis suggested that miR-7, miR-16, miR-125a-5, miR-141, miR205, miR-452, miR663, and miR-3646 were involved in docetaxel resistant breast cancer, whereas miR-21, miR-200c, miR-210, miR-221, miR-375, miR-630, and miR-5423p were found to be trastuzumab resistant BC cells by regulating respective drug-regulated pathways (Tian et al. 2021). However, miRNAs play a vital role in drug resistance and can be novel drug targets for drug-resistant BC patients.

7.6 Progress of Breast Cancer Biomarker Research: Limitations, Challenges, and Future Perspective

It is undeniable that biomarker-based technologies into breast cancer diagnosis and prognosis have numerous advantages over the last few years. Despite these new promising therapies, efforts must be addressed to improve health outcomes before finishing the gaps in translating knowledge to clinical practice. Till now, all the reported studies are at the discovery phase, without clinical trials. There are a number of drugs that have been designed using molecular docking techniques for several cancers (Juneja et al. 2021) but still it requires further analysis of the pharmacogenomics approach. Furthermore, overlapping findings resulted due to lack of standardization of various methods including sample storage, processing, and data normalizing.

Moreover, most of the studies performed with small sample size and the results were not being considerable, hence it needed to be validated in a large cohort with both analytical and clinical reliability. Combinational therapy using multiple biomarkers has shown better response than single one, it needs to establish a minimal number of biomarkers with higher specificity and ability to detect cancer. Overall more polishing touch required for standardizing protocol, sample collection, storage, and analysis as it is the key parameter for reliability, reproducibility, and quality assurance. Additionally, validation should be there in larger cohorts to establish them as an biomarker for early detection of BC (Yeen et al. 2018).

7.7 Conclusion

In the present chapter, we have discussed a comprehensive overview aimed to the development of noninvasive biomarkers for early detection of breast cancer. Blood and other body fluids such as tears, apocrine sweat, breath, nipple aspirant fluid, and urine all consist of promising biomarkers for breast cancer screening. Noninvasive methods for the detection of BC can overcome the limitation of traditional screening method mammograms. Advanced molecular techniques including metabolomic analysis, mRNA expression profiling, protein profiling, etc., have been used for the identification and characterization of various significant biomarkers for the screening, diagnosis, and prognosis of breast melanoma. Among them some of the biomarkers have been authenticated by clinical trials but some promising biomarkers still need proper validation.

Notably macromolecules and cells are also involved in tumor cell growth, proliferation, angiogenesis, metastasis, and drug resistance. Therefore, these biomarkers might be used as novel therapeutic targets to achieve precise treatment for the patient having breast cancer. As this is the era of personalized medicine, the incorporation of noninvasive biomarkers into a new therapeutic strategy would have great impact on clinical practices.

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Epigenetics Involvement in Breast Cancer

8

Mariam Ashfaq Khan

Abstract

Breast cancer is the most common cancer among women worldwide, costing the lives of millions of women around the world. Despite the enormous research by scientists, screening programs, and awareness campaigns, we are still far away from the goal of lowering the global burden of breast cancer. For the precise drug designing and better clinical management of breast carcinoma, it is essential to decipher the underpinning molecular mechanisms of breast carcinogenesis. Due to enormous efforts to understand tumor biology, it had been unveiled that transformation of normal to malignant cells involves not only genetic mutations but also epigenetic mutations. The interplay of both types of mutations initiates malignant transformation and is responsible for cancer development and progression.

The distinguishing property of epigenetic mutations is that epigenetic modifications do not alter the sequence of the DNA, making them not only an attractive candidate as a prognostic and diagnostic biomarker but also a potential therapeutic target. Aberrant epigenetic modifications, namely, histone modifications, DNA methylation, and non-coding RNAs (ncRNAs) have a crucial role in initiating breast carcinogenesis, progression, and drug resistance. Moreover, distinct epigenetic mutations are involved in every stage of breast cancer and can be differentiated between the tumor and normal tissues. In this chapter, we will discuss in detail the three types of epigenetic modifications involved in breast cancer and will give a detailed account of their remarkable potential as diagnostic, prognostic, and predictive biomarkers. Lastly, we will also shed light on why epigenetic mutations hold a promising future as a therapeutic target for precision medicine.

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Keywords

Epigenetic modifications · Histone modifications · DNA methylation · Non-coding RNAs (ncRNAs)

8.1 Introduction

Transformed malignant cells are the product of a plethora of accumulated genetic mutations. It has been long believed that normal cells are transformed into malignant counterparts due to the accumulation of genetic mutations over time. However, in the early 2000s, it has been found that epigenetic mechanisms are involved in the initial key events of carcinogenesis. Since then, extensive research has been carried out globally to investigate the role of epigenetic involvement in various tumors. Technological advancements due to the development of sophisticated techniques in molecular biology such as polymerase chain reaction and genome sequencing have led to our better understanding of molecular biology. Henceforth, we are getting a better insight into tumor biology, and it is now evident that epigenetic modifications play a pivotal role in tumor development and progression. The role of epigenetics is evident in each phase of cancer, such as initiation and progression, invasion and metastases, response, and resistance to the therapy. The fact that epigenetic modifications do not alter the structure of the DNA opened a new and fascinating avenue for the research of new diagnostic and prognostic markers. Now, epigenetics is one of the most commonly sought and important parts of cancer research for diagnosis, prognosis, and in search of therapeutic targets. After years of immense research on cancer epigenetics, recent advances have indicated that genetic and epigenetic factors are associated with each other at all stages of cancer development to promote cancer progression.

8.2 Epigenetics and Breast Cancer

Likewise, in other cancers, epigenetics has been the area of substantial research in breast cancer as well, and significant advancement has been made in this area deciphering epigenetic modifications in the initiation, development, and progression of breast carcinoma, the role of epigenetic markers in the diagnosis of the breast carcinoma, and response to the treatment. This section of the chapter will elaborate on the various epigenetic mechanisms responsible for the development and progression of breast cancer.

8.3 Epigenetic Mechanisms in the Breast Cancer

Epigenetic modifications involved in breast carcinogenesis include DNA methylation, histone modifications, and RNA-mediated gene editing. These mechanisms modulate various molecular, biological, and cellular pathways which are associated with the various steps of breast carcinogenesis (Dawson and Kouzarides 2012). Recent studies have stipulated the role of deregulation of epigenetic mechanisms with hallmarks of breast cancer such as stemness of cancer cells and resistance to the therapy (Pasculli et al. 2018). Herein, we will explicate molecular mechanisms underpinning the epigenetics of breast cancer, and how these epigenetic modifications play a part in the pathogenesis of breast cancer including genetic reprogramming of tumor-suppressor genes and proto-oncogenes. Figure 8.1 demonstrates the effect of mutations in three types of epigenetics mechanisms involved in breast carcinogenesis.

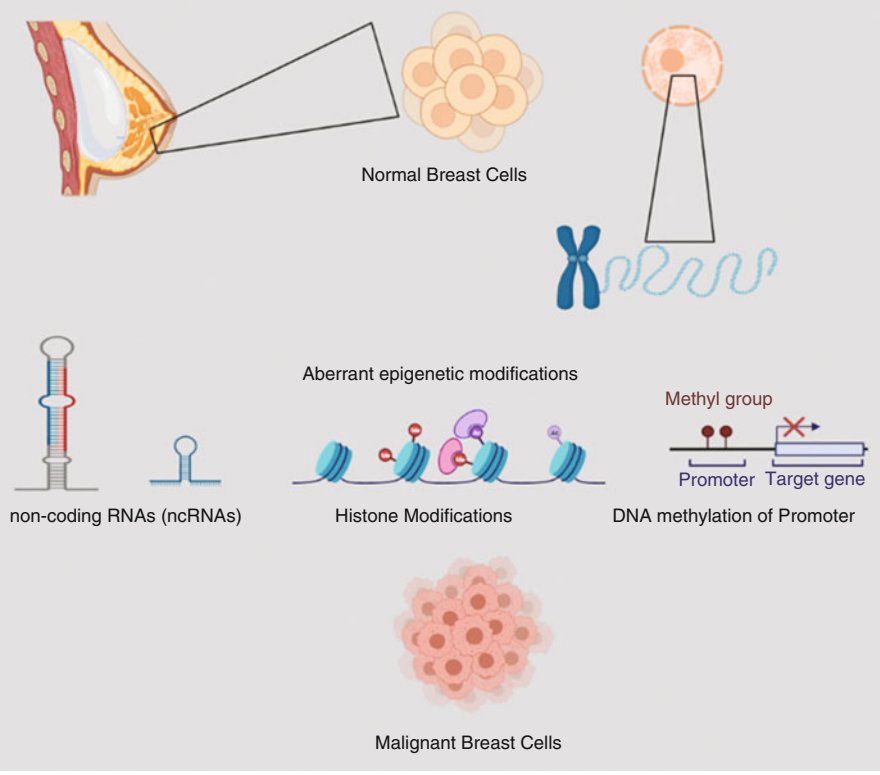

Fig. 8.1 General illustration of normal breast cells transformation into the malignant cells by epigenetic mutations through DNA methylation of promoter regions of the genes, histone modifications, and non-coding RNAs (ncRNAs)

8.3.1 DNA Methylation

To date, the most studied epigenetic modification is DNA methylation; it is a critical, post-replication enzyme-driven chemical modification of DNA bases, involving the covalent addition of the methyl ($-CH_3$) group primarily onto the fifth carbon of the pyrimidine ring of the cytosine base within the CpG dinucleotide (Liu et al. 2015). This covalent addition is mediated by a family of enzymes called DNA methyltransferases (DNMTs) including DNMT3A, DNMT3B, and DNMT1 (Robertson 2005). While DNMT1 is responsible to maintain the already methylated DNA, DNMT3A and DNMT3B mediate the de novo methylation of DNA, targeting the unmethylated or semi-methylated CpG sites (Lyko 2018). DNA methylation regulates several important processes such as transcription, post-transcription, and post-translational processes, chromatin remodeling, imprinting of genome, inactivation of repeating elements of the DNA, and inactivation of X-chromosome (Robertson 2001; Kurihara et al. 2008).

As a result of the DNA methylation, specific gene regulatory proteins are recruited to the DNA, inhibiting the transcription factors from accessing the chromatin, thus influencing the gene expression. Moreover, DNA methylation establishes the restricted and closed form of the chromatin, this restricted modified form of chromatin is not responsive to the nuclease digestion which ultimately results in the decrease of the acetylation of histone proteins on the chromatin (Pasculli et al. 2018). Conversely, the intragenic region of the DNA, which regulates the elongation of transcription and alternative splicing, is reported to have densely methylated sequences (Jones 2012). A CpG dinucleotide is comprised of a cytosine residue preceded by a guanine nucleotide, whereas p indicates the phosphodiester bond between them.

CpG rich regions which are widely known as CpG islands are the continuation of 500–2000 base pairs of CpG dinucleotide which are mainly found in the adjacent region of promoter of the genes, including housekeeping and tissue-specific genes, transcriptions sites, and the repetitive sequences. Even though CpG islands are generally found in 5′-untranslated regions and the first exon of the coding genes, they are present in 3′ region and within the body of the gene as well. Likewise, CpG methylation in promoter regions, CpG islands in typical locations (exogenic regions) are prone to methylation (Nguyen et al. 2001). Generally, these CpG islands are unmethylated, thereby permitting transcription factors to bind and express the genes. However, most of the genome is hypermethylated, which is required for chromosomal stability, compactness, and integrity (Deaton and Bird 2011). Henceforth, in this way, hypermethylation and hypomethylation occur simultaneously genome-wide depending upon the region, i.e., regions of the genome where the genes are located hypomethylation cause loose packaging of the genomic region, thus facilitating the exposure of genomic sequences for the transcription machinery. On the other hand, the same hypermethylation of the genomic region (heterochromatin) causes close packaging of the genome facilitating stability and compactness. In this way, hypomethylation and hypermethylation regulate various processes including disease outcomes together.

The significance of unregulated methylation of genes and regulatory proteins is well-established in many cancers including breast carcinoma. Various endogenous and exogenous mutagenic processes are responsible for aberrant DNA methylation on the CpG islands within the promoter regions, leading to the silencing of various tumor-suppressing genes and expression of several proto-oncogenes. Therefore, carcinogenesis is associated with turning on the methylation of transposable elements and tuning off the methylation of tumor-suppressing genes. Figure 8.2 illustrates that how DNA hypermethylation at the promoters of antitumor genes and hypomethylation at the promoters of oncogenes affect the overall gene expression in breast cancer cells contributing to carcinogenesis.

It is a well-established fact that a vital step in breast carcinogenesis is the epigenetic reprogramming of breast cancer-initiating cells and subclonal evolution, subsequently leading to the clinical and pathological diversity of breast cancer cases (Hur et al. 2014). Stemness is the feature of cancer that is associated with tumor relapse, and breast cancer stems cells (BCSCs) have different methylomes as compared to non-cancerous breast stem cells. (Leick et al. 2012). Moreover, TGF-β signaling is the fundamental epigenetic regulator in driving the differentiation in methylation pattern in breast cancer stem cells and non-cancerous breast cells (Severi et al. 2014).

Furthermore, there is a distinct methylation pattern during the transition of multiple stages of pre-malignancy to carcinoma in situ and invasive carcinoma (Zhang and Xu 2017; Bennett and Licht 2018; Joo et al. 2018; Salas et al. 2020). For instance, there is a difference in the methylation pattern of a promoter of *RASSF1A* and *RARβ* during the transition of pre-malignancy to malignancy (Liu et al. 2016a, b). There is a different pattern of DNA methylation at the promoter region of CDH1, CTNNB1, and APC in breast tumors (ADH, DCIS, and IDC) when compared with healthy tissues (Tang et al. 2016).

Deregulated methylation pattern is evident in various aspects of the pathogenesis of breast cancer including tumor stage, histological grade (Győrffy et al. 2016), metastases, and invasion. For instance, global hypomethylation of the genome may lead to the loss of imprinting which plays an important part in the initial stages of transformation and carcinogenesis. For example, insulin-like growth factor-2 (IGF-2) has an important role in cellular growth, whereas loss of imprinting for IGF2 leads to upregulated growth and genomic instability. Nevertheless, progression to malignancy and genome-wide hypermethylation and hypomethylation of CpG islands stem either from the over activity or mutations of DNMTs (Zhang and Xu 2017).

Studies focusing on the methylation analysis of tumor cells have explored different underlying mechanisms of methylation and how it triggers tumor pathogenesis. As hypomethylation of TRIM27, LDHA, LIMD2, and SEPTIN7 have been associated with proliferation, invasion, and metastates (Salas et al. 2020). On the other hand, RARB, DAPK, APC, and SFN are frequently methylated in breast carcinoma (Tang et al. 2016). In breast cancer, nearly 100 genes have promoter hypermethylation (Robertson 2005) including the genes with crucial roles in significant cellular processes such as DNA repair (BRCA1, GSTP1), cell-cycle regulation

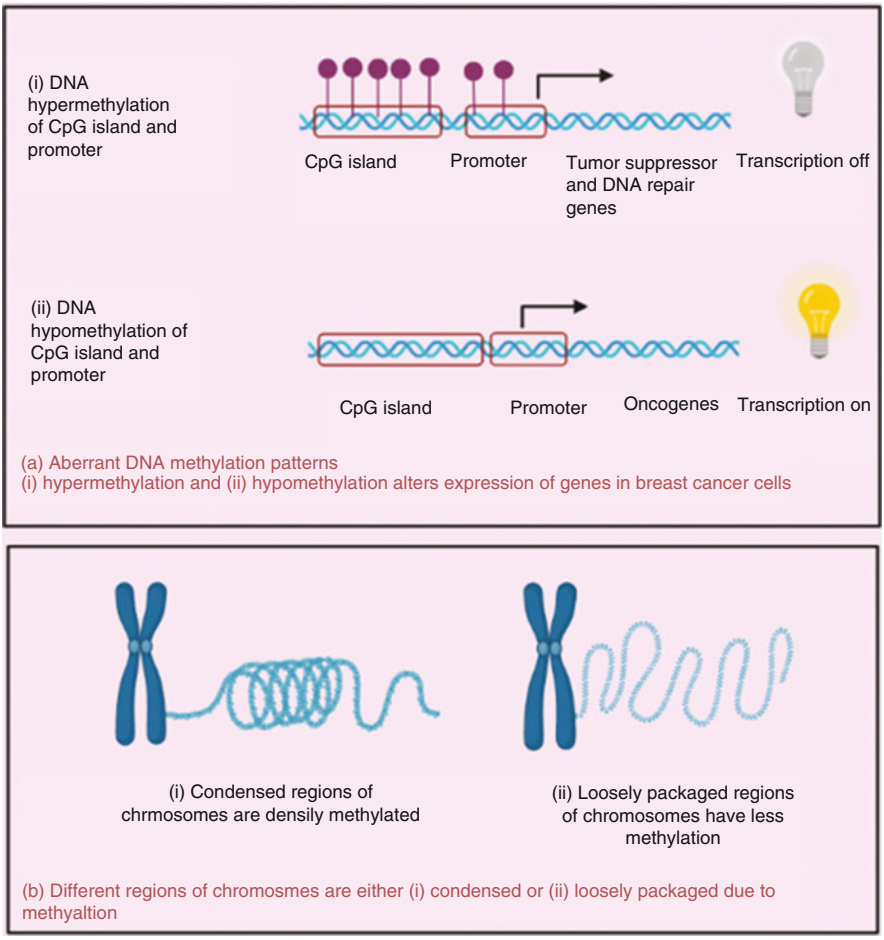

Fig. 8.2 Effects of aberrant DNA methylation in the initiation of breast carcinogenesis. (**a**) Aberrant DNA methylation in CpG island and promoter regions leads to alteration in gene expression contributing to breast carcinogenesis (1) hypermethylation of promoter regions of genes such as tumor-suppressor genes and DNA repair genes turn off their transcription, (2) hypomethylation at the promoter regions of oncogenes turns on their transcription, subsequently modification of these types of genes significantly contributes to the early stages of the breast carcinogenesis. (**b**) (1) condensed and (2) loosely packed region of the chromosomes are affected by the DNA methylation and subsequently regulate the chromatin integrity in breast cancer cells

(CDKN2A, CCND2), tissue invasion, and metastasis (HIN1, TWIST, RARβ, RASSF1A), cell-adhesion (CDH1), regulation of transcription (HOXA5), apoptosis (DAPK, BCL2), hormone-mediated cell signaling (THRβ, ERα, and ERβ). Moreover, one of the common features of the breast tumor includes genome-wide hypomethylation which usually occurs in segmental duplications regions (Lyko 2018). Hypomethylated genes in breast cancer include NAT1, FEN1, MDR1,

IL-10, urokinase, JAGGED1, NOTCH1, Synuclein (Robertson 2001; Kurihara et al. 2008; Jones 2012).

Moreover, one of the hallmarks of the cancer cells is metabolic reprogramming, that has also been modulated by several signaling regulatory circuits which are affected and regulated by abnormal DNA methylation. A co-activator associated arginine methyltransferase cause methylation mediated reprogramming of an important glycolytic enzyme pyruvate kinase M2 (PKM2), thereby enhancing proliferation, energy, migration, and metastasis in breast tumor cells (Liu et al. 2017). Association of DNA methylation and anti-oxidant gene expression had been demonstrated in the development of breast cancer; epigenetic silencing of superoxide dismutase (SOD3) due to methylation of its promoter is linked with aggressive subtypes of breast cancer, triple-negative breast cancer (TNBC), and Her2+ breast cancer (Griess et al. 2020).

Epigenetic regulation is also involved in the chemoresistance of breast cancer cells. (Boettcher et al. 2010). Epigenetic regulatory mechanisms, such as reversible histone modifications and DNA methylations, result in the generation of variable transcript states, giving rise to a heterogeneous population of tumor cells. Subsequently, epigenomes confer survival benefits to the tumor cells due to aberrant transcription of pro-apoptotic factors, DNA repair enzymes, and drug transporters, thereby, rendering cytotoxic and targeted drugs ineffective, and allowing the selection of few drug-resistant tumor cells(Wilting and Dannenberg 2012). The epigenome confers survival benefit to the cancer cells in the presence of drugs in different transcripts of several important genes like drug metabolism. Epigenetic silencing of regulatory genes via hypermethylation leads to the inactivation of genes involved in uncontrolled cellular growth, while hypomethylation gives rise to activation of genes involved in metastasis and drug resistance (Benevolenskaya et al. 2016). For instance, hypermethylation of the promoter of MSH2 causes epigenetic silencing, leading to doxorubicin resistance in breast cancer cells (Ponnusamy et al. 2018), hypermethylation of BRCA1 is associated with ER−ve breast cancer and poor clinical outcomes (Downs and Wang 2015). There is a contrasting relationship between hypermethylation and tamoxifen resistance. Overall, hypermethylated tumors have greater resistance to drugs as compared to their normal tumors (Chang et al. 2005).

8.3.2 Histone Modifications

In eukaryotes, genomic material exists in the form of chromosomes, which are compacted along with proteins, namely histones. Chromatin is made of repeating subunits of nucleosomes, which are composed of an octameric core of duplicate copies of histone proteins, namely H2A, H2B, H3, and H4, the strands of DNA and H1 linker protein are wrapped around this octameric core. Histone proteins are alkaline, whereas the DNA is negatively charged thus they bind together strongly through hydrogen bonding and salt bridges. Alteration in the conformational structure of the chromatin demonstrates the genome into two separate regions, non-dense

and dense/compact regions which greatly influence the gene expression (Müller et al. 2014).

Post-translational modification of histones, involving the addition of chemical groups to the N-terminal tails of histones, ultimately alters the chromatin structure. The charged nature of histone proteins is influenced when other groups such acetyl or methyl are added resulting in the alteration of dense nucleosomes either to be closed or relaxed. Consequently, these modifications of histones have a tremendous effect on DNA packaging ultimately modulating crucial processes such as recombination, repair, replication, and transcription (Wang et al. 2009). The most widely acknowledged of these modifications are acetylation and methylation, regulated by numerous enzymes, for instance, histone acetyltransferases (HATs), deacetylases (HDACs), demethylases (HDMs), and methyltransferases (HMTs), respectively; methylation and acetylation mostly occur in the regions which are close of promoters and enhancers (Swygert and Peterson 2014).

Depending upon which residue in histone is modified, histone acetylation and methylation turn on or turn off the gene expression. For example, trimethylation of lysine 9 (H3K9me3) and lysine 27 (H3K27me3) on histone 3 represses the gene transcription, whereas trimethylation of lysine 4 on histone 3 (H3K4me3) activates the gene expression. It is speculated that the methylation of arginine also activates gene expression.

HATs are considered as ‘‘writer” and HDACs as ‘‘erasers” add or remove acetyl group to the lysine or arginine on the tails of H3 and H4 histones, thereby regulating the packaging of the DNA; acetylation results in the relaxation of chromatin and allowing transcriptional enzymes and other factors to bind to the DNA and perform transcription. In this manner, modification of histones controls the transcription of genes by activating or repressing the gene expression.

Acetylation of the histones on lysine in the promoter region results in the relaxation and opening of chromatin, allowing transcription factors and transcription enzymes to bind, thereby activating the genes (Meeran et al. 2010).

Other histone modifications include ubiquitination, phosphorylation, ADP ribosylation, deamination, citrullination, propionylation, formylation, butyrylation, O-GlcNAcylation, proline isomerization, and crotonylation (Tweedie-Cullen et al. 2012). Figure 8.3 depicts how histone modifications mainly acetylation and methylation regulate the chromatin packaging and gene expression of antitumor genes and oncogenes.

Studies have found out that histone acetylation has a significant role in breast carcinogenesis. Aberrant histone acetylation has been associated with gene expression modulation and reprogramming involved in metabolic reprogramming, stemness, breast cancer pathogenesis, and resistance to drugs. Accumulation of acetylated mitochondrial reactive oxygen species (mtROS) and mitochondrial superoxide dismutase 2 (SOD2) results in the promotion of hypoxia signaling of hypoxia-induced factor 2 alpha (HIF-2α), enhancing the tumor stemness. Expression of 1A histone methyl eraser, lysine-specific demethylase 1 (LSD1) is higher in breast cancer, acts on H3K4 and H3K9 (Gomez-Moreno 2009; Lim et al. 2010); furthermore, high expression of LSD1 is associated directly with the breast cancer

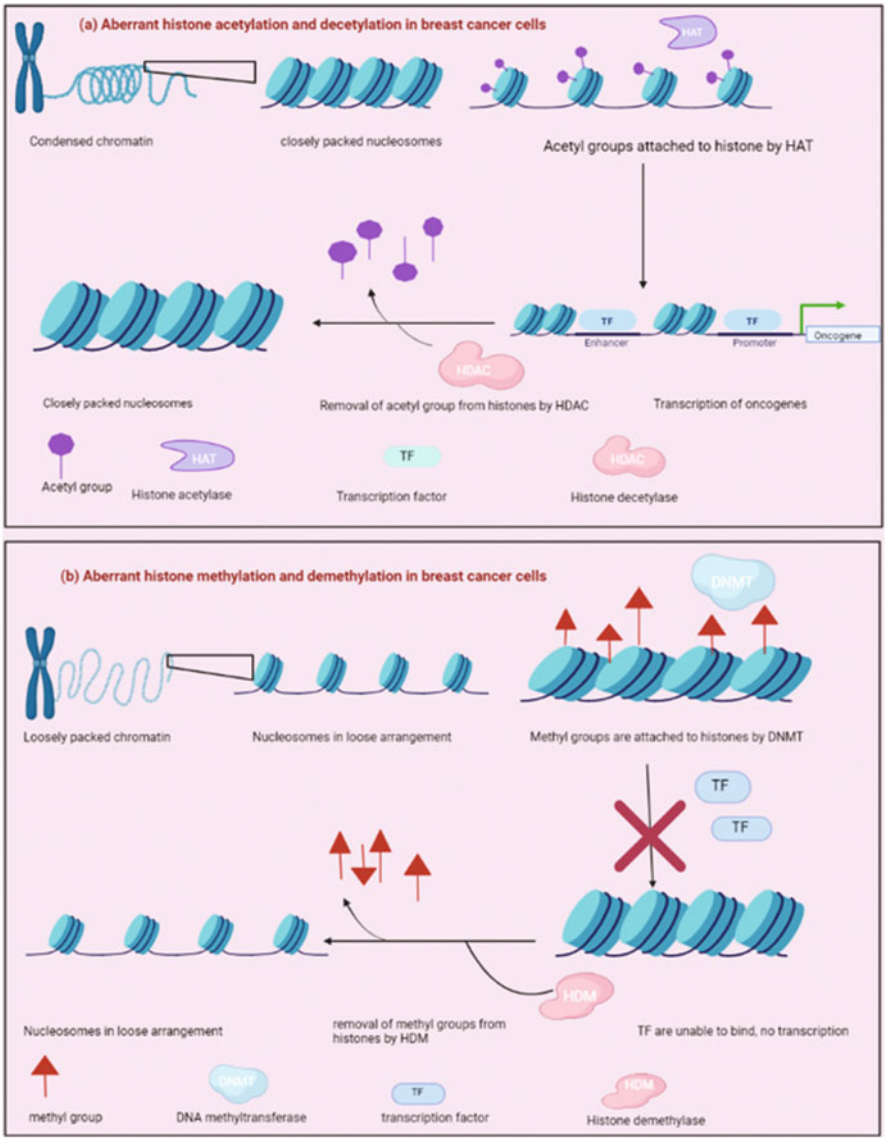

Fig. 8.3 Aberrant histone modifications in breast cancer cells; (**a**) acetylation of histone loosens the binding of DNA and nucleosomes thereby allowing to expose the promoter region of oncogenes and allowing TF to bind and transcribe oncogenes, while deacetylation causes histones and DNA to bind tightly. (**b**) methylation of histone causes the dense packaging of nucleosomes, thereby hindering of TFs to bind the promoter region of antitumor genes and subsequently turning off their expression, while demethylation causes these nucleosomes to be in loose packaging

progression (Lim et al. 2010; Serce et al. 2012). Likewise, enhancer of zest homolog 2 (EZH2), an HMT, which transfers a methyl group to the lysine 27, is overexpressed in many tumors including breast tumors; EZH2 is associated with more aggressive subtypes of breast carcinoma. Decrease in CAF1 (histone chaperone proteins) associated histone mark, H3K27me3, results in the reduction in the expression of EZH2 and consequently upregulating the expression of thrombospondin type 1 motif 1. This CAF1 mediated reduction of thrombospondin is associated with tumor invasiveness.

In a recent finding, it has been reported that interaction of bi- or trimethylated lysine 4 of histone 3 with Pygopus 2 (Pygo2) which is a co-activator of Wnt/β-catenin signaling is crucial for breast cancer development and metastasis, therefore inhibiting this interaction could be the potential therapeutic option for the breast cancer management (Saxena et al. 2020). Moreover, epigenetic modifications due to aberrant expression of LSD1 are associated with reprogramming the stemness features of breast cancer. KDM7A, a histone deacetylase is crucial for the growth and maintenance of breast cancer stem cells by the upregulation of BCL2, C-MYC, and KLF4 which are related to stemness (Meng et al. 2020).

Epithelial to mesenchymal transition (EMT) crucial for tumor metastasis and drug resistance is also affected by the epigenetic reprogramming via modulation of HDACs, TET hydroxylase together with Mbd3/NuRD complex subsequently make the cells in a state of highly mesenchymal metastasis, thereby indicating that combinatorial interference might prove to be efficient in suppressing the metastasis of the breast cancer (Kilinc et al. 2020). Increased stabilization of SIRT1, histone deacetylase leads to the increased expression of nicotinamide methyltransferase (NMT) which are involved in drug resistance in breast cancer, inhibition of SIRT1 may help to overcome the resistance against paclitaxel and Adriamycin(Wang et al. 2019). Moreover, a transcriptional co-activator of BRCA1, p300/CBP (CREB binding protein) facilitates the cross-talk between NF-κB and ER signaling pathways (Nettles et al. 2008). Furthermore, it also induces EMT epigenetically in breast cancer metastasis by associating with the DOT1L-cMyc complex. Breast cancer cells accumulate stem cell-like features by the increased levels of p300/DOT1L-c-Myc (Pao et al. 2000).

8.3.3 Non-coding RNAs (ncRNAs)

It is a well-known fact that a large part of the eukaryotic genome is transcribed but not translated; and only 2–3% of the genome is coding for the proteins, while 80% is genome codes for non-coding RNAs, ncRNAs (Barata et al. 2016; Chen et al. 2016). Based upon their size, ncRNAs can be classified into two broad categories: small ncRNAs, varying size range less than 200 nucleotides; while ncRNAs with a size greater than 200 nucleotides (>200 nts–100 kb) are categorized as lncRNAs. Based on the functions, ncRNAs are classified as transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), regulatory molecules like small-interfering RNA (siRNAs), micro RNAs (miRNAs miRs),

piwi-associated RNAs (piRNAs), and lncRNAs (ENCODE Project Consortium 2012; Djebali et al. 2012).

In carcinogenesis, miRNAs and lncRNAs act as tumor-suppressor genes and oncogenes as they might be found downregulated or upregulated. In this respect, in cancer cells, global downregulation of miRNAs is found as compared to healthy tissues (Di Leva et al. 2014), whereas as an expression of lncRNAs is found to be upregulated overall as compared to their low expression in normal physiological conditions (Chandra Gupta and Nandan Tripathi 2017). Particularly, the expression of ncRNAs in breast cancer is dysregulated owing to the aberrant epigenetic modulation of their gene promoters. In breast cancer, mostly abnormal methylation is found in the promoter regions of ncRNAs as compared to protein-coding genes, especially promoters of lincRNA are the most methylated ones. Five distinguished patterns of aberrant methylation of promoters of ncRNAs have been identified in cancers, including methylation, not on CpG islands, but also the flanking sites of the CpG islands, and CpG lacking promoters as well (Li et al. 2015). There is a correlation between ncRNA promoter methylation and transcriptional regulation that subsequently alters the key cellular signaling pathways like the MAPK signaling pathway(Pasculli et al. 2018).

We will discuss first the lncRNAs; lncRNAs constitute the largest class of ncRNAs, including enhancer ncRNAs (miRNAs), natural antisense transcripts (NATs), long intergenic RNAs (lincRNAs), and others. Similar to protein-coding mRNA, RNA polymerase II undergoes co- and post-transcriptional processing (Guttman and Rinn 2012). They can fold in complex secondary and tertiary structures corresponding to their functions and interactions with different types of molecules such as proteins, DNA, and RNA; henceforth, they participate in multiple regulation networks constituted by transcriptional and post-transcriptional gene regulation, chromatin remodeling, alternative splicing, and molecular decoys for miRNAs (Cheetham et al. 2013). The idea that ncRNAs are categorized as epigenetic regulators comes from the aforementioned fact that they modulate the gene expression without altering the DNA sequences.

The mechanisms by which these lncRNAs are involved in carcinogenesis appear to be diverse and well-organized (Huarte 2015). lncRNAs are differentially expressed in breast tumor cells and normal mammary cells reinforcing their roles in breast carcinogenesis (Zhao et al. 2014). The first lncRNA reported to be involved in breast carcinogenesis was HOTAIR (HOX antisense intergenic RNA), where it has high levels of expression as compared to nearby tissues (Gupta et al. 2010). Particularly, this lncRNA is involved in the transcriptional control of HOXD10, which is the target of metastasis promoting miR-200b, thereby promoting metastasis in breast cancer patients (Ma 2010a, b). HOTAIR is engaged in chromatin reprogramming, causing the occupancy of polycomb repressive complex-2 on promoters of the genes which are involved in the inhibition of tumor progression, including HOXD10 (Rinn et al. 2007; Gupta et al. 2010). Likewise, in primary breast cancer, there is upregulation of metastasis-associated lung adenocarcinoma transcript 1, (MALAT1), its levels are further elevated in corresponding metastasis (Arun et al. 2016). MALAT1 is the co-transcriptional splicing scaffold that works

together for the transcription and splicing of pro-tumorigenic genes such as integrins, ECM proteins, and other genes which are responsible for metastasis and tumor migration throughout the tumor progression. Moreover, alternatively spliced variant of MALAT1 can be differentially expressed about a full-length transcript and independent prognostic marker for median-free survival (MFS) (Meseure et al. 2016), indicating the underpinning complexity of regulatory mechanisms of lncRNAs to modulate the cancer phenotype. Recently, a study reported that in a luminal subtype of breast cancer, six lncRNAs markers notably increased its prognosis(Chen et al. 2020). The lncRNA DANCR, (differentiation antagonizing non-protein coding RNA), has an important part in the inflammatory breast cancer-associated phenomena: EMT-mediated inflammation and stemness in late-stage triple-negative breast cancer; DANCR also downregulates SOC3 with the help of epigenetic mechanisms of EZH2 (Zhang et al. 2020), and lncRNA cancer susceptibility candidate 9 (CASC9) binds to EZH2 and control MD2 gene leading to drug resistance in breast cancer(Zhang et al. 2020).

miRNAs are highly conserved small RNAs that modulate the gene expression by the inhibition of translation and degradation of protein-coding mRNA, by the imperfect base pairing to complementary sequences on the target mRNA (MREs; miRNAs response elements). Breast cancer is one of the tumors in which dysregulation of miRNA was reported by the comparison of 76 breast tumor tissues with 10 normal mammary tissue through microarray (Guttman and Rinn 2012). In this pioneering study, some miRNAs were upregulated, while others were downregulated in breast tumor tissues in comparison with the healthy tissue indicating that each miRNA has a different role in the pathogenesis of breast cancer. Table 8.1 summarizes the significance of deregulation of miRNA and its effects on cancer pathogenesis (Pasculli et al. 2018).

8.4 Epigenetics as a Diagnostic Tool for Breast Cancer

Breast cancer is a diverse disease and despite many efforts to develop tools for better diagnosis, treatment, and control of the disease, a rise has been witnessed in its prevalence globally; being the most prevalent cancer worldwide, its morbidity and mortality rates have been raised to 20% and 14%, respectively, during the last decade (Bray et al. 2018). Better treatment outcomes of breast cancer are linked with the earlier diagnosis of tumor, driving the scientists to make enormous efforts constantly in search of better diagnostic markers and diagnostic methods to detect breast cancer at the earlier stage.

Timely diagnosis of breast cancer relies mainly on mammography, which is the gold standard for breast cancer screening, nevertheless, it has certain shortcomings, such as the lack of sensitivity and specificity, and over-diagnosis, or misdiagnosis due to its inability to diagnose tumors less than 1 cm (Zubor et al. 2019). Moreover, it is not a good option for women with dense breasts and young girls (Moss 2004; Qaseem et al. 2007).

Table 8.1 Deregulated miRNAs in breast cancer, their expression in cancer, targets, and molecular effects

miRNA	Expression in breast cancer	Targets	Molecular effects	The outcome in tumor pathogenesis	References
miR-205	Decreased	ZEB1/ZEB2, VEGF-A, HER3	Activation of HER2 mitogenic pathway	Activating metastasis and invasion	Iorio et al. (2009)
miR-10b	Increased	HOXD10, Neurofibromin MT1-MMP KLF4	Activation of RhoC, urokinase plasminogen activator receptor, MT1-MMP, α3-integrin	Activating invasion and metastasis	Ma et al. (2007), Ma (2010a, b)
miR-155	Increased	RHOA, SOCS1C, SOCS1C/ EBPβ	Induction of STAT3 mediated inflammatory signaling SOCS1. Induction of HK2	Tumor-promoting inflammation, dysregulation of cellular energetics	Volinia et al. (2006), Jiang et al. (2010), Jin et al. (2010), Jiang et al. (2012)
miR-21	Increased	Bcl2, PDCD4, TIMP3, TPM1	Blocking apoptosis and activation of cyclin-dependent kinase inhibitors (e.g., p21), and dysregulating anchorage-independent growth	Evasion from growth suppressors, resistance to cell death, activation of invasion and metastasis	Si et al. (2007), Zhu et al. (2007), Frankel et al. (2008)
miR-200 family	Decreased	FOG2, ZEB1/ ZEB2, fibronectin, moesin, BMI-1	Loss of epithelial marker (e.g., E-cadherin)	Activation of invasion and metastasis	Gregory et al. (2008), Park et al. (2008), Tryndyak et al. (2010), Gregory et al. (2011)
miR-26b, miR-107	Decreased	CDK8	E-cadherin expression inhibition. Activating invasion and metastasis	Activating invasion and metastasis. Sustaining proliferative signaling	Li et al. (2014a, b)

Better diagnostic markers are crucial not only for the accurate and earlier diagnosis of breast cancer but also for better clinical management of the disease, as the right diagnosis at the early stage would increase the possibility for a right choice of treatment modalities, hence enhancing the likelihood to achieve better prognosis and overall survival rate. On the contrary, the wrong diagnosis would mislead the treatment and affect the disease course.

Currently, early and accurate diagnosis of breast cancer is the biggest challenge for the successful management of the disease and unfortunately hampering the efforts for decreasing the prevalence of breast cancer. This necessitates the development of efficacious and robust diagnostic tools for breast cancer screening, diagnosis, and monitoring the progression of the disease during and after the treatment (Xiang et al. 2013).

This section of the chapter will provide a detailed account of the significance of epigenetics for the breast cancer diagnosis.

A biomarker is defined as the quantifiable biochemical entity found in body tissues, body fluids, and blood as a response to disease development and progression. The protein, RNA, DNA, and epigenetic markers are included as a biomarker. A specific tumor biomarker stipulates the presence of the disease, helps to monitor the prognosis, and could guide the treatment options (Costa-Pinheiro et al. 2015).

Breast cancer is a diverse disease, and carcinogenesis is an intricate process that involves the interconnection of multiple genetic and epigenetic factors regulating the different stages of breast carcinoma. Owing to their crucial role in breast carcinogenesis, genetic and epigenetic markers are of key importance for diagnostic markers development; therefore, a lot of research has been focused to unravel the details of epigenetic mechanisms, and the extent of their influence on induction and modulation of tumorigenesis, to decipher the potential of the epigenetic marker as a diagnostic marker.

8.4.1 DNA Methylation as Potential Diagnostic Biomarker

It is a well-established fact that aberrant histone modification and DNA methylation at the promoter region of genes are responsible for gene silencing of various genes engaged in distinct processes such as (DNA damage repair, and cell-cycle inhibitors, pro-apoptotic proteins), thereby inducing the initial events of carcinogenesis. As DNA methylation is extensively involved in modulating the expression of various genes, it is regarded as an attractive tool for the diagnosis of breast cancer. Moreover, liquid biopsies, it is also an attractive option for diagnosis due to the factors such as its cancer specificity, early-onset, biological stability, and bioavailability in body fluids.

Furthermore, as DNA has greater stability than RNA and protein; it is highly stable and can be detected in circulating cell-free tumor DNA (ccfDNA) from liquid biopsies, increasing the probability of implementation of DNA methylation as a reliable, fast, robust, noninvasive, and cost-effective tool for the diagnosis of breast cancer. The release of cell-free DNA from tumor cells and its circulation in plasma

was first found during the analysis of mutations in *P53* and *KRAS* (Yamada et al. 1998; Jackson et al. 2001). Even though there is variation, levels of circulating cell-free DNA are greater in the plasma of cancer patient in comparison with healthy individuals. Generally, a healthy individual's plasma has 50 ng/ ml of DNA, while in cancer patients it ranges from 0 to 1 μg/mL. In healthy individuals, increased circulating cell-free DNA is due to inflammation, exercise, and tissue injury, while in cancer patients levels of cell-free DNA are increased due to the necrosis and apoptosis of the tumor cells. While analyzing cell-free DNA for mutations, it was also found that ccDNA contains a DNA methylation pattern similar to that of primary tumors, indicating its potential to utilize as a blood-based diagnostic marker for tumor detection (Gormally et al. 2007; Breitbach et al. 2012; Haber and Velculescu 2014).

It had been found out that selected markers such as RARβ2 and RASSF1A have DNA hypermethylation during the early stage of breast carcinogenesis, implying the potential use of plasma ccDNA as an early-stage diagnostic marker of cancer. Various studies focusing on the DNA methylation pattern of more frequently used genes such as CDH1, RASSF1A, APC, BRCA1, GSTP1, RARβ, and others (Van De Voorde et al. 2012; Ma et al. 2013; Schricker et al. 2018)have shown consistent results. Nevertheless, owing to the heterogeneity of breast carcinoma, analyzing DNA methylation pattern of the single gene often generates results with low sensitivity, and may be associated with specific subtypes; therefore, a panel of epigenetic markers would be useful for detecting cancer at the early stage with higher sensitivity and specificity (Van De Voorde et al. 2012). Table 8.2 summarizes the data of studies that were conducted to analyze the importance of DNA methylation of selected genes as potential diagnostic biomarkers for the early breast cancer diagnosis (Sher et al. 2020).

Another considerable factor while analyzing DNA methylation for cancer diagnosis is the effect of treatment on the DNA methylation which could result in the wrong inference. Therefore, studies were carried out to take a blood sample for DNA methylation analysis before the diagnosis of cancer, and it was found out that DNA methylation was detectable before the clinical diagnosis of breast cancer, henceforth abnormal DNA methylation profile has the potential to serve as a valuable diagnostic biomarker.

The prominent potential of epigenetic biomarkers from blood would add the benefit to breast cancer screening along with mammography and MRI, taking into account that markers are specific and sensitive. According to the guidelines of the National Comprehensive Cancer Network (NCCN) (Network 2017) and National Cancer Institute (NCI) (PDQ Supportive and Palliative Care Editorial Board 2015), women who are at greater risk of breast cancer need to be screened for breast cancer screening at the early age of 25 years. Although criteria to define the high-risk group vary among the guidelines, it includes BRCA1 and BRCA2 carrier, 5-year risk of greater than 1%, and lifetime risk greater than 20% depending upon family history. It indicates that women who are *BRCA1* and *BRCA2* mutations carriers, and due to this having lower DNA repair capability, might have about 15–-20 mammograms by the age of 40 years. However, if sensitive and efficient plasma epigenetic biomarkers

Table 8.2 The most studied potential epigenetic markers in blood as potential diagnostic markers for breast cancer

S. No.	Epigenetic marker	Category	Sensitivity %	Specificity %	Role in detection	Sample	References
1	SFN, P16, hMLH1, HOXD13, PCDHGB7, & RASSF1A	Panel markers	82.4	78.1	Detection and monitoring of breast cancer patients	Serum	Shan et al. (2016)
2	RAR*β*, RASSF1A	Panel markers	94.1	88.8	Invasive and in situ ductal breast cancer	Serum	Kim et al. (2010)
3	APC, FOXA1, & RASSF1A	Panel markers	81.82	76.92	Detection and monitoring of breast cancer patients	Plasma	Salta et al. (2018)
4	ALU247	Single marker	>99	88	Metastatic breast cancer	Plasma	Agostini et al. (2012)
5	FHIT	Single marker	93.4	95.4	Early ductal breast carcinoma	Serum	Liu et al. (2015)
6	APC, RAR*β*	Single marker	95.6	92.4	Invasive TNBC	Serum	Swellam et al. (2015)
7	HYAL2	Single marker	64	90	Early stage of breast carcinoma	Peripheral blood (leukocytes)	Yang et al. (2015)

would be coupled with MRI for breast cancer screening, it will save this high-risk group from exposure to higher radiation doses. Nonetheless, for blood-based epigenetic diagnostic markers to be included in screening for cancer, a cohort of prospective studies is needed. These studies would analyze the panel of epigenetic markers from the different types of samples collected so that accurate and precise evaluation could be made, and only specific markers would be selected (Duffy et al. 2013; Siegal et al. 2014). Henceforth, adding useful blood-based epigenetic markers would help to make the findings of mammography more valuable and decrease the chances of false positives, it would be greatly effective to help to reduce breast cancer-associated mortality.

A critical link was found between the methylation status of *RASSF1A* and breast cancer development, whereas a strong association was found between the *BRCA2* mutations and hypermethylation of *CDKN2A*. However, to evaluate the significance of DL as a noninvasive and promising tool for the detection and diagnosis of breast cancer, further prospective epidemiological studies are required with a large panel of more genes (Antill et al. 2010).

Similarities between the DNA methylation pattern of primary breast cancer tissues and sample from plasma have been reported, indicating the potential of the use of methylation status from blood in the diagnosis of breast cancer.

To conclude, there are multiple reasons which prompt the significance of DNA methylation as a promising diagnostic biomarker. These reasons include:

1. Its prevalence in tumorigenesis, as mentioned earlier, is the most frequent epigenetic mutation for cancer initiation and development, abnormal hypermethylation of approximately 600–1000 genes per tumor has been reported (Ushijima and Asada 2010).
2. DNA hypermethylation is involved in the early phase of carcinogenesis, therefore, it is of great importance for the early diagnosis of breast cancer.
3. DNA methylation is shown by the tissues surrounding the tumor as well (Fabian et al. 2005; Yan et al. 2006; Radpour et al. 2011; Wong et al. 2011).
4. It is stable, making its detection relatively easy and less laborious, and a small sample is required than other methods of detecting mutations such as gene expression profiling (Lo 2000).
5. DNA hypermethylation produces strong positive signals as compared to the unmethylated background, making its detection easier as compared to other methods of genetic mutation analysis such as loss of heterozygosity (Herman and Baylin 2003).
6. It can be detected from the plasma with a sensitivity of greater than 90%.

So far, DNA methylation markers are not included for a breast cancer diagnosis; however, utilizing the prognostic value of DNA methylation, PCR-based prognostic methods for breast cancer have been developed. In 2018, Therawis and Qiagen developed *therascreen* ®PITX2 RGQ, which is the first validated clinical test for DNA methylation. In Europe, this test is available for the prediction of the response of a specific high-risk group of breast cancer patients (HER2−, ER+, and LN+) to

anthracycline-based chemotherapy with or without hormonal therapy (Schricker et al. 2018). Apart from this, in the USA a validated DNA-methylation-based test, Ivy Gene is available, which is used for the diagnosis of early stage of four tumors (colon, lung, liver, and breast). This assay is based on a panel of 46 makers, thus can quantify the existence of these aforementioned cancers in blood samples of suspected cancer patients.

Although there is convincing evidence of DNA methylation as a promising diagnostic biomarker, it is crucial to do evaluation and quantification of potential gains of its use for the early breast cancer diagnosis in suspected or asymptomatic and healthy individuals. For this purpose, large prospective epidemiological studies are required which may investigate the effect of DNA methylation markers for prediction of the disease incidence and overall survival (OS) after diagnosis.

8.4.2 Non-coding RNAs as Potential Diagnostic Biomarker

Just like ccDNA, many studies have focused on the potential of circulating ncRNA in blood for the diagnosis of breast cancer, and it has been found out that alongside ccDNA, circulating non-coding RNAs, especially miRNAs possess the stability to be utilized as diagnostic biomarkers for the breast cancer from the body fluids (Tong and Lo 2006; Bird 2007; Wang et al. 2010; Schwarzenbach et al. 2014; Xu et al. 2015; Wang et al. 2016). For example, the first study in this regard has reported that expression of let-7 and miR-195 was significantly higher in breast cancer than healthy individuals (control), and this expression of these circulating ncRNAs diminished after the surgical removal of the tumor(Heneghan et al. 2010). Furthermore, elevated levels of circulating miR-21 and miR-10b were linked with ER-negative status of breast cancer, while the association of PR positive status of breast carcinoma was found to be associated with the higher expression level of miR-155 (Zhu et al. 2009), miR-155 upregulation in breast cancer patients sera has been validated by other studies as well (Roth et al. 2010; Wang et al. 2010). Interestingly, miR-155 expression was distinguishable between metastatic and primary tumor patients from the healthy controls, while miR10b and miR34a expression were able to distinguish expressed between metastatic and healthy controls. Furthermore, several studies have demonstrated a difference in expression levels of circulating miR-181a-5p in healthy and diseased individuals, breast cancer patients have lower expression of circulating miR-181a-5p, representing it as a promising and reliable biomarker candidate for the breast cancer diagnosis (McDermott et al. 2014). Large cohorts of breast cancer patients have also confirmed the circulating miRNAs signature as a potential biomarker to help diagnose breast cancer (Chan et al. 2013). Moreover, various studies reporting lncRNAs can be detected from the body fluids, gained the attention of oncology researchers searching for cancer diagnostic biomarkers. In this regard, a study had found that expression levels of circulatory lncRNA in serum RP-11445H22.4 were markedly higher in breast cancer patients, identifying breast cancer cases with 74% specificity and 92% sensitivity (Xu et al. 2015). Interestingly, a strong association has been found between the

expression levels of lncRNA prostate cancer gene 3 (PCA3) to prostate cancer, and it became the first lncRNA to be included in the routine clinical practice for identifying prostate cancer from urine samples, (Progensa PCA3 urine test), thus avoiding the invasive tumor biopsies (De La Taille 2007). This provides evidence that ncRNAs hold the promising potential to be utilized as reliable biomarkers for cancer diagnosis, management, and care of cancer patients.

Lastly, keeping in view the compelling evidence of epigenetic markers as potential biomarkers through enormous studies, and constant efforts that are being made to unleash the potential of epigenetic biomarkers in diagnosing cancer, we may anticipate that epigenetic markers (DNA methylation and ncRNAs) hold the promising future of noninvasive diagnosis of breast cancer.

8.5 Epigenetics as Predictive Biomarkers

In early-stage breast cancer, mastectomy followed by radiotherapy represents the standard of care (Coates et al. 2015a, b; Harris et al. 2016; Gradishar et al. 2017); decisions to give patients adjuvant systemic therapy depend upon the clinical and pathological characteristics of the breast tumor. Not provided with adjuvant therapy, 12–58% of breast cancer patients face the relapse of cancer within 5 years (Fisher et al. 1997, Early Breast Cancer Trialists' Collaborative Group (EBCTCG) 2005, Peto et al. 2012); therefore, adjuvant therapy has opted for most of the breast cancer patients.

ER, +ve breast cancer patients receive hormonal therapy, and patients with HER2-*neu* amplified status receive anti-HER2 treatments as a standard of care. In locally advanced tumors, systemic therapy comprising hormonal therapy, chemotherapy, and anti-HER2neu therapy is used to reduce tumor mass before surgery. Taxanes and anthracyclines are included in the most commonly used chemotherapy regimen of breast cancer patients (Coates et al. 2015a, b; Senkus et al. 2015; Gradishar et al. 2017).

8.5.1 DNA Methylation as Predictive Markers

Studies analyzing global methylation have been conducted on large scale to evaluate the association of global methylation differences with different subtypes of breast cancer and clinical characteristics of the tumor. Findings from such studies have greatly helped in the better understanding of molecular mechanisms underpinning the behavior and characteristics of cancer and hold significant potential to identify the patients who can get an advantage from the better treatment modalities for precision medicine.

Currently, for routine clinical practice, the standard predictive and prognostic biomarkers are established on the recommendations of the multi-disciplinary panels and include assessment of PgR, Ki67 immunoreactivity HER2, and ER, along with clinicopathological variables such as tumor size, grade, and status of lymph nodes

(Coates et al. 2015a, b; Senkus et al. 2015; Harris et al. 2016; Giuliano et al. 2017; Gradishar et al. 2017). In a study of profiling of 200 receptor-positive tumors, 10 genes were identified to be associated with resistance to hormonal therapies (Martens et al. 2005; Harbeck et al. 2008). Methylation of PIXT2 has been associated with ER-positive, lymph node-negative patients receiving hormonal therapy only (Harbeck et al. 2008; Nimmrich et al. 2008). Furthermore, promoter methylation of PITX2 had been linked with poor patient outcomes in lymph node-positive, ER-positive, and HER-2/neu negative patients who are receiving anthracycline-based chemotherapy treatments (Marie-Hélene Quentien et al. 2011). Although the role of *PITX2* in breast cancer is unclear, as it is engaged in the basal and hormone-regulated activity of prolactin, henceforth, when the expression of *PIXT2* is decreased due to promoter methylation, it might influence levels of the prolactin within breast tissues and affect the sensitivity of the cells for the anti-estrogen therapies(Marie-Hélene Quentien et al. 2011; Kavarthapu et al. 2014) Although specific monoclonal antibodies are effective for the successful treatment of HER2-positive breast cancers, resistance to therapy develops in 30% of breast cancer. A high frequency of *HSD17B4*, *CDH13,* and PGR methylation has been involved in breast cancer (Fiegl et al. 2006). A link between promoter hypermethylation of *HSD17B4* and complete response (CR) to anti-Her2 treatment has been reported.

BRCA1 DNA methylation is correlated with chemosensitivity in TNBC. Germline *BRCA1* mutations are associated with 2% of overall 10–55% of familial breast cancers. However, in 13–40% of sporadic breast cancers, promoter hypermethylation of *BRCA1* has been found (Esteller et al. 2000; Xu et al. 2013; Cai et al. 2016). *BRCA 1* is a useful biomarker of response to the chemotherapy in TNBC patients; as per the study reported, for subgroup of 167 TNBC patients, who were given adjuvant chemotherapy, methylated BRCA1 was an independent favorable predictive biomarker for 10 years disease-free survival in multivariable analysis. Contrastingly, in another subgroup of patients, who had TNBC and were given adjuvant chemotherapy, methylated BRCA 1 was a predictive marker of worse survival in the univariable analysis(Xu et al. 2013); thereby, compelling the importance of BRCA 1 methylation as a predictive biomarker for the chemotherapy sensitivity in TNBC. Pre-clinical studies on ovarian and breast cancer clinical trials had illustrated that Poly (adenosine- diphosphate)-ribose polymerase (PARP) displays efficient inhibition in *BRCA2* and *BRCA1* mutant breast cancer patients (Ledermann et al. 2016; Robson et al. 2017). As *BRCA1* promoter hypermethylated tumors display a similar molecular phenotype as BRCA mutated cancers (Lord and Ashworth 2016), it has been postulated that hypermethylated *BRCA1* may predict the response to PARP inhibitors, it has been proven by an in vitro study; however, no clinical data is supporting the role of methylation of *BRCA1* methylation for the prediction of response to PARP inhibitors in non-*BRCA* mutated cancers.

Taxanes in chemotherapy regimens improve the clinical outcomes in breast cancer patients (Van Poznak et al. 2015; Cardoso et al. 2017). In a cohort including 102 patients, it was reported that methylation of the KEAP gene promoter is associated with better Overall Survival (OS) in the patients' in univariable and

multivariable analysis, this group received sequential therapy with taxanes preceded by cyclophosphamides and anthracyclines (Barbano et al. 2013).

8.5.2 Histone Modifications as Predictive Biomarkers

The correlation between histone modifications and resistance to anthracyclines had been evaluated by utilizing a panel of breast cancer cell lines representing its different subtypes. In all the epirubicin-resistant cell lines, H2 and H3 histones were upregulated as compared to their epirubicin-sensitive cell lines. Furthermore, epirubicin resistance could be over-turned by treating with small molecules histone deacetylase inhibitors. Moreover, in an adjuvant clinical trial, histone modification was associated with anthracyclines resistance by utilizing gene expression analyses. Specifically, patients who had low expression of 18 genes histone module got more benefit from the treatment with anthracycline than patients with high expression (Braunstein et al. 2016).

An integrative approach was performed to identify the genes responsible for proliferative response to the estrogen E2, this approach included analysis of gene expression, whole-genome sequencing of transcription binding sites, and chromatin immunoprecipitation. It was identified that E2 inducible histone variant H2A.B was greatly correlated with worse overall prognosis and lymph node metastasis (Hua et al. 2008). The serine 139 phosphorylation of H2AX leading to its constitutive activation is associated with BRCA1 mutations, TNBC, or basal-like phenotype in cancer cell lines; it is linked with worse prognosis in lymph node-negative and TNMC patients in multivariate analysis. Phosphorylation at serine 19 at H2AX is one of the earliest events upon DNA damage, leading to the DNA Double Strands Break (DSB) repair system. Therefore, constitutive activation of H2AX in TNBC and basal-like breast carcinoma is anticipated to govern the sensitivity to the conventional radio and chemotherapy (Nagelkerke et al. 2011).

HDACs have a controversial role as a predictive biomarker in breast cancer. Higher expression of HDAC1 significantly predicted better disease-free survival (DFS) in the multivariable and univariable analysis (Gao et al. 2015). However, another study did not observe any association between increased levels of HDAC1 and HDAC2 and prognosis (Müller et al. 2013). Moreover, increased HDAC6 is linked with small tumors, hormone receptor-positive status, and low histological grade. Consequently, patients who had higher expression levels of HDAC6 mRNA and protein were associated with better DFS in comparison with patients with low expression levels of HDAC6 mRNA and protein (Zhang et al. 2004).

8.5.3 ncRNAs as Potential Predictive Biomarkers

Lastly, studies have been carried out to assess the significance of ncRNAs as predictive biomarkers, but only a few ncRNAs have shown the association with response to the therapy by a large cohort of patients. For instance, in a small cohort

of breast cancer patients, higher miR-21 has been associated with increased MFS (Tavazoie et al. 2008), and with increased relapse-free survival [RFS] in the patients with tamoxifen-treated patients (Jansen et al. 2012), might be due to the interrelated targeting of pro-apoptotic genes *MERTK, PITPNC1*, and *IGFBP2* (Khew-Goodall and Goodall 2012; Png et al. 2012). Moreover, mi*R*-30c and mi*R*-342 are correlated with decreased distant relapse in breast cancer patients with ER-negative status (Jansen et al. 2012), whereas both are correlated with clinical advantage in the patients who were treated with tamoxifen(Rodríguez-González et al. 2011; Jansen et al. 2012). Besides this, mi*R*-301 has been shown to correlate with an elevated risk of relapse and mediation of resistance to tamoxifen (Shi et al. 2011). Although the exact mechanisms of tamoxifen resistance by ncRNAs are not known yet, mi*R*-301 targets *PTEN* in breast cancer suggesting that the miR-301/PTEN/Akt axis might be involved in the development of tamoxifen resistance. In the same way, the resistance of other therapies, miR-128 mediates the sensitivity to chemotherapeutics by ABCC5 and Bmi-1 regulation (Zhu et al. 2011); plasma levels of miR-221 have an association with the overall response rate (ORR) in chemotherapy-treated patients (Zhao et al. 2011), while chemotherapy resistance was predicted by circulatory miR-125 (Wang et al. 2012); resistance to anti-Her2 Trastuzumab is mediated by higher levels of miR-210 in plasma(Jung et al. 2012) while the increased plasma levels of miR-155 mediate the sensitization of breast tumor cells to radiotherapy (Gasparini et al. 2014).

The role of lncRNAs as predictive biomarkers for the treatment of breast cancer has also been focused on intense research for the years and some remarkable results have been achieved. For instance, tamoxifen resistance has been predicted by lncRNA, breast cancer anti-estrogen resistance 4 (BCRA4); contrastingly, in ER+ breast cancer lncRNA, LINC01016 and LINC00160 were downregulated as compared to ER-negative and predicted the response to anti-estrogen therapy. Moreover, downregulation of colon cancer-associated transcript 2 (CCAT2) in a subset of patients has been associated with benefit to adjuvant therapy including methotrexate, 5-fluorouracil, and cyclophosphamide(Redis et al. 2013). Higher levels of lncRNA-ATB, which is activated by TGF-β, have been found in breast cancer patients and associated with resistance to trastuzumab. The underlying mechanism to resistance is the competitive binding to the miR-200C, leading to the upregulation of ZNF-217 and ZEB1 and inducing epithelial to mesenchymal transition (EMT) (Shi et al. 2015).

Lastly, to date, several clinical trials have included the measurement and evaluation of miRNAs as their potential ability to utilize as predictive biomarkers as primary and secondary end points (Pronina et al. 2017).

To conclude, keeping in view the great potential of predicting response to the treatment in breast cancer patients, and remarkable progressive results being added up from the on-going extensive research, it might be anticipated those epigenetic markers, histone modifications, DNA methylation, and non-coding RNAs hold a promising future as predictive biomarkers for treatments in breast cancer.

8.6 Epigenetics as Prognostic Markers in Breast Cancer

Owing to its highest mortality and morbidity rates in women globally, with the incidence of 1.7 million cases every year (Smith et al. 2019), there is always room for the betterment of current treatment interventions for breast cancer. In curative treated patients, there is a higher risk of cancer recurrence, and this happens in 10% of patients with hormone (ER and PR) positive cancers within 5 years, and they are at risk with a yearly rate of 1.2–2.4% for over next 20 years (Dowsett et al. 2015; Pan et al. 2017). Adjuvant systemic therapies decrease the risk of recurrence but have side effects negatively impacting the quality of life (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) 2012). In clinical oncology practice, the risk of recurrence is calculated with the use of classical prognostic factors utilizing nomograms like a UK-based PREDICT tool New Adjuvant Online (Olivotto et al. 2005; Wishart et al. 2011; Coates et al. 2015a, b), although these predictive tools are good at predicting the risk of cancer recurrence depending on the clinical features, these prediction models are at the population level. Therefore, at the individual patient level, there is either overtreatment or under treatment (Denduluri et al. 2016).

Prognostic biomarkers might be helpful to improve the risk assessment, enabling better distinguishing patients who are at greater risk of recurrence from the low-risk patients, subsequently, treatment may be included or omitted accordingly, benefiting the patients (Hudis 2015). This effect has been recently demonstrated in both the oncotype DX biomarker and MammaPrint assays by the TAILOR and MINDCAT trials (Cardoso et al. 2016; Sparano et al. 2018).

In this section of the chapter, we will focus on the potential of epigenetic markers for prognosis of the breast cancer.

8.6.1 DNA Methylation as a Prognostic Biomarker

With the advancements in molecular biology techniques and ever-growing data from TCGA, the importance of epigenetic markers for cancer prognosis is increasingly evident. Core histone modifications and DNA methylation profiles have been assessed in breast tumor tissues aiming to further clarify the molecular classification of breast tumors and upgrade the predictive and prognostic potential in clinical settings. In this regard, the first study evaluated the methylation at 145 CpG sites (correlating with 803 genes) in 189 paired normal samples and primary breast tumors. In this study, it was found out that luminal A and luminal B, and basal-like subtypes of breast cancer bear specific methylation profiles. Specifically, basal-like/ TNBC subgroup has been defined by the methylation patterns of five genes, namely *MGMT, CDKN2B, RB, CD44,* and *P73*, and hypermethylation of 11 genes, namely *TWIST1, GSTP1, MSH2, PMS2, MLH1, MSH3, DLC1, ID4, CACNA1G, MSH6, CACNA1A, and 1D4* (Holm et al. 2010). Later, numerous studies have combined DNA methylation profiles with expression analyses to find out the molecular signatures that might better account for the clinical and pathological heterogeneity of breast cancer.

Some studies have identified the methylation profile of specific subtypes of breast cancer. Particularly, in a large cohort of TNBC, the methylome analysis stratified the population into high, medium, and low-risk groups; it also identified the noteworthy association of 17 differentially methylated regions with patient's survival in both the multivariable and univariable analyses (Holm et al. 2010). For luminal breast cancers, the application of a powerful integrative network algorithm for matching the RNA-Seq and DNA methylation data from the TCGA led to the identification of the two methylation clusters correlating with the luminal A and luminal B subtypes. Same epigenetically dysregulated traits were shared by subtypes luminal A and luminal B, nevertheless, luminal subtype B has a greater level of hypermethylation than normal tissues (Gao et al. 2015).

8.6.2 Histone Modification as a Biomarker

Post-translational modification of histones for the classification has been evaluated using IHC. Post-translational modifications on Histones 3 and 4 had been analyzed in a large series of breast tumors ($n = 808$). Particularly, lysine methylation (H3K20me3 and H3K4me2), lysine acetylation (H3K18ac, H3K9ac, H4K16ac, and H4K12ac), and arginine methylation (H4R3me2) were investigated.

High levels of global histone methylation and acetylation were solely correlated with the luminal-like subgroup of the breast cancer subgroup. On the other hand, triple-negative/basal-like and Her2 amplified subtypes were linked with moderate to lower levels of arginine(H4R3me2) and lysine methylation (H4K20me3 and H3K4me2), lysine acetylation (H3K9ac, H4K12ac, and H3K18ac). Loss of methylation at lysine Histone 4 (H4K20me3) was linked with luminal subtypes and was independently correlated with worse disease-free survival (DFS) in the multivariable analysis (Elsheikh et al. 2009).

8.6.3 Non-coding RNAs as Prognostic Biomarkers

A distinct number of miRNAs have been correlated with breast carcinoma prognosis, whereas few miRNAs have also been validated by large cohorts of patients. For example, higher levels of miR-21 have been linked with worse OS and DFS in breast cancer patients at an early stage (stage I and stage II), expression of miR-21 was found to be strongly linked with histological grade in this cohort (Mulrane et al. 2012). Likewise, miR-10b was significantly associated with DFS, worse OS, and distant metastases, independently of other routinely used prognostic factors for the stratification of patients following their risk of disease progression (Parrella et al. 2014). However, the statistical significance of miRNAs panels over single miRNA has been illustrated by the integrated miRNA/gene signature in a group comprised of 466 patients of breast cancer from The Cancer Genome Atlas (TCGA). Thus, the final signature comprising 7 miRNAs (miR-103, miR-93, miR-148b, miR-484, miR-328, miR-1307, and miR-874) and 30 mRNAs has proved to notably predict

the OS in a multivariable model independently of other clinic pathological features, moreover, for stage I and II breast cancer patients, this signature has demonstrated the highest distant relapse-free survival. Moreover, eight independent breast cohorts, with 2399 patients have validated the superior performance of this signature to stratify the risk regarding other RNA predictors, including those encompassed in the Oncotype DX panels and MammaPrint.

As for the lncRNAs, expression of HOTAIR has been demonstrated to predict the metastasis risk independently in 164 breast cancer patients with ER-positive status (Sørensen et al. 2013). While, in a study alternatively spliced variants of MALAT1 were differentially expressed as compared to full-length transcripts and illustrated the independent prognostic factor for median-free survival (MFS) (Meseure et al. 2016). Other lncRNAs having prognostic potential include both lncRNAs signature (Zhao et al. 2014; Liu et al. 2016a, b) and individual transcripts such as LINC00472 (Shen et al. 2015), LINC00978 (Deng et al. 2016), and SPRY4 intronic transcript 1 (SPRY4-IT1) (Magee et al. 2015). In a genome-wide analysis to investigate the lncRNA landscape across 172 normal breast tissues and 835 breast tumor tissues samples, 215 abnormally expressed lncRNAs had been identified in breast tumors in comparison with normal samples. Particularly, the lncRNA profile was able to differentiate ER− from ER+ breast tumors. Furthermore, it also allowed to stratify into various molecular subtypes, therefore signifying the potential as a prognostic marker (Van Grembergen et al. 2016). For example, the lncRNA H19, which is the first to be identified as an imprinted long non-coding transcript (Van Grembergen et al. 2016), is responsible for EMT and regulation if different miRNAs (Raveh et al. 2015) were found to be upregulated in luminal A subtype of breast cancer. The same study also found an overall reduction of lncRNAs in comparison with other subtypes; in the basal-like subtype, LINC00993 was the most downregulated. Moreover, HOTAIR was the most increased lncRNA of the HER2 positive signature (Berteaux et al. 2008).

8.7 Epigenetics Modifications as Therapeutic Target

As mentioned in earlier sections of this chapter, the interplay of epigenetics along with genetic mutations has a crucial part in breast carcinogenesis. What made epigenetic mutations an ideal target, a ray of hope for a better future of cancer medicine? It lies in its ability to be reverted as epigenetic mutations do not alter the DNA sequence. Therefore, restoration of normal phenotype is possible theoretically by the use of epigenetic modifying drugs (Abdel-Hafiz and Horwitz 2015; Kamińska et al. 2019).

Mounting evidence suggests that epigenetics modifying drugs could be used synergistically when with and/or other chemotherapeutic drugs, thereby increasing the therapeutic effects. Various trials have included the histone deacetylase (HDAC) and DNA methyltransferases (DNMT) inhibitors to evaluate their therapeutic potential to control the epigenetic alterations and hormone resistance for the treatment of breast cancer (Sher et al. 2020).

Table 8.3 List of DNMT inhibitors, their clinical trial status, and indicated use

Drug name	Status	Current use
N-(2-hydroxyphenyl)-2-propylpentanamide	Pre-clinical studies	Multiple sclerosis
Azacitidine	FDA approval in 2004	Myelodysplastic syndrome
Fingolimod	FDA approval in 2010/18	Adult/Pediatric multiple sclerosis
Decitabine	FDA approval in 2006	Myelodysplastic syndrome
Ferrocenyl	Pre-clinical studies	Solid & soft cancers
5-Fluoro-2-deoxycytidine	Under trials	Solid tumors
Entinostat	Undertrial	Hodgkin lymphoma, BC, kidney
CUDC-101	Undertrial	Solid tumors
Hydralazine	FDA approval 1997	Hypertension
Belinostat	FDA approval in 2014	Peripheral T-cell lymphoma
Abexinostat	Undertrial	Follicular lymphoma, solid tumors

Table 8.4 List of HDACis and HMTis, their status, and current use

Class of inhibitors	Drug	Status	Current use
	YCW1	Pre-clinical studies	BC and lung cancer
	Romidepsin	FDA approval in 2009/12	Peripheral/cutaneous T-cell lymphoma
	Vorinostat	FDA approval in 2006	Cutaneous T-cell lymphoma
HDAC	Santacruzamate A	Pre-clinical studies	Solid tumors
	Valproic acid	FDA approval in 2008	Epilepsy/mania/migraine
	Sodium butyrate	Under trial	Solid tumors
	Trichostatin A	Under trial	Solid tumors
	Tetrahydrouridine	Under trial	Leukemia and solid tumors
HMT	EPZ004777	Pre-clinical studies	Mixed lineage leukemia
	UNC0638	Pre-clinical studies	Lung cancer and TNBC

Tables 8.3 and 8.4 enlist the potential epigenetic drugs for breast cancer treatment including drugs with FDA approval and investigational epigenetic drugs (HDAC and DNMT inhibitors). For example, vorinostat, belinostat, romidepsin, and panobinostat are FDA-approved HDAC inhibitors; and cytidine analogs (decitabine and azacitidine) are approved DNMT inhibitors and can induce DNA demethylation (Sher et al. 2020).

Various clinical trials have evaluated the use of a combination of epigenetic modifiers and have demonstrated remarkable antitumor effects against breast cell carcinoma; in addition, these studies have reported the greater benefits of combined epigenetic drugs with/without anticancer treatment as compared to the single-agent

treatment. For example, phase I and phase II trials have been carried out with HDAC inhibitors (panobinostat, entinostat, and vorinostat) alone and in combination with other therapies like immunotherapy, endocrine therapy, and chemotherapy (Damaskos et al. 2017). Data generated from the concluded trials ranged from no response to 55% response (Sher et al. 2020).

High expression of HDAC5, HDAC1, HDAC3 in tumor cells of the breast indicates that these HDACs may serve as a novel prognostic biomarker and discriminating and crucial drug target(Li et al. 2016). In vitro studies have demonstrated that targeting HDAC1 in breast cancer cell lines reversed the immune evasion leading to sensitivity to T-cell mediated lysis (Gameiro et al. 2016; Eckschlager et al. 2017). Furthermore, various HDACis such as N-(2-hydroxyphenyl)-2-propylpentanamide, panobinostat, sodium butyrate, vorinostat, mocetinostat, and entinostat have shown therapeutic effects against TNBC (Garmpis et al. 2017).

While HDACis in combination with other therapeutic agents have demonstrated remarkable outcomes, as a single agent they have limited effects. For instance, a new HDAC5 inhibitor, LMK-235 in combination with bortezomib has provided a novel therapeutic strategy for breast cancer treatment of breast cancer. In addition to this, combination therapy consisting of tamoxifen and vorinostat in ER+ advanced metastatic breast cancer patients has shown the promising potential of reversal of hormone resistance(Munster et al. 2011).

Potential epi-drugs mentioned in the table have shown promising results against breast carcinoma; in a clinical trial phase I when the combination of HDAC and DNMT inhibitors (tetrahydrouridine and 5-fluoro-2′-deoxycytidine) was given, it was found to be well-tolerated. Moreover, this combination has a partial response of 16 months and the potential of drug resistance reversal in a breast cancer patient (Newman et al. 2015). Subsequently, in clinical trial phase II, ClinicalTrials.gov identifier: NCT00978250, when the response of this combination was investigated against advanced breast carcinoma, efficacy results of the combination HDAC and DNMT inhibitors indicate that the further testing of this combination of drugs is unwarranted in breast cancer (Geraldine et al. 2020). On the other hand, in clinical trial phase II, the efficacy of this combination of HDACis (azacitidine) and DNMTis (entinostat) was evaluated, findings from this study indicated that breast cancer patients with hormone resistance might get an advantage with epigenetic therapy and/ or endocrine therapy may be restarted afar progressions (Geraldine et al. 2020).

At present, the implementation of epigenetic drugs for breast cancer is yet in its early stages and has not entered routine clinical practice. The HDACis and DNMTis which have been investigated have illustrated promising results in breast cancer treatment. However, these epi-drugs suffer the issue of relative toxicity, and as gene modulators, their pharmacodynamics is unclear raising concerns as a major challenge. In addition to this, there are other limitations as well which are impeding their use as prognostic, predictive, and diagnostic markers; these limitations include the fact that conflicting results have been reported by different studies due to the different methodologies adopted across the various studies. Moreover, there is a low concentration of an epigenetic substance in specimens and a requirement of additional purification steps while dealing with ncRNAs and histones. Lastly,

epigenetic modifications are generally cell-specific which are affected by other external factors including environment and aging as well, consequently, these histone modifications would be non-functional then. All these elements must be taken into account while seeking epigenetic modification as biomarkers and therapeutic targets (Lorincz 2011; Thomas and Marcato 2018; Roberti et al. 2019; Sher et al. 2020).

8.8 Conclusion

Extensive research on cancer epigenetics has led to the generation of compelling data demonstrating the promising role of epigenetics almost at every stage of breast cancer. Epigenetic mutations are specific to cancer cells making them a suitable diagnostic biomarker; these mutations can be identified from ccDNA and their ease of isolation from peripheral blood promoted the potential they hold as a diagnostic biomarker. Although significant findings have been reported for DNA methylation biomarkers, there is still a need for the large prospective cohort to make the inclusion of these diagnostic biomarkers in the screening for the early diagnosis of breast cancer.

Moreover, it is evident from the findings of numerous studies that epigenetic mutations can be utilized for the prognosis of breast cancer and prediction of drug response, thereby can play a critical role in directing the better clinical management of breast cancer. Epigenetic mutations are reversible, this finding speculated the hope for better targets and ultimately a better clinical outcome. Although there are several drugs approved by FDA and many others in the trial phase, reversal of mutations is not the exact mechanism. To get more advantage of the potential of these crucial mutations in carcinogenesis, further exploration of the underlying mechanisms of these mutations is needed. Lastly, it is evident that epigenetic mutations are important therapeutic targets, we may anticipate that this aspect of cancer pathogenesis holds a promising future for precision cancer medicine.

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9 Role of Fibrinolytic Mechanisms in Breast Cancer Diagnosis, Prognosis, and Treatment

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Abstract

Fibrinolysis is identified to play a crucial role in pathological and physiological processes. It counteracts excessive blood clotting to maintain hemostatic balance. However, its association with cancer is known from many years with the evidence that aberrant expression of any of its components leads to enhanced tumor growth, invasion, and progression. Malfunctioning of fibrinolytic system is found associated with various pathologies that majorly include inflammation, neuropathies, thrombosis, and metastasis. For this reason, fibrinolytic system can also be considered in designing cancer therapies. In breast cancer, with the disease progression, malignant cells invade within the blood stream and reach to the distant non-breast tissues. Although it is a complex process, yet homeostatic elements are considered major factors that facilitate the invasion, cellular transformation, tumor cell survival, proliferation, angiogenesis, and metastasis of breast cancer cells.

A pool of preclinical evidence is available for the therapeutic potential of fibrinolytic system yet it lacks in clinical trial-based evidence. Through this chapter, we aim to highlight importance of targeting main oncogenic components of fibrinolytic system and to provide comprehensive overview of the roles played via fibrinolytic component and their activation. Furthermore, we will discuss possible diagnostic and therapeutic strategies of fibrinolytic system during progression and spread of breast cancers.

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Keywords

Fibrinolytic system · Breast cancer · Pathologies · Diagnostic and therapeutic targets

9.1 Introduction: Fibrinolysis and Fibrinolytic System

Proteases and protease inhibitors regulate the activation process of the fibrinolytic system that is involved in the triggering of fibrinolysis, a process of dissolving blood clots that prevents blood vessels obstruction (Mahmood and Rabbani 2021). Besides maintaining a hemostatic balance by thrombolytic function, the fibrinolytic system also plays crucial roles in different physiological and pathological events like immune responses, tissue remodeling and cancer progression, invasiveness, and metastasis. A key enzyme of this system is plasmin, which dissolves the accumulated fibrin into fibrin degradation products (FDP) soluble in blood and base membranes (BM) as well as extracellular matrices (ECM) to promote remodeling and migration of tissues and cells, respectively (Lin et al. 2020). The action of urokinase-type or tissue-type plasminogen activators (tPA/uPA) and the plasmin inhibitors 2-antiplasmin (2-AP) and 2-macroglobulin balanced plasmin activities to produce plasmin from inactive plasminogen by neutralizing the free plasmin concentration (Duffy 2004). Besides tPA and uPA, proteases like plasma kallikrein (PK) or coagulation factor XIIa also abbreviated as (FXIIa) may activate plasminogen in the contact activation system (Duffy and Duggan 2004). The plasminogen activator inhibitor-1 (PAI-1) and -2 (PAI-2) modulate the activities of plasminogen activators (Castellino and Ploplis 2005).

Urokinase-type plasminogen activator (UPA) is a serine protease, with an epidermal growth factor-domain (GFD) at N-terminus that is generated like an inactive single-chain (sc) zymogen (Castellino and Ploplis 2005). This complex is activated by binding with the membrane-bound uPA receptor (uPAR). In cancer progression, a type II transmembrane (serine) protease, matriptase, is discovered to activate uPA. Plasmin is reported to be the most proficient activator among these sc-uPA activators (Santibanez 2018). Tissue-type plasminogen activator (TPA), is another serine protease with N-terminus fibronectin type II domain, a growth factor similar domain, kringle domains, 2 in numbers and a catalytic domain with C –terminus and act as the main plasminogen activator in blood (Santibanez 2018; Kanellopoulos et al. 2002). The tPA like uPA has no known physiological substrate other than plasminogen and it specifically binds with fibrin via its fibronectin type II domain and both kringle domains (Melchor and Strickland 2005). TPA, a single-chain zymogen (sc-tPA) is produced and stored in endothelial cells and released in the blood stream upon stimulation by histamine, thrombin, bradykinin, and/ or other molecules (Melchor and Strickland 2005). Proteolytic cleavage then transforms Sc-tPA in two-chain form (tc-tPA). The proteolytic activity of sc-tPA and tc-tPA is highly comparable, unlike inactive sc-uPA (Medcalf 2017). The uPAR is a glycosylphosphatidylinositol-receptor anchored with homologous domains (D1,

D2, and D3) (Ngo et al. 2011) which are extensively glycosylated and provide accommodation to the GFD domain of uPA (Huai et al. 2006). Extracellular uPAR binding causes signal transduction via interactions with membrane proteins like epidermal growth factor receptor (EGFR) with G-protein-coupled receptors (GPCR) and integrins (Liu et al. 2002; Resnati et al. 2002). The uPAR is found in biological fluids in a soluble form (su PAR) and in cleaved form (cu PAR) that do not possess D1 domain (Montuori and Ragno 2009). In fibrinolytic system, cu PAR (D2-D3) has a negative feedback mechanism, that do not possess binding affinity for vitronectin or uPA after plasmin mediated proteolytic cleavage (Montuori et al. 2002). Phospholipase cleavage releases suPAR from cells or plasma membranes, which can be found as compact or cleaved su PAR (D1-D2-D3, C-suPAR, D2-D3) (Wilhelm et al. 1994). Compact suPAR can receive all external membrane bounded-anchored proteins uPAR. As a result, su PAR competes for physiological activities with membrane-anchored uPAR including peri-cellular uPA decreased activity and binding of ECM (Wilhelm et al. 1994). Also, suPAR has no influence on uPA- and vitronectin-independent uPAR activities (Montuori et al. 2005).

9.2 Breast Cancer Etiology

The cancer starts with the ducts of epithelium (85%) and/or lobules (15%) of the breast glandular tissue, and it grows asymptomatically in the duct or lobule at first (Feng et al. 2018). Eventually, this cancerous mass progresses, invades, and spreads in the neighboring breast tissues (invasive type) and subsequently spreads to the nearest lymph vessels and nodes, also called as regional metastasis and when it spreads to other organs in the body, is called as distant metastasis (Feng et al. 2018). This cancer is one of the frequently diagnosed cancers in the women throughout the globe and is one among the most complicated of all cancers (Ferlay et al. 2010). About 2.3 million women were diagnosed with different types of breast cancers and 685,000 deaths in 2020 around the world. The data of the past 5 years shows that there were 7.8 million alive women diagnosed with breast cancer, making it most prevalent cancer (American Cancer Society 2020). No single cause is responsible for this, but multiple factors can influence the likelihood of breast cancer development. The risk of cancer prevalence increased with age, Approximately 77% women with age over 50 are diagnosed with breast cancer each year, and 40% are over 65. (Breast cancer n.d.). A woman's chance is increased, if she has first line relations (mother, sister, or daughter) who has had the cancer. About 5–10% breast cancer is inherited (Breast cancer n.d.). Women with inherited mutations of *BRCA1* and *BRCA2* genes have a higher potency of getting breast cancer. Besides this, mutations in the *TP53* gene are also reported as links to increase risks of breast cancer. Woman with dense breasts is more likely to get breast cancer due to fatty, fibrous, and glandular breast tissues (Breast cancer n.d.). Long-term estrogen exposure appears to increase breast cancer risk due to high estrogen levels between these times. Being overweight, smoking, alcohol intake, exposure to certain chemicals and radiations, a family history are other risk factors of breast cancer (Risk factors for breast cancer n.d.).

Treatment options for breast cancer depend on number of factors like, patient age, overall health, type, and stage of cancer. Current available treatment modalities are surgery, hormone therapy and biological therapy, or targeted drug therapy (Ke and Shen 2017).

9.3 Pathologies Associated with Fibrinolytic System in Breast Cancer

The counterpart of the coagulation system was first recognized as fibrinolytic system. Compare to common thrombotic disorders caused by coagulation system dysregulation, though, aberrant fibrinolytic system activation is clinically uncommon. Extravascular fibrinolysis, in contrast to intravascular fibrinolysis, appears to have a significant impact in different clinical diseases, according to mounting data (Lin et al. 2020). Fibrinolysis is a vital physiological function that prevents excessive thrombosis and maintains hemostatic balance. The fibrinolytic system's components are well-known and have been linked to a various physiological and pathological events. Tumor growth at increased rate, invasiveness, progression, and metastasis have been linked to abnormal level of expression of numerous components, including uPA, with its cognate receptor uPAR, and PAI-1 (Mahmood and Rabbani 2021). As a result, cancer biologists have become very interested in targeting the fibrinolytic system for disease diagnostic and therapeutic. Despite promising preclinical verification of the therapeutic potential of fibrinolytic system's basic oncogenic components targeting, still a lot of research work is required in bringing bench to the bedside results due to a lack of clinical studies (Mahmood and Rabbani 2021).

9.3.1 Breast Cancer Progression and Role of Hemostatic Function

Since the early age, series of event are involved in the spread of breast cancer from initial stage to metastatic spread in different sites of body through blood stream. In this process, clonogenic tumor cells that have potential to proliferate and make colony accumulate in non-breast tissues. Hemostatic elements including platelets, coagulation, and fibrinolysis play a pivotal role in the breast cancer progression (Lal et al. 2013). The progression of breast cancer proceeds by cells transformation, propagation, survival, angiogenesis, and homeostatic system components that regulate all these process. Thus novel therapeutics approaches are in the process of development to target components of hemostatic system (Lal et al. 2013). A clear direct effect of platelets in the spread and invasion of breast cancer was elucidated by a rise in the circulation of platelets count in breast cancer patients (Taucher et al. 2003). Breast tumor cells can also induce aggregation of platelets. Moreover, breast cancer cells produce matrix metalloproteinases that help in platelets and coagulation system activation (Alonso-Escolano et al. 2004). The activated platelets release factors that help in survival and ultimately growth of cancerous cells at metastatic sites (Tokyol et al. 2009). Furthermore, activated platelets also produce

micro-vesicles that promote breast tumor cells adhesion, proliferation, chemo-invasion, and chemotaxis (Janowska-Wieczorek et al. 2006). Coagulation system also shows enhanced activity in breast cancer due to rise in venous thromboembolism rates systematically (Janowska-Wieczorek et al. 2006). Coagulation activation is an important phenomenon of regulating cellular transformation, angiogenesis, and metastasis in breast cancer progression. Tissue factor and thrombin are main elements of coagulation system that are active in breast cancer spread (Lal et al. 2013; Janowska-Wieczorek et al. 2006). The fibrinolytic system has a key role in normal physiological processes, and its possible function in the progression of breast cancer is being studied by researchers The fibrinolytic system stimulates cancer growth by many diverse mechanisms like apoptosis, angiogenesis, proliferation, and degradation of tumor cells and extracellular matrix, respectively (Caine et al. 2003). One example is uPA system as mentioned above. It performs important function in the progress of breast cancer and its components are uPA, uPAR, and PAI-1 and PAI-2 (Caine et al. 2003). Clinical studies in breast cancer patients have revealed a direct relation in poor survival with increased expression levels of uPA and PAI-1. Particularly, combined levels of uPA and PAI-1 can be used as a prognostic biomarker in untreated lymph node-negative breast cancer. It can predict the need of chemotherapy (Han et al. 2005).

9.4 Components of Fibrinolytic System

Fibrinolysis which results in degradation of fibrin is a complex process and involves sequential interactions of multiple components. Each component is of core importance and plays a key role in clots dissolution (Bharadwaj et al. 2021). Herein, the major components of the fibrinolytic system are discussed.

9.4.1 Plasminogen and Plasmin

Plasminogen is a protein present, abundantly, in plasma. Originally, it is produced in liver and circulates in blood at 200 μg/mL concentration (Castellino and Ploplis 2005) and exists in various zymogenic forms with a biologic 2.2 days half-life (McMahon and Kwaan 2008). Plasminogen, when secreted, consists of full form and contains glycosylated protein with single-chain and an glutamic acid (Glu) at N-terminal Pan-apple domain, kringle regions (Kanellopoulos et al. 2002) with lysine-binding domains, that modulates the plasminogen activation and binding, and a C-terminal protease domain (Sanderson-Smith et al. 2012). Plasminogen can reside as two conformations, closed and open (Law et al. 2013). After proteolytic degradation, it is converted into active form of plasminogen, plasmin. Glu-plasminogen is a closed form that cannot be freely stimulated by plasminogen activators. After binding with fibrin via cell surface binding Glu-plasminogen can get open conformation. Deletion of Pan-apple domain during the process of pre-activation by plasmin results in the production of alternative zymogen form,

known as Lys-plasminogen, an open conformation (Miles et al. 2003). Both, open conformation and activation loop adopt a flexible formation and open cleavage by activators and inhibitors. All inclusive, a tangled interplay among the activators and inhibitors of plasminogen regulates the level of fibrinolysis, tissue remodeling, invasion, and spread. Plasminogen activators trigger its cleavage at Lys-77 abd Lys-78, thus forming Lys -plasminogen (Ponting et al. 1992). Next cleavage at Val-561 and Arg-560 peptide bond leads to formation of plasmin (Miles et al. 2003). Lysine enhances the binding of plasminogen to 2-antiplasmin, annexin II, thrombospondin, fibrin, and to other anti-fibrinolytic agents, etc. However, how lysine binding to plasminogen elicits conformational modification, remains unclear. Interestingly, in closed plasminogen crystal structure, the Lys77 site has been found inaccessible (Horrevoets et al. 1995; Xue et al. 2012). The data recommend that full-length conformation change is required for full-length plasminogen.

Different activators are responsible for cleavage of plasminogen. The uPA and tPA are reported as the most important ones. tPA is a glycoprotein of 70 kDa, which is put together by the endothelial cells and maintains vascular patency as a result of intravascular fibrin formation (Bharadwaj et al. 2021). The uPA is a serine protease, pro-uPA of 53-kDa zymogen. It exists at the cellular surface and binds to the receptor, i.e., Glycosylphosphatidylinositol -anchored receptor (GPI-uPAR) (Bharadwaj et al. 2021). In tumor cells, both activators (tPA and uPA) co-exist. The uPA and its receptor act as a modulator of cellular processes, while tPA with its binding receptor annexin II controls fibrin deposition. High levels of uPA is considered as prognostic factor in breast cancer (Dovnik and Takac 2017). Furthermore, studies reported that uPA elevated expression is directly associated with aggressiveness and poor prognosis with different types of cancers, Paluchowski et al. also reported that increased levels of uPA act as independent predictive factor which reduce the overall breast cancer's patients survival rate with known status of circulating tumor cells (Banys-Paluchowski et al. 2019). Interestingly, they found that raised uPA levels in serum associates with extent of the cancer (visceral metastasis ($p = 0.036$) and multiple metastatic sites ($p = 0.016$)). No link was found associated with other typical clinical parameters, like tumor grade/stage or status of the receptor (Banys-Paluchowski et al. 2019). Moreover, uPA overexpression in serum was also found to be associated with elevated levels of HER2, VEGF, CAIX, RAS p21, and TIMP1 in serum of breast cancer patients (Banys-Paluchowski et al. 2019; Breuss and Uhrin 2012). For RAS family members, uPA and uPAR contributed to the activation of Ras/extracellular signal-regulated kinase (ERK) signaling pathway and to boost the invasiveness of tumors with RAS-mutations (Di Mauro et al. 2017). Apart, due to different binding site, other than uPA aids uPAR to interrelate with integrins and transmembrane receptors to activate intracellular signaling cascades facilitated by well-known effectors including Akt, src, and focal adhesion kinase (FAK) (Montuori et al. 2005). Thus, uPA can act as the best prognostic markers for breast cancer patients (Duffy et al. 2014).

Plasminogen upon activation via above said proteolytic activity converts to plasmin which is a serine protease. Plasmin is a multipurpose enzyme having different physiological substrates, including proteins from blood and other

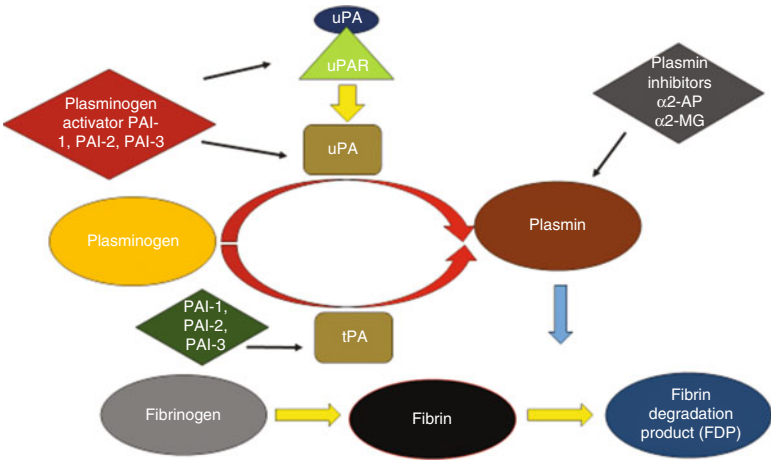

Fig. 9.1 Schematic representation of component of urokinase plasminogen activator system (uPA) and their role in plasmin formation. tPA and uPA both can be inhibited by plasminogen activator inhibitor (PAI-1 and PAI-2). uPA together with uPAR can facilitate in plasminogen conversion to plasmin while plasmin can be inhibited by α2-antiplasmin and α2-macroglobulin

extracellular proteins (Hervio et al. 2000). Plasmin acts as an activator of proteins involved in different functions. Plasmin has affinity for fibrin, fibrinogen, and matrix proteins. Major extracellular matrix proteins include fibronectin and laminin. Activation might occur directly or indirectly via activation of latent metalloproteinases (McMahon and Kwaan 2008). In tumor cells, it modulates wide range of pathologic processes including growth of tumor and metastasis. Deryugina and Quigley reported that plasmin modulates the generation of over 7 matrix metalloproteinases (MMPs) (Deryugina and Quigley 2012), thus playing a role in tissue remodeling, cancer progression, etc. Other key substrates include growth factors and definite cleavage sites of plasminogen-generating angiostatin. Thus, its association with other important proteins proves that plasmin is an intercessor protease and performs many important functions (Fig. 9.1).

9.4.2 Thrombin

There are many factors which act as the point of intersection between coagulation and fibrinolytic system, thus maintaining the homeostasis. Thrombin is a potent factor which catalyzes conversion of plasma fibrinogen to fibrin, contributing to tumor metastasis. This is unique regarding its function in clotting; as it regulates both anticoagulation and pro-coagulation, making it the part of both coagulation and fibrinolytic system (Esh and Ri Narayanan 1999). Thrombin activates platelets via its receptor, present on the surface of platelets, during its procoagulant (coagulation) function. Burst thrombin formation is regulated by thrombin medicated modulation of coagulation factors V, VIII, and XI (Esh and Ri Narayanan 1999). The activation

of XI prevents fibrin clots from undergoing fibrinolysis. Thrombomodulin binds to thrombin and medicates its anticoagulant function. Thrombomodulin is an endogenous anticoagulant membrane receptor protein existing on the endothelial membrane of the blood vessel and originates a chain of reactions that consequently leads to fibrinolysis (Esmon and Owen 1981). Thrombomodulin, activated protein C, and thrombin-activatable fibrinolysis inhibitor (TAFI) work in collaboration and maintain the anti-inflammatory and anticoagulant state of endothelial microenvironment. In terms of structure, thrombomodulin is separated into 5 domains and consists of extracellular component (496 amino acids), cell membrane component (23 amino acids), and intracellular component (38 amino acids) (Sugano et al. 2021). The lectin-like domain (D1) located in the N-terminal has anti-inflammatory potential by adsorbing high mobility group box 1 or lipo-polysaccharide (Abeyama et al. 2005). EGF-like domain (D2) is thrombin binding domain and contains six epithelial growth factor (EGF)-like components and after binding to this domain l, coagulant activity of thrombin is lost. Thus, D2 is critical for the protein's anticoagulant cofactor activities (Abeyama et al. 2005). The complex inhibits coagulation via modulation of activated protein C and inhibition of coagulation factors V, VIII. Moreover, thrombomodulin has an anti-fibrinolytic effect through the activation of TAFI (Bajzar et al. 1996). Furthermore, thrombomodulin aids in maintaining balance between fibrinolysis and coagulation. The importance of thrombomodulin in malignant tumors considered as multifaceted and has not been fully elucidated. Reduced expression of thrombomodulin correlates to less survival or enhanced metastasis in many tumors including breast cancer (Hanly et al. 2005, 2006). Kim et al. also reported that thrombomodulin might play a key role in cancer metastasis and invasion, and proposed it as new prognostic biomarker in invasive breast cancer (Kim et al. 1997).

9.4.3 Tissue Factors

The activities of fibrinolytic system can be modulated by many other contributors that play roles in either activation and/or inhibition of components of the system. One factor include is plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2). Among two inhibitors, PAI-1 acts more 10–100 times quick and rapid than PAI-2, confirmed using in vitro studies (Thorsen et al. 1988). PAI-1 is considered as an important modulator of the fibrinolysis. PAI-1 suppresses the fibrinolytic system and inactivates plasminogen activators, i.e., tPA and uPA, as mentioned earlier in the chapter too. The PAI-1 is a glycoprotein belonging to the serpin family (Jensen and Gettins 2008). After synthesis in endothelial cells, PAI- 1 is stored in platelets. It binds to tPA and uPA and limits the generation of plasmin, thus promote coagulation (Brogren et al. 2004). PAI-1 exists mainly into two forms, the latent and the active. Within few hours, the active PAI-1 changes to the latent form, irreversibly (Kindell et al. 2015). Reactive central loop domain of active form covalently blocks the plasminogen activators at their active site after proteolytic cleavage (Lang et al. 1992). Latent PAI-1 loses its inhibitory activity due to embedded reactive central

loop. PAI-1 is also involved in cell adhesion, migration and acts as the inhibitor of uPA. Several studies have documented that raised levels of PAI-1 in breast tumor tissue are predictors of poor responses and adverse outcomes in the patients with lymph node-negative breast cancer (Jänicke et al. 2008). Several studies also documented that uPA and PAI-1 serve as an independent prognostic biomarkers for breast cancer patients (Duffy et al. 2014).

Coagulation factor XII (α-FXIIa) is another factor which implies its impacts on fibrinolytic system. The factor binds to fibrin and enhances the thickness and hardness of the fibrin clot. Contrariwise, proteins of the contact system and the fibrinolytic system share the homology, and α-FXIIa can also contribute to conversion of plasminogen into plasmin, thus leading to degradation of fibrin (Konings et al. 2015). Konings et al. found that α-FXIIa contributes to conversion of plasminogen into plasmin, directly, and reduces time of clot lysis, at all the tested concentration of tPA (15–1500 pM) (Konings et al. 2015). The group found that in the existence of α-FXIIa, simultaneous assessment of plasmin generation and fibrin degradation/formation show an earlier onset of fibrinolysis. Furthermore, under flow conditions, the fibrinolysis of clots formed, revealed that incorporation of α-FXIIa enhanced the clot breakdown by additional plasmin generation on top of formation by tPA. Konings et al. noticed that in the presence of plasminogen, α-FXIIa enhances fibrinolysis, irrespective of presence tPA. The team postulated that FXIIa first strengthens structure of the clot during its formation and then contributes to fibrinolysis in later stage (Konings et al. 2015).

9.5 Fibrinolytic System as Diagnostic Target

In early 1970s, it was brought into attention that fibrinolysis plays a pivotal role in cancer spread and invasion (Rabbani and Mazar 2007). The increase in the fibrinolytic activity is associated with plasminogen activator system that has multiple components uPA, receptor; uPAR, plasminogen activator inhibitors PAI-1 and PAI-2, all these provided major role in tumor progression and cancer spread. Increase in fibrinolytic activity system with plasminogen activator system shows a positive association, for this reason both the term can be used interchangeably (Duffy and Duggan 2004).

The inactive form, plasminogen converted to active form plasmin after binding of uPA and uPAR, initiating proteolytic cascade to debase extracellular matrix and tumor cell invasion and migration to secondary sites (Pillay et al. 2007). Certain studies also suggest that PA system plays multiple roles in different stages of cancer especially invasion and metastasis. The increased expression of uPA and uPAR system has been found related to adverse patient outcome in various cancers. The system is also recognized to have diagnostic, prognostic, and therapeutic potential (Duffy and Duggan 2004).

9.5.1 Prognostic Biomarkers

A wide range of cancers has been found to have abnormal expression in the components of the fibrinolytic system (Mahmood et al. 2018b; Kwaan et al. 2008). This opened up new opportunities for developing prognostic and therapeutic targets to improve cancer patient's clinical outcome and currently some have entered in clinical trial for cancer treatment (Berkenblit et al. 2005). In aggressive cancers, fibrinolytic factors such as uPA, uPA receptor (uPAR), and PAI-1 are more noticeable, especially the presence of uPA is more prominent (Berkenblit et al. 2005).

In 1985, O'Grady et al. discovered that malignant breast cancers have more uPA activity than benign tumor (O'Grady et al. 1985). Well along, various studies have demonstrated a link between uPA activity and tumor growth, invasion, and metastasis in primary breast cancers (Duffy et al. 1988) Breast cancer patients with higher uPA activity had a shorter disease-free interval than those with lower uPA activity, according to Duffy et al., suggesting that uPA could be a potential prognostic marker in breast cancer (Duffy et al. 1988). More specifically, Jänicke et al. studied 556 breast cancer patients and found higher levels of uPA and PAI-1 proteins in initial breast cancers were linked to the patients' poor prognosis (Jänicke et al. 2001). Since then, extensive study has been conducted to estimate and analyze the role of uPA and all of the uPA system's components in mediating breast cancer metastasis and progression.

In a detailed and pooled examination of nearly 8000 individuals with breast cancer, the elevated uPA and PAI-1 levels were found to be more predictive of poorer prognosis than estrogen receptor status or tumor size (Look et al. 2002). When 3000 patients with high uPA/PA-1 levels found to benefit more from adjuvant treatment, these findings were clinically validated (Harbeck et al. 2002). According to the authors, the levels of uPA and PAI-1 in primary tumor tissue provide evidence-based data on relapse risk and treatment response, which will aid in the tailoring of personalized adjuvant therapy approaches for breast cancer. Using uPA and PAI-1 as risk classification biomarkers of 4149 patients with node-negative breast cancer, it was discovered that in those with low risk, adjuvant chemotherapy might be avoided (Kantelhardt et al. 2011). Another large prospective trial on a 10-year follow-up of nearly 8000 patients of breast cancer found the levels of uPA and PAI-1 were utilized to estimate adjuvant chemotherapy (Harbeck et al. 2013). The actuarial 10-year relapse rate for patients with high uPA/PAI-1 levels in the observation group was 23.0% (without any adjuvant systemic medication), compared to only 12.9% for patients with low uPA/PAI-1 (Harbeck et al. 2013). Almost half of patients with node-negative breast cancer had a lower risk of recurrence and did not require chemotherapy, despite having a decent long-duration disease-free survival rate (Harbeck et al. 2013).

The uPA inhibitor, such as PAI-1 elevated levels has been associated with poor outcomes in breast cancer patients. This is supported by research work of Foekens et al. used a cohort of 2780 breast cancer patients (Foekens et al. 2000). They access the prognostic value of major components of the four uPA system [uPA, the receptor uPAR (CD87), and the inhibitors PAI-1 and PAI-2] and the antigen levels were

estimated by enzyme-linked immunosorbent assay (ELISA). It was showed that the levels of uPA and PAI-1 can be utilized as independent predictors of poor relapse-free survival and overall survival (Foekens et al. 2000). These indicators could be useful in determining a patient's specific risk, selecting different types of personalized adjuvant treatment, and identifying people who might benefit from personalized medicines currently being developed (Foekens et al. 2000). Increased uPAR expression has been associated with a poor prognosis and metastasis in later stages of breast cancer (Pierga et al. 2005). Surprisingly, the American Society of Clinical Oncology (ASCO) suggests using ELISA to estimate the optimum adjuvant therapy for patients with breast cancer (Harris et al. 2007). Furthermore, the recent advancement in molecular biological techniques such as Oncotype DX (Paik et al. 2009) and MammaPrint (Knauer et al. 2010), which are based on mRNA, has shown potential in predicting breast cancer outcome in recent years. However, the ELISA-based assessment of uPA and PAI-1 protein is less time-consuming and expensive than these tests (Nicolini et al. 2018). As a result, it is feasible to avail and study this method to forecast breast cancer outcome on patients.

9.5.2 Diagnostic Biomarkers

As mentioned earlier, the components of fibrinolytic system can be used in cancer diagnosis at multiple stages of cancer invasion and progression. It was found for breast cancer patients by Duffy et al. that they have higher uPA activity with shorter disease-free span as compared with patients having lower uPA activity (Mahmood et al. 2018b; Duffy et al. 1988). In another study, data of 8377 patients suffering from breast cancer has confirmed that higher level either for uPA or PAI-1 correlates with cancer aggressiveness and relapse free, poor overall survival of breast cancer patients (Look et al. 2002; Jänicke et al. 2008). For the clinical evidence and detecting uPA and PAI-1 protein in patient's sample, ELISA kits have been utilized as they are available commercially. FEMTELLE® kit is one such example which has been validated by multicenter quality assessment programs, and having wider detection range of 6.2–8.2% for uPA and 13.2–16.6% for PAI-1 (Hayes et al. 1996; Simon et al. 2009; Benraad et al. 1996). However, one of the demerit of using ELISA kit is the requirement of sample tissues in fresh or frozen form, which is challenging logistically. For this reason, paraffin embedded formalin fixed tissues are considered a straight forward solution to overcome the challenge (Benraad et al. 1996; Sweep et al. 1998). Another challenge is the overlapping of uPA and PAI-1 presence in stroma cells and tumor cells as well, that makes immune-histochemical scoring bit difficult for disease diagnosis (Schmitt et al. 2008). Moreover, it is considered that machine learning algorithms and AI–artificial intelligence technology may be used in future to differentiate the cells, overcoming the major hurdle in smooth diagnosis of the disease.

Another method studied for breast cancer diagnosis is quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in which uPA (PLAU gene) and PAI-1 (SERPINE1) mRNAs were quantified along with nucleic acid sequence

based amplification assays (NASBA) (Biermann et al. 2008; Lamy et al. 2007).Yet, discrepancies and incompatibilities were observed among cross laboratory results. However, targeted sequencing of uPA and PAI-1 mRNA with advancement may establish strong evidence for their use as cancer biomarkers.

It is known that DNA methylation is a cancer hallmark (Mahmood and Rabbani 2019) and anomalous DNA methylation-based biomarkers are apparent as diagnostic biomarkers in breast cancers. Epigenetic modification through anomalous DNA methylation holds for transcriptional regulation of gene expressions. The DNAs are stable and isolated efficiently isolated from paraffin embedded formalin fixed tissue samples. These properties make DNAs more suitable candidate for diagnosis (Locke et al. 2019; Xing and Rabbani 1999).

It was also demonstrated that uPA promoter anomalous DNA methylation and its RNA expression are inversely correlated with the tumor progression to advance aggressive state (Xing and Rabbani 1999; Pakneshan et al. 2004b) which indicates that assessment of uPA promoter is more suitable as early stage diagnostic biomarker. Similarly, it was also demonstrated for PAI-1 gene, for the ocurance of targated sequencing that will make the methylation site assessment easier on specific genome location (Gao et al. 2017) aberrant DNA methylation is observed in almost all cancers. Hypermethylation inactivates tumor suppressor genes, whereas hypomethylation activates pro-metastatic genes which make DNA as more stable biomarkers for cancer diagnosis (Rabbani and Gladu 2002).

Among other components of fibrinolytic system, uPAR is present at very low levels in healthy tissues but its expression level increases with the cancer aggressiveness (Boonstra et al. 2011), this characteristic helps in making uPAR a concrete candidate for cancer diagnosis. uPAR based imaging strategy is currently in use for estimating stage of cancer and level of aggressiveness. Use of antibodies targeting uPAR for diagnosis is more advantageous for the longer half-life of antibodies in serum and can prolong the cancer imaging timeframes. uPAR-based oncological imaging has a powerful potential in the field of cancer diagnostics (Persson et al. 2015; Yang et al. 2016). This multimodal imaging has also conjugate the single target to radionuclide near infrared fluorescent dye for distinguishing tumor tissue with other non-tumorigenic tissues (Boonstra et al. 2015). Taken together, a combination of studies have already been done for validating and improving diagnosis of breast cancer.

Distant differences have been identified between breast cancer tissue samples in occurrence and distribution of fibrinolytic components. uPA is usually found in nonmalignant breast tissues and secretions (Mahmood et al. 2018b). In normal breast tissues, PA system leads to growth and maintenance of ductal structures under physiological and pathological condition. The PA system is also associated with microsomal fraction of tissue homogenate (McMahon and Kwaan 2015). Higher the level of uPA advance will be the stage of cancer, greater number of positive lymph nodes, greater invasive and proliferative activities, rapid metastasis, and enhanced chances of reoccurrence (Stoppelli 2013).

Based on the provided evidence, it can be postulated that uPA is a marker for aggressive advanced stage breast cancer.

9.6 Fibrinolytic System as Therapeutic Target

This system has garnered importance as a putative facilitator of breast cancer. Recently, researchers have started to untangle and resolve the pathophysiologic relevance of breast cancer and fibrinolytic system (Lal et al. 2013). There are different fibrinolytic system that plays a crucial role in various cancers (including breast cancer) including uPA, its receptor uPAR, and PAI-1 (Han et al. 2005). In the physiological and pathological states, uPA as a therapeutic target has given the pathophysiological significance (Mondino and Blasi 2004). The cancer biomarkers and their clinical relevance for cancer diagnostic, prognosis, and therapeutic response prediction increased levels for both uPA and PAI-1, which have been linked to relapse-free, poor overall survival rate in breast cancer patients (Look et al. 2002). Increased levels of uPA and PAI-1 in cancer patients with untreated, lymph node-negative breast cancer showed good predictive value, and increased levels of uPA and PAI-1 in breast cancer patients that could be used as a biomarker for illness at a higher risk and urgency for identification and need for therapy (Look et al. 2002).

9.6.1 Inhibitors of Coagulation

In breast cancer, various inhibitors of coagulation have been studied in the clinical trials, which include heparin and warfarin, low molecular weight inhibitors (Kakkar et al. 2004; Levine et al. 1994). But the limitation with these clinical trials was that breast cancer patients were a minority and were enrolled patients with other cancers. The work done by Levine et al. 1994 was the particular trial employing therapy with warfarin that involved only patients with breast cancer. They found no survival advantage with this drug (Levine et al. 1994). Recently, Pang et al. investigated the level of fibrinolysis and coagulation-related markers in the plasma of patients with breast cancer following surgery (Pang et al. 2021). They selected 63 patients from May 2016 to May 2019 and found significant differences in blood pressure, platelet count, diabetes history, and tumor metastasis (Pang et al. 2021). According to the available evidence, it is unclear if anticoagulants would benefit patients and, if so, at what stage of the disease these treatments would be most successful.

9.6.2 Transcriptional Repression

The use of diverse gene therapy based techniques such as antisense oligonucleotides or RNA interference (RNAi), ribozymes, and other methodologies to repress fibrinolytic system (uPA or uPAR gene) expression have all been studied in various forms of cancer to reduce cell proliferation and metastasis (Mohan et al. 1999; Gondi et al. 2007).

The uPA/uPAR genes have also been subjected to RNA interference (RNAi) such as adenovirus-mediated delivery construct which enhanced the expression of uPAR in glioma significantly abridged tumor development (Karikó et al. 1994).

In case of ribozyme, Karikó et al. created a 37-mer hammerhead ribozyme, targeting the mRNA of uPAR and used lipofectamine to deliver it into osteosarcoma cells (Karikó et al. 1994). According to this, the artificially made ribozymes reached cancer cells cytoplasm, cleaved mRNA uPAR, and caused reduction in mRNA to form protein (Karikó et al. 1994). In the future, clinical efforts to inhibit uPA, uPAR, and numerous other known oncogenes by transcriptional repression may become more common.

9.6.3 Small Molecules and Epigenetic Agents

The earlier attempts to stop uPA activation were focused on developing inhibitors of its catalytic activity as Vassalli et al. reported the first attempts to block uPA activation in 1987, focusing on creating inhibitors (amiloride) of its catalytic activity (Vassalli and Belin 1987). In 1991, other in vivo work involved using an antibody-based method by Ossowski et al. shown that it disrupt the activity of enzymes of uPA, which was successful in preventing general invasion, but not metastasis by injecting mice with human squamous cell carcinoma (Ossowski et al. 1991). Small-molecule inhibitors were later found to be more successful in attaining inhibition in later experiments. For this reason, Towle et al. developed a family of uPA inhibitors known as the 4-substituted benzo(b)thiophene-2-carboxamidines by altering the molecular structure of amiloride (Towle et al. 1993). Advance research of B-428 and B-623, two compounds belong to this class, was shown to inhibit uPA with median inhibition concentrations (IC50: 0.32) and 0.07 M; with inhibitory constants (Ki) of 0.16 and 0.53 mM, respectively (Towle et al. 1993). Hence, small molecule inhibitors of uPA have been widely employed to reduce proliferation, invasion, and metastasis by disrupting its enzymatic activity.

Rabbani et al. used B-428 in in vivo experimental conditions to diminish prostate cancer growth and metastasis (Rabbani et al. 1995). More investigation has revealed that when combined with tamoxifen, B-428 inhibits breast cancer development and metastasis in a synergistic manner (Xing et al. 1997). Interestingly, WX-671 (MESUPRON®), a comparable agent is a prodrug of WX-UK1. This drug has completed clinical trial phase Ib for treating head and neck cancer patients. (Goldstein 2008; Setyono-Han et al. 2005) A biopharmaceutical company (Wilex AG0) discovered a number of small molecule and powerful uPA inhibitors such as Wilex AG's WX-UK1, greatly reduced breast cancer invasion and spread in vivo model (Setyono-Han et al. 2005). In clinical trials for breast cancer treatment, MESUPRON and its prodrug have showed encouraging outcomes (Schmitt et al. 2011).

During the *last decade*, a significant knowledge has been gained about a wide range of *epigenetic changes* that crucially contribute to some of the diseases and targeting such abnormalities using epigenetic agents (Garcia-Martinez et al. 2021).

Interestingly, DNA hypermethylation-mediated inhibition of *uPA* that blocks invasion, metastasis, and spread of breast cancer has been shown by Pakneshan et al. (Pakneshan et al. 2004a, b). They used the universal methyl group donor *S*-adenosylmethionine (SAM), to reverse the hypo-methylated state of *uPA* with decreased *uPA* expression (Pakneshan et al. 2004a, b). Furthermore, Mahmood et al. 2018a, b have shown that treatment with SAM decreases cancer cell invasion, proliferation, spread, and metastasis of highly invasive triple-negative breast cancer (MDA-MB-231) xenografts implanted into the immunocompromised mice (Mahmood et al. 2018a, b). Regardless, more research is needed to see if exogenous SAM delivery gives cancer cells an edge in terms of survival.

9.6.4 Toxin Conjugates

Levive van et al. described the importance of toxins for cancer treatment in early nineteenth century (Van Mellaert et al. 2006). Later William B. Coley found the curative effect of toxin conjugated on sarcoma patients. He had also developed a vaccine using two bacterial strains of Serratia marcescens and Streptococcus pyogenes against cancer (Coley 1898, 1909). Toxin-based cancer therapeutics provides more selective delivery system as bacterial toxins may increase therapeutic response if used with conventional standard cancer therapies (Liu et al. 2014). In PA system, the most common strategy is targeting uPAR expressing cells with suitable toxin conjugate. For example, ATF conjugates with toxins of Pseudomonas species (ATF-PE) (Rajagopal and Kreitman 2000). In another study, Zuppone et al. revealed that conjunction of ribosome inactivating saporin protein with ATF reduces the breast cancer and bladder cancer viability (Zuppone et al. 2020).

Further, ATF and saporin conjugates (ATF-SAP) were found selective for cancer cell treatment with no observable effect of uPAR expressing non-tumor cells. Lot of research is still required in this field for translation of abovementioned results in human clinical trials. Major disadvantage in toxin conjugate-based therapy was found as immunogenicity and septic shock in the host (Weerakkody and Witharana 2019) (Fig. 9.2).

9.6.5 Antibodies

Antibodies play a promising role in treatment of various diseases. In the past two decades, it has been greatly observed that antibody-based targeted therapies for cancers are causing marked decrease in cancer spread and metastasis. For example, in preclinical settings, polyclonal rat anti-uPAR antibodies bring about notable decline in primary growth of breast cancer (Rabbani and Gladu 2002). Another monoclonal antibody ATN-658 can target human uPAR protein and it was found greatly effecting prostate cancer metastasis and invasiveness. This antibody is completely humanized (huATN-658) and recent in vivo studies have revealed the significant decline in breast cancer development and progress (Rabbani et al. 2010;

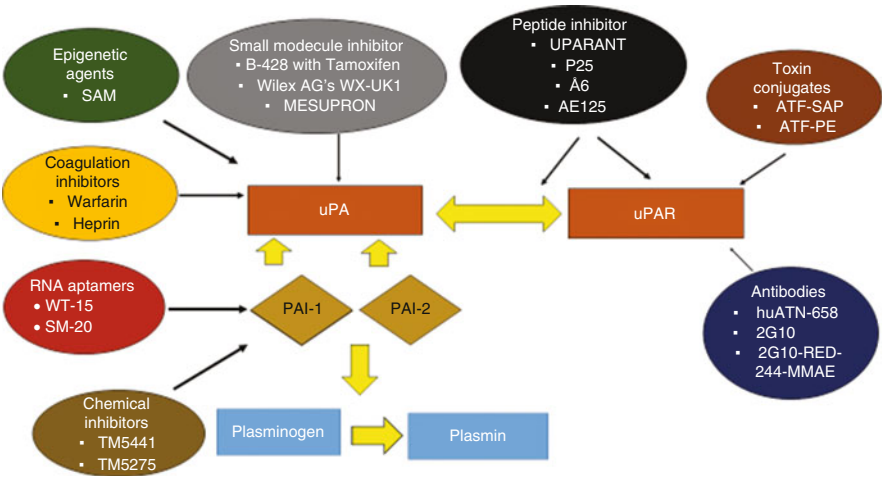

Fig. 9.2 The inhibitors of fibrinolytic system targeting major components (uPA, UPAR, PAI-1) of plasminogen system which are deregulated during breast cancer progression

Mahmood et al. 2020). 2G10 is another uPAR-based antibody with anti-cancer therapeutic potential especially in breast cancer (Duriseti et al. 2010). Also, the 2G10 conjugate (2G10-RED-244-MMAE) has the potential to decrease breast tumors compared to 2G10 monotherapy. Duriseti et al. identified that 2G10 antibody blocks the uPA–uPAR interaction after binding to uPAR and suppresses the cancer cell invasiveness in vitro (Duriseti et al. 2010; LeBeau et al. 2013; Harel et al. 2019).

Moreover, when human breast cancer cell MDA-MB-231 and bone metastatic variant breast cancer cells MDA-BoM-1833 were implanted in tibia of immunocompromised animal, the human huATN-658 has reduced the growth of breast tumor in bone microenvironment. This therapeutic effect is further enhanced by combining bisphosphate zoledronic acid, which is Food and Drug administration (FDA) approved medication for treating multiple bone related diseases and different types of cancers (Mahmood et al. 2020; Harel et al. 2019).

9.6.6 Peptide Inhibitors

Various small molecules have been produced with peptide inhibitors that block the interaction which can cause significant reduction in tumor progression. Plasmin is a protease that can cleave uPAR, uPA, and metalloproteases. The most receptive area of uPAR is in between D1 and D2 domains linker region. If the D1 domain is missing, then uPAR do not bind to uPA's function in cell migration. Similarly, a non-competitive inhibition of uPA–uPAR interaction may cause decrease in tumor growth and metastasis (Guo et al. 2000).

Another peptide inhibitor of uPAR, UPARANT (cenupatide) can block VEGF directed angiogenesis (Carriero et al. 2014). Moreover, Å6 is capped peptide which

is noncompetitive antagonist of uPA-uPAR that can enhance anti-cancer effect if given in combination with tamoxifen and cisplatin (Boyd et al. 2003; Piotrowicz et al. 2011). Also, peptide-based inhibitor of uPAR is AE120 that can block the uPA-uPAR binding and reduce the invasiveness of human carcinoma cells (Piotrowicz et al. 2011; Guo et al. 2002).

In addition to all mentioned above, there are studies revealing peptide inhibitors against uPAR including P25 (van der Pluijm et al. 2001), M25 (Simon et al. 2000), α325 (Chaurasia et al. 2006), and m.P243–251 (Alexander et al. 2012).

9.6.7 Natural and Chemical Products

Natural products derived from certain herbs are believed to have therapeutic potential against cancer. For example, alkaloid derivatives of Chinese herb Sophora flavescens possess anti-proliferative potential against cancer (Wang et al. 2017). One of the alkaloid compounds is oxymatrine that has the ability to reduce PAI-1 expression effecting component of TGF-β signaling and inducing the E-cadherin (epithelial cell marker), and on the same time decreasing α-Smooth muscle actin (mesenchymal marker) expression reversing the epithelial-to- mesenchymal state (EMT), thereby, reducing cancer cell migration and spread of cancer (Wang et al. 2017). Yet, the understanding of complete process and how it takes place at every step warrant further explanation.

Chemically, it is known that PAI-1 levels have been increased in cancers and it was in 1990s that first class inhibitors were developed to some extent (Bryans et al. 1996); however, their potential use in cancer therapeutics was barely known. Tiplaxtinin (PAI-039), a chemical product has the ability to reduce cancer cell proliferation and angiogenesis. It also elevates apoptosis by halting PAI-1 expression in cancers (Gomes-Giacoia et al. 2013). XR5967 is a di-keto-piperazine, has the potential to decrease cell migration, invasion, and angiogenesis among cancer cells (Brooks et al. 2004). Two anti-PAI-1 agents (TM5441 and TM5275) possess antiproliferative ability against cancer cells (MDA-MB-231 breast cancer cell line) (Masuda et al. 2013).

9.6.8 Aptamers

RNA aptamers are called as single stranded oligonucleotide that can bind to particular site and fold into complex structures. They have the high affinity and specificity for binding and hence can be used for therapeutics and in some cases for diagnostics too (Zhou et al. 2012). It was identified by Blake et al. that two RNA aptamers SM-20 and WT-15 can disrupt PAI-1 disruption with vitronectin and heparin after binding with high affinity and specificity (Blake et al. 2009). Whereas, disruption of PAI-1 and vitronectin interaction shows anti-metastatic potential as without disruption they promotes metastasis by detaching tumor cells from extracellular matrix (Sundaram et al. 2013).

The two aptamers are also known to reduce invasion and migration of cancer cells in highly invasive breast cancer cells (Fortenberry et al. 2016). To inhibit PAI-1, an alternate way is using peptide paionin-4-D1D2 that can convert PAI-1 into inactive form (Mathiasen et al. 2008). Still, in vivo and preclinical studies are required to understand role of RNA aptamers in cancer therapy.

9.7 Conclusion and Future Directions

Through the research studies done so far, it is clear that uPA and uPAR axis has multiple effect at numerous stages of cancer and they possess diagnostic and therapeutic potential as well. Apart from this various abnormalities in the component level of uPA system, it has also been noted that they possess multiple roles in various malignancies particularly in case of breast cancer. It has been observed that they have association with suppression of tumor and metastasis.

However, further analysis of pathways for metastasis needs to be elucidated further so that their specific prognostic, diagnostic, and therapeutic role can be identified to get the maximum benefits with or without combination of different targeted therapies and chemotherapeutic agents. Based on the preclinical data provided here, in future improved cancer associated diagnostic and therapeutic potential can be evaluated further which can improve the morbidity and mortality associated with breast cancers.

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MicroRNAs and Noncoding RNAs as Gene Regulators and Potential Therapeutic Agents

10

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Abstract

Breast cancer is highest prevailing cancer that incredibly affects women also globally it is the prominent reason of cancer-related mortality. The untranslated transcripts, called noncoding RNAs can be classified in short, mild, and long according to their length 19–31 nucleotides, 20–200 nucleotides, and >200 nucleotides, respectively. Among them, microRNAs are crucial in breast cancer. MicroRNAs efficient to regulate gene expression by regulating diverse cellular pathways. Breast cancer develops and progresses via either oncomiR (oncogenic miRNA) or else tsmiR (tumor suppressor miRNA). It is involved in certain regulatory pathways including PI3 kinases, Wnt/β-catenin, STAT, and HIF 1α which are hallmarks for tumor suppression or progression. MicroRNAs control the manifestation of multiple genes via sequence precise hybridization at 3′ untranslated region (UTR) of mRNAs. Expressively enhanced miRNAs manifestation is capable for alterations in cancer progression, initiation, invasion, migration, metastasis, and drug resistance. In the current scenario, miRNAs signature for drugs known as miRNA therapeutics is under investigation. Based upon the antisense technology very potential oligonucleotide targeted against miRNA which enhanced treatment effect of disease. miR-125b, miR-21, miR-155, and miR-145 frequently dysregulated and associated with the breast cancer. In future, miRNA-targeted therapeutics develop as a potential therapeutic as it is appropriate for delivery system of miRNA through improved efficacy and also specificity,

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miRNA-based therapeutics evolve on or after phase I/II to phase III. The present chapter will showcase combinatorial approaches with miRNAs therapy aimed at breast cancer.

Keywords

Breast cancer · Noncoding RNA · miRNAs · Gene regulation · Therapeutic agent

10.1 Introduction

Cancer states as a complex disease associated with abnormal genetic change of the tumor cells. Several oncogenes and tumor supressor genes plays important role in cancer development and progression and also involves in degradation of various cell signalling pathways (Shah et al. 2015; Shah 2016; Shah et al. 2017, 2018). Neoplastic transformation includes a multistep process including combined genetic and epigenetic changes at multiple levels (Reddy 2015). Malignant tumor occurs in the cells of breast known as a breast cancer. Commonly, breast cancer arises into cells of the lobules or the ducts, respectively, it is a milk secreting gland and the passages which are draw off milk from lobules to nipple. Globally, breast cancer is frequently identified cancer in female. As per WHO (World health organization), there are 22, 61,419 (11.7%) new cases of breast cancer arise, about 6, 84,996 (6.9%) mortality observed worldwide in 2020 only. Globally, 24.5% female suffering from breast cancer (Sung et al. 2021). There are 1,78,361 (13.5%) breast cancer cases which are diagnosed and 90,408 (10.6%) mortalities reported due to breast cancer in India. It is a leading malignant disorder in Indian women and around 26.3% women distress with breast cancer in India (Mathur et al. 2020). Possible risk factors associated with breast cancer development are aging, sex, heredity, estrogen, unhealthy lifestyle as well as gene mutations (Sun et al. 2017). Oncogenic viruses like Human Papillomavirus (HPV) also plays vital role for onset of cancer (Turakhiya et al. 2018; Patel et al. 2021b). Recent study reveals various plant-based natural inhibitors and metabolites for the cancer (Juneja et al. 2021; Patel et al. 2021a).

MicroRNAs belong to small noncoding RNAs (ncRNAs), generally 20–25 elongated nucleotides and proficient in regulating gene expression next to posttranscriptional level. miRNA mediated gene expression takes place via mRNA cleavage, mRNA disintegration and translational repression also control apoptosis, differentiation, and cell division (Filipowicz et al. 2008). Attachment to the coding region and untranslated regions (UTRs) of miRNAs exhibits their regulatory efficiency, and this binding influences and prevents translation or ruin of miRNAs (Singh and Mo 2013). MicroRNAs playing an important role in metabolism, apoptosis, differentiation, development, and several human diseases including cancer. miRNAs serve as tumor suppressive or oncogenic and regulate progression of breast cancer through regulation networks (Xia et al. 2020). The oncomiRs are over-expressed and suppressed the translation of the tumor suppressor gene which accelerates tumor growth. OncomiR significantly enhances the cell proliferation, migration, and

invasion (Yang and Liu 2020). Tumor suppressor miRNAs typically inhibit oncogenes translation and prevent tumor formation and inhibit progression (Jansson and Lund 2012). Epigenetics plays crucial role in cancer prognosis, CRISPR-CaS9 teachnology is need to cure genetic disorder including cancer (Nalla and Shah 2021).

10.2 Biogenesis of miRNA

miRNA biogenesis is initiated through processing of RNA polymerase II/III transcripts via post- or co-transcriptionally (Ha and Kim 2014). Currently recognized miRNAs state as an intragenic and processed via introns and comparatively limited exons of protein encoding genes, whereas left over transcribed individually and regulated via own promoters are known as intergenic (Kim and Kim 2007). Occasionally miRNAs transcribes as a long transcript recognized as clusters, which might look alike seed regions, in this state it means by family (Tanzer and Stadler 2004). miRNA biogenesis categorized as canonical and non-canonical pathways.

10.2.1 Canonical Pathway Aimed at miRNA Biogenesis

Canonical biogenesis pathway known as foremost mode via miRNA is processed. In the canonical pathway, pri-miRNAs transcribe as of their respective genes then through microprocessor complex, comprising of DiGeorge Syndrome Critical Region 8 (DGCR8) that is RNA binding protein and a ribonuclease III enzyme, Drosha pri-miRNAs translate into pre-miRNAs (Denli et al. 2004).

10.2.2 Non-canonical Pathway Aimed at miRNA Biogenesis

Non-canonical pathway includes diverse groups of the proteins involved mostly Drosha, exporting 5, AGO2, and Dicer (Fig. 10.1). Commonly non-canonical way of miRNA biogenesis divided as following: (1) Dicer-independent pathways and (2) Drosha/DGCR8-independent (Ruby et al. 2007). Pre-miRNAs formed via Drosha/DGCR8-independent pathway similar like a Dicer substrate (Babiarz et al. 2008).

10.3 MicroRNAs as a Gene Regulator

miRNAs attach to exact sequence on 3′ UTR of aimed mRNAs also enhance translational suppression, decapping, and deadenylation of mRNA. Binding sites for miRNA also identified in other mRNA regions comprising 5′ UTR also coding regions reported for the enrichment of the transcription (Cannell et al. 2008).

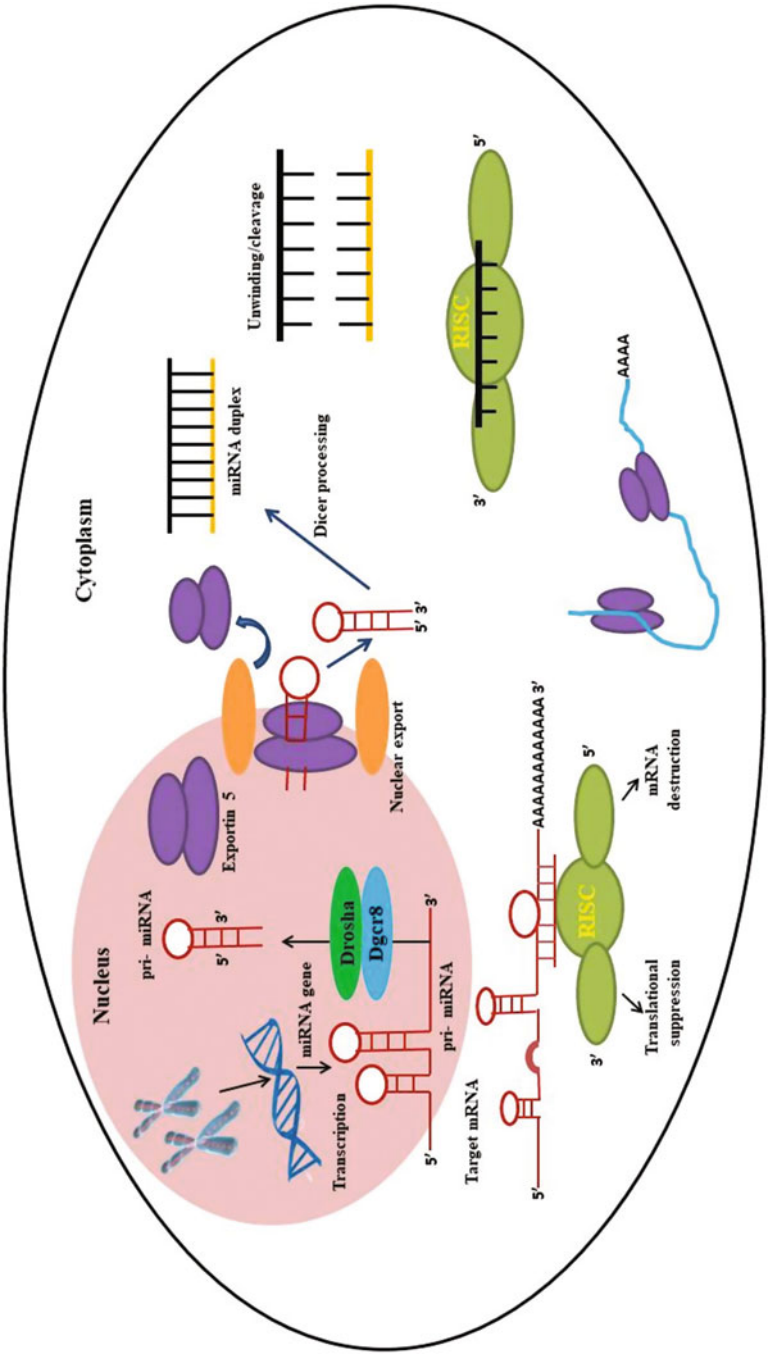

Fig. 10.1 Biogenesis of miRNA

10.3.1 MicroRNAs Mediated Gene Silencing

MicroRNAs (miRNAs) serve by means of posttranscriptional controllers for the expression of gene which is essential for processes containing cell growth, differentiation, proliferation, development, and metabolism (Jo et al. 2015). MicroRNAs (miRNAs) state as preserved type small noncoding RNAs and accumulate in miRNA-induced silencing complexes (miRISCs) via Argonaute protein for posttranscriptional silencing of the complementary mRNA targets. Silencing accomplished through various translational suppression and mRNA destabilization afterward it impart to the steady-state suppression in animal cell cultures. mRNA target degradation started by deadenylation along with decapping and 5′-to-3′ exonucleolytic decay (Ameres et al. 2010).

10.3.2 MicroRNA-Mediated Translational Activation

Although ultimate research describes on the mechanism of miRNAs gene expression inhibition, some describe upregulated expression of gene through miRNAs. AGO2 with additional protein associated with miRNA-protein complex (microRNPs), Fragile-x-mental retardation related protein 1 (FXR1) related by means of AU-rich elements (AREs) next to 3′ UTR and stimulate translation in serum starving cell. Various miRNAs, together with let-7, related to AGO2 and FXR1 and initiate translation in the course of arrest of cell cycle and prevent translation of multiplied cells (Vasudevan and Steitz 2007) In quiescent cell (oocytes), there is upregulated expression of gene through miRNAs.

miRNA driven translational initiation includes AGO2 and FXR1 rather than GW182 (Truesdell et al. 2012). At the time of amino acid starvation, gene activation via miRNAs involves attachment towards 5′ UTR of mRNAs encoding ribosomal proteins; consequently signifying that miRNA-guided upregulated gene expression takes place under particular circumstances (Orom et al. 2008).

10.4 Significance of Noncoding RNA into the Breast Cancer

10.4.1 miRNA and Breast Cancer

microRNAs (miRNA/miRs) play a consequence role in cellular regulation. Several miRNAs are reported as oncogenic and are associated in breast cancer progression. This oncogenic miRNAs are responsible for metastasis and cancer development (Braicu et al. 2013). Circulating miRNAs detection also predicts cancer at early stage (Thermann and Hentze 2007). miR-200c, miR-10b, miR-34, let-7, and miR-155 decrease, while miR-221, miR-21, and miR-195 increase in the blood plasma. miR-21, miR-125b, and miR-145 significantly upregulated, whereas let-7, miR-221, and miR-210 downregulated in breast cancer (Gezer et al. 2014). Breast cancer patients including in postmenopausal state miR-146a, miR-499, and

miR-196a-2 whereas miR- 196a-2 detected in female with premenopausal condition and varies after healthy persons (Alshatwi et al. 2012). Furthermore, miRNAs co-express in the serum as well as in tumor cells and miR-23a, miR-23b, miR-24, miR-25, miR-29a, miR-103, and miR222 upregulated. High level of miR-222 observed in serum which serves as a precision biomarker (Wu et al. 2012).

10.4.1.1 miRNAs Serve as a Tumor Suppressor

10.4.1.1.1 Let-7 Family

Lethal-7 (let-7) family able to suppress the tumor growth and including ten subtypes let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7 g, let-7i, miR-202, and miR-98. Typical function of family let-7 includes cellular linkage, muscle formation as well as embryogenesis. Lin 28 regulated the let-7 biogenesis as a posttranscriptional repressor. DNA methylation by DNA methyl transferases like DNMT3 and DNMT1 is one of the reasons that change let-7 expression (Brueckner et al. 2007). Downregulation of let-7 was observed at an early stage of the breast cancer. Let-7 family adversely regulates numerous well-known oncoproteins like RAS, c-Myc, HMGA2,CDK6, CDC25A, and cyclin D2. Let-7 family targets several signaling pathways like JAK/STAT3 and c-Myc which are vital in cell development (Wang et al. 2010).

10.4.1.1.2 miR-200 Family

miR-200 family comprises miR-200c, miR-200b, miR-200a, miR-429, and miR-141. miR-200 together suppresses the EMT that is epithelial to mesenchymal transition with the help of ZEB1 and also ZEB2 E-cadherin transcriptional repressor. E-cadherin accompanied with miR-200 family proficient to alter cell morphology. miR-200c regulates the migration, invasion, elongation, and cell fiber formation, and its downregulation has shown correlation with the drug resistance (Jurmeister et al. 2012). It also regulates several pathways PLCG1, TGF-β2, ZEB, FAP-1, and BMI1, which plays a crucial role in tumor suppression. Higher expression of miR-200 family promotes secretion of the IGFBP4 also Tinag1 like metastasis suppressors (Korpal et al. 2008).

10.4.1.1.3 miR-205 Family

miR-205 family found on chromosome 1q32.2 and acts as oncosuppressive miRNA in breast cancer. miR-205 expression is higher in the lobules and normal mammary ducts as compared to cancerous tissue. In metastases, miR-205 family is downregulated. Relating miR-205, it is downregulated in the serum of breast cancer signifying that miR-205 is convenient for the clinical diagnostic biomarker for the breast cancers (Zhang et al. 2015). It targets ZEB1, SIP1, HMGB3, HMGB1, RunX2, ITGA5, VEGF-A, Ubc13, and FGF2. Both E2F1 and LAMC1 are experimentally validated target for miR-205. In PI3K/Akt pathway, it targets Her3 and also enhances the response towards gefitinib and lapatinib drugs which are tyrosine kinase inhibitors (Piovan et al. 2012).

10.4.1.1.4 miR-145 Family

Downregulated miR-145 observed in breast cancer tissue might play as the potential biomarker for early detection as it expresses at early stage of breast cancer. miR-145 targets estrogen receptor- α (ER- α) protein manifestation as well as enhances apoptosis. miR-145 directly targets renowned oncogene c-Myc and inhibits the tumor cell growth. As well have shown significance in P53 regulation (Kim et al. 2011). miR-145 represses metastasis gene mucin 1 (MUC1), MUC1 suppression cause decreases β-catenin and cadherin 1 both have high oncogenic potential. Adenovirus constructed miR-145 and 5-FU combination shows anti-tumor effect. RAS and VEGF-A target and regulate by miR-145 and prevent tumor angiogenesis (Zou et al. 2012).

10.4.1.2 miRNA Serves as an Oncogene

Various miRNAs repress the manifestation of anti-oncogenes in metastasis, invasion, cell proliferation, and apoptosis and upregulated and extremely expressed in BC (Zhang et al. 2007). The oncogenic miRNAs include miR-10, miR-15, miR-16, miR-17, miR-18, miR-19, miR-20, miR-155, miR-21, miR-92, and miR-569.

10.4.1.2.1 miR-10

miR-10 family includes subtypes miR-10a as well as miR-10b where both actively involve in the development and metastasis, respectively. In the metastasis, high expression of miR-10b extremely express and encourage migration cell and invasion via targeting HOXD10 gene and E- cadherin probable target for the miR-10b it's effect the tumor size, clinical staging and tumor grading. miR-10b hinders Tiam 1-(T lymphoma invasion and metastasis) mediated Rac activation as well as regulates cell adhesion and EMT via E-cadherin and reduces metastasis. Thus, miR-10b established as an advance progression biomarker for the BC (Ma et al. 2010a, b; Moriarty et al. 2010).

10.4.1.2.2 miR-17 ~ 92

miR-17 ~ 92 situated at part of DNA which amplifies in human B-cell lymphoma subtypes containing miR-19b, miR-18b, miR-93, miR-106, miR-20a, and miR-92 (Fassina et al. 2012). Higher expression of miR-17-92 is elevated in triple negative breast cancer (TNBC) but reduced in estrogen receptor (ER)-positive breast cancer (ERPBC). In vitro analysis reveals that miR-17 ~ 92 highly expresses in invasive BC but not into non-invasive cell. Likewise, downregulated miR-17 ~ 92 represses metastasis in invasive MDA-MB-231 cell (Li et al. 2011).

10.4.1.2.3 miR-21

miR-21 is crucial miRNA related towards breast cancer as it is vital in cell migration, invasion, and tumor progression. Tumor suppressor tropomyosin 1 (TPM1) is probable target for miR-21 and it enhances the breast cancer development via suppressing Programmed cell death protein 4 (PDCD4) and maspin expression (Si et al. 2007).

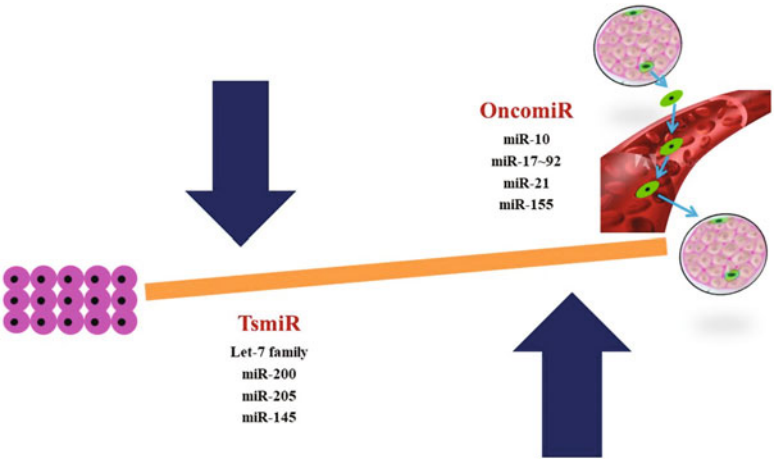

Fig. 10.2 miRNAs as a tumor suppressor and oncogenes

10.4.1.2.4 miR-155

miR-155 upregulated in various human cancers. miR-155 targets repressor of cytokine signaling 1 (SOCS1) into human breast cancer and negative regulation of the SOCS1 through miR-155. miR-155 aids by means of potent target for the breast cancer management (Jiang et al. 2010) (Fig. 10.2).

10.4.1.3 Metastatic MicroRNAs in Breast Cancer

Migration of cancer as of primary tumor towards the various body parts states as a metastasis cancer. Mesenchymal to epithelial transition (MET) and epithelial to mesenchymal transition (EMT) are responsible for the metastasis (Yang and Weinberg 2008). The miR-9, miR-29a. miR-15547, miR-10b, miR-21, and miR-373/520 families stimulate the metastasis in breast cancer. miR-9 plays a role in cell focusing on E-cadherin, enhances vascular endothelial growth factor (VEGF). EMT and metastasis of breast cancer regulate by tristetraprolin (Ma et al. 2010a, b). Invasion and migration enhance by miR-373/520 with the assistance of CD44. miR-373 and CD44 connected with each other and enhance the breast cancer metastasis. Various subgroups including miRNA-17/20, miRNA-7, miRNA-22, miRNA-31, miRNA-30, miRNA-145, miRNA-126, miRNA- 146, miRNA-206, miRNA-335, miRNA-205, miRNA-193b, and let-7 prevent metastasis. Several cellular processes regulate by epidermal growth factor receptor (EGFR) and EGFR associated with miR-7 and enhance the metastasis and migration in BC (Webster et al. 2009). miR-17/20 targets cyclin D1 at G1-S phase so cyclin D1 is overexpress in breast cancer. miRNA and cyclin D1 inhibit the invasion, cytokeratin 8 and connect IL-8 (Reddy et al. 2008). Upregulation of miRNA reduces the cell mortality via CDK6, Sp1, and SIRT1. Estrogen receptor α targets by miR-22 and hinders the cell proliferation (Pandey and Picard 2009). Metastasis prevents by miR-145 via targeting c-myc, mucin-1, IRS-1, and JAM-A. EGFR induces via miR-146 and

prevents the metastasis also it downregulates interleukin and TNF associated factor 6 and controls NF-kB. Highly expressed miR-30 suppresses the cell growth via targeting Ubc9 and are a significant role for the cancer progression (Wu et al. 2009a, b).

10.4.2 Role of lncRNAs into Breast Cancer

Several lncRNAs (long noncoding RNAs) include LUCAT1, lncRNA ES1 NEAT1, FGF13-AS1, and lncRNA-Hh stated to be commonly involved and upregulated signaling pathways and modulate the stem cell factors (Hedgehog, myc, Wnt/β-catenin, KLF4, NANOG SOX2, and OCT4). Zheng et al. (2019) have shown potentiality for the enhancement of stemness in breast cancer and consequent invasion, tumor progression, and metastasis (Ma et al. 2019) (Tables 10.1 and 10.2).

10.5 miRNAs-Based Targeting Therapy Aimed at Breast Cancer

10.5.1 Nucleic Acid Dependent Therapies

Chemically altered nucleic acid uses to re-establish common action of miRNAs. Nucleic acid-founded therapeutic approaches divided into two classes: (1) miRNA replacement and (2) anti-miRNA.

10.5.1.1 Therapies for miRNA Replacement

10.5.1.1.1 miRNA Mimics

miRNA replacing approach appears to be a favorable therapy for developing tools to substitute downregulation of tsmiRs and overcome breast cancer (Cui et al. 2014; Sun et al. 2014). Downregulated Let-7 causes metastasis of breast cancer cell, to overcome this lentiviral let-7, miRNA vector can be used to decrease the cell proliferation. BRCA1 upregulates the tumor suppressor miR-145 and miR-205. Elimination of BRCA1 decreases these tumor suppressor miRNAs. Function of BRCA1 restores by miR-145 and miR-205 mimics (Chang et al. 2011). Via miRNA replacement therapy reform the function of downregulated tumor suppressors like miR-205, miR-335, miR-126 miR-451 and Let-7.

10.5.1.2 Anti-miRNA Therapy

To suppress the function of oncomiRs, anti-miRNA therapy is useful. There are four strategies for the suppression of oncomiRs: (1) anti-miRNA oligonucleotides (AMO), (2) miRNA sponges, (3) miRNA antagomirs, (4) locked nucleic acid (LNA).

AMOs are 17–22 nucleotide long, single-stranded, chemically altered antisense oligonucleotides and synthesized to the complementary of the miRNA of interest. LNA is known as a modified AMO (Vester and Wengel 2004). LNA-modified

Table 10.1 miRNA in breast cancer with associated pathways

miRNA	Type	Level of expression	Targets	Pathways	Reference
miR-155	TsmiR	↑	FOXO3A, RHOA, SOCS1	STAT3, proliferation, TGFβ signaling	Jiang et al. (2010); Kong et al. (2010); Kong et al. (2008)
miR-145	TsmiR	↑	ERA, RTKN, MUC1	Apoptosis, invasion, proliferation	Sachdeva and Mo (2010); Spizzo et al. (2010); Wang et al. (2009)
miR-31	TsmiR	↑/↓	RDX, RHOA, ITGA5	Metastasis	Valastyan et al. (2009, 2010)
miR-125b	TsmiR	↑/↓	ERA, RTKN, HER2, CRAF, BAK, MUC1	Apoptosis, migration, and proliferation	Scott et al. (2007); Zhou et al. (2010); Hofmann et al. (2009)
miR-21	OncomiR	↑	PDCD4, PTEN, TPM1, BCL2 MASPIN, RHOB, MMP3	EMT, invasion, inflammatory signals, migration, apoptosis	Carpenter et al. (2015); Song et al. (2010); Qi et al. (2009); Huang et al. (2008); Qian et al. (2009)
miR-205	TsmiR	↓	HER3, EMT, VEGFA	Invasion, proliferation	Iorio et al. (2005); Wu et al. (2009a, b); Gregory et al. (2008)
miR-210	OncomiR	↑	RAD52, MNT	Hypoxia	Camps et al. (2008); Zhang et al. (2009)
miR-196A	OncomiR	↑	ANXA1	Proliferation, apoptosis	Luthra et al. (2008)
miR-3646	OncomiR	↑	GSK-3β	β-catenin	Zhang et al. (2016)
miR-34A	OncomiR	↑	CCND1, BCL2	Apoptosis	Kastl et al. (2012)
miR-222	OncomiR	↑	PTEN	PTEN, Akt/FOXP1	Shen et al. (2017)
miR-944	OncomiR	↑	BNIP3	Cell proliferation, invasion, migration	He et al. (2016)
miR-141	OncomiR	↑	EIF4E	Apoptosis	Yao et al. (2015)
miR-34	TsmiR	↓	NOTCH, BCL2	Apoptosis, NOTCH	Kato et al. (2009)
miR-520h	OncomiR	↑	DAPK2	PI3K/Akt	Su et al. (2016)

(continued)

Table 10.1 (continued)

miRNA	Type	Level of expression	Targets	Pathways	Reference
miR-22	TsmiR	↓	HER3, CDC25C, SP1, ERα, CDK6	Estrogen signaling	Pandey and Picard (2009)
miR-146	TsmiR	↓	NF-kB	Inflammatory signal	Bhaumik et al. (2008)
miR-335	TsmiR	↓	SOX4, PTPRN2, MERTK, TNC	Metastasis	Tavazoie et al. (2008)
miR-221	TsmiR	↓	P57, P27	Wnt/β-catenin	Rao et al. (2011)
miR-191	OncomiR	↑	BDNF, SATB1, CDK6	Estrogen signaling	Nagpal et al. (2013)
miR-148a/152	TsmiR	↓	DNMT1, IGF-IR and IRS1	PKM2/IGF-IR	Xu et al. (2013)
miR-20	OncomiR	↑	E2F	Proliferation	Trompeter et al. (2011)
miR-126	TsmiR	↓	PIK3R2 and VEGFA	VEGF/PI3K/AKT	Zhu et al. (2011)
miR-98	TsmiR	↓	MMP11, ALK4	Angiogenesis, invasion	Siragam et al. (2012)
miR-519c	TsmiR	↓	HIF-1α	Hypoxia	Cha et al. (2010)
miR-140-5p	TsmiR	↓	VEGFA	Metastasis, angiogenesis	Lu et al. (2017)
miR-494	TsmiR	↓	PTEN	Akt, NF-kB, mTOR	Liu et al. (2012)
miR-19	OncomiR	↑	Tissue factor	Angiogenesis, metastasis	Zhang et al. (2011)
miR-29A	OncomiR	↑	PTEN	Apoptosis	Zhong et al. (2013)
miR-129-3p	OncomiR	↑	CP110	Cell cycle, apoptosis, cell explosion	Zhang et al. (2015)
miR-218	TsmiR	↓	BRCA1	DNA repair, cell enhancement, invasion	He et al. (2016)
miR-302b	TsmiR	↓	E2F1	E2f1-ATM axis	Cataldo et al. (2016)
miR-638	TsmiR	↓	BRCA1	DNA repair, invasion, and	Tan et al. (2014)

(continued)

Table 10.1 (continued)

miRNA	Type	Level of expression	Targets	Pathways	Reference
				cell proliferation	
miR-199a-3p	TsmiR	↓	TFAM	Mitochondrial biogenesis	Fan et al. (2017)
miR-16	OncomiR	↑	CCNJ, FUBP1	PI3K/Akt	Esteva et al. (2010)
miR-139-5p	OncomiR	↑	Notch1	Cell growth, apoptosis	Zhang et al. (2015)
miR-214	OncomiR	↑	UCP2	Autophagy	Yu et al. (2015)
miR-100	OncomiR	↑	mTOR	Cell progression, survival	Zhang et al. (2016)
miR-210	OncomiR	↑	RAD52	Invasion, proliferation, migration	Jung et al. (2012)
miR-451	OncomiR	↑	Bcl-2	Apoptosis	Gu et al. (2015)
miR-892b	TsmiR	↓	TRAF2, TAK1, and TAB3	NF-kB	Jiang et al. (2016)
miR-196B	OncomiR	↑	HOXD10	Hox pathway	Plummer et al. (2013)
miR-205	TsmiR	↓	YAP1	miR-205/ YAP1, angiogenesis, metastasis	Du et al. (2017); Zhang and Fan (2015)
miR-200	OncomiR	↑	ZEB1, ZEB2	EMT	Tomar et al. (2020)
miR-143	OncomiR	↑	FOSL2	EMT, metastasis	Tomar et al. (2020)
miR-18	OncomiR	↑	SMAD7	EMT, metastasis	Tomar et al. (2020)
miR-467	OncomiR	↑	TSP-1	Angiogenesis	Bhattacharyya et al. (2012); Bishnoi et al. (2016)
miR-17-92	TsmiR	↓	HIF-1α	Angiogenesis, hypoxia	Taguchi et al. (2008)
miR-19a	OncomiR	↑	PTEN	Cellular progression, Th1 immune response	Anfossi et al. (2014)
miR-206	TsmiR	↓	MAPK3, VEGF, and SOX9	Angiogenesis and invasion	Liang et al. (2016)

Table 10.2 lncRNA in breast cancer with associated pathways

lnc RNA	Type	Level of expression	Targets	Pathways	References
HOTAIR	Oncogene	↑	BRCA1, PTEN	PI3K/AKT-BAD pathway, HOXD10	Hansji et al. (2014)
ACNR	Tumor suppressor	↓	TGF-β	Metastasis, invasion	Li et al. (2017)
MEG 3	Tumor suppressor	↓	p53	p53	Youness and Gad (2019)
PLNCRNA-1	Oncogene	↑	TGF-β	Metastasis, invasion, apoptosis	Youness and Gad (2019)
NKILA	Oncogene	↑	NF-kB	EMT	Wu et al. (2018)
EPIC 1	Oncogene	↑	Myc	Cell cycle	Wang et al. (2018)
PTENP1	Tumor suppressor	↓	PTEN	Apoptosis	Youness and Gad (2019)
PVT-1	Oncogene	↑	KLF-5, β-catenin	WNT/β-catenin	Youness and Gad (2019)
MALAT-1	Oncogene	↑/↓	AKT, p53	Apoptosis	Meseure et al. (2016)
PVT-1	Oncogene	↑	KLF-5, β-catenin	WNT/β-catenin	Youness and Gad (2019)
LINK-A	Oncogene	↑	HIF-1α	Hypoxia pathway	Youness and Gad (2019)
CCAT2	Oncogene	↑	ERK	MAPK	Caia et al. (2016)
NEAT	Oncogene	↑	ZEB1, RAS	RAS, MAPK, RSF1	Shin et al. (2019)
GAS5	Tumor suppressor	↓	PTEN	Apoptosis	Li et al. (2016)
UCA1	Oncogene	↑	mTOR, β-catenin	mTOR, WNT/β-catenin	Saunders-Hastings et al. (2016)
BCAR4	Oncogene	↑	SNIP1, PNUTS	Hedgehog/GLI 2 signaling transduction	Youness and Gad (2019)

antisense oligonucleotides exhibit advanced affinity and thermal firmness with target molecules.

Chemically modified antagomirs proficient to altered ss-RNA molecule which is 23 nucleotide long and corresponding to desired miRNAs enrich RNA stability and inhibit it from ruin, miR-10b silencing with the help of antagomir prevent metastasis in mouse tumor model, through antagomirs expressively reduced miR-10b levels and enhance miR-10b target, HOXD10 (Ma et al. 2010a, b).

miRNA sponges comprise multiple binding sites and this sites contest with endogenous miRNA target for binding and inhibit the oncomiRs. miR-9 upregulated in breast cancer and inhibits manifestation of CDH1 that is tumor suppressor. miRNA sponges made up of 4 miR-9 binding positions proficiently inhibit activity

of the miR-9 in addition re-established endogenous manisfection for the CDH1 and subsequently suppress the metastasis (Hildebrandt et al. 2010).

10.6 Obstacles in Emerging miRNA Founded Therapeutics

Role of miRNA in cancer treatment would add abundant impact for the survival of the cancer patients. Although it also have several challenges over the advantages for effective delivery. Major obstacle of miRNA therapy for cancer treatment is to transport miRNA mimics or miRNA antagonists aimed towards tumor with appropriate penetration in cancerous cell (Jain and Stylianopoulos 2010).

Furthermore challenges with respect to miRNA based delivery that is tolerate miRNAs integrity and stability into flow. Unmodified or unprotected miRNAs promptly ruined by RNase A-type alike nucleases and vanish in blood circulation and naked miRNAs quickly vanish through the renal excretion and effects small half-life in circulation (Yu et al. 2009). Moreover, miRNAs proficient to enhance immune toxicity. miRNA systemic delivery stimulates innate immune system, subsequent into the rapid toxicities and substantial side effects. Systemic administrated miRNA duplexes stimulate the excretion of type I interferons (IFNs) as well as inflammatory cytokines via toll-like receptors (TLRs).

Off-target consequence for the miRNAs is a major issue concerning with miRNA therapy. Subsequently they are intended to aim several pathways through inadequate match in the 3′ UTR, miRNAs proficient for the unexpected gene silencing of the several tumor suppressor genes. It may enhance latent toxicities and consequently decrease therapeutic effect. A combinative approach could improve miRNA therapy for the inhibition of undesirable off-target impact (Van Dongen et al. 2008). Multifunctional nanoparticles transport miRNA as well as siRNA and trigger various tumor suppressor miRNAs to evade off-target phenomenon and inhibit several oncogenic pathways (Chen et al. 2015).

10.7 Strategies of Delivery for the miRNA Dependent Therapeutics

To increase of effectiveness for miRNA delivery, there are two major approaches might be used: local delivery and systemic delivery. Local delivery perhaps useful as it requires lower miRNA doses, selective deliver miRNAs to the target and less toxicity (Bader et al. 2011). Moreover, it is the only method for solid tumors, not applied for the leukemia like hematological malignancies, and not appropriate to the metastasis cancer cells. Hence, systemic delivery is an ideal way for administration and offers superior competence of the biodistribution of drugs to cancer tissues. Substantial development is prepared in initial systemic miRNA delivery approaches. At present, viral as well as nonviral miRNA delivery systems are convenient; also definite advantages and disadvantages intended for each and every approach describe in Table 10.3.

Table 10.3 Strategies for miRNA dependent delivery

No.	Techniques for delivery	Benefits	Drawbacks
#	**Nonviral vectors**	Less immunogenicity, easy to use, low cost	Lower proficiency
1.	Lipid-based vectors		
(a)	Liposomes		
•	Cationic	Formation of steady nucleic acid lipid particles, it shields RNA molecules inside vesicles	Less in vivo strength
•	Anionic		
•	Neutral		
2.	Polymeric vectors		
(a)	Polyethylenimine (PEI)	Elevated structural and configuration variability, lesser toxicity and relevant to use and regulate without any trouble, increase stability, tissue specific and cellular intake	Poorly biodegradable and toxic (PEI), accumulated in the liver (PAMAM)
(b)	Poly lactic-co-glycolic acid (PLGA)		
(c)	Poly amidoamine (PAMAM)		
3.	Inorganic nanoparticles	Lower cytotoxicity, non-immunogenic, greater in vivo firmness	Extended duration colloidal stability in aqueous solution in the lack of surfactants, non-specific binding affinity
#	**Viral vectors**		
	Adenovirus, lentivirus, and retrovirus	High transfection effectiveness, steady manifestation	Highly immunogenic, higher toxicity, huge scale making is complicated, costly

10.8 Future Perspectives

Breast cancer states as complex and heterogeneous illness. miRNA-based therapeutics seems emerging field because miRNA functions as pleotropic molecules and moderate numerous dysregulated genes and pathways. However, pleotropic role denotes ambiguity because till all the promising molecules targets of each miRNA will not be completely known. For the therapeutic purpose, their activation or inhibition will not entirely controllable from clinical perspective. miRNA-based therapeutics looks undeveloped field yet not reach to clinical approach. Hence, key obstacle in the miRNA-based therapeutics needs to enlarge the knowledge of target and respective pathways. Consequently miR-based therapeutics over the phase II.

10.9 Conclusion

Involvement of miRNAs plays an essential role for the breast cancer development. miRNAs is important in gene expression regulation to accomplish homeostasis. And deregulation of their activity leads to inclusive variety of pathways which are related to various diseases including cancer, therefore numerous prospective for miRNA founded therapeutics in place of precise approach for targeted therapies in breast cancer. In vitro studies reveal that miRNA-based therapeutics restrain the targeted gene expression. However, there are numerous challenges to defeat for successful translate promising in vitro research to effective therapies in clinical practice. Among dysregulated miRNA, highly expressed miRNAs are significant in initial stage breast cancer detection and also support in identification of most relevant treatment targets and accentuate on developing of initial detection and treatment for breast cancer.

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Lipid Biomarkers for Breast Cancer Diagnostics

11

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Abstract

The accurate, early, and fast detection of breast cancer is important for diagnosis and targeted therapies in cancer patients. Traditionally, after examination through mammograms and other tests (ultrasound, magnetic resonance imaging (MRI)) that confirm alterations in breast tissues, a biopsy is performed for breast cancer diagnosis. A biopsy specimen is a tiny piece of the suspicious breast area that is taken out and tested. However, correct and authentic classification of a breast tumor is a difficult challenge even for experienced pathologists. By the recent advancement in new analytical techniques, it is possible to find new ways in cancer research and diagnostics with promising results. Specifically, the search for lipid biomarkers that appear, increase, or decrease in breast cancer patient compared to the normal healthy person can help in cancer diagnosis. In lipidomics study, all lipids present in a confined biological sample are quantified with the help of analytical instruments. Lipidomics provide knowledge about promising biomarkers for detecting the type and degree of breast cancer. This chapter focuses on the study of lipid biomarkers from breast cancer tissues/plasma samples compared to normal tissues/plasma samples using analytical

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techniques. The lipid profile study can open new doors for oncology research with prospects to become a tool for routine analyses in diagnostic centers.

Keywords

Lipidomics · Breast cancer · Cancer diagnostics · Biomarkers · Lipids

11.1 Lipidomics: A Promising Biomarker for Cancer Diagnosis

Metabolomics is a fast-emerging area to offer valuable information about diseases of which a subclass is lipidomics. Lipidomics may be defined as "a study about the content and function of whole lipids in living systems" (Belhaj et al. 2021). Lipids are essential in various cellular mechanisms like cell survival, growth and proliferation, intercellular communications, and apoptosis (Ayala et al. 2014). It has been established that disruption of lipids metabolism is a consequence of various illnesses such as hypertension, diabetes, neurodegenerative diseases, and cancer (Heindel et al. 2017). Lipidomics is the quantitative systematic analysis of lipids and their interaction in cells, tissues, fluids, or organism at a scheduled time. Extensive research in the field of lipidomics suggests that lipids play significant functions in living organisms, particularly in the transformation, advancement, and metastasis of cancer (Yan et al. 2018). Studies found that certain cancer cells thrive off of the energy generated from oxidation of fatty acids. Some types of lipids have been discovered to increase in cancer, for example, ovarian cancer is associated with increased levels of lysophospholipids (Zhao et al. 2019), hepatocellular carcinoma with glycerophospholipids (Cotte et al. 2019), prostate cancer with glycerophospholipids and arginine (Giskeødegård et al. 2015), whereas breast cancer with sphinogolipid-1-phosphate (Aoyagi et al. 2012). The levels of lipids and phospholipids containing choline increase during metastasis of cancer. A correlation of lipid metabolism has been established with colorectal cancer, the serum levels of cholesterol, lipoprotein cholesterol, apolipoprotein A1, and apolipoprotein B were found to decline in colorectal cancer condition, whereas free cholesterol was found on the rise (Zhang et al. 2014). Hence, lipidomics is suggested as a feasible way to monitor the prognosis, diagnosis, and treatment of cancer, and therefore can be considered as a new cancer biomarker.

A recent study compared the plasma lipid profile of healthy individuals and lung cancer patients with different subtypes. A diversity in the lipidomics was observed among different subtypes, and the data was correlated with lipid protein-associated genomic expression (Yu et al. 2017). In general, lipidomics profiling can be extensively utilized for identification and confirmation of biomarkers specific to a certain disease. These biomarkers are sensitive to the subtype and severity of the illness.

Healthy individuals and cancer patients have a variety of lipids that can be assorted for qualitative and quantitative lipidomic profiling. An increase in accuracy and sensitivity with advancement in technology is leading to rapid development in the field of lipidomics. A commonly used method for the analysis and study of

lipidomics is mass spectrometry, a technique which has the capacity to analyze different lipids with varying physico-chemical properties (Yang and Han 2016).

The application of lipids as biomarkers for the detection of cancer has started gradually at clinical level. An abnormal lipid metabolism was found in patients with prostate cancer (Zhou et al. 2012). A recent investigation revealed that only 1 mL of a patient's urine contains various lipid components and can be employed for the diagnosis of breast cancer (Li et al. 2020).

Lipidomics is also helpful in exploring new remedies for cancer and overcoming challenges like drug resistance. Metabolomics of lipids contribute to the resistance phenomenon displayed by the anticancer drugs. Drug-resistant cells have low levels of phosphorylcholine, whereas high levels are found in drug-sensitive cancer cells (Germain et al. 2020). Cell membranes contain lipids as essential components that interact with the drug before triggering death to the cancerous cells. Lipid metabolism of anticancer drugs leads to changes in cancer patients. Lipids can link with proteins directly or indirectly, modifying their structure and function (Casares et al. 2019). Strong antitumor and antimicrobial activities are shown by the substance obtained by complexation of α-lactalbumin and oleic acid. Lipid rafts are microdomains in membranes and are of immense importance in life cycle of microorganisms that initially colonize or induce inflammation. A new strategy suggested to modulate lipid rafts and/or regulate the signaling pathway based on these rafts, for efficiently combating diseases. A huge challenge in clinical application of lipidomics is the complex structure of lipids that lead to their diverse physico-chemical properties (Van Der Meer-Janssen et al. 2010). Lipidomics-based analyses are emerging as an improved method for identification, assessment, and treatment of diseases. Lipid metabolism and their regulation play a role in the development and progress of illnesses. A comprehensive insight on mechanism of lipid metabolism in cancer patients can be obtained by lipidomics, and it also helps in developing personalized drugs for the treatment of diseases (Yan et al. 2018).

11.2 Introduction to Lipids and Lipidomics

Lipids play several important roles in living systems. Lipid bilayer structures around the cell make them relatively independent of the external surroundings (Watson 2015). Lipids are responsible for providing a hydrophobic medium for membrane proteins for efficient functioning and interactions. Lipids containing species give rise to secondary messengers as a result of enzymatic reactions. Besides, aberrant lipid metabolism characteristic of various diseases enticed a lot of research in the field (Giusto et al. 2010). Recent advancements in the field of mass spectrometry and chromatographic techniques have contributed exponentially to the development of lipidomics. Major research in the field of lipidomics focuses on two crucial points; firstly, establishing a link between lipid metabolites/pathways and one's metabolic health and secondly, interpretation of variations in lipid metabolic system or the regulation of these pathways linked to diseases from a physio- and pathological standpoints (Avela and Sirén 2020). That is why, lipidomic studies usually focus on

the extent of modifications/variation of lipids that are indicative of an illness, environmental change, or reaction to a specific diet, medication, genetics, etc.

Very often, the lipid profiles in clinical surveys of patients suffering from a specific disease or individuals with specific genetic profiles serves for detection of a possible biomarker associated with an illness or a particular gene expression when compared to those of healthy controls (Mayeux 2004). A large quantity of analytical data related to lipid profiles has been generated over time. A major challenge now is to statistically analyze the available databases to derive useful information.

11.2.1 Diversity of Lipids

Lipids consist of a large number of discrete molecular structures with diverse functions that might be polar (e.g., phospholipids), apolar (e.g., sterol esters), or neutral (e.g., triglycerides) (Graeve and Janssen 2009). This huge diversity makes it challenging to classify lipids; however, a scheme for lipid classification and nomenclature was put forward by Fahy and coauthors (2009). Lipids are characterized into eight categories based on their chemical structures, hydrophobicity, and hydrophilicity by virtue of different elements. The categories include fatty acyls, glycerophospholipids, glycerolipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides discussed in detail in this chapter.

11.2.2 Lipid Analysis

Traditional approaches for lipid analysis involves pre-fractionation of lipids into classes using techniques like normal phase liquid chromatography, thin layer chromatography, and solid-phase extraction, etc. followed by separation of these lipid classes into individual molecules by high performance liquid chromatography coupled with a detector (Ruiz-Rodriguez et al. 2010). Nonetheless, these traditional techniques are not very sensitive and often utilize large amounts of the sample. Besides this, complex procedures of sample preparation and low resolution make them less desirable.

Gas chromatography (GC) is also used for lipid analysis; however, it involves very lengthy processes like hydrolysis and derivatization, without which lipids are not GC-suitable (Řezanka et al. 2016). GC-based approaches fulfill the requirements of lipidomics with respect to wide distribution of molecular composition and physical properties and extensive array of lipids concentrations. Mass spectrometry-based detection techniques are often employed (Quehenberger et al. 2011). New ionization technologies like matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) for mass spectrometry coupled to liquid chromatography are fast and sensitive approaches for lipidomics (Li et al. 2014). Presently, lipid analysis strategies make use of direct-infusion ESI–MS and ESI–MS/MS, MALDI combined with Fourier transform ion cyclotron resonance and MS (MALDI–FTICR–MS) or

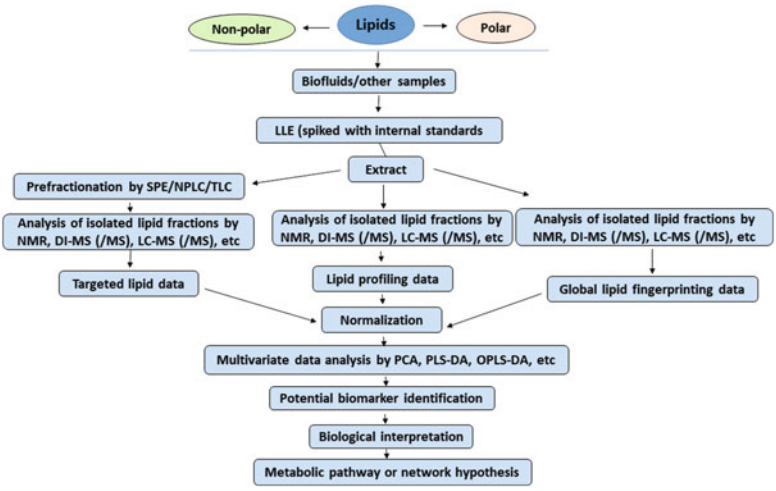

Fig. 11.1 Systematic approaches in lipidomics for biomarker discovery

time-of-flight–MS (MALDI–TOF–MS), LC coupled with ESI–MS or MS/MS, and LC coupled with APCI–MS (Cozzolino and De Giulio 2011). Nuclear magnetic resonance spectroscopy has also been useful for lipid analysis (Yabsley et al. 2012). Despite all these developments, it is nearly impossible to aim at a single technique for measuring and identifying lipids in a sample.

Figure 11.1 summarizes different methods in lipid research which mainly include targeted lipid analysis, lipid profiling, and global lipid fingerprinting. Target lipid analysis focuses on certain lipids that are anticipated to be important, and lipid profiling approach is focused on specific lipid metabolites, a specific class, or a certain pathway. Global lipid profiling includes analysis of as wide a range of lipids as possible. The workflow of all these approaches is almost the same with a little difference. Biological samples with internal standards are extracted. The crude lipid extracts are fractionated into different fractions of lipids. Mass spectrometry detection is employed, using either straight infusion of the sample or chromatographic techniques like gas chromatography or liquid chromatography for the lipid samples analysis. In this fashion, a list of lipid metabolites is established with absolute or relative amounts from varying samples of diseased and healthy individuals. The data is then subjected to analysis after normalization, for identifying metabolites which are characteristics of a particular disease. The acquired results are interpreted and linked with existing biological knowledge so as to link the potential biomarkers with metabolic processes in the living systems. This last step is quite challenging as the new results cannot always be linked to the previous existing knowledge and hence a clear picture of the bioactivity cannot be obtained.

11.3 Metabolism of Lipids

Lipid metabolism involves the synthesis of lipids (both structural and functional lipids) and their degradation to provide for the metabolic requirements of a body. Metabolism of lipids is always in a state of dynamic equilibrium, which means constant oxidation of lipids for energy production goes hand in hand with the synthesis and storage of lipids. Animals obtain their fats from food or synthesize them in their liver. The process of synthesis of fats is called lipogenesis. Lipid metabolism generally refers to the digestion and absorption of dietary lipids for production of energy used for the normal functioning of a body. For metabolism of lipids, they need to be solubilized as they are hydrophobic in nature. The first step involves hydrolysis in the presence of some enzymes in the digestive system. It involves degradation of triglycerides to monoglycerides with the help of an enzyme known as lipase. The degradation continues as the food is transported across the digestion system, along with further mechanical processes until fatty acids are obtained. This is followed by the second step, i.e., absorption of fatty acids. Fatty acids (FAs) are absorbed by the epithelial cells of the intestine. In the cytosol of epithelial cells, FAs and monoglycerides are converted back into triglycerides, where they are packaged with cholesterol and transported in the form of chylomicrons. Chylomicrons are particles that transport digested lipids through bloodstream to the adipose tissues and others where required. Transport proteins called as lipoproteins which are amphipathic in nature are utilized for the transport of hydrophobic membrane lipids, cholesterol, and triglycerides. Chylomicrons are one such class of lipoproteins. The densities of different lipoproteins determine the type of fats they transport, for example, very-low-density lipoproteins transport the triglycerides synthesized by the body and low-density lipoproteins carry cholesterol to the peripheral tissues. Some lipoproteins are produced in the liver, however, not all of them originate from liver.

After the transport of chylomicrons, they undergo a degradation phenomenon at the expense of lipoprotein lipase found in the luminal surface of the endothelial cells, and triglycerides are released as a result. Triglycerides are further decomposed into FAs and glycerols before diffusing into the cells. The residual cholesterol is transported back to the liver through the blood.

In cytosol, glycerol is converted into glyceraldehyde-3-phosphate which participates in the process of glycolysis, where it undergoes oxidation and produces energy. The key step of catabolism of fatty acids occurs in mitochondria. Long fatty acid chains are converted into fatty acyl-CoA so that it passes through the mitochondrial membrane. The catabolism process that starts in the cytoplasm as acyl-CoA synthetase uses the energy from ATP for the catalysis of the addition of coenzyme A to the FAs. The acyl-CoA participates in beta-oxidation. Beta-oxidation includes acetyl-CoA, FADH, and NADH. A number of enzymes take part in the beta-oxidation process, such as acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase.

Besides the dietary fats, lipids stored in the body are also a source of energy. Triacylglycerols, membrane lipids, and cholesterol can be bio-synthesized via various pathways in the body.

11.4 Types of Lipid Biomarkers in Plasma/Serum

Lipids mainly comprise both hydrophobic and hydrophilic molecules which possibly are formed due to condensation of carbocation group of thioesters and/or of isoprene units (Guo et al. 2020).

There are many different techniques for breast cancer diagnosis and evaluation; however, plasma containing lipid biomarkers steal the show due to its promising and specific profiling characteristic proficiency. Lipid biomarkers which are normally found in plasma can be outlined using mass spectrophotometry tools. For comprehensive understanding towards lipid biomarkers, LIPID MAP alliance (Fahy et al. 2009) has been developed for lipid characterizations on the basis of structural and functional differences, for international lipid classification and nomenclature. The enlisted plasma lipid biomarkers can be followed, which were further identified and distinguished statistically using partial least squares regression (PLS regression) method for early breast cancer diagnosis.

1. Fatty acid (FAs)
2. Glycerophospholipids (GPs)
3. Glycerolipids (GLs)
4. Sphingolipids (SPs)
5. Sterol lipids (STs)
6. Prenol lipids (PRs)
7. Polyketides (PKs)
8. Saccharolipid (SL)

On the basis of PLS regression analysis, six different types of lipids were recognized in which PC (20:2/20:5), PC (22:0/24:1), TG (12:0/14:1), and DG (18:1/18:2) were observed in high abundance, while PE (15:0/19:1) and *N*-palmitoyl were detected in lower levels among the samples of breast cancer patients and healthy individuals (Jiang et al. 2017).

11.4.1 Fatty Acids

The chain elongation of an acetyl-CoA primer and malonyl-CoA conjugate makes up a vast group of molecules named as fatty acids (FAs). Fatty acids are the simplest class of lipids and a basic unit of all other lipids. They are saturated or unsaturated hydrocarbons with a carbon chain length of 14–24, with double bonds ranging from 0 to 6 in numbers. They are precursors for a number of other bioactive lipids, for example, eicosanoids work as signaling molecules via special receptors and have

arachidonic acid as their precursor. The key lipid-building blocks of complex lipids are normally represented by fatty acyl structures and the repeated series of methylene group has characteristic property to interfere with hydrophobicity for such type of lipids (Paola Donato et al. 2017).

11.4.2 Glycerophospholipids

Glycerophospholipids include numerous molecular structures produced by glycerol with a functional polar group at sn-3 position through a phosphodiester bond esterified with many combinations of different fatty acids at the sn-1 and sn-2 position of the glycerol. These are also called phospholipids, which might have subdivided into subclasses on the basis of polar headgroup sn-3 position of the glycerol backbone both in eukaryotic organisms and bacteria with typical cell wall and flagella. GPs are also found in archaebacteria with the sn-1 position on glycerol backbone. There are certain precursor molecules of GPs like *Phosphatidic acid* or 1,2-diacyl-*sn*-glycerol-3-phosphate, a cancer biomarker *lysophosphatidic acid* or 1-acyl-*sn*-glycerol-3-phosphate and cardiolipin, a heart muscle constituent named as *diphosphatidylglycerol also belongs to GPs family* (Donato et al. 2013). Glycerophospholipids can be further categorized into glycerophosphatidic acids, glycerophosphocholines, glycerophosphoethanolamines, glycerophosphoserines, glycerophosphoglycerols, and glycerophosphoinositols, depending on the different polar head group. Additionally, lyso-glycerides, with one of –OH groups at the sn-1/sn-2 position of the glycerol backbone intact and the other one esterified to fatty acid, are also counted in the category of glycerophospholipids. All these glycerophospholipids are diverse in their structures and functions and are important components of the cell membranes. They take part in different cellular processes including cell signaling, transport of substrates, and membrane anchoring. Molecules such as lyso-glycerophosphocholines, glycerophosphocholines, glycerophosphoethanolamines, and glycerophosphoinositols are potential biomarkers for illnesses like pancreatic and ovarian cancer, and obesity.

11.4.3 Glycerolipids

The characteristic property of *Glycerolipids* is its presence as main cell's component, primarily as core cell membranes factor and target attachment site for intra- and extracellular proteins. The involvement of glycerolipids is not only included in the participation of cell metabolism but also in cell signaling in eukaryotic cell, either as precursor or as second messenger which originates from membrane.

The fatty acids connected to different positions along the glycerol backbone give rise to different stereoisomers. Monoglycerides exist as 1-, 2-, and 3-isomers, whereas diacylglycerides occur as sn-1,2-, sn-1,3-, and sn-2,3-isomeric forms. The triglycerides are mainly found as a component of animal fats. Three different fatty acids esterified with glycerol give rise to various distinct biological molecules. The

natural fatty acids in the triglycerides are long hydrocarbon chains comprising even the number of carbon atoms and are very critical for biological functions. Triglycerides are crucial for energy storage in the cells and also perform a role as a mediator in metabolic processes and diseases. Alterations and modifications in the triglycerides synthesis and metabolism are indicative of disease pathology.

11.4.4 Sphingolipids

SPs are common in structural similarity and divided into a variety of major classes, which are produced by de novo synthesis with the combination of serine and a long-chain fatty acyl-CoA to develop sphingoid backbone which is further converted into species with variable complexities. Sphingoid bases and its derivatives, ceramides, phosphosphingolipids, glycosphingolipids, and proteins are secondary parts of sphingolipids. The sphingolipids from plant sources are called *Phytosphingosine* (long-chain structures), whereas the mammalian types are called *D-erythro-sphingosine* and *Sphing-4-enine*. In the sphingoids, the change in structure and addition of hydroxyl groups becomes the cause of functional modifications, for example, it may alter the cell membrane permeability, which acts as the main skin barrier.

Ceramides comprise of long-chain bases of FAs. They are key constituents of skin and help in the synthesis of other complex FAs with variable heads combined in different fashion. Examples include phosphosphingolipids and glycosphingolipids. The acidic type of glycosphingolipids is *Gangliosides* which is complex in nature and is the combination of more than one sialic acid groups (*N*-acetyl or *N*-glycolyl neuraminic acid).

They are part of composition of animal tissues like central nervous system immune cells. There are certain adducts of SPs formed by attachment with exterior skin proteins, i.e., hydroxyceramides and glucosylceramides. Ceramides are precursors for the sphingomyelins and are found in stratum corneum, functioning as an epidermal barrier. Skin diseases such as psoriasis and dermatitis are accompanied by aberrations in ceramides metabolism, which endorses their function as an epidermal barrier. Besides this, sphingomyelins play a role in signal transduction and get accumulated in a condition known as Niemann–Pick disease.

11.4.5 Sterol Lipids

The significant part of membrane lipids are *sterol lipids* (STs) which are mainly cholesterol and their derivative, a tetracyclic ring combined with double bond and a free hydroxyl group. The main property of these lipids is they play an important role in maintaining membrane fluidity and composing most part of animal tissues. The examples of plant-associated SPs are sitosterol and ergosterol. Cholesterols are found in mammals and contribute to cardiovascular complications when present in elevated amounts.

Another SP is *steroids*, which is a four-ring fused structure—the characteristic feature of steroids are they serve as hormones and participate in cell signaling. C18 subclass of steroids belongs to estrogen family, on the other hand C19 steroids are related to the group of androgens which comprises testosterone and androsterone. Another subclass, C21, consists of *progestogens*, such as glucocorticoids and mineralocorticoids.

There are some vitamin D-producing steroids as well, which can cleave the main structure of B ring that forms *secosteroids*.

11.4.6 Prenol Lipids

5-carbon precursors, isopentenyl diphosphate and dimethylallyl diphosphate, are responsible for the synthesis of *prenol lipids* (PR), which is able to serve as origin for the production of vitamin A; moreover, it possesses antioxidant properties. Some of the PR contain complexes which comprise more than 40 carbon atoms known as *polyterpenes*.

Other examples of this PR molecules are known as *quinones* and *ubiquinones*, which resemble vitamin K- and E-like molecules.

11.4.7 Saccharolipids

Saccharolipids are group of lipids in which FAs are directly bound with a sugar backbone substituting glycerol spine support that is likely present in glycolipids and glycoproteins structures. These lipids serve as precursor molecule for the synthesis of lipid A which is present in lipopolysaccharide component in Gram-negative bacterial cell membrane named as acylated glucosamine. It is found in disaccharide form of glucosamine and is responsible for enhancing the immunogenicity and producing waxy material outside the cell wall.

11.4.8 Polyketides

The synthesis of *polyketides* occurs as a result of the polymerization of acetyl, and propionyl subunits can be extracted from plant, animal, fungal, and aquatic origin. A large number of secondary metabolites and natural products are composed of polyketide lipids with great structural range. Many of them form cyclic compound which can be used for the treatment of different diseases caused by microorganisms (bacteria and virus), parasites (ticks and insects), and cancers (due to mutation or virus) (Paola Donato et al. 2017). Figure 11.2 demonstrates the types of different lipid structures and their role in cell membrane and breast ductal carcinoma (human body).

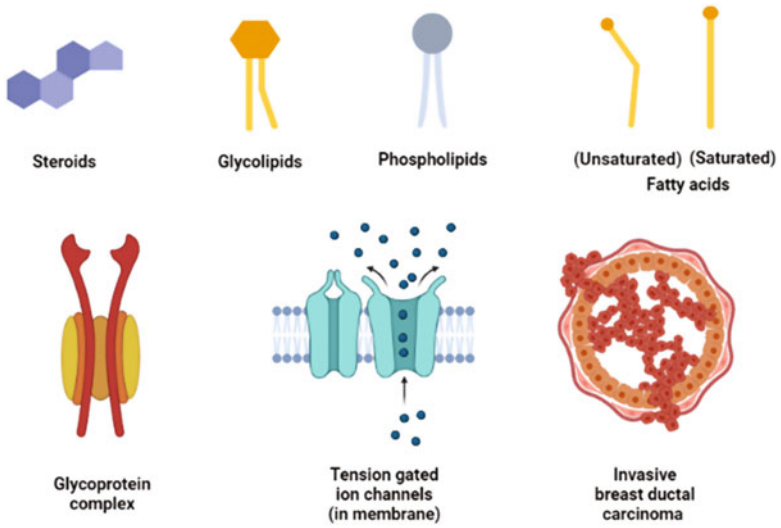

Fig. 11.2 Types of lipids in human body

11.5 Role of Lipids in Breast Cancer Progression

An increase in breast cancer cases is the worldwide problem among women, which is a result of obesity and excess accumulation of adipose cells; in this regard, deeper understanding of fat metabolism and its association with breast tumor cell is important to study.

In previous study, Blücher and Stadler (2017) reviewed that the role of extracellular lipids is very significant in the progression of breast cancer as they serve as substrates for the oxidation of fatty acids which are mainly structured as signaling molecule for oncogenesis of lipid. The upregulation of de novo lipid acquisition is the key reason behind the cancer development and progression. In case of obesity, dysfunctional adipose tissues are playing an important role while the dietary lipid consumption and its consequences are important to consider. Additionally, it was demonstrated that both main components of breast tumor stroma, breast cells and adipose cells, interact with each other for continuous exchange of growth factors, immune cell markers like interleukins and chemokines, reciprocally. Therefore, adipocytes are the main providers of lipids to breast cancer cell for energy generation and cancerous cell development (Blücher and Stadler 2017; Guo et al. 2020).

Another aspect discussed in several studies, i.e., an increase in lipid droplet molecules facilitates the cancerous cells to escape from adaptive immune response of host body or during unfavorable conditions. However, the need of keen understanding towards the cancer cell metabolism is important in targeting therapeutic design and management of cancer disease and its treatment (Lim and Kwan 2018; Liu et al. 2017).

11.6 Importance of Lipid Biomarker in Breast Cancer

Lipid biomarkers can be identified using triple quadrupole liquid chromatography electrospray ionization tandem mass spectrometry (XXXQ-LC-ESI-TMS). By using these techniques, large data of lipids can be profiled, which can result in comparable data among breast cancer patients and healthy individuals. The predictive models tend to combine to detect lipid species which show more elevation among the breast cancer patient's samples than normal healthy individual's samples. In the study, plasma lipidomic was carried out to differentiate the lipid profile among patients with breast cancer using benign lesions for the early-stage diagnosis of breast cancer evaluation. They observed significant higher similarity in lipid species which can be supportive for the breast cancer diagnosis in the early stage of cancer development (Chen et al. 2016).

There is another type of breast cancer reported, triple-negative breast cancer (TNBC) with high mortality rate and fatality as compared to normal breast cancer among women in the United States. Low survival rate in this type of cancer is due to high metastasis rate. This highlighted the need to track the early-stage TNBC to tackle the high level of pathological condition. Researchers detected two types of diagnostic biomarker groups for both TNBC and early-stage TNBC. They revealed that marked disturbance appeared in metabolism of choline and glycerophospholipids and sphingolipid signaling during the analysis of enrichment pathway. This work has been honored to share a great knowledge about TNBC detection for the first time in the diagnostics of lipid biomarkers (Eghlimi et al. 2020; Pralea et al. 2020).

Certain enzymes which participate in lipid metabolism and alteration in their functionality may alter the lipid functionality, for example, FASN, FABPs, and LSCR1. The alteration reflects the idea of using these proteins as biomarkers for the diagnosis which is directly involved in lipid metabolic cycles thus in tumor progression (Butler et al. 2020). In this context, phospholipase A2 enzyme activity was studied in depth for the understanding of lipid metabolism, which directs the role of phospholipase A2 as breast cancer cells sensors. A decrease in IC50 value of drugs (oxorubicin and tamoxifen) used for the anticancer therapy was observed. On the other hand, the function of other phospholipases like C and D was discussed with the involvement in the cell signal transduction, which helps in cancer establishment and growth (Perestrelo et al. 2021).

11.7 Metabolic and Lipidomic Profiling for Human Breast Cancer

The initiation and proliferation of cancer have been discussed in several studies in relation with the involvement of various functionally altered metabolic enzymes; therefore, these enzymes are now receiving the title to be called as *oncoproteins*. Though the genetics of cancers is very complicated, heterogenicity demonstrates the changes in some processes accompanied with cancer development. However, the

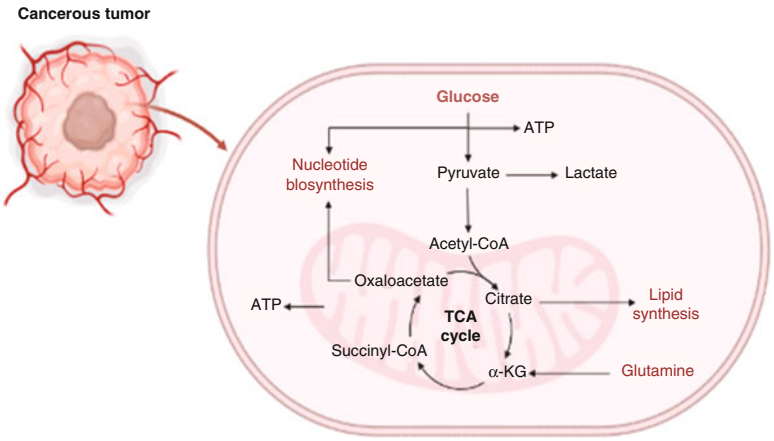

Fig. 11.3 Cancer cell metabolism associated with TCA cycle and nutrient-rich environment

selection of noninvasive methods for the identification and quantification of cancer-related lipid biomarkers is potentially emerging to monitor disease progression and prognostication in cancer patients.

To simplify the understanding towards tumor metabolism, one must gain in-depth knowledge of biochemical pathways involved in lipid metabolism. Lipid metabolism initiates the consumption of glucose during the aerobic glycolysis pathway, which is normally facilitated by the enzymes altered by mutagenesis during TCA cycle. This TCA cycle disruption response to create the metabolism phenotypically changes regulates glucose carbon on the way to anabolic synthesis; this condition facilitates NADPH breakdown thus achieving the stability of glutathione level and cell oxidative stress. In this scenario, the role of mitochondria takes the lead as facilitator of cancer cells taking advantage of blocking TCA cycle. Mitochondria participates in tumor anabolic biosynthetic pathway and contributes to de novo synthesis of fatty acids which are the main causes of cancer development (Armitage and Southam 2016). Figure 11.3 illustrates the cancer cell metabolism during TCA cycle disruption and nutrient-rich condition facilitated by biosynthesis of fatty acids.

The role of elevated level of cholesterol in lipid metabolism was studies diversely using epidemiological parameters which relates that high cholesterol level tends to increase the rate of breast cancer cells growth. In the meta-analysis study of cholesterol consumed from diet and breast cancer development, a strong association of the two was found in in vivo animal model, which suggested hypercholesterolemia due to tumor-promoting effect of cholesterol. Additionally, the identification of primary metabolites such as oxysterol of cholesterol exhibited the role of promoting BC cell growth and metastasis, i.e., higher levels of 2-OHC were observed in human with estrogen-positive breast tumor when compared to the nearby breast cancer-negative cells, indicating the importance of 2-OHC biomarker in endocrinological therapy of BC patients (Blücher and Stadler 2017).

Another extensively studied biomarkers are omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) in BC as well as in other human diseases. PUFAs display anticancer effects, while saturated FAs and monosaturated FAs play role in elevation of cancer development. PUFAs are essential fatty acids which are found in fish, as an important dietary component. In western world, the low consumption of omega-3 in diet in correlated with high risk of cancer development. Omega-3 and omega-6 derivatives, resolving and protecting have anti-inflammatory effect, utilize eicosanoid precursors for resolving inflammation (Blücher and Stadler 2017).

11.8 Function of Lipid Molecules in Drug Resistance

De novo lipogenesis is a well-recognized hallmark which facilitates cancer cell to acquire protection against free radicals and chemotherapy with the help of lipid saturation mechanism thus, maintaining aggressive cancer stage and rapid cell proliferation. Cancer cells manage to resist oxidative stress-induced cell death by undergoing modulation of de novo lipogenesis with the help of change in cell membrane saturation. Moreover, the drug entry inside the cell was studied using doxorubicin drug uptake by using fluorescently labeled phospholipid which was tagged with lipid bilayer and observed cell extract under fluorescent microscopy and fluorometric analysis. The instant quenching of nitrobenzoxadiazole (NBD) fluorescence was observed after the supplementation of doxorubicin, which indicates the coupling of doxorubicin with lipid outer leaflet of phospholipid bilayer and its translocation inside the cell.

11.9 Recent Advancements in Lipidomics

There are several analytical and molecular strategies which encompass the evaluation of wide range of biological molecules, i.e., blood, serum, plasma, urine, and animal tissue derived from animal origin or from patients from clinical pathology. Before the selection of analytical tool appropriately, the most important is to analyze the nature and chemical properties of the given biological sample for lipid analysis. In this regard, NMR and MS are rapidly emerging fields which seem to practically solve the issue of lipid identification in various disease pathological conditions compared with normal healthy persons. In addition, the further advancement of MS has been extended with more analytical tools such as matrix-assisted laser desorption and ionization (MALDI) and electrospray ionization (ESI).

Raman spectroscopy (RS) is another promising approach in the application of lipidomics; in this technique, infrared rays have been utilized to identify molecular markers for different types of diagnostics using both in vitro and in vivo testing. RS helps to study different structural characteristics of molecule (lipid) by using light scattering phenomenon to achieve information. Spectroscopy is capable of being coupled with various other techniques for analysis of different biological fluids and samples, i.e., endoscope probed with spectroscopic tool and atomic force

microscopy-infrared spectroscopy AFM-IR for nanoscale molecular interaction imaging and structural categorization (Perrotti et al. 2016).

In the study conducted by Min et al., four different types of PLs (PS, PI, PG, and PA) were identified from the common cancer patient's urine samples using nanoflow LC-ESI-MS-MS technique and compared with healthy controls. After evaluation, an increased level of two PS (18:1/18:1 and 18:2/18:00) species were observed, whereas PI (18:0/20:4) showed significant decline in the sample reported for breast cancer and suggests the utilization of urine samples for early breast cancer diagnosis (Min et al. 2010).

MALDI-IMS is a high-resolution imaging technique which was sourced by a group of researchers, and they performed lipidomics of cancer cell extracted from clusters and healthy breast cells. It was concluded that there is high heterogenicity which is very prominent in the cancer cell cluster and is significantly expressed as (PI 18:0,18:1) and (PI 18:0,20:3). While some other lipid species were also studied, the relationship between characteristic lipids and and their involvement in breast tumors was established. This lipidomics profiling was a great contribution which describes the spatial distribution of phosphatidylinositol group of lipids and highlights its involvement in different perspective (Kawashima et al. 2013).

The quantitative lipidomics approach was exploited for the characterization of BC tissues in comparison with adjacent healthy tissues through HILIC-HPLC/ESI-MS. In this study, tumor tissues lipidome expressed differential quantification of lipid classes compared to healthy tissues and revealed PI, PE, PC, SM, and LPC in higher levels. In addition, they have demonstrated the subclasses association among C16:0 and C18:0 PLS classes (Cifkova et al. 2015).

Furthermore, cancer cell lines utilization is one of the promising developments which is helpful to study different cell metabolic pathways in vitro using lipidomics. In this framework, Singer et al. execute lipidomics using BC cell lines to study different characteristics of BC cells isolated from primary, metastasis, and normal breast cell lines. They witnessed 16–19-fold elevation in phosphocholine (PCho) and 27-fold increase in primary BC and metastasis cell lines, respectively. In contrast, normal breast cells were reported with no elevated level of PCho. They also documented the differential correlation of tumor features with the alteration in phospholipid profiles with respect to hormone sensitivity and resistance. They resumed that PC and PE lipid species were totally absent or low in cell with hormone sensitivity and remarkably elevated in cell lines high hormone resistance (Mistry and French 2016).

Indication of prognostics marker is also a great development which could help treating BC patients in early stage in disease management. The relationship of lysophosphatidylcholine (LysoPC) with low-risk breast cancer progresses. They concluded that particularly 18:0 lipids of LysoPCs are associated with cancer propagation, whereas an increased level of PC C30:0 is related to higher chances of BC or other common cancer expansion. This study can be indicative of dietary management and selection of LysoPcs supplementation to lower the cancer risks or disease severity (Kuhn et al. 2016). In similar perspective, breast cancer cells were screened in early-stage cancer cell lines using LC-MS. Cell lines being noninvasive

tools gaining more attention for the diagnostics of BC-associated lipid subtypes. The study represents abundancy in triglycerols (TG) $\geq$ C-48 in which moderate different fatty acyl chains were found. Nevertheless, ether-phosphatidylethanolamines (PE) downregulation was majorly reported tumor subtypes in the cell lines which signifies with estrogen receptor and progesterone receptor. HER-2 overexpression was also exploited in cell lines as tumor subclass and spotted elevated level of TG ($\leq$C-46), PC, and PE fatty acids. Triple-negative breast cancer (TNBC) cell lines showed increased levels of PC $\geq$ C-40 subtype. This study reflects the need for further understanding towards subtype-based studies to potentiate the biological relevance (Eiriksson et al. 2020).

In situ technology is an emerging approach for molecular subtype prediction in the field of precision medicine designing. Understanding of origination and propagation of breast cancer subtypes is insufficient. In recent advancement, desorption electrospray ionization-mass spectrometry imaging (DESI-MSI) added great capacity in the field of analytical and disease pathological state. After DESI-MSI molecular subtype characterization, different regions were selected from IBC, DCIS, and ABT cancer tissues in a study conducted by Santoro et al. with the help of data they were able to characterize: the different types of breast cancers and their related molecular basis pathologies. They were able to categorize selected molecular subtypes and their role established and expressed under the development of types of BC (Santoro et al. 2020).

When metabolomics is combined with lipidomics, it advances the quantification and characterization of biological molecules that are involved in metabolism and metabolic signaling pathways. Health-related metabolic changes and their regulation insights are being enhanced with the help of these tools (Gallart-Ayala et al. 2020; Lam et al. 2021). By the same considerations, the use of ion-mobility mass spectrometry (IM-MS) analysis added advanced parameters for metabolomics and lipidomics evaluations for the lipids and metabolites separation and identification which are difficult to find in biological samples with complex nature. Collision cross-section (CCS) is a derivative of IM-MS that helps finding out the physicochemical properties of metabolites and lipids and its computational modeling tools have prediction capability with the help of machine learning approach. CCS generates large-scale information through precise CCs database which could be facilitating both metabolomics and lipidomics analysis (Zhou et al. 2018).

Sample preparation and enrichment methods should comply with analytical technique that tends to facilitate the overall procedure and related outcomes. In particular, the application of solid-phase microextraction (SPME) enhances the results of mass-spectrometry metabolomics and lipidomics for the targeted and untargeted analysis (Reyes-Garcés and Gionfriddo 2019).

11.10 Diagnostic Potential of Lipid Biomarkers for Breast Cancer

The evolution and spread of cancer are complex processes. The direct relationship between lipids and breast cancer diagnosis has been proved by studies based on metabolomics and lipidomics. While studying lipid profiles of diseased samples, most part of the researcher's focus is on total levels of lipids present in the samples. Studies show that tumors are linked to the increase or decrease of certain molecules that can be considered as biomarkers. Min et al. analyzed four different categories of phospholipids from urine samples of breast cancer patients (Min et al. 2010). In comparison to the control samples (healthy ones), two phosphatidylserine [PS] molecules (18:1/18:1 and 18:2/18:0) were noticed to mark a significant increase in breast cancer samples. It was also observed that the postoperative level was decreased to the normal concentration. On the other hand, phosphatidylinositol PI molecule (18:0/20:4) concentration was considerably reduced in the breast cancer samples compared to the normal ones (Min et al. 2010).

Literature shows that very few studies have been conducted on benign breast cancer samples. Yang et al. evaluated plasma lipid profiles of malignant and benign breast disease patients and showed the diagnostic ability and efficiency of the lipid biomarkers present in the samples (Yang et al. 2015). In another study, a group of 15 lipid species from plasma samples was spotted as important biomarkers for breast cancer diagnosis at an early stage. This study also showed that these biomarkers helped in differentiating the early-stage diseased samples from benign ones (Chen et al. 2016).

It has been known that lipids and lipoproteins in blood samples have been involved in carcinogenesis through inflammation, oxidative stress pathways, insulin resistance, and propagation of signaling compounds (Giovannucci 2007).

The fact that lipids are involved in different pathways for initiating various human health issues is not new and breast cancer is one of those health issues (Baumann et al. 2013). Polar lipids have oncological power that may participate in growth and propagation of cancer metastasis (Luo et al. 2017).

Some studies have shown the relationship of some lipids like apolipoprotein A-I (Apo A-I) and apolipoprotein B-100 (Apo B-100) with some types of cancer development (Chandler et al. 2016). However, such studies are few in number with regard to breast cancer study and data shows some inconsistent facts. For example, Han et al. showed direct relation of Apo A-I increased levels with breast cancer risk (Han et al. 2005), while Chang et al. studies showed completely opposite results (Chang et al. 2007).

11.11 Mass Spectrometry for Lipidomics Research for Breast Cancer

Mass spectrometry is an analytical tool that helps in explaining disease mechanisms of lipidomics for cancer diagnosis and treatment. High-resolution mass spectrometry is an ideal tool for testing lipid profiles of a sample (Rui Guo et al. 2020). It can help

in detecting different stages of the disease including the earlier stages with mild or even without any symptoms. The commonly used laboratory methods are:

11.11.1 Electrospray Ionization (ESI) Mass Spectrometry (MS)

In this method, there is an electrospray ionization source with high sensitivity through which sample in the form of a solution is passed. It is usually coupled with liquid chromatography device. Fourier transform ion cyclotron resonance and quadrupole time-of-flight (QTOF) are the most advanced mass spectrometry analyzers for lipidomic studies (Jelonek et al. 2013). From small fatty acids to massive lipids, ESI-MS is capable of analyzing lipidomics of the desired sample.

11.11.2 Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

In this method, sample analytes are co-crystallized with the matrix (mainly small organic molecules). The matrix molecules primarily absorb the laser energy and help in the formation of charged analytes (Jelonek et al. 2013). TOF appears to be more convenient for lipidomic studies although studies report other kinds of analyzers as well. This technique is suitable for nonpolar and hydrophobic lipids such as phospholipids (Rauser et al. 2010).

11.11.3 Gas Chromatography-Mass Spectrometry (GC-MS)

In this method, the sample with lipid molecules is first transformed into gaseous state by derivatization into the less polar methyl esters for boosting volatility. The GC is mostly coupled with ion-trap or quadrupole analyzers (Jelonek et al. 2013).

11.11.4 Nonaqueous Capillary Electrophoresis-Mass Spectrometry (NACE-MS)

This method is quite efficient in high separation and quantification of lipid profile in a sample and is relatively low cost. Sample preparation is not complicated for applying this technique. It is adequate for analyzing phospholipids and inactivated fatty acids (Azab et al. 2019).

11.11.5 Mass Spectrometry Imaging (MSI)

Mass spectrometry imaging (MSI) is a unique technique that has the capability of showing spatial distribution lipid maps, not only for identifying but also for

quantifying different lipid molecules, on biological tissue sample surfaces of laboratory-prepared histological slides. Among many techniques of ionization for MSI, MALDI and DESI seems more promising. Compared to MALDI, where matrix is added, desorption electrospray ionization (DESI) has an advantage of having no matrix interference. It is widely used for imaging as very little or no sample preparation is required; sample ionization is external and analyses are direct. The ion formation depends on collision of charged microdroplets of solvents with the tissue surface. This technique is quite effective for analyzing a wide variety of lipids, for example, fatty acids, glycerolipids, glycerophospholipids, sphingolipids, and steroids (Rennó et al. 2017).

11.12 Breast Cancer Xenograft Metabolism and Lipidomics

Metabolic changes are a sign of cancer which can be used for cancer diagnosis as well as treatment purposes (Hanahan and Weinberg 2011). In breast cancer, lipid metabolism is significantly altered (Fernández et al. 2020). The ongoing membrane synthesis while tumor cell growth (Glunde et al. 2011) is due to augmented levels of phospholipids.

Studies on metabolic pathways have improved our vision about cancer and aided the identification of biomarkers linked to disease prognosis and prediction (Graça et al. 2020). Various analytical techniques that are in use for cancer study are highly sensitive, however require homogenization and tissue extraction. These techniques are not able to perform spatially resolved metabolic scanning (Yuan et al. 2012).

Mass spectrometry imaging (MSI) is a fresh breeze of air in cancer metabolomics as it allows (Sun et al. 2019):

1. Identification of various chemical species
2. Provides spatial resolution
3. Accompanies histopathological analysis to record metabolic and biological features

Mostly fresh frozen tissue samples are used for MSI, but formalin-fixed paraffin-embedded (FFPE) tissue is also a way for storing clinical biopsies for years. The FFPE studies allow to check the correlation between biological features and survival rate among cancer patients that contributes to the learning of cancer heterogeneity (Engstrøm et al. 2013). Denti et al. investigated lipidic alterations in patient-derived xenograft of breast cancer (Denti et al. 2021). They used matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) technique, with evaluation studies of spatial metabolic differentiation within tissue compartments, reproducibility, and treatment response induced by a glutaminase inhibitor (CB-839). Several lipids were able to distinguish necrotic and tumor regions among the experimental replicates. Also, they were successful in distinguishing changes in the tissue lipidome of xenograft treated with glutaminase inhibitors

(Denti et al. 2021). This study is a step-forward towards a robust and reproducible technique that can be used in preclinical and clinical applications.

11.13 Breast Cancer Subtypes in Lipidomic Studies

Blood cancer has many subtypes and the treatment with later outcome depends on the subtypes. The diversity in cancer subtypes in linked with genetical, molecular, or clinical variations. This leads to contrasting proliferation rates and the possibility of metastatic secondary tumors. Therefore, proper treatment at an early stage requires new methods and techniques for the detection of cancer subtypes (Eiriksson et al. 2020).

The main types of invasive breast cancer include (Feng et al. 2018):

1. Ductal carcinoma: Type of cancer that starts from the ducts present inside the breast. It is the most common type that exists.
2. Lobular carcinoma: Type of cancer that starts from the lobes of the breast. It is the second most common type that exists.

Sometimes, invasive breast cancer is a mixture of both carcinoma types mentioned above. There exist some types that are less common, however are invasive, for example, inflammatory breast cancer. When the abnormal tumor cells are present in the duct or lobe of the breast but do not or have not spread, they are classified as noninvasive or in situ. They can be both ductal carcinoma in situ (which is a precancer stage that may lead to breast cancer) and lobular carcinoma in situ (which is a precancer that rarely spreads).

Transcriptomics analyses are performed to know an organism's transcriptome, i.e., the complete sum of all its RNA transcripts. The DNA records all information content of an organism and expresses it via transcription (Lowe et al. 2017). On the basis of transcriptomic analysis, blood cancer is classified into five main subtypes.

During cancer study, transcriptome information is interpreted for identifying cancer subtypes on the basis of the progesterone receptors (PgR), hormone receptor status of the estrogen receptors (ER), and human epidermal growth factor receptor 2 (HER2/*neu*).

The common cancer subtypes are (Eiriksson et al. 2020):

1. Luminal A (ER+, PgR+, HER2−)
2. Luminal B (ER+, PgR+, HER2−/+)
3. HER2-overexpressing (ER−, PgR−, HER2+)
4. Triple-negative breast cancer (TNBC; ER−, PgR−, HER2−)
5. Normal-like subtype

Although majority of breast cancers are categorized on the basis of above-mentioned subtypes, many studies classify them differently. Therefore, consistency on breast cancer subtype categorization is lacking in the literature (Dai et al. 2017). It

has also been observed that high heterogeneity occurs among individual samples of cancer subtypes due to various other factors (Joseph et al. 2018; Sørlie 2016).

In the recent studies by Eiriksson et al., lipidomics of six cell lines of breast cancer were investigated to validate the subtype defined by the transcriptome (Eiriksson et al. 2020). A liquid chromatography-mass spectrometry (LC-MS) equipment was used for this purpose. In the cell lines that represented positive subtypes of estrogen receptor (ER) and progesterone receptor (PgR), researchers observed an escalating amount of triacylglycerols (TG) $\geq$ C-48 along with average or multiple unsaturation in fatty acyl chains. Moreover, suppressing response of ether-phosphatidylethanolamines (PE) (C-34 to C-38) was seen. In a breast cancer cell line representing human epidermal growth factor receptor 2 (HER2) overexpressing subtype, an uplifted amount of TG ($\leq$C-46), phosphatidylcholines (PC) and phosphatidylethanolamines (PE) having small-chained ($\leq$C-16) saturated or mono-unsaturated fatty acids were detected. High abundance of PC $\geq$ C-40 was reported in cell lines of triple-negative blood cancer. Moreover, variations were seen in lipidomes of the previously stated breast cancer subtypes (Eiriksson et al. 2020).

In another study by Santoro et al., DESI-MSI technique was used for identifying chemically the molecular subtypes of breast cancer (Santoro et al. 2020). In invasive breast cancer (IBC) regions, major ions identified were deprotonated glycerophospholipids, polyunsaturated fatty acids, and sphingolipids. Highly saturated lipids as well as antioxidant molecules [taurine (*m/z* 124.0068), ascorbic acid (*m/z* 175.0241), uric acid (*m/z* 167.0210), and glutathione (*m/z* 306.0765)] helped in distinguishing invasive breast cancer (IBC) from adjacent benign tissue (ABT). More complex lipid profiles were observed in case of luminal B and triple-negative subtypes compared with luminal A and HER2 breast cancer subtypes. Ductal carcinoma in situ (DCIS) and invasive breast cancer (IBC) were differentiated by cell signaling and apoptosis-related ions such as fatty acids (341.2100 and 382.3736 *m/z*) and glycerophospholipids (PE (P-16:0/22:6, *m/z* 746.5099, and PS (38:3), *m/z* 812.5440)). This study shows that DESI-MSI was able to identify different lipid composition in breast cancer types and molecular subtypes (Santoro et al. 2020).

11.14 Significance of Lipidomics in Early-Stage Breast Cancer Diagnosis

Preliminary stage identification is very important as it can help in taking necessary measures for controlling prognosis of breast cancer. Mostly, mammography is used as a reliable technique for screening of cancer. Although the sensitivity level is from 54% to 77% (Güth et al. 2008), there are many reports of false-negative or false-positive results (Gøtzsche and Jørgensen 2013). Therefore, in case of positive results, additional diagnostic exams such as magnetic resonance imaging (MRI) and/or biopsy are performed. These tests have their own limitations, for example, with MRI test, it is difficult to differentiate between malignant and benign breast lesions. This takes the patient to have further biopsies. In case of benign lesions,

patient suffers physiological and mental stress, with additional expenses (Yahalom 2013). Thus, highly sensitive and accurate techniques need to be investigated for early diagnosis of breast cancer and to differentiate malignant breast lesions from benign lesions, so that needless, costly, and invasive examination of benign patients can be avoided. Tumor biomarkers from blood samples are a different approach to address these challenges. However, biomarkers from serum didn't reach to the level of clinical trials yet.

Studies by Chen et al. have led to discover some relevant lipid biomarkers for early diagnosis of breast cancer. They have investigated a group of lipid molecules and identified 15 lipid subtypes from plasma samples that are promising for distinguishing the early stage of breast cancer from benign tumors (Chen et al. 2016). It was observed that plasma levels of phosphatidylcholine (PC) and ether-linked PC classes increase in concentration in breast cancer patients. Moreover, studies showed that the plasma levels of lysophosphatidylcholine (LPC) and cholesterol ester (CE) were observed to reduce in breast cancer patients, compared to the benign samples (Chen et al. 2016). This might be linked with high metabolic rate in breast cancer patients. Literature shows that lipidomics is a cancer detection technique that involves just a minimal invasive method with quick, efficient, and reliable results.

11.15 Relationship of Obesity, Lipids, and Breast Cancer

In 1979, first time obesity was investigated for its influence on breast cancer. Obese patients with >20% body weight over the standard weight (cm of height − 100) × 0.9, reported large size of the primary tumors and bad survival rates in comparison to cancer patients with standard weight (Abe et al. 1976). Another study has reported that obese women can have up to 11% reduced survival in case of breast cancer (Protani et al. 2010). In case of survival, health outcomes are complex due to obesity. For instance, type 2 diabetes, metabolic syndrome, and heart diseases may even cause death of the patient (Bradshaw et al. 2016; Smith and Ryckman 2015). Complications are expected during surgical operations, chemotherapy, and radiotherapy with higher risk on recurrence as compared to normal weight patient. Chemotherapy is also reported to be with less positive outcomes even when dosage depends on weight. Endocrine therapy is less effective and breast reconstruction is also not very common due to complications faced by the patients later on. Obese patients have greater challenges in patient care and overall cancer treatment management compared to the rest of the population (Lee et al. 2019). There are many reasons for the bad effects of body fat on breast cancer. Many factors including physical, biological, and psychosocial present higher risk factors for this disease and worsen the clinical outcomes in the obese population (Lee et al. 2019). Further studies are needed to optimize therapies for such patients.

Several mechanisms have been reported for associating obesity with breast cancer progression. These mechanisms include inflammatory signaling, adipokines, chemokines, and insulin. In more advance studies, it has been shown that breast

cancer growth and progression is due to extracellular lipids that play a very important role by providing substrates for oxidation of activated fatty acid or by acting as building units for carcinogenic lipid-signaling compounds. Deregulated adipose tissue is known to provide extracellular lipids for breast cancer cells, by food intake pathway or by direct interaction with adipocytes from the tumoral stroma. There exist emerging proofs that indicate the flexibility of breast cancer cells for getting adapted to their metabolic environment. Extracellular lipids can be utilized as a target in breast tumor cells for opening new ways for tumor treatment (Blücher and Stadler 2017).

11.16 Conclusions

Lipidomics is a developing field which systematically studies a wide range of lipids and alterations in their metabolism in connection with diseases. This unravel new insights into metabolic and inflammatory illnesses. Current developments in MS technologies and advances in chromatography have significantly boosted the progresses and applications of lipidomics for breast cancer. The lipid profiling can help understand pathology of breast cancer and other diseases, discover new potential biomarkers, drug reaction monitoring, etc. In mammals, studying metabolic pathways is complex, especially when it is concerned with disease progression due to altered metabolism and mutations in genes. Lipidomics application in animal models in combination with coupling tools like imaging and spectrometry can efficiently add potential for solving problems related to the treatment of breast cancer and disease management.

It is established that modifying unhealthy habits into a healthy lifestyle by dietary changes (Estruch et al. 2006; Foster et al. 2003) and including proper exercise routine (Febbraio 2017) can help in the prevention of breast cancer. Smoking cessation (Gossett et al. 2009) is also effective in increasing high-density lipoprotein (HDL) cholesterol (that is actually "good" cholesterol).

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Part III

Breast Cancer Treatment

Therapeutic Options in BRCA1-Linked Breast Cancer and Systemic Approaches

12

Amir Khan, Ali Jan, and Muhammad Qaiser Fatmi

Abstract

BReast CAncer gene 1 (*BRCA1*) has been established as a regulator of DNA repair, cell cycle, and transcription in response to any damage to DNA. Individuals at the age of 40–50 years, most of the females, carrying mutant genes are at very high risk for developing ovarian and breast cancers, whereas the pathways through which tumor development takes place varies from person to person; the same goes for their prognosis and survival. Various therapeutic interventions are opted for breast cancer patients; these include surgical removal of the defected area, chemotherapy, or immune therapy. Surgical interventions include lumpectomy, mastectomy, and bilateral salpingo-oophorectomy. Lumpectomy is carried out if the size of the tumor is small or if it is in a region where only a small surgery can easily be performed. On the other hand, mastectomy is performed if tumor size is large. It involves the removal of certain parts of the skin, some tissues, and lymph nodes from the chest wall for histopathological studies to find out whether there are any other cancerous cells. Removal of both ovaries and fallopian tubes due to the presence of ovarian cancer in case of hereditary mutation of *BRCA1* and *BRCA2* genes are known as a bilateral-salpingo-oophorectomy. Chemotherapy is effective for the non-surgical removal of tumors. Chemotherapy involves the use of drugs like taxanes, platinum agents, and poly ADP-ribose polymerase (*PARP*) inhibitors. Immunotherapy involves

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the use of anti-*PD-1* and anti-*CTLA4*. Radiotherapy is also commonly used where radiologists use high-energy radiations to kill cancerous cells.

Keywords

Breast cancer · BRCA1 · Surgery · Chemotherapy · Immunotherapy · Radiotherapy · Combination therapy

12.1 Introduction

BReast CAncer gene 1 (*BRCA1*) has been established as a regulator of DNA repair, cell cycle, and transcription in response to any damage to DNA. *BRCA1* and *BRCA2* genes are the most altered genes that are associated with a high risk of breast tumor. Among these, BRCA1 is a tumor suppressor gene and the mutations of the *BRCA1* gene meaningfully enhance the probability of acquiring various forms of epithelial malignancies, specifically ovarian and breast tumor (Greer and Whitcomb 2007). Genetic factors, involving *BRCA1* and *BRCA2* alterations, are accountable for about 5–10% of breast cancer incidents. Hereditary breast and ovarian cancer syndrome (HBOC) associated with *BRCA1* and *BRCA2* genes mutation is inherited in an autosomal dominant fashion and makes up approximately half of the malignancy incidents associated with inherited genetic risk (Rebbeck et al. 1996; Casey 1997).

BRCA1 is solely accountable for roughly 40–45% of hereditary breast cancer, whereas its expression is decreased in the case of sporadic cancers. Rapid genetic lab testing can be helpful in the early identification of the genetic status of the carcinogenesis and may help in planning the treatment of the patient (Rosen et al. 2003; Francken et al. 2013).

12.1.1 Role of *BRCA1* in Tumorigenesis

BRCA1 is the major tumor suppressor gene, but its loss of function caused by somatic mutations leads to the development of malignant tumors. The loss of transcriptional activation domain that plays a role in tumor suppression helps in the tumor growth. Genes linked with breast cancer tumorigenesis due to *BRCA1* are cyclin *D1*, *MYC*, *JAK 1*, *STAT 1*, *ID 4*, etc. (Welcsh et al. 2002; McPherson et al. 2004; Wu et al. 2010). It is reported that *P53* deficiency and *Chk2* pathway activation can also lead to the development of tumorigenesis, which is caused by DNA damage signaling pathway responses in BRCA1-deficient cells. The *AKT* oncogenic pathway in these deficient cells also plays a significant role in tumorigenesis (Xiang et al. 2008).

Evidence has been found that heterodimeric *BARD1* plays a significant role in directing cellular responses to DNA damage. The *BRCA1*/*BARD1* heterodimer consists of a ligase, known as E3-ubiquitin (Ub) ligase, which in response raises the likelihood of ubiquitylation specifically at certain targets that may allow *BRCA1*/

BARD1 to cause cellular and DNA damage. E3-ubiquitin (Ub) ligase is hence, in some cases, responsible for causing mutation in *BRCA1*, which leads to tumorigenesis (Boulton 2006).

12.1.2 Relation of *BRCA1* with Other Potential Hereditary Breast Cancer Genes

Initially, it was thought that only *BRCA1* and *BRCA2* genes mutation or their absence was responsible for the generation of malignancies in breast and ovarian cancers, later it was found that there are various genes and factors which play a vital role in the advancement of hereditary breast malignancy (i.e., *PALB2*, *PTEN*, *TP53*, *HER-2*). Genes present in the 17q region of the chromosome are mainly involved in breast cancer like *HER2* (oncogene), *HOX2* (homeobox 2), *EDHB17* (estradiol-17β dehydrogenase), *WNT3* (integration site of mouse mammary tumor virus), *NM23* (associated with metastasis), and *RARA* (retinoic acid receptor α), while *BRCA1* is present in 17q21.3 region (Mehrgou and Akouchekian 2016). Various cellular processes are involved in *BRCA1* tumorigenesis: these include transcriptional regulation of genes involved in DNA repairment, formation of heterochromatin on the X chromosome, and ubiquitination. *BRCA1* gene binds to *BRCA2* gene in the presence of *TP53*, and *RAD51* helps in the DNA repairment; therefore, the absence of these genes or mutation will lead to the development of cancer (Obermiller et al. 2000; Godet and Gilkes 2017; Tung et al. 2020).

12.1.3 *BRCA1* Mutations and Prognosis

BRCA1 is directly responsible for the causing breast cancer in males and females. Therefore, mutations in *BRCA1* gene evidently make individual susceptible to the development of breast and ovarian cancer. Germline mutation leads to prolonged survival and effective solution after taking proper therapeutic measurements. More than 1600 mutations have been identified in the *BRCA1* gene, which mostly progress towards the development of ovarian cancers in females and prostate cancers in males (Godet and Gilkes 2017; Huszno et al. 2019; De Talhouet et al. 2020).

12.2 Clinical and Pathological Risk Factors Associated with *BRCA1* Mutations

There are various risk factors associated with *BRCA1* mutation. Through various studies, it has been found that women carrying these genetic mutations are on high-risk level for suffering from breast cancer at very early stages of their life, and the tumors that usually develop are triple-negative tumors. The exposure to estrogen, a sex hormone, is associated with this mutation; hence, the mothers who breastfeed have lower chances of developing breast cancer. However, it was noted that the

feeding time in these carriers (mothers) was less than that in normal females (Rubin et al. 1996; Jouhadi et al. 2016).

12.2.1 Age, Grade, and Histological Types

There is no specific age for the tumor or development of cancerous cells as they can happen whenever the mutational proliferation starts occurring. Mostly, it is seen that tumors associated with *BRCA1* appear at an early age as compared to *BRCA2*. At the age of 40–50 years, most of the females carrying mutant genes are at very high risk for developing ovarian and breast cancers, whereas the pathways through which tumor development takes place varies from person to person; the same goes for their prognosis and survival (Honrado et al. 2004; Eerola et al. 2004, 2005). There are various histological types of *BRCA1*. These include serous tumors, mucinous tumors, and endometrioid tumors (Lakhani et al. 2004).

12.2.2 Estrogen *ER*/Progesterone *PgR* Expression Status

The expression of estrogen *ER* and progesterone receptors *PgR* is usually seen in *BRCA1*-associated cancers, as they depend upon cancerous cells for their growth. Before analysis of breast cancer, the patients are tested for the presence of these receptors, as they are used as biomarkers and represent the presence of malignant cells. In *BRCA1*-associated breast cancer, at early ages, the expression of sex hormones is low whereas *TP53* is usually positive in other tumors (Eerola et al. 2005).

The proportion of breast cancer cells expressing *ER* and *PgR* are higher in all *BRCA1*-linked genetic cases, whereas their presence was comparatively lower in sporadic tumors. Testing the presence of these hormones is very important as it gives a basic understanding of treatment and hormonal therapy. Apart from *BRCA1*-linked cases, the expression of *PgR* is lower in sporadic cases of breast cancer, whereas the presence of *PgR* receptor is seen on the marginal surfaces of these tissues that are present in the close surrounding to the tumor. The expression of *PgR* helps to find the localization of the tumor in the breast more significantly. Expression of *PgR* is reported to be noticeably higher in *BRCA1*-linked patients of cancer than in *BRCA2*-linked cases. But it had also been detected that the expression of *PgR* in *BRCA2*-linked tumors is not significantly different from the expression that is found in sporadic tumor cancer patients (Honrado et al. 2004; King et al. 2004).

12.2.3 Association of *BRCA1/2* Mutations with Overall Survival

In breast cancer patients, these mutations play a significant role in developing treatment plans. These germline mutations mainly develop cancers of two types. *BRCA1* mutational cancers are known as triple-negative breast cancers with high

estrogen and progesterone receptors, whereas tumors in *BRCA2* are difficult to treat because it is hard to identify them, as they show the same features as sporadic cancers. The survival rate of patients harboring this gene can be improved when the tumors are detected in the early stages of tumor formation or even metastasis, for example, at stage 0 to stage 2. These genes are highly sensitive to chemotherapeutic agents when used for destroying cancerous cells. Ovarian cancer that develops in carriers of *BRCA1/2* genes are usually high-grade serous ovarian carcinomas (HGSOC). Chemotherapeutic drugs such as platinum agents or taxanes are used for the treatment of such cases which results in prolonged overall survival rate (Feng et al. 2018; De Talhouet et al. 2020).

12.2.4 Chemoresistance and Response to Poly ADP-Ribose Polymerase (*PARP*) Inhibitors

Chemoresistance is developed in patients when they use single-drug therapy for the treatment of expanding cancers. It is evident from various studies that a combination drugs therapy gives better results and increases the survival rate of cancer patients. There is often resistance against one type of drug. Before the start of treatment, it is also suggested that patients should be screened for poly ADP-ribose polymerase inhibitors (PARPi), as their expression is present in more than 60% of tumors. *PARP* protein inhibitors show desired result in patients carrying a genetic mutation of *BRCA1/2* genes. Due to defective DNA, these tumor cells are more sensitive to these inhibitors. Nowadays, due to significant prognostic results that are obtained through *PARP* inhibitors, these drugs have been approved for the treatment of breast and ovarian cancers. It is also found that patients without *BRCA* mutations show low response to these inhibitors and 40% fail to respond because of prolonged usage of PARPi. HRD, homologous recombinant DNA repair-deficiency, plays a vital role in killing tumor cells while homologous recombination repair therapy is the cause of resistance of PARPi (King et al. 2004).

12.3 Therapeutic Interventions

With the evolving curative therapies, there is a better chance of survival for cancer patients, yet they need to be dealt with extreme care and a strong supportive environment so that they can get out of distress. Various therapeutic interventions could be opted for the treatment of breast cancer patients; these include surgical removal of the defected area, chemotherapy, or immune therapy. After these therapies, proper rehabilitation is done. Moreover, various exercises, specifically for breast cancer patients, are recommended so that they could find some relief from pain and long-term side effects (Glanz and Lerman 1992; Binkley et al. 2012).

12.3.1 Surgery

Three types of surgical interventions which are opted for are described as under:

12.3.1.1 Lumpectomy (Partial Mastectomy)

Breast cancer is the most common type of malignancy. When the doctors observe that the size of a tumor is <4 cm, they prefer a small surgery at the site of the tumor in which the breast remains intact but only the diseased portion is removed (Fig. 12.1). Lumpectomy is carried out if the size of the tumor is small or if it is located in a region where only a small surgery can easily be performed. Furthermore, lumpectomy is also the first choice when the tumor is present only at one specific region inside the breast tissue. After lumpectomy, radiation therapies are prescribed to reduce the chances of cancer relapse and to destroy if any other cancer cells are present in the surrounding area. It was found that the chances of recurrence of cancer in patients with lumpectomy are 40%, while for those who took radiation after the first surgery the chance of their getting a second relapse is only 14% (Gottlieb 2002). For this process, FNAC or fine-needle aspiration therapy is carried out to indicate the borderlines of the tumor, and a biopsy sample is taken to identify the type of tumor present. Studies show that after the surgery, patients with partial mastectomy had intact body image and a greater sense of sexual desirability as compared to the patients undergoing complete mastectomy (Wellisch et al. 1989; Tartter et al. 2000). After lumpectomy, when the margins are not clear then the mastectomy is carried out (Morrow et al. 2017).

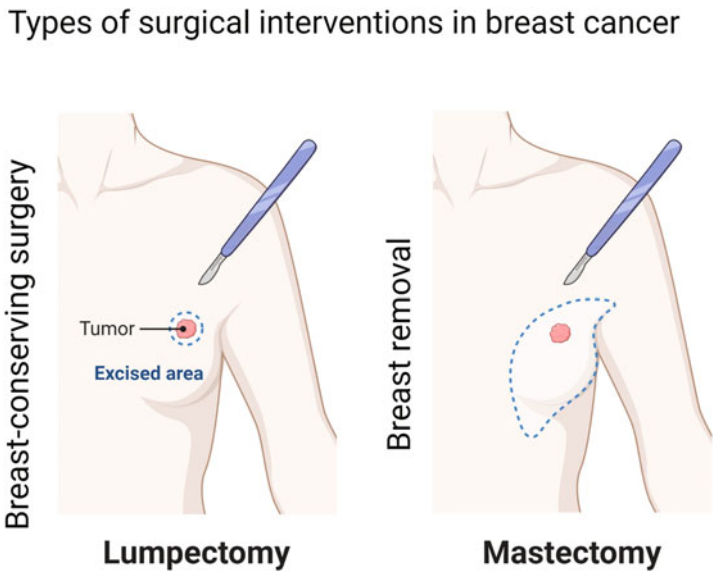

Fig. 12.1 Surgical interventions for the treatment of breast cancer

12.3.1.2 Mastectomy

Mastectomy is carried out for the advanced stages of cancer where tumor has spread in more regions and cannot be removed with the help of lumpectomy (Fig. 12.1). In this procedure, certain parts of the skin, some tissues, and lymph nodes are removed from the chest wall for histopathological studies to find out whether or not there is any other cancerous cells. This surgery is important for recurrence-free survival, tumor control, and overall survival (Blichert-Toft et al. 2008). The radical mastectomy has been practiced for at least a century now for the treatment of breast cancer patients. In the modern era, after the mastectomy, another surgery is performed to reconstruct the breast so that they can appear as much close to normal as possible. Breast implants or tissues of any other part of the skin can be used for this purpose (Carlson et al. 1997; Blichert-Toft et al. 2008; Alaloul et al. 2019).

12.3.1.3 Bilateral Salpingo-Oophorectomy (BPSO)

Removal of both ovaries and fallopian tubes due to the presence of ovarian cancer in case of hereditary mutation of *BRCA1/2* genes are known as a bilateral salpingo-oophorectomy (BPSO). BPSO is one of the surgeries that helps in pertaining cancer-free survival in women who have suffered from ovarian cancer due to these mutations. This is the most effective surgery in treating females at a higher risk of getting breast and ovarian cancer. Hysterectomy is also mostly done with BPSO, as a result, there is the complete removal of the reproductive organ that leads to surgical menopause, which has high climacteric symptoms than those females who undergo normal menopause. However, this surgery do not affect the quality of sexual life (Domchek et al. 2006; Benshushan et al. 2009; Berek et al. 2010). At the age greater than 40 years, there are higher chances of having positive genetic results due to which this surgery becomes a necessity for survival in the mutant gene population (Schmeler et al. 2006).

12.3.2 Chemotherapy

Apart from surgery, chemotherapy is effective for the non-surgical removal of tumors. The cases of nodular metastasis of breast cancer warrant chemotherapeutic intervention. It restricts the cancer it to a certain region. Chemotherapy uses anti-cancerous drugs that are taken orally, injected into the bloodstream, and sometimes these are also injected into the spinal fluid. These drugs can be used before or after the surgery, and sometimes these are used as a source of main treatment when there is metastasis in breast cancer. Some effective chemotherapy methods are discussed below:

12.3.2.1 Taxanes

One of the most effective combination of drugs used for chemotherapy is known as taxanes. It is a combination of two drugs docetaxel and paclitaxel. These drugs inhibit the proliferation of cancerous cells in tissues and decrease the rate of progression of cancer. Various adverse effects have also been observed due to

these medications which include loss of hair, nausea, vomiting, and some allergic reactions. This chemotherapeutic agent has been used for the last three decades as a standard therapy for malignant and metastatic tumor cases of breast cancer. They provide significant prognostic outcomes and give effective results in its fight against breast cancer (Nabholtz and Gligorov 2005; Ghersi et al. 2015). Due to its chemical composition, taxane comes with few side effects which include a mild risk of febrile neutropenia, fatigue, and neuropathy; however, it does not cause cardiotoxicity. Various studies revealed that taxanes are most effective in those metastatic cases where lymph nodes are also affected than those cases where lymph nodes are yet not damaged by cancerous cells. Overall, taxanes provide a chance of cancer-free life to females with breast cancer in early stages (Willson et al. 2019).

12.3.2.2 Platinum Agents

Platinum is a cytotoxic agent which is used for the treatment of triple-negative breast cancers (TNBC). TNBC do not express estrogen or progesterone receptors. Besides TNBC, platinum is also used for the treatment of pancreatic, lungs, head and neck, and ovarian cancers. Platinum works to reduce the severity of disease; however, it has a short-term efficacy. Its combination with taxanes has shown higher efficacy in containing the spread of the disease (Liu et al. 2013; Egger et al. 2017; Pandy et al. 2019). Higher efficacy of this chemotherapeutic agent also brings along adverse effects on the patient's body. Most people taking these drugs suffer from hair loss, anemia, kidney damage, vomiting, nausea, hand and feet syndrome, and even leukemia in the worst scenarios (Egger et al. 2017).

12.3.3 *PARP* Inhibitors

Emerging chemical agents increase the quality of life for patients dealing with breast cancer. *PARP* inhibitors belong to a new innovative class of inhibitors that target the tumor with DNA repair defects. *BRCA1/2*-associated tumors have shown their high sensitivity to these chemical reagents. These are used in the population who are at higher risk of getting gene mutations leading to breast and ovarian cancers. These inhibitors effectively shrink the size of a tumor while blocking the DNA repair in the tumor cells (Fig. 12.2). As a result, these inhibitors kill tumors and increase the progression-free survival in breast cancer patients. *PARP* inhibitors reduce the mortality rate by 13%, while it decreases the risk of disease progression by 37%. The chances for the shrinkage of the size of the tumor is also increased by 66.9% (Vinayak and Ford 2010; Al-Ejeh et al. 2013; Taylor et al. 2021; Yan et al. 2021).

12.3.4 Immunotherapy

Immunotherapy helps to fight against cancerous cells by invoking the immune system of the human body. It helps in T cells proliferation to activate immune response. But in some cases, immune therapy may worsen the condition of

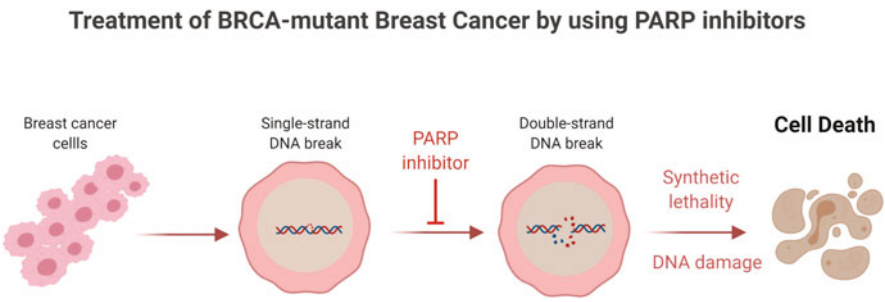

Fig. 12.2 Schematic overview of treatment of BRCA-mutant cancer by using PARPi

autoimmune disorders in patients with rheumatoid arthritis. It is only recommended in the initial stages of the disease. In patients with breast cancer, it can also cause inflammation in the overall body along with skin changes, chest pain, and hypothyroidism. Yet advancements in immunotherapy are a ray of hope for breast cancer patients, as it has the potential for the eradication of cancerous cells.

12.3.4.1 Anti-programmed Cell Death Protein 1 (Anti-*PD-1*) Therapy

In 2019, anti-programmed cell death protein 1 (anti-PD-1) antibody—known as atezolizumab—was used with chemotherapeutic drugs for metastatic breast cancer patients, who had *PD-L1* protein-expressing tumors. Programmed death-ligand 1 (*PD-L1*) agents have been evaluated in breast cancer, particularly in the triple-negative subtype, with hopeful outcomes when delivered as monotherapy or in combination with other conventional treatments (Rosato et al. 2018; Planes-Laine et al. 2019). In metastatic *PD-L1*-positive TNBC, the preliminary overall survival from IMpassion130 trials provides level I evidence supporting atezolizumab (anti-*PD-L1*) plus nab-paclitaxel as a standard first-line therapeutic approach. It was recommended for first-line therapy for patients with a greater than 12-month time span in a distant recurrence-free interval of cancerous cells and *PD-L1*-positivity. Biomarker assessment for breast cancer and chemotherapy plus anti-*PD-1*/*L1* can be used in the future for prevention and in early diagnosis of the tumor if the sample is treated in immunohistochemistry (IHC) (Page et al. 2019).

12.3.4.2 Anti-*CTLA4* Therapy

CTLA4 or cytotoxic T lymphocyte-associated protein 4 inhibitory checkpoint is seen on activated T cells, which are a reliable source of cancerous cells destruction. One of the drugs targeting *CTLA4* has been introduced. When anti-*CTLA4* was introduced, drastic positive changes were observed because of monotherapy. Unfortunately, it was effective in some cases but not in others. For instance, 50% of the cases showed no response. Later, the combination of anti-*CTLA4* and anti-*PD-1* blockers were given together in patients which showed good response and enhanced efficacy in breast cancer patients (Persson et al. 2011; Rotte 2019; Pilones et al. 2020).

CTLA-4 and *PD-1* blockers combination has a very strong synergistic effect in initiating anti-tumor immune response that enhances the positive outcome in patients. This is not only limited to the treatment of breast cancer but other cancer types as well. It showed great efficacy in treating cancerous cells. The medication which was used as blockers for this purpose was a combination of nivolumab and ipilimumab. This combination was also studied for the treatment of other types of cancers such as esophagogastric cancers, non-small cell lung cancer, sarcoma, and mesothelioma and showed great responses as a result (Rotte 2019).

12.3.5 Combination Therapy

As breast cancer is the most common occurring pathological condition in females, the combinatory approach is taken to give promising and lifelong effects and survival without remission or relapse of the breast cancer. Various combinations of therapeutic techniques are used for this process. For example, in the early-stage tumors, lumpectomy is performed followed by radiation for complete removal of cancerous cells. Similarly, when chemotherapeutic are used, the combination of two or more anti-cancerous drugs shows promising results. Although, they have some side effects, they decrease the progression rate of disease. Besides, the combinations are also necessary because multiple drug resistance is also observed in cancer patients hence monotherapy is no longer effective for the proper treatment of breast cancer patients (Zanardi et al. 2015; Lai et al. 2018; Fisusi and Akala 2019; Gadag et al. 2020).

Combination therapy is also used in chemo- as well as immunotherapy where a combination of two drugs is given that produces better outcome. This helps in shrinking the size of tumor that has been metastasized and in decreasing its progression which prolongs the survival of patients (Rotte 2019).

12.3.6 Radiotherapy

Usage of high-energy radiations to kill cancerous cells that are left even after a surgery is known as radiotherapy. It is one of the most important steps that is needed to be done after conservative surgery for carcinoma. As a result of radiotherapy, the chances of relapse decrease up to the maximum level. Hormonal therapy is required in response to radiotherapy in females who just recovered from the surgery. In the recent innovations, radiation of lymph nodes, especially axillary lymph nodes, are done. A large group of patients is benefitted from radiation therapy. During the past few decades, new techniques have been developed for breast cancer patients to decrease their mortality and morbidity rate. These techniques include the heart sparing technique in breast radiotherapy and neoadjuvant radio chemotherapy (Hennequin et al. 2016; Haussmann et al. 2020). Proper treatment after surgery or after chemotherapy provides long-lasting and valuable results (Wang 2013).

12.4 Conclusion

After lung cancer, breast cancer is one of the leading causes of death in females. Every year hundreds and thousands of cases are reported. Some cases are congenital while others are environmentally induced. Individuals with inherited mutation in one of the breast cancer susceptibility genes, *BRCA1* or *BRCA2*, are prone to breast cancer. These two genes are also responsible for causing ovarian cancer in females, which is also very common after 40 years. Various risk factors affect the development and progression of breast cancer, for example, age, immunity, and genetic mutation presence. Multiple techniques have been used for decades for the treatment of breast cancer.

In this chapter, we mainly described breast cancer and possible genetic mutations, their occurrence, and development of innovative ways for killing those unmanageable proliferating cells. Surgical interventions involve the usage of lumpectomy, mastectomy for surgical removal of cancerous cells. Chemotherapy involves the use of chemical compound or drugs to kill cancerous cells. Immune therapy is a newly evolved technique to kill cancerous cells. Studies show that combination therapy gives far better results than using monotherapies. Radiology also plays a very critical role in the complete and ultimate eradication of tumors. In case of metastasis, chemotherapy and immune therapy are used to control the spread of cancerous cell to shrink the size of the tumor. Furthermore, there is still a need to develop technologies for the early diagnosis of breast cancer, detection of mutations in *BRCA1/2* before the onset of proliferation of malignant cells and its treatment.

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Transcriptional Control Leading to Clinical Outcomes in Breast Cancer Cases

13

Ayesha Aftab, Ranjha Khan, Uzma Hameed, Tanveer Abbas, and Bashir Ahmad

Abstract

Transcription is an important event that use information from a gene and make its protein. Specific transcription factors are involved in the process that plays a role in generating gene expression. One of the major occasions that control the gene expression at transcription level is via epigenetics process, which include histone modifications, DNA methylation, and RNA-mediated processes. While other steps including translation also regulates the epigenetics. The expression of the gene can be controlled by two major events, one is via controlling the amount of mRNA at transcriptional level and other is by controlling the post-transcriptional events. The epigenetic regulators can affect the hormonal signaling in breast cancer, which provide a prospective target in the treatment of breast cancer. In breast cancer, complex and widespread pathways are involved that intermingle with these epigenetic elements. This chapter focuses on the epigenic events and

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their interaction with transcription pathways, which can lead towards the targeted therapies. The preclinical and clinical transcriptional control drugs/agents are also discussed in detail. Eventually, this chapter will provide a comprehensive overview of the transcriptional control events that might lead towards the successful clinical outcome.

Keywords

Breast cancer · Epigenetics · Transcription · Targeted therapy · Signaling pathways

13.1 Introduction

Previously, cancer was thought to be triggered by genetic mutations only, now it is well known that epigenetics has increased the etiological complexity of cancer pathogenicity. Epigenetic changes are heritable changes that effect the gene expression without alteration in DNA sequences. These changes result in gene expression and activity alteration and can be inherited to the next generation. These effects may be part of normal development or dietary, lifestyle, and environmental factors (Herceg 2007; Ballestar 2011; Soreide 2017; Nebbioso et al. 2018). The epigenetic changes involve different mechanisms such as histone modification, DNA methylation, and non-coding RNAs (ncRNAs) (Kanwal et al. 2015; Soreide 2017).

The epigenetic alterations in tumor-related genes lead to the imbalance of multiple gene expression, resulting in abnormal transcription regulation and loss of dynamic equilibrium in cell differentiation, migration, survival, and invasions. The well-investigated changes include DNA methylation and histone modification that target the promoter regions of genes and subsequent increase or decrease in expression of the target genes (Zhuang et al. 2020). Histone modifications are epigenetic alterations that influence the structure of chromatic and subsequent gene regulation, expression, and oncogenesis. Chromatin is composed of nucleosomes and each nucleosome is a packed structure in which DNA (147 bp) is wrapped around an octamer of histone proteins. Each octamer core consists of two subunits of four different H2A, H2B, H3, and H4 histone proteins. The center of each nucleosome is formed by the globular part of the histone proteins, with the N-terminal tail bulging from the nucleosome. The nucleosomes are stabilized by linker histone H1 and coiled chromatin fiber is formed. The histones undergo various post-translational modifications (PTS), including methylation, acetylation, phosphorylation, glycosylation, ribosylation, carbonylation, ubiquitination, and histone tail clipping. However, N-terminal tails are more PTS modification rich (Bannister and Kouzarides 2011; Kanwal et al. 2015; Kalashnikova et al. 2016; Soreide 2017; Zhao and Shilatifard 2019; Buocikova et al. 2020).

Several histone-modifying enzymes like histone methyltransferase (HMTs), histone demethylases (HDMs), histone deacetylase (HDACs and sirtuins), histone acetyltransferase (HATs), ubiquitin ligases, deubiquitinates, kinases, phosphatases,

and proteases work together for the PTS (Simo-Riudalbas and Esteller 2014; Kanwal et al. 2015; Soreide 2017). Several combinations of these modifications in specific genomic regions prime to the closing and opening of chromatin structures responsible for the repression and activation of the gene, respectively (Kanwal et al. 2015). In humans, at least 18 different types of HDACs have been identified. HATs, also a very diverse group, are classified into five families, i.e., TAFII250, MYST, SRC, p300/CBP, and GNAT. Bromodomain proteins recognize acetylation present within many HATs, effector enzymes, and other chromatin-associated proteins. Members of the bromodomain family can increase oncogenes proliferation and expression (e.g., Myc). HDMs and HMTs remove or introduce or methyl groups in proteins other than histones (Buocikova et al. 2020).

Alternations in histone-modifying enzymes either by mutation or misregulation can also develop cancer (Soreide 2017). For example, various alterations including overexpression of HDAC1, HDAC2, HDAC3, HDAC6, p300 and HBO1, depletion of H3K9 trimethyl-demethylase (JMJD2B), amplification and overexpression of EZH2 (enhancer of zeste homolog 2), downregulation of LSD1 (lysine-specific histone demethylase 1A), and others have been associated with breast cancer. However, histone modification in cancer is less studied than an alteration in ncRNAs or DNA methylation due to more demanding methodological approaches. Many epigenetic changes involve the alteration in histone methylation status by various methyltransferases and demethylases. Histone modification occurs at lysine and arginine residues and is a reversible process and some of these (H3K4, H3K36, and H3K79) are linked with transcription activation while others (H3K9, H3K27, and H4K20) are linked with transcriptional repression under normal cellular conditions (Jenuwein and Allis 2001).

Global loss of lysine K16 acetylation and tri-methylation on lysine K20 is characteristic of various cancers. In breast cancer, decreased levels of arginine methylation (H4R3me2), lysine methylation (H3K4me2, H4K20me3), and lysine acetylation (H3K9ac, H4K12ac, and H3K18ac) have been associated with poor prognostic tumors (Buocikova et al. 2020). For example, H4K20 methylation is catalyzed by KMT5 family acts as a transcription repressor. In breast cancer, H4K20me3 is decreased significantly and is an independent predictor of poor prognosis. Furthermore, breast cancer invasiveness is increased with a decrease in H4K20me3. This effect can be retreated by increased expression of SUV420H1/SUV420H2 (Li et al. 2021a). Similarly, H3K36 is demethylated by KDM4 (H3K36me2/3), KDM_2 (H3K36me1/2), and JMJD5 (H3K36me2/3). KDM2A expression in breast cancer is upregulated and it regulates the rRNA transcription. On glucose starvation, KDM_2 inhibits the rRNA transcription by demethylation of H3K36me2 at the promoter region and can lead to breast cancer cell suppression. Moreover, in $JHDM1B^{-}$ breast cancer cells significant increase in methylation at rDNA loci (H3K36me2 levels) results in the significant 45S pre-rRNA transcription and process and subsequent increase in the ribosome biogenesis (RiBi) that confers aggressiveness in breast cancer cells (Li et al. 2021a). In most of primary breast tumors, downregulation HAT (hMOF) and subsequent H4K16ac represent an early sign of breast cancer (Buocikova et al. 2020). Similarly, HAT can increase catechol-

O-methyltransferase (COMT) gene expression that is considered as breast cancer risk factor (Zhuang et al. 2020). However, the detailed role of DNA epigenetics in breast cancer will be discussed later in this chapter.

13.2 Transcriptional Regulation Effect by Acquired Factors in Breast Cancer Cases

13.2.1 Breast Cancer Incidence by Acquired Factors

Breast cancer oncogenesis is not always genetic but is also associated with non-genetic acquired risk factors. There is a long list of non-genetic risk factors related to lifestyle and personal behavior. These predominantly include birth control and contraceptive use, hormone replacement therapy (HRT), excessive alcohol consumption, obesity, not having children or not breastfeeding, early menarche, menopause, lack of physical activity, and radiation exposure (Feng et al. 2018).

Certain major acquired risk factors such as gender, aging, and genetic predisposition are associated with breast cancer and are not in control of an individual. For example, being a woman and aging increases the risk of breast cancer. Similarly, the risk of having breast cancer becomes double with first-degree relatives diagnosed with breast cancer. About 5–10% of the breast cancers are related with inherited genetic mutations in *BRCA1* or *BRCA2* gene and women with a genetic predisposition in these genes have 55–65% and 45% lifetime risk for breast cancer development, respectively. Women with these mutations are more likely to get the breast cancer at a younger age, whereas they have 70% chances of breast cancer development by age 80 (Hulka 1996; Collaborative Group on Hormonal Factors in Breast Cancer 2001; Cipollini et al. 2004; Polyak 2007; Colditz et al. 2012). Increased incidence of breast cancer has been related to lifestyle and consumption habits such as high-fat diets, alcoholism, and smoking (Martin 2000). These are the high-risk factors for this disease as shown in Fig. 13.1, which are described in detail below.

Multiple factors can cause cancer by altering the gene expression program within cells. Among these are the most easily detectable and prominent high penetrance mutations that are either acquired or inherited. In the case of breast cancer, these mutations are generally associated with BRCA1, P53, RB1, PTEN, PIK3CA, GATA-3, and MAP3K1 genes (Pérez-Solis et al. 2016). However, the etiology of breast cancer associated with these penetrance mutations does not exceed 10% (Cipollini et al. 2004). The breast cancer incidence in modern women without high penetrance mutations implies that acquired factors related to lifestyle habits are associated with mammary adenocarcinoma. Based on epidemiological studies, there is continuously growing evidence on the positive correlation between diet, smoking, alcoholism, and breast cancer in women. These findings have prompted further research to fully elucidate the role of these factors in the dysregulation of the expression of different genes associated with breast cancer (Pérez-Solis et al. 2016).

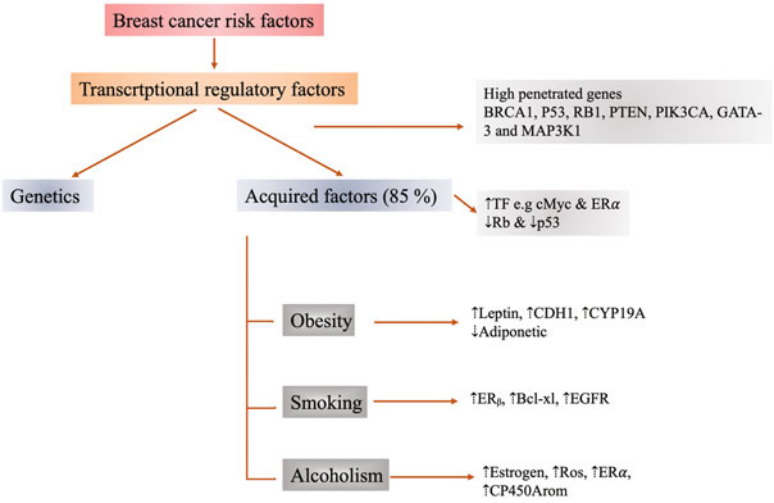

Fig. 13.1 Transcription regulatory factors responsible for onset of breast cancer

13.2.2 Transcriptional Regulation in Breast Cancer

The breast cancer prompting sources are distinct and may be genetic, epigenetic, or post-translational, resulting in the alteration of expression or functions of specific proteins, DNA-binding transcription factors, histones, coregulators, DNA, and histone-modifying enzymes that are the leading players in the regulation of transcription (Whyte et al. 2013; Pérez-Solis et al. 2016). Multiplatform genomic analysis of breast cancer tissues revealed 93 frequently mutated genes in breast cancer. Out of these, 49 genes were directly or indirectly related to transcription (Zacksenhaus et al. 2017). The upregulation of transcription factors such as c-Myc and estrogen receptor alpha (ERα) expression and downregulation of Rb (retinoblastoma) and p53 are common alterations of transcription regulation that are associated with breast cancer (Varley et al. 1989; Tripathy and Benz 1993).

13.2.3 Obesity and Effect of Obesity on Transcriptional Regulation in Breast Cancer

The proliferative effect of obesity in the mammary gland's healthy and malignant epithelial cells is prominent. Obesity and fat distribution are also influenced by endocrine hormones and hyperinsulinemia (Pujol et al. 1997). During postmenopause adipose tissue are the primary source of estrogens as ovaries stop producing the estrogen. The overconsumption of high-fat food can result in hypertrophy and hyperplasia of adipose tissues (Lipkin and Newmark 1999). The higher production of estrogens by adipose tissues contributes to increased cell proliferation, especially those carrying the mutation within mammary glands (Pérez-Solis et al.

2016). Leptin is an essential hormone produced by adipocytes that acts both in paracrine and autocrine manners. It signals the brain to inhibit hunger and thus regulates the energy balance. The leptin receptors (Ob-R) are expressed in different cell types, including mammary epithelial cells and adipose-derived stromal cells (ADSCs) (Pérez-Solis et al. 2016). Hypertrophy and hyperplasia in adipose tissues result in high leptin expression (Couillard et al. 2000) that induces many signaling pathways that culminate in the stimulation of cell proliferation (Vona-Davis and Rose 2007). The signaling networks include the IGF/insulin/Akt signaling system, the leptin/JAK (Janus kinas)/STAT (signal transducer of activators of transcription) signaling pathway, and other inflammatory cascades that have been related to obesity and cancer. Hyperglycemia, for example, has been demonstrated to activate NF-B, which could relate obesity to cancer (Anand et al. 2008; Atoum et al. 2020). Furthermore, higher C-reactive protein levels, inflammatory cytokines such as TNF-a, IL-6, IL-8, and MCP-1, and leptin contribute to the chronic inflammatory state associated with obesity in cancer (Kern et al. 2018).

High leptin levels in adipose tissues and serum, more estrogens, and high mRNA expression of leptin receptors predict a generally increased breast cancer risk and poor prognosis breast cancer patients (Atoum et al. 2020). In multiple investigations, leptin has been established as an independent predictor of breast cancer pathological tumor size and TNM stage (Simone et al. 2016).

Obesity-related metabolic abnormalities cause changes in numerous pathways, and these are the target for an anticancer drug. Among the physiological estrogen, E2 is biologically active in post-menopausal women. It has several essential roles in addition to the activation of many cell proliferation genes. It is associated with the initiation and development of breast and endometrial cancers (Zhao et al. 2016). The ADSCs can synthesize and secrete biologically active E2. The production process of E2 involves the aromatization of δ-4-androstenedione (δ4A) by CP450Arom (cytochrome P450 aromatase). During the biosynthesis of estrogen, aromatase acts as the rate-limiting enzyme. The transactivation cytochrome P450 gene (CYP19A) is crucial for the development and survival of Erα expressing malignant tumors (Bulun et al. 1994). The key mechanism for the expression of transcription of CYP19A involves binding of the transcription factor CREB (cAMP response element-binding protein) with CYP19A proximal promoter (PII). This recruitment requires CREB binding with its coactivator CRTC (CREB-regulated transcription coactivator) (Brown et al. 2009). CRTC is regulated by phosphorylation via AMPK signaling pathway. Thus, under obese conditions, a high level of leptin is responsible for inhibiting CRTC phosphorylation through Ob-R and subsequent high expression of CP450Arom and local E2 synthesis shown in Fig. 13.2. The increased level of E2 results in the upregulation of cell proliferation genes in the breast ductal epithelium of the mammary gland (Catalano et al. 2003).

Leptin also stimulates Cadh-1 production, a protein involved in adherent junctions formation and is supposed to be correlated with breast carcinoma and metastasis. Leptin interaction with Ob-R also activates the ERKs pathway, which results in the nuclear translocation and binding of Erα and CREB to SP1 (specific protein) and CRE (cAMP response element), respectively. This interaction is E2

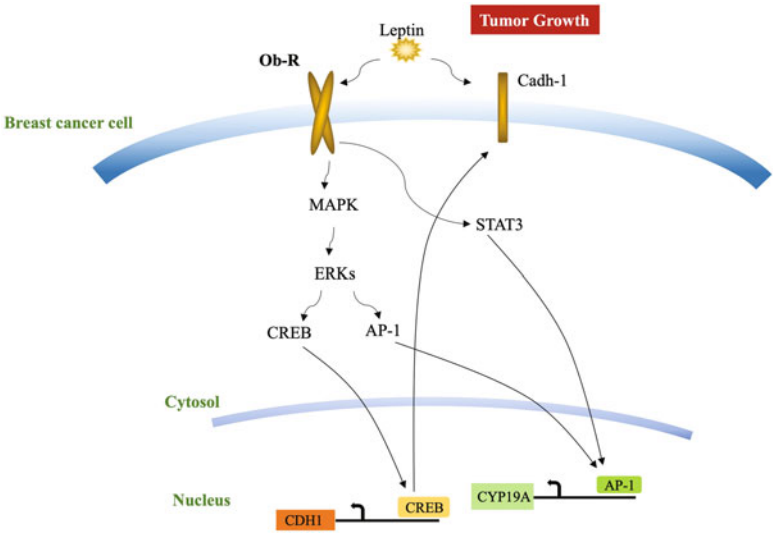

Fig. 13.2 The pathway that obesity can affect during the development of breast cancer

independent so, leptin also increases the non-classical genomic pathway of ER via Cadh-1 transcriptional activation mechanism (Mauro et al. 2007; Safe and Kim 2008; Pérez-Solis et al. 2016).

Besides leptin, another adipokine, adiponectin regulates E1 and CP450Arom levels in ADSCs. This cytokinin inhibits the cell proliferation, exerts pro-apoptotic effects on cell via p53 expression, stimulates AMPK pathway, activates the tumor suppressor complex, and inhibits growth via mTOR suppression. It also activates the PPAR-γ pathway that many other genes involved in cell proliferation and differentiation. It also inhibits the aromatase expression in breast cancer cells and low adiponectin serum level is linked with breast cancer risk (Gulcelik et al. 2012; Nalabolu et al. 2014; Simone et al. 2016).

13.2.4 Smoking and Effect of Smoking on Transcriptional Regulation in Breast Cancer

The part of smoking in cancer incidence and mortality is unarguable as it is responsible for 25% of all cancers in men and 4% of all cancers in women (Khani et al. 2018). In case of breast cancer, it is the most central risk factor (Zhang et al. 2019b). Epidemiological and clinical studies indicate a conclusive and strong correlation between active smoking exposure and breast cancer incidence (Jones et al. 2017; Zhang et al. 2019b). Numerous chemicals have been detected in mammary glands tissues of both healthy and breast cancer patients generated by cigarette consumption. Among these many chemicals such as nicotine, phenanthrene, benzo(a)pyrene, 1-methylanthracene can alter the epigenetic and

transcriptional mechanisms of different genes associated with epithelial-mesenchymal transition (EMT) in breast cancer (Pérez-Solis et al. 2016). The function of EMT is associated with initial events of metastasis that lead to the loss of epithelial properties of cancer cells to acquire mesenchymal nature and become invasive and motile. EMT is key process in breast cancer metastasis and leads to breast cancer-related death (Zhang et al. 2019b).

13.2.4.1 Effects of Smoking on Transcription Regulation

Nicotine, an important compound of cigarette smoke, interacts with the AChR (ionotropic acetylcholine receptor) of nerve cells (Albuquerque et al. 1995) and epithelial cells of other tissues that express AChR (Minna 2003; Shin et al. 2005; Guo et al. 2008). In cancer cells, nicotine influences multiple signaling pathways. The scientific outcomes in this area have revealed that nicotine provokes epithelial-mesenchymal transition (EMT) and increases the aggressiveness of breast cancer cells (Zhang et al. 2019b). However, all the mechanisms linking smoking to the development of breast cancer are not entirely understood yet.

Due to the large variety of chemicals in cigarette smoke, it is difficult to establish the etiology of cancer regarding smoking. In vitro comparison between healthy epithelial cells and breast cancer cells exposed to cigarette smoke extract (CSE) revealed dysregulation of many genes. However, a significant decrease in mRNA and proteins levels of Claudin-1 and Occludin was observed due to increased methylation. A significant increase in methylation of Erβ gene was also observed (Di Cello et al. 2013). However, the specific compound and underlying mechanism for this epigenetic change are still unclear.

The smoke-mediated transcriptional mechanism in breast cancer involves C/EBPβ (CCAAT element-binding protein beta), which transactivates the Breast cancer l-XL (anti-apoptotic gene) and promotes its transcription. Breast cancer l-XL, a marker of aggressive metastatic tumors, is involved in apoptotic caspase pathway suppression. In vitro study revealed exposure of CSC (cigarette smoke condensate) simultaneously increased the Breast cancer l-XL and C/EBPβ expressions in non-malignant cell line MCF-10A. The increase in CSC concentrations increased the expression of both Breast cancer l-XL mRNA and protein. The exact mechanism for CSC action is still unknown, but smoking may induce high penetrance mutations that lead to the aggressiveness and survival of breast epithelial cells (Olopade et al. 1997; Connors et al. 2009). Furthermore, nicotine stress in breast cancer cell induces epithelial-mesenchymal transition (EMT).

Nicotine exposure stimulates CEBPB-dependent transcription of GFA. Nicotine induces the remarkable increase in UDG-GlcNAC in breast cancer cells which in turn glycosylates the CHOP, a negative regulator of CEBPB that prevents its binds with GFAT (glutamine: fructose-6-phosphate aminotransferase) promoter. Glycosylation disrupts the transcriptional repressor CHOP (C/EBP homologous protein) and CEBPB interaction and augments the binding of CEBPT to GFAT promoter and subsequently enhances the expression of GFAT. In addition, the increase in UDP-GlcNAc cellular flux through HBP (hexosamine biosynthetic pathway) is also governed by GFAT, which is the rate-limiting step in this process. Thus, in

breast cancer cell, GFAT-governed HBP flux may directly influence the EMT by cellular OGlcNAcylation alteration. Therefore, hyper-O-GlcNAcylation plays an important role in EMT, and the development of smoking facilitated breast cancer.

Breast cancer growth is promoted by nicotine via another mechanism. On nicotine exposure, aAChR induces activation of EGFR (tyrosin kinase receptor) signaling via Scr kinase. The activation of EGFR results in the phosphorylation of ERK1/2, Src, Akt, and PKB that leads to E2F1, and Breast cancer l-2 upregulation involved in the growth and survival of breast cancer cells. The simultaneous activation of PKB (protein kinase B) and ERKs via crosstalk between the EGFR and AChR occurs. The E2F1 is a very definite transcription factor of the cell cycle regulatory CDC25A phosphatase gene. The phosphorylated PKB pathway induces the Breast cancer l-2 transcriptional expression. The crosstalk between EGFR and nAChR via Sr provides new insight into the carcinogenic effect of smoking in breast cancer (Nishioka et al. 2011).

13.2.5 Alcoholism and Effect of Alcoholism on Transcriptional Regulation in Breast Cancer

Consumption of alcohol is a recognized risk factor linked with breast cancer in women. About 10 g per day alcohol intake is linked with about 8–9% increase in breast cancer risk. The enduring alcohol consumption will increase the risk to 15% and 51% with 5–9.9 g/day and 30 g/day, respectively. Estrogen receptor ER-negative (ER^{-}) and (ER)-positive (ER^{+}) tumors show a positive association with alcohol consumption, but the latter appears strongly associated (Wang et al. 2017). The time window between menarche to first pregnancy when breast tissue is particularly inclined to carcinogens is also important. Epidemiological data shows that early life alcohol consumption increases the risk of both pre- and post-menopausal breast cancer. Similarly, more prolonged exposure to alcohol before first pregnancy may predispose to breast cancer (Liu et al. 2015).

The exact mechanism of alcohol and breast cancer association is not fully understood, but it is hypothesized to be associated with estrogen metabolism, at least in part (Wang et al. 2017). Other proposed mechanisms included the acetaldehyde and reactive oxygen species (ROS) generation associated with alcohol metabolism. IARC (International Agency for Research on Cancer) has classified acetaldehyde as a carcinogen. It can form DNA adducts that promote mutagenesis and cell malignancy. It has been verified experimentally that alcohol consumption leads to acetaldehyde accumulation in rat mammary tissues (Pérez-Solis et al. 2016; Wang et al. 2017). The clinical studies revealed an increased risk of breast cancer is associated with moderate alcohol intake (15–30 g/day) in a group of premenopausal women homozygous for $ADH^{1-1}{}_{3}$ (alcohol dehydrogenase 3^{1}) allele. This phenotype is linked with increased alcohol dehydrogenase activity and thus subsequent acetaldehyde production, implying the association of acetaldehyde produced from alcohol with increased breast cancer risk (Pérez-Solis et al. 2016). The ROS, generation by ethanol metabolism, promotes several aspects of tumor development

and progression. Furthermore, the DNA and/or DNA and/or histone hypomethylation and disruption of folate metabolism have also been proposed to be involved in alcohol-mediated carcinogenesis (Wang et al. 2017).

13.2.5.1 Effects of Alcohol in Transcription Regulation

Alcoholism can cause changes in the expression of genes by different pathways. The change in the transcriptional expression of genes by as low as 0.06% ethanol concentration is associated with malign proliferation in epithelial cells of mammary glands. Nearly, 75% or breast cancer patients are ERα-positive and the role of the ethanol-induced estrogen-mediated cellular proliferation, metastasis (Wong et al. 2012), and survival is already demonstrated in many studies (Singletary et al. 2001; Brown et al. 2009). The overexpression of ERα and CP450Arom is critical to early mammary adenocarcinoma development. The ethanol induces the synthesis of ERα mRNA and subsequent proliferation of breast cancer cells when used in moderate doses. However, complete transcriptional regulation is still not clear. However, the transcription effect of the acute and chronic exposure of ethanol concentrations >0.5% revealed that ethanol provides greater stability to AC (adenylyl cyclase) by inhibiting the function of Gαi. This inhibition increases in cAMP level (Blumenthal et al. 1991; Nagy and DeSilva 1992; Singletary et al. 2001; Yoshimura et al. 2006) that in turn promotes the transactivation of genes such as CP450Arom and Amph (Amphiregulin) are the target of CREB and Amph (Amphiregulin). Amph belongs to the endothelial growth factor family and is a mitogen agonist (Meng et al. 2000; Mauro et al. 2007; Willmarth and Ethier 2008). In breast cancer, there are two alternates but linked routes for the EGFR signaling pathway-mediated production of E2. The first and most widely described pathway is via the expression of the Amph and TGFα by ERα-dependent transcriptional activation pathway (Meng et al. 2000; Ciarloni et al. 2007; Kenney et al. 1993; Levin and Pietras 2008). The second pathway involves GCPR (membrane-bound classic estrogen receptors or G protein-coupled estrogen receptor) that overstimulates the EGFR signaling pathways. GCRP activates (Bates et al. 1988) metalloproteinases that are involved in the discharge of EGFR agonists that are attached to the cell membrane (Levin and Pietras 2008; Filardo 2002). Furthermore, in breast cancer cells, EGFR can also induce Erα activity both in ligand-dependent and independent pathways via inhibitor of kappaB kinase α (IKKα) phosphorylation and PKA, respectively. Both the pathways converge and transactivate in several genes, including Myc, Breast cancer l-XL, cyclin D1, CDKN1A, and AP1, which are correlated with tumor proliferation, aggressiveness, and survival. Thus, this bi-directional feedback between estrogenic activity and EGFR is critical for cell proliferation in breast cancer cells (Pérez-Solis et al. 2016). In another in vitro study, unlike leptin, ethanol increased cell migration at 12% conc. by inhibiting the Cadh-1 expression in breast cancer cell line (Meng et al. 2000; Mauro et al. 2007) by recruiting master repressor factors, Slug and Snail. Interestingly, these two factors are activated by AKT, p38, and ERK which are themselves targets of EGFR (Fearon 2003). However, the exact mechanism of how ethanol promotes cell migration needs to be confirmed experimentally.

Moreover, increased dose-based exposure of ethanol resulted in a corresponding increase in expression of Erα and a decrease in BRCA1 level in HER2-positive mice tumors and ERα-negative cell lines. As the repression of transcriptional activity of Erα via BRCA1 interaction is critical in breast cancer cell proliferation, the dysregulation in the level of both implicates the direct involvement of ethanol in transcriptional regulation of these genes. However, experimental verifications are still required (Fan et al. 1999; Meng et al. 2000; Wong et al. 2012). Epidemiological and clinical studies have recounted that ethanol abuse is also linked with systematic folate deficiency in chronic disorders such as certain cancers, hepatosteatosis, pancreatic diseases, and megaloblastic anemia (Savage and Lindenbaum 1986; Levin and Pietras 2008; Wani et al. 2011). Ethanol interferes with the absorption and assimilation of folate and subsequent methionine synthesis in cells (Hamid et al. 2009; Christensen et al. 2010). The methionine deficiency reprograms the genome activity and promotes the expression of oncogenes (Halsted et al. 2002; Giovannucci 2004; Portela and Esteller 2010) like ethanol-induced expression of ERα in breast cancer but further studies are still required to confirm it. The expression and activity of DNA methyltransferases altered ethanol can be explained by ethanol-induced global hypomethylation (Lopatina et al. 1998). However, in tumor cells, the ethanol-induced specific hypermethylation of tumor suppressor genes (such as silencing of BR CA1 in breast cancer) needs to be established experimentally (Pérez-Solis et al. 2016). In this regard, the integration of alcohol-induced DNA methylation and gene expression data can be helpful to explore the underlying mechanisms involved in the role of ethanol in breast cancer (Wang et al. 2017).

13.3 Ribosomal RNA Transcription in Breast Cancer

13.3.1 Ribosome's Biogenesis

The ribosomes, a ribonucleoprotein complex, are universally conserved molecular machines responsible for proteins synthesis in all living cells. It is consisting of two subunits, i.e., small and large subunits. Each of these is formed by the interaction of numerous distinct ribosomal proteins (r-proteins) and ribosomal RNA (rRNA). The large subunit catalyzes the peptidyl transferase reaction during protein synthesis (Ban et al. 2000). The small subunit has a decoding function and involves in the pairing of codon on mRNA and anticodon on tRNA. The 60S ribosome subunit in humans comprises 18S rRNA a33 ribosomal proteins (RPs). The small 40S subunit comprises three rRNAs (5S, 5.8S, and 28S rRNA) and four distinct RPs (Ban et al. 2000; de la Cruz et al. 2015; Panse and Weirich 2016; Pelletier et al. 2018).

The formation of ribosomes, i.e., ribosomes biogenesis (RiBi), is a complex and crucial cellular process and is ultimately responsible for synthesizing all cellular proteins. In eukaryotes, it takes place both in nucleolus and cytoplasm. The nucleolus (plural nucleoli) is the most conspicuous and dynamic structure within the nucleus. In human cells, nucleoli are formed around nucleolar organizing regions (NORs) of short arms of acrocentric chromosomes (13, 14, 15, 21, and 22) that

contain ribosomal RNA genes. These regions contain clusters of hundreds of rRNA genes that are present in the form of tandem repeats with several palindromic units (Pederson 2011; Pelletier et al. 2018; Harold et al. 2021). Structurally, the nucleolus has a specific tripartite architecture as it consists of subcompartments, i.e., FC (fibrillar center), DFC (dense fibrillar component), and the GC (Glandular component).

rRNA transcription-associated factors are localized in FC region and the transcription of rRNA genes occurs at the interface of DFC and FC by RNA Polymerase 1 (RNAP1), resulting in the 47S pre-rRNA transcript. This primary transcript is further treated to produce mature 5.8S, 18S, and 28S rRNAs within DFC region. The fourth rRNA (5S) gene, located on chromosome 1, is transcribed in nucleoplasm by RNA polymerase III (RNAPII). The genes of RBs are located on X, Y, 20, and 22 chromosomes. The assembly of the pre-ribosomal subunit (90S) takes place in the GC region. This process involves a very ordered co-transcriptional association between primary 47S pre-RNA transcript with 5S rRNA, most RPs and numerous assembly factors. The assembly factors such as ~200 *trans*-acting factors, including snoRNAs (small nucleolar RNAs), assembly factor proteins, endonucleases, exonucleases, base modifying, and ribose-modifying enzymes all help to modulate the processing, modification, and proper folding of the pre-rRNAs into pre-40S and pre-60S subunits. Both subunits are then transferred to the cytoplasm where both subunits undergo final maturation steps and assemble into 80S ribosomes and are ready to perform protein translation (Thiry et al. 2000; Pelletier et al. 2018; Tiku and Antebi 2018; Harold et al. 2021).

The number of ribosomes directly reflects the translation capacity that is the fundamental cellular process required to sustain the cell. RiBi is an rDNA transcription-dependent process, so it is crucial to maintain fundamental cellular processes. rDNA transcription by RNAPI represents approximately 60% of nuclear transcription. This high rRNA synthesis is satisfied by the arrangement of rDNA loci in tandem repeat and high copy number, which is about 300 rDNA repeats in human diploid cells. However, not all these genes, but only a subset of these genes, is transcribed at a given time (Conconi et al. 1989; Moss and Stefanovsky 2002; McStay 2016).

Eukaryotic RNA Pol I is a protein (590 kDa) composed of 14 subunits. It requires three basal transcription factors (SL1 complex, UBF, and Rrn3) for efficient transcription of 47S pre-rRNA gene (Fernández-Tornero et al. 2013; Campbell and White 2014; Pelletier et al. 2018). UBF (upstream-binding factor), which is a nucleolar transcription factor (Pelletier et al. 2018), binds to rDNA at multiple sites leads to the assemblage of PIC (pre-initiation complex) and rDNA chromatin remodeling. SL1 (selectivity factor 1) complex is composed of TBP (TATA-binding proteins) and TAFs (TBP-associated factors). SLI and UBF interact with each other and bind with promoter sequences of rDNA. The third cofactor RRN3 (TIF-A1) recruits RNA Pol I via UBF/SL1 complex-mediated interaction that starts the transcription process (Russell and Zomerdijk 2005; Campbell and White 2014).

13.3.2 RNA Polymerase I Transcription Activity in Breast Cancer

Dysregulation in ribosome biogenesis in human cells results in various diseases and disorders such as ribosomopathy, Treacher Collins syndrome and cancer (Hannan et al. 2013). Interestingly, the rDNA copy number is variable in metastatic breast cancer cells where both loss/gain of mutation events have been spotted. For malignant transformation, increased quantities of ribosomes are required that reflect the increased activity of all the RNA polymerase (Valori et al. 2020; Harold et al. 2021). However, RNAP1 activity is the hallmark of the malignant transformation, and the most substantial evidence comes from the expression of ΔN-netrin-1(nucleolar N-terminal truncated isoform of netrin 1) in malignant cells. In normal cells, netrins are involved in axon guidance. But ΔN-netrin-1 binds with RNAPI transcriptional machinery components in cancer cells and drives rRNA synthesis, pre-rRNA, processing, and subsequent increase in the number of mature ribosomes in tumor cells. Furthermore, ECT2 (epithelial cell-transforming sequence 2 oncogenes), a guanine nucleotide exchange factor, when phosphorylated by PKC1 (protein kinase C1 type), activates rDNA transcription by binding with UBF1. The auranofin, a specific inhibitor of PKC1, exerts an anti-proliferative effect in the cancer cells by inhibiting ECT2 phosphorylation and subsequent rDNA transcription (Pelletier et al. 2018).

13.3.2.1 Nucleolar Remodeling in Breast Cancer

The growth and health of cells are dependent on the nucleolar ultrastructure that is susceptible to genetic and environmental changes. Abnormalities such as increased nucleolar numbers, hypertrophy, and irregular morphology and mirror increased RNAPI transcription are hallmarks of many types of cancers, including breast cancer. It reflects increased quantities of ribosome and protein translation to accommodate the high metabolic activity of the proliferating cancer cells. Even though the morphology of nucleolus in slowly proliferating malignant cancerous tissues remains unaltered and is of no diagnostic utility (Derenzini et al. 1990; Derenzini and Ploton 1991), the link between the nucleolar adaptions and cancer cannot be disregarded. Instead, nucleolar morphology has been employed frequently as a prognostic factor in breast cancer.

For example, screening for the nucleolar size from 1600 patients with invasive breast cancers using hematoxylin and eosin-staining showed that higher nucleolar scores of tumor cells are associated with breast cancer-specific survival (breast cancer SS) and shorter distant metastasis-free survival (DMFS). It is also a highly significant and promising parameter for breast cancer grading and other clinicopathological parameters such as patient age (Elsharawy et al. 2020). Similarly, AgNORs (silver staining of argyrophilic nucleolar organizer regions) analysis of breast cancer tissues showed the correlation with smaller nucleolar areas and more robust survival rates of patients and vice versa (Derenzini et al. 2009). rDNA transcription is the driving factor for nucleolar formation as well as is the rate-limiting step for cellular proliferation. Therefore, understanding pathways and molecules concerned in the regulation of RNAPI activity and subsequent

pathogenetic mechanisms of breast cancer will lead to identifying the new anti-tumor drug targets (Harold et al. 2021).

13.3.3 Signal Transduction Pathways that Modulate RNA Polymerase I Activity

RNAP1 transcriptional activity dysregulation is a cause of many cancers, including breast cancer. The Myc, PI3K/AKT/mTOR, and Wnt are critical signaling pathways that modulate the activity of RNAP1 and are often dysregulated in malignant tissues.

13.3.3.1 MyC

The MYC family of cellular proto-oncogenes consists of three but highly related nuclear phosphoproteins C-Myc, N-Myc, and L-Myc. These transcription factors are the downstream target of many signaling pathways and have a vital role in the transcription of many genes involved in ribosomes biogenesis, cell cycle progression, proliferation, immortalization, differentiation, cell adhesions, metabolism, and apoptosis (Adhikary and Eilers 2005; Campbell and White 2014). In >50% of human cancers, Myc family oncogenes are deregulated and frequently linked with poor prognosis (Chen et al. 2018b). c-Myc overexpression in mammary glands is associated with induced tumor development, progression, high increase in tumor size, and poor relapse-free survival (Stewart et al. 1984; Schoenenberger et al. 1988; Qu et al. 2017). MYC enhances ribosome biogenesis by dysregulating RNA Pol I and III activities (Adhikary and Eilers 2005; Campbell and White 2014; Harold et al. 2021).

Dysregulation of RNA Pol I-derived transcription in breast cancer involved enhanced rRNA genes transcription by affecting the interactions of UBF, RRN3, and RNA Pol I subunits. The binding of c-Myc to promoter rRNA genes facilitates the enrolment of the SL1 complex to activate RNA Pol I and initiate PIC formation. Overexpression of c-MYC is also linked with nucleolar hypertrophy depicting a role in maintaining the nucleolar structure (Campbell and White 2014; Harold et al. 2021). RNA Pol II-associated chromatin interactions in the Myc oncogene subchromosomal region are involved in prostate cancer. It involves the AR and FOXA1-mediated activation of enhancers. The activated enhancers modulate RNA Pol II activity by interacting with the Myc promoter (Ramanand et al. 2020).

13.3.3.2 PI3K/AKT/mTOR Pathway

The PI3K/AKT/mTOR signaling pathway is a very intricate intracellular pathway that orchestrates a broad spectrum of cellular activities, including the metabolism, regulation of cell growth, and autophagy (Paplomata and O'Regan 2014; Harold et al. 2021). The dysregulation in this pathway can lead to cell growth and tumor proliferation. It also shows an important role in endocrine resistance in breast cancer.

Lipid kinase P13K, a heterodimer from class IA of P13Ks, plays a prominent role in this pathway. It consists of two subunits, i.e., regulatory (p85) subunit and catalytic (p110) subunit. P85 regulates the activation of p110 in response to

stimulation of RTKs (receptor tyrosine kinases) by different growth factors (Paplomata and O'Regan 2014), such as fibroblast growth factor receptor (FGFR), epithelial growth factor receptor (EGFR), and vascular endothelial growth factor receptor (VEGFR). RTK recruits an adaptor protein to indorse the binding of p85 and p110 to activate PI3K. Activated PI3K converts PIP2 (phosphatidylinositol 3,4-bisphosphate) into PIP3 (3,4,5-triphosphate), which acts as secondary messenger and binds to PDK1 (phosphoinositide-dependent kinase-1) and phosphorylates AKT. AKT is the key signal transduction protein, and it can also be phosphorylated by mTORC2. After activation, AKT phosphorylates numerous substrates and downstream effectors, involving MMP (matrix metalloproteinase), VEGF, mTOR (mammalian target of rapamycin), and CDKs (cyclin-dependent kinases). Furthermore, in breast cancer, the gene of PDK1 is the most frequently mutated and the critical determinant of anticancer therapy resistance (Liu et al. 2009; Cidado and Park 2012; Dong et al. 2021).

Breast cancer development and endocrine resistance are associated with the hyperactivation of this pathway which in turn results in the upregulation of the effector mTOR. mTOR, a serine/threonine kinase is, present downstream of PI3K and Akt, and enhances cellular proliferation by increasing rRNA synthesis (Lauring et al. 2013; Paplomata and O'Regan 2014; Harold et al. 2021). mTOR can be activated by many signals, including environmental, nutritional, and growth factors, thus modulates the transcription of rDNA through several mechanisms. For example, mTOR helps in PIC formation through its downstream target ribosomal protein S6K1 (S6 kinase 1). S6K1 activates the C-terminus domain of UBF by phosphorylation leading to binding of SL1 at rDNA promoter. mTOR also enhances rRNA synthesis by binding to the rDNA promoter directly and induction of MYC translation indirectly. Furthermore, mTOR phosphorylates RRN3 (TIF-1A) to modulate its activity and localization (Mayer et al. 2004; Tsang et al. 2010; Chen et al. 2018b). RRN3 is a chief rate-limiting factor for the initiation step of rDNA transcription and is a shared target of both mTOR and c-Myc oncogenic pathways. In breast cancer cells, frequent upregulation of RRN3 genes and concomitant increase in pre-RNA levels, both in advanced and early stages of breast cancer, depict that overexpression of RRN3 is enough to increase rRNA transcription. mTOR is referred to two different complexes, mTORC1 and mTORC2 and both display different modes of action. mTORC1 is better studied and characterized for its role in tumorigenesis and is also the target of rapamycin and rapamycin analogs. However, recently the role of mTORC2 has been described in cancer cell growth. ATK, the most commonly hyperactivated protein in cancer, is a crucial substrate of mTORC2. After activation by mTORC2, ATK activates mTOR signaling, which adds more complexity to this signaling pathway (Paplomata and O'Regan 2014; Kim et al. 2017). Moreover, AKT can also regulate RRN3 binding to rDNA loci independently from mTOR and augment RNAPI activity via CK2-mediated phosphorylation (Nguyen and Mitchell 2013). Thus, in breast cancer cells, enhanced rRNA synthesis driven by overexpressed RNAPI transcription-associated factors can be considered a key contributor to mammary oncogenesis initiation (Harold et al. 2021).

13.3.3.3 Wnt Signaling Pathways

Wnt signaling includes a group of highly evolutionary conserved and important signal transduction pathways that regulate cell proliferation, cell polarity, and differentiation during embryogenesis (Komiya and Habas 2008; Zhan et al. 2017; Weeks et al. 2021). The Wnt signaling is distinguished into three major cascades, i.e., canonical, noncanonical planar cell polarity and Wnt/Ca^{2+} (Komiya and Habas 2008). The canonical Wnt/β-catenin pathway is critical for the development of mammary glands, especially mammary ductal epithelium development, mammary ductal progenitor cell population maintenance and luminal differentiation (Weeks et al. 2021; van Schie and van Amerongen 2020). The dysregulation of Wnt signaling leads to breast cancer and in over 50% of breast cancer patients, Wnt signaling is activated and associated with reduced overall survival (Zhan et al. 2017; Weeks et al. 2021). Wnt/β-catenin signaling pathway regulates the expression of rDNA transcription-associated factors, including putative regulator PPAN (peter pan homolog) and c-Myc. Thus, this pathway affects the activity of RNAP1 indirectly. β-catenin regulates the c-Myc expression in non-basal like breast cancer cells (Xu et al. 2016). In triple-negative breast cancer (TNBC) cell lines, more nucleoli per cell are associated with upregulated Wnt/β-catenin signaling pathway than non-TNBC cell lines. The treatment of TNBC cells with a highly specific inhibitor of β-catenin-driven transcription (catenin-related transcription inhibitor) at sub-lethal doses significantly reduced nucleolar number. Furthermore, significantly reduced expression of LAS1L (LAS1-like ribosome biogenesis) factor in the nucleoli of β-catenin-inhibited TNBC cells was observed. LAS1L protein expression is functionally critical for invasive tumor growth in mammary glands and is significantly high in TNBC (Weeks et al. 2021). Thus, a significant reduction in nucleolar number by inhibitors of β-catenin-driven transcription implicates the reduced rDNA transcription and ribosome biogenesis, in addition to highlighting the novel β-catenin function in orchestrating nucleolar activity in TNBCs (Weeks et al. 2021).

In cancer cells, nucleolar stress over-activates the canonical Wnt pathway and stimulates PPAN (Peter Pan) expression to maintain rRNA synthesis. PPAN is an evolutionarily conserved and essential ribosome biogenesis factor is localized to the nucleolus and mitochondria. The cell cycle needs to stabilize UBF and help in 47S rRNA processing. High expression of PPAN has been identified as a novel prognostic marker of TNBC and associated with poor overall survival of TNBC patients (Pfister et al. 2015; Bao et al. 2020).

Noncanonical, β-catenin-independent Wnt pathway involves negative regulation of rDNA gene transcription by Wnt5a in breast cancer. Wnt5a is para- and autocrine β-catenin-independent ligand that induces tumor suppression in breast cancer. In 45–75% of breast cancer patients, reduced expression of Wnt5a is observed, and this protein is associated with reduced disease free-survival and early relapse (Prasad et al. 2018; Harold et al. 2021). Wnt5a suppresses tumor growth via impaired cell migration and invasion. It has anti-proliferating activity due to rDNA transcription repression capacity in breast cancer. It was evident in the $Wnt5a^{-/-}$ transgenic mice model of breast cancer which exhibited both increased AgNORs and high expression of tumor proliferation marker Ki-67 compared to wild-type tumors.

Furthermore, human breast cancer cell line MCF7 expressing exogenous Wnt5a showed decreased nucleolar area, 47S pre-tRNA level and cellular proliferation, indicating the overall antagonist effect of Wnt5a on rRNA synthesis. This Wnt5a repression of rRNA synthesis was explicitly due to NORs localization and subsequent binding of Dishevelled 1 (DVL1) to rDNA chromatin in breast cancer cells. It is due to an obligate downstream effector of the Wnt5a signaling pathway, and it leads to the deacetylase SIRT7 dissociation from the RNAPI machinery (Dass et al. 2016). SIRT7 catalyzes the deacetylation of PAF53, which is a subunit of RNAPI and enhances its binding with rRNA genes (Chen 2016).

13.3.4 Tumor Suppressor Protein Inhibits Ribosomal DNA Transcription

The product of tumor suppressor genes (TSGs) are tumor suppressor proteins which are crucial for cell survival. These are involved in inhibition of cell division, induction of apoptosis, DNA damage repair, and suppression of metastasis. Therefore, loss-of-function mutations within these TSGs would result in beginning and development of cancer (Wang et al. 2018). The p53, pRb, PTEN, and ARF are important tumor suppressors that suppress breast tumors by inhibiting rDNA transcription.

13.3.4.1 p53

TP53 (p53) being common mutated gene in cancers including breast cancer which appear in all up to 35% and 80% in invasive breast cancer and triple negative, respectively (Duffy et al. 2018). P53 is a 53 kDa protein that was first discovered in 1979 (Vogelstein et al. 2010). It regulates the cell cycle by arresting the G1/S phase in response to cellular stress and DNA damage (Chen 2016). The loss-of-function mutation is associated with abnormal proliferation and more aggressive breast cancer notably in basal-like breast cancer (Kumar et al. 2012). In addition to cell cycle regulation, P53 arrest cell proliferation and growth by inhibiting rRNA synthesis. It represses RNAP1 transcription by association with TBP and TAF_I110. It also interferes with PIC formation by associating with the TBP subunits of the SL1 complex thus abrogating SL1 and UBF interaction and subsequent inhibition of RNPI transcription. Furthermore, loss of p53 expression is linked with increased AgNOR mean area values leads to a worse prognosis (Treré et al. 2004; Grummt 2010; Harold et al. 2021).

Ribosome biosynthesis is also sensitive to nucleolar stress response (NSR) that can cause cell cycle arrest and apoptosis in response to cellular stressors such as hypoxia and heat shock. MDM2 (mouse double mint 2: an E3 ubiquitin ligase) is a negative regulator of p53. In normal cellular conditions, MDM2 binds to N-terminus of the p53 and stops its transcriptional activity. The binding also initiates the ubiquitination of p53 and subsequent degradation by the proteasome system. Furthermore, MDM2 also contains a nuclear export signal that interacts directly with P53 and induces its nuclear export (Geyer et al. 2000; Lohrum et al. 2001; Dai et al.

2004; Dong et al. 2021) and prevent it from interacting with DNA elements. Thus, MDM2 suppresses p53-mediated apoptosis and cell growth arrest. However, under stressed conditions, NSR leads to the release of ribosomal ribonucleoproteins (mRNPs) that bind to MDM2 and inhibit its binding with P53 as well as E3 ligase activity (Dai et al. 2004; Grummt 2010). p53-dependent cell cycle arrest inhibits RiBi by disrupting rRNA processing. Thus, there is a clear link between the cell cycle, RIBI and P53. The nucleolus acts as a stress sensor and maintains a low level of p53. Under stress conditions, the nucleolar function is impaired, which automatically leads to elevated levels of P53 and subsequent cell death. However, nucleolar stress can also be P53-independent (Rubbi and Milner 2003; Bursac et al. 2014) but in cancers cells with p53 mutation, nucleolar activity targeting is a viable option.

13.3.4.2 pRb

Tumor suppressor protein pRb (retinoblastoma protein) has the same functional role as p53. pRb belongs to the pocket protein family that are negative growth regulators. pRb induces stress-mediated cell cycle arrest via E2F dysregulation. However, it can also control chromatin structure, chromatin cohesion, differentiation, cell death, and tumorigenic proliferation through mechanisms beyond the influencing E2F (Ciarmatori et al. 2001; Witkiewicz and Knudsen 2014). In the differentiated cells or cells with cell cycle arrest, pRB accumulates in nucleoli and repress rDNA transcription (Cavanaugh et al. 1995; Voit et al. 1997; Hannan et al. 2000; Grummt 2003). pRb also dysregulates the rRNA transcription by directly repressing the RNAP I activity. This repression is mediated by the interaction between pRB C-terminal part and HMG boxes 1 and 2 of UBF leading to inactivation of UBF by deacetylating (Grummt 2003; Pelletier et al. 2018). However, an overlapping function of pRb family members is required as rRNA synthesis remained unchanged in $Rb^{-/-}$ cells compared to the cells lacking either all three or pRb and p130 proteins. pRB and pocket protein p130 share the ability to repress RNAP 1 transcription via UBF interaction both in vivo and in vitro (Ciarmatori et al. 2001; Grummt 2003).

In general agreement, pRb must be inactivated via phosphorylation to proceed with the cell cycle. However, the inactivation of Rb involves a plethora of other mechanisms. In vivo and in vitro loss-of-function pRb gene mutations have been reported in many cancers, including breast cancer. For example, ~40% and ~20% of DCIS of TNB and ductal carcinoma *in situ* (DCIS) are *pRb* negatives. However, different subtypes of breast cancer are associated with differential mechanisms of Rb pathway inactivation. For example, ER (estrogen receptor)-positive breast cancer commonly displays CDK4/6 dysregulation due to aberrant expression or amplification of cyclin D_1 (Witkiewicz and Knudsen 2014).

13.3.4.3 PTEN

PTEN gene encodes phosphatase, and it is a dual phosphatase of protein and lipid and was first reported in 1997 as a TSG. It displays the regulatory role in major cellular processes, including signaling pathways, growth, proliferation, survival, and apoptosis directly or indirectly. As a lipid phosphatase, it is an antagonist of

phosphatidylinositol 3-kinase and regulates the AKT pathway. *PTEN* inactivation cause activated intensities of AKT, thus promoting cell growth, survival, migration, and proliferation via multiple downstream effectors (Majumder and Sellers 2005). Loss in the expression of PTEN is associated with the development of many types of cancers including breast, lung, glioblastoma, and prostate cancer (Lebok et al. 2015; Chen et al. 2018a; Harold et al. 2021). Dominating biological effects of PTEN is dephosphorylation of PIP_3 into PIP_2. PIP_3 accumulation serves as a major signal for the stimulation growth factor AKT and protein kinase C (PKC) (Chen et al. 2018a). PTEN is a negative regulator of the PI3K/AKT/mTOR pathway and therefore, ultimately decreases the rRNA synthesis and cell growth. It also directly represses the RNAP1 transcription by interfering with RNAP 1 binding at the promoter site. This involves the differential reduction in the occupancy of SL1 subunits on the promoter of rRNA gene and dissociation and disruption of SL1 complex from promoter site (Weng et al. 1999; Zhang et al. 2005).

A noteworthy correlation between loss of PTEN expression and aggressive BC, including lymph node metastasis, larger tumor size, later TNM stage, and poor differentiation, has been recorded (Li et al. 2017). The sufficient expression of *PTEN* is vital for normal cell function and prevents uncontrolled proliferation partially mediated by decreased transcription of the rRNA genes.

13.3.4.4 ARF

A key upstream controller of p53 is the tumor suppressor ARF, which provides the first line of defense against hyperproliferative signals that are provoked by oncogenic stimuli. ARF is sequestered in the nucleoli of unstressed cells. Nucleolar sequestration of ARF depends on continuous transcription, and the release of ARF from the nucleolus is a plausible mechanism for transmission of the stress signal. ARF activity is induced upon nucleolar stress, which increases p53 concentrations by binding to MDM2/HDM2 and inhibiting its ability to trigger p53 degradation. ARF has been reported to downregulate RNAPI transcription through interaction with UBF and inhibition of pre-rRNA processing, possibly by lowering the level and/or activity of the endonuclease NPM, thereby blocking a specific step in the maturation of rRNA. Thus, ARF not only triggers a p53 response that represses RNAPI transcription but also blocks the production of mature rRNA by inhibiting the processing of pre-rRNA. Presumably, the primordial role of ARF is to slow ribosome production in response to hyperproliferative stress provoked by oncogenic stimuli. Its subsequent linkage to p53 may have evolved to improve its efficiency and provide an adequate checkpoint for coupling ribosome production with p53-dependent inhibitors of cell cycle progression. Moreover, a recent study demonstrated that ARF inhibits the nucleolar import of transcription termination factor I (TTF-I), causing TTF-I accumulation in the nucleoplasm (Savkur 1998; Németh et al. 2008; Grummt 2010; Lessard et al. 2010).

The p14 alternative reading frame (p14Arf) is a tumor suppressor protein and an upstream regulator of p53. It regulates the cell cycle during the G2 phase and provides the defense to the cells against hyperproliferative signals of oncogenic stimuli (Normand et al. 2005; Grummt 2010). In the normal cells, ARF is

sequestered in the nucleoli, from where it is released and activated on the transmission of the nucleus stress signal. In the active forms, it bins with MDM2/HDM2 and increases the concentration p53 by inhibiting its degradation. p14Arf-Topo 1 complex (in complex with topoisomerase 1) also downregulates the RNAP1 by interacting directly with the rDNA promoter (Ayrault et al. 2004). In another p53-independent pathway, p14Arf hypophosphorylates the UBF and consequently interrupts its efficiency to recruit the transcription complex (Ayrault et al. 2004, 2006; Grummt 2010). In another pathway, p14Arf disrupts the subnuclear localization of TTF-1, which shuttles between nucleoplasm and nucleolus and inhibits its nucleolar import. The depletion of TTF-1 in nucleolar inhibits ribosomal RNA synthesis. TTF-I also promotes the nascent rRNA release by facilitating the termination of transcription and re-initiation by RNAPI. p14Arf blocks pre-mRNA processing and maturation of mRNA (Normand et al. 2005; Németh et al. 2008; Lessard et al. 2010).

The role of p14Arf in breast cancer is not well described. The expression of this protein alters with the stage of cancer. An increased expression of p14Arf is prominent in both invasive and preinvasive breast cancer. An increased expression of p14Arf along with another senescence marker (p16INK4a) has been associated with reduced prognosis and increased threat of recurrence in invasive ductal carcinoma (Silva et al. 2003; Pare et al. 2016). The high initial expression p14Arf followed by a decrease with further breast cancer tumor progression may counteract cancer (Wazir et al. 2013). Furthermore, this elevated expression of p14Arf is primarily localized in the cytoplasm instead of the nucleolus, indicating functional inactivation or a dual function of p14Arf to show tumor suppressor activity in some tumors and oncogenic activity in some others (Pare et al. 2016).

13.3.5 Regulation of rRNA and Transcriptional Control by Non-coding RNAs

Advancement in molecular biology elucidated the greatest surprise with the discovery even though 90% genome in human is dynamically transcribed but below 2% of the total genome represents the protein-coding genes only. Now it has become evident that non-coding RNAs (ncRNAs) that were initially described as accumulated evolutionary debris or spurious transcriptional noise play chief biological roles in cell physiology, development, and pathologies. The differential expression of ncRNA is recognized as hallmarks of cancer cells, and these employed as novel prognostic, diagnostic, and predictive biomarkers (Sana et al. 2012).

microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) add an additional layer of rDNA transcription control (Harold et al. 2021; McCool et al. 2020). miRNAs constitute a class of short (~21 nucleotides), ncRNAs that regulate post-transcriptional stability and translatability of mRNA. The progression of many cancers correlates with dysregulation of miRNA expression. Currently, about ~3662 reports are available on miRNA involved in initiation, progression, and metastasis of breast cancer. Several miRNAs directly regulate the rRNA

transcription via Myc and P53 interactions (McCool et al. 2020; Harold et al. 2021). For example, dysregulation of Let-7 family miRNA has been reported in breast cancer. Let-7 family miRNAs under normal cellular conditions downregulate Myc. Rpl22 (miRNA Ribosomal protein L22)-mediated downregulation of Let-7 family miRNA is reported in invasive breast cancer cells. Inactivation of Rpl22 upregulates stemness factor Lin28B, which downregulates the Le-7 family miRNA that increases the activity of Myc and subsequent Myc-mediated rRNA transcription in cancer cells (Rao et al. 2012; Thammaiah and Jayaram 2016). On the other hand, Myc is also downregulated by microRNAs. For instance, miR-24, miR-130a, and miR-145 interact with RPL5, RPL11, and RPS14 and suppress the Myc. miR-504 increases the expression of the nucleolar protein FGF13 and downregulates p53 thus, increased expression of miR-504 interrupts the rRNA synthesis (Bublik et al. 2017; Harold et al. 2021). MiR-504 has been reported to downregulate the p53 expression both at protein and mRNA levels in the MCF7 breast cancer cell line. Likewise, miR-424-5p downregulates rRNA and protein synthesis by interacting POLR1A and UBTF components of the transcriptional machinery (Hu et al. 2010; Harold et al. 2021).

LncRNAs are non-coding RNAs with a length that ranges from 0.2 to 100 kb and lack ORFs (Zhang et al. 2019a). These have complex heterogeneous 3D structures and can have different shapes. Thus, these can interact with various intracellular components (miRNA, proteins, DNA regulatory sequences, etc.) and modulate their activities and location. Furthermore, lncRNAs also act as sponges (ceRNAs) for miRNAs and block their activities. These are involved in the development, differentiation, mRNA splicing, protein stability, gene imprinting, chromatin modification, and antiviral drug responses (Klinge 2018) and are also associated with breast cancer metastasis (Zhang et al. 2019a; Liu et al. 2020). However, the detailed role of these lncRNs is still to be explored (Klinge 2018) in cells, including breast cancer. LncRNAs are involved directly or indirectly in regulating RNAP1 activity and rRNA synthesis (Li et al. 2018; Harold et al. 2021).

A well-known lncRNA species member, *ZFAS1* antisense RNA 1 (*ZFAS1*), is aberrantly overexpressed in various tumors and functions as an oncogene. However, ZFAS1 is downregulated in breast cancer cells and may act as a TSG. In the breast cancer cell line, overexpression of *ZFAS1* significantly suppressed cell proliferation by arresting the cell cycle and apoptosis induction. The overexpressed ZFAS1 also inhibited cell migration and invasion via epithelial-mesenchymal transition regulation (Fan et al. 2018). However, the exact mechanism of ZFAS1 involved in breast cancer, and the reason for different roles is yet to be explored. However, it may have a role in ribosome biogenesis (Hansji et al. 2016).

Several lncRNAs are involved in rRNA transcription regulation. LoNA (long nucleolar RNA) can reduce both pre-rRNA and mature rRNA levels. It interacts with nucleolin (a nucleolar protein) and inhibits the rDNA euchromatin modification. RNAPI transcribes the pRNA (promoter-associated RNA) that leads to NoRC (nucleolar remodeling complex)-mediated silencing of rDNA transcription. SLERT is snoRNA-ended lncRNA that develops pre-rRNA transcription and interacts with RNA helicase DDX21 to reduce its inhibition of rDNA transcription.

IGS transcripts, including PAPAS (promoter and pre-rRNA antisense transcripts), regulate chromatin modifications under various stress conditions and inactivate rDNA transcription. However, the RiBi regulatory role of many novel non-coding RNAs is still being explored (McCool et al. 2020).

13.3.6 Therapeutic Targeting

The final target of all anticancer therapies is to knock out the critical and preferentially required sustained support by the cancer cells (Drygin et al. 2010). Dysregulated ribosomes biogenesis, nucleolar function, rRNA synthesis, and RNAP1 are the hallmark of cancer cells. The RiBi is a multistep process including rRNA transcription, processing, RP synthesis, and ribosomal assembly that can be potential targets of therapeutic inhibition (Ferreira et al. 2020; Kanellis et al. 2021).

High selectivity of RNAP1 for 47S rRNA transcription can be exploited to avoid the potential side effects of anticancer drugs specifically designed to target RNAP1 activity compared to the anticancer drugs that target RNAPII activity. The low RiBi level in healthy somatic cells renders these cells less sensitive to RNAP1 inhibitor than malignant cells (Ferreira et al. 2020). Thus, targeting the Pol I machinery components is a prime target for the rational design of anticancer drugs. Therefore, inhibitors of RNAP1 activity and subsequent RiBi are of immense interest as a therapeutic target for many cancers, including breast cancer (Drygin et al. 2010; Ferreira et al. 2020). Moreover, selective rRNA transcription inhibition in the nucleolus is an effective strategy to promote p53 activation in cancer cells (Hein et al. 2013).

13.3.6.1 Current RNAP1 Activity Inhibiting Antineoplastic Drugs

The regulation of RiBi involves several steps which also includes transcription and processing of rRNAs, synthesis of RP, and ribosomal assembly, and all these are helpful for potential antineoplastic drugs. RiBi and RNAP1 activity inhibiting drugs are already in use for cancer treatment are both in the market and clinical trials. These drugs repress rRNA transcription and cancer growth. These drugs can be grouped as non-selective and selective rRNA transcription inhibitors (Table 13.1). Valuable information can be gained from mechanistic details, therapeutic potential, and mode of action of these drugs to develop new and better RNAP1 inhibitors (Ferreira et al. 2020).

13.3.6.2 Non-selective Antineoplastic Drugs

Alkylating agents Cisplatin and its analogs are platinum-based alkylating compounds (Dasari and Tchounwou 2014). These form DNA adducts to DNA repair proteins bind irreversibly, caused the inhibition of protein synthesis, cell cycle arrest, and subsequent cell death. But these also affect normal cells and cause side effects. Cisplatin and oxaliplatin displace UBF from rDNA loci and inhibit the transcription (Ferreira et al. 2020; Harold et al. 2021). These drugs are commonly used to treat a range of cancers, especially with a high mortality rate

Table 13.1 Chemotherapeutics affecting Pol I transcription (Ferreira et al. 2020)

Drug type	Drug	Effect on Pol I transcription	General mechanism of action	NSP activation	Clinical use
Alkylating agent	Cisplatin	*Displacement of UBF from the rDNA loci*	DNA cross lining and damage DNA synthesis inhibition	Yes	Yes
	Oxaliplatin	*Displacement of UBF from the rDNA loci*	DNA synthesis inhibition, depletion of the pre-Rrna, increase in RPL11 expression	Yes	Yes
Anti-metabolite	5-Fluorouracil	Intercalation into rRNA, inhibition of rRNA processing	Uracil analog Incorporates in 47S pre-rRNA inhibits processing of pre-rRNA Thymidine Synthetase inhibitor Intercalation into DNA and RNA	No	Yes
	Methotrexate	Inhibition Undetermined mechanism	Folate analog Thymidine synthesis inhibitor	Yes	Yes
Antibiotics	Actinomycin D	RNAP1 transcription elongation inhibition	DNA intercalation at GC-rich regions rDNA, interfere with transcription by stabilizing the G4 structures	Yes	Yes
	Mitomycin C	Inhibition by undetermined mechanism	DNA intercalation DNA alkylation	Yes	Yes
	Doxorubicin	Inhibition—likely prevention of transcription initiation	Topoisomerase II inhibitor, DNA intercalation	Yes	Yes
	Mitoxantrone	Inhibition likely prevention of transcription initiation	Topoisomerase II inhibitor, DNA intercalation	Yes	Yes
Plant alkaloids	Camptothecin	Inhibition by an undetermined mechanism	DNA intercalation, topoisomerase I inhibitor	Yes	Yes
	Irinotecan	Inhibition by undetermined mechanism	Topoisomerase I inhibitor	Yes	Yes

(continued)

Table 13.1 (continued)

Drug type	Drug	Effect on Pol I transcription	General mechanism of action	NSP activation	Clinical use
	Etoposide	Inhibition by undetermined mechanism	Topoisomerase I inhibitor	Yes	Yes
	Ellipticine derivatives	Inhibition of transcription initiation	Topoisomerase II inhibitor, SL-1 displacement	Yes	Failed clinical trial phase II
Specific Pol I inhibitors	CX-3543	Inhibition by undetermined mechanism	Dissociation of Nucleolin-rDNA G-quadruplex complexes	Yes	Clinical trial phase II
	CX-5461	Inhibition of transcription initiation	Disruption of the interaction between Pol I and SL-1 at the rRNA promoter	Yes	Clinical trial phase II
	BMH-21	Inhibition of transcription elongation	Degradation of RPA194 and displacement of RRN3	No	No

NSP nucleolar surveillance pathway

(Siddik 2003). Cisplatin is a more commonly used chemotherapeutic agent for breast cancer treatment. Recently, oxaliplatin has been reported to inflict RiBi defects by depleting the pre-rRNA and increasing the expression of RPL11 (Bruno et al. 2017).

Antimetabolites Antimetabolites drugs are the structural analog of cellular metabolites and cause inhibition of specific enzymes: commonly used 5-fluorouracil (5-FU) and methotrexate which are uracil and folate analog, respectively. 5-FU can cause cell damage and death by incorporating into the DNA and RNA as well as by inhibiting the dTTP production via thymidine synthetase inactivation. It affects rRNA synthesis by blocking 47S rRNA maturation. It is used to treat breast and colon cancer (Burger et al. 2010; Ferreira et al. 2020).

Methotrexate is used to treat breast cancer, leukemia. Methotrexate binds and inactivates dihydrofolate reductase leading to a deficiency of folate that renders thymidine synthetase incapable of converting uracil to thymine. Thus, this results in DNA synthesis inhibition. Methotrexate also reduces RNAPI transcription and subsequent nucleolus disruption (Burger et al. 2010).

Antibiotics Antibiotics are also being used as chemotherapeutic agents in cancer, and they act as intercalating agents. These become intercalated between the DNA bases of rapidly dividing cells and induce DNA damage. Frequently used Actinomycin D (ActD) represses the RNAP1 activity at low concentrations. This drug stalls the RNAP1 at the replication fork by intercalating with GC-rich regions of rDNA

(Sobell 1985; Tanaka and Tsuneoka 2018). This intercalation also results in a stable topoisomerase 1-DNA complex which blocks the rRNA transcription (Trask and Muller 1988). Mitomycin C (MMC) preferentially inhibits rDNA due to its ability to alkylate the DNA as well as intercalate with GC-rich rDNA regions and introduce cell damage and death just like AtD (Tomasz 1995; Burger et al. 2010).

Doxorubicin and mitoxantrone act as topoisomerase II poisons. DNA adducts that are formed due to the intercalation of these antibiotics within DNA, inhibit the topoisomerase II enzymes to re-ligate the DNA strands, thus causing irreparable double-strand break within DNA (Pommier et al. 2016). Both doxorubicin and mitoxantrone, just like the methotrexate, induce a marked decrease in 47S pre-rRNA maturation and processing and are being used in breast cancer treatment (Taymaz-Nikerel et al. 2018; Awad et al. 2019).

Plant Alkaloids These are natural products of plants that can intrude with DNA synthesis and the cell cycle. For example, Camptothecin (a traditional Chinese medicine) and its derivatives Topotecan and Irinotecan are being used in several different categories of cancers (Wecker et al. 2010; Pommier et al. 2016). These alkaloids target RNAP1 associated-Type I topoisomerase and form topoisomerase I-DNA complex, which blocks rRNA transcription, increases DNA breaks and thus, promotes DNA damage (Thomsen et al. 1987; Rose et al. 1988; Pondarre 1997; Delgado et al. 2018). Ellipticine (planner plant alkaloids) can inhibit topoisomerase IIα and act as a selective inhibitor of RNAP1 transcription because it can displace the SL1 complex from the rRNA promoter. It also activates NSP. Ellipticine and its derivatives (e.g., hydroxyl-methyl-ellipticine and 9-hydroxy-ellipticine (9HE)) were failed at clinical trial due to adverse side effects (Stiborová et al. 2011; Andrews et al. 2013).

13.3.6.3 Selective Inhibitors of Pol I Transcription

The drugs that specifically target RNP1 activity might lead to new anticancer therapies with reduced toxicity and increased potency. In the wake of search and identification of drugs with substantive anti-Pol I transcription activity, Cylene Pharmaceuticals used high throughput technology to screen small molecules. They identified a series of fluoroquinolone derivatives with the ability to disrupt nucleolin/rDNA G4 complexes. This led to the discovery of CX-3543 and later on, a second direct and more selective compound, CX-5461 was discovered. Pimera Inc. reported BMH21 and PMR-116. These drugs are much anticipated in terms of their potential in cancer treatment (Drygin et al. 2009; Bywater et al. 2012; Peltonen et al. 2014; Ferreira et al. 2020).

CX-3543 (Quarfloxin) Nucleolin binds and stabilizes the G4 quadruplex structures, which when present at rDNA, promote rRNA transcription. The CX-3543 binds rDNA G4 structures with a high affinity that results in the dissociation of nucleolin from putative G4 structures at rDNA loci. This directly inhibits the RNAP1 activity and results in nucleolin accumulation in the nucleoplasm, leading to

apoptosis. The CX-3543 has been reported to inhibit cancer cell growth in a variety of cell lines (Drygin et al. 2009; Rhodes and Lipps 2015). This compound passed the Phase 1 clinical trial but failed at Phase II due to bioavailability issues (Harold et al. 2021).

CX-5461 CX-5461 is a very effective compound that exerts selective inhibition of RNAP1 at low concentrations in a variety of cancer cell lines. This compound inhibits the PIC formation specifically by targeting the interaction between SL1 and RNAP1. It has been reported to elicit p53-dependent apoptosis (in murine models of lymphoma and leukemia) and p53-independent apoptosis (in lymphoblastic leukemia cells) via the noncanonical ATM/ATR pathway. The cell death mechanism is proposed to involve topoisomerase II poisoning. CX-5461 has also been reported to block the transcription initiation by blocking the release of RNAP1-RRN3. It induces DNA damage via BRCA1/2-mediated homologous repair in breast cancer cells. The multiple action mechanisms of CX-5461 may be cell context-dependent, but it is still needed to be explored (Harold et al. 2021; Ferreira et al. 2020). The drug has successfully passed clinical phase 1 and has shown the potential to treat breast cancer with abnormal BRCA1/2 in addition to other malignancies (Harold et al. 2021; Ferreira et al. 2020).

BMH compounds BMH compounds (such as BMH-7, BMH-9, BMH-21, BMH-22, and BMH-23) were screened for p53 activation by a cell-based high throughput screening. Among these BMH-9, BMH-22, and BMH-23 were able to induce RNAP1 transcription stress, proteasome-dependent destabilization of the RPA194, and inhibition of rRNA synthesis. These compounds were tested and verified for their anticancer potential against cancer cell lines for many tumor types. However, BMH-21 was the leading compound, and it was able to intercalate at GC-rich sites of rDNA and subsequent decrease in rRNA transcription. It also induced depletion of the largest subunit of RNAPI (Peltonen et al. 2010, 2014). However, these are currently not in the clinical trial for these drugs.

13.4 Epigenetic Alternation

Cancer was long thought to be caused by a genetic mutation. However, the control of the genome through epigenetic modification has contributed to the complexity during the last decade. The involvement of epigenetic alteration in normal growth-related activities such as imprinting and X-chromosome inactivation has long been recognized (Barbara et al. 2017). Aberrant epigenetic changes have just lately been discovered to have a key function in neoplasia. Epigenetic modifications are heritable cellular information that is a non-genetic in nature and can be transferred during cell division (Feinberg and Tycko 2004). The two main epigenetic modifications discussed are DNA level methylation where CpG in genome experience covalent bonding with methyl group that results in controlling expression of gene and histone

modification where histone proteins go through deacetylation or methylation that results chromosomal packing regulation (Feinberg and Vogelstein 1983; Issa 2004).

Methylation changes at the DNA level can be divided into two categories, namely hypomethylation and hypermethylation, strongly affect expression of gene. Hypomethylation occurs once the first methylated gene in mature DNA is demethylated, causing gene expression. Expression of frequently suppressed genes (such as HRAS oncogenes and other genes) can lead to cellular dysfunction and consequent tumor progression (Feinberg and Vogelstein 1983). On the contrary, hypermethylation impacting gene transcription happens when methylation silences CpG islands in a gene's regulatory or promoter sites. There is a compound succession of moves for methylation and silencing of gene to happen that involve the enrolment of many regulatory proteins and biochemical reactions that eventually lead to changes in histone status and chromosome folding (Jones and Baylin 2002). One of the most extensive examples of cancer-related hypermethylation studies is the silencing of hMLH1 in colorectal cancer and BRCA1 in breast cancer (Herman et al. 1998). Both lead to the exclusion of important TSG, in this situation, the protein is involved in DNA maintenance and repair (Herman et al. 1998). DNA methylation is a breast cancer risk factor, and it is a potential biomarker for early diagnosis. Therefore, identifying DNA methylation changes and their association with breast cancer has been of great interest (Johnson et al. 2017; Joo et al. 2018; Ennour-Idrissi et al. 2020). In one study, 368 individual CpGs were identified that were differentially methylated in breast cancer tumors compared to healthy breast tissues. Among these, 56% of hypermethylated sites were present in upstream promoter regions and 66% of hypomethylated sites were localized within genes. Hundreds of genes have been stated to be hypermethylated in breast cancer that are a critical cell cycle regulation (e.g., FOXA2, CCND2, AK5), DNA repair (e.g., MGMT, MLH1, BRCA1), cell adhesion (e.g., CDH1), apoptosis (e.g., breast cancer L2, APC), hormone-mediated cell signaling (THRB, ESR1, and ESR2), and tissue invasion and metastasis (e.g., RASSF1A, HIN1, TWIST, RARβ) (Buocikova et al. 2020). Many genes with altered methylation states have been reported as biomarkers. For example, women with BRCA1 methylation at the promoter site have a 3.5-fold increased breast cancer risk. Similarly, methylation status as a potential biomarker for breast cancer of several other genes is documented. Some of them are *ATM*, *PALB2*, *VTRNA2-1 BRCA2*, *HYAL2*, *S100P, TP53*, HIN-1, MGMT, RASSF1A, *CDH1*, *CHEK2*, and *MLH1* (Sebova et al. 2012; Spitzwieser et al. 2015; Scott et al. 2016; Joo et al. 2018; Guan et al. 2019; Zeng et al. 2020). The epigenetic silencing of tumor suppressor genes has been associated with breast cancer. For example, hypermethylation-mediated silencing of BRCA1 in baseline/TNBC tumors (Feng et al. 2007; Foulkes et al. 2010; Stefansson et al. 2011). PTEN is another important tumor suppressor in breast cancer, linked to epigenetic modifications. Loss of function of PTEN tumor suppressor genes is associated with hypermethylation in the promoter region. This gene negatively regulates PIP3-Akt pathways. PTEN silencing leads to stimulation of the pathway and subsequent destruction of cell apoptosis and increased survival rate (Lu et al. 1999; García et al. 2004; Khan et al. 2004). The DNA methylation status of

hormonal receptors has also been associated with breast cancer (Widschwendter et al. 2004).

In the field of epigenetics, histone alterations are becoming increasingly important. Histone proteins can be acetylated, phosphorylated, or methylated, all of which regulate chromosomal stability and packing. Acetylation causes chromosomal packing to relax, enabling transcription factors to access and activate gene transcription (Herman and Baylin 2003). On the contrary, deacetylation by histone deacetylases (HDACS) and subsequent methylation of histone residues, on the other hand, causes histones to tighten, limiting regulating transcriptional protein access. Several studies show a complicated relationship among DNA level epigenetic alterations and histone level variations. Some records suggest modifications in DNA methylation might precipitate histone residue modifications and chromatin packing (Kass et al. 1997; Herman and Baylin 2003; Esteller 2008). Some researchers provided evidence to support this conclusion and has indicated that histone demethylation by HDAC inhibition was insufficient to reverse DNA methylation and result in gene expression (Cameron et al. 1999). On the other hand, some researchers argued that histone-mediated chromatic modification is not the chief driver of epigenetic silencing of gene (Timp and Feinberg 2013). Associates research data for this hypothesis came from investigation showing independent DNA methylation gene silencing by histone modification only. Although significant progress has been made in colon cancer, glioma, and leukemia (Esteller 2008), more advanced cancers, such as breast cancer, require further research.

13.4.1 Methylation of Gene Promoter CpG Islands

Hypermethylation of promoter CpG islands is another method of gene inactivation that can occur early in the development of breast cancer. In primary breast tumors or breast cancer cell lines, more than 100 genes have been shown to be hypermethylated (Hinshelwood and Clark 2008). Numerous abnormally methylated genes are involved in tumor suppression and cell cycle control, apoptosis, angiogenesis, tissue invasion, and metastasis (Bediaga et al. 2010).

13.4.1.1 CpG Island Hypermethylation and Breast Cancer Progression

Hypermethylation of promoter CpG islands has been linked to breast cancer progression. Recently, during important stages of breast cancer development, methylation levels of TSG *RAR2* and *RASSF1A*, *MINT17*, and *MINT13* were investigated, and the researchers discovered a noteworthy rise in the expression levels of these genes throughout the progression of breast cancer (van Hoesel et al. 2013). Hypermethylation of *RAR2* and *RASSF1A* promoter CpGs have been shown in various research to have a part in breast cancer and regarded as primary epigenetic processes in breast cancer. RAR2 and RASSF1A methylation was detected in lesions from both lobular carcinoma in situ (LCIS) and ductal carcinoma (DCIS) by Jovanovic and colleagues (2010). Another study looked at 57 promoter CpG loci in 20 IDCs and their associated normal breast tissues and showed that a stepwise

increase occurred in methylation of 15 different genes from normal to atypical ductal hyperplasia (ADH)/flat epithelial atypia (FEA) to ductal carcinoma in situ (DCIS) (Park et al. 2011).

The methylation significance in normal, pre-malignant lesions and tissues of breast can be compared to see if there is a link between promoter CpG gene hypermethylation and breast cancer development. The ideal way to prepare DNA for these investigations is to employ formalin-fixed paraffin-embedded (FFPE) tissues using laser capture microdissection, which guarantees the correct kind of tissue is obtained for DNA extraction and methylation tests. DNA methylation abnormalities have also been linked to clinical and pathologic features of breast cancer (Jovanovic et al. 2010). The size of the tumor, lymph node status, distant metastasis, and hormone receptor status have all been linked to epigenetic alterations (Klajic et al. 2013). The MSDK, which was developed by the K. Polyak team at Dana Farber Cancer Institute and Harvard Medical School, is a comprehensive DNA methylation profiling tool that enables impartial and complete methylation profiling (Hu et al. 2005).

13.4.1.2 DNA Methylation in Hormone Receptor-Positive and Negative Breast Cancer

Diverse epigenetic profiles among hormone receptor-positive and negative tumors have been identified (Hu et al. 2005; Feng et al. 2007; Bediaga et al. 2010; Jovanovic et al. 2010; Park et al. 2011; Klajic et al. 2013; van Hoesel et al. 2013). Array-based methylation analysis has various drawbacks, even though it can assess many genes at once. Due to the inability of array-based analysis to give quantitative measurements of CpG methylation, further experiments are required to confirm and validate findings. As a result, new approaches like pyrosequencing are defensible. Pyrosequencing is a synthesis-based sequencing technology for quantifying DNA methylation at CpG sites within a target area. Using pyrosequencing methylation analysis, Feng et al. identified two panels of methylation patterns that linked with hormone receptor expression in breast cancer (Widschwendter et al. 2004) and found 12 tumor suppressor genes in 90 pairs of malignant/normal breast tissues (ARHI, RASSF1A, HIN-1, RAR2, hMLH1, 14-3-3, RIZ1, p16, E-cadherin, RIL, CDH13, and NKD2).

Data from the study showed that 5 of the 12 genes tested (*RIL*, *HIN-1*, *RASSF1A*, *CDH13*, and *RARβ2*) were methylated in tissue of breast cancer but not in normal breast tissue. Nearly 70% of breast tumors are diagnosed ER-positive. Patients with ER-positive breast malignance goes for tamoxifen treatment, which strives with estradiol for binding of ER. Post-menopausal women with ER-positive breast tumors are also candidates for aromatase inhibitor (AI) treatment. AIs decrease estrogen production by inhibiting enzymes aromatase, which synthesizes estrogen from testosterone and androstenedione (Brodie and Njar 1998). Although the benefits of hormone therapy for patients with ER^+ breast cancer have been well documented, not all patients with ER-positive tumors respond to treatment (Musgrove and Sutherland 2009).

13.4.1.3 Promoter Hypermethylation of TSGs in Breast Cancer

Epigenetic dysfunction of TSGs, i.e., BRCA1, is associated with severe sporadic breast cancer cases (Birgisdottir et al. 2006; Esteller 2008). Preliminary reports suggest that epigenetic silencing is an important mechanism for the loss of BRCA1 expression (Feng et al. 2007). Moreover, loss of BRCA1 expression has recently been associated with baseline/TNBC tumors (Foulkes et al. 2010). Stephenson et al. tested BRCA1 methylation in 111 sporadic breast tumors, previously tested for bacterial BRCA1 mutations showing that BRCA1 CPG island hypermethylation is significantly linked with basal/TNBC tumors (Stefansson et al. 2011). Recently, other researchers, Hsu et al., used methylation-specific PCR (MSP) to analyze the methylation of BRCA1 promoters in 139 early-stage breast cancer tissues. Their findings revealed a link between BRCA1 promoter methylation and TNBC tumor types (Hsu et al. 2013). PTEN is another important tumor suppressor in breast cancer has been linked to epigenetic mediated loss (García et al. 2004; Khan et al. 2004).

This gene encrypts phosphatase PIP3 and negatively regulates the PIP3-Akt pathway. Loss of PTEN can activate Akt pathways, suppress cell apoptosis, and increase cell survival (Lu et al. 1999). It was found that the expression of the PTEN protein is lost or decreased in 38% of cases of invasive breast cancer. Promoter hypermethylation is believed to be a key mechanism of PTEN gene loss in breast cancer. Khan et al. used MSP to examine PTEN promoter methylation in 44 invasive breast tumors. Studies have shown that 34% of tumors had PTEN hypermethylation and tumors with PTEN promoter hypermethylation had 60% of samples loss of PTEN protein. Moreover, Garcia et al. investigated promoter hypermethylation of the PTEN gene in 90 invasive breast cancers and discovered that the PTEN promoter was hypermethylated in 48% of the tumor tissues (García et al. 2004).

13.4.2 Non-coding RNA

Like DNA methylation and histone modifications, non-coding RNAs (ncRNAs) are modulators of gene expression and chromatin regulation and are fundamental to development and embryogenesis. Many of these ncRNAs families have highly conserved sequences across the species. These are involved in transcriptional and post-transcriptional gene silencing via complementary base pairing with the target genes. It has been predicted by computational studies that approximately 1000 miRNA genes in the human genome target multiple protein-coding transcripts. About 60% of the protein-coding genes translation is regulated by miRNA. The miRNAs can inhibit the expression of multiple genes and thus have fundamental roles in regulating different cellular processes, including proliferation and differentiation. The dysregulation of miRNA is associated with cancer and other diseases (Kanwal et al. 2015; Soreide 2017; Zhuang et al. 2020). During breast cancer progression, aberrant expression of these ncRNAs can imbalance the miRNA cellular levels and ultimately worsen the disease. The specific miRNAs that are reported to be present in blood samples of cancer patients can be a potential

biomarker of cancer. Therefore, extensive research is being carried out to discover more miRNA as novel biomarkers of tumors for early diagnosis and treatment (Zhuang et al. 2020). For example, miR-34 and miR-21 both have opposite roles but are dysregulated in cancer. miR-21 is upregulated in multiple cancers and can be considered a proto-oncogene and potential biomarker for malignancy. miR-21 can promote EMT (epithelial-to-mesenchymal cellular transitions) and involve the maintenance of pluripotency. High miR-21 expression is correlated with increased tumor proliferation and invasion in cancer, including breast cancer (Asangani et al. 2008; Qian et al. 2009; Kumarswamy et al. 2011; Wang et al. 2019; Kumar et al. 2020). Conversely, the miR-34 family is considered a tumor suppressor because the downregulation of the miR-34 family is reported in multiple cancers. The expression of miR-34 family is observed in gastrointestinal tract, lungs, and brain cells as a part of positive feedback, with p53 inhibiting the tumorigenesis via cycle arrest and apoptosis. Breast cancer L2, CDK4/6, and cyclin E2 are targets of miR-34 that facilitate tumorigenesis by inhibiting apoptosis and promoting cell cycle progression. In breast cancer, miR-34a targets E2F1/E2F3, stem cell-associated transcription factors E2F1/E2F3, are upregulated in breast cancer patients. Reduced miR34a expression correlates with aggressiveness of the breast cancer and decreases in patient survival (Tarasov et al. 2007; Hermeking 2012; Zhang et al. 2019a; Han et al. 2020; Kumar et al. 2020).

13.4.3 DNA Methylation and Histone Modification in Breast Cancer

The enzymes DNMTs methylate the cytosine residue in CpGs. There are four primary kinds of DNMTs, namely DNMT1, 2, 3a, and 3b, while DNMT1, DNMT3a, and DNMT3b being the most dynamic (Huang 2002). The most well-studied DNMT inhibitors are 5-azacytidine (VidazaTM) and 5-aza-2′-deoxycytidine (decitabine) (Esteller 2008). These compounds covalently trap DNMTs by incorporating into DNA and substituting cytosine during DNA replication. Because the stuck DNMTs cannot continue methylation, the cell loses DNMT activity, which leads to DNA demethylation (Juttermann et al. 1994). The related activity of DNA and histone levels epigenetic alteration indicates that to effectively treat the reversal of silencing, dual targeting of DNA and histone modifications should be applied. Clinical trials with 5-aza-2′-deoxycytidine and HDAC inhibitors (such as trichostatin A) in various cancer systems have been conducted, focusing on leukemias and myelodysplastic syndromes (Bhalla 2005). In solid tumor systems, such as breast, prostate, and colorectal cancer, further research is needed. The methylation of histone H3 (Lys27) was involved in silencing the CASP8 gene in MCF7 using a chromatin immunoprecipitation (ChIP) test. The gene silencing was reversed, and Caspase 8 expression was dramatically restored only after a combination of 5-aza-2′-deoxycytidine and HDAC inhibitors, trichostatin A (Wu et al. 2010).

This method might be used to reactivate other repressed genes linked to breast cancer tumorigenesis and disease resistance. The ESR1 gene that may be demethylated to rebuild ER expression and consequent tumor responsiveness to

targeted therapy like tamoxifen would be a great target. Indeed, Fan and colleagues proved that joint treatment restores ER expression in ER breast cancer cell lines and reinstates response to ER-targeted therapy (Fan et al. 2008). Furthermore, the ChiP test might be helpful in future investigations looking into the effectiveness of these drugs. The ChiP test is an effective method for assessing the state of histone modifications. When presenting the ChIP experiment, cells are first reversibly fixed to cross-link the protein–DNA interactions in the cell nucleus. After that, the cells are lysed, and the chromatin is extracted and broken using either sonication or enzymatic digestion. Then, antibodies specific to a certain protein or histone alteration are used for immunoprecipitating the chromatin. Any DNA sequences connected to the protein or histone modification of interest will co-precipitate as part of the cross-linked chromatin complex, and the immunoselection process will enrich the relative quantity of that DNA sequence. The protein–DNA cross-links are reversed, and the DNA is purified after immunoprecipitation. A variety of approaches can then be used to identify the enrichment of a certain DNA sequence or sequences.

13.5 Regulation of Gene Transcription

The expression of gene is strongly controlled and well coordinated at various levels in a cell. Tissue-specific gene expression is regulated by coordination of *cis*-acting and *trans*-acting elements at transcription level (Ohler and Wassarman 2010). Genes are regulated at their immediate vicinity by *cis*-acting elements. Core and proximal promoter elements are typical examples of *cis*-acting elements that are restricted to a few hundred base pairs of transcription start sites (Maston et al. 2006). While distal *cis*-acting elements are more than 1 kb or sometimes 1 MB away on either side of the transcription start site (Lettice et al. 2003). Moreover, findings recommend that genes are also regulated by *trans*-acting elements located on different chromosomes (Williams et al. 2010). Currently, transcription regulating elements are classified into four main classes, most of them are distal, for example, silencers, locus control regions (LCRs), enhancers, and insulators. Enhancers are the sequences that activate transcription at any location of element relative to the promoter. Enhancers usually consist of many transcriptions factor-binding sites that regulate tissue specific and temporal genes by recruiting transcription factors. Enhancers are very flexible in their function and many enhancers can act on a single promoter. On contrary, silencers are sequence-specific elements that have negative effect on transcription of a gene by silencing or suppressing its transcription (Ogbourne and Antalis 1998). Repressors confer negative effect on transcription factors and bind on silencers. There are two main classes of silences, silencer elements and negative regulatory elements. Silencer elements directly interfere with transcription factor assembly and they are position independent, while negative regulatory elements prevent binding of transcription factors to *cis*-regulatory elements and are position dependent (Gerasimova and Corces 2001). Insulators are protein complexes that prevent gene transcription from inapt signals. Insulators act as enhancer blocker to prevent

enhancer–promoter interaction or restrict spread of heterochromatin (Dorman et al. 2007). So far only one mammalian insulator protein, CCCTC-binding factor (CTCF) is known. LCRs consist of clusters of *cis*-acting elements that regulate gene expression in development and cell differentiation. LCRs are also position independent and most of them are strong transcription enhancers. Long-range DNA interactions are involved in transcription regulation and chromatin loop act as mediator as most of the distal-acting regulatory elements are >1 kb away from the genes they regulate (Sexton et al. 2009). In chromatin loop model, tethering proteins help the regulatory elements to contact the promoter. Mammalian b-globin locus was the first site where intrachromosomal looping was observed and LCR activates expression of gene from more than 50 kb upstream. Studies showed that distal *cis*-acting elements and target genes follow loop interaction model (Sexton et al. 2009). Moreover, several proteins establish intrachromosomal loops, for example, transcription factors, structural proteins, and chromatin remodeling factors. Physical interactions between elements located on different chromosomes have also been reported (Williams et al. 2010). Although the exact mechanism of intrachromosomal interaction is yet to be determined but CTCF and cohesion mediator complex might be involved in these interactions. Identification of tether gene loop and their role in mediation of long-range interactions is a future challenge. Current study, however, will focus specifically on the potential role of long-range regulation in breast cancer.

13.5.1 Long-Range Transcriptional Enhancers

Currently, a little is known about long-range enhancers that regulate the gene expression involved in breast cancer, reflecting lack of strategies in identification of distal regulatory elements. Numerous estrogen receptors (ER)-mediated long-range promoter enhancers interactions have been reported. ER has emerging role in long-range interactions and effects of estrogen in breast cancer. Recent studies showed that expression of MYC oncogene is stimulated by estrogen via distal enhancer that is 67 kb far from the TSS. Activation protein-1 (AP-1) and ER are required for transcriptional induction of MYC by chromatin looping (Wang et al. 2011). Expression of Cathepsin D (CTSD) is also induced by estrogen via distal enhancer located 9 kb upstream of the TSS. Overexpression of CTSD in breast tumors is a marker and linked to metastasis. Occupancy of transcription factor and transcription activation depends on DNA methylation of CTSD promoter and enhancer which concludes that any defect in long-range transcription regulation is important for clinical diagnosis of breast tumors (Bretschneider et al. 2008). It has not been determined that increased CTSD level in patients with breast tumors have similar DNA methylation of CTSD promoters and enhancers. Estrogen-responsive enhancer that regulates the expression of carbonic anhydrase XII (CA12), a zinc metalloenzyme, is located 6 kb upstream from the TSS. In breast cancer cells, interaction of ER-binding enhancer with CA12 promoter by interchromosomal looping, results in recruiting of RNA polymerase II (RNA pol II) and steroid coactivators SRC-2 and SRC-3. Remarkably, this enhancer is present in mice

homolog and on estrogen treatment its expression is rapidly activated (Barnett et al. 2008). ER-independent long-range promoter–enhancer interactions of some genes involved in breast cancer are reported, for example, B-cell lymphoma 2 (breast cancer L2) is important for early diagnosis of breast cancer. The transcription enhancer of breast cancer L2 is located on 200 kb downstream of TSS. Studies showed chromatin looping between breast cancer L2 promoter and enhancer mediated by SATB1 is required for epigenetic modifications and breast cancer L2 expression by allowing CREB1 (Gong et al. 2011). Breast cancer L2 transcription is regulated by two estrogen-responsive elements (EREs) found in coding region of the gene via ER-mediated chromatin looping. These two independent pathways involved in breast tumorigenesis may provide a comprehensive explanation of *breast cancer L2* expression in breast tumor cells. In breast cancer cells, *CDKN1A* tumor suppressor can also be regulated by vitamin D receptor (VDR) via long-range chromatin looping. Looping between three vitamin D response elements (VDREs) is induced by VDR ligand 1a,25-dihydroxy vitamin D3. These VDREs are located 7 kb upstream of TSS and *CDKN1A* transcription is increased by 1a,25-dihydroxy-induced looping (Saramäki et al. 2006). Accumulation of *CDKN1A* transcript and chromatin looping depends on histone deacetylase 4 (HDAC4), mediator protein subunit MED1, and the lysine demethylase LSD1. The association of reduced expression of *CDNK1A* in breast cancer is a poor prognosis. It is yet to be determined that cells with low *CDKN1A* have low expression of VDR. Furthermore, *cis*-activation of genes involved in breast cancer, there are evidences that variants identified by GWAS frequently fall within enhancer elements.

13.5.2 Long-Range Transcriptional Silencers/Insulators

A gene silencing-associated chromatin interaction has been recognized between the promoter and terminator region of major breast cancer susceptibility gene *BRCA1*. In mice models, the same gene silencing communication was noticed in mammary epithelial cells at lactational growth and was undetectable during pregnancy, when Brca1 levels are low and when BRCA1 expression is induced. Remarkably, an evolutionarily conserved putative enhancer and silencer element, referred to as CNS1 and CNS2, was previously found within this intron approximately 5 kb from the TSS (Wardrop and Brown 2005). The non-coding region of BRCA1 presented transcriptional silencer elements which might be involved in BRCA1 repression.

A gene silencing loop also exists between the human IGF2 promoter, and an imprinting control region (ICR) located approximately 80 kb downstream. Numerous studies on ICR methylation have shown that the ICR is maternally unmethylated and paternally methylated, and that CTCF orchestrates an intrachromosomal loop by binding to both the maternal ICR and IGF2 promoter which silences the maternal allele (Zhang et al. 2011). Many human tumors including breast tumors have demonstrated abnormal bi-allelic expression of IGF2. This suggest that a dysfunctional CTCF regulatory pathway and subsequent chromatin looping could promote

tumorigenesis. Also, a lack of correlation between DNA methylation and IGF2 imprinting status has been observed in several studies. A recent study showed that binding of decoy CTCF proteins to the ICR failed to recruit the appropriate TFs, thus abolishing the loop which results in reactivation of the normally suppressed maternal allele. Although several studies supported CTCF-controlled allelic regulation of IGF2, it is still not known whether de-regulation of the pathway will be a potential cause in breast cancer.

Recently, it has identified and characterized another silencing loop present at Mammary Carcinoma Susceptibility-5A (MCS5A/Mcs5a) gene, a breast cancer susceptibility locus which is conserved in both human and mice (Smits et al. 2012). In order to confer mammary carcinoma resistance, Mcs5a harbors two non-protein coding, synthetically interacting elements (Mcs5a1 and Mcs5a2), located on the same chromosome. These two elements then interact physically via a long-range insulator loop requiring the binding of CTCF and cohesin. In rat model, this interaction resulted in reduced expression of the E3 ubiquitin ligase gene Fbxo10 in the thymus and reduced tumor multiplicity. However, in the human orthologous loci the germline variants associated with breast cancer risk are located on either side of the looped structure and can interact to alter transcriptional activity. In absence of evidence suggesting direct transcriptional effect on FBXO10, a similar mechanism underlying the rat and human susceptibility alleles has been suggested. However, the comparative genetics analysis shows that the MCS5A variants resemble GWAS-identified risk alleles, with the disease-associated SNPs being intergenic and conferring a low relative risk. Functional variation within distal *cis*-elements with an increased breast cancer risk highlights the potential importance of long-range regulation in breast cancer (Smits et al. 2011).

13.5.3 Other *Cis*-Regulatory Elements

Although little is known about UCRs, several studies suggest a functional role of UCRs in long-range gene regulation with enhancer-like activity and transcriptional activation. Yang et al. did genotype analysis to show link of germline SNPs in two intergenic UCRs (uc.353 and uc.140) with predisposition to breast cancer (Yang et al. 2008). Importantly, both UCRs have been previously known to function in vivo as tissue-specific enhancers of gene expression and act as long-range enhancers of genes outside this region. However, to define their mechanism of action and confirm their target genes further studies are needed. Because two subsequent studies, using different ethnic cohorts, could not establish a significant association between these two SNPs and breast cancer susceptibility. These conflicting studies could be explained by differences in allele frequencies in the studied ethnic groups and/or by statistical fluctuations when using a small sample size.

13.5.4 Long-Range *Trans*-Regulatory Elements Implicated in Breast Cancer

In mammals interchromosomal (or *trans*-) gene regulations, in addition to *cis*-interactions has also been reported. Although less is known about the physiological significance of these interactions. At present, only one such interaction associated with breast cancer is well reported, which is between the estrogen-regulated genes TFF1 (trefoil factor 1) located on human chromosome 21 and GREB1 (gene regulated by estrogen in breast cancer 1) on chromosome 2 (Hu et al. 2008). By implication of a modified 3C technique, an estrogen-induced interaction between TFF1 and GREB1 genes has been observed in HMEC mammary epithelial and MCF7 breast cancer cell lines with a striking observation of chromosome repositioning of territories within the nucleus, which conflicted with the popular belief that chromatin has limited mobility in mammalian cells. However, a recent article used similar experimental conditions reported that estrogen induced no such long-range interactions or chromosome reorganization (Kocanova et al. 2010). Furthermore, ER-mediated chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) also did not find any reproducible ER-mediated interchromosomal interactions at this locus, or indeed anywhere else in the genome, suggesting these may be a rare or weak events. In almost 50% of human breast tumors, TFF1 and GREB1 are abnormally expressed. Although both genes can be activated via ER binding to proximal and distal EREs but the mechanisms regulating their expression are not well established. Long-range transactivation represents an interesting possibility, in which estrogen induces a coordinated enhancement of gene expression, but this phenomenon requires supporting experimental evidence. It is important to experimentally resolve this discrepancy because this locus is potentially the best example to date of a functional interchromosomal interaction linked with breast cancer.

13.6 Therapeutic Strategies for Transcription Control in Breast Cancer

13.6.1 Epidrugs as Cancer Therapeutics

The reversible epigenetic modification involved in cancer has sparked interest in epidrugs. The epidrugs possess epigenetics modulatory activities. These drugs target HDACs and DNMTs and modulate their activities leading to the restoration of normal epigenetic landscapes in cancer cells (Shukla et al. 2019). USFDA has already approved epidrugs for several different cancers. The approved epidrugs include DNMT inhibitors including Decitabine and Azacitidine and HDAC inhibitors including Panobinostat, Belinostat, Romidepsin, and Valproic Acid. Several other epidrugs alone or in combination are in the clinical trial. With recent advancements, the horizon of epidrugs-based therapies has widened. Several other inhibitors of class I, II, and IV-specific HDACs, HATs, BRD (BET), KMTs, KDMs,

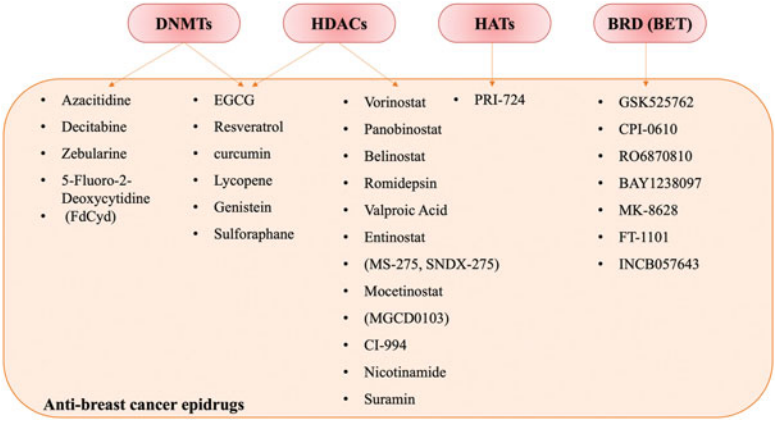

Fig. 13.3 Anti-breast cancer epidrugs

and multiple kinases are being considered for antineoplastic activity. Another DNMT inhibitor Zebularine is active against multiple cancers and is in preclinical studies. Different phytochemicals are also actively being explored for their anticancer potential (Shukla et al. 2019). Bioactive polyphenols such as EGCG (epigallocatechin-3-gallate), which is a common component of green tea have been reported for anticancer activity through different mechanisms, including DNMTs and HDACs inhibition (Ciesielski et al. 2020). Resveratrol, a phytoalexin present in grape skin, berries, *Polygonum cuspidatum*, can act as an anticancer molecule. It showed the ability to repress multiple tumors initiation and progression by a different mechanism, including modulating the activities of DNMs and HDACs (Zhu et al. 2012; Farhan et al. 2019; Shukla et al. 2019). Similarly, other phytochemicals such as curcumin (a polyphenol present in rhizome of turmeric), Lycopene (a plant carotenoid present in many fruits and vegetables), Genistein (an isoflavone found in several plants, including soybean) (Stefanska et al. 2012; Sundaram et al. 2018) Sulforaphane (a sulfur-rich compound present in Brassicaceae family vegetables (Su et al. 2018) are also reported to have anticancer activity by modulating enzymes involved in epigenomic alteration along with other mechanisms (Fig. 13.3). These phytochemicals, along with many other natural biomolecules, may lead to the development of some potent anticancer drugs with fewer side toxic effects.

13.6.1.1 Epigenetic Regulation of Hormonal Pathways and the Potential Drug Targets

Breast cancer is a heterogeneous disease characterized by complex molecular behavior (Green et al. 2013). Breast cancer can be categorized into different phenotypes based on molecular criteria such as estrogen (ER)/progesterone (PR) receptor expression, human epidermal receptor 2 (HER2/ERBB2) expression and TNP (triple-negative) lack the expression of all these receptors. Different

molecular subtypes of these phenotypes are present (Hervouet et al. 2013). Both the genetic and epigenetic alterations are involved in these breast cancers. Compared to genetic mutations, which are challenging to correct, epigenetic alterations are reversible and easy to target by the small molecular compounds. Moreover, the increased sensitivity of epigenetic modulation in cancer cells to the immune system makes them an attractive target for immunotherapy (Roulois et al. 2015; Deblois et al. 2020; Garcia-Martinez et al. 2021).

Estrogens have normal biological roles in reproduction and development. The estrogen subtypes are estrone (E1), 17-β estradiol (E2), estriol (E3), estetrol (E4), and estrone-sulfate (E1s). E1 serves as the main estrogen reservoir and can easily be transformed into biologically more active E2 by a reversible reaction (Hervouet et al. 2013; Garcia-Martinez et al. 2021). High estrogen levels with prolonged exposure can lead to constitutive activation of genes predominantly related to metabolism and cell cycle regulation and subsequent increased risk of breast cancer risk. Estrogen receptors (ER) belong to a family of transcription factors. Two isoforms of estrogen receptors (ER) exist, i.e., ERα and Erβ, which are involved in the modulation of hundreds of estrogen-targeted genes. However, stimulation of genes lacking an ERE (estrogen response element) is also evident that is achieved by ERα interaction with specific TFs (transcription factors) such as AP-1, SP1, FOS, and JUN; NF-kB; and C/EBPβ. About 75% of estrogen-responsive genes require EREs or ERE-like sequences for their transcription (Hervouet et al. 2013; Garcia-Martinez et al. 2021). E2 stimulation of genomic signaling pathways begins with homodimerization of ERα followed by binding to chromatin either directly or indirectly by TFs via its AF domain. ER^+ breast cancer TFs like GATA3, AP-2γ, PBX1, and FOXA1 facilitate ERα binding to condense chromatin. After activation also recruits a cohort of corepressor and coactivator-mediated gene repression or activation. ERα interaction with chromatin is mediated via hundreds of coregulators in a highly coordinated manner to regulate targeted genes and have both epigenetic and oncogenic roles. The members of the p160 family, PRMTs, SWI/SNF complex, P300/CBP, and the mediator complex are prominent epigenetic coactivators of ERα. The coactivators of p160 family (SRC-1, SRC-2, and SRC-3) act as a platform after binding directly with ERα and recruit many other chromatin remodeling complexes and activating enzymes that lead to the epigenetic alterations of enhancers and promoters. ERα also utilizes HAT for acetylation (H3K27ac) of enhancer via SRC-3. This acetylation results in the recruitment of SWI/SNF chromatin remodeling complex implying further remodeling and activation of enhancers (Garcia-Martinez et al. 2021).

The BRCA1, NCoR1, NCoR2, and LCoR are among the notable epigenetic corepressor of ERα transcriptional activity and downregulation of targeted genes (Fig. 13.2). For example, BRCA1 represses ERα transactivation function by direct binding, and it also degrades it by mono-ubiquitination (Wang and Di 2014; Garcia-Martinez et al. 2021).

13.6.2 Breast Cancer Therapies

The treatments for breast cancer vary depending upon the tumor size and stage. Treatments involve surgery, hormonal therapy, chemotherapy, and radiotherapy, or a combination of two or more of the preceding methods. The most common chemotherapeutic agents used to treat this malignancy available in the market are methotrexate, fluorouracil, cyclophosphamide, doxorubicin, capecitabine, and mitomycin. After surgery, breast cancer metastasized to lymph nodes, so paclitaxel is often prescribed (Maira et al. 2007). For ER^+ breast cancer patients, endocrine therapy is standard care to suppress the estrogen and ERα. The main therapies are SERMs (selective estrogen receptor modulators), SERDs (selective estrogen receptor degraders), and AIs (aromatase inhibitors). Some already in use endocrine targeting drugs include Tamoxifen, Toremifene, Raloxifene, Fulvestrant, Anastrozole, Letrozole, Exemestane, and Goserelin (Table 13.2).

The tamoxifen is an SERM target ERα by competing with E2 for the receptor. It was the first clinically approved drug. For both early and advanced breast cancer patients, it is a primary treatment option. Tamoxifen, an SERM, competes with E2 for receptor binding and can reduce E2-stimulated in vitro. Despite the attainment of tamoxifen therapy, disease recurrence has been observed in one-third of treated patients (Liu et al. 2001; Anbalagan and Rowan 2015). However, most of these patients remain sensitive to SERDS, such as Fulvestrant, a frequently used drug in endocrine therapy. It degrades ERα by disrupting its dimerization and nuclear localization and blocks ERα-mediated transcriptional activity. The clinical trial for luminal breast cancer Fulvestrant therapy as the first hormonal therapy of patients showed better progression-free survival than AIs. However, many combination therapies of SERM and SERD are tested in clinical trials. As E2 is no more formed in the ovaries in post-menopausal women. Instead, subcutaneous fat, liver, breast epithelial, and stroma surrounding normal breast cells and fibroblasts of primary breast tumors are the main producers of estrogen. In them, AIs, which bind with and

Table 13.2 Commonly used endocrine therapeutic drugs for breast cancer (Maira et al. 2007; Sheikh et al. 2015)

Sr. No	Drug	Mechanism
1.	Tamoxifen	Selective estrogen receptor modulators (SERM)
2.	Toremifene	Selective estrogen receptor modulators (SERM)
3.	Raloxifene	Selective estrogen receptor modulators (SERM)
4.	Fulvestrant	Selective estrogen receptor degrader (SERD)
5.	Anastrozole	Aromatase inhibitor
6.	Letrozole	Aromatase inhibitor
7.	Exemestane	Aromatase inhibitors
8.	Goserelin	Luteinizing hormone-releasing hormone analog
9.	Leuprolide	Luteinizing hormone-releasing hormone analog
10.	Abemaciclib	CDK inhibitor selective for CDK4 and CDK6
11.	Megestrol	Synthetic progestin, inhibit intracellular androgen action

Table 13.3 FDA-approved monoclonal antibodies and combinational therapies for breast cancer

Name	Antigen	Format	Indications (Year of First Approval) 1
Unconjugated antibodies			
Atezolizumab	PD-L1	Humanized IgG1	Triple-negative breast (2019), cancers (2019), bladder and non-small cell lung (2016)
Pertuzumab	HER2	Humanized IgG1	Breast cancer (2012)
Trastuzumab	HER2	Humanized IgG1	Breast cancer (1998)
Antibody–drug conjugates (ADCs)			
Trastuzumab emtansine	HER2	Humanized ADC	Breast cancer (2013)
Trastuzumab deruxtecan	HER2	Humanized ADC	Breast cancer (2019)
Sacituzumab govitecan	TROP2	Humanized ADC	Triple-negative breast cancer (2020)

inhibit aromatase, are used. In most post-menopausal cases, AIs and Fulvestrant, alone or in combinational therapy with additional endocrine targeted vehicles such as CDK4/6 inhibitors, are prescribed (Fanning and Greene 2019; Burstein 2020; Garcia-Martinez et al. 2021).

Even though the primary driver in ER^+ cases is ERα, but there are other genetic alterations that are also present in addition to mutations within ER1 such as overexpression of cyclin D1, CDKN2A loss, PI3K pathway alterations, MAPK pathway alterations, alterations in transcriptional regulators (*FOXA1, CTCF*, *TBX3,* and *MYC*), aberrant cofactor activity, and epigenetic variations are also reported (Hanker et al. 2020). All these alterations and others contribute to endocrine therapy resistance. Therefore, the combinational therapies depend on the additional contributor factors in ER^+ cases are used. For example, cyclin D1 is overexpressed in 50% of breast cancer cases, leading to increased CDK4/6 activation and RB phosphorylation and subsequent cell cycle progression (O'Leary et al. 2016; Burstein 2020). Therefore, CDK4/6 inhibitors alone or in combination with or AIs such as Fulvestrant or letrozole are recognized choices for managing both endocrine-sensitive and endocrine-resistant $ER^+/HER2^-$ metastatic breast cancer. Furthermore, after periods of endocrine monotherapy, the endorsement of aimed therapies against PI3K (Alpelisib) and CDK4/6 (Palbociclib, Ribociclib, Abemaciclib) and mTOR (Everolimus) led to noteworthy improvement in the managing of breast cancer. Numerous clinical trials have established the efficacy of inhibition CDK4/6. Furthermore, after the approval of target therapies against CDK4/6 (Palbociclib, Ribociclib, Abemaciclib), PI3K (Alpelisib), and mTOR (Everolimus) multiple clinical trials are in progress for combinational treatment (Table 13.3) (Hanker et al. 2020).

One of the emerging fields in breast cancer treatment is immunotherapy. In this therapy, the patient's own immune system is used to destroy cancer cells effectively.

The idea to utilize the immune system to fight cancer is over 100 years old. The immune system can suppress tumor growth or inhibit outgrowth. But it can also promote cancer cell growth by allowing selective cancerous cells that are more fit to survive by establishing a tumor microenvironment. Cancer cells evade the immune system by several mechanisms. However, breast cancer is considered more responsive to immunotherapies. Breast cancer metastasis can be prevented by appropriate activation of the immune system. For example, ER^- breast cancer patients' treatment with anthracycline monotherapy showed highly modulated immune scores associated with anthracyclines sensitivity. Furthermore, the immune system is critical to determine the response to tyrosine kinase inhibitors, mAbs, and endocrine treatment (Bayraktar et al. 2019; Li et al. 2021b). Immunotherapies include monoclonal antibodies (mAbs), CAR T-cell therapy immune checkpoint inhibitors, cytokines, immunomodulators, and vaccines.

MAbs has proven successful and nowadays is considered as the main component of standard for care of cancer therapy along with surgery, chemotherapy, and radiations. The antigens that are unique to overexpressed by cancer cells can be targeted by mAbs and subsequent tumor cell death by various mechanisms. The one mechanism by which many antibodies induce tumor cell death is the blockade of growth factor receptor signaling. For example, EGFR is overexpressed and lead to tumor proliferation, metastasis, and invasion. mAb Cetuximab is an anti-EGFR mAb and induce apoptosis in tumor cells by receptor dimerization and blocking ligand binding. Indirect mechanisms such as CDC, ADCP, and ADCC are also used (Zahavi and Weiner 2020).

Despite the success, clinical resistance to mAbs has become an issue as the majority of patients develop the refractory disease within a year after treatment. There are many resistance mechanisms, including induction of alternative growth signaling pathways, mutations of the antibody target, impaired effector cell responses, and EMT (Zahavi and Weiner 2020). This has shifted the treatment paradigms towards combinational therapy with other chemotherapy, radiation, and targeted inhibitors. Many combinational therapies undergo clinical and preclinical trials, while others have already been approved for breast cancer.

13.6.3 Transcriptional Controlling Drugs

Transcription is one of the fundamental cellular processes required for cell survival. The process is upregulated in the cancer cell to accommodate the increased ribosomes biogenesis and protein production that is the hallmark of cancer cells, including breast cancer. For transcription to start, the DNA should be in an open conformation; proper enzyme-mediated DNA methylation and histone modifications are required. These enzymes responsible for these epigenetic modifications are the first target for therapeutic drugs (Table 13.4) for cancer (Cheng et al. 2019). The transcription is a highly regulated and multistep process that can be divided into the following steps: the pre-initiation complex (PIC) formation that involves recruitment of RNA polymerase and several TFs at promoter site, initiation is followed by

Table 13.4 Transcription controlling drugs for cancer

	Drug	Mechanism	Status
Transcription inhibitors			
	CX-5461 (Pidnarulex)	RNA Pol I inhibitor	In clinical trial (NCT04890613)
	BMH-21	RNA Pol I inhibitor	
	Metarrestin (ML246)	RNA Pol I inhibitor disrupts the function of the perinuclear compartment	In phase 1 clinical trial (NCT04222413)
	α-Amatinin	RNA Pol II and III inhibitor	–
		Non-selective competitive inhibitor of all three RNA polymerases	Terminated at phase II (NCT00737360)
Transcriptional complex disruptors			
	BMS-986158	BET inhibitor: Inhibition of the interaction between BET proteins and acetylated histones and TF	In phase 1 clinical trial (NCT03936465)
	Triptolide (Minnelide)	BET inhibitor: block transcription elongation, binding to TFIIH and inhibitor of RNA Pol I and II transcription	In clinical trials (NCT04896073)
	RO6870810	BET inhibitor	Phase I completed (NCT02308761)
Premature transcription chain terminators			
	Fludarabine	Purine analog, inhibitor of DNA ligase and DNA primase, incorporate into RNA and chain termination	FDA approved
	8-Cl-Ado	Adenosine analogs, reduces ATP level by adenosine kinase-mediated phosphorylation	Suspended (NCT02509546)
CDK inhibitors			
	Palbociclib	Inhibitor of CDK4 and CDK6	FDA approved
	Ribociclib	Selective inhibitor of CDK4/CDK6	
	Abemaciclib	Selective inhibitor of CDK4/CDK6 and n-specific inhibition of other kinases	FDA approved
	Alvocidib (Flavopiridol)	Inhibitor of CDK1, CDK2, CDK4, CDK6, and CDK9	Phase II completed (NCT00058240)
Post-transcription inhibitors			
	BP1002 (L-Breast cancer l2)	Antisense oligonucleotides targeting breast cancer l-2	In clinical trial (NCT04072458)
	Trabedersen (AP 12009)	Antisense oligonucleotides targeting TGF-β2	Clinical trial terminated (NCT00761280)
	BP1001-A	Liposomal antisense oligonucleotide targeting Grb2	Clinal trial NCT04196257
	AZD4785	cET-ASO targeting oncogene KRAS	Phase 1 completed (NCT03101839)
	siG12D LODER	RNA interference, siRNA targeting KRAS	In clinical trial NCT01676259

(continued)

Table 13.4 (continued)

	Drug	Mechanism	Status
	EphA2-Targeted siRNA	RNA interference, siRNA targeting EphA2	In clinical trial (NCT01591356)
	MRX34	Tumor suppressor microRNA-34a (miR-34a) downregulates the expression of >30 oncogenes	Terminated (NCT01829971)
	SY-1365	CDK inhibitor, decrease expression of multiple oncogenic transcription factors	Clinical trial terminated (NCT03134638)

elongation that consists of the creation of RNA copy and termination when polyadenylation occurs. After that, primary RNA transcripts undergo post-transcriptional processing where RNA-binding proteins play a critical role (Kornberg 2005; Pereira et al. 2017; Cramer 2019; Laham-Karam et al. 2020). These steps and components of transcription machinery are potential targets for drug development especially small molecules are of interest that can target multiple steps and components such as TF, including TFIID, TFIIH, subunits of TFs, cofactors, RNA polymerase, mediator complexes (Mandel et al. 2008; Villicaña et al. 2014; Laham-Karam et al. 2020). Inhibitors of bromodomain and extra-terminal motif (ETM) can reversibly inhibit their binding to acetylated histone and slow down PIC formation (Alqahtani et al. 2019). CDK inhibitors have been extensively used as a target for the treatment of cancer and other diseases due to their pivotal role in the cell cycle (Malumbres and Barbacid 2009; Blachly and Byrd 2013; Galons et al. 2013). In addition to epidrugs several other transcriptional controlling drugs are in clinical trials (Table 13.4).

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Utility of Personalized Medicine in the Treatment of Different Subtypes of Breast Cancer

14

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Abstract

Breast cancer (BC) is a multifaceted disease caused by the progressive accumulation of multiple gene mutations combined with dysregulation of critical genes and protein pathways. The personalized medicine vision, where genomic and proteomic of individual is used to predict risks of diseases and responsiveness to drugs, revolutionized the medical management of BC. The phenomenon has increasingly gained popularity due to its potential for developing successful treatment regimens and boosting overall survival. Number of genes and molecular targets are being identified that can be incorporated clinically as biomarkers for different subtypes of BC. These prognostic markers not only provide information in determining the paramount drug choice/dosage for personalized treatment, but also in identifying molecular targets and gene signatures for invention of new therapeutic approaches. This would eventually lead to the creation of more tailored treatments for effective cancer therapy. The personalization of BC care is a major factor in improving outcomes, highlighting the significance of personalized medicines.

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Keywords

Personalized medicine · Breast cancer · Biomarkers · Pharmacogenetics

Abbreviations

BC	Breast cancer
BCT	Breast-conserving therapy
DCIS	Ductal carcinoma in situ
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
IDC	Infiltrating ductal carcinoma
LCIS	Lobular carcinoma in situ
LRR	Local-regional recurrence
miRNAs	MicroRNAs
PR	Progesterone receptor
TNBC	Triple-negative breast cancer

14.1 Background

Breast cancer (BC), among different cancers, is the most prevalent cancer of women and despite extensive investigations, its incidence continues to rise. BC is a biologically and clinically highly heterogeneous disease, involving many genetic and environmental factors. Many features such as type of BC, histological score, size, metastasis lymph node metastasis, progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2/neu) influence the prognosis of BC and treatment response. These patterns provide a unique portrait of each tumour and are a challenge for tumour classification to reach an appropriate clinical outcome. The typing of invasive BC, its histological types and their grading system is considered to be well established. At the other end, almost 20% of BC patients die despite prescribed maximum treatments. Moreover, adverse drug reactions due to "one-dose-fits-all" approach, also contribute to treatment failure (Spear et al. 2001). Almost 16% of approved drugs in the USA are reported to show adverse reactions (Spear et al. 2001). With new approach to treat cancers, medicine is moving towards a personalized therapy. Personalized medicines involve tailoring targeted treatment to every patient, built on the molecular characteristics, medical conditions, and the patient's personal preferences. Personalized approaches are needed for BC because of its heterogeneity characterized by distinct molecular aberrations (Reis-Filho and Pusztai 2011). Providing the most effective and appropriate treatment to BC patient with minimal side effects is of paramount importance

in personalized medicine (Wang et al. 2014). Concept of personalized medicine has transformed the healthcare standards by assimilating genetic information of individual, improving the efficacy of treatment, remodelling the practices of medicine, and providing opportunities for new economic and business models. Moreover, recent progresses in high-throughput omic technologies further improved the understanding of underlying molecular networking of BC, thus, uncovering particulars that lead to variable in clinical outcomes and/or differential drug responses. With the incorporation of the personalized concept the clinicians can make optimal picks to maximize the prospect of effective treatment regime and to reduce adverse drug responses; scientists can make the breakthroughs in the drug discovery routes, and pharmaceutical industries can manufacture devices to predict prognosis, thus facilitating early detection of disease.

In this chapter, we will initially discuss histopathological and molecular classification of BC. Next, we will highlight the benefits of personalized molecular subtyping in case of chemotherapy, surgery, or radiotherapy. Next section will be devoted to different strategies for personalized therapies in BC. This will include both mutation and expression profiling in detail, with respect to personalized treatment. Furthermore, how high-throughput technologies are assisting in understanding the dynamics of BC tumourigenesis and its progression with the ambition of emerging precision medicine methods. Finally, this chapter also presents challenges related to introducing personalized medicine in BC. All these matters are sightseen within the framework of precision medicine from bench-top to clinical settings.

14.2 Histopathological Classification of Breast Cancer

Being highly aggressive and is a collection of diseases with diverse clinical presentations, risk factors, pathological features, clinical appearances, response to treatment, and different outcomes. BC is classified using different systems and these classification schemes contribute to treatment and prognosis of BC. Unfortunately, due to lack of defined markers it is very hard to define the types of BC carcinoma (Stingl and Caldas 2007). BCs are usually epithelial tumours and the World Health Organization contributed to the classification of BC. The BCs can be classified into different subgroups based on histological type (HT) (Ellis et al. 1992) and histological grade (HG) (Elston and Ellis 1991). HT refers to the growth pattern of the cancers and on the basis of HT BC can be categorized into in situ and invasive carcinoma. BC in situ is sub-classified as ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) (Henry and Cannon-Albright 2019). Histopathological characters such as cell type, cells number, location of secretion, immunohistochemical profile, and architectural characteristics determine if the tumour is DCIS or LCIS. The DCIS began developing in milk ducts and invade to the fatty or fibrous tissues of the breast. Cribriform, comedo, micropapillary, solid, and papillary are further subtypes of DCIS. If the DCIS spread throughout the ducts, producing extensive lesions is called lobular cancerization. The LCIS has low histopathalogical variation. Invasive

carcinoma is highly heterogeneous type and is classified as invasive lobular carcinoma (ILC), infiltrating ductal carcinoma (IDC), ductal/lobular, mucinous, medullary ductal carcinoma (MDC), papillary and tubular carcinomas. Most common is the IDC type of BC and accounts for 70–80% of all newly diagnosed invasive lesions (Li et al. 2005). MDC is rare with a percentage of only three to five. The tumour usually appears on a mammogram with spongy tissue feel. Mucinous ductal carcinoma occurs when tumour cells within the breast produce mucous. Papillary carcinoma has good prognosis that mainly occur in women with age > 60. Tubular ductal carcinoma is also rare, and cancer looks like hundreds of tiny tubes under the microscope. Furthermore, HG is a prognostic factor and is an assessment of the degree of aggressiveness and proliferative activity based on mitotic index of a tumour. In a broad generalization, "low grade" cancers tend to be less violent than "high grade" cancers. IDC is further classified as grade 1, grade 2, or grade 3 refers to well, moderately, and poorly differentiated tumours, respectively. The grade and type provide complementary information regarding tumour (Weigelt et al. 2010b).

14.3 Molecular Subtyping of Breast Cancer

Classification of BC aims to provide an exact diagnosis and prediction of tumour behaviour to enable oncologic decision. Traditional BC classification may not contribute to varied clinical courses of distinct BCs. With the advancements, molecular analytical systems help to determine the predictive and prognostic landscapes of cancer. In the new era, new methods applied with new skills provide definition of various characteristics of BC in a different way and allows associating them with morphological appearance of BC (Eliyatkın et al. 2015). The pivotal study proposed five intrinsic molecular subtypes of BC based on transcriptomic and genomic profiling (Perou et al. 2000; Sørlie et al. 2001; Foulkes et al. 2003). Classification is based on the expression of four markers (ER, PR, human epidermal growth factor receptor-2 (HER2 and Ki-67)). Subtypes include (a) Luminal A or human epithelial receptor type 2 (HR+/HER2)− (HR-positive/HER2-negative) (b) Luminal B or HR +/HER2+ (HR-positive/HER2-positive) (c) Triple-negative/basal-like (TNBC) or HR−/HER2− (HR/HER2-negative) (d) HER2-enriched and normal-like (Perou et al. 2000; Foulkes et al. 2003; Sorlie et al. 2003). Luminal A and luminal B are ER-positive enriched BCs, whereas HER2-overexpressing, basal-like BCs and normal-like are ER−. The key features of different molecular subtypes are mentioned in Table 14.1. This typing reflects the biological diversity of BC and differs in their genomic complexity, main genetic variations and prognosis (Gatza et al. 2010). Among different types, TNBC is highly heterogeneous compared to other types; six distinct TNBC subtypes are identified via gene expression analyses.

In addition to the assessment of standard biomarkers, other factors also contribute to classification, including proliferation rate and cytokeratin expressions, etc. Ki67 was also included as a marker of prognosis according to St. Gallen 2013 system classification (Gerdes et al. 1983). Gene expression profiling further gives the detailed information about distinct characters and behaviours of BC subtypes

Table 14.1 Percentage frequency, characteristics, and personalized therapy responses of different subtypes of breast cancer

Molecular subtypes	Frequency (%)	Morphology	Immunohistochemical profile	Genetic mutations	Expression profile	Chemotherapy response	Personalized therapy	References
Luminal A	30–40	Grade 1 or 2; well differentiated; no special type, tubular, neuroendocrine, classical lobular, mucinous, and cribriform carcinomas	ER-positive, PR high expression (≥20%), HER2-negative, and low Ki-67	PIK3CA (48%), RUNX1 (11%), MAP2K4 (7%), TP53 (10%), FOXA1 GATA3 mutations; gain of 1q, 8q, loss of 8p, 16q	High expression of ESR1 XBP1	Low	Aromatase inhibitor, Fulvestrant, tamoxifen	Carey et al. (2006), Subik et al. (2010), Vuong et al. (2014)
Luminal B	20–30	Grade 2 or 3; less differentiated cancers, mostly invasive ductal carcinomas with no special type, some invasive micropapillary carcinomas	ER-positive, <20%. Expression of PR, HER2-null and higher level of Ki67 labelling index (>14 or 20%)	TP53, JAK2, PIK3CA, MAP2K4, RUNX1, KRAS, BRAF, GNAS, PTEN mutations MDM2 amplification, ATM loss, enhanced genomic instability, focal amplifications	High expression of FGFR1, HER1, PI3K, and Src	Intermediate	Tamoxifen, aromatase inhibitors, Fulvestrant	Harvey et al. (1999), Cheang et al. (2009), Goldhirsch et al. (2013)

(continued)

Table 14.1 (continued)

Molecular subtypes	Frequency (%)	Morphology	Immunohistochemical profile	Genetic mutations	Expression profile	Chemotherapy response	Personalized therapy	References
				(e.g. 8p12, 11q13)				
HER 2 enriched/ normal-like	12–20	Grade 2 or 3; infiltrating carcinoma, apocrine and pleomorphic lobular carcinomas	HER2-enriched subtype (ER and/or PR-negative/HER2-positive) and luminal HER2 subtype (ER and/or PR-positive, HER2-positive); and further divided into two phenotypes based on PR expression: ER-positive, PR-positive, HER2-positive and ER-positive, PR-negative, HER2-positive	HER2, TP53, PIK3CA mutations, cyclin D1 amplification, high genomic instability	High expression of FGFR4 and EGFR	High	Lapatinib, Rastuzumab, Pertuzumab, Adotratuzomab Emtansine	Perou et al. (2000), Sørlie et al. (2001), Sorlie et al. (2003), Staaf et al. (2010), Fountzilas et al. (2012)
Basal-like/ triple-negative breast cancer	15–20	Invasive ductal carcinoma of no special type	No expression of ER, PR, and HER2. High Ki67 expression	Mutations in cytokeratin genes, cell cycle and DNA replication, and DNA damage response pathways.	High expression of P-cadherin, fascin, caveolins 1 and 2, alpha-beta crystallin and (EGFR), high myoepithelial	High	PARP1 inhibitor (Olaparib and Iniparib), cisplatin	Yersal and Barutca (2014)

			BL2 tumours also manifest as invasive ductal carcinoma NST, mutations of BRCA1, PTEN, ERBB2, TP53, PIK3CA, and RB	markers, such as CK5, CK 14, CK 17, and laminin			

(Reis-Filho et al. 2010; Weigelt et al. 2010a). Gene expression profiling also contributed to division of BC to other subtypes which include acinar cell carcinoma, small cell carcinoma, adenoid cystic carcinoma, PR-positive, PR-HER2-positive, normal-like carcinoma, neuroendocrine, metaplastic, medullary carcinoma, luminal NS carcinoma, carcinoma not specified, ER-PR-HER2-positive, based on different mutation profile of each type using COSMIC database (Jeibouei et al. 2019).

14.4 Personalized Oncology for Breast Cancer Subtypes

Personalized medicines lead to breakthroughs in treatment of BC, as well as the fact that breast tumours are detected at an earlier and more curable stage. Over the last two decades, the above-mentioned subtypes of BC have been used primarily to personalize treatment and play a key role in improving outcomes. However, current basic science and clinical research are focusing on further refining these classifications in order to obtain improved outcomes in patients throughout the risk within each broad-spectrum category. Advanced personalized medicine has the ability to tailor treatment for the finest effective response and the utmost safety edge, resulting in improved BC patient care. BC treatment is a leading model for genuinely individualized diagnosis and treatment in the current era of precision medicine since this highlights the revolution in precision medicine. Personalization in breast oncology is not only playing the role in improving health care, but also contribute to cutting costs by allowing each patient to obtain earlier diagnosis, risk assessments, and effective treatments. Personalized medicines are already dictating systematic therapy for BC patients for tumour recurrence or all stages advancements of care, from the first diagnosis to surgery, treatment, and follow-up. This section highlights the latest developments and approaches for personalizing BC treatment with radiation, surgery with chemotherapy and vaccines.

14.4.1 Personalization in Radiation Oncology

Precise radiation therapy has the prospect of creating a number of diagnostic, predictive, and prognostic assays to predict the likelihood of distant metastases and local-regional recurrence as well as the intrinsic radio-responsiveness. For many years, surgical and radiation treatment for lymphatic drainage of breast tumours has been considered standard of care. Previously, radiation therapy in BC was applied in a consistent manner in terms of target volumes and dose. Treatment has improved in recent years as a result of novel surgical techniques, systemic therapy alternatives, and a greater knowledge of the disease's biology. After breast saving surgery, radiation therapy is an important part of a multidisciplinary strategy to BC treatment that yields comparable oncologic results to mastectomy alone (Fisher et al. 2002). In clinical stage, personalization based on the size of tumour and the presence of positive lymph nodes, with additional pathologic indicators

associated with positive nodes, as well as other pathologic markers (margin width), influenced radiotherapy treatment options as listed below.

In BC patients, cost-effectiveness and patients' convenience priors are the most common factors for personalized utilization of hypofractionated radiotherapy. Hypofractionated whole breast radiation has been thoroughly verified in a number of randomized experiments and appears to be equally effective as conventional fractionation regimens while also being potentially less harmful (Haviland et al. 2013). In other study, fractionation sensitivity was investigated in a big cohort of population with prospectively derived outcomes, like rate of local recurrence, with long-term follow-up. Report showed no evidence of a connection between molecular subtype of BC and fractionation procedure (Lalani et al. 2021). Nguyen et al. investgated 793 individuals with BC, who had lumpectomy and radiation therapy as part of their BC therapy and luminal A was compared to all other subtypes (Nguyen et al. 2008). According to multivariate analysis, the adjusted hazard ratio of local recurrence was found to be 9.2 for HER2-positive and 7.1 for basal type. In univariate analysis, the adjusted hazard ratio for distant metastases was 5.3 for HER2-positive, 4.6 for basal subtype and 3.9 for luminal B, and cancer patients (Nguyen et al. 2008). Only the basal and luminal B and groups had a higher incidence of distant metastases. In other study, Kyndi et al. also established that patients with robust prognostic markers (HER2 negativity and ER/PR positivity) had a significantly better overall survival rate after post-mastectomy radiation therapy, whereas the patients of ER/PR-negative, and HER2-positive had no significant survival after post-mastectomy radiation therapy (Kyndi et al. 2008).

In terms of effective care, TNBC is the most difficult and aggressive subtype to treat. Even though the overall survival was the same, TNBC was connected to a greater risk of locoregional recurrence and distant metastasis than the luminal subtypes (Wang et al. 2013). TNBCs in women are known to contain the BRCA1 gene mutation, and tumours without BRCA1 functional are weak in homologous recombination to repair double-strand DNA breaks, making them radiosensitive. Hidden BRCA1-deficient tumour foci in the breast and surrounding tissue could be removed if conservative breast surgery is followed by radiation, limiting locoregional spread (Abdulkarim et al. 2011). Interestingly, niraparib has already been demonstrated to radiosensitize several human tumour xenografts, including the triple-negative human BC (Wang et al. 2012). As a result, combining these medicines with RT appears to be promising, albeit more research into the efficacy and safety of this combination is needed.

14.4.2 Personalization in Surgery and Chemotherapy

Surgical management innovation has aimed to reduce the quantity of surgery performed over the last two decades (Barnard and Klimberg 2017). As it was widely assumed that much more invasive surgery was connected with a great outcome or survival. Breast-conserving therapy (BCT), which comprises of lumpectomy and radiotherapy, has improved dramatically in the previous decade, with the majority of

women now having the choice of choosing between mastectomy alone, mastectomy with fast reconstruction, or BCT. The Early Breast Cancer Trialists' Collaborative Group series of meta-analyses of these trials came after multiple significant randomized studies established breast-conserving surgery further confirmed it (Darby et al. 2011).

The type of systemic therapy is mostly determined by the molecular subtype of BC. Interestingly, Mazouni et al. looked at 1194 patients with primary BC to see how surgical treatment affected their outcome and which molecular subgroups of BC they had (Mazouni et al. 2013). They discovered that molecular subgroups have an influence on the frequencies of BCS and nodal surgery. They reported that BC surgery is better in luminal A cancer (70.6%) than in TNBC (66.2%) and 60.9% of HER2+ tumours (Mazouni et al. 2013). According to a growing amount of research, the likelihood of local recurrence differs by subtype. This is supported by the study done by Caudle et al. found that patients with HR+/HER2 and HR+/HER2+ subgroups had brilliant local-regional recurrence (LRR) and free survival was determined regardless of tumour response to neoadjuvant treatment in 595 patients (Caudle et al. 2012). Patients with HR/HER2 and HR/HER2+ subgroups who had a poor neoadjuvant chemotherapy response had a lower LRR-free survival after BCT (Caudle et al. 2012). TNBC, that has narrow range of treatment choices, with high rate of metastasis and recurrence and has a poor prognosis when compared to other kinds of BCs (Yin et al. 2020).

In response to treatment, the patterns of each molecular subtype differ. Chemotherapy has become the mainstay treatment for TNBC as no expression of ER, HER2, and PR, making specialized endocrine and targeted therapy ineffective and poor prognosis (Ismail-Khan and Bui 2010). This is attributed to a shorter disease-free interval in the neoadjuvant and adjuvant setting, as well as more aggressive progress in the metastatic situation. Various studies have recently emerged indicating that employing neoadjuvant chemotherapy regimens in TNBC treatment can improve patients' prognosis. This includes using a combination of different chemotherapy agents such as cisplatin, taxane, anthracycline, fluorouracil, and cyclophosphamide in treating TNBC (Berrada et al. 2010). Despite the fact that anthracycline-based chemotherapy has been demonstrated to be most effective in patients with HER-2 overexpression, its efficacy in patients with TNBC is still debatable (Shah and Gradishar 2018). Thus, selection and personalization of appropriate chemotherapy combinations is crucial for ensuring a favourable treatment outcome and prognosis for TNBC patients.

14.4.3 Personalization in Therapeutic Cancer Vaccines

Vaccines targeting a variety of tumour antigens have been developed and demonstrated to trigger an anti-tumour immune response. BC subtypes that are HER2-positive and TNBC are the most immunogenic. Due to this, stimulating the patient's immune system is a feasible treatment for certain BC subtypes (Arab et al. 2020). Although progress has been gradual and this knowledge's clinical use has

been difficult, preclinical studies have offered significant support for vaccines of cancer, and there have been some successes.

HER2 is one of the most promising antigens for vaccination (Arab et al. 2020). Patients with resectable HER2+ BC were registered in a phase I trial to develop a destruct HER2 epitope-based vaccination comprising of four human leukocyte antigen class II-restricted epitopes combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) showed that for each peptide, the percentage of patients who replied with enhanced T cell immunity was elevated (68–88%). HER2-specific antibodies were also boosted by the vaccine, which resulted in prolonged immunological activity for the next 2 years after vaccination (Knutson et al. 2020). In individuals with HER2-expressing BC, a study compared the ability of E75 vaccination (Nelipepimut-S, NeuVaxTM), a human leukocyte antigen (an A2/A3-restricted HER2 peptide), and GM-CSF, in 108 patients found vaccine based on E75 is nontoxic and have clinical effectiveness (Mittendorf et al. 2014). But its phase III trial showed no evidence of a meaningful clinical benefit in terms of avoiding recurrence of BC (Mittendorf et al. 2019).

A randomized phase II trial (NCT00524277) registered 456 node-positive and HER2-derived peptides will be given to high-risk node-negative individuals as an alternate strategy to target HER2 (GP2 and AE37), but overall 5-year difference was not found in the primary analysis (Brown II et al. 2020). E37 immunization may be beneficial for patients with HER2 low-expressing malignancies and advanced stage, according to a subset analysis. Additional research may be required to gain approval for this method.

Novel immune system modulators, as well as adaptive cellular treatment, chimeric antigen receptor (CAR) T cell treatment, and vaccines, have all shown promising results in TNBCs (Kim et al. 2019). In a TNBC mouse model, a whole-cell cancer vaccine based on the oncolytic vesicular stomatitis virus boosts natural killer and CD8+ T cell activity, improving TNBC prognosis (Niavarani et al. 2020). More study with TNBC patients in a randomized trial is required. More research in a randomized trial with TNBC patients is needed.

14.5 Strategies to Personalized Therapy Development

Different strategies for the development of personalized therapy are followed, as shown in Fig. 14.1, are discussed below.

14.5.1 Pharmacogenetics in Breast Cancer Subtypes

Pharmacogenetics is the differential responses of patients to drug therapy which is based on their genetic makeup. These studies are conducted with two main designs: genome-wide association studies (GWAS) and candidate-gene approach. In GWAS, variants across the entire genome are investigated for a particular result, however in the candidate-gene approach, genes related to metabolism, excretion, transport, or

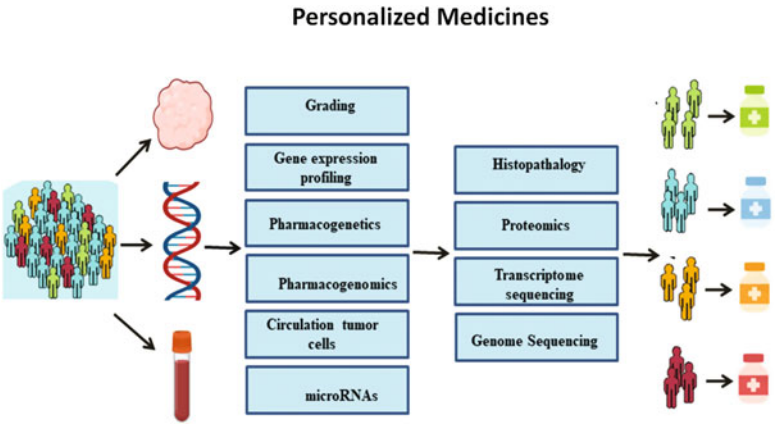

Fig. 14.1 Strategies and high-throughput technologies for the development of personalized medicines

drug targets are investigated for biomarkers that demonstrate strong correlations with specific outcomes such as response or adverse event. Here we will discuss role of different molecular genetic markers in the treatment of different subtypes of BC.

14.5.1.1 Estrogen Receptor-Positive (ER+)

ER+ BC is a major type that can be treated with personalized hormonal therapy like artificial estrogen analogue, i.e. tamoxifen. Tamoxifen binds to ER thereby blocking the stimulation of the classical signalling pathway which eventually causes ductal hyperplasia. Some other signaling pathways associated with ER upregulation are not hormone dependent. In this regard, ER acts via growth factor signalling that involve activation of intracellular phosphatase and kinase. Kinases affect in the phosphorylation of ER protein that leads to resistance of hormonal therapy. Moreover, the ER interaction with transcription factors such as C-Fos/C-Jun (AP-1), NF-KB can lead to angiogenesis, tumour proliferation, and metastasis (Barone et al. 2010). In addition to tamoxifen, fulvestrant is also used as the ER downregulator in BC treatment by inhibiting its dimerization and promoting degradation. In postmenopausal woman who could get benefit from fulvestrant, anastrozole can be used to block estrogen conversion from adrenal androgen. However, tamoxifen activity is independent from menopausal status (Russell 2014). In epithelial cells, PR gene expression is linked with ER expression that is why in almost half cases ER+ tumours are also PR+. It is evident from earlier work that PR− patients shows worse prognosis as compared to PR+ patients when treated with tamoxifen. Anastrozole is beneficial for these patients (De Abreu et al. 2013). Primary and acquired endocrine resistance can develop in these patients due to many reasons including mutation rate, methylation, acetylation, overexpression of ERβ, downregulation of ERα, as well as crosstalk between signalling pathways (Barone et al. 2010). Conventional hormone therapy is not sufficient in cases where mutations occur in the ER gene (ESR1). Hotspot mutations positioned in the

ligand-binding domain of ERα include D538G, Y537C, Y537N, and Y537S. These mutations are associated with acquired resistance to endocrine therapy and represent >80% of ESR1 mutations. This is the main resistance mechanism in metastatic BC patients treated with hormonal therapy. These mutations can be assessed in plasma cell-free DNA for selecting treatment strategies, such as ARN-810 drug is effective that persuades proteasomal-mediated destruction of ERα and is effective against tumours exhibiting ESR1 mutation, where hormonal therapy is not effective. Fulvestrant alone or in combination with palbociclib in metastatic BC also benefit patient with ESR1 mutations (Fuqua et al. 2014). The joint action of the drugs leads to better prognosis. However, such targeted therapies are not effective in all patients (Jeibouei et al. 2019). It is reported on COSMIC database that Y537S mutation in ESR1 affects insulin-like growth factor-I receptor (IGFIR) phosphorylation. This is linked with resistance to target therapy and a shorter overall survival. Another mutation was reported to decrease sensitivity to tamoxifen via phosphorylation of protein kinase B (AKT) is K303R. It is also reported in COSMIC database that PIK3CA mutations are found in most of the ER+ cases. Studies have shown the relevance of PIK3CA and ESR1 mutations with clinical features. While detecting mutation in ESR1 and PIK3CA, a high heterogeneity was noted in ESR1 as compared to PIK3CA. PIK3CA mutations usually occurs in tumours during early stage of development while ESR1 mutations come later during hormonal therapy and in metastatic stage. So it can be concluded that mutation might occur as a result of pressure on tumour due to hormonal therapy. Furthermore, methylation of PITX2 methylations is also associated with tamoxifen resistance. Thus, methylation analysis of candidate genes is also important to prevent patients from ineffective treatment by administrating demethylation agents. New drugs based on pharmacogenetics, maintenance of systemic haemostasis, and resistance profile are needed to treat BC patients (Jeibouei et al. 2019).

14.5.1.2 HER2-Positive (HER2+)

For treatment of HER2+ different types of anti-HER2 mediators are available such as monoclonal antibodies (pertuzumab and trastuzumab), which bind directly to the HER2 extracellular domain, tyrosine kinase inhibitors (neratinib, lapatinib, and afatinib), that bind to intracellular domains of various HER family members and antibody–drug conjugates (trastuzumab emtansine), that contain monoclonal antibody with cytotoxic agent. All these drugs are FDA and EMA approved for HER2+ BC patient's treatment. However, many novel agents are being tested in clinical trials (Goutsouliak et al. 2020). Trastuzumab was the first monoclonal antibody against HER2+ BC. Though studies showed that some patients developed resistance to this therapy related to signalling pathways of HER2 and due to mutations RAS, PIK3CA, Src, NF-KB, and PTEN (Jeibouei et al. 2019).

14.5.1.3 Triple-Negative Breast Cancer (TNBC)

Unfortunately, overall survival of TNBC patients is less than other BC subtypes. However, with the advancement of technology our knowledge about the genomic and molecular basis of TNBC has been increased which will impact the therapeutic

interventions. Studies have shown that TNBC is a heterogeneous condition with different molecular aberrations (Azim et al. 2020). There are three subtypes of TNBC, namely basal-like, normal-like, and non-basal-like. About 80% of basal TNBC display p53 mutations whereas homologous recombination repairs (HRR) and homologous recombination deficiency (HRD) are also present in basal and non-basal TNBC patients. Homologous recombination repair is important in the prediction of therapeutic response (Jeibouei et al. 2019). Mutations in other genes like BRCA1, RB, PIK3CA, and PTEN have been reported in COSMIC database that are associated with TNBC. The TP53 gene mutations have been associated to cisplatin treatment resistance. Although TP53 mutations are related to methylation rate of BRCA1, but there is no association between BRCA1 and TP53 mutations (Foedermayr et al. 2014). Phosphoinositide 3-kinases (PI3K) pathway is activated by loss of PTEN or INPP4B, mutations in PIK3CA gene, and overexpression of EGFR. P53 high expression is associated with increased proliferation while PTEN, ERBB2, and BRCA1 mutations are associated with high risk of metastasis. Alterations in genetic and epigenetic DNA repair mechanism may result as initial contributor of cell transformation. As anticancer agents, treatment medications that target cells' impairments in the repair of double-strand DNA breaks are extremely important. Anti-tumour agents targeting cells with DNA breaks are available like platinum-based compounds and PARP inhibitor. Inhibitor of PARP is active for cancer cells that are deficient in DNA repair of double-strand DNA breaks. This inhibitor blocks PARP enzyme like PARP1 that cause accumulation of unrepaired single-strand breaks. This eventually leads to double-strand DNA breaks in replication fork that BRCA1- and BRAC2-deficient cells cannot repair and leads to cellular death (Gudmundsdottir and Ashworth 2006). Initially promising results were obtained by using PARP inhibitors in TNBC, but such findings could not obtain in subsequent trials. Thus, it is not clear that PARP inhibitors like iniparib and olaparib treatment is beneficial for which subset of TNBC or for which type of BRCA mutations. Studies have also shown that BRAC1 methylation status can be used as a biomarker for response to PARP inhibitors (Stefansson and Esteller 2013). Platinum-based agents like cisplatin can be effective for cells with BRCA1 and BRCA2 mutations and methylation. This drug can crosslink with DNA and target ineffectively repaired lesions having double-strand DNA breaks. Further studies and clinical trials are needed to develop TNBC-targeted drugs, which will be mutation specific and based on molecular profiles of patients.

14.5.2 Pharmacogenomics in Predicting Chemotherapeutic Response

Biomarkers to predict toxicity and efficacy of chemotherapy are discovered by pharmacogenomics studies. However, due to very limited number of such studies in case of BC, it is not possible for regulatory bodies to warrant guidelines for specific dosing. The only available guidelines are for *DPYD* testing before the use of fluoropyrimidines (Al-Mahayri et al. 2020). This testing is related to inherited

dihydropyrimidine dehydrogenase (DPD) deficiency and its effect on cancer patients treated with fluoropyrimidines. There is a genetic risk of toxicity after treatment with fluoropyrimidines. A wealth of knowledge has been yielded by working on inherited DNA variation in the DPD gene (DPYD) that leads to genetic testing in the clinic (Innocenti et al. 2020). A total of four DPYD variants, that cause DPD deficiency, were selected by Clinical Pharmacogenetics Implementation Consortium. For these variants, recommendations were provided to help in fluoropyridine therapy and dosing decision (Innocenti et al. 2020).

Research based on pharmacogenomics studies is underway to discover new biomarkers in case of BC, but the results are not consistence. Such studies that were conducted vary in their selected outcome, used chemotherapeutic agents and their selected genes. Some researchers used the grade of toxicity as the endpoint, whereas others decided to measure the frequency of dosage delay, modification, or therapy discontinuation owing to toxicity. Furthermore, few studies concentrated on explicit symptoms such as gastrointestinal indications, or events such as infection at a particular grade. However, in these studies very few biomarkers in genes encoding ATP-binding cassette family members active in cytotoxic agents' efflux and transport or the metabolizing cytochrome p450 enzymes were investigated. Due to heterogeneity in the design of study, we cannot compare their results. One such GWAS study was designed and directed to find out genomic biomarkers to investigate caused by chemotherapy in BC patients. In this study, 303 BC patients with docetaxel-induced alopecia were compared with 880 patients of BC without docetaxel-induced alopecia. At the end of study, only one SNP in the calcium channel voltage-dependent subunit beta 4 (*CACNB4*) with significant association was found along with many other suggestive SNPs. However, to find strong associations, more studies with strong study design, representative sample size, with a focus on incidence rate of the systemic side effect, and with the use of techniques that cover all genes in focused pathways are warranted.

14.5.3 Gene Expression Profiling

Personalized medicine treatment is gaining attention for its prospect of developing effective cancer regimen. One of the strategies for personalized therapy development is gene expression profiling, which is the determination of the expressed genes pattern at transcription level and to identify transcriptional variations between normal and malignant cells (Sørlie et al. 2001). In any specific cell, gene expression profiling can predict complete cellular functions and activity of 1000 genes at once with molecular portrait of BC spread. These profiles can also reveal cells responses to a particular treatment (Arce-Leal and Bautista 2020). Gene profiling can also be helpful in determining optimal drug dosage and drug choice for use in personalized treatment. It may also help in identifying novel molecular targets for defining cancer management strategies (Jabato et al. 2021).

It is documented previously and as we mentioned about genes linked to the development of cancer with mutational changes that causes altered responses against

chemotherapies due to variable protein expression. One such example is glutamic acid-to-lysine mutation in AKT1 (AKT1 E17K mutation), which is important component of PI3K-AKT—mammalian target of rapamycin (mTOR) pathway, at position 17 among advanced level cancer patients which results in enhanced expression of whole pathway (de Bruin et al. 2017). This target is cancer promoting phosphoinositide 3 kinase-AKT-mammalian target and designing inhibitors to this target may assist as a promising approach to inhibit tumour growth. Another example is everolimus in BC treatment, which is one of the analogues of rapamycin and as allosteric inhibitor of mTOR1 that targets the PI3K-AKT-mTOR pathway and causes AKT1 E17K as a diagnostic mutation for the BC (Rudolph et al. 2016). Along with genomic profiling, there is a feasibility of phospho-profiling for biomarker identification targeted by PI3K-AKT-mTOR pathway inhibitors based on abundance in number of phosphoproteins. It also enables the determination of these pathway inhibitors efficacy based on phosphorylation level of biomarkers (Andersen et al. 2010). The profiling strategies also provide basis for optimizing personalized therapy and a vivid profile of drug resistance developed by the activation of compensatory pathways in cancer therapies. This also affects the overall survival rate of BC patients and quality of life. For example, PI3K-AKT-mTOR pathway, cyclin-dependent kinase 4/6 signalling pathway had depicted effective prolonging in survival of patients yet it does not increase overall survival rate (Jeibouei et al. 2019). This observation deemed the need of developing personalized medicine approach while prescribing cancer treatment.

Turnbull and colleagues had identified biomarkers to a specific aromatase inhibitor known as letrozole through their developed model for biomarker identification which can also identify drug-specific biomarker for tailoring personalized therapy. They identified two upregulated genes, IL6ST and NGFRAP1 which were implicated in immune response and apoptosis induction respectively. Also, two genes were found downregulated post-cancer treatment, ASPM, and MCM4, which were implicated in cellular proliferation (Turnbull et al. 2015). These gene's expression was suggested as a useful tool in developing personalized treatment among BC patients. For example, HER2 overexpression was found to be associated with BC progression. HER2 expression status assessment has a direct relation in prescribing trastuzumab (chemotherapeutic drug) across the various stages of cancer trajectory. It was observed that HER2 expression can be increased by 20% with tumour progression from primary to metastatic stage (Chen et al. 2017). So it is advisable to have specific genes assessment prior commencement of personalized therapies.

Another genomic biomarker that can participate in gene profiling is mitochondrial DNA (mtDNA) with their common deletions in BC patients. These mutational changes can be due to elevated level of reactive oxygen species (ROS) during oxidative phosphorylation and can serve as biomarker for optimizing cancer therapies (Nie et al. 2013). Apart from these, modifications in metabolome were also considered as a potential biomarker to predict cancer incidence and disease status. Role of these metabolites can spark the future research involving hormonal therapies against cancer and in designing personalized medicine therapy. For

example, expression level of phosphatidylcholine in TNBC patients was found associated with disease reoccurrence (Hosokawa et al. 2017). Similarly, high testosterone level was found associated with increased risk of cancer reoccurrence (Secreto et al. 2017). So, while designing personalized treatment regular check on hormonal level will ensure the effective therapy design with reducing risk of cancer reoccurrence.

In order to complete the clinical activity assessment of cancer therapies, reliable method like gene expression profiling can be warranted to detect even rare mutations causing cancer. This will also pave the way for designing personalized medicine therapy for BC patients. There are several commercially available GEP tests such as MammaPrint, Oncotype DX, EndoPredict, and Prosigna (PAM 50). They have prognostic ability, while Oncotype DX has also demonstrated predictive ability.

14.5.4 Circulating Tumour Cells

In nineteenth century, the mechanism of haematogenous spread of single tumour cell from primary tumour site was initially confirmed. The shredded tumour cells then reach to secondary homing sites at bone marrow and peripheral blood, they may serve as surrogate biomarkers for precursors of distant metastasis and minimal residual disease. The emergence of liquid-based biomarkers including circulating tumour cells have revolutionized the palliative care of cancer patients. It is well known that metastatic cascade is a combination of multiple steps that enables the primary tumour cells to reach to secondary organs over a certain period of time, sometimes require decades for the complete spread of disease. Upon completion of surgical procedure and the required adjuvant therapy, still certain patients suffer from distant recurrence due to activation of metastatic cascade long before diagnosis (Chan et al. 2017). Evidence recommend that tumour microenvironment and immune system also influence the detachment of primary tumour cell and homing at secondary organs. However, significant proportion of circulating tumour cells die in a process called metastatic inefficiency leaving a small subpopulation of these cells behind. The dead cells rapidly get cleared by macrophages, natural killer cells, monocytes, and neutrophils. It has also been suggested that tumour-circulating cells also stimulate the development of pre-metastatic niche in secondary organs (Steinert et al. 2014). Different mechanisms have been proposed in this context. For example, expression of vascular signal proteins and circulating cells may attract the vascular endothelial growth factor receptor haematopoietic stem cells influencing fibroblast at the homing site, creating tumour microenvironment for metastatic growth at the later stage (Kaplan et al. 2005). The microenvironment created at different BC subtypes can provide the scientist with new therapeutic targets for designing personalized medicine treatment.

14.5.4.1 Prognostics Significance in Early and Metastatic Breast Cancer

In primary stage BC, tumour cells circulate after entering the blood stream and spread in the peripheral blood may relapse in the course of disease. In multicentre

pooled analysis, circulating tumour cells presence was confirmed in early stage of BC as an independent predictor of distant disease-free survival for four survival endpoints like grading, tumour stage, nodal stage, hormone receptor status, and in some cases HER 2 status as an independent significant prognostic factor (Janni et al. 2016). Other non-significant factors for early-stage BC association with reoccurrence and survival rate includes menopausal status, histologic type, non-adjuvant chemotherapy, and adjuvant chemotherapy. However, in low-risk patients with small node-negative tumours cancer could be treated successfully. This prognostic index helps the oncologist in designing personalized medicine. Moreover, tumour cell dissemination and spread patterns vary according to the biological aspects of the disease. For example, in patients with HER2-positive and hormone receptor-negative tumours predictive survival due to circulating cells is not present but can be estimated for TNBCs and luminal subtype (Hwang et al. 2012).

Studies revealed that circulating tumour cell not only have the prognostic role but the changes in their count also effect the treatment options and therapy response along with characterization of these cells (Bidard et al. 2014). In a study by Budd et al., it was observed that in metastatic BC patients, circulating tumour cells remain persistent and can predict the impaired clinical outcome even after the radiological response. In this case, a non-invasive blood analysis by liquid biopsy presents an attractive too for monitoring disease progression and therapy response (Budd et al. 2006). In one study, 107 patients initiating a new line of therapy was evaluated and it was found that HER2 status did not influence the overall survival (Wallwiener et al. 2013). Clinical significance of circulating tumour cells is still under study in the German DETECT trials (NCT01619111). Characterization of circulating tumour cells at the molecular level could support to identify mechanism of resistance for optimization of systemic treatment.

14.5.4.2 Monitoring of Therapy in Early and Metastatic Breast Cancer

Chances of subsequent relapse among BC patients are more prominent beyond adjuvant chemotherapy. SUCCESS trial demonstrated that circulating tumour cells correlated with shorter, recurrence-free survival. However, the presence of circulating tumour cells after 2 years post-chemotherapy predicted worst survival in clinically disease-free patients (Janni et al. 2018). However, tumour cells with enhanced resistance mechanism have chances to survive longer leading to metastatic growth. In a study by Hall et al. from 57 patients in TNBC patients, it was found that upon completion of neoadjuvant therapy statistical significant association was established between circulating tumour cells and relapse-free overall survival (Hall et al. 2015). In adjuvant therapy, this response observing tool is not available anymore for the reason that systemic treatment is directed after surgery. With a median follow-up for 71 months over 237 patients, it was observed that taxane-based adjuvant therapy has longer disease-free survival rates (Xenidis et al. 2013). Since circulating cells depict decreased clinical outcome and they appear to reflect treatment response in metastatic BC, a prominent query was raised to evaluate circulating tumour cells-based therapy with its clinical outcomes. The first large clinical trial SWOG S0500 (NCT 00382018) has addressed this issue. It was a phase III trials

with metastatic patients and high levels of circulating tumour cells after first cycle of chemotherapy. Strong prognostic power was confirmed during this trial for circulating tumour cells. However, the clinical outcome was poor in patient with continuously raised circulating cells levels. These patients could be from chemo-resistant population that may require alternative treatment approach (Smerage et al. 2014). In another phase III trial CirCeo1 (NCT 01349842) on therapy guidance by institute Curie, France, it was observed that in circulating tumour cell-positive patients, disease progression can be analysed by clinical test, imaging or by enumeration of circulating tumour cells. Patients with an insignificant decrease in levels of circulating tumour cells can be switched to another circulating tumour cells-based regime of chemotherapy. This practice may be a guide in avoiding inefficient and toxic chemotherapy.

14.5.5 MicroRNAs in Personalized Medicine

MicroRNAs (miRNAs) are considered as an embracing tool for designing personalized medicine. They possess a number of desirable properties for the type of rigorous analysis required in clinical practise. They are very stable and can be detected easily and to date, more than 2000 human miRNAs have been identified. miRNAs have the ability to tune the expression of genes which sensitize these markers for follow-up of disease pathology. Important factors that limit the use of miRNA in personalized medicine include source of miRNA and influence on recovery with final outcome. This is specifically absolutely true for mRNA extracted from bio-fluids. Despite the fact that mRNAs are a promising tool with a bright future in personalized medicine, it is still challenging to produce an authorized clinical diagnostic test due to technical challenges in robust and comparable mRNA profiling. Pre-analytical, analytical, and post-analytical stages are where most technical issues arise. Therefore, source of extraction, working conditions, data processing all lead to variable results and unease in designing mRNA-based personalized medicine (Blenkiron et al. 2007).

In the pre-analytical phase, composition of sample, patient's condition, sample handling, titre of mRNAs, secretion and sorting of mRNAs are the factors effecting the combined results. Variability among the concentration of mRNAs reaches up to 23,000 copies/μL, according to Miotto et al. (2014). Presence of contaminant mRNAs is another challenge posed to the developing process. It is well known that circulating mRNAs (c-mRNAs) comes from different cellular sources with a major contribution from blood cells; therefore, haemolysis and variation in blood cell count affects the mRNA signatures up to 30-fold (Pritchard et al. 2012). In post-analytical phase relative quantification of detection method, endogenous control over the process, normalization analysis of signals is required to consider the biological and technical aspects of the process. Still the process control has to be discovered in terms of disease stage, treatment method, and tissue type (Chevillet et al. 2014).

It is believed among the scientific community that universal endogenous control is hard to discover keeping all the steps, phases into consideration under different biological and technical condition of the samples. However, importance of mRNAs in personalized medicine treatment can't be ignored (Detassis et al. 2017).

14.6 High-Throughput Technologies to Assist Personalized Therapies in Breast Cancer

14.6.1 Genome Sequencing

High-throughput sequencing for genetic and genomic variation could be confined to a specific gene (targeted gene panels) may include all coding regions of the genome (whole exome sequencing) or can focus on all intronic and exonic regions of genome (whole genome sequencing). The decision for the use of any of these techniques is influenced by a variety of factors such as tumour testing in research vs. clinical, cost, the result required, and technological efficiency (Moorcraft et al. 2015). So far, WES and WGS have mostly been limited to the research sector, with the goal of accumulating massive amount of genetic data for translational research applications that will help us better understanding of cancer biology with time. Targeted gene panels, on the other hand, are preferable in clinical settings. This is because targeted gene panels provide greater depth of coverage in specific region of interest (e.g. hotspot regions with known actionable mutations), more clinically relevant data, and faster turnaround (Yip et al. 2019). These techniques have become an essential component of BC genetics. Now it is possible to sequence bulk of targeted genes involved in the susceptibility of BC at a cheaper cost as compared to testing *BRCA1/2* alone. Multi-gene panels, containing 6 to more than 100 genes, are available for detection of mutations related to cancer predisposition. BC gene panels are high in demand for women with an apparent BC predisposition (Catana et al. 2019). This testing is done to find out hereditary nature of BC and holds great health benefits for the patient. In this way, patients can be diagnosed at an early stage of BC and with personalized therapy the survival rate of patients will be increased. Furthermore, tumour genomic profiling for detecting somatic mutations is also becoming important for advanced BC patients and HER2 and PIK3CA are targetable mutations. It will be possible to use these multi-gene panel tests in clinical setting after the availability of clear guidelines (Cragun et al. 2017; Catana et al. 2019). At the same time, it is also feasible to perform WES and WGS to identify novel gene responsible for the predisposition of BC.

14.6.2 Transcriptome Sequencing

Over the last decades, transcriptome profiling emerged as one of the most powerful approaches in oncology, providing prognostic and predictive utility for cancer management. Transcriptome sequence is considered important as most humans

undergo a process of alternative splicing. In this process, two or more rearrangements like removal of introns and rejoining of exons may find in mRNA in different stages of cell growth. Different proteins are produced from one mRNA however, occurrence of errors at the level of alternate splicing may lead to many diseases like cancer and usually these defects are not detectable at the genomic level (Scotti and Swanson 2016). This technique is involved in determining the genetic code contained in the transcriptome and analysing function of cells across the wide range of biological conditions. Differential expression of genes can be measured via microarray analysis via complementary probe hybridization which helped in the discovery of many breast cancer-related genes.

Non-coding RNA is another important aspect of transcriptomic. With the advantage of gene expression analysis of non-coding genes and fusion genes, allelic imbalance, viral integrated genes, pseudogenes, post-transcriptional regulation, splicing and RNA editing also gets investigated. Moreover, advances in RNA sequencing (RNA-seq) technologies upgraded the knowledge regarding breast cancer. Using enormous mRNA sequencing, differential expression of many transcripts between TNBC and non-TNBC have been identified. mRNA typing revised the sub-classification of TNBC into distinct molecular subtypes with unique transcriptional features is helpful for therapeutic decision-making and prognostic prediction (Pan et al. 2008).

14.6.3 Proteomics

High-throughput mass spectrometry provides a draft of the human proteome. Proteomic analysis in cancer patients is a useful technique in biomarker monitoring and discovering the unknown molecules with high mass accuracy and sensitivity. Moreover, proteomic analysis also monitor the therapeutic effect, drug regimen activity, finding unknown aetiology, and providing treatment options for personalized medicine design (Wilhelm et al. 2014). For biomarker monitoring and identification proteomics analysis deals with two major categories, one is disease severity and other one is specific diagnosis of the disease with the limitations of number of analytes and sensitivity. Clinically, assays performed quantitate biomarkers and analyse single molecule at a time to rule in or out the disease. Mass spectrometry can quantitate several biomarkers from a single sample in a single test. Multiplexing the sample analysis will reduce the required time for patient diagnosis.

The challenge for new biomarker development makes use of the exquisite sensitivity of mass spectrometry for detecting biomarkers at the lower level. Also it may include post-translational modification, metabolites, metabolic flux, and identification of isoform from a complex mixture. Proteins also exist in multiple states either enzymatically cleaved or post-transnationally modified. Activation of protein in the disease state or while developing the disease state may find some precursor form of protein in relative abundance (Prakash et al. 2012). These analyses may also lead to the identification of novel biomarker. This untargeted approach for

novel biomarker identification may eliminate a priori research or systematic research before identification. This may lead to provide platform for developing personalized medicine treatment for BC patients (Zhang et al. 2013).

Proteomics is a vast field, and it also provides analysis of small molecule metabolites. The change of metabolite concentration in the samples, metabolic flux can signify any alterations in the functioning during disease state. Alterations in energy consumptions and the presence of onco-metabolites linked to cancer spread which is not easily detectable by conventional means. Furthermore, monitoring, activation, and clearance of therapeutics could also be helpful for oncologist in providing real-time functional information about the success of treatment administered to the patients. As tumours re-occurred usually, the analysis of tumour unresponsiveness to treatment would show no change. Protein kinetics, clearance, and rate of protein synthesis effect overall concentration of target proteins which are helpful determinants in determining dosing strategies (Zheng et al. 2014). One of the goals of personalized medicine is to specifically match the patient best course of therapy with best optimal outcome and minimal risks for each individual patient.

14.7 Challenges in the Personalized Era of Breast Cancer

Regardless of the advances made in targeted therapy, personalized treatment, however, does not help all patients, and oncologists still face numerous problems in implementing it (Dey et al. 2017). To date, some roadblocks have been discovered in the successful adoption of individualized treatment among BC patients. Variations in response among individuals to various cancer treatment regimens (radiation, chemotherapy, or surgery) as well as cancer heterogeneity (Smith et al. 2017) creates translational, analytical, and ethical issues (Lheureux et al. 2017). It has been suggested that ethnic differences in single nucleotide polymorphisms (SNPs) in specific cancer-related genes could be a factor in treatment response discrepancies (Alwi 2005). These differences cause difficulties in the development of personalized therapies, which require the selection of therapeutic agent and their dosages to be accurately suited to each individual. Due to the need to tailor therapies for multiple genes with some still unidentified SNPs, based on the modified treatment responses of various individuals caused by these SNPs, the formulation of the optimal personalized therapy for different individuals possessing various SNPs will be costly and time-consuming.

Combining genetic variant information on environmental and lifestyle-related risk variables might result in a more accurate risk assessment. Even though the evaluation of these risk variables using retrospective self-reporting and questionnaires is of poor reliability and vulnerable to evoke bias (Garcia-Closas et al. 2014), epigenetic modifications capture the interplay among noticed and unnoticed risk factors in each individual at the cellular level (Pashayan et al. 2016). Creating a personalized healthcare system is a multistep procedure that poses a variety of obstacles for policymakers as well as the people they serve. To provide equality of access to risk evaluation and interventions, training the

workforce, developing an infrastructure for monitoring the efficiency of tests and services, and designing IT platforms and data storage capacity are just a few of the organizational challenges that must be addressed. The absence of data from varied demographics and the limited evidence base available to guide clinical use are among the implementation hurdles (Korngiebel et al. 2017).

Ethical Challenges: The major ethical concern with personalized medicine is whether everyone around the world will be able to access and meet the expense of diagnostic tests and required treatments, or whether only the developed countries and rich people will be able to afford specific tests and medicines, thus increasing discrimination among certain groups. Another major concern is about privacy and confidentiality of data, for instance who will have access to personal information and what will be the potential consequences. Another problem of personalized treatment is the ethics of inadvertent results.

14.8 Future Perspective

The identification of new mutations and driving genes has clearly enabled the creation of novel targeted therapeutic techniques in combination with established chemotherapies for BC treatment. Different regulatory approaches will be required, as well as rejuvenated cooperative groupings, increased international collaboration, and realization that medicine must be available to all people suffering from the disease in order to be effective.

Personalized BC prevention and treatment will not be achieved by a single technique, but through combinatorial approaches that work together. Electronic medical records are being built in most hospitals and at physician clinics; the oncology workforce will evolve breakthrough technology to give individualized therapy at lower prices during the next decade. To improve the patient's care, it is possible that negative drug reactions will be recorded and shared with other doctors. Because we recognize that BC is a diverse set of diseases, novel-targeted therapy for various subtypes of BC will be available in the future decade. Patients who, based on their tumour profile and individual genotypes, are unlikely to respond to a specific dosage of a treatment may be spared unnecessary exposure to the agent once we understand why new medications respond differently. This is because we know that drug metabolism is heterogeneous, resulting in a wide range of pharmacological efficacy and toxicity. Drugs will be dosed more accurately as pharmacogenetics and pharmacodynamics become more understood and similarly, there is heterogeneity in cancer risk. Identification of high-risk individuals will be a critical component of cancer control, as will the implementation of cancer prevention strategies that will lower total mortality rate. Patients who require MRI screening will be given it based on their individual risk, not the risk of the entire community. Individualizing BC surveillance based on customized cancer risk assessments has proven to be a good strategy. The American Cancer Society's recommendations for high-risk BC patients screening which emphasize shared decision-making in weighing the risks and advantages of various screening technologies, particularly for younger women,

imply such a customized screening approach (Smith et al. 2006, 2008). By avoiding unnecessary toxicities and expediting early identification and prevention, these personalized techniques are likely to save money in the long run (Olopade et al. 2008).

14.9 Conclusion and Recommendations

While targeting the ER and the HER2 oncogene has resulted in considerable breakthroughs in the prevention and treatment of BC over the last decade, the options for women with triple-negative illness remain limited. Novel biomarkers will need to be identified for the targeted molecular therapy of less responsive subtypes of BC. Because of the availability of novel technologies such as microRNA profiling and genome-wide association studies, we are only now beginning to understand the role of host genetics in the optimization of therapy and resistance. MicroRNA technology is rapidly advancing, revealing new information about BC. MicroRNA interference may constitute a potential treatment option for specific subsets of BC in the future, given the accumulating evidence that microRNAs can be associated with either "oncogenic or antioncogenic" tumour-suppressive functions. A trans-disciplinary system biology approach is required to characterize novel susceptibility loci, understand the role of epigenetics in carcinogenesis and tumour progression, develop predictive and prognostic biomarkers, and identify novel therapeutic targets that are more efficacious and specific, to BC. Personalized strategies to BC prevention and screening are promising, but they will require international collaboration between multidisciplinary teams with expertise in omics, bioinformatics, epidemiology, public health, economics, decision analysis, ethics, law, risk communication, and scientific community engagement with healthcare professionals. To address the multiple scientific hurdles, collaborative research involving multinational consortia is required to overcome the various scientific challenges associated with personalized cancer medicine.

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Molecular Progression of Breast Cancer and Personalized Medicine in Terms of Clinical Trials

15

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Abstract

Breast cancer (BC) is well-known for its diversified clinical behaviors despite the similar histopathological characteristics at diagnosis. Research studies on molecular pathogenesis of BC reveal that it is a multitude of various diseases having variable molecular basis that regulate therapeutic responses, long-term survival, and disease-free intervals. Remarkable similarities between the molecular progression of BC and normal development suggest that BC might be caused by mammary cancer stem cells. Various signaling pathways such as the HER2, ER, and PR regulate mammary stem cells and normal breast development by controlling stem cell motility, differentiation, proliferation, and cell death. Recent studies suggest that non-coding RNAs and epigenetic regulations might play an important part in the metastasis and heterogeneity of BC specifically for triple-negative BC (TNBC). Traditionally used therapeutic strategies depend upon the expression levels of PR, ER, and HER2. Even though the methods used for clinical classification help choose targeted therapies, the prediction of patient responses and their long-term survival remains difficult. Recent advances in the field of molecular biology (multigene assays, next-generation sequencing) have led to innovations in BC diagnostics and therapeutics. Numerous multigene assays like Oncotype DX, MammaPrint, etc. have been developed for better prediction and prognosis at the early stages of BC. The concept of personalized

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medicine is gaining attention due to its potential role in developing effective BC treatment regimens. The recent identification of various molecular biomarkers via gene expression profiling might help in the prediction of drug response and intensity of cancer-related symptoms. The molecular biomarkers can also help in determining optimal drug choice/drug dosage and to identify more molecular targets leading towards the development of more personalized treatment strategies. For the practical implementation of personalized BC therapies, proper evaluation and analysis of molecular specifications of BC in each patient is needed. Moreover, the epigenetic and genetic changes should also be considered in management of BC patients. Finally, clinical trials are the link between chains of knowledge and determine the role of therapeutic advances. Out of the various clinical trial designs being used, adaptive clinical trials are most frequently used as they aim at reducing the resources, lessen the completion time, and improve the possibility of detecting the effects of treatments.

Keywords

Gene sequencing · Immunotherapy · Lymphangiogenesis · Prognosis · Tumor markers

15.1 Introduction

Breast cancer (BC) is a multifarious medical condition divided into various subcategories based upon certain cellular organizations, molecular transformations, and clinical behavior (Ferlay et al. 2015). The prognosis and the body's response to cancer treatment depend upon various factors, i.e., type and size of tumor, lymph node metastasis, histological grade, progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2). On a molecular basis, human BC is divided into four major categories, i.e., normal-like, luminal-like, basal-like, and HER-2 positive (Perou et al. 2000). Subsequently, a fifth class was added by the division of luminal class into luminal-A and luminal-B subclasses (Carey et al. 2006). The classification of molecular subtypes of BC can be done as follows: luminal-A (ER+/PR+/HER2−/lowKi-67); luminal-B (ER+/PR+/HER2−/+/high Ki-67); triple-negative breast cancers/TNBCs (ER−/PR−/HER2−), basal-like and HER2-enriched (ER−/PR−/HER2+) (Goldhirsch et al. 2011).

Luminal/hormone-regulated pathways and cell cycle-regulated pathways are used to distinguish the luminal subtypes of BC. In the case of luminal-B tumors, cell cycle-related genes, for example, Aurora kinase A (AURKA) and Marker of proliferation Ki-67 (MKI67) are highly expressed, whereas hormone-regulated genes, for example, forkhead-box A1 (FOXA1) and progesterone receptor (PR) have a lower expression (Prat et al. 2013). The estrogen receptor (ER) is similarly expressed in two luminal subtypes and is used to distinguish luminal and non-luminal disease (Prat et al. 2013). Comparison of luminal-A and luminal-B tumors at the DNA level shows that luminal-A tumors have a lesser mutation rate which means fewer

mutations in TP53-tumor protein (12% vs. 29%), lower amplification rate of cyclin D1 (CCND1), similar mutation rate for GATA3-GATA-binding protein 3 (14% vs. 15%) and high mutation rate for MAP3K1—Mitogen-activated protein kinase kinase kinase 1 (13% versus 5%) and PIK3CA-Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (45% vs. 29%) (Koboldt et al. 2012). Interestingly, a subtype of luminal-B tumors possesses hypermethylation, and a subtype of luminal-A (7.8%) and luminal-B (20.8%) tumors express higher rates of HER2-amplification (Hashmi et al. 2018).

The characterization of HER2-enriched subtype is done by the higher expression rates of cell cycle-related and HER-2-related proteins and genes, for example, HER2, erythroblastic oncogene B-2 (ERBB2), growth factor receptor-bound protein-7 (GRB7), intermediate expression rates of hormone-related genes and proteins, for example, progesterone receptor (PR), estrogen receptor-1 (ESR1), and lower expression rates of basal-related genes and proteins, for example, forkhead-box C1 (FOXC1) and keratin 5. A high mutation rate has been observed for HER-2-enriched tumors, i.e., 39% and 72% mutated PIK3CA and TP53. The HER2-enriched subtype is enriched for tumors having higher Apolipoprotein B mRNA-Editing Enzyme Catalytic Subunit 3B (APOBEC3B)-associated mutations frequency (Roberts et al. 2013). APOBEC3B is a subclass of APOBEC (Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide) cytidine deaminases, which converts cytosine into uracil and is a major mutation source in different cancer types (Kuong and Loeb 2013).

The characterization of basal-like subtype is done at the RNA and protein level by increased expression of cell cycle-related genes, for example, MKI67 and keratins mainly expressed by the basal skin layers, for example, keratins 5, 14, and 17, intermediate expression of HER2-related genes, and extremely low expression of hormone-related genes. Genomic analysis indicates the second highest mutation rate among which most of the mutations are due to hypermethylation and contain PIK3CA (9%) and TP53 (80%) mutations, respectively. Breast cancer due to mutated BRCA-1 gene causes basal-like disease (Prat et al. 2014; Foulkes et al. 2003).

In the case of triple-negative breast cancer (TNBC), the genes associated with PR, ER, or HER-2 are not expressed, and this is a major challenge as TNBC patients don't respond to endocrine therapies or other targeted agents. TNBC possesses similar metastatic potential as compared to other subtypes but TNBC tumors have a short median time to relapse and death (Hudis and Gianni 2011).

In the past few decades, significant substantial progress has increased our knowledge regarding the molecular basis of different cancer subtypes in addition to the recognition of novel therapeutic targets to facilitate the implementation of personalized medicine to treat different tumor types (Froeling et al. 2021). The technology for implementing molecular subtyping in clinical practice will help evaluate the patient-specific prognosis, relapse risk, and possibility of pathological response. The most important advantage would be to identify the cases in which the risks of neoadjuvant therapy are greater than the advantages (Al-Thoubaity 2020).

15.1.1 Molecular Alterations Defining Administration of Approved Targeted Agents

In estrogen-positive BC, estrogen targeting is the most commonly used method for the inhibition of the estrogen signaling pathway because the principal factors required for the progression of BC are the estrogen and estrogen receptors. The estrogen-dependent subtype of breast cancer can be treated by the suppression of tumor growth using selective estrogen receptor modulators (SERM). Tamoxifen was the first drug (approved) used for treating the estrogen-positive metastatic BC reducing the recurrence rate by almost 40–50% (Den Hollander et al. 2013). Aromatase inhibitors, i.e., exemestane, letrozole, and anastrozole are also used as a substitute for the treatment of estrogen-dependent BC because they inhibit the aromatase enzyme and block the androgen biosynthesis. This results in reduced levels of estrogen in tumor cells (Den Hollander et al. 2013). Various treatment strategies are used for the hormone-independent types of BC. The HER2 protein is the most overexpressed receptor marker in BC, and it is considered to be the related biosignature for BC treatment. Trastuzumab (Herceptin) was the first FDA-approved recombinant antibody to treat HER2-positive BC (Slamon et al. 2001; Pegram et al. 1998). Other targeted agents, i.e., lapatinib and pertuzumab have not proved to be effective in developing resistance mechanisms, and they have also shown numerous side effects (Swain et al. 2015; Maximiano et al. 2016; Moasser and Krop 2015). The HER2-positive BC can also be treated with the help of conjugated monoclonal antibody TDM1 (trastuzumab emtansine) because it effectively delivers the DM1 drug (microtubule inhibitor) into BC cells and inhibits growth. TNBC that lacks HER2 and hormone receptors might target HER1 and might respond to PARP1. The combination of cetuximab antibody with cisplatin chemotherapy in the Phase-II study indicated encouraging results. It might be due to the sensitivity of a few TNBC subtypes to epidermal growth factor receptor (EGFR) inhibition (Higgins and Baselga 2011). The conventional therapies, i.e., anthracycline and taxol derivatives are still used until the identification of further "druggable" targets (Higgins and Baselga 2011).

The approval of targeted therapies for the vascular endothelial growth factor (VEGF) has been carried out and bevacizumab in combination with docetaxel or paclitaxel has been effective in treating advanced metastatic BC (Kawalec et al. 2015; Miller 2005). Few therapies include the inhibition of pathways like PI3K/AKT/ mTOR and RAS/MEK/ERK. Furthermore, factors against tyrosine kinases, i.e., SRC, poly-ADP ribose polymerase (PARP) inhibitors, insulin-like-growth-factor [IGF/IGF-receptor (IGFR)], and matrix metalloproteases (MMPs) are required for tumor cell metastasis (Kalimutho et al. 2015; Wei and Lewis 2015; Wilks 2015; Dominguez-Brauer et al. 2015; Jamdade et al. 2015; Redmond et al. 2015; Munagala et al. 2011). The therapeutic resistance in BC cells is because of the alternative molecular pathways developed by the survival pathways including elevated levels of phosphatidyl-inositol-3-kinase (PI3K) signaling (Azim et al. 2016; Chamberlin et al. 2016; Weinberg 2016; Yang et al. 2016), receptor tyrosine kinase signaling outside

the HER/ErbB family and participation of some other HER receptors (Dey et al. 2015).

15.1.2 Manifestations of Targeted Therapies for Breast Cancer

Targeted therapies have been used to treat BC patients in which few specific proteins are overexpressed resulting in abnormal growth patterns. Antibodies are used as targeted therapy because their mode of action is like the human immune system. Until now, the targeted therapy for HER2 overexpression on the cell surface is the most effective for BC treatment. Out of all targeted therapies, seven BC-targeted therapies efficient at blocking various molecular pathways, are widely used, i.e., Bevacizumab or Avastin block the nutrient and oxygen supply to tumor cells by inhibiting the growth of blood vessels supplying the cancerous cells (Gianni et al. 2013; Chan et al. 2010); Trastuzumab or Herceptin make the cancer cells incapable of receiving the growth signals (Giordano et al. 2014; Denduluri et al. 2016); Everolimus or Afinitor inhibits the mTOR pathway and hence blocks the energy supply to cancerous cells (Linda et al. 2016; Nicolini et al. 2015; Lee et al. 2015; Johnston 2015) and T-DM1 or Kadcyla is the combination of Emtansine and Herceptin (Gan et al. 2014; Moasser and Krop 2015). Herceptin acts as a delivery method for carrying Emtansine towards tumor cells. Other therapies include Pertuzumab or Perjeta inhibit the cancer cells from responding to growth signals (Giordano et al. 2014; Baselga et al. 2012), and Lapatinib or Tykerb inhibit the HER2 protein resulting in blockage of cell growth signals (Kawalec et al. 2015; Clavarezza et al. 2016).

15.1.2.1 The HER2 Protein

Almost 10–12% of 2500 cases of BC are due to overexpression of HER-2 proto-oncogene which causes malignant transformation and reduced survival rate in tumors containing metastatic lymph nodes (Moore et al. 2014). The HER2/neu oncogene (erbB2) belongs to the EGFR family of tyrosine kinases and is located on chromosome 17 (17q12). It is the commonly overexpressed receptor in BC and is a potential therapeutic target (Denduluri et al. 2016; Asif et al. 2016; De Mattos-Arruda et al. 2016; Esparis-Ogando et al. 2016; Kanaya et al. 2017; Marchio et al. 2017; Sartore-Bianchi et al. 2016; Tsiambas et al. 2016). The EGFR family consists of four receptors, i.e., EGFR/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. The common domains of these receptors include leucine-rich repeats in the extracellular domain, intracellular domain with cysteine-rich repeats, a small juxtamembrane region, kinase region, a transmembrane spanning domain, and the cytoplasmic tail containing numerous tyrosine phosphorylation sites (Rimawi et al. 2015; Elster et al. 2015). The ligands bind to the extracellular domains of EGFR, HER3, and HER4 to produce kinase active homodimers and heterodimers that bind to HER2, a preferred partner. Six docking sites of PI3K are used by HER3 to activate PI3K/AKT signaling pathway. The survival of HER-2 producing cells depends upon the HER3/PI3K axis

because HER3 loss decreases the survival rate of BC cells that overexpress HER2 (Holbro et al. 2003a, b).

15.1.2.2 Trastuzumab Resistance Mechanisms

Trastuzumab or Herceptin was the first approved antibody to treat HER2-positive BC followed by lapatinib and pertuzumab. Trastuzumab inhibits the cytotoxicity and downstream signaling by uncoupling of HER2/HER3 heterodimers resulting from the attachment of trastuzumab with the juxtamembrane portion of HER2 receptor tyrosine kinase. The HER2 gene amplification and overexpression of protein/RNA results in the development of resistance mechanisms against trastuzumab. Despite bypassing the action of trastuzumab, the cancerous cells overexpressing HER2 continuously depend upon the HER2 oncogene. This might be due to receptor tyrosine kinase (RTK) signaling outside ERbB family, HER2 forms that are not recognized by trastuzumab, and elevated levels of PI3K signaling. The resistance against trastuzumab may be due to the modulation of p27 (Cdk-inhibitor) by IGF-1 because overexpressed IGF-1 activates the PI3K pathway and hence affects Akt (Ahmed et al. 2015; Arteaga and Engelman 2014). The suppression of miR-375 (tumor suppressor gene targeting IGF1R) expression plays an important role in trastuzumab resistance in HER2-positive BC (Ye et al. 2014). The complete blockage of IGF1R resulted in the restoration of trastuzumab sensitivity in HER2-positive BC cells (in vitro). The miR-375 loss and subsequent epigenetic alterations, i.e., histone deacetylation and DNA methylation might cause the overexpression of IGF1R resulting in trastuzumab resistance. For restoring trastuzumab sensitivity in HER2-positive BC cells, targeting the miR-375 gene might prove to be a possible therapeutic target (Ye et al. 2014). The newly developed trastuzumab DM1 (TDM1) has been used to treat HER2-positive BC as it is effective in the suppression of trastuzumab-resistant and sensitive HER2-positive BC cells (in vitro). TDM1 inhibits the microtubule assembly by driving mitotic and apoptotic catastrophe in Jimt-1 (trastuzumab-resistant BC cell line). The major characteristic of these cells includes the coexistence of trastuzumab resistance mechanisms, i.e., moderate HER2 receptor expression, low expression of PTEN, PIK3CA-mutated gene, and NRG1 overexpression. HER2 accumulation in lysosome-like organelles was observed in the JIMT-1 cell line which indicates the segregation of the protein in these specific intracellular granules (Barok et al. 2011). The integrin αvβ6 is essential for developing trastuzumab resistance as it promotes the survival, migration, and invasion of tumor cells. In the case of HER2-positive BC, the development of trastuzumab-resistant tumors can be slowed down by targeting αvβ6 with a 264RAD antibody (Moore et al. 2014). Various soluble factors secreted by the pre-adipocytes and adipocytes in adipose tissue located near BC cells are involved in developing BC cell resistance to trastuzumab moderated cytotoxicity. The resistance mechanisms in the following case are developed by inhibiting trastuzumab-induced tumor lysis by adipose tissue (in vivo) and by the natural killer cells (in vitro). An increased trastuzumab antitumor effect was observed in mice having distantly located tumors and lipoma, whereas the reduced antitumor effect was recorded for the mice having tumors located near lipoma. The hypoxic condition

of adipocytes enhanced the obstruction of antitumor activity indicating obesity as a factor in developing trastuzumab resistance mechanisms (Duong et al. 2015). The use of antibody therapy to target the HER family receptors might help to overcome cetuximab resistance mechanisms developed by tumor cells. The combined administration of U3-1287 (monoclonal antibody) and cetuximab against EGFR and HER3 indicated a significant reduction in proliferation of cetuximab-sensitive cancerous cells. This is because of the obstruction in AKT and MAPK pathways as well as the reduced signals from HER receptors (Iida et al. 2014). Various factors affecting the efficiency of trastuzumab to inhibit the multiplication of BC cells include the density of HER family receptors, presence of the ligands that activate HER receptors, i.e., heregulin-β1 and EGF (Hurrell and Outhoff 2013). An unpredicted resistance mechanism is found in association with the downstream mutations in tristetraprolin (TTP), an mRNA-binding protein. The TTP mRNA generated because of TTP gene germinal mutation is ineffectively translated in the form of protein. The reduction in TTP, as well as the increased concentration of TTP competitor HuR (embryonic lethal abnormal visual system-ELAV like protein 1), causes an increase in the half-life of mRNA-encoding genes, angiogenic and inflammatory factors. TTP mutation can be considered as a prognostic factor for trastuzumab resistance (Griseri et al. 2011). Therefore, TTP is usually considered to be the tumor suppressor gene in case of BC (Favre et al. 2010; Brennan et al. 2009; Griseri and Pagès 2014b). Similar kinases phosphorylate human antigen R (HuR) and TTP, but phosphorylation oppositely affects both proteins, i.e., TTP inactivation and HuR activation. Hence, the activated intracellular signaling pathways usually increase the concentration of proteins linked with oncogenic characteristics (Griseri and Pagès 2014a). The major side effect of trastuzumab therapy include the cardiovascular side effects because HER2 mutation analysis predicts cardiac toxic effects (Beauclair et al. 2007). Trastuzumab administration induces the risk of heart failure and cardiac dysfunction because of the HER2 expression in human myocardium. Before the prescription of an adjacent chemotherapy along with trastuzumab, one must consider the anthracycline-linked cardiotoxicity followed by the inhibition of erbB2/HER2 receptor to ensure patient's safety. Various trastuzumab-associated cardiotoxic side effects might be reversible whereas in few cases, the administration of monoclonal antibody followed by radiotherapy or chemotherapy may reduce the risk of cardiotoxic effects. It can be concluded that trastuzumab therapy is beneficial for the patients and should be administered as a standard treatment to inhibit erbB2/HER2 while minimizing its side effects (Beauclair et al. 2007).

15.1.2.3 Endocrine Therapy Resistance Mechanisms

In the case of hormone-sensitive breast cancers, resistance against hormone therapies is a major challenge despite the effective improvement in quality of life (QOL) by PR- and ER-targeted therapies. The endocrine resistance mechanisms are developed by several pathways, i.e., insulin receptors/IGF1, VEGF and FGF, HER tyrosine kinase receptor, AKT, SRC, and the stress-associated kinases play an important role in developing resistance against endocrine therapy during overexpression of their

ligands. The cross-linking between the hyperactivated PI3K pathway, GFR signaling, and ER is also linked to endocrine resistance (Di Leo et al. 2015). The post-translational modification of androgen receptors and nuclear receptors makes them alternative growth inducers enabling the diversion in ER inhibition. To circumvent the endocrine resistance, simultaneously targeting the HER2 and EGFR pathways are considered to be the most effective strategy because these two factors are essential in this specific resistance scenario (Osborne and Schiff 2011).

15.1.2.4 The mTOR Pathway

Being a master regulator of cell physiology, the mTOR pathway might play an important role in BC targeted therapy (Hatem et al. 2016). After the discovery of rapamycin (antifungal agent) in the 1970s, later studies proved that it could halt the growth of various eukaryotic cells due to its immunosuppressive function. Rapamycin associates with FKBP12 and the resulting complex binds to a protein known as mTOR (De et al. 2013). mTOR is a threonine/serine kinase that resembles the kinase region of the PI3 kinase and its associated enzymes. The mTOR pathway is an important element in the mammalian cell cycle because it integrates the incoming signals and mechanisms like protein synthesis and glucose import. It is also responsible for the phosphorylation of two kinases, i.e., 4E-BP1 and S6 kinase (S6KI) involved in translation (Ni et al. 2016; Rojo et al. 2007). The small 40s ribosomal subunit activation follows the S6KI activation, and this initiates the synthesis of proteins after association with the large subunit of ribosomes. mTOR being the controller of the AKT signaling pathway is essential for the proliferation and apoptosis. Inhibition of the mTOR complex causes the shutdown of the AKT signaling stream hence hyperactivating PI3K/PTEN expression loss (Chaffer and Weinberg 2015; Thomas et al. 2011). In 70% of cases of BC, the mTOR/PI3K/AKT pathways are overactivated and the protein kinases associated with these pathways might be the possible drug targets for BC treatment. Selective silencing of the mTOR/PI3K/AKT pathway might prove effective in treating patients who have developed resistance mechanisms against previously prescribed therapies.

The combined use of mTOR and other targeted therapies might prove effective to overcome the resistance mechanisms developed in various cancer patients. To recover the BC cells' sensitivity to conventional therapies and to overcome the resistance mechanisms, mTOR pathway inhibition by everolimus drug administration along with ER inhibitors or HER2 may prove to be a promising strategy (Grunt and Mariani 2013). The uncontrolled cancer cell proliferation is due to BRAF, PI3K, AKT, or EGFR mutations. It has been observed that BC cell sensitivity to conventional drugs can be increased by targeting the epithelial cell line of breast containing knocked-in mutations and by the use of mTOR and EGFR inhibitors (Glaysher et al. 2014). The combined administration of drugs, i.e., erlotinib/gefitinib and sirolimus/ZSTK474 also effectively blocks the EGFR and mTOR pathways (Glaysher et al. 2014).

15.1.2.5 RTK Inhibitors Resistance Mechanisms

Lapatinib is the ATP competitor that inhibits HER2/EGFR tyrosine kinase. It is usually administered along with paclitaxel to treat patients having HER2-positive metastatic BC. Unfortunately, the resistance mechanisms are thought to emerge after lapatinib therapy, specifically when AKT phosphorylation inhibition results in elevated levels of ER signaling and ER-α transcription. This resistance mechanism can be overcome by the administration of fulvestrant (ER-down regulator) that prevents lapatinib-resistant cells proliferation. Additionally, the HER2 protein mutations especially the insertion of YVMA at G776 in the exon 20 might cause de novo resistance to trastuzumab and lapatinib (Arteaga and Engelman 2014). The amplification of downstream signaling might bypass the inhibition effect of lapatinib and the activated HER3 upregulation reduces the inhibitory effect of tyrosine kinases. The HER3 upregulation causes PI3K/AKT pathway activation resulting in elevated levels of transcription factors (FoxO3A) that control neoplastic transformation, epithelial to mesenchymal transformation, and cell cycle (D'Amato et al. 2015). The use of antibodies to target HER3 is found to be effective in clinical and preclinical studies. However, with time, the tumor cells become resistant to the antibody because the antibody only blocks the signals rather than changing the HER3 expression. Novel techniques, i.e., EZN-3920 (antisense oligonucleotide) and entinostat (HDAC inhibitor) are aimed to reduce the HER3 levels (Ma et al. 2014). The hepatocyte growth factor receptor (HGFR)—c-Met tyrosine kinase might be essential in developing resistance mechanisms against targeted therapies specially trastuzumab and lapatinib. It has been observed in the preclinical studies that c-Met inhibition in gastric cancer circumvents the resistance mechanisms and restores growth inhibition (Chen et al. 2012). Poor prognosis, as well as aggressive phenotypes, have been observed in thyroid, colon, ovarian, esophageal, breast, and lung cancers due to AXL (receptor tyrosine kinase) overexpression. This might be responsible for the lapatinib acquired resistance in the in vitro preclinical BC studies. The activity of SRC tyrosine kinase also affects lapatinib resistance because SRC overexpression in BC cell lines causes increased EGFR interaction instead of HER2. EGFR inhibition with cetuximab and SRC inhibition with saracatinib results in restoration of sensitivity and death of lapatinib-resistant BC cells (Formisano et al. 2014). The administration of both cetuximab and lapatinib in the in vivo and in vitro conditions causes reduced HER2/EGFR signaling (Formisano et al. 2014). The elevated levels of receptor tyrosine kinase-ligand might be due to the production of autocrine tumor cells and neuregulin-1 (HER3 ligand) induces complete lapatinib resistance (Wilson et al. 2012). The crosstalk between HER2 and ER pathway develops another lapatinib resistance mechanism. The ER upregulation induced by lapatinib due to PI3K/AKT pathway inhibition results in elevated expression levels of Bcl-2 (anti-apoptotic protein) leads to the development of resistance against lapatinib and escape from cell death (Giuliano et al. 2015).

15.1.2.6 The VEGF and VEGF Resistance Mechanisms

Tumor angiogenesis is mainly modulated by the VEGF as well as its surface receptors. VEGF receptor kinase inhibitors along with bevacizumab or Avastin

(anti-VEGF antibody) has an essential role in the anti-angiogenic treatment of cancer (Miller et al. 2007). The VEGF ligand has a homodimeric structure consisting of monomers made up of β-strands that are balanced by disulfide knot as well as a pair of symmetrically disposed bridges that link the monomers together. Seven immunoglobulin resembling structures (Ig domain) are present on the extracellular domain of VEGF receptors (VEGF-1,2,3) (Shibuya and Claesson-Welsh 2006; Ferrara et al. 2003). The placenta growth factor and the four members of the VEGF family attach to the three endothelial cell tyrosine kinase receptors each having a different function. VEGFR1 promotes vascular system maintenance and differentiation, VEGFR2 induces vascular permeability and endothelial cell multiplication and VEGFR3 stimulates lymphangiogenesis. Neuropilin-1 and neuropilin-2 might attach to the class 3 semaphorins responsible for axonal growth as well as too few VEGF1 isoforms in the form of co-receptors, and this causes additional binding of VEGF to VEGFR2 (Djordjevic and Driscoll 2013). Endothelial cell multiplication increases vascular permeability, survival as well as tubular formation are the cellular effects resulting from the modulation of receptor sites as a result of heparin activation. The VEGFR usually has an endothelial origin, but they might also be present in the tumor cells and in stroma as macrophages. The hypoxia-inducible factor (HIF) plays a significant role in gene transcription, i.e., VEGF under hypoxic conditions. Under normoxic conditions, ubiquitylation results in the degradation of α-subunit of HIF heterodimer (α,β). This proteasome-dependent degradation occurs by the formation of the E3-ubiquitin ligase complex resulting from the binding of HIF-α to p-VHL (von Hippel-Lindau tumor suppressor protein). Under hypoxic conditions, the heterodimerization of hypoxia response elements (HRE) and HIF-beta with HIF-α subunit results in the stabilization of the HIF-α subunit. The stabilization of the HIF-α subunit results in the activation of essential components of the HIF target genes, i.e., VEGF, genes regulating cell multiplication and metabolism (Kaelin and Ratcliffe 2008; Mole et al. 2001). The upregulation of VEGF occurs in hypoxic microenvironments displaying specific vascular phenotypes and elevated levels of VEGF expression is a commonly known prognostic factor for human BC. This depicts VEGF as a major therapeutic target. Recent data indicates that the combined administration of bevacizumab (anti-angiogenic) and chemotherapy agent, i.e., paclitaxel is highly unfavorable due to the side effects such as neutropenia, GIT perforations, hemorrhage, arteries blockage and stroke (Ranpura et al. 2011).

15.1.2.6.1 VEGF Resistance Mechanisms

Various mechanisms are involved in developing resistance against anti-angiogenic therapy. The most important mechanism is related to the cancer cell promiscuity due to which it produces different types of angiogenic signals limiting the drug efficacy. The cancer cells might not respond to anti-angiogenic drugs due to the amplification of angiogenic genes, diverting to vessel co-option from vessel sprouting, vasculogenesis to secure nutrient supply. The pro-angiogenic factors, i.e., ephrins, angiopoietin, or fibroblast growth factor might be secreted in response to bone marrow-derived cell recruitment by cancer cells. A cytokine cascade might be induced by VEGF receptors resulting in an inflamed microenvironment favorable

for tumor metastasis and extravasation. Various alternative targets to control the resistance mechanisms against anti-angiogenic therapies might include targeting Bv8 (*Bombina variegata*) and placental growth factors for reducing tumor inflammation, moderating hypoxia, reducing vessel leakiness, and reducing angiogenesis. Other targets include the Notch pathway associated with anti-delta-like ligands 4 (DII4) and the secretase inhibitors for reducing excessive sprouting of vessels and leaky vessels. Vessel normalization can be done by the inhibition of PHD2 to improve vessel function, reducing hypoxia and metastasis. Lymphangiogenesis might be treated by neuropilin-2 (Npn-2) inhibition while angiogenesis and tumor growth can be reduced by targeting neuropilin-1 (Loges et al. 2010). The development of resistance against anti-angiogenic therapy might induce some other pathways through acquired resistance or intrinsic tumor resistance. It includes angiogenic redundancy that produces pro-angiogenic factors, i.e., tumor necrosis factor-α (TNF-α), fibroblast growth factors (FGFs), placenta growth factor (PGF), and platelet-derived growth factors (PDGFs). Synergistic targeting of the pro-angiogenic factors is appropriate as they allow tumor vasculature growth despite the inhibited VEGF pathway. The increased tumor hypoxia resulting from anti-angiogenic therapy is involved in angiogenic repetition. HIF-1 overexpression correlates with aggressive cancer cell selection and chemotherapy resistance because it directly induces the transcription of angiogenesis-related genes. The HGF (hepatocyte growth factor) activates the c-MET (tyrosine kinase receptor) during angiogenesis. This results in the downstream activation of AKT, STAT3, SRC, and MEK with increased VEGF expression by the endothelial cells. The collaboration of c-MET/HGF results in invasive cancer phenotypes and enhanced metastasis. More invasive tumor cells might be selected because hypoxic conditions force the cancer cells to move towards normoxic conditions. The adaptive resistance mechanisms might be due to inflammatory cell invasion and bone marrow-derived pro-angiogenic cells because hypoxic conditions induce the cells to release high quantity of pro-angiogenic factors. The resistance mechanism against anti-angiogenesis therapy resulting from the crosstalk b/w angiogenic pathways might be due to changes in endothelial cells and pericytes. This results in the simultaneous VEGF pathway inhibition and the inhibition of PDGF receptor by tyrosine kinase inhibitor, providing an effective strategy to increase treatment efficacy. Vessel co-option might produce cancer cells with a normal vasculature that has low sensitivity to anti-angiogenic therapy. The early-stage tumors grown in angiogenesis-independent manner might escape inhibition (Giuliano and Pagès 2013). The prospects of anti-angiogenic therapy seemingly depend upon the vascularization of different tumors as well as the pathways by which they overcome the therapeutic effects. Clarification of the complex biology of angiogenesis along with the knowledge regarding the functions of key biomarkers might prove effective in enhancing the advantages of anti-angiogenic therapy for achieving vascular normalization and enhancing chemotherapy effects.

15.1.2.7 TNBC and PARP Inhibitors

TNBC cases account for 10–20% of the total invasive BCs in general population, and it prevails in the American-African ethnic group where 28% of the patients are affected by this disease (Boyle 2012). In almost 80% of the breast tumors lacking HER2 protein overexpression, the ER and PR are categorized as TNBC. Their categorization might be done by the increased concentration of PARP enzymes, and they frequently develop from basal-like cells. TNBC is the most aggressive type of BC and targeted therapies for its treatment are not available. Claudin-low subtype accounts for 12% of TNBC cases, and it is identified by using DNA microarray expression profiling.

TNBC tumors seemingly react to molecules targeting the repair systems of DNA for inducing lethality when administered with other drugs. The PARP inhibitors are a curative choice if one gene in the synthetic lethal pair is already dysfunctional, causing cell death. PARP-isoenzymes are a group consisting of 18 molecules that play a central role in the base excision repair pathways of the single-stranded DNA fragments. For example, BRCA1–2 mutation in BC allows the targeting and blockage of the DNA repair system by the PARP inhibitors resulting in selective death of tumor cells (Banerjee and Kaye 2013). In the case of TNBC, resistance against chemotherapy is developed by nuclear basic fibroblast growth factor (bFGF) protein found in the TNBC subset (Li et al. 2015). The residual TNBC subpopulation remains alive following short-term chemotherapy and proliferation resumes over time. The number of TNBC cells reduces after knocking down bFGF in the residual cancer cells. This phenomenon is associated with the suppression of DNA-dependent protein kinase (DNA-PK) that accelerates the process of DNA repair. It might be concluded from the study that bFGF expression in TNBC cells can be possibly used to predict incomplete chemotherapy and tumor recurrence in TNBC patients (Li et al. 2015). The survival rate of TNBC patients is significantly lowered by the major challenges, i.e., development of substituent resistance mechanisms and circumventing the resistance mechanisms induced by treatment because they frequently show incomplete pathological response (Grunt and Mariani 2013). Sunitinib seemingly suppresses tumor proliferation, angiogenesis, growth, and migration of basal-like BC cells. Xenograft models show a decline in tumor volume due to sunitinib action, but an increased level of BC stem cell multiplication was observed because of its effects on hypoxia and Notch-1 protein expression through HIF-1. The combined administration of γ-secretase inhibitor with sunitinib might prove to be effective in TNBC treatment while targeting angiogenesis and cancer stem cells (Chinchar et al. 2014). In the case of TNBC, VEGF might be expressed at elevated levels, and this might be the reason for considering Sunitinib as an effective treatment choice. High VEGF levels might be a prognostic factor in the TNBC subtype because the vascular pathway is an important component while targeting this rare BC subtype (Linderholm et al. 2009). Targeted therapies for TNBC treatment have not been discovered yet, so conventional chemotherapy by drugs like taxane and anthracycline can be used. There is a need to investigate the multiple pathways responsible for the progression of TNBC for the isolation of specific therapeutic targets. BRCA1 and BRCA2 mutations are found in 20% of

TNBC cases, and these patients might be sensitive to the combined administration of PARP inhibitors and chemotherapy (Zanardi et al. 2015). A clinical trial (Phase-II) designed to evaluate the effect of combined administration of iniparib (PARP inhibitor) along with gemcitabine and cisplatin showed that iniparib significantly enhanced the cytotoxic effect of gemcitabine and cisplatin in addition to enhancing the antiproliferative effect (O'Shaughnessy et al. 2011).

15.1.2.8 Immunotherapy for BC

BC was considered to be non-immunogenic until recently, data suggests that HER2- and TNBC-positive subtypes have an immune infiltrate that might be an effective target for complementing the role of synergistic drugs. Sipuleucel-T and ipilimumab have successfully been administered as a vaccine against castration-resistant prostate cancer. Immunotherapy aims to activate the human immune response for the recognition of the tumor as an antigen eventually killing the tumor cells. The TME (tumor microenvironment) consisting of T-Reg (tumor regulatory) cells has a complex intercellular communication, and this represents a promising research area aiming to isolate the immunogenic targets that might enhance the effectiveness of existing therapies (Mittendorf and Peoples 2016). PD-1 receptor (immune-checkpoint) is expressed upon TILs (tumor-infiltrating lymphocytes) having the role to inhibit the effector T-cells activity resulting in the prevention of inflammatory and autoimmune response. The receptor is upregulated by the attachment of PDL-1 (PD-1 ligand) with T-cells. The immune response is suppressed by the inhibition of kinases involved in activating the immune response (Mittendorf et al. 2014). The clinical blockage of the PDL1/PD-1 axis should enhance the function of antibodies highlighting the significance of further research in this specific area of BC research. The inhibitory ligand PDL1 overexpression and the pro-inflammatory cytokines are essential in developing cancer immune resistance mechanisms. This results in a tolerant or exhausted immune T-cell response highlighting the importance of further research on PDL1 as resistance biomarker (Ramsay 2013). Nivolumab (anti-PD-1 antibody) has successfully targeted the immunoregulatory proteins resulting in enhanced tumor response in melanoma as well as in lung cancer patients. Several other potential targets, i.e., B7-H3, B7-H4, B7-H6, CD80, CD86, ICOS-L, and PDL2 have also been investigated. The prospects in BC immunotherapy highlight the significance of combined checkpoint blockage for maximizing the clinical response (Callahan et al. 2015).

15.2 Gene Expression Profiling for BC

Technological advances in the past few years have enabled researchers to examine the simultaneous expression of various genes. Multiple markers can be rapidly analyzed with the help of these methods. Another advantage of these methods is the recognition of gene expression patterns, and they might serve as the tumor markers. Various methods to observe several hundred to thousand gene messages have also been developed. Gene expression profiling aims to provide a preferable

prediction of the clinical outcome as compared to the traditional pathological and clinical parameters. This tool aids the clinicians to estimate the outcome of local treatment and expected advantages from systemic adjuvant chemotherapy and endocrine therapy. The characterization of low-risk patients that do not require adjuvant tailoring therapy and chemotherapy regarding RNA transcripts synthesized by the tumor cells remains a challenge. Tumor gene expression profiling appears to be an important tool for providing further information related to the behavior and biology of BC.

15.2.1 Methods

15.2.1.1 Oncotype DX

Oncotype DX is a quantitative reverse PCR-based method that examines the expression of 21 genes (5 reference genes and 16 cancer-related genes). The formalin-fixed paraffin-embedded tissues are used for RNA extraction. The Oncotype assay is prescribed to the patients having non-overexpressed HER2 axillary lymph node-negative BC at an early stage and hormone receptor-positive BC. The requirements of this test include ER assessment and the use of alternative methods to assess HER2 status (Paik et al. 2004). Oncotype DX is the most commonly used gene expression assay in the United States (Fisher et al. 1997). The validation of Oncotype DX has been performed on a large cohort of patients with ER-positive node-negative BC, enrolled in NSABP B-14 clinical study after treatment with tamoxifen. The study indicated recurrence rates at 10 years for the low-risk group (6.8%), intermediate group (14.3%), and high-risk group (30.5%), respectively (Paik et al. 2004). The recurrence score (RS) ranges from 0 to 100 and it measures the risk of disease relapse within 10 years. RS is the independent prognostic factor for the patients having ER-positive node-negative BC treated with adjuvant tamoxifen. The study indicated that the low-risk group of patients seemingly do not benefit from the adjuvant chemotherapy whereas, the patients in the high-risk group received a benefit from the adjuvant chemotherapy (Habel et al. 2006).

15.2.1.2 MammaPrint

MammaPrint is an RNA gene expression profiling assay based upon the microarray technique. The test comprises DNA extraction from a freshly frozen sample of human tumor tissue and the identification of 70 genes from an initially unselected set of more than 25,000 genes (Van De Vijver et al. 2002). Various study trials have indicated that the test can be performed by RT-qPCR in formalin-fixed paraffin-embedded tissue and freshly frozen tissues with similar results (Espinosa et al. 2005). The genes are linked to all the distinctive features of cancer, i.e., angiogenesis, proliferation, and invasion (Van De Vijver et al. 2002). The validation of this signature has been carried out on various groups of node-negative patients, and it has provided independent prognostic information apart from the standard variables, i.e., pathological stage, age, and histological grade (Van De Vijver et al. 2002; Buyse et al. 2006; Cardoso et al. 2008).

MammaPrint is the gene expression assay test for patients having node-negative BC, and it categorizes the patients into two groups, i.e., low-risk group and high-risk group. After adjustment for the lymph nose status, MammaPrint can be used to predict the overall survival as well as distant disease-free survival. The low-risk group patients possess a distant metastasis-free survival rate of 90% without any additional systemic chemotherapy. The test can also be used to predict the effect of chemotherapy on different groups, i.e., patients in high-risk groups get an advantage from the adjuvant chemotherapy (Van De Vijver et al. 2002). In a retrospective study, patients having the locally advanced disease were sampled and the low-risk group patients did not show complete pathological response to the neoadjuvant chemotherapy (Straver et al. 2010). In another research study, the combination of BluePrint and MammaPrint resulted in the identification of four molecular subtypes including the HER2 type, basal type, luminal-A (Luminal type/MammaPrint low-risk), and luminal-B (Luminal type/MammaPrint high-risk). Luminal-A patients (MammaPrint low-risk/BluePrint luminal) have an excellent survival with a good baseline prognosis and may not get an advantage from chemotherapy. BluePrint categorizes more patients into basal type ($n = 120$) category with 42% PCR rate as compared to clinical subtyping ($n = 93$) with 31% PCR rate. Molecular subtype identification improves patient stratification in the neoadjuvant setting. It can be concluded that MammaPrint establishes a clinical correlation between treatment outcomes and molecular subtyping (Gluck et al. 2012).

15.2.1.3 Mapquant DX Genomic Grade Index

Mapquant DX is a predictive and prognostic signature established to stratify the tumors based on histologic grade, therefore, diminishing interobserver variability. This test differentiates grade 1 tumors from grade 3 tumors and also subdivides grade 2 tumors into two subclasses, i.e., low vs. high recurrence risk. Mapquant DX demotes 70% of tumors in grade 2 to grade 1 (Sotiriou et al. 2006). The test composes GGI (genomic grade index) with the help of 97 genes that are responsible for cell proliferation and regulation of the cell cycle. Adjuvant chemotherapy is recommended for patients in high-risk groups. For the patients receiving anthracycline and taxane chemotherapy, elevated levels of GGI are associated with an effective response to the chemotherapy (Sotiriou et al. 2006). The prognostic information obtained from GGI can only be applied to ER-positive tumors. The initially required tumor tissue for Mapquant DX is in freshly frozen form just like MammaPrint, but another RT-qPCR-based version has also been developed (Toussaint et al. 2009).

15.2.1.4 Rotterdam Signature

The development of the Rotterdam 76 gene biomarker was performed based upon the microarray data analysis of 115 BC patients. The evaluation of ER-negative and ER-positive tumors was done separately, resulting in the identification of 16 genes in ER-negative tumors and 60 genes in ER-positive tumors. This prognostic biomarker can facilitate the prediction of distant metastasis development within 5 years in the lymph node-negative BC patients who have not received systemic chemotherapy,

without considering the tumor size and age of the patient (Wang et al. 2005). The 76 gene signature proved as effective prognostic factor in 198 patients, and it outperformed the NCI and St Gallen's guidelines for the identification of patients with good prognoses, not requiring adjuvant chemotherapy. The median research duration was 14 years showing distant metastasis n 51 patients (26%) out of which, 35 patients (18%) showed disease progression in 5 years. The results of the Adjuvant! Online software indicated that 152 (77%) patients were considered at high risk, whereas 46 (23%) patients were in the low-risk category. High genomic risk (72%) and low genomic risk (28%) were observed by the 76 gene signature. Interestingly, the low genomic risk group contained 21.9% (14 of 64) of all ER-negative patients, whereas the clinical low-risk group did not contain any. The chances of distant metastasis of 5 and 10 years were 98% and 94% for the low genomic risk group, whereas 76% and 75% for the high genomic risk group (Desmedt et al. 2007).

15.2.1.5 PAM50

A neoadjuvant research study on 157 early-stage BC patients has shown a low pCR (pathologic complete response) and npCR in the tumors with luminal signature. The basal subtype and HER2-enriched tumors specifically having p53 mutation show a high pCR rate after four rounds of preoperative chemotherapy (Glück et al. 2012). PAM50 (Prediction Analysis of Microarray) was proposed in 2009 to standardize BC subtype classification using a set of 50 genes (Parker et al. 2009). PAM50 is the clinical representation of this gene set by using a qRT-PCR assay. The validation of the qRT-PCR assay has been done on formalin-fixed paraffin-embedded tissues. The test measures the expression rates of 5 control genes and 50 classifier genes for the identification of intrinsic BC subtypes known as basal-like, HER2 enriched, luminal-A and luminal-B. Multivariate analysis using the clinical data (ER status, node status, grade) and PAM50 subtypes have shown PAM50 as an independent prognostic survival test in the case of BC (Nielsen et al. 2010). The additional information provided by the PAM50 test includes tumor biology as well as the quantitative data regarding the biomarkers used for treatment decisions until now. In addition to the categorical classification of BC subtypes, the PAM50 test provides quantitative figures for luminal gene expression, ERBB2, PGR, proliferation, and ESR1. Luminal-A tumors generally possess normal to high ESR1 expression, ER-related genes and rarely show high expression of ERBB2. Luminal-B tumors generally possess normal to high ESR1 expression, ER genes and frequently show higher proliferation rate as compared to luminal-A tumors. HER2-enriched tumors possess normal-to-high ERBB2 gene expression, normal-to-low ESR1 expression and ER genes. Basal-like tumors normally possess low expression of PGR, ERBB2, ESR1, and ER genes, whereas high proliferation rate has been observed.

15.3 Improvements via Next-Generation Sequencing

Rapid developments in the field of medicine have resulted in advances in the study of diseases not only at the individual, cell, or even molecular level. After the successful completion of the human genome project, more people have become concerned with the study of genes and the genetic basis of diseases. Various researchers have been associated with the study of genes and diseases, achieving good results for complex diseases (diabetes, cancer, autoimmune diseases, etc.), and some commonly observed abnormal indexes (weight, blood lipids, blood glucose, etc.). These research studies have resulted in the discovery of a large number of gene loci linked to disease pathogenesis and have brought novel ideas for the treatment and diagnosis of these diseases. The outcomes of genome-based research in the area of cancer excite the cancer researchers and in the past decade, a significant number of genetic research on cancers of various types have been performed (lung cancer, liver cancer, prostate cancer, breast cancer, etc.)

Sanger sequencing method is the traditionally used method for gene sequencing, and it includes the double end termination method that was used as a standard method for the detection of genes. The disadvantages of the Sanger sequencing method include deviation from the target, high cost, and low throughput but the next-generation sequencing (NGS) method has overcome the disadvantages of the conventional method (Moorcraft et al. 2015). The next-generation sequencing method is similar to deep sequencing and high throughput sequencing. As compared to the conventional Sanger sequencing method, the advantages of NGS include high accuracy, high speed, and high throughput (Fan et al. 2015). The higher sequencing and diagnostic sensitivity of next-generation sequencing have been confirmed in a research study where NGS in detecting BRCA1/2 gene mutation is more reliable as compared to the conventional Sanger sequencing method (D'Argenio et al. 2014, 2015). Hereditary susceptibility has been observed in two leading BC genes, i.e., BRCA1 and BRCA2. A new mutation in BRCA2: c.8946_8947delAG (p. D2983FfsX34) has been identified in a Chinese woman (Ma et al. 2017). While considering the molecular diagnostic rate, exome sequencing of NGS is at a higher rate as compared to the Sanger single gene sequencing, microarray analysis, chromosome analysis, and other conventional methods (Ahmadloo et al. 2017). The next-generation sequencing method no longer requires the in vivo amplification of *Escherichia coli*, but it employs the in vitro sequencing directly by polymerase and ligase. The major technologies used in next-generation sequencing include Illumina/Solexa technology, Helicos technology, ABI/SOLID technology, and Roche/LS454 technology (Geskin et al. 2015). The Illumina/Solexa technology is frequently used in GWAS and exon sequencing and its advantages include simple operation, minute deviation in homopolymer detection, and low cost. GWAS involves genome-wide association studies to identify sequence variations linked to diseases in a wide range of human whole-genome SNPs (single nucleotide polymorphisms). Exon sequencing involves sequencing of a large number of exons to identify the disease-related areas and to verify the SNPs.

15.3.1 Applications of NGS in BC Research

In the past decade, the next-generation technology has been used by various researchers for the interpretation of BC genome diversity hence, revealing the genomic landscape of BC. The applications of NGS in BC research mainly cover three aspects, i.e., RNA transcription group sequencing (small RNA sequencing, whole transcriptome analysis, and non-coding RNA analysis), genomic DNA analysis (targeting gene sequencing, whole-genome sequencing, exon sequencing), and epigenetic sequencing (methylation analysis sequencing, chromatin immunoprecipitation sequencing). NGS plays a significant role to study extranuclear heredity and RNA transcription groups, for instance, studies have shown that particular miRNA anomalies are linked to specific BC types, i.e., miRNA linked to invasive papillary carcinoma (Li et al. 2012). Various types of miRNAs, i.e., miR-9, miR10b, miR31, miR126, and miR335 are found to be linked with BC metastasis, whereas distinctive miRNA expression has not been observed in TNBC (Farazi et al. 2011).

15.3.1.1 Genome-Wide Association Studies (GWAS) and Risk Prediction in BC

Genome-wide association studies (GWAS) is a very important tool to study human genes, approximately 2000 gene loci or SNPs have been identified by this method. Besides the advantages of next-generation sequencing, low-frequency mutation, and fewer sample requirements are the advantages of whole-genome association studies. Various epidemiologic studies have resulted in the identification of lifestyle and environmental risk factors, i.e., body mass index (BMI), menarche and menopause age, exogenous hormone use, and age at first birth. In addition to these risk factors, BC is closely linked to the risk gene (Lin et al. 2014). The association of more than 90 risk loci with BC has been confirmed and since 2007, large-scale GWAS studies have reported five more risk loci linked with BC. According to a prediction, more than 1000 risk loci might be linked to BC, and these genes have not been discovered yet (Ghoussaini et al. 2013). A whole-genome association study of the female BC in African ancestry has confirmed the close association of two gene loci (rs10510333 at 3p26; rs4322600 at 14q31) with BC occurrence in African women (Chen et al. 2013). The GWAS study of BC in East Asia resulted in the identification of three gene loci linked with BC in Asian women (Cai et al. 2014). Even though the identification of a single SNP by GWAS has a minute impact on the BC mechanism, it should not be ignored because multiple SNP superpositions can be used for BC risk prediction. Another research study identified a close correlation between 4 SNP of intron 2 of tyrosine kinase receptor (FGFR2) and BC occurrence (Hunter et al. 2007). BCs associated with BRCA1 have a high proliferation rate and are TP53 mutated, lacking ERBB2 and ESR1 expression. Moreover, various new mutations have also been identified by NGS in patients with familial ovarian/breast cancer. In addition to BRCA1/2, PALB2 (partner and localizer of BRCA2) is found to be the second most commonly mutated gene (Kraus et al. 2017). Several genes have been found to be linked with BC; however, BC incidence is due to the combined effect of multiple factors. The ratio of risk genes in cancer causing factors is low and needs

further exploration. Prominent differences in the non-coding and coding RNA profiles in MDAMB-231 and MCF-7 cell lines have been observed in a research study. Most differential expression among the ncRNAs and mRNAs, i.e., SOD2, SLP1, miR-7, miR-143, and miR-145 have a high expression level in MCF-7 cells, whereas KRT17, CD55, miR-9, miR-10b, miR-21, PICSAR, and NEAT1 were highly expressed in MDA-MB-231 cells. mRNAs having differential expression are involved in the process of biological adhesion, focal adhesion, locomotion, and ECM–receptor interaction pathway. The miRNAs and mRNAs associated with the differentially expressed RNAs are linked to tumor metastasis, but the role of ncRNAs is still uncharacterized (Shi et al. 2017).

15.3.1.2 Whole-Genome Exon Sequencing and BC Research

A large number of non-silent mutations (85%) occur in the proteins encoding region of the exon that accounts for only 1% of the human genome (Kaur et al. 2013). Therefore, whole-genome exon sequencing is found to be significant in detecting the gene and risk prediction for malignant tumors. Recent research on BC have used whole-genome exon sequencing, and it has promoted the genomic studies of BC. Earlier studies have revealed a risk ratio of 50–80% in women that carry BRCA1 or BRCA2 mutations (Pan et al. 2014). It has been found by exon sequencing that the rarely found male BC risk was closely associated with BRCA2 gene deletion (de Souza Timoteo et al. 2015). Even though the BC patients are evenly distributed in a population, 15% of these patients are found as family aggregation. The whole-genome exon sequencing has resulted in the identification of BLM and FANCC as the susceptibility alleles (DNA repair gene) of BC (Thompson et al. 2012). The whole-genome exon sequencing has also been used in the identification of the FANCM gene to be the susceptible gene locus in the case of TNBC (Kiiski et al. 2014). A research study on familial BC with negative BRCA has found that seven mutations (TH, XCR1, SRL, ACCS, CCNF, DLL1, and SPPL3) might be linked with potential BC (Noh et al. 2015). Another research study using whole-genome sequencing and exon sequencing has found that SF3B1 mutation has proved to be another target of anticancer therapy (Maguire et al. 2015). In addition to the enrichment of BC genes research, the discovery of new mutations also brought novel ideas for the treatment and diagnosis of BC. Exon sequencing can be widely used by researchers to study multiple aspects of BC patients, i.e., genetic susceptibility, stem cell differentiation, and so on.

15.3.1.3 Targeted Sequencing and BC Research

Targeted sequencing targets specific genomic regions or genes for the targeted sequencing analysis. In BC studies, some other gene loci sequencing research have been carried out to identify the loci that have already been found. For instance, MED 12 gene is linked to BC. The comparison of targeted sequencing to identify the mutation volume between phyllodes tumors and mammary gland fibroma has shown a higher mutation volume in phyllodes tumors as compared to mammary gland fibroma (Mishima et al. 2015). It has also been reported that harmful mutation genes in BC and ovarian cancer can be easily detected via targeted sequencing (Rajkumar

et al. 2015). Concurrently, BC targeted therapy can also be improved with the help of targeted sequencing. It can be said that after exon sequencing and whole-genome sequencing, targeted sequencing is a very important supplement to BC research.

15.4 Clinical Utility of Molecular Profiling

Molecular profiling is the method for comparing various tissue types at the molecular level, i.e., mRNA, DNA, proteins, etc., on a global scale. The clinical utility of this field has been hindered due to difficulty in final data interpretation as various methods have been designed to achieve this goal. Until now, a number of research studies have focused on the interpretation and manipulation of cDNA arrays. cDNAs are produced by the conversion of mRNAs from various tissue types into cDNAs. The quantitation of these cDNAs is done by fixing them onto a solid substrate. The researchers can interpret the biology of different tissue types by evaluating the comparative expression of cDNA from each tissue.

Molecular profiling of BC has been used to address three main issues: (1) comparing the biology of breast tumors with normal tissue as well as with other breast tumors. (2) Accurate prediction of the clinical outcome for similar types of tumors provides a chance for better-informed decisions. (3) Accurate prediction of the tumor response to specific treatment type for improving the benefit/risk ratio for the treatment of each patient.

Valuable data regarding the transcriptomic, proteomic, genomic, and epigenomic aspects of different cancer types have been reported by The Cancer Genome Atlas (TCGA) project (Cancer Genome Atlas Research Network 2008, 2011, 2012, 2013; Cancer Genome Atlas Network 2012; Levine 2013). The data collected by large-scale molecular profiling might provide valuable information regarding various aspects of oncology practice. Accurate prognosis is the key application for patients at an early stage of the disease. It helps to categorize the patients into various risk groups that help them choose accurate surveillance and treatment strategies. Tumor stage and age are the clinical variables that affect the prognosis. Recently, molecular information has been incorporated for a better prognosis. For instance, HER2 protein levels, PR, ER, and HER2 gene amplification are the major biomarkers in BC that have high importance in clinical use (Weigel and Dowsett 2010). However, previous research considered only a specific number of genes or a single platform genomic data (microarrays), as molecular profiling on large scale is very costly. These studies are limited to a specific cancer lineage. Choosing a targeted therapy based upon the alteration spectrum of a patient's tumor is another application of molecular profiling. Various efforts have been made to apply the high throughput sequencing data into clinical strategies (Garraway 2013; MacConaill et al. 2009) although changes in the clinically targetable genes have not been completely cataloged yet. The information provided in this catalog might help in the informed selection of targets for drug development and clinical trial design. It can also help to identify the patient populations that might get an advantage from the emerging targeted therapies.

15.5 Adaptive, Basket, and Master Protocol Trials

Clinical trials are essential to find out the efficacy of various therapeutic agents. An adaptive clinical trial is a novel approach towards clinical trials, and it allows the modifications in trial design characteristics throughout the trial (Fig. 15.1). The choice to bring a modification in a clinical trial is based upon the data gathered during the trial. It is different from the conventional clinical trial designs in which modifications cannot be incorporated during the enrolment and patient's follow-up as the safety and efficacy of the trial can only be calculated when the trial ends. The adaptive clinical trial design aims to decrease completion time, decrease the number of participants, reduce the resources, and enhance the probability of detecting the effect of treatment in a clinical trial. A massive increase in the number of adaptive clinical trials has been observed during an analysis of the registered trials on ClinicalTrials.gov. It has been reported that only 10 adaptive clinical trials were registered before 2006, but 133 adaptive clinical trials were registered from 2006 to 2013, and the majority of the trials were carried out in oncology (Hatfield et al. 2016). Pharmaceutical drug development is the principle use of adaptive clinical trials in oncology (Mistry et al. 2017). Various reviews of the adaptive clinical trials have suggested an increased reliance on this study design, specifically in pharmaceutical research (Mistry et al. 2017; Hatfield et al. 2016).

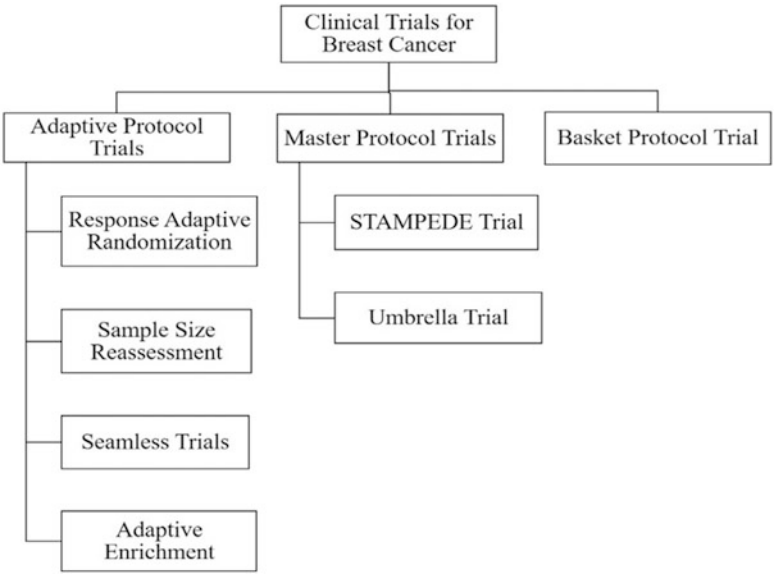

Fig. 15.1 Clinical trials for breast cancer

15.5.1 Adaptive Protocol Trials

The increased use of adaptive clinical trials should be recognized because of the differences in conventional and adaptive protocol trials. The effectiveness of adaptive protocol trials has been observed by the regulatory authorities, i.e., European Medicines Agency and the US Food and Drug Administration, and they have also provided guidelines for the investigators to consider the regulatory considerations while the planning and trial stages (Elsäßer et al. 2014). The appreciation and understanding of adaptive trials are typically limited among researchers and clinicians (Meurer et al. 2016). The commonly used adaptive clinical trials include sample size reassessment (SSR), adaptive enrichment, response-adaptive randomization (RAR), and seamless design.

15.5.1.1 Response-Adaptive Randomization

RAR-adaptive designs authorize changes in the treatment allocation ratio according to interim analysis during the trial. In RAR, the treatment allocation ratio is adapted in favor of the treatment arm providing more beneficial results. RAR trial can decrease the harmful clinical outcomes in the trial and might decrease the sample size without affecting statistical precision. "Play-the-winner" is the best-known RAR design (Bartlett et al. 1985). This RAR design begins with two urns, each representing treatment, and each ball represents the initial 1:1 allocation ratio. Changes are made to the allocation ratio whenever success is being observed for a treatment, addition of a ball to the corresponding urn is being made. The disadvantages of this RAR design include the increased chances of extreme allocation in a treatment group having efficient initial results that might be because of chance event. RAR trials include the application of computational algorithms (i.e., Bayesian predictive probability) and sophisticated rules to maintain a stable rate of allocation ratio (Saville et al. 2014).

15.5.1.2 Sample Size Reassessment

Sample size reassessment (SSR) has been used for decades, and it was the main focal point during the early years of the adaptive trial design method. The main stimulus behind SSR development is to reduce the negative effect of incorrect estimation of sample size during the planning phase because it results in an underpowered clinical trial (Bauer et al. 2016). If a trial fails in meeting the pre-specified endpoints, simple enrolment of more patients is not a good option because false-positive findings' risk will not be under control. In this situation, SSR can be pre-planned for controlling the risk of false-positive (Bauer and Koenig 2006). SSR is not aimed at restoring the failed trials, but it reduces the risk of a trial being over- or underpowered at an earlier stage.

SSR can also be used to decide whether to terminate or continue a trial. For example, if the newly required sample size shown by the SSR is too large, the researchers might decide to end the trial or change the trial objective from superiority to non-inferiority. The adjustment of sample size can be done after a few interim looks at the data as determined by the pre-specified plan (Bauer and Koenig 2006).

The use of simulations and analytical approaches is recommended for pre-planning, defining the allowed and intended adjustments.

15.5.1.3 Seamless Trials

Conventionally, the evaluation of an intervention being tested on humans passes through three phases: Phase-I involves testing the tolerability of healthy individuals towards the intervention, Phase-II involves the identification of tolerable dose ranges in people having health condition of interest, and Phase-III tests the safety and efficacy of the intervention in people having health condition of interest. Additionally, few research studies might also include Phase-IV trials where patients are being tested in a setting that corresponds more to the real world as compared to the much-controlled setting in Phase-III trial. In the case of conventional randomized clinical trials (RCTs), the findings from a phase can only be used after the phase has been completed.

Seamless study trial is a study design that involves the immediate continuation from one phase to the next phase. In the clinical settings, Phase-II and Phase-III are similar, therefore, these designs are used for the seamless combination of Phase-II and Phase-III trials (Cuffe et al. 2014). A seamless Phase-II/III RCT is favorable with few alterations in the design during the phases, but it also requires repetition in various important components. For instance, a Phase-II trial to examine the tolerability of five doses, ends by the termination of three of these doses, the Phase-III trial can be pursued with the two remaining doses, or it may also add another treatment arm, i.e., placebo group. Seamless trials aim at the rapid analysis and interpretation of early data for an informed decision regarding the transition into the next phase (Cuffe et al. 2014). The advantage of seamless design involves the reduction of time relapse from the first Phase-II randomized patients to reach a definite conclusion about the safety and efficacy by the end of Phase-III trial (Cuffe et al. 2014).

15.5.1.4 Adaptive Enrichment

Adaptive enrichment is a modification to the eligibility criteria of the trial (Simon and Simon 2013). For instance, if the interim analysis indicates different responses of pre-specified patient subgroups, the eligibility criteria of the trial can be modified by including only the favorable group (Simon and Simon 2013). In this case, SSR can also be performed independently for modification in the requirement of sample size by each subgroup. Contrary to this, if new evidence during the trial suggests that broader population as compared to the one established by considering the trial's eligibility criteria may be advantageous, the eligibility criteria of the trial might be broadened (Antoniou et al. 2016).

The emerging trends in the field of precision medicine have emphasized biomarkers and an increase in the demand to examine the effects of biomarker subgroup has been observed. The BATTLE and I-SPY2 are the adaptive trials in which enrichment acted as an important component of the design. I-SPY 2 is a Phase-II trial that evaluated the efficacy of 12 neoadjuvant therapies against 10 biomarkers (Park et al. 2016; Rugo et al. 2016). The treatment group having a high predictive probability to treat patients (bearing corresponding biomarker)

effectively, continued to Phase-III trial. The BATTLE trial is also a biomarker-driven enrichment adaptive trial (Kim et al. 2011). At an early stage, BATTLE aimed to identify the predictive biomarkers for the randomization of the non-small cell lung cancer (NSCLC) patients into one of the four possible treatment arms. Based upon the first stage, BATTLE-2 emphasized the evaluation of targeted therapies for the NSCLC with KRAS mutations.

15.5.2 Master Protocols

The master protocol is a multi-arm trial consisting of fixed number of treatment arms in a particular disease setting. The interim monitoring is being used for making decisions about the early discontinuation of arms for futility or efficacy. The master protocol allows the addition of treatment arms (in new or existing patient subgroups) during the trial in which various treatments are being tested. A trial in which master protocol is used is also known as a "platform" trial (Redman and Allegra 2015).

15.5.2.1 STAMPEDE Trial

STAMPEDE trial is an example of master protocol in which multiple treatments are developed in the disease setting. For instance, STAMPEDE trial is used to evaluate the effect of numerous agents (added to the standard hormone therapy) on advanced prostate cancer by the addition of new treatment arms when new effective treatments are available and dropping the treatment arms when sufficient activity is not observed (James et al. 2016). The advantage of the STAMPEDE trial approach is the addition of new treatment arms and still using the existing multi-arm trial. This leads to faster testing of the newly developed treatments for a specific disease. However, the randomized entry of the new experimental arms decreases the statistical efficacy of multi-arm design because the randomly assigned patients to these arms can only be compared to the randomized control arm patients. The challenges in conducting a multi-arm master protocol trial with fixed number of treatment arms include: convincing different industry partners for participation, funding for the trial, sharing information between the industry partners, and various extra-regulatory complexities (Redman and Allegra 2015).

15.5.2.2 Umbrella Trial

Master protocols have additional benefits with tumor biomarkers (Simon 2016). Umbrella trial involves the enrolment of patients having one tumor type into various treatment arms based upon the molecular specifications of their tumors. Lung-MAP is an umbrella trial for patients having advanced squamous cell lung cancer (Herbst et al. 2015). With the development of new targeted agents, the addition of new treatment arms can be made for the patients having tumors with appropriate molecular targets.

15.5.3 Basket Trial

In a basket protocol trial, the enrolment of patients into different treatment arms is based only upon the molecular characteristics of the tumor and not upon the histology. With the discovery of new actionable targets and their associated drugs, they are being added to the trial. For instance, the National Cancer Institute Molecular Analysis for Therapy Choice (NCI-MATCH) trial is an example of a basket trial (Conley and Doroshow 2014).

Screening component is a major part of basket and umbrella trials, and it is used to direct the patients towards the treatment strategy from which they can benefit the most. Both the basket and umbrella designs allow the addition of experimental arms and single treatment arms to their corresponding control arms for random comparisons.

Master protocols having biomarker-defined treatments are highly effective because most of these groups represent a small ratio of the patient population that can be captured with the help of the screening component of the trial. This is particularly advantageous to the patients who have screened their tumor once because they can find the potential treatment and trial based upon the molecular characteristics of their tumors. The efficacy of master protocol design is further increased by the adaptive factors of the deletion or addition of treatment arms based on interim monitoring results. The evidentiary requirements for the addition of new treatments to master protocol might be different for the definitive and early phase studies so, the requirements need to be clearly defined. This will help to keep the master protocol active by avoiding the pressure of adding new treatment arms having weak credentials.

15.6 Conclusion

Recent advances in the biology and molecular pathogenesis of BC have improved our understanding of the diverse clinical behaviors shown by different types of invasive BC. Even though the traditional histopathological techniques are important for classifying BC based upon the phenotypic characteristics, i.e., HER2 and ER expression, the real complications of the disease cannot be understood without considering BC proteome, genome, and transcriptome. According to the available proteomic, genomic, and transcriptomic data, every BC can be predicted as a distinct entity. The recent data reported by the Cancer Genome Atlas Network indicates the molecular biomarkers of each BC to be unique when compared to the closest neighbors or other BCs of the same classification or being compared across all the cases. The amount of observed molecular diversity increases with the number of genes being evaluated.

The molecular changes noticed in any BC type might be due to some driver events essential for cancer progression or due to passenger events that have secondary importance and are not essential for disease progression. The available data indicates that BC is due to a large number of drivers or driver pathways, and each

pathway represents a minute percentage of cancers. The variable responses to a particular drug in a similar cohort of BC represent underpinning molecular alterations that make treatment ineffective or develop resistance against it. Additional studies are required to elaborate on the relationship between BC biology and its response to a particular treatment strategy.

Next-generation sequencing of cancer-derived DNA and RNA can be used to study gene mutation status, gene expression patterns, and copy number variations. This technology will surely help to investigate the individual biology of BCs and to identify new biomarkers for disease prognostication. Additional detailed molecular data of the patients being exposed to therapeutic strategies might facilitate the correlation analysis to identify the molecular lesions that can guide treatment (by developing sensitivity) or may confound treatment (by developing resistance).

The major challenge to current clinical, basic science, and translational researchers in the evaluation of large numbers of BCs (having known clinical and treatment response measures) for linking quantitative and qualitative molecular traits with the responses to targeted and non-targeted drugs. With the recent advances in the knowledge of BC molecular biomarkers, personalized medicine can be implemented to various cohorts of patients via prospective clinical trials which increase the evidence-based knowledge regarding the pharmacogenomics of breast cancer.

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Chemotolerance of Breast Cancer and Its Management by Personalized Medicine

16

Yoser Al Rawi, Saima Shakil Malik, and Nosheen Masood

Abstract

Breast cancer is the commonly reported cancer among women around the globe. Breast cancer patients experience a repertoire of symptoms that are detrimental to the development of therapeutic options with least side effects and better quality of life. Many patients become resistant or tolerant to a wide range of chemotherapeutic drugs; therefore, researchers are digging into personalized medicine for the management of chemotolerance to increase cancer survivors with better quality of life. Recently, various genes/polymorphisms have been explored to be used as predictors of drug response and severity of cancer-linked symptoms. This will lead us to the identification of optimal drug dosage as personalized medicine and finding respective molecular targets which can help in disease management and introduction of effective therapeutics. Research is at the beginning stage and needs a lot to explore to optimize personalized therapeutics for chemotolerant breast cancer patients.

Keywords

Breast cancer · Chemotolerance · Personalized medicine · Molecular targets

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16.1 Introduction

Chemotolerance also known as chemoresistance, or drug resistance, is a term used to describe the insensitivity of cancer cells to therapy (Nikolaou et al. 2018). It is defined as the decrease in the drug's efficacy and potency to produce therapeutic effects (Chan et al. 2017). It represents a major obstacle to successful cancer treatment particularly metastatic cancer in where the primary management modality involves systemic therapy resulting in treatment failure in over 90% of patients with metastatic cancer. This phenomenon was first described by Gillman and his co-workers who noticed that the patients who were improving initially after starting nitrogen mustard therapy for advanced malignant lymphoma began not responding well with time (Rueff and Rodrigues 2016). Chemotolerance could develop either in the later phase of the treatment or be present from the beginning. The former is known as extrinsic resistance or acquired resistance and the latter as intrinsic resistance. Each classification is thought to have different mechanisms. Intrinsic is commonly related to the person's genetic makeup. It is also seen in certain types of malignancies such as renal cancer, hepatocellular carcinoma, and malignant melanoma having a poor initial response to anticancer treatment (Masood and Malik 2020). However, extrinsic resistance is acquired by tumors that were initially sensitive to cancer therapy. Such tumors initially respond well to cancer treatment but with time started to become insensitive to it or resulting in treatment failure and poor survival. Chemoresistance could also be classified into single drug resistance of multidrug resistance or based on the resistance mechanism. Understanding the mechanisms of resistance is essential to developing better treatment regimes, for cancer patient particularly those with metastatic disease leading to increasing survival rates and decreasing mortality (Eccles et al. 2013). This chapter will review our current understanding of chemotolerance-linked mechanisms in breast cancer and further discuss the potential of novel clinical strategies including targeted therapies and personalized medicine to manage and overcome chemoresistance.

Current forms of systemic therapies are chemotherapies, immunotherapies, and antiangiogenic agents and chemotolerance encompasses all those therapies. The interaction between drugs and the tumor microenvironment is dynamic and complex. Like how bacteria when faced with the stress of medications tends to develop mechanisms to overcome the drugs, so does cancer cells. This results with the development of resistance or tolerance to the therapy which is seen with chemotherapies targeting different molecular pathways (Cao et al. 2021). Studies have been trying to depict the pathways through which cancer cells develop resistance to the drugs.

16.2 Chemotolerance in Breast Cancer: An overview

Breast cancer (BRCA) is the most common malignancy and the most frequent cause of cancer-related death among women worldwide. However, metastatic cancer and chemoresistance, especially in TNBC are still inevitable and lead to poor prognosis

(Cao et al. 2021). Systemic therapy is the main treatment for metastatic breast BRCA. Chemotherapy is one of the major systemic treatments of BRCA and resistance to it has been a major obstacle in successful treatment of BRCA. However, even more new targeted therapies are found to develop resistance to treatment. Research in chemoresistance is expanding focusing on mechanisms of the development of resistance and ways to overcome it (Masoud and Pagès 2017).

16.3 Mechanisms of Chemotolerance in Breast Cancer

Multidrug resistance is the development of tolerance to multiple, structurally unrelated anticancer drugs and should be differentiated from the resistance to targeted and immune therapies (Bugde et al. 2017).

16.3.1 Enhanced Efflux of Drugs

One of the mechanisms of development of MDR is the expression of efflux transporters that pump the drug outside cancer cell decreasing the intracellular accumulation of anticancer drugs. One of the first transporters to be discovered in drug resistance is the P-glycoprotein that was later found to belong to the class ATP-binding cassette transporters (ABC transporters). This group of transporters is big group of transporters encoded by 48 human genes and classified into seven subfamilies—from ABCA to ABCG (Robey et al. 2018). Several ABC transporters (19 out of the 48) that have been shown to efflux cancer drugs to some extent (ref 43 from paper 6). The first subset of ABC transporters that were reported as multidrug efflux pump were the ABCB1 (P-glycoprotein/P-gp/MDR1), ABCG2 (BRCA Resistance Protein/BCRP), and ABCC1 (multidrug resistance protein1/MDRP1) (Robey et al. 2018). Their physiological role involves transporting substances across cell membrane for excretion and/or for protection. Consequently, those transporters can also affect the pharmacokinetics of drug including drug absorption, distribution, metabolism, excretion, and drug toxicity (Bugde et al. 2017).

The P-glycoprotein was the first transporter to be identified in 1976 by Victor Ling and was later found to be part of the ABC transporters subfamily ABCB1 (Gottesman and Ling 2006). The human gene MDR1 (later named as ABCB1) was reported in 1989 after Gros and colleagues reported a drug resistance gene cloned from multidrug resistant Chinese hamster cells, and later it was found that this gene induces resistance when transferred to sensitive cells (Borst 2020). A wide range of chemotherapeutic drugs including Taxol, Vincristine, Etoposide, and Daunorubicin have been susceptible to resistance through P-gp-mediated efflux (Akhtar et al. 2011).

The second member of the ABC transporter family, MRP1 (later named as ABCC1) was reported by Cole and colleagues in 1992 (Gillet et al. 2007). It was implicated in the development of resistance to Doxorubicin, Etoposide, and

Vincristine. However, its ubiquitous expression made it unlikely to be a suitable target for anticancer therapy (Robey et al. 2018). It has been shown to transport various neutral and anionic hydrophobic compounds and products of phase 2 drug metabolism, including many glutathione and glucuronide conjugates. In addition, ABCC11/MRP8 was overexpressed in TNBC and produced resistance to 5-fluorouracil and methotrexate (Nedeljković and Damjanović 2019).

The third ABC transporter identified is the breast cancer resistance protein (BCRP; subsequently renamed as ABCG2). It was found to be strongly involved in the chemotolerance of TNBC. It excretes various anticancer drugs including Mitoxantrone, Doxorubicin, SN-38, and several TKIs. One small number of inhibitors to BRCP has been developed and tested in clinical trials (Dey et al. 2019; Malik et al. 2020a).

16.3.2 Cancer Stem Cells

Cancer stem cells are the most commonly studied in acute myeloid leukemia and is known to possess CD 34^{+} and CD 38^{-} phenotype (Blatt et al. 2018). The cancer stem cells have the ability to form cancer lesions in mice. Studies have shown that cancer stem cells have the potential to metastasize making condition worse, and they may cause resistance to chemo- or radiotherapy making treatment problematic. Cancer stem cells are known to play an important role in EMT (epithelial to mesenchymal transition) leading to metastasis. EMT and cancer stem cell plasticity have a strong correlation, but it was reported that most of the metastasized cells lack EMT property and somehow they gain this ability when cells reach to distant body regions (Lambert and Weinberg 2021). Cancer stem cells are potently dangerous as they remain in a quiescent state when patients are undergoing chemo- or radiotherapy and once the treatment ends these cells regain their active state resulting in recurrence. This dormancy of cancer stem cells is reported in liver, prostate, and breast cancer cases. TGF beta in BRCA patients are the main factors that decrease the rate of proliferation of stem cells to achieve quiescence (Sulaiman et al. 2021). Most of the therapies encounter the active proliferating stem cells and overlook the dormant cells. Moreover, apoptosis mediated by p53 inhibit radiation-induced EMT leading to resistance for radiotherapy (Farhood et al. 2020).

16.3.3 Signaling Pathways

Cancer stem cells have self-renewal ability and are a type of multi-potent cells. They are dynamic by controlling pathways like Wnt, Notch, and hedgehog that help them by playing their roles in sustaining this ability in stem cells (Han et al. 2018). These cells survive in blood streams by escaping from apoptosis and travel in the body and find a suitable cancerous microenvironment far from the primary site. On their surface, they contain CD44 markers in addition to many others like ALDH1, Trop2, alpha2beta1, Sca1, Lgr4, CD117+, Lin, CD133+, p63, Nkx3.1, and many

others. Depending upon the markers, the cancer stem cells have many subpopulations that result in different response to treatments (Scioli et al. 2019). Many of the genes that are involved in preserving the cancer stem cell properties are oncogenic and genes that restrict self-renewal are tumor suppressor genes. Normally, these genes exhibit protective effects but during replication mutations accumulate in these genes leading to cancer. Oncologists have been working to create such compounds that interact with these molecular pathways and reduce or diminish the power of cancer stem cells to metastasize (Fiorentino et al. 2020).

The PI3K/AKT/mTOR and RAS/MAPK/ERK pathways are involved in chemotherapeutic resistance to breast tumors/BRCAs (Lee et al. 2015), whereas PI3K/AKT/mTOR is stimulated in HER2+ tumors (Khan et al. 2013; Ruchi Sharma et al. 2017). The PI3K/AKT/mTOR pathway is liked with the management of tumor aggressiveness, apoptosis, and bad prognosis (Paplomata and O'Regan 2014; Ortega et al. 2020). It is well known that JAK/STAT is associated with tumorigenesis, malignancy, angiogenesis, proliferation, metastasis, and survival (Groner and von Manstein 2017; Tabassum et al. 2019), whereas RAS/MAPK/ERK is implicated in cellular proliferation along with survival (Xu et al. 2015).

Genetic alterations in various genes such as BRCA1, BRCA2, PTEN, TP53, STK11, MLH1, PALB2, HIF1A, MSH2, and JAK2 are linked with greater risk of BRCA development (Schon and Tischkowitz 2018; Lipsa et al. 2019; Malik et al. 2020b, 2021; Costa et al. 2020; Lainetti et al. 2020; De Talhouet et al. 2020). Additionally, mutations in certain genes are found to be associated with poor prognosis like AKT1, BRCA1, BRCA2, c-KIT, and KDR (Pop et al. 2018; Lainetti et al. 2020; Vidula et al. 2020). Moreover, PIK3 alterations are more specifically related with poor prognosis in triple-negative tumors (Sobral-Leite et al. 2019).

Literature has shown that breast cancer cases with allelic alterations particularly losses in RAD51B, BRCA1, ERCC, and PALB2 genes are resistant to chemotherapeutic treatment options. Chemoresistance has been observed among BRCA patients with mutations specifically amplifications in ERBB2, ESR1, MYC, FGFR1, PIK3CA, TP53, and CCND1 genes (Lainetti et al. 2020).

Genetic alterations like loss or gain of function or allelic frequency changes have already been linked with chemoresistance, survival time, and chemosensitivity (Longley and Johnston 2005). One of the case control studies has reported that BRCA patients with BARD1 and ERBB3 polymorphisms have greater recurrence rates with no response to polychemotherapy treatments in comparison with the ones who do not encompass these genetic polymorphisms (Coté et al. 2018).

Signaling pathways have a vital role in the initiation, metastasis, progression, and chemoresistance of BRCA. Substantial collaboration and crosstalk exist between signaling pathways, their roles, genetic alterations, and chemotolerance; therefore, a lot more research is required to understand the underlying mechanisms and management strategies for patients.

16.3.4 Epithelial Mesenchymal Transition

Epithelial to mesenchymal transition is marked as the lost cell adhesion and E cadherins and conversion of cell to mesenchymal (Lee et al. 2019). The phenotype of cancer stem cells is never constant, instead it is dynamic. Cancer stem cells have property of EMT transition allowing them to metastasize. They both have same pathways of molecular involvement leading to invasion as well as migration of CSC from the site of tumor origin. Both express microRNA, hedgehog signaling and TGF beta expression in esophageal and colon cancers poor prognosis had been attributed to EMT (Peng et al. 2021). BRCA cells showing EMT express higher levels of CD44 that is a marker gene for identifying cancer stem cells (Sousa et al. 2019).

In epithelial mesenchymal transition, adhesion and polarity of epithelial cells lose and they attain migratory and invasive characteristics leading to the formation of specialized cells like mesenchymal ones. Basically, as a response to extracellular stimuli some of the transcriptional events and protein variations cause alterations in the phenotype of epithelial cells which is either reversible or not. CDH1 is an E-cadherin gene, with highest expression among epithelial cells it plays a predominant role in epithelial mesenchymal transition and is found to be poorly expressed among mesenchymal cells (Lombaerts et al. 2006). Expressional loss or reduction of CDH1 causes invasion of noninvasive cells, demonstrating its significant part in tumor's invasion capability and metastasis (Ling et al. 2011). Research has shown that breast carcinoma cells with mesenchymal phenotype become resistant to chemotherapeutic drugs at higher rate and linked with cancer stem cells.

16.4 Markers Linked with Chemotherapeutic Response

NGS has led to the identification of novel biomarkers which acts as chemoresistance/antitumor response predictors. Researchers have reported that BRCA cases with rs6484711 polymorphism showed poor response to neoadjuvant treatment with epirubicin and docetaxel causing increased expression of ABTB2 gene (Gong et al. 2020). According to our research, nine different studies have explored various chemoresistance-linked biomarkers just in 1 year as shown in Table 16.1.

16.5 Role of Personalized Medicine in the Management of Chemotolerance

The concept of personalized medicine is a dream come true using bioinformatics and DNA sequencing. This concept has increased the overall survival rate of BRCA patients more remarkably compared with NSCLC or colorectal cancer (Xie et al. 2020). However, the success of personalized medicine is limited due to tumor heterogeneity and variations in response to treatment options and not every person gets benefit from it. Genetic variations among different ethnic groups may be one of the factors for this response. Genetic screening of multiple genes is costly, and many

Table 16.1 Studies with chemoresistance in breast tissue samples

Drugs	Markers	Expression	Reference
Paclitaxel	ATIP3	Overexpression	Rodrigues-Ferreira et al. (2020)
Cyclophosphamide + methotrexate + fluorouracil Fluorouracil + paclitaxel + epirubicin + cyclophosphamide	NNMT	Overexpression	Wang et al. (2019)
Paclitaxel/docetaxel, epirubicin, cyclophosphamide	2,3-Dioxygenase Indoleamine	Overexpression	Zhao et al. (2020)
Anthracycline	FKBP12	Downexpression	Xing et al. (2019)
Doxorubicin	TWIST1	Overexpression	Demir et al. (2019)
Doxorubicin	lnc-TRDMT1–5	Overexpression	Chen et al. (2020)
Epirubicin/cyclophosphamide	TCHH, PLXNA1, CACNA1C, S100PBP, ZFHX4, SYNE1, FLG2, CENPF, ABL1, and ARAP2	Somatic variance	Al Amri et al. (2020)
Trastuzumab	NCAPG	Overexpression	Jiang et al. (2020)
Epirubicin and docetaxel	ABTB2	Overexpression	Gong et al. (2020)

SNPs are still unidentified and act as a barrier for success of tailored medicine (Nabirotchkin et al. 2020).

Treatment choice for most of the breast cancer patients is chemotherapy, but unfortunately there is a long list of associated side effects with this method. The problem is further complicated as the level of response to chemotherapeutic drugs vary greatly among patients. Age of patient and the stage effect the decision of treatment, both factor as high, the lower will be the response rate (Shrestha et al. 2019). A major challenge for oncologists always lies in choosing the effective and safe treatment drug as well as dosage for cancer treatment.

One of the mechanisms explained above included the P-gp-mediated efflux of anticancer drugs. Several clinical trials have looked at the effectiveness of inhibitors to ABC transporters in the hope of overcoming the MDR but were not very successful and thus people stopped trying to conduct more clinical trials using them. However, contemporary understanding of target biology and biomarker development could identify setting in where transporters involved in MDR could be considered important therapeutic targets. Newer technology that is now available was not there at the time of conduction of those trials (Thomas and Coley 2003).

There is ample evidence to support the fact that genetics play an important role in chemotherapeutic response. AKT1 mutation E17K although rare is known to effect the PI3K AKT mTOR pathway that is target of rapamycin (Tan 2020). Therefore, inhibiting this pathway with drug like everolimus can result in tumor inhibition (Du et al. 2018). Although rare but if this gene is screened before treatment, it can lead to precise medicine that will be effective. Phospho profile for identification of biomarker that are target of PI3K AKT mTOR pathway inhibition can be used for personalized treatment (Wang et al. 2020). If these phophoproteins can inhibit the pathway so it can inhibit BRCA progression (Khan et al. 2019). The problem lies in the fact that drug may activate compensatory pathways to overcome the effect and leave the drug ineffective. Researchers have not found increased overall survival from this strategy, but prolonged progression-free survival of BRCA is established. Already known genes like BRCA1, BRCA2, Neu, and HER2 have important roles in cancer treatment suggestions.

Cancer therapeutic efficacy can also be enhanced by profiling mitochondrial DNA (Yamazaki et al. 2020). Higher levels of reactive oxygen species cause oxidative damage leading to mitochondrial DNA mutations. Such mutations are known to cause drug resistance in cancer patients and need to be screened before that. Metabolome varies from patient to patients and is reported to have an effect on efficiency of drugs (Zidi et al. 2021). Recurrence of disease was found to be higher in patients that are triple negative and have higher levels of phosphatidylcholine. Similarly, higher testosterone levels are also associated with recurrence of breast cancer (Ecker et al. 2019). An outline of most of the options that need to be considered before treatment are presented in Table 16.2.

Table 16.2 Personalized treatment strategies for overcoming chemotolerance

Sr. no.	Strategy	Targeted use
1.	Gene expression profiling	Optimization of drug choice for cancer treatment
2.	Monitoring of circulating tumor cells	Effectiveness of drug
3.	Use of pharmacogenomics	Predicting response to chemotherapeutic drugs
4.	Use of MicroRNA	Triggering drug release
5.	Use of biomedical engineering tools	Intervention design
6.	The use of genomic-adjusted radiation dose (GARD)	Optimizing radiation dose in radiotherapy

16.6 Personalized Medicine in Providing Better Quality of Life to Patients Undergoing Chemotherapy

Most of the patients experience adverse side effects with the use of chemotherapeutic drugs that basically decreases their quality of life and overall performance (Lindley et al. 1992). So, personalized medicine comes up with different approached not only to increase the efficacy of respective treatment processes but also provide ways to manage the side effects and manage chemotolerance among patients (Boos et al. 2022). Now, we are going to explain the available information related to genomics, proteomics, and metabolomics that might help to attain advancement in personalized medicine improving quality of life for the patients having chemotherapeutic treatment.

With reduced normal functioning of ovaries among women aged 40 years or below causes decreased estrogen production leading to premature menopause (Okeke et al. 2013). Therefore, finding estrogen biosynthesis and metabolism-associated genetic polymorphisms can provide better insight into biomarkers used to address premature menopause/chemotherapy-induced menopause among BRCA patients. Researchers have identified various genetic polymorphisms that explain the link between premature menopause, chemotherapy-induced menopause, and sex hormone production; but unfortunately, they are unable to find any association or direct link between them (Abrahamson et al. 2007; Riancho et al. 2008). One of the studies has reported the association of polymorphisms in sex hormone-binding globulin (SHBG) gene with SHBG serum levels, but these polymorphisms were found not to be linked with bone fractures and fragility, a predominating symptom for premature menopause. SNPs among ESR1 and ESR2, estrogen receptors genes, were recently reported to be associated with premature ovarian failure, a characteristic of premature menopause (Cordts et al. 2012). This kind of findings leads us to discover allelic biomarkers for treating or managing BRCA cases having premature menopause and provide ways for future research. This might bring fruitful results regarding the development of personalized medicine and strategic ways and combining them with available chemotherapeutic drugs to get such drug dosage or

treatment regimens that would help in managing and treating chemotolerance among BRCA cases.

It has been observed that BRCA cases having amenorrhea, clinical characteristic of premature menopause, showed better survival and disease-free survival outcomes after chemotherapeutic treatment (Swain et al. 2010). Therefore, it has been concluded that future research should consider all the pros and cons of respective treatment options when exploring personalized medicine.

Recently, various genetic markers have been identified among cancer cases undergoing chemotherapeutic treatment and showed their association with chemotherapy-induced peripheral neuropathy (Brewer et al. 2016). It also sheds light on the induction of neurotoxicity because of chemotherapeutics and its control and management among patients. ABCB1 gene codes for an important protein of ATP-binding cassette subfamily reported to have 3435 TT polymorphism causing substantially increased neurotoxicity among BRCA cases enduring chemotherapeutic treatments with docetaxel and paclitaxel (Kus et al. 2016).

Cognitive impairment is one of the known outcomes of chemotherapeutic treatment among cancer patients. Still the underlying mechanisms are not fully explored, researchers have suggested that inflammation would be one the potent fundamental mechanisms (Loh et al. 2016). IL1R1 was found to have a drastic polymorphism meant to promote inflammation and found to linked with greater level of perceived cognitive function in survivors of breast carcinoma. It basically explored a probable marker for the identification of cases encompassing chemotherapy with lesser cognitive dysfunction risk. Tailored interventions focusing cognitive impairments can be executed to those who are at greater cognitive dysfunction risk, thus saving assets for intervention execution (Myers et al. 2017; Lange and Joly 2017).

Depression is considered as an important psychological symptom experienced by breast cancer patients undergoing adjuvant cancer treatment (So et al. 2010) and becomes more drastic when combined with other related symptoms such as fatigue, sleep disturbance, and pain leading to poor physical, emotional, social, and functional health effects (Thornton et al. 2010). Thus, personalized medicine and tailored therapeutics focusing depression might prove to be a useful approach for better quality of life among BRCA patients.

16.7 Conclusion

With the rapid advancement in molecular biology, researchers have attained remarkable development in BRCA therapeutics; nevertheless, some groups of BRCA still have considerable challenges of metastasis chemoresistance. BRCAs involve extremely complex pathways; therefore, one of the greatest difficulties or challenges is the complete understanding of all the BRCA-related molecular mechanisms, but it is crucial to explore all of them in order to identify novel treatment targets.

Presently, new therapeutic regimens come up as more competent answer for BRCA resistance than the conventional ones. Findings of new drug delivery systems have presented potent attitude for improved efficacy of anticancer agents in cancers

with chemoresistance. Additionally, another promising approach is the use of immunotherapy for drug-resistant or -tolerant cancers and more research is required in this domain.

Although cancer stem cells and autophagy regulation are not commonly used in clinics, it is much hopeful to improve the prognosis of BRCA with metastasis and chemoresistance and metastasis. More future research is required on BRCA considering underlying molecular mechanisms producing ways toward new clinical strategies for cancer management and treatment leading to better survival outcomes.

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Advances in Breast Cancer Pathology

17

Zaineb Akram

Abstract

Modern medicine is a multidisciplinary field in which the role of pathologist has been highly redefined for accurate diagnosis of the disease, prognosis, and optimal treatment modalities. The pathologists identify the potentially targetable lesions on their phenotypes and by assessment of related biomarkers that help predict response. Several molecular methods have been adapted by the surgical pathologists including immunohistochemistry, fluorescence in situ hybridization, conventional and real-time PCR, Sanger sequencing, next-generation sequencing, microarrays that can be applied to patient's tissue or blood sample. Basically, the role of pathologist is to integrate morphology, genetics, epigenetics, and molecular tests of the patient to help reach a diagnostic, prognostic, and therapeutic decision by the treating physician. The advancement in technological aspect of the new world has also made it possible to develop software that can study a patient's sample based on artificial intelligence algorithms. These helps minimize the human error and intrapersonal differences. This is a unique and modern field and still requires more work to be done. It will be possible by the intense cooperation among engineers, pathologists, and clinicians.

Keywords

Pathologist · Immunohistochemistry · FISH · NGS · Breast cancer

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17.1 What Is Surgical Pathology?

Pathology most commonly applies to the study of disease. It involves examining body organs, tissues, body fluids, and sometimes autopsy. It helps detailing information to the patient and their treating physician regarding the diagnosis and treatment of the disease. Pathologists can sub-specialize in various areas like gastroenterology, blood diseases, microbiology, breast cancers, etc. They work in close collaboration with the surgeons, radiologists, and oncologists.

The most important branch of pathology is surgical pathology in which pathologist examines surgically removed tissues with naked eye first along with microscope to diagnose the disease. The specimens can be from small skin biopsies or core biopsies. It involves macroscopic (gross) and microscopic (histologic) analysis of tissues along with many laboratory-based tests to study their molecular properties (Cree et al. 2014).

17.2 Molecular Pathology and Diagnostics in Breast Cancer Diagnosis

A great majority of breast cancers are detected by patients themselves. This is based on appreciating the physical change in the structure of breast mostly in the form of a lump, thickening, or any other visually perceivable change. The patient then visits the healthcare system where the diagnostic team starts its work. The first step for any such patient is the clinical breast assessment (CBA) or clinical breast exam (CBE). This provides important information including the duration of abnormality that hints towards the nature of underlying pathology of the disease, whether it is congenital, ominous, or benign. The second important aspect is the changes in radiographical or clinical size of abnormal patch. Changes in characteristics and features of the concerned part along with the symptoms associated are also very important to note. Symptoms may include pain, tenderness of lymph nodes, pain, dysphagia, etc. The pathologist should also get a detailed medical, familial, and social history of patient (Harris and McCormick 2010).

After the detailed physical exam, "triple-test" is performed which includes clinical examination, diagnostic mammography, and fine needle aspiration cytology (FNAC). The diagnostic accuracy of breast cancer reaches to 100% when triple test is performed. For women above 40 years of age, 2D digital diagnostic mammography is suggested because of its high sensitivity and specificity. It should be enough for diagnosis but in more difficult cases tomosynthesis (3D mammography) or Magnetic Resonance Imaging (MRI) may also be used. For biopsies, FNAC is mostly the first choice in which a fine ultrasound-guided needle is inserted in the area of concern and few cells are aspirated. Second option is a core biopsy in which a small sample or core of the concerned tissue is removed from the solid lump. Specially in situations where it is tough to identify the specific area of concern stereotactic mammography, core biopsy is performed which exactly points out the region of breast with abnormality by using computer analysis performed on X-rays

of different angles. The application of high-throughput techniques have increased the understanding of disease biology along with improving the prognostic and predictive ability of the treating physician (Khoda et al. 2015).

17.3 Biological Markers in Breast Cancer

In early times, many biological markers were studied but eventually only human epidermal growth factor receptor-2 (Her2), progesterone receptor (PR), and estrogen receptor (ER) have sustained their position and are routinely and mandatorily employed in management of breast cancer. Her2 is a protein that is normally present on all breast cells and functions in promoting growth. Breast cancer is regarded as Her2-positive or -negative based on overexpression or normal expression of Her2 gene in the patient. It has been proven that Her2 overexpression is linked to rapid tumor growth, higher recurrence risk after treatment, poor response to chemotherapy and reduced survival. These connections lead to the development of Her2 antagonist, a monoclonal antibody known as trastuzumab. Similarly, estrogen plays an important role in normal breast as well as the progress of breast cancer (Wolff et al. 2018). Estrogen receptor (ER), nuclear hormone receptor acts as a transcription factor. ER-positive breast tumors do not show good response to cytotoxic chemotherapy but benefit largely from targeted endocrine therapy like tamoxifen, aromatase inhibitors etc. Similarly, progesterone receptors (PR) help in ER signaling and ER regulates PR expression. PR acts as a driver for breast cancer, especially in women in post-menopausal stage (Collins et al. 2005). Tumor responsiveness to endocrine therapy correlates with PR expression even if present in low amount. However, in case of ER-positive breast cancer, PR assessment may not have any additional prognostic information. It is a standard practice to evaluate HER2 and ER/PR expression for breast cancer patients and most commonly immunohistochemistry methods (IHC) are used in which fixatives play a role in tissue processing, decalcification of specimens by acids and internal/external assay controls. IHC method measures the level of protein synthesis in tumor sample as it identifies the number of cancer cells that synthesize specific protein. Intensity of staining represents the level of protein synthesis (Nadji et al. 2005). Another advance technique, fluorescence in situ hybridization (FISH) is often regarded as gold standard because of its better reliability and validity (Hicks and Tubbs 2005). The reason of preferring immunohistochemistry methods is low cost, ease of performing, and easy availability. There are commercially available IHC-based kits including Mammostrat® test (Clarient Inc., US) for 5 IHC markers and IHC4 that measures 4 IHC markers. According to recommendations from The American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP), the final report should represent positively stained tumor cells percentage, overall staining intensity, and hormone receptor status. If $\geq$1% tumor cells stain positive, then the ER/PR expression is labeled as positive (Hammond et al. 2010). Triple-negative breast cancers represent the class with absence of ER, PR, and HER2 expression. They are not

treated by endocrine therapy but have to reside on conventional chemotherapy option (Ginter et al. 2020).

Gene expression profiling techniques can also be used to identify the level and quantity of mRNA of specific genes that indirectly represents the expression level of protein in cancer cells. In this method, many genes can be observed at a single time, and it mostly uses real-time reverse transcription-polymerase chain reaction (RT-PCR) and deoxyribonucleic acid (DNA) microarrays. These tests report HER2, ER, and PR status along with some other genes that are different for various platforms. Some of the specialized RT-PCR kits used for gene expression profiling include OncotypeDX™ (Genomic Health Inc., Redwood City, CA, USA—http://www.oncotypedx.com) measures expression of 21 genes, PAM50 gene expression assay (ARUP Laboratories, Salt Lake City, UT, USA) is for 50 genes, MapQuant (Ipsogen) is for 9 genes, MammaPrint® (Agendia, the Netherlands—http://www.agendia.com) measures expression of 70 genes (Wesolowski and Ramaswamy 2011).

17.4 Genetics of Breast Cancer

With the development and improvement of genetic technologies, the identification of several breast cancer predisposing genes has become easier. These panels include BRCA1/2 along with many other associated genes. With increased awareness regarding genetic testing, its demand has increased causing a decrease in the cost. Almost 20% of breast cancers are hereditary (Kwong et al. 2016), out of which two thirds are caused by mutation in BRCA1/2 (Coppa et al. 2014). Non-BRCA genes that have been identified as predisposing factors for breast cancer include *ATM*, *PTEN*, *CHEK2*, *TP53*, *PALB2*, and others (Crawford et al. 2017). Ataxia-telangiectasia mutated (*ATM*) gene encodes a protein that activates cellular responses to DNA double-stranded breaks thus making its role crucial in DNA damage pathway (Angèle and Hall 2000). Checkpoint kinase 2 (*CHEK2*) gene is a tumor repressor gene thus is involved in DNA repair and apoptosis (Apostolou and Papasotiriou 2017). *PALB2* (Partner and Localizer of *BRCA2*) acts as tumor repressor and its loss of activity have been seen in Fanconi anemia as well as breast and pancreatic cancer (Antoniou et al. 2014). *PTEN* (phosphatase and tensin homolog) is also a tumor suppressor gene involved in cell survival, apoptosis, and proliferation (Noh et al. 2011). With the increased use of Next-Generation Sequencing (NGS) and the discovery of new genes involved in breast cancer pathology, multigene panels have been developed that give more information than a single test. They have proven their importance for the assessment of breast cancer risk by maximizing health benefits, early detection for easy and cheaper treatment and increasing survival rates (Cragun et al. 2016). Before recommending NGS testing, some considerations should be kept in mind including huge amount of data consists of variants of unknown significance (VUS), low or incomplete penetrant mutations, high costs and emotional impact on patient and family (Lizard et al. 2016). The advantages of using an NGS multigene panel for breast cancer patients are that it allows to

sequence a large number of fragments in a single run, the turnaround time is shorter, and it has lower cost associated to it as compared to comprehensive profiling on Sanger sequencing (El-Deiry et al. 2019). Mostly, the only hurdle is that the interpretation of results requires specialized trained individuals for biostatistical analysis (Fassan 2018).

17.5 Role of Surgical Pathologist in the New Molecular Targeted Therapies for Cancer

The area of precision/targeted medicine is shifting towards multidimensional approach that includes genomics, transcriptomics, proteomics, and metabolomics analysis of markers involved in cancer development, progression, and prognosis (Dienstmann et al. 2017). This resulted in immense revolutionization in the strategies and practices of surgical pathology. Or it can be said that the role of surgical pathologist has become the backbone of decision-making regarding the targeted therapies. The aim of surgical pathology has been widened which includes (Yates et al. 2018):

1. They work to gather as much predictive information as they can regarding the tumors like prognosis of the diseases, response to specific drugs, metastasize state, etc.
2. They help the treating physician to choose the appropriate drug based on specific characteristics of the tumor like expression levels of proteins involves genetic mutations, etc.
3. To help improve and widen the list of biomarkers that will define the bases of targeted drug development by research groups and pharmaceutical industries.

17.6 New Image Guided, Multimodality Theranostic Agents

One of the important aspects of surgical pathologist's job is to perform imaging of the biopsied sample which will help identify specific cancer targets. These images will then help design specific targeted agents, visualize the delivery and response, and alongside save normal tissues. This is called theranostic imaging which combines diagnosis and therapy. The focus is on developing theranostic agents that help in targeted therapy and minimizing the collateral damage to the neighboring normal cells. The theranostic agents require to be widely researched upon before being approved by Food and Drug Administration (FDA) as they have immunogenicity problems, cGMP synthesis issues, and associated high costs for synthesis and clinical trials as well.

The aim of theranostic imaging is to deliver specific therapeutic agent to the specific target and track the effect by noninvasive imaging. In breast cancer patients, 20–30% are positive for HER2 marker and Trastuzumab acts as a targeted therapeutic agent for extracellular domain of HER2 (Rayson et al. 2014). Many imaging

techniques are being explored for theranostic imaging including magnetic resonance imaging/spectroscopy (MRI/S), positron emission tomography (PET), and single photon emission computerized tomography (SPECT). Theranostic imaging will tremendously improve the area of personalized targeted therapy for cancers. Right now, the cancer cell receptors and antigens are being targeted, but in future it can be expanded to targeting microenvironments niches, stromas, and even cancer stem cells.

17.7 Digital Pathology: A Way Forward

The role of a surgical pathologist in physical exam of histopathological samples is to visually recognize the morphological patterns of the affected tissues. This evaluation is affected by interobserver and intraobserver variability even after using standard practices according to guidelines. To omit these differences, high-resolution imaging along with artificial intelligence and machine learning can be used as this is the era of computing. For example, in breast cancer IHC tested positively stained cells can be counted through software to improve accuracy and decrease human error (Griffin and Treanor 2016). For detection of morphological features of the biopsy samples, artificial intelligence software can be used to identify the cellular and subcellular structures, intratumor heterogeneity, differentiation between malignant and benign areas and giving grades according to severity level of disease (Bera et al. 2019). In order to shift from traditional pathology to digital pathology, a high integration level is needed to be developed between the engineers and the pathologists. They need to work in close connections to design, develop, and improve software that can perform crucial image analysis for diagnosis and research (Gallo Cantafio et al. 2018).

17.8 Role of Pathologists in Integrating Genotype and Phenotype for Optimal Patient Care and Management

The role of modern pathologists has become increasingly important in this era to provide interpretation of patient's state based on interlinking the results from morphology, genetics, molecular and clinical information (Walk 2009). With the revolution of personalized medicine, each and every aspect has improved including disease diagnosis, characterization, and treatment. Each aspect involved in precision medicine from morphology to molecular pathology has a significant role. Morphological evaluation confirms the subtype of lesions based on histology, distinct characteristics of the representative sample (necrotic tissue percentage, inflammation, etc.), choosing molecular analysis required (most efficient, cost-effective), detect any artifacts in previous analysis and evaluate intratumor heterogeneity (Fassan 2018). Similarly in molecular pathology, the critical role of molecular pathologist is to choose most appropriate test and platform for the sample according to the guidelines in order to reach to the accurate and clinically relevant information (Lindeman et al. 2018). The role of pathologist is to contextualize the molecular

information according to the histopathological evaluation. No matter how much the phenotype and genotype appear to be independent fields from each other the morphological appearance of a lesion is almost always the by-product of the molecular makeup underground (Klauschen et al. 2015). Thus, the report from a pathologist should represent a plethora of information combining morphological and molecular information that has been interpreted for diagnostic, prognostic, and predictive value.

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Modern Radiation Therapy Techniques and their Toxicities for Breast Cancer

18

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Abstract

Breast cancer (BC) is the major cause of death in women worldwide. It is a global issue with nearly 2.3 million patients diagnosed with BC and 0.685 million deaths in 2020 which greatly affects human resources as well as healthcare costs. Besides the development of systemic therapies, radiotherapy (RT) provides long life expectancy and high survival rates in breast cancer patients. With the advent of current radiotherapy planning systems, irradiation of breast cancer has undergone a drastic change. Historically, conventional fractionation techniques using tangential field protocol were considered a benchmark in RT for breast cancer. During the past 15 years, advances in treatment techniques, specifically, RT allows subsequent decline in treatment-related complications. The transition from two-dimensional to three-dimensional treatment planning has drastically decreased long-term cardiac toxicity. Herewith, it is prudent that treatment using radiations is carried out with utmost efficiency. This chapter provides practical as well as theoretical insight into the advances in radiotherapy techniques that are recently used in clinical practice. Besides three-dimensional RT planning, Intensity Modulated Radiotherapy (IMRT), Volumetric Modulated Arc Therapy (VMAT), and proton therapy are other modern treatment options. The other

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attractive approach which has resulted in shortened treatment duration is the hypofractionated RT technique. In addition, Accelerated Partial Breast Irradiation (APBI) and Deep Inspiration Breath Hold (DIBH) are also reasonable options for low-risk patients and maximal heart protection, respectively. Advanced techniques focus on cardiac sparing or deep breath-hold approaches which provide maximum safety and a decrease in long-term cardiac toxicity. This chapter will provide an up-to-date guide and resource for radiation oncologists, clinicians, and fellows seeking to learn and practice breast cancer radiotherapy.

Keywords

Radiotherapy · Breast cancer · Hypofractionated radiotherapy · Deep Inspiration Breath-Hold Radiotherapy · Cardiac toxicity

18.1 Positioning and Immobilization Techniques

Adjuvant RT, which is the fusion of irradiation of breast and breast-conserving therapy (BCT), has become state-of-the-art therapeutic option for BC. Breast irradiation not only contributes to improved outcomes in patients but also reduces the fatality rate. Radiotherapy (RT) of breast cancer can be variable and difficult based on the patient's anatomy, i.e., axilla depth and concavity of chest wall. The first step is to perform CT simulation for scheduling conformal RT or IMRT using heart-sparing techniques like heart blocking or breathe hold, particularly for patients with left breast cancer (BC). Treatment fields are a combination of the whole breast, axillary fields, internal mammary artery, and supraclavicular. The predominant goal of breast radiotherapy fields is to keep away from hot and cold dose areas among adjoining fields while decreasing radiation dose to healthy surrounding tissues, i.e., heart and lungs. Treatment fields have to be adjusted according to the anatomy of the breast, which because of its irregular surface can originate inhomogeneity in dose. Also, a setup must be designed which can easily be reproduced.

Most patients receiving radiotherapy post breast-conserving therapy (BCT) are treated in the supine position, which is considered as topmost natural position for women. It has advantages such as accuracy, comfort, repeatable positioning, and optimum surgical access to the chest wall. During this position, the mammary gland extends over the chest wall, particularly in heavy-breasted women. Consequently, irradiation of the heart and lungs is unavoidable and the area of skin fold expands. Therefore, radiation-induced toxicity is inexorable (Yu et al. 2018).

Usually, a prone position is recommended for patients with large sagging breasts to reduce late and acute toxicities. The development of modern CT planning techniques has made this position reproducible. The prone technique is advocated not only for patients with large breasts but in most BC patients as it largely reduces the lung and heart within the field (Griem et al. 2003; Cross et al. 1989). However, there are conflicting data on healthy tissue efficiency reduction. Modern RT techniques have eased the exploration of the hypofractionation scheme with the

associated boost to the tumor bed in the prone position (Huppert et al. 2011). Initially, a prone position is suggested for heavy-breasted women. Later, reports of decreased doses to lung and heart resulted in the utilization of prone breast irradiation for patients with or without heavy breasts. At the same time, refinements in RT techniques and the advent of DIBH methods, that moves the heart away from the radiation field, result in a significant decrease in radiation doses to OARs even with supine position (Haffty 2018).

According to Würschmidt et al. (2014), the ancillary dose to the LAD (left anterior descending) arterial blood vessel, once the left breast was irradiated in a prone position, was 33.5 Gy versus 25.6 Gy in a sitting position. In comparison, Kirby et al. (2010) also predicted prone position to decrease the heart doses to about 64% of 30 patients treated with whole breast irradiation (WBI) and 24 percentage of the same number of patients treated with partial breast irradiation (PBI) with median reduction is 29.3 Gy, along with decreasing ipsilateral lung (mean) in the whole breast and 61 of 65 PBI cases, and chest wall V (50 Gy) in all WBI cases. According to Varga et al. (2009) randomized clinical trial, the displacement range was greater in this position. In the prone position, the main concern is setup errors and reproducibility in contrast to the standard supine position. Setup errors were lower for supine positioning as compared to prone positioning in such a way: systematic errors: 3.1–4.3 mm (prone) ($p = 0.02$) and 1.3–1.9 mm (supine) and random errors: 3.8–5.4 mm (prone) ($p = 0.02$) and 2.6–3.2 mm (supine). Even patient treatment time and comfort scores were compared. Reported clinical target volume and planning target volume margins were determined to be smaller for the supine position (10 mm) than for the prone position (12–16 mm) (Kirby et al. 2011).

Patients with large breasts are recommended to take up the lateral chronic leg position, which is a side-lying posture. Women undergoing breast irradiation have only been treated in this way by experienced centers since it is cumbersome to provide full coverage to the lymphatic region. More than 500 women have been dosed with 50 Gy of WBI using lateral decubitus position, which has been employed at the Curie Institute (Paris). Thin carbon fiber supports and special devices for patient positioning were designed specifically for this position. Suggested techniques have been indicated acceptable dose homogeneity for BC treatment volume, with a very small radiation dose to the underlying organs at risk (Campana et al. 2005). Despite the valid single-center outcomes, this position has not been extensively undertaken for everyday clinical practice.

Although there remain advocates for both prone and supine positions for breast irradiation depending on tumor location, breast anatomy, or other clinical considerations, in which one position may have benefited over another, patients are treated in both positions at most radiation centers. Choice of positioning preference may depend on differences in body habitus, training biases, physician preferences, and tumor size and shape. There is no proof that one positioning technique is superior to the other, but the main concern is that RT is effective and safe whether it is delivered in the supine position or prone position (Haffty 2018).

For the treatments of BC patients, special immobilization devices are designed which are widely used and are commonly available in routine. The well-known

devices are recorded as alpha cradle, Vac-fix bag-Vacuum Cradle Bed, Board-Wing Butterfly Board, inclined plane, and breast boards. In the supine position, the simplest and most preferred arrangement has been made with the breast board in conjunction with an inclined plane having an armrest. The patient's head should be facing to the opposite side, where the arms abduct (90°–120°) and rotated externally. On the stable board, the woman is lying on her back, and the breast board is angled to confirm the breastbone parallel to a table. According to clinical requirements, this angle could be modified, but larger angles can lead to an elevated radiation dose in the patient's lungs demanding a supraclavicular field. The edge between the supraclavicular field and chest wall is commonly placed at the clavicular head bottom. Radiopaque wires have been exercised to describe breast borders and incisions (Griem et al. 2003). To minimize the dose to healthy surrounding organs, the use of a thermoplastic bra has also been explored. Results suggest that the use of thermoplastic bra provide shallower girder setting for the left-sided BC (medial with bra 288°–315° vs. without thermoplastic bra 302°–325°) and decreased radiation dose to lungs by 30.6% without any specific eligibility criteria for everyday clinical usage (Piroth et al. 2016).

18.2 Modern Planning and Delivery Techniques

BC is very common in females after cancer of the skin. Timely diagnosis, customized approach to therapy, and more understanding of the disease have all contributed to an increase in BC survival rates and continuous reduction in the number of fatalities associated with the disease.

It is one of the diseases for which the use of radiation for therapeutic purposes has progressed substantially over the last century, such as brachytherapy and EBRT. The use of advanced methods in EBRT has increased largely with time. As a result, the choice of radiation method is critical to cure the tumor and to reduce toxicities. The number of planning approaches have progressed over the past 20 years from 2D to 3D techniques, including IMRT, VMAT, and as well as proton therapy.

Modern radiotherapy techniques have reduced complications such as scarring of the tissues and long-term cardiac toxicity, while also improving loco-regional control rates, and coronary sparing with 4D-breath-hold techniques. The main purpose to use any appropriate therapy and planning is to deliver a high radiation dose to the tumor while reducing exposure to the surrounding healthy tissues or

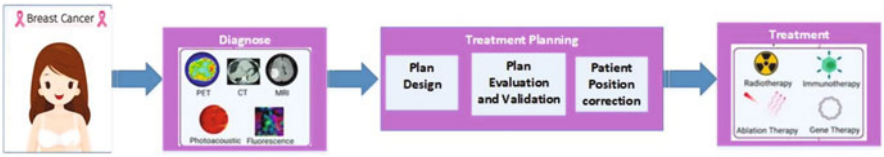

Fig. 18.1 The treatment planning process for breast cancer

organs (such as the lung and heart) which are at risk. Figure 18.1 displays the complete process of treatment planning of BC.

18.2.1 Hypofractionation

In the middle of the 1980s, the hypofractionation (HF) approach has been familiarized and used in numerous departments of radiotherapy. Over the years, the HF approach was known by the number of statements. This term is defined as "fewer treatments" while conventionally 5–6 fractions are administered per week.

HF radiation therapy is the one in which total radiation dose is broken into large fractions and radiation doses are delivered often every day. Similar to conventional RT, the objective of HF is to destroy breast cancer cells, but through larger doses of radiation in fewer sessions. Its main advantage is to increase the dose of biological radiation to cancer without increasing the radiation dose to late responding healthy normal tissues. HF is the form of EBRT that releases X-ray beams of high-energy carefully targeted at the breast. Conventional radiations are supplied via the same machine, but with HF, tumors receive a higher radiation dose for each treatment session. So the course of RT is completed more rapidly using HF. When breast cancer patients experience a type of surgery that's planned to keep as much of their breast tissue as possible, HF RT can also be used as follow-up therapy.

The two important benefits for employing the HF approach are:

First of all (Friberg and Ruden 2009), once megavolt machines have been familiarized, very deep tumors might be treated which increases demand for radiotherapy. No consistent increase in total treatment units was observed so it takes short time for treatment. With fewer fractions for each patient, additional patients might be treated over time. Secondly, HF can ease the problem for cancer patients that they don't have to travel to the hospital every single day for treatment purpose.

With the passage of time, it was obvious that these treatment schedules might also harm patients. From the identified patients, radiation-induced injuries were noted, i.e., paralysis of the arm, edema of hand or arm, fractures in arms and thoracic skeleton, immobilization of shoulder, heart disease, respiratory distress, horner syndrome (due to damage to the sympathetic chain), and hoarseness. The main advantage is convenience because in fewer sessions' patients can achieve full radiation treatment. Breast swelling (breast edema) as well as fatigue, skin itchiness, and skin irritation were less common among the women who received HF compared to other forms of RT techniques. Moreover, there is a reduced demand on equipment, time, and staff making HF a very resource-efficient approach while providing identical result for the patients. Gilbert Fletcher (1988) has defined the HF approach as: "The maximum time of patient treatment is consumed on arrangements, but the real-time for treatment is simply a part of total time, it has been reasonable to reduce the machine time by fewer large fractions."

18.2.2 Whole Breast Irradiation

For the EBC, the standard of care has advanced from the modified-radical mastectomy (MRM) to breast conservation therapy (BCT), which consists of nodal basin evaluation, lumpectomy (partial mastectomy), and 5 weeks (Goldberg and Whelan 2020) or 5–7 weeks (Sanders et al. 2007) of the WBI (standard dose of almost 50 Gy). Based on patients and the tumor characteristics, an extra increased dose of 10 or 16 Gy is provided to the tumor bed (Bartelink et al. 2015). Today, HF regimes with a dose of 15–16 fractions are the favorite regimes (Miranda et al. 2019). Whole breast irradiation, as part of BCT, has low toxicity, good cosmesis, and well-established results. Current examinations have observed tumor control rates and the toxicities related to the shorter radiation delivery options. HF-WBI includes treating the WB with a greater dose of radiation on a daily basis. The following study state HF-WBI as an accelerated WBI (Shaitelman et al. 2014). Theoretical arguments in support of shorter radiation therapy courses expected that these treatments lead to more patient conformity, greater choice of breast conservation over the mastectomy, improved life quality, and cost efficiency to the patient and the wider national healthcare organization (McCarthy et al. 2006). In APBI, the area in which radiation is being targeted is different compared with WBI, so this is examined that an approach concerns to different toxicity rates as well as local control rates. Several consensus guidelines were reported for HF-WBI and APBI to give guidance to the physicians on the best patients for these different approaches outside the medical trials settings (Smith et al. 2011; Shah et al. 2013). As most of the BC recurrences occur near the cavity of lumpectomy, APBI administered entirely to a small volume of tissues near an original lumpectomy position has achieved greater attention and is discussed now as a replacement to WBI.

18.2.2.1 HF Whole Breast Irradiation (HF-WBI)

WBI treats a whole breast with 2.5 or 3.20 Gy every day for the total dose of about 39 or 42.5 Gy/13–16 fractions over 3–5 weeks (Whelan et al. 2002, 2010; Yarnold et al. 2011) or hypofractionated WBI treats with 40 or 42.5 Gy in 15–16 fractions for 3 weeks (Smith et al. 2011). Hypofractionation, as compared to conventional fractionation, requires a shorter course of treatment, reduced number of fractions, and total dose, and results with lower costs and improved patient convenience. In several past years, so many randomized trials have tested hypofractionation in contrast to conventional fractionation after BCS for the patients of BC (Whelan et al. 2010; Yarnold et al. 2011). For appropriately selected patients, these experiments initially described in 2005 revealed that HF yields overall survival and disease-free survival rates compared to conventional fractionation (Whelan et al. 2010; Yarnold et al. 2011; Haffty and Buchholz 2013). Moreover, recently reported follow-up of 10 year of these trials have revealed that conventional and hypofractionation have similar long-term toxicity profiles (Haffty and Buchholz 2013; Whelan et al. 2010). As a consequence, clinical practice guidelines were published recently, establishing HF as a suitable therapeutic option mostly for early breast cancer patients (Smith et al. 2011). The reasons for the slow adoption

of HF-WBI are multifactorial, together with concerns about struggles achieving the dose homogeneity and reducing acute toxicity while supplying a greater dose per fraction for women with large breasts.

18.2.3 Partial Breast Irradiation (PBI)

WBI is related to the dose-dependent, lung cancer (Taylor et al. 2017) and higher occurrence of cardiotoxicity, with the gradual increase in the hazard over time after exposure. It is very important to give proper attention to long-term toxicity in women who were cured of early breast cancer. PBI is a limited form in which radiation is focused on a tumor volume, the place for the majority of recurrences (Smith et al. 2000). By supplying radiation dose to a reduced selected volume, PBI lowers radiation exposure to organs at risk comprising skin, lung, ribs, heart, and contralateral breast tissue; thereby minimizing potentially late adverse effects. The number of studies have described the higher risk for toxicity (Huo et al. 2016) and local recurrence (Liu et al. 2017; Korzets et al. 2019). Previous meta-analyses have revealed that PBI is related to less number of deaths as well as the absence of BC recurrence (Liu et al. 2017). The main outcome IBTR and chronic and acute toxicities at 5 years with partial breast irradiation are compared with whole breast irradiation in numerous randomized trials. Ipsilateral breast tumor recurrence rates remained similar among WBI and PBI with no notable changes observed with PBI techniques. However, partial breast irradiation has fewer chronic and acute toxicities than WBI (Shah et al. 2021). During past decades, partial breast irradiation has appeared to replace WBI.

18.2.4 Accelerated Partial Breast Irradiation (ABPI)

APBI technology was initiated in the 1990s (Ribeiro et al. 1990) into clinical practice together with many techniques, such as 3D conformal radiotherapy, intraoperative RT with photons or electrons, single brachytherapy, multicatheter, and intensity-modulated radiotherapy. APBI offers several benefits over traditional radiation therapy. Because in APBI, the radiation beams are focused so narrowly, therefore exposure to healthy tissues and organs in the adjacent area containing the lungs, heart, muscles, ribs, and skin can be reduced. With APBI, acute radiation side effects (ARS) are usually minor. After APBI, late radiation side effects (LRS) are fairly less common but comprise adverse breast cosmesis, or fat necrosis, fibrosis, telangiectasia's, and focal skin pigmentation variations (Correa et al. 2017).

BCS together with the whole breast irradiation was gold-standard therapy for the early EBC patients, which can produce cancer outcomes similar to the mastectomy (Formenti et al. 2012a). Whereas whole breast irradiation is mostly well tolerated, it brings the risk of late effects, for example, radiation pneumonitis, secondary malignancy, and cardiotoxicity. Whole breast irradiation is typically carried once a day over several successive weeks, making access to effective radiotherapy challenging

for women suffering from some socioeconomic obstacles. After BCS, recurrence patterns recommend that maximum local recurrences take place mostly at or adjacent to the breast tissue or nearby post-excision lumpectomy cavity (Veronesi et al. 2001). It is predicted that 15–25% (Wenz et al. 2015) of candidates undergoing BCS may be eligible for the APBI, i.e., patients with minor invasive ductal breast cancer (IDBC) without involvement of lymph node. The standards of patient selection having ages above 40, status post-lumpectomy, negative clinical lymph nodes, BC (in situ OR invasive disease) measuring <3 cm, negative margins (at least 2 mm), and no lymph vascular space invasion (LVSI). Women with EBC and their caretakers must be familiar with this favorable treatment option. APBI irradiates only tumor beds in 1–3 weeks (Correa et al. 2017), as it decreases the problem of care so it is a favorable treatment for many patients. Moreover, owing to the reduced irradiation range of accelerated partial breast irradiation, it is predicted to decrease harms and increase life quality and cosmetic effect than WBI (Mouw and Harris 2012).

18.2.5 Deep Inspiration Breath Hold (DIBH)

The BH approach was first reported in treatment BC in 2001 (Sixel et al. 2001) and became well-known (Lai et al. 2020) only recently. Its greatest benefit is that it delivers less amount of radiation dose to the lungs and heart (Lai et al. 2020; Latty et al. 2015). However, the extent of advantages varies individually according to the lung capacity and anatomical features (Latty et al. 2015) of the patients; with the rarity of cases having no benefits (Sixel et al. 2001) or even higher heart doses (Dell'Oro et al. 2019) have been reported. In BC radiation therapy, deep inspiration breath-hold is a very dominant heart-sparing technique.

In BC management, RT has played an important role for decades. On an individual basis, important changes have been observed in the radiotherapy practice focusing on optimized care, owing to the convenience of modern RT technologies. Maximum of the candidates after BCS require postoperative whole breast irradiation with or without a boost to the tumor bed, while PBI is sufficient in low-risk cases. The irradiation of the chest wall (CW) is needed infrequently unless combined with the post-mastectomy irradiation and nodal irradiation.

Most patients with breast cancer are long-term survivors, therefore therapeutic interventions do not endanger the candidates' well-being and overall health. The main dangers due to radiations are lung damage and radiogenic heart resulting in important morbidity numerous decades or years after RT (Darby et al. 2005). The additional risk of cardiac mortality or secondary lung cancer was valued as 0.04 and 0.11 per 1 Gy rise of the radiation dose to a heart and whole lung, respectively (Taylor et al. 2017). Radiation-induced heart disease (RIHD) mostly shows the damage of capillary and coronary vessels of the heart which makes a progressive process of fibrotic leading to circulatory changes with possibly fatal ischemic heart disease (IHD) (Andratschke et al. 2011). The harmful effect of dose-dependent radiation disclosure to the heart has been revealed in retrospective analyses and

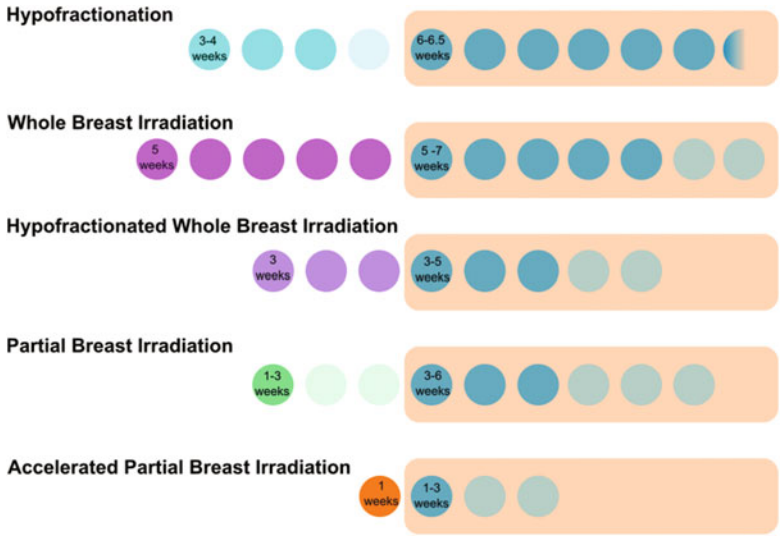

Fig. 18.2 Length of treatments for Hypofractionation, Whole Breast Irradiation, Hypofractionated Whole Breast Irradiation, Partial Breast Irradiation, and Accelerated Partial Breast Irradiation

simulations of radiotherapies of the patients of BC, with IHD (van den Bogaard et al. 2017). The radiation dose to the heart and therefore radiation-induced heart disease (RIHD) incidence is greater in the case of left sides and the hazard is more probable in the young patients (Jacobse et al. 2019). It is expected that every 1Gy dose to the heart increases the IHD incidence by 7.4% that might be enhanced by smoking (Taylor et al. 2017) and pre-existing cardiovascular hazard issues (Darby et al. 2013).

In the systematic evaluation of contemporary publications, a 3.7 and 5.2 Gy dose to heart in right and left sides, respectively, whereas the radiation dose of 9 Gy (Taylor et al. 2017) to an ipsilateral lung is recommended. Hence, a lot of struggles have been put to avoid or reduce the heart exposure.

There are numerous methods to save the heart from exposure to radiation. Breathe hold and prone positioning techniques work by sparing the heart from the radiation fields. The advanced proton irradiation and IMRT approaches are not applied extensively, but the small volume to be treated during PBI or exclusion of RT are choices in case of low risk. These approaches have changeable effects on heart and lung exposures: whereas prone RT lessens lung doses dramatically (Kahán et al. 2018). Figure 18.2 shows the treatment length of five radiotherapy techniques used in the treatment of BC.

18.2.6 Three-Dimensional Conformal RT (3D CRT)

Better computerized treatment planning systems make it easier to develop several treatment plans for a patient. The long-term cardiac toxicity of breast irradiation is one of the most significant issues. The risk of long-term severe cardiac problems linearly increases with the mean radiation dose to the heart, with an estimated risk of 7.4% reported with each 1 Gy rise in mean heart dose. Covering the whole breast parenchyma with a standard 2D tangential beam approach is sometimes difficult. This difficulty can be overcome using the conformal technique (Darby et al. 2013). 3D-CRT treatment is a sophisticated approach that employs imaging technology to produce a three-dimensional image of a patient's tumor as well as surrounding organs and tissues. As a result, malignant cells might be exposed to a larger and more effective dosage of radiation.

A major problem is to improve the radiation dose uniformity to the target tumor volume while reducing the risk of complications related to treatment. In current years, particularly conformal RT, inverse or forward IMRT, which is a sophisticated and more advanced method of 3DCRT, is becoming attractive for breast irradiation because it gives improved normal tissue sparing and reduced inhomogeneity (Cheung 2006). Simultaneously, the radiation dose obtained by the surrounding healthy normal tissues might be considerably lowered. 3D-CRT is utilized to treat cancers that were previously thought to be too near to essential organs and structures for radiation treatment. For example, 3DCRT permits radiation to be administered to malignancies in the neck and head while reducing exposure to the salivary glands, spinal cord, optic nerve, and other vital tissues.

If 3D-CRT is indicated for a patient, a radiologist will use one of the following imaging methods to produce three-dimensional pictures of the tumor and surrounding tissues:

- Computed tomography (CT)
- Positron emission tomography-computed tomography (PET-CT)
- Positron emission tomography (PET)
- Magnetic resonance imaging (MRI)

Because 3D CRT provides for more precise and accurate radiation treatment delivery, it may be suggested for malignancies near critical organs.

18.2.7 Intensity-Modulated Radiation Therapy (IMRT)

IMRT is a new excellent form of 3DCRT and is increasingly adopted for the treatment of BC. As the likelihood of dose inhomogeneity increases for women with large breasts, IMRT is the technique of choice. Patients of left-sided BC are more likely to benefit from this technique to decrease dose to heart, contralateral breast irradiation, and deep-seated tumor bed irradiation. Anatomical complexity and variations in-depth of regional nodal regions have made the breast a particularly

difficult area to treat with radiation. IMRT is a kind of cancer therapy that uses sophisticated computer algorithms to calculate and administer radiations directly to cancer cells from a variety of angles. It enables cancer patients to get greater, more effective radiation doses while reducing harm to healthy tissues and organs surrounding them. This improves the chances of a cure while decreasing the probability of adverse effects.

Firstly, the patient will undergo an imaging test known as a CT scan, which will map her tumor in 3D. Then, by using modern computer algorithms a team of radiation treatment professionals, including physicians and physicists, will calculate and administer radiations directly to the tumor from various angles. A radiation therapist will place the candidate on the treatment table at the start of each treatment session, putting marks on the skin to determine where the radiation therapy will be delivered. Treatment sessions are not painful. The 3D CT scan determines the form, size, kind, and position of the tumor, which instructs the clinician on how to alter the IMRT beam to attack the tumor while avoiding healthy tissues. A moveable gantry of the linear accelerator will provide an X-ray beam to the tumor, and a computer within the accelerator will modify the form of the beam to match the shape of the tumor using a device known as a multi-leaf collimator. By adjusting or modulating the direction and intensity of the radiation beam, the collimator will enable increased dosage to the tumor while protecting healthy tissue from the radiation. As compared to traditional RT techniques, IMRT reduces toxicity to OARs and improves the quality of life. Computerized dose estimates are used in combination with 3D computed tomography (CT) scans to calculate how much radiation should be applied based on tumor shape. Multiple fields of IMRT coming from dissimilar beam directions are commonly combined to produce a custom-tailored dose of radiation that boosts tumor dose while decreasing exposure to normal organs.

IMRT corrects 3DCRT flaws and improves dosage distribution.

- IMRT plans are computer-generated plans that give rise to complicated field forms by using several tiny fields termed beamlets to avoid key normal structures.
- IMRT plans adjust the number of fields and the intensities within each field to provide more precise doses to cancer while saving normal tissues from damage.
- To improve treatment results, IMRT also aids in dosage escalation to the tumor.
- Reduced toxicity also improves the quality of life for many people.

Even when dosages are not raised, IMRT has the potential to minimize treatment toxicity. Thus, IMRT may be a therapeutic option if the patient has already had conventional radiation therapy and is having recurring tumors in the treated region. Radiation treatment, especially IMRT, inhibits cancer cell division and proliferation, therefore reducing or halting tumor development.

Each IMRT treatment plan can be customized for individual cases by varying the collimator angle, gantry angle, and shaping the tangential fields with MLC leaves so that primary tumor volume is adequately covered while OARs are excluded from high dose region simultaneously. Treatment field modification for each patient should be applied with great caution while taking the location of the primary

tumor area, the contour of the breast, and normal tissue anatomy into consideration. Forward treatment planning using the field-in-field method gives outstanding dose homogeneity in the irradiated regions. However, a more advanced IMRT technique, i.e., inverse planning is used in the case of the highly complex target volume (Vicini et al. 2002). Several other IMRT techniques, forward planned step-and-shoot method, arc therapy, topotherapy, static or dynamic MLC-based IMRT, and tomotherapy, can all improve dose homogeneity (Caudrelier et al. 2009). The superiority of IMRT over 3DCRT in terms of dosimetric results is documented in the literature, providing excellent tumor coverage and better sparing of OARs (Fong et al. 2009; Yim et al. 2015; Hall and Wuu 2003). Moreover, dosimetric studies have successfully documented better cosmesis and low skin toxicity using the IMRT technique (Freedman et al. 2006; Pignol et al. 2008). The risk of moist dermatitis was found to be associated with the size of the breast. These trials report a significant reduction in moist dermatitis using IMRT. It was found that more skin toxicity is reported in patients with medium breast volume as compared to large or small breast volume when treated with IMRT (Pignol et al. 2008). Some IMRT approaches are considered better at sparing one structure, while others are good at sparing other structures. There is no disagreement in the amount and orientation of radiation beams being used among different planning studies. An arc of 180°–360° and up to 11 beams are recommended. Because of the use of sharp dose gradients in this technique, an effect due to breathing motion and errors in patient setup procedure for dose distribution must be taken into consideration, when evaluating the benefits of IMRT over the 3DCRT technique (Erven et al. 2008). Expertise in treatment planning beginning from immobilization of tumor to treatment confirmation, rigorous quality assurance (QA), and toxicity evaluation is crucial before executing the IMRT approach to avoid unessential high doses to OARs to obtain tumor coverage.

18.2.8 Volume Modulated Arc Therapy (VMAT)

VMAT is a novel RT technique that supplies a continuous dose of radiation during the rotation of the treatment machine. This is a kind of IMRT where the dosage is delivered in arcs ranging from 0° to 360° as it rotates. As a result, dosage compliance improves as a result of this increase in flexibility. Due to its ability to administer radiation at faster rates, VMAT reduces the entire treatment time to less than 5 min (compared with IMRT treatment which takes approximately 20–30 min). VMAT may be used to create complex plans that are difficult to develop using IMRT. VMAT planning and treatment are precisely challenging and necessitates a high level of accuracy. The VMAT technique, like a three-dimensional conformal therapy, generally takes approximately 20 min. The majority of this time is spent precisely situating the patient. Daily X-ray scans are obtained to ensure that everything is as planned. VMAT employs the same sort of radiation as other forms of radiotherapy.

RT is an essential element of a multidimensional approach to treat BC and, depending on the number of factors, a variety of fractionation schemes and treatment

approaches have been examined and analyzed successively. As reported by published data, VMAT finds limited clinical applications for breast cancer treatment, yet it is possibly the preferred solution applicable to partial or whole breast treatment and conventional or modified fractionation schemes. Also from a technological standpoint, attractive alternatives of the VMAT technique have been tested and tried out by computer software or simulation to investigate upcoming prospects (Cozzi et al. 2017; Liu et al. 2016).

18.2.9 Proton Therapy

Photons employed in conventional radiation therapy have different dosimetric characteristics than protons. Currently, photon (X-ray)-based intensity-modulated external beam radiotherapy is the most frequent radiation treatment for most malignancies. Charged particles, especially intensity-modulated proton therapy, are being used in more effective and noninvasive radiation for malignant diseases as a result of recent technological, scientific, and clinical research developments. Most of the energy of proton beams is concentrated in their outermost range (the Bragg peak), leading to an increase in radiation dose to clinical targets and a decrease in radiation doses to nearby normal tissues. The linear energy transfer (LET) of photon beams is low but in proton beams the spread-out Bragg peak (SOBP) is high. Due to this, proton beam treatment has specific biological advantages over photon radiation (Mohan et al. 2013).

Researchers investigated both the likelihood of recurrence and cardiac toxicity induced by poor radiation dose coverage on lymph nodes (LN) targets in BC (Stick et al. 2017). Forty-one candidates with left-sided BC required adjuvant complete nodal irradiation were compared to "realistic" photon programs. Darby et al. (2013) models were used to estimate cardiotoxicity risk. Twenty randomized controlled trials were used to estimate recurrence risk following a compromise in LN coverage. The increased absolute cardiac morbidity was low. Using photons, the probable further risk of BC recurrence after 10 years was 0.1%, while using protons, it was just 0.02%. It was found that proton therapy can lower the predicted cardiac toxicity risk up to 2.9% and the chance of the return of BC by 0.9% for individual candidates. Several further studies confirmed the dosimetry benefits of protons over current photon techniques (Cuaron et al. 2015; Mast et al. 2014; Bradley and Mendenhall 2018). The lung volume getting 20 Gy (V-20) when using photons was 30% greater than when using protons, and the heart dosage was 4–10 Gy as compared to 1 Gy when using photons (MacDonald et al. 2013). According to available studies, based on normal dosage procedures, proton treatment can retain the mean cardiac dose below or at 3 Gy (RBE), which includes lymphatics, even the most complex chest wall irradiation. It's not clear whether photon-based RT can keep these individuals' dosages at the same lower level. Numerous organizations use different approaches to maximize the distribution of doses. Heart and coronary artery dosage vary widely based on the candidate anatomy, such as the heart's closeness to a chest wall and the chest wall curve.

18.3 Toxicity Concerns

Although BC is the second major cause of cancer mortality among women worldwide, the annual rate of death from this disease has declined steadily over time (Breasted 1930; Rahusen et al. 2002). This substantial decrease is believed to have resulted from increased use of screening mammography and immense improvement in treatment. The use of mammography results in early detection of abnormal growth of tissues is limited to lactiferous ducts, known as ductal carcinoma in situ (DCIS). Soon after the breakthrough of X-rays by Roentgen in 1895, a second-year medical student in Chicago, Emil Grubbe, have claimed to irradiate breast cancer patient in 1896. He safeguarded the adjacent skin surrounding the tumor with tinfoil. Later on, German scientist Herman Gocht delivered radiations to two women with advanced BC cases while guarding the skin with flexible lead (Gocht 1897; Bland et al. 2018). Although toxic after-effects of radiotherapy are generally mild in most women, well-being may be impaired by these effects, at the minimum in the initial period after radiotherapy. One of the major obstacles in the analysis of radiation dose delivered to various parts is that the multitude of parameters is variable, such as patient's weight and height, shape and size of the breast, and treatment modality. Thus, retrospective study of radiations administered to different parts of breast should be interpreted with caution (Schnur et al. 2011; Raj et al. 2006). One study suggested the factors that impact RT-induced toxicity are woman's buildup, such as breast size and weight bring about additional skin folds and spreading within these folds, rather than radiation dose inhomogeneity. So, careful patient positioning is necessary at the time of simulation to eradicate these skin folds as much as can be achieved. Supine position is advocated to remove these folds and decrease spreading (Formenti et al. 2012b). Women undergoing treatment are recommended to routinely use cotton pads placed in the inframammary fold (IMF) to lessen the effect of skin reaction (Back et al. 2004).

18.3.1 Breast Cancer Screening Techniques

The American Cancer Society recommends regular mammographic screening in women ages 55 years and older (Berry et al. 2005). However, in younger women, it is a concern that mammographic screening may be less sensitive due to dense breast tissues. Magnetic resonance imaging is currently under consideration as an alternate screening technique for young women. Nonetheless, investigators are concerned about the False-Positive Rate (FPR) of MRI screening. Although screening mammography does involve an added dose of radiation, the exposure is negligible, so it is a widespread inexpensive early detection tool to aid in the diagnosis of BC. It is reasonable to screen patients for second cancers before their expected development. Most often RT-induced cancers develop within 10–15 years after radiotherapy, so a patient should be screened about 8–10 years after being treated with radiations between ages 20 and 30 years. The likelihood of developing secondary breast cancer before ages 25–30 is very rare (Aisenberg et al. 1997), so a woman treated before

20 may start undergoing screening at the age of 25 (Raj et al. 2006). With new techniques, less radiation is delivered to the heart even to patients which have tumors on the left side. Though new treatment techniques such as IMRT, breathe hold technique, and hypofractionation have limited the radiation-induced cardiac toxicities to large extent, still the skill of radiation oncologist to use the technique remains one of the most important factors in reducing radiation dose to the heart. Evidence from numerous studies suggests that patients irradiated during adolescence have a higher risk of developing secondary BC since the rampant growth of breast tissues during puberty time subject these patients to DNA damage in mammary epithelial cells (MECs). For patients of ages greater than 30 years, only a little increase in the probability of secondary cancer was observed (Aisenberg et al. 1997; Swerdlow et al. 2000). Various studies regarding mantle radiation have demonstrated that for low dose range, i.e., 5 Gy, there is the linear rise in the risk of breast cancer with dose (Mattsson et al. 1993; Little et al. 1999; Boice 2001; Preston et al. 2002). For high radiation dose, i.e., 20 Gy, it was found that high doses were co-related with a high probability of breast cancer development.

18.3.2 Skin Toxicity

Skin toxicity is the most typical acute impact of breast carcinoma. It ranges from erythema (redness) to desquamation (peeling of the skin), necrosis, and ulceration. This scale of skin irradiation is called "skin toxicity." Skin toxicity is observed in 75–100% of patients undergoing radiotherapy treatment. During radiotherapy treatment of breast cancer, skin toxicity may bring about physical irritation, emotional suffering, and body image concerns (Schnur et al. 2011).

Skin toxicity occurring within 3 months post-irradiation is called early reaction and if a reaction occurs after 3 months, it is known as a late reaction. There are approved process of assessment of toxic effects on the skin using Radiation Therapy Oncology Group (RTOG) scoring as depicted in Fig. 18.3 (Wong et al. 2011).

18.3.3 Cardiac Toxicity

Particular awareness has been devoted to the possible cardiac toxicity of breast radiotherapy in recent years. Many young women receiving radiotherapy demonstrate the risk of long-term cardiotoxicity and increased probability of developing secondary breast cancer. While many breast cancer patients completely recover from the disease, there is a serious concern about the long-term risks of treatment. Detection of early-stage disease and improvements in treatment modalities have increased breast cancer survival, but cardiotoxicity, which is one of the major reactions of BC therapy, remains the area of concern. Side effects of cancer treatment using anthracyclines have been established for 30 years, but acute effects of cardiotoxicity due to radiotherapy and chemotherapy have been investigated more recently. Pericarditis, pericardial effusion, and arrhythmias are commonly diagnosed

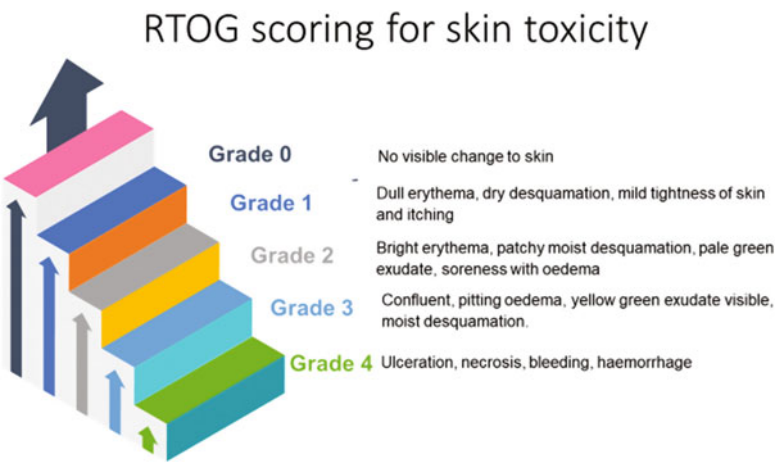

Fig. 18.3 RTOG grading system for skin toxicity

cardiac problems post-radiation therapy. If the radiation syndrome to intimal coronary endocytes and pericardial is severe, it finally leads to fibrosis and myocyte ischemia in the long run. Radiation toxicity to coronary endocytes causes inflammation, ultimately leading to the blood clot. It has been observed that cardiac diseases post-radiotherapy are more frequent in women receiving radiation dose to the left breast, but the situation has changed a lot with current treatment modalities (Yeboa and Evans 2016).

The major drawback of using the older RT approach for treating BC and draining lymph nodes is that a fairly high radiation dose is delivered to heart volume. There exist clear and strong evidence of cardiovascular morbidity and mortality in patients dosed with old techniques. Although radiation exposure to the cardiovascular system is considerably reduced by the use of modern radiotherapy techniques, it is not eliminated. Numerous studies of Hodgkin's disease survivors have indicated the risk of developing secondary BC post-mantle field radiation therapy for this disease. The risk of BC development after mantle field radiotherapy appears to be dependent on factors such as radiation dose administered to healthy surrounding tissues, patient's age at the time of breast irradiation, and if chemotherapy is included in the entire treatment planning process. It is noteworthy that besides late cardiac complications associated with tangential breast radiotherapy; others include rib fracture, lymphedema, pneumonitis, brachial plexopathy, and poor cosmesis. These concerns have led investigators in the modification of radiotherapy techniques to reduce not only the volume but also the dose of incidental cardiac irradiation (Raj et al. 2006).

Recent researches have proposed that the magnitude of heart toxicity has decreased due to the use of more conformal techniques (Giordano et al. 2005). However, perfusion defects are detected even in patients suffering from BC treated with modern RT techniques (Marks et al. 2005). Several studies indicated the hike in the risk of ischemic cardiac events post-radiotherapy for left-sided BC though the

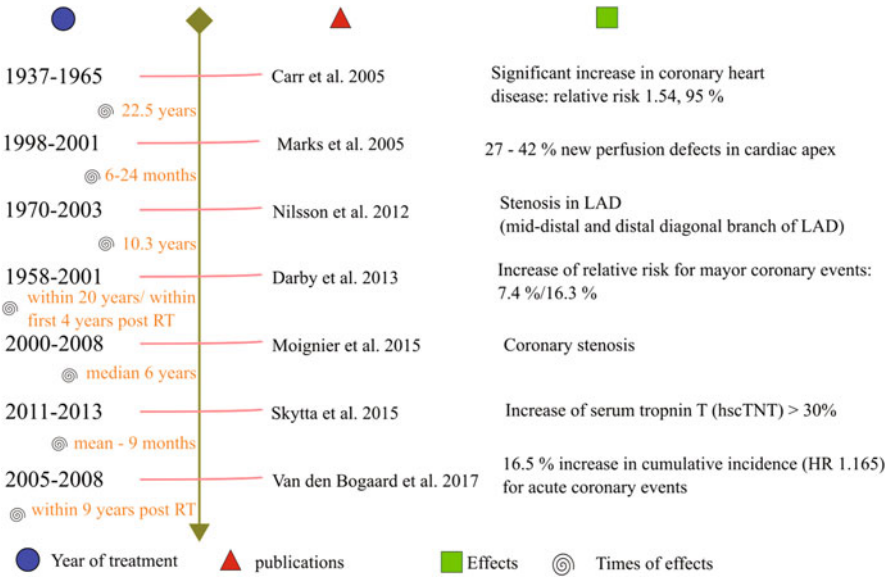

Fig. 18.4 Schematic illustration of publication summary representing radiation-induced cardiac toxicity based on various conclusions (Carr et al. 2005; Marks et al. 2005; Nilsson et al. 2012; Darby et al. 2013; Moignier et al. 2015; Skyttä et al. 2015; van den Bogaard et al. 2017)

exact magnitude of this risk is lowered (Feng et al. 2011). Current explorations have also indicated that other heart risk factors such as smoking, hypertension, and radiation-induced damage may be synergistic in their effects (Harris et al. 2006; Hooning et al. 2007). Therefore, women with a considerable probability of net gain should not avoid radiotherapy just on account of the concerns related to cardiac toxicity. Since minimizing radiation dose to the heart is a worthy endeavor, cautious treatment planning is necessary to ensure that patient's risks are minimized (Jagsi 2014). Figure 18.4 represents a summary of publications to investigate clinical evidence of radiation-induced cardiac toxicities for breast cancer patients.

18.3.4 Comparison of RT Techniques for Breast Cancer

Approaches to decrease radiation dose to the heart are currently under investigation. These techniques encompass respiratory gating and deep inspiration breath-hold, heart blocks, tomotherapy, mixed electron/photon beams, and IMRT. Oftentimes, breast treatment is inevitable for suitable coverage of axillary lymph nodes. There is a dire need to search for different strategies to prevent the development of cancer in such patients. For the present, it is crucial to screen these patients carefully for the development of secondary BC. It is expected that modern treatment methods and modalities, such as mixing photon and electron beams, use of protons, and

cone-beam CT, will cause the decrease in PTV margins, which will eventually result in better sparing of healthy organs (Vicini et al. 2007).

With the increase in the treatment of BC using radiations over time, it is necessary to be well informed about the long-term cardiotoxic effects. Meta-analyses reported the increase in mortality due to cardiovascular causes for women undergone radiotherapy treatment from the 1960s to 1980s (Group 2000). Thus, radiation oncologists and medical physicists face the challenge to develop novel ways of breast cancer treatment without delivering excessive doses to the heart. There have been a lot of advancements in cardiac avoidance which include careful patient positioning and verification, heart blocking, APBI, DIBH, active breathing technique, hypofractionation, IMRT, and use of protons. DIBH is one of the most beneficial techniques in BC RT as it involves gating radiation doses to deliver treatment when the least cardiac volume is in the field (Yeboa and Evans 2016).

Comparison of different modalities reveals that median cardiac volume receiving dose higher than 50% of was reduced from 19% to 3% with DIBH (Lu et al. 2000). Hence, DIBH or inspiratory gating greatly reduces the heart volume and toxicity in the current era. APBI is another modality of cardiac avoidance and treats the fraction of normal breast. Hypofractionation of WBRT has equivalent cosmesis and clinical outcomes. Proton therapy is also widely used as it is also reported to reduce the cardiac dose. Comparison of proton therapy with DIBH photon IMRT reveals that the mean radiation dose to the heart was decreased from 1.6 to 0.009 Gy with the use of proton therapy (Yeboa and Evans 2016).

Technological advancements in radiotherapy have played a crucial role in improving disease control and toxicity outcomes. The aim is to minimize radiation exposure to non-targeted tissues, mainly lung and heart, while enhancing the dose to critical breast cancer tumors, including regional lymph nodes. The choice of treatment modality depends largely on the laterality of breast cancer and patient anatomy. Prone positioning or DIBH is used to achieve physical displacement of the heart from the breast. IMRT and VMAT are preferred over 3D-CRT in cases when a high dose is to be delivered to the target region and at the same time smaller doses to contralateral breast and lung tissues. Proton therapy also improves target coverage and reduces integral doses to OARs. Hypofractionation scheme further improves the outcomes, but at least 10 years of follow-up is required to confirm the expectations (Erven et al. 2008).

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Nanoparticles: Emerging Diagnostic and Therapeutic Agents for Breast Cancer Treatment

19

Ramish Riaz and Abdullah Ahmad

Abstract

Despite considerable advances in chemotherapeutics, mortality rate of breast cancer is still on the rise. This is largely due to shortcomings associated with chemotherapeutics medicines. Firstly, majority of medicines lack specificity, second most of them get solubilized before reaching the target resulting in reduced bioavailability of drug at target site. Introduction of nanotechnology in medicine offers some promising solutions. Since nanoparticles have small size with at least one dimension less than 100 nm, they have unique interaction with biological systems at molecular level. Their large surface to volume ratio gives an excellent opportunity to manipulate them for desired properties, i.e., targeting cancer cells, loading drug and genetic materials, controlled release, increasing cellular uptake thereby increasing selectivity and bioavailability to target site. By targeting cancer cells, nanomedicine can help in reducing effects of systemic toxicity of chemotherapeutic drugs. Both metallic and non-metallic nanoparticles are currently studied as emerging therapeutic platforms against breast cancer therapy. Not only this, but nanoparticles can also be used as a platform to construct a delivery system where therapeutics and diagnostics can be integrated leading to the development of theranostic (Therapy + Diagnostic) nanoparticles. In this chapter, we have discussed in detail the potential of these nanoparticle-based treatment, its pharmacokinetics, their mechanism of controlled release, and their application as theranostic agents in breast cancer.

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Keywords

Breast cancer · Nanomedicines · Nanoparticles · Theranostic agent · Active targeting · Passive targeting

Scientists have been struggling to understand the pathophysiology and molecular mechanism to diagnose and treat breast cancer since its first documentation 3500 year ago by Hippocrates and Galen. Till now, the disease is not unraveled fully and is still a topic of research (Thorat and Bauer 2020). Even in the year 2020, incidence of breast cancer was highest compared to other cancers in females and remained second leading cause of death among cancer-related deaths in females. Though changes in mortality trend have been noted. Survival of breast cancer is much higher in developed countries compared to under-developed countries, where it is still the leading cause of death. There is wide disparity in mortality due to breast cancer among different nations (Sung et al. 2021). Developed nations like the USA have 5-years survival rates up to 90% (Siegel et al. 2021). This is largely attributed to nationwide screening coverage as well as advances in therapy. There had been dramatic advancement in systemic breast cancer therapy in the last 20 years. Introduction of aromatase inhibitors, monoclonal antibodies and immunotherapy have shown promising results in controlling breast cancer. However, stage at time of diagnosis still remained the strongest predictor of prognosis and survival of breast cancer (Trimboli et al. 2020). Once the cancer metastasizes, rate of mortality increases dramatically and availability of treatment options starts diminishing either due to non-suitability for systemic therapy or lack of effectiveness as a result of drug resistance. Therefore, we are still in dire need of new therapeutic regimen that will help in tackling this problem (Wu et al. 2017).

To tackle these issues, nanotechnology offers some solutions. Since nanoparticles have small size with at least one dimension less than 100 nm, they have unique interaction with biological systems at molecular level. Their large surface to volume ratio gives an excellent opportunity to manipulate them for desired properties, i.e., targeting cancer cells, loading drug and genetic materials, controlled release, increasing cellular uptake thereby increasing selectivity and bioavailability to target site (Wu et al. 2017). By targeting cancer cells, nanomedicine can help in reducing effects of systemic toxicity of chemotherapeutic drugs. Moreover, by entrapping drug inside nanocarrier can help in increasing stability, increasing bioavailability, and controlled release kinetics. Their excellent carrier properties lie in their high tissue penetration capability, enhanced permeability and retention effect, biocompatibility, non-toxicity, and prolonged circulation time (Wang et al. 2020a). This chapter discusses in depth about types of nanomedicines, their mechanism of targeting, their role as carriers, as diagnostic and theranostic agents.

19.1 Types of Nanomedicines

Over the last few years, nanotechnology has been introduced in multiple fields and has revolutionary impact with increasing number of applications and products based on nanomaterials being available. Similarly, the field has also dramatically impacted medicine and pharmaceutical research. The term nanomedicine is specifically defined as the use of nanomaterials for diagnosis, prevention, control, and treatment of diseases. Research in nanomedicine is currently recognized as "Key Enabling Technology" capable of providing innovative solutions to medical problems by European Union (Pita et al. 2016). Currently, nanomedicines are classified into two major classes depending on the type of material, i.e., organic nanomedicines and inorganic nanomedicines (Fig. 19.1) (Ahmed Hamed Khalil et al. 2020).

19.1.1 Organic Nanoparticles-Based Nanomedicines

Organic nanoparticles have been widely explored as nanocarriers for drug delivery and targeted therapy due to their unique properties, i.e., the ability to entrap drugs and functionalization property by manipulating surfaces via conjugating moieties like targeting ligands, fluorescent dyes, and therapeutic agents. Most commonly applied organic nanoparticles include liposomes, polymers, and dendrimers (Bor et al. 2019).

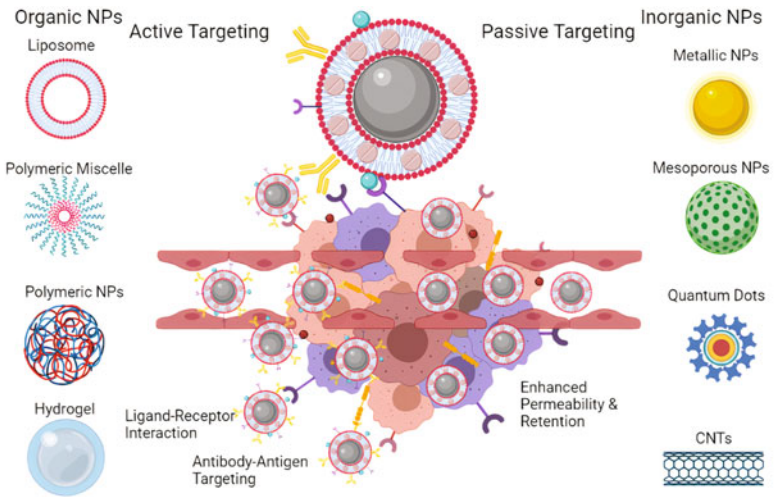

Fig. 19.1 Types of nanoparticles and their mechanism of targeting; active targeting via ligand–receptor or antigen–antibody interaction; and passive targeting via enhanced permeability and retention effect

19.1.1.1 Liposomes-Based Nanomedicines

Liposomes are lipid vesicles having lipid bilayer with core usually having aqueous solution. Mostly composed of phospholipids and cholesterol. Phospholipid bilayer entraps the drugs and increases its solubility, bioavailability, and therapeutic efficiency. Cholesterol helps in reducing fluidity and stabilizes the structure in blood (Afzal et al. 2021). Liposomes stay longer in blood stream compared to non-liposomal drugs thereby provide extended release of drug increasing the bioavailability of drug for a longer time. Liposomes have the ability to carry both hydrophobic and hydrophilic drugs. They can accumulate naturally at the site of tumor and can deliver higher amounts of drug at target. They are usually cleared via hepatobiliary system. Nearly 50–70% of the drug uptake occurs by reticuloendothelial system (Lee Ventola 2012).

Liposomal drugs are the oldest among nanoparticle-based therapy. The first drug to get approved by FDA in 1995 was Doxil®, a PEGylated liposomal formulation of doxorubicin. Doxorubicin is an effective chemotherapeutic drug however has been known for cardiotoxicity. Encapsulation by liposome resulted in significant reduction of cardiotoxicity without effecting its therapeutic efficiency. Coating with PEG helps in increasing its half-life and reduction in RES uptake. Drug is known to be one of the successful products of nanotechnology with marked tumor suppression activity and increased survival (Park 2002; Wu et al. 2017).

With success of Doxil® many chemotherapeutic drugs have been encapsulated in liposomes like lipoplatin® liposomes having cisplatin. Currently, 10 out of 20 approved nanomedicines are liposome based (Ahmed Hamed Khalil et al. 2020). Another interesting application in targeted delivery is making immunoliposomes. Anti-HER2 (Human Epidermal Growth Factor) liposomes have shown positive results in HER-positive breast cancer. Study by Tang et al. formulated doxorubicin entrapped in HER2-targeted immunoliposomes. They also studied combinational regimen by combining these immunoliposomes with another liposomal drug encapsulating bevacizumab an anti-angiogenic drug. Both in vitro and in vivo results showed significant reduction in tumor size and great potential to be used in HER+/MDR double-positive breast cancer (Tang et al. 2017).

19.1.1.2 Polymer-Based Nanomedicines

Polymer-based nanocarriers have been widely investigated for drug and gene delivery. Both synthetic and natural polymeric nanoparticles have been explored for drug delivery. Polymeric nanoparticles provide better stability and prolong circulation when compared to liposomal formulations. This makes them promising candidates as nanocarriers. However, till now none of the polymer-based drug is approved for breast cancer (Ahmed Hamed Khalil et al. 2020). Though they are approved for other types of cancer. Studies are undergoing to check the effect of these drugs on breast cancer. One such study on poly(lactic-co-glycolic acid)-block-polyethylene glycol (PLGA-PEG) nanoparticles conjugated with AXT050, a collagen-based peptide with anti-angiogenic and anti-tumor properties showed inhibition of adhesion and proliferation in triple-negative breast cancer cell line via binding to integrin $\alpha_v\beta_3$ (Bressler et al. 2018). Apart from polymeric nanoparticles, polymeric micelles

are also currently investigated. Micelles are usually amphiphilic block copolymers with hydrophobic core and hydrophilic shell therefore has the capability to encapsulate both hydrophobic and hydrophilic drugs. They can carry high pay load and shows enhanced permeation and retention (EPR) effect at the site of tumor (Afzal et al. 2021). One such study involving doctaxel-loaded poly (styrene-maleic acid)-poly (amide-ether-ester-imide) copolymeric nanomicelles-targeted Raloxifene receptor showed significant inhibition of tumor activity and increased animal survival (Enteshari et al. 2018).

19.1.1.3 Protein-Based Nanomedicines

Protein-based nanomedicines are famous for their biocompatibility and biodegradability. A variety of proteins have been employed as nanocarriers including ferritin, apoferritin, albumin, gelatin, heat shock protein, collagen, and whey protein. First drug to get approval by FDA in 2005 for breast cancer treatment was Abraxane®, Paclitaxel containing albumin nanoparticle (Bor et al. 2019). Protein cages are the recently developed carriers used for drug delivery. They are based on protein building block composed of limited number of sub-units which assembles to form ribbons, chains, or nanosphere-like structures. Protein cages compared to other protein-based structure have uniform size which allows uniform drug distribution and shows less agglomeration. Protein cages in nanometer size can escape macrophages and stick on cancer cells providing prolong effective treatment (MaHam et al. 2009).

19.1.2 Inorganic Nanoparticle-Based Nanomedicines

Inorganic nanoparticles due to their unique properties have recently gained attention. Their unique properties make them suitable for both imaging and therapeutic applications. Though they have limited capacity to carry drug, they show advantage of control of drug release via provocation. A variety of inorganic nanoparticles are currently under trials. Broadly they are divided into metallic and non-metallic nanoparticles.

19.1.2.1 Metallic Nanoparticles-Based Nanomedicines

Use of metals like gold and silver in therapeutics dates back to 2600 BC, when Egyptians believed in curing power of gold for all types of physical, mental, and spiritual illnesses. In last decades, with advancement of nanotechnology, gold nanoparticles (GNPs) became a topic of research with lots of papers focusing on diagnostic and therapeutic properties of gold nanoparticles (Faa et al. 2017). Gold nanoparticles show very high absorbance and fluorescence properties as compared to bulk material. Their optical properties, the phenomenon of surface plasmin resonance, and ability to generate ions upon interaction with radiation makes them an ideal material for as optical sensors and photoablation therapy (Lee et al. 2014). Recent study showed promising results of nanophotolysis therapy in breast cancer. The study utilized gold nanoparticles that irradiate with laser beam in visible region

for the production of ions. Increased cell death was observed with an increase in the number of gold ions.

Another common metal currently under trials is superparamagnetic iron oxide nanoparticles. Since the particles show superparamagnetic behavior, they act as both contrast agent for magnetic resonance imaging and agents to be stimulated in magnetic hyperthermic treatment (Lee Ventola 2012). They have long circulation time, are biodegradable, and have low toxicity. Magnetic hyperthermia is already under clinical trials for cancer treatment. For breast cancer, different SPION formulations are under study (Moreira et al. 2021). They are used as drug carrier or alone as hyperthermic agent, both via optical stimulation or magnetism (Talluri and Malla 2019).

Quantum dots also have gained recent attention in oncology. They are semiconductor nanocrystals with size range of 2–10 nm. They are famous for their fluorescence properties. They emit 20–50 times more light than conventional fluorophore which makes them ideal for in vivo applications. They have large surface area for attachment of drugs and functionalization with other ligands. Most commonly studied quantum dots are cadmium selenide and cadmium telluride. However, their amount to be administered in body is limited due to toxic nature. Currently, studies are focused to reduce its toxicity via coatings, and generation of other novel QDs to utilize its full potential in therapy (Lee Ventola 2012).

19.1.2.2 Non-Metallic Nanoparticles-Based Nanomedicines

Among non-metallic nanoparticles, carbon nanotubes have fascinated extensive attention. The CNTs being famous for their high surface area can bear up high payloads. Also, they have unique mechanical and optical properties (Afzal et al. 2021). Liu et al. studied hyaluronic acid (HA)-modified amino single-walled CNTs for delivery of doxorubicin. Since HA specifically binds to CD44 cells, formulation showed a significant increase in delivery of doxorubicin in CD44 overexpressing MDA-MB-231 cells and also inhibited their proliferation and induced apoptosis resulting in decreased breast cancer cells growth (Fabbro et al. 2012).

19.1.3 Hybrid Nanoparticle-Based Nanomedicines

Combining the organic and inorganic nanoparticle-based nanomedicine provides advantages of both and overcomes the limitations of individual nanomedicines and helps in achieving desired results. Especially, with an increase in trend of theranostic nanoparticles, hybrid particles are ideal materials. In most of the scenarios, drug-loaded polymeric nanoparticles or liposomes are functionalized and attached with metallic nanoparticle to make them both therapeutic and diagnostic agents. Though none of them are FDA approved, many of them are under clinical trials which hold potential to change conventional breast cancer therapy (Afzal et al. 2021).

19.2 Pharmacokinetic of Nanomedicines

Pharmacokinetics of nanomedicines, i.e., ADME, adsorption, distribution, metabolism, and elimination vary considerably from conventional therapies. The pharmacokinetic properties of nanomedicines depend on physicochemical composition and routes of administration.

19.2.1 Routes of Administration and Adsorption Methods

Nanomedicines can administer all the routes including oral, systemic, transdermal, and nasal. However, for nanomedicines used in breast cancer majority are either administered orally or given via intravenous injection. Orally administered drugs need to get absorbed from intestinal lumen to reach systemic circulation. For this, first barrier for nanomedicines is mucosal membrane covering the luminal surface of intestine (Moss and Siccardi 2014). Studies have shown that nanoformulations with size less than 200 nm can cross mucus membranes much easily compared to large-sized formulations. Not only physical barriers, nanoformulations also have to survive harsh variations of pH in GI tract, metabolizing enzymes and surfactants. Nanomedicines trapped inside the coating, i.e., polymeric capsules, gels, prevent premature release and degradation of these drugs. Most of the hydrophobic nanoformulations with size less than 300 nm get adsorbed by enterocytes while more than 500 nm enter via payer's patches in duodenum (Wang et al. 2020b). Macropinocytosis, caveolae, and clathrin-mediated endocytosis are the most common mechanisms of absorption of drugs administered via oral route.

19.2.2 Distribution of Nanomedicines

Once inside the systemic circulation, distribution of nanomedicine to tissues starts. Distribution of drug to various tissues depends on nanoparticle's size, shape, charge, chemical composition, coating, and also on individual variability. Nanoparticles are rapidly phagocytosed by the macrophages of liver, therefore to increase the circulation time and bioavailability of nanomedicines, most of them are coated with hydrophilic polymers like PEG. Inside the circulation, nanomedicine comes in contact with a variety of proteins. It is difficult to predict the nature of interaction between nanomedicines and circulatory proteins due to a variety of protein and their binding affinity with a variety of formulations. However, tissue distribution of nanomedicines usually occurs via diffusion through process-like enhanced permeation and retention (Moss and Siccardi 2014). Delivery of nanomedicine to breast cancer tissue can be achieved via both active and passive targeting (Fig. 19.1).

19.2.2.1 Passive Targeting to Breast Cancer

Nanomedicines tends to accumulate in cancerous tissues compared to normal ones. This occurs due to the increase in leaky vasculature inside the cancerous cells. Due to

repeated angiogenesis and inflammation, tumor vasculature has increased permeability which increases the accumulation of nanomedicine in the target area. This is also accompanied by poor lymphatic drainages inside the cancerous tissue resulting in less clearance of nanomedicines. Tumor vasculature has pore size of 100 nm to several hundred nanometers, rendering the nanomedicines to easily pass through them. This is not possible for conventional medicines. This EPR effect alone increases the bioavailability of nanomedicines several times compared to standard chemotherapeutic drugs. Within tissue, cellular uptake of nanomedicine occurs via passive endocytosis mechanism like micropinocytosis. Majority of currently available nanomedicines rely on passive targeting though research is undergoing on active targeting to further enhance its cellular delivery (Attia et al. 2019).

19.2.2.2 Active Targeting to Breast Cancer

To increase the cancer targeting and increased delivery, active targeting of nanomedicine is the most studied technique. For targeting, nanomedicines can be decorated with targeting moieties, i.e., receptor ligands, monoclonal antibodies having affinity for target which could be tumor or tumor microenvironment. Expression of an ideal target should be higher in cancerous tissues than normal ones so that it should be accessible to nanomedicine such that surface receptors rather than intracellular receptors, also it should preferably support delivery process, i.e., transport inside the cell and correlate with malignant behaviors (Wu et al. 2017).

In breast cancer research, active targeting of nanomedicines has gained much attention. Many overexpressing receptors have been tried to be targeted. In one such study, Tang et al. designed a targeted dual drug delivery system, where he encapsulated anti-angiogenesis drug bevacizumab in liposomes and chemotherapeutic drug doxorubicin inside immunoliposomes decorated with Human Epidermal Growth Factor Receptor 2 (HER2) antibody. The combinational therapy was tested both in vivo and in vitro and results showed promising growth inhibitory effect (Tang et al. 2017).

In another study by Mamot et al., immunoliposomes against multidrug-resistant cancer cells overexpressing epidermal growth factor receptor were tested. Immunoliposomes were loaded with doxorubicin and conjugated with monoclonal antibody fragments (cetuximab and matuzumab) directed against EGFR. Targeted immunoliposomes showed 4–8 times more accumulation inside the cell compared to free doxorubicin which was effluxed out by MDR-resistant cells. Also, the designed construct showed 20–200 times more cytotoxicity to cancerous tissue compared to free drug (Mamot et al. 2012).

Active targeting is now not only limited to receptors expressed by cancerous cell, but also towards targeting breast cancer stem cells and tumor microenvironment. This along with growth inhibition also helps in preventing cancer recurrence which is one of the goals in breast cancer therapy (Wu et al. 2017).

19.2.3 Nanomedicines Metabolism and Elimination

Most of the nanomedicines especially non-metallic nanoparticle-based medicines are biodegradable and undergo chemical or enzymatic degradation once inside the body. Degradation kinetics also determine the release profile of drug contained in nanovesicles. Non-degradable carriers get eliminated via renal or hepatobiliary clearance. Inorganic small nanomedicines usually undergo renal clearance via glomerular capillaries having endothelium with basement membrane having podocytes for phagocytosis. Usually, particles less than 5.5 nm easily undergo renal clearance; however, bulk of nanomedicines ranging from 10 to 100 nm undergo hepatobiliary excretion. Research indicate that nanomaterials undergo transcytosis into hepatocytes, from where they enter biliary canaliculi and getting releases in bile ultimately leading to elimination via feces. This process can take hour to days (Poon et al. 2019). Since there is large variation in clearance mechanism of nanomedicine, there is need of further studies to compare the mechanism of clearance depending on types of nanomedicines.

Few studies have demonstrated another phenomenon known as accelerated blood clearance (ABC) of nanomedicines occurring in response to interaction of nanomedicines with immune cells. Liver is a pool of macrophages. The phenomenon has been studied in depth in response to administration of second dose of PEGylated nanomedicines. It was first time explained by Dams et al. who observed that administration of second dose of PEGylated liposomes with 5–20 days of first dose in rhesus monkeys resulted in rapid clearance of PEGylates liposomes (Dams et al. 2000). Later studies demonstrated that this occurs due to the formation of anti-PEG IgM antibodies inside spleen. If second dose is administered within few days when blood has levels of circulating antibody, then it will bind with PEGylated nanomedicines and will activate complement system. This will result in the formation of C3 fragments leading to opsonization of nanomedicine by Kupffer cells resulting in accelerated clearance. Recently, it has been found out that spleen is not the only source of production of these antibodies. Lymphoid tissue could also play a role in the formation of anti-PEG antibodies. This speculation is based on the production of these antibodies in patients with splenectomy. The phenomenon is not only restricted to PEGylated nanomedicines (Abu Lila et al. 2013). It has been observed that some other nanomedicines also undergo rapid clearance due to the formation of anti-drug antibodies. Since clearance of nanomedicines is a complicated phenomenon depending on size and type of nanocarrier used, generalized approach is not applicable. And there is need to do further studies on the type of nanomedicines and their clearance mechanisms (Ilinskaya and Dobrovolskaia 2016).

19.3 Nanoparticle as Drug Delivery Vehicles for Breast Cancer

Currently, breast cancer is treated by combinational therapy including mastectomy, radiation therapy, and chemotherapy. Mastectomy and radiation therapy are usually offered to patients with local diseases; however, advance disease usually requires

chemotherapy. Standard chemotherapeutic drugs suffer from drug resistance. Nanomedicine-based Doxil and Abraxane has been successfully applied for the treatment of breast cancer since over the last few years. However, both of them are general anti-cancer delivery carriers not specifically designed for the treatment of breast cancer. Recently, with successful introduction of humanized anti-HER2 monoclonal antibody, Trastuzumab interest has been grown in targeting specific molecular pathways. Nevertheless, the challenges for conventional targeted therapy remained same. Major hurdles involved in efficient treatment includes lack of bioavailability of drugs due to undesirable pharmacokinetics, drug resistance due to efflux transport, ineffectiveness in tumor microenvironment due to hypoxia, low pH, and cancer cross-talk and lack of capability in eradicating breast cancer stem cells. Nanoformulations offer solutions to overcome these challenges by improving cellular uptake, simultaneous targeting of cancer cells and microenvironment, triggered release mechanisms and improved bioavailability of drugs (Wu et al. 2017).

One such study was conducted to normalize tumor microenvironment and improve response to immunotherapy by Panagi et al. He encapsulated Doxil with TGF-β inhibitor Tranilast. Tranilast is an approved anti-fibrotic and anti-histamine drug which improves perfusion and oxygenation thereby improving anti-tumor immunity. Introduction of this combinational nanomedicine in triple-negative breast cancer mouse models significantly improved the oxygenation and treatment efficiency depicted by reduction in tumor size. Also improvement in immune response was noted by infiltration of immunostimulatory macrophages M1 and T cells in the cancerous tissue which usually migrate away in immunosuppressive environment created by cancer-associated fibroblasts (Panagi et al. 2020).

Breast cancer stem cells have been found to induce resistance and disease relapse after chemotherapy. Conventional chemotherapeutic treatments are found to be ineffective in treating breast cancer stem cells. Different studies have shown potential of nanomedicines against stem cells (Gao et al. 2020). Yang et al. fabricated PLGA nanoparticle-based co-delivery system for paclitaxel and curcumin targeted to breast cancer stem cell by attaching a lipoid (HA-HDA) to the surface of nanoparticles. HA-conjugated nanoparticles interact with CD44 receptors on breast cancer stem cells thereby help in delivering drug to these cells. This targeted co-delivery system killed both breast cancer cells and breast cancer stem cells. It resulted in reduction of mammosphere formation of breast cancer stem cells and inhibited their migration. Combinational therapy also suppressed EMT signaling. Results indicated strong therapeutic efficiency both in vivo and in vitro (Yang et al. 2017).

Given the heterogeneity of breast cancer, cocktail therapies hold more potential than single regimen-targeted therapies. Yet there are several factors needed to be considered before designing combination therapy. Firstly, all the selected drugs must be synergistic without overlapping toxicity and mechanism of action. They should be able to target both cancer cells and cancer stem cells. There should not be any cross-resistance (Gao et al. 2020). One such triple nanodrug delivery system was designed recently by Sahli et al. against Triple-Negative Breast Cancer (TNBC), one of the most aggressive breast cancers. He co-encapsulated paclitaxel, combretastatin

A4 (CA4), and verteporfin in lipid-polymer hybrid nanoparticles. Paclitaxel is a known chemotherapeutic drug with anti-proliferative potential but results in multi-drug resistance and induction and migration of cancer stem cells resulting in chemoresistance, relapse, and remission. Combretastatin A4 is vascular disrupting agent and is under Phase III trials, so far it has shown potent anti-angiogenic activity. CA4 has also shown to be effective tubulin-binding chemotherapeutic agent and has shown anti-cancerous activity. Verteporfin is an FDA-approved agent for the treatment of macular degeneration and is found to have anti-tumor activity by inhibiting cellular proliferation via cytoplasmic sequestration of Yes-Associated Protein (YAP). YAP has been found to be potent driver of cancer stem cells. Its expression is directly linked with metastatic potential of breast cancer. This cocktail approach not only inhibited TNBC cellular viability and migration but also inhibited paclitaxel-induced CSCs enrichment. This is attributed to the effect of verteporfin which downregulated the expression of YAP. Triple nanodrug delivery system effectively reduced tumor growth, eliminated cancer stem cells, and angiogenesis. In vivo results were quite promising in terms of inhibition of cancer with tolerability of drug and no toxic effects (El-Sahli et al. 2021).

Another major advancement with nanodrug delivery systems is controlled release of drug at target site which not only increases the bioavailability of drugs at target tissue but also helps in reducing side effect to other tissues. Most of these systems are based on triggered release of drug once it reaches the target by some external stimuli.

19.3.1 Triggered Release of Drugs by Nanocarriers

The delivery of drugs via nanocarriers ensures more bioavailability, less initial concentration, and even controlled release. The development of different types of biocompatible nanocarriers in previous decades have opened avenues to deliver targeted, and more efficient delivery of drugs inside the body as these nanoscale carriers provide multiple advantages as compared to conventional macroscale drug delivery systems. Nanocarriers provide high surface to volume ratios and thus a high concentration of drugs or ligands can be attached to these nanocarriers. Similarly, the small size ensures higher tissue penetration rate as compared to macro-sized delivery systems and also ensure long systemic circulation times. Tunable nanoparticles can be designed to deliver drugs in a controlled fashion, and this ensures better pharmacokinetics. Different mechanisms involved in controlled release of drug includes:

19.3.1.1 Controlled Degradation

Polymeric nanoparticles are the primary choice for the development of drug delivery systems as they are usually "biodegradable," easy to synthesize, biocompatible, and provide multiple ways to control drug release. Primarily, drug release from polymeric nanoparticles takes four different routes. The first is diffusion through pores in which the nanocarrier is designed in such a way that there is multiple water-filled pores within the nanoparticle and the drug molecules encapsulated within the polymeric nanoparticle diffuse out via these pores (Sánchez et al. 2020). This

route is usually taken where the polymer matrix is degradable, and the water-filled pores develop and increase in size over time as the polymer degrades. The second one is again diffusion but this time via the polymer matrix. In this mechanism, the drug molecules directly diffuse out of the polymeric system as the polymer is usually non-degradable. In this case, the rate of release is influenced primarily by the nature of the polymeric material and not by the concentration gradient and thus the rate of release remains constant (Kamaly et al. 2016). The third way of drug release is via osmotic pumping of drug outside the nanocarrier. The water is allowed inside the pores of the nanocarrier via convection force and the osmotic pressure build up drives the drug molecules out of the system (Keraliya et al. 2012). The last one is via erosion of nanocarrier which can either be surface erosion or bulk erosion. The surface erosion, as the name suggests, is the erosion of the nanocarrier from outside to the inside. The surface tends to erode slowly thus releasing the encapsulated drug. Surface erosion has many advantages as it is reproducible and since it is not dependent on water infusion, hydrophobic drugs remain stable. In case of bulk erosion, water penetrates the bulk of the polymeric nanocarrier leading to erosion and drug release. It is much less reproducible and predictable as compared to surface erosion (Kamaly et al. 2016).

19.3.1.2 pH-Triggered Release Mechanism

Both organic and inorganic drug release can be controlled by pH. In case of pH-responsive systems, the concept of altered pH at infection sites is utilized. The altered pH at target sites results in morphological changes in liposomes resulting in drug release. These morphological changes are induced by protonation/deprotonation depending on the target site pH. Tumor microenvironment is slightly acidic which supports drug release. Studies are focused to design nanomedicines responsive to acidic pH. To achieve this, cationic polyamines have been extensively studied since acidic environment induces electrostatic repulsion in dendrimers due to protonation of amine groups. Another technique is to build nanomedicines with pH-responsive cross-linkage, i.e., hydrazine bond which dissemble at specific pH resulting in drug release (Liu et al. 2016).

19.3.1.3 Enzyme-Sensitive Release Mechanism

The other way is drug release via enzymatic stimulus. In elevated enzyme expression systems such as tumor sites, this mechanism is efficiently employed. The esterase and proteases are usually the means to cleave amides and peptides within the DDS resulting in drug release. Lipase- or protease-sensitive prodrugs are developed which upon contact with high expression levels of these enzymes (usually in the tumor microenvironment) are hydrolyzed and form cytotoxic by-products (drug) which are detrimental to surrounding cancer cells. For example, PMA-KP9 which is a conjugate of glutathione reductase, and a disulfide-linked-polymer-oligopeptide was encapsulated into the liposomes for the delivery of cargo through an enzyme-responsive cascade-release system. Glutathione has been known to be a significant cellular antioxidant which helps to cleave the disulfide-linkages (S–S) and also prevents the free radical damage. Glutathione reductase (GR) is involved in the

oxidation of glutathione disulfide (GSSG) as a result of which reduced sulfhydryl form of glutathione (GSH) is obtained. Glutathione disulfide was converted into its sulfhydryl form through the catalysis of encapsulated glutathione reductase along with the subsequent GSH-mediated cleavage of peptide-conjugate-disulfide bonds in order to release the encapsulated oligopeptides. This enzyme-cascade system resulted in 50–70% release as compared to the standard samples where only 20% release was observed at 37 °C even after 24 h. Also, it must be noted that GSSG converts to GSH only at 37 °C (Chandrawati et al. 2011).

19.3.1.4 Redox-Responsive Release Mechanism

Redox-responsive liposomes have also been recently investigated which exploit electron transfer reactions for numerous drug delivery applications. Different parameters such as potential difference, removal of the cross-links, change in hydrophilicity of the amphiphiles, and chemical reducing agents can be optimized to destabilize such vesicles (McCarley 2012). The activating stimulus for intracellular drug delivery as well as for tumor targeting can be both activated through high redox potential differences (100–1000 folds) existing between the reducing intracellular environment and oxidative environment of extracellular space (Candiani et al. 2010). The most commonly used redox-oxidation system involves the disruption of thiolytic reducing agents through disulfide-linkages within the amphiphilic region, for example, dithiothreitol (DTT). Critical micelle concentration of thiolytically cleaved amphiphilic by-products is increased significantly following the reduction reaction (McCarley 2012).

19.3.1.5 Physical Stimuli-Triggered Release Mechanisms

The release of nanomedicines can also be initiated by some physical stimuli such as light. When using light as a stimulus, the activating photos should pass safely through the living tissues so that they can start the initiation process. Typically, light within wavelength range of 600–900 nm considered as transmissible to penetrate deep into the living tissues because of small absorption coefficients and low scattering (Conceição et al. 2013). Photodynamic therapy is another advanced therapeutic strategy for the treatment of cancer, and it utilizes photo-sensitizing molecules having different intensities, wavelengths, and pulse durations as activating agents for direct killing of the cancerous cells or selective release of the drug from a carrier vehicle (Lee and Thompson 2017).

19.3.1.6 Diffusion Mechanism in Nano-Emulsions

Nano-emulsions are basically made after the biphasic dispersion of two liquids which are immiscible, either oil in water or water in oil which is then stabilized through an amphiphilic surfactant. These have been investigated as an ultrafine dispersion which can be applied for a wide variety of biomedical applications such as drug delivery because of their viscoelasticity, visual properties, and differential drug-loading capabilities. The drug release from nano-emulsions passively is triggered according to the Fick's first law as given by the following equation:

$$t_{1/2} = 1/4 \times 0.0585 r_2 \, K_{OW} \, D$$

where, $t_{1/2}$ is time which is required by half of the drug to diffuse out of the emulsion, r is radius of droplet, D is diffusion coefficient of the drug, and K_{OW} is oil-water partition coefficient (Singh et al. 2017). In case of highly lipophilic drugs, the release is possible only when the oil component of the emulsion is digested away.

Nano-emulsions are currently the most successful route of drug administration among all the nanocarriers with multiple drugs currently under clinical trials. The applications of nano-emulsions in drug delivery can take all the possible routes of drug administration, i.e., nasal, topical, oral, ocular, and injections (Tayeb and Sainsbury 2018). The reason for such extensive applications in drug delivery is the increased bioavailability as well as a better medium for hydrophobic drugs, for example, Quercetin is a natural compound of the flavonoid family and is used in wound healing, but it has very low skin penetration, bioavailability, and aqueous solubility. However, its skin permeation and bioavailability is significantly improved when loaded with nano-emulsions formed via spontaneous emulsification (Fasolo et al. 2009).

19.4 Nanoparticles as Theranostic Agents for Breast Cancer Treatment (Diagnosis + Therapy)

As described in previous sections, nanoparticles are an effective means of drug and gene delivery against cancer. Nanoparticles are also currently being employed for diagnosis of various types of cancers including breast cancer. Currently, mammography is the most commonly employed technique for the diagnosis of breast cancer; however, 20% of new breast cancers are not visible on a mammogram (Astley 2014). Mammography also has varied diagnostic ability in one type of breast cancer as compared to the other (Núñez et al. 2018) After mammography, MRI is the preferred technique for diagnosis of breast cancer. Both T1 and T2 contrast-enhanced MRI helps in diagnosing breast cancers (American Cancer Society 2019) Many nanoparticles have been employed as contrast agents for MRI, out of which superparamagnetic iron oxide nanoparticles are the most studied contrast material (Kandasamy and Maity 2015). By loading both drug and gene along with any fluorescent moiety or contrast agents, nanomedicines can be used as theranostic agents (Fig. 19.2).

19.4.1 Super Paramagnetic Nanoparticles Loaded Theranostic Agents

Super paramagnetic nanoparticles are nanoparticles that are composed of a metallic core of iron oxide, cobalt, or nickel, and the core is encapsulated with layers of organic polymers like polyethylene glycol (PEG), dextran, starch, etc.

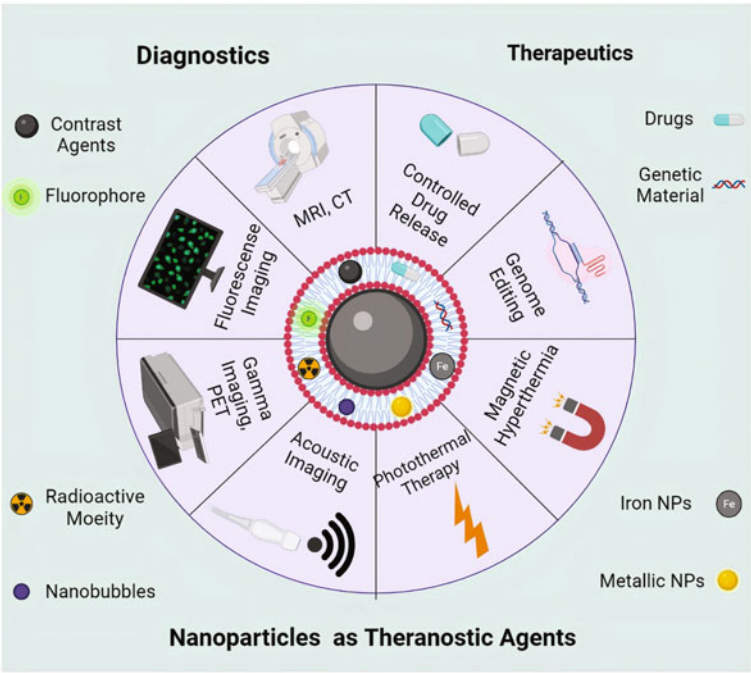

Fig. 19.2 Commonly employed theranostic approaches in breast cancer

The magnetic properties of these nanoparticles make them an effective contrast agent in MRI imaging for diagnosis of tumors. Peptides targeting the breast cancer cell receptors conjugated with superparamagnetic iron oxide nanoparticles (SPIONs) are extensively studied as a T2 contrast agent for MRI imaging of breast cancer. For example, gastrin-releasing peptide (GRP) is overexpressed in breast cancer and SPIONS coated with dextran and conjugated with bombesin (BBN) DSPION-BBN are studied as a T2 contrast agent for breast cancer detection. DSPION-BBN showed good targeting of GRP receptors in T47D breast cancer cell lines and also showed good diagnostic ability of breast cancer in mice as a contrast agent for MRI (Jafari et al. 2015). Moreover, arginine-glycine-aspartic acid (RGD) peptides-modified SPIONs, biotin-ligated PEG-SPIONs, human transferrin-ligated ultrasmall SPIONs (USPIONs) are some examples employed against multiple breast cancer receptors both in vitro and in vivo (Kresse et al. 1998; Yan et al. 2013; Zhang et al. 2007).

Superparamagnetic nanoparticles are commonly employed for simultaneous diagnosis and therapy of breast cancer. In one study, therapeutic siRNA was encapsulated in hollow manganese oxide nanoparticles coated with polyethylenimine (PEI), and these nanoparticles were ligated via anti-HER2 (Herceptin) ligands to actively target breast cancer cells. The results of this study showed increased uptake due to active targeting thus increased therapeutic effect of

siRNA as well as a strong positive T1 contrast on the MR image (Bae et al. 2011). Another such example is the theranostic agent studied by employing SPIONS with anti-CD44 as a targeting moiety and gemcitabine (GEM) as a therapeutic agent against CD44+ breast cancer (Aires et al. 2016). Multimodal systems for both the optical and MRI imaging and simultaneous therapy of breast cancer have also been developed. One such example is the development of iron oxide nanoparticles coated with a fluorescent dye Cy5 for both MR and optical imaging These particles were then coated with targeting moiety urokinase-type plasminogen activator (uPA) and a fluorescent drug doxorubicin. In vitro experiments revealed increased delivery of doxorubicin to 4T1 and MDA-MB 231 cells and 4T1 mouse mammary tumor model was employed for in vivo studies (Cao et al. 2010).

19.4.2 Quantum Dots Loaded Theranostic

Quantum dots are fluorescent nanoparticles which are composed of a typical core-shell structure. The core of the quantum dots consists of usually hard metals like cadmium selenide (the most common), technetium, tantalum, etc. covered with a zinc-sulfide shell. Quantum dots are extensively used in imaging studies owing to their size-dependent tunable wavelength emission. They have also been widely employed in breast cancer studies in vitro and in vivo.

Quantum dots have been used to detect breast cancer via both MRI and optical imaging. Targeting moieties such as anti-HER2 antibody (Tada et al. 2007), anti-HER2/neu scFv antibodies (Balalaeva et al. 2012), anti-estrogen receptor (ER) antibodies (Chen et al. 2010), anti-progesterone receptor (PR), anti-EGFR, and anti-mTOR antibodies (Yezhelyev et al. 2007) have been extensively employed for optical imaging of breast cancer cells in vitro and in vivo.

Quantum dots with magnetic cores are also employed in MRI as well as their inherent optical properties provide optical imaging avenues as well. Multilayered core-shell nanoprobes (MQQ-probes) were employed in a study whereby iron oxide magnetic nanoparticles, and multi-sized quantum dots were embedded in different silica layers to develop a nanoprobe with both applications as contrast agents for MRI and optical imaging. These MQQ-probes were then ligated with anti-HER2 antibody to actively target and image breast cancer cells in vitro and in vivo (Ma et al. 2012).

Quantum dots have also been developed for theranostic applications. These usually include a complex of quantum dots and magnetic nanoparticles conjugated with doxorubicin for simultaneous applications of this complex in MRI and optical imaging for detection of breast cancer and doxorubicin for therapy (Park et al. 2008).

In another study, complex nano-polymersomes made of PEG-PLGA were developed containing fluorescent hydrophilic MSA-capped quantum dots and doxorubicin and were effectively employed to develop a theranostic platform for simultaneous optical detection and therapy of breast cancer.

19.4.3 Gold Nanoparticles as Theranostic Agents

The unique properties of gold nanoparticles and their contributions in the tumor destruction for the treatment of breast cancer has been used extensively over the past few years. Different formulations of gold nanoparticles can be made for different treatment procedures having distinct shape, size, and surface properties depending on the clinical applications. These nanoparticles exhibit unique electronic, optical, and magnetic properties which makes the excellent candidates to be used for various detection and diagnostic techniques (Lee et al. 2014).

19.4.3.1 Tuning Optical Properties of Gold Nanoparticles for Diagnosis

Gold nanoparticles exhibit distinct SPR (Surface Plasmon Resonance) and optical properties due to which these nanoparticles are extensively used in ultra-sensitive detection techniques which are required for the early diagnosis and treatment of breast cancer. The scattering properties and absorbance of gold nanoparticles can be tuned according to the size parameter (Durr et al. 2007). The scattering properties increase for larger sized nanoparticles and gold nanorods are capable of exhibiting two absorbance bands, so that they can correspond to the commercial lasers when synthesized with a suitable ratio. The shift of plasmon band towards infrared region shows that gold nanorods penetrate deeper into the living tissues which is relatively deeper than that of visible light (Durr et al. 2007). Gold nanoparticles also have been shown to undergo a color change from dark red to purple due to the change in refractive index around the nanoparticles' surface. Specific antibodies can be attached to the surface of nanoparticles and when specific analytes bind to these antibodies, their interaction results in color change corresponding to the concentration of analyte.

19.4.3.2 Fluorescence Behavior of Gold Nanoparticles

Gold nanoparticles exhibit excellent anti-photobleaching behavior under strong light illumination and show strong fluorescence despite their low-quantum yields. When cells stained with gold nanoparticles are illuminated, the fluorescence of nanoparticles inside the living tissues is collected for cell imaging (He et al. 2008). Thus, gold nanoprobes can be successfully exploited in the fluorescence-based breast cancer diagnostics.

19.4.3.3 Magnetic Resonance Properties of Gold Nanoparticles for MRI

Gold nanoparticles are especially attractive for photo-imaging diagnostics because of magnetic properties and their ability in providing enhanced spatial and temporal resolutions for imaging (Kumar et al. 2011). In photo-imaging technique, functionalized gold nanoparticles are injected specifically at the target site, where they bind to tumor cells and scatter, due to which cancerous cells are easily identifiable from the normal cells by the surgeons, for example, HER2-positive SK-BR-3 cell lines were visualized under laser power in TPIP imaging (two-photon-induced photoluminescence), resulting in the visualization of tumor cells in the breast (Menon et al. 2013).

19.4.3.4 From Diagnostics to Theranostics

Gold nanoparticles are also extensively exploited in theranostics, where diagnosis and treatment are combined. Multifunctional gold nanoparticles get accumulated in the tumor cells to enable the detection through luminescence and thus increase the survival rate through photo-thermal treatment.

19.4.3.4.1 Drug Delivery

In chemotherapy for breast cancer treatment, anti-cancer drug resistance can be primary (intrinsic) or secondary because of repetitive cycles of chemotherapy (acquired). Also, chemotherapeutic drugs alter drug-specific targets or fail to activate them. Gold nanoparticles can be used as complex functional biomaterials for nanocarrier-mediated therapies in order to maximize therapeutic efficacy and drug release to overcome the problems of drug resistance in breast cancer treatment (Lee et al. 2014).

19.4.3.4.2 Photo-Thermal Therapy

Most of the photo-thermal therapies for breast cancer treatment fail because of the effects of tumor microenvironment on light to heat conversion efficiency. Gold nanostars effectively covert light to heat under photo-thermal therapy due to their heating efficiency and aqueous dispersion depending on the particle size and laser wavelength. For example, PEGylated gold nanoparticles have exhibited increased absorption and accumulation at cancerous site in NIR region and showed no re-occurrence of disease after a single 10 min laser exposure (Espinosa et al. 2016).

19.4.3.4.3 Trastuzumab Conjugates

These are novel antibody-drug conjugates and have two potential benefits in breast cancer therapy, one they have the ability to uptake gold nanoparticles and second to overcome the trastuzumab resistance. The application of 300 peak kilovoltage (kVp) radiations onto HER2 overexpressing SK-BR-3 breast tumor cells lead to five times more breaks in double-stranded DNA in case of PEGylated gold nanoparticles (Bozzer et al. 2021).

19.5 Nanoparticles-Mediated Gene Delivery for Breast Cancer Treatment

The surface-modified gold nanoparticles have been recognized as potential vehicles for gene delivery for the treatment of breast cancer because of their remarkable biocompatibility and their distinct and excellent structural and optical properties (Chattopadhyay et al. 2010).

19.5.1 Antisense Oligonucleotides

Antisense oligonucleotides act by binding with the target mRNA, splicing of heteronuclear RNA to mature mRNA, blocking the expression of target gene by signaling its cleavage and translation of mRNA through steric hindrance. Gene silencing involves the delivery of specific nucleic acids to the tumor site which ultimately downregulates the expression of target genes. Gene silencing therapy is the most commonly utilized therapy through the delivery of siRNA into the cancer cells, designed specifically to target a selective mRNA of the target gene resulting its degradation by blocking the translation process.

Gold nanoparticles are used extensively in gene silencing because of their unique properties, and they have been proven excellent candidates for the breast cancer treatment. RNAi (RNA interference) is one of the most promising gene therapy techniques because of its high efficiency and capacity. Over the past few years, the detection and gene knock-down have been done successfully using RNA interference strategy. The expression of telomerase increases significantly in many types of tumor cells; its activity inhibition through hTERT (human telomerase reverse transcriptase) has also been investigated recently. However, there are some challenges which inhibit the efficient delivery of hTERT at intracellular level as well as tissue level. The successful delivery of RNA interference lies in an effective release of siRNA from endosomes so that it can degrade the target mRNA inside the cytoplasm and ultimately inhibit the target protein expression. The post-conjugation of siRNA and mRNA also result in the degradation of target mRNA of specific complementary sequences, thereby greatly reducing the target protein expression. eEF-2K is a eukaryotic elongation factor 2 kinase, and it has been identified as an important part of oncogenic pathways to promote the breast cancer (Kumar et al. 2019). Modified gold nanoparticles can be formulated as highly stable and mono-dispersed nanoformulations and then conjugating them with siRNA of eEF-2K can result in successive downregulation of the triple-negative breast cancer cells (Kumar et al. 2019).

19.5.2 CRISPR/Cas9-Based Transcriptome Reprogramming

Almost all aspects of an organisms' life from early developmental stages till death are controlled by transcriptional programs. Cancer is an abnormal state which has its own specific transcriptional program, and it can be validated by CRISPR in different tumor types including breast cancer. Cancer cells are found to have a unique cell-specific transcription regulation pathway in which epigenetic modulations are the main key factors. This program can be destroyed through the inhibition of CDK7 receptors, potential therapeutic targets for breast cancer. The most remarkable feature is that the inhibition of CDK7 not only distort cell proliferation of breast cancer but also the resistance of MCF7 breast cancer cells. CRISPR/Cas9 delivery system consisting of PEGylated nanoparticles is now considered an efficient strategy for breast cancer treatment. Also, CRISPR-mediated editing of HER2 gene can be

enhanced in the presence of PARP inhibitors which are involved in cell death (Jubin et al. 2016).

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Breast Cancer and Next-Generation Sequencing: Towards Clinical Relevance and Future

20

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Abstract

Breast cancer, the world's most commonly diagnosed cancer, is a heterogeneous disease that demands appropriate diagnosis for better patient management. Thus, the understanding of the molecular mechanisms of tumor heterogeneity, as well as the characterization and precise monitoring of cancer, have been central themes of research in oncology. In this framework, NGS emerged as one of the most important tools for genetic studies in oncology. Since then, NGS has revolutionized the field of precision oncology, improving cancer detection, prevention, and treatment. This technique can be used to characterize tumor types, screen hereditary cancer, identify appropriate therapeutic agents, and indicate disease prognosis. This chapter reviews NGS-guided applications in breast cancer research, diagnostics, and treatment, starting with the history of NGS in oncology, including a review of NGS technique and heredity in breast cancer, following through the challenges of variants classification, and highlighting some available databases. Thereafter, clinical NGS testing and the importance of genetic counseling are discussed. The benefits, limitations, and future prospects of NGS are also explored. In the era of precision medicine, molecular tests have reaffirmed their usefulness in directing treatment decisions and improving patient survival and quality of life. Understanding the main features of NGS in breast cancer could help scientists from basic to clinical genomics areas in using NGS for specific applications in precision oncology.

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Keywords

Breast cancer · NGS · Precision oncology · Sequencing

20.1 Breast Cancer and Next-Generation Sequencing

20.1.1 History of NGS in Oncology

Advances in DNA sequencing techniques have allowed for a more comprehensive study of genetic alterations. The sequencing method developed by Sanger and colleagues (1977) is the forerunner of current sequencing techniques, which have been improved over the years, making possible several developments including the Human Genome Project (HGP; 1990–2003) (International Human Genome Sequencing Consortium 2004; Venter et al. 2015). Initially, Sanger sequencing was used routinely in the medical care of breast cancer (BC) patients to study partial sequences of genes such as *BRCA1* and *BRCA2* described as high-risk susceptibility genes for BC by linkage disequilibrium (LD) studies and positional cloning between 1994 and 1995 (Kamps et al. 2017). However, at that time, Sanger sequencing was limited due to the high cost per sequenced base and the huge protocols runtime.

The completion of the HGP and great advances in technologies, mainly boosted by re-sequencing, allowed the beginning of next-generation sequencing (NGS) or massive parallel sequencing (Mardis 2008). Thus, NGS represents one of the major milestones for genetic studies, especially in oncology, expanding the capacity to generate data and improving human genomic knowledge (Cho et al. 2015; Kamps et al. 2017). In addition, NGS is a more accessible technology owing to the reduced costs and improved speed (Meldrum et al. 2011; Kamps et al. 2017).

NGS involves the following three main steps: (1) library construction, (2) nucleic acid sequencing, and (3) data analysis (Fig. 20.1). Briefly, library construction refers to the process of DNA sample preparation and involves two common methods, namely, capture-based (e.g., Illumina) and amplicon-based approaches (e.g., Ion Torrent) (Fig. 20.1a). Nucleic acid sequencing techniques vary between sequencing platforms, mainly based on the type of clonal amplification (e.g., bridge amplification or emulsion PCR, Fig. 20.1b) and the detection method (e.g., sequencing by synthesis or semiconductor sequencing, Fig. 20.1c). Data analysis consists of sequencing data processing and interpretation using numerous bioinformatics tools and software available (Mardis 2008; Slatko et al. 2018).

In view of the ability of NGS to generate large volumes of information, applied in cancer genetics, allowed the emergence of large consortia and collaborative projects. Some examples include the International Cancer Genome Consortium ARGO (ICGC ARGO) (Argo 2021) and The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Network et al. 2013). These aim to assemble large amounts of sequencing data that can help in understanding the various types of cancers and their mechanisms. Thus, the identification and documentation of germline and somatic genetic variants in databases such as the COSMIC (Forbes et al. 2008)

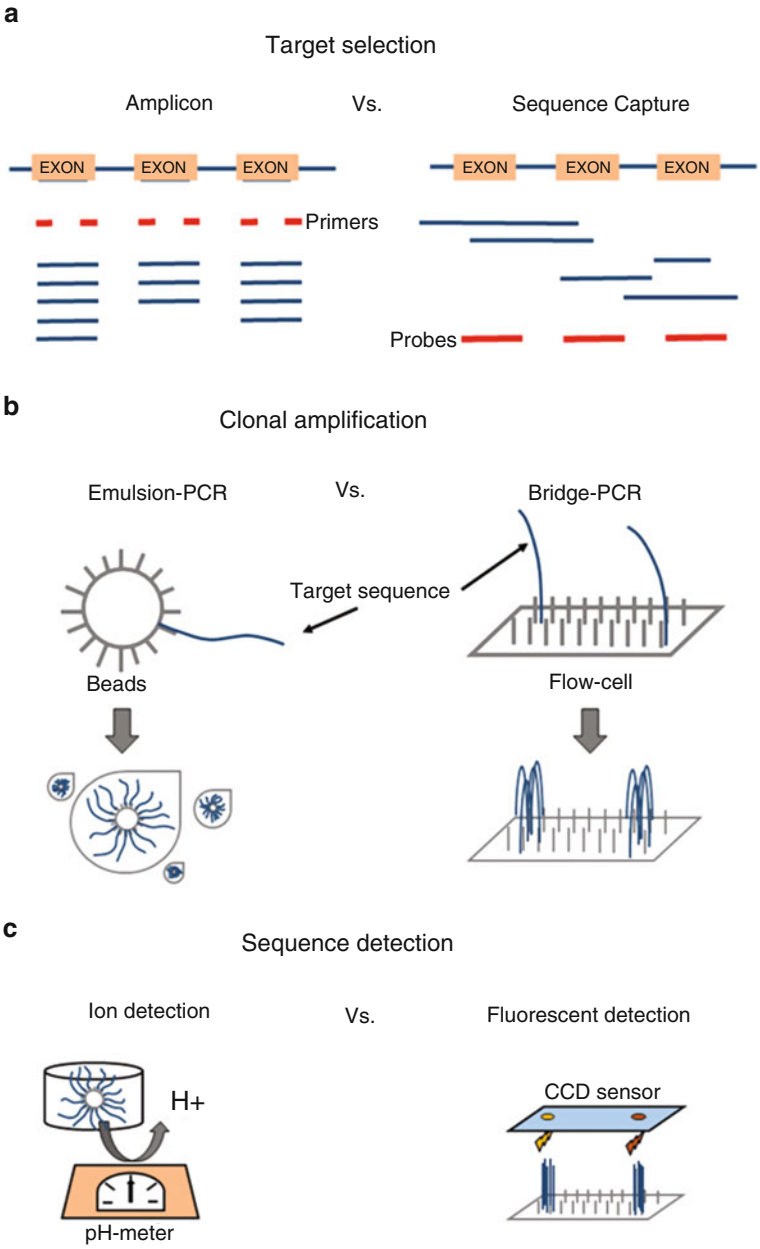

Fig. 20.1 Summary of main NGS techniques. Regardless of the methods used in each platform, NGS generally involves three steps—library preparation, nucleic acid sequencing, and data analysis. (**a**) In the amplicon method, specific primers are used to define the region of interest for target selection, while in the sequence capture method, insert size is defined by mechanical or enzymatic fragmentation and selected by specific probes. (**b**) Clonal amplification generates approximately 1000 copies of the target fragment, through emulsion PCR or bridge PCR, from a single molecule fixed on beads or flow-cell, respectively, to amplify the signal to be detected by the sequencers. (**c**) The detection in Illumina sequencing occurs through a fluorescent light sensor according to

(see Sect. 20.1.3) allows knowledge obtained from basic research to be applied in clinical oncology, following the approach of translational medicine (Beigh 2016).

Massively parallel sequencing technologies have played an important role in cancer research in identifying new candidate genes and understanding disease progress (Shah et al. 2009; Jones et al. 2010; The International Cancer Genome Consortium et al. 2010; Yachida et al. 2010). This versatility has rendered NGS an important tool in clinical oncology because of the development of target panels for BC testing with relatively low cost-effectiveness (Walsh et al. 2010; Hoppman-Chaney et al. 2010; Castéra et al. 2014; Schroeder et al. 2015). For example, studies involving the whole genome sequencing (WGS), the whole-exome (all protein-coding genes—WES), and also of selected genes independently or across with hot-spot regions. BC was one of the first types of cancer investigated by WGS (Foley et al. 2015). Considering this, some of the numerous advances in techniques based on massive parallel sequencing, as well as their applications in cancer diagnosis and prognosis will be discussed later in this chapter.

20.1.2 Heredity and Breast Cancer

BC involves the uncontrolled growth of breast cells forming a malignant tumor. The loss of cell proliferation control mechanisms is caused by mutations and represents the essence of tumorigenesis (Hanahan and Weinberg 2011). Mutations comprise changes in the DNA sequence in germline or somatic cells. Germline mutations, also known as hereditary mutations, occur in germ cells and can be passed onto offspring. These mutations are of great interest in the investigation of hereditary syndromes, including hereditary BC, and genetic counseling. In contrast, somatic mutations occur in cells of specific tissue and are not transmitted to the progeny (non-hereditary) (Milholland et al. 2017; Lindsay et al. 2019).

Genetic variations can be grouped into the following two distinct categories: (1) gain-of-function mutations in proto-oncogenes (e.g., *HER2*), which induce cell growth, or (2) loss-of-function mutations in tumor suppressor genes (e.g., *BRCA1/2* and *TP53*) that normally help prevent uncontrolled cellular proliferation, promote DNA repair, and control cell cycle checkpoints. Mutations in tumor suppressor genes are more common in hereditary BC (Lee and Muller 2010; Sheikh et al. 2015).

BC susceptibility genes can also be classified according to their genetic penetrance and can be grouped into the following three classes: (1) high penetrance genes (e.g., *BRCA1/2*, *CDH1*, *NF1*, *PTEN*, *STK11*, and *TP53*), (2) moderate penetrance genes (e.g., *ATM*, *BRIP1*, *CHEK2*, *HRAS1*, *NBN*, and *PALB2*), and (3) low-penetrance genes (e.g., *MEN1*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *PPM1D*) (Hall et al. 2014; Palmero et al. 2018). Accurate penetrance estimation is

Fig. 20.1 (continued) incorporated nucleotide (reversible terminator chemistry), whereas detection in ion sequencing occurs by an ion sensor, which captures the release of the H^+ ion during the nucleotide incorporation

critical for a more assertive assessment of the lifetime risk of developing BC, as well as to support management strategies such as increased surveillance and risk-reducing interventions, including prophylactic surgery (Fan et al. 2021).

20.1.2.1 Hereditary Breast Cancer

Pathogenic germline mutations that are transmitted over generations confer a high risk of developing cancer and are responsible for 5–10% of BC cases (Cancer 2001; van der Groep et al. 2006; Daly et al. 2010). Such patients often have an early age of onset and exhibit an autosomal dominant inheritance pattern (Daly et al. 2010).

A large proportion of the pathogenic variants in hereditary BC are in the *BRCA* genes (*BRCA1* and *BRCA2*); however, approximately 30% of patients with a personal or family history of BC are *BRCA*-negative and show alterations in other genes (Coppa et al. 2018; White et al. 2018). Catana and colleagues conducted a retrospective study in over 87,000 patients who underwent multigene panels for hereditary BC and identified the prevalence of up to 12% mutations in non-*BRCA* genes (*ATM*, *CHEK2*, *PALB2*, *PTEN*, *TP53*, and others) (Catana et al. 2019). Similar results have been reported in other studies (Kurian et al. 2015; Tung et al. 2016; Couch et al. 2017). Other genes and rare syndromes have also been identified and associated with predisposition to BC, including, *TP53* (Li-Fraumeni Syndrome), *PTEN* (Cowden Syndrome), and *STK11* (Peutz Jeghers Syndrome) (Lindor et al. 2008; Daly et al. 2010; Lalloo and Evans 2012; Bodian et al. 2014; Maxwell et al. 2015).

Populational differences have also been described in the distribution patterns of germline variants and affected genes (Brovkina et al. 2018; Yedjou et al. 2019). Caswell-Jin and colleagues compared the rate of pathogenic variants in different genes in white, Asian, and Hispanic patients and observed a significantly higher percentage of *CHEK2* mutations and a lower percentage of *APC* mutations in whites than in non-whites (Caswell-Jin et al. 2018). Furthermore, founder mutations are another important factor influencing BC risk in different ethnic groups. These mutations are inherited and occur at a high frequency in specific populations as a consequence of their genomic location in a region of LD (Ashton-Prolla and Vargas 2014; Ossa and Torres 2016; Gomaa Mogahed et al. 2020).

20.1.2.2 Sporadic or Somatic Breast Cancer

Most cases of BC are sporadic (90–95%) and are associated with the acquisition of somatic mutations in breast cells during an individual's lifetime (van der Groep et al. 2006). Cancer of somatic origin results from the combined effects of low-to-moderate susceptibility risk alleles and environmental factors.

Several risk factors, which can be endogenous (age at first menstruation, menopause, first pregnancy, lactation, number of children, etc.) or exogenous (obesity, high cholesterol, diabetes, hormone replacement therapy, physical activity, exposure to radiation, serum vitamin D levels, alcohol consumption, active and/or passive smoking, cumulative exposure to estrogen, estrogen metabolites, etc.), can be associated with BC (CALAF et al. 2015; Miricescu et al. 2020). Since intrauterine life, we are exposed to many known toxicants as well as several potentially

hazardous chemicals, the risks of which are less well characterized. Several chemicals are persistent organic pollutants owing to their resistance to degradation, environmental persistence, and bioaccumulation in the food chain. Despite being banned in many countries because of negative human health effects, they still accumulate in soils, air, and biota. Therefore, humans are still exposed to such chemicals through several routes (Lee et al. 2017a; Koual et al. 2020). Studies have investigated the role of environmental disruptors in the initiation, invasion, and metastasis of BC, and more recently, in their influence on the response to chemotherapy (Koual et al. 2020).

Besides investigations about risks and susceptibility, studies concerning the genetics of BC have been highlighted to sub-classify and describe clinical particularities, such as response to target therapy, associated with genetic heterogeneity. In this regard, several studies have used NGS to investigate somatic mutation signatures, including the single nucleotide variants (SNVs) in clinically significant BC genes such as *AFF2*, *ARID1B*, *BCL2*, *CASP8*, *CBFB*, *DDR2*, *EGFR*, *FGFR1*, *FGFPR2*, *GATA3*, *JAKP1*, *KDM6A*, *KDR*, *MAP3K1*, *MYC*, *NCOR1*, *NOTCH3*, *PDK1*, *PIK3CA*, *RB1*, *RETCD1*, *RUNX*, *TBX3*, and copy number changes in *CDKN2A*, *ERBB2*, *MAP2K4*, *MLL3*, *PIK3CA*, *PTEN*, *RB1*, and *TP53* (Marotti et al. 2017; Low et al. 2018; Miricescu et al. 2020).

20.1.3 Variant Classification and Databases

Classifying variant pathogenicity is challenging. For standardizing variants interpretation, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP), in 2015, published a classification system for germline variants, from benign to pathogenic, based on factors such as population frequencies, computational predictions, and functional and inheritance studies (Richards et al. 2015). They have proposed specific recommendations for interpretation of variants, such as for genes *CDH1* (Lee et al. 2018), *PTEN* (Mester et al. 2018), and *TP53* (Fortuno et al. 2021), to reduce the number of variants with uncertain clinical significance (VUS). Later, in 2017, the AMP, the American Society of Clinical Oncology (ASCO), and the College of American Pathologists (CAP) proposed a normalization for somatic variant classification, in Tier, based on the existence of target therapies and clinical applications for each cancer type (Li et al. 2017). A comprehensive explanation of this topic was recently discussed by Pereira and colleagues (2020).

Guidelines have also been formulated that disseminate the best bioinformatics practices for analyzing genetic variants (Kluk et al. 2016; Soukupova et al. 2018). The implementation of previously published pipelines for more comprehensive panels can require additional adjustments in clinical practice (Hynst et al. 2021). The bioinformatic analyses cover at least one step to retain high-quality sequences (Martin 2011; Bolger et al. 2014; Chen et al. 2018), a step for alignment against a multigenic reference or a genome (Li and Durbin 2009; Lunter and Goodson 2011; Ruffalo et al. 2011; Langmead and Salzberg 2012; Thankaswamy-Kosalai et al.

2017), the identification of germline variants (Li et al. 2008; Qi et al. 2010; McKenna et al. 2010), and the annotation of these variants with subsequent application of specific filters (Wang et al. 2010; Cingolani et al. 2012; McLaren et al. 2016).

Regardless of the chosen pipeline, the following must be considered carefully during analysis: (1) capture-based methods tend to ensure better sequencing uniformity than amplicon-based methods and may be more suitable for copy number variation (CNV) investigation (Samorodnitsky et al. 2015) (see Sect. 20.2.3); (2) the quality control step must be efficient to ensure a greater distinction between biological variants and sequencing noise (Soukupova et al. 2018); (3) some questions may require additional wet experiments with the use of dual indexing with unique molecular identifiers (UMIs), or specific probes/primers to support the detection of variants in low-sensitivity regions (McConnell et al. 2020).

20.1.3.1 Databases

There are many data sources for BC studies (Pavlopoulou et al. 2015; Clare and Shaw 2016; Mirbagheri et al. 2020; Sisti et al. 2020). To provide an overview of high-throughput databases, omic data repositories (which can be filtered for a specific cancer type) to more focused compendiums (with links to the main journals of interest on this topic) are listed in Table 20.1.

Some examples of generic hubs include the TCGA Program (National Cancer Institute) and canSAR. The TCGA Program contains the molecular characterization of over 20,000 primary cancer samples and corresponding normal samples spanning 2.5 PB of omic data (Tomczak et al. 2015). The canSAR database integrates multidisciplinary data (biology, chemistry, pharmacology, structural biology, networks, and clinical annotations) with a focus on the prediction of drug targets for different cancer types (Mitsopoulos et al. 2021).

On the other hand, as an example of specialized data, the Department of Surgical Breast Oncology at the University of Texas MD Anderson Cancer Center provides a database with clinical information from about 30,000 patients (https://www.mdanderson.org/cancer-types/breast-cancer.html). Besides, the BC Database (BCDB) was developed to provide researchers a quick overview, based on scientific literature, of genes involved in BC, their functions, and drugs for treatment (Mohandass et al. 2010).

With the increasing generation of data, especially due to the advent of NGS techniques, the organization of bench-top data together with the associated clinical information represents a necessity and a challenge. Thus, several websites provide statistical information, screening, prevention, treatment, and clinical trials in BC (e.g., https://www.cancer.gov/types/breast). In this sense, databases have been crucial importance in studies in the area of oncology, allowing to store and share information from various studies conducted by the scientific community around the world.

Table 20.1 Databases for breast cancer research

Database	Description	URL	Curated	Variants annotation	Genes, samples, or variants	Cancer types	Breast cancer	Update frequency (last update)
Clinical Genome Resource (ClinGen)	Central resource that defines the clinical relevance of genes and variants for use in precision medicine and research	https://clinicalgenome.org/	X	Germline and somatic	2221 genes 2727 variants	Uncategorized by cancer type		Unscheduled (2021)
Leiden Open Variation Database (LOVD)	Database that include information about genes, variants, and inherited genetic disorders	https://www.lovd.nl/	X	Germline and somatic	22,998 genes 837,502 variants	Uncategorized by cancer type		Unscheduled (2021)
The Human Gene Mutation Database (HGMD)	Collection of germline mutations in nuclear genes, underlying or associated with human inherited disease	http://www.hgmd.cf.ac.uk/ac/index.php	X	Germline	12,233 genes 323,661 variants (total) 210,341 variants (public access version)	Uncategorized by cancer type		Every 3 months (2021)
CanDL	Potentially genomic drivers based on literature categorized by	https://candl.osu.edu/search/	X	Somatic	56 genes 330 variants	Uncategorized by cancer type		Unscheduled (2015)

	levels of evidence (tier)							
CIViC	Clinical Interpretation of Variants in Cancer	https://civicdb.org/	X	Somatic	455 genes 2870 variants	316	Uncategorized by cancer type	Weekly (2021)
COSMIC	Catalogue of Somatic Mutations in Cancer. It can also be accessed on a BioMart portal	http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/	X	Somatic	237 genes (total) 49 genes (curated) 149,089 samples 520 variants	Uncategorized by cancer type		Monthly (2021)
HemOnc	Wiki of medical knowledge relevant to oncology and hematology	https://hemonc.org	X	Somatic	5525 variants	Around 27	7 breast cancer conditions	Monthly or less (2021)
JAX-CKB	Interpretation from Jackson Laboratory of cancer genomic profiles in the context of protein impact, therapies, and clinical trials	https://ckb.jax.org/	X	Somatic	1840 genes (total) 50 (public access version) 41,535 (total) 8632 (public access version)	Uncategorized by cancer type		Monthly (2021)

(continued)

Table 20.1 (continued)

Database	Description	URL	Curated	Variants annotation	Genes, samples, or variants	Cancer types	Breast cancer	Update frequency (last update)
MdAnderson	Information about potential therapy by specific tumor biomarkers from MD Anderson Cancer Center	https://pct.mdanderson.org/	X	Somatic	32 genes <1000 variants	Uncategorized by cancer type		Yearly or more (2021)
OncoKB	Comparison between genetic alterations and clinical data on cancers analyzed by Memorial Sloan Kettering Cancer Center (MSK) experts	https://www.oncokb.org/	X	Somatic	682 genes 5670 variants	125	22 genes, 40 drugs	Monthly (2021)
PMKB	Clinical interpretations structured by cancer variants	https://pmkb.weill.cornell.edu/	X	Somatic	610 genes 2247 variants	156	1126 generic interpretations	Unscheduled (2019)
ClinVar	Public archive of reports of the relationships among human variations and phenotypes	https://www.ncbi.nlm.nih.gov/clinvar/	–	Germline and somatic	>1 million of variants	Uncategorized by cancer type		Unscheduled (2021)

dbVar	Database of human genomic structural variation, larger than 50 bp	https://www.ncbi.nlm.nih.gov/dbvar/	–	Germline and somatic	>7 millions of variants	Uncategorized by cancer type		Unscheduled (2021)
Single Nucleotide Polymorphism Database (dbSNP)	Catalog that includes both common and rare short variations, less than 50 bp, in nucleotide sequences for human	https://www.ncbi.nlm.nih.gov/snp/	–	Germline and somatic	>1 billion of variants	Uncategorized by cancer type		Unscheduled (2020)
Online Mendelian Inheritance in Man (OMIM)	Catalog of human genes and genetic disorders and traits, with particular focus on the relationship between phenotype and genotype	https://www.omim.org/	–	Germline	16,578 genes 4804 variants	Uncategorized by cancer type		Daily (2021)
Cancer Hotspots	List of statistically significantly variants identified in large-scale cancer genomics data	http://www.cancerhotspots.org/#/home	–	Somatic	247 genes 24,592 tumor samples 1165 variants	Uncategorized by cancer type		Unscheduled (2017)
cBioPortal	Interactive visualization of multidimensional cancer genomics data	http://www.cbioportal.org/	–	Somatic	137,374 samples >5000 tumor samples	>20	20 studies, 10,920 samples	Quarterly or less (2021)

(continued)

Table 20.1 (continued)

Database	Description	URL	Curated	Variants annotation	Genes, samples, or variants	Cancer types	Breast cancer	Update frequency (last update)
MyCancerGenome	Precision cancer medicine information, focused on mutations and related therapeutic implications	https://www.mycancergenome.org/	–	Somatic	6871 molecular biomarkers	955	1081 Biomarkers, 68 Drugs, 5974 Clinical Trials	Unscheduled (2019)
TCGA	The Cancer Genome Atlas. Assessed from GDC (Genomic Data Commons) Data Portal	http://cancergenome.nih.gov/	–	Somatic	23,621 genes >20,000 primary cancer and matched normal samples	33 cancer types, 67 primary sites	9115 cases, 56,794 files	Quarterly or less (2021)

20.2 NGS Testing

As discussed throughout this chapter, NGS is an important clinical tool that allows the identification of different mutations (SNVs), insertion-deletions (indels), CNVs, structural variations (SVs), and rearrangements. Figure 20.2 summarizes the main steps involved in NGS testing in clinical applications.

To guide healthcare providers in identifying high-risk cancer patients, the National Comprehensive Cancer Network (NCCN) developed the Clinical Practice Guidelines in Oncology (NCCN Guidelines®). The NCCN Guidelines update for Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic (version 1.2022) (Daly et al. 2021) was developed based on knowledge of advances in molecular genetic applications, and recommended approaches to genetic risk assessment, genetic testing and counseling, and patient management with pathogenic or likely pathogenic variants. The following topics will discuss some of the main applications of NGS in genetic testing, as well as future perspectives regarding the use of the technique in gaining knowledge that can benefit the care of BC patients.

20.2.1 Multigene Panels

The understanding of cancer heterogeneity coupled with recent improvements in DNA sequencing technology has enabled the development and incorporation of multigene panel tests by NGS into clinical practice. Multigene panels perform the simultaneous sequencing of a considerable number of genes in a large number of individuals and offer significant benefits when compared to sequential testing of a single gene—they are faster, cheaper, and more efficient for differential diagnoses, such as in cases of overlapping clinical manifestations among distinct cancers or individuals with a personal and family history suggestive of an inherited susceptibility but tested negative for one particular syndrome (Kurian and Ford 2015; Catana et al. 2019).

In addition to BC risk management, multigene testing panels can be used to orient the choice of target therapy choice. Currently, several FDA-approved therapies are administered based on biomarkers that can be detected by NGS, such as poly ADP ribose polymerase (PARP) inhibitors for *BRCA1/2* mutations (see Sect. 20.2.2), larotrectinib and entrectinib for *NTRK* fusions (see Sect. 20.2.4), and pembrolizumab for tumor mutation burden (TMB) (see Sect. 20.2.5) (Daly et al. 2021).

Currently, different multigene testing options are available in the market. Commercial multigene panel tests analyze a pre-established set of genes, usually defined by medical societies such as the NCCN guidelines (version 1.2022), which recommend as multigene panel testing the investigation of the genes *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CHEK2*, *EPCAM*, *MSH1*, *MSH2*, *MSH6*, *PMS2*, *NF1*, *PALB2*, *PTEN*, *RAD51C*, *RAD51D*, *STK11*, and *TP53* for BC risk management (Daly et al. 2021). Other genes such as *RECQL* and *NBN* are also analyzed (Fountzilas and Kaklamani 2018; Beitsch et al. 2019; Sorscher 2019; Dominguez-Valentin et al. 2019). In addition to pre-designed commercial tests, some companies

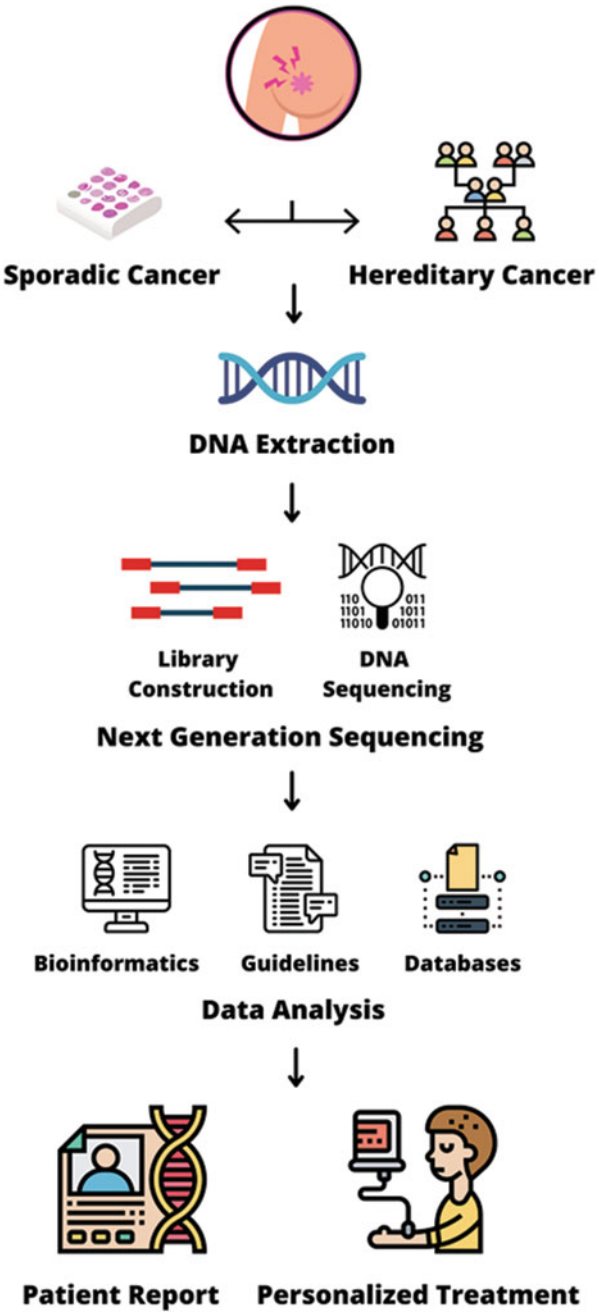

Fig. 20.2 Main steps involved in clinical NGS Testing. First, it is important to investigate whether the origin of the tumor is related to sporadic or hereditary events. The DNA from samples is extracted and target regions are selected (library construction). Libraries are sequenced and then analyzed by bioinformatics tools. The interpretation of the sequencing data is converted into a report to be used by healthcare providers for choosing the best treatment for the patient and genetic counseling, within the personalized medicine approach

also customize NGS panels. A custom panel allows the customer to select a set of specific genes to be analyzed. This flexibility allows the investigation of particular clinical conditions or cases with distinct clinical presentations (Castellanos et al. 2017).

Multigene panels can also be categorized into (1) cancer-site-specific panel testing or (2) pan-cancer panel testing. The cancer-site-specific panel test allows testing of a range of genes that are clinically associated with increased risk for a specific cancer type. In contrast, the pan-cancer panel test can include a large group of genes related to multiple types of cancer, of hereditary or somatic origin (Easton et al. 2015; Maga et al. 2017). Table 20.2 summarizes NGS multigene panels for BC patients offered by genetic testing companies.

Despite the advantages of NGS panels, several issues must be considered regarding their use. Multigene testing usually has a longer turnaround time and different tests may analyze different numbers of genes. They can also identify an extensive number of genetic variants, including mutations with low or incomplete penetrance, as well as VUS variants, which add complexity that may make interpretation difficult for health providers (Buys et al. 2017; Colas et al. 2019; Jones et al. 2021). Therefore, genetic counseling is recommended before and after multigene panel testing, and tests are currently recommended only for patients who meet well-defined risk criteria (Hampel et al. 2015; Shiovitz and Korde 2015) (see Sect. 20.3).

20.2.2 Homologous Recombination Deficiency (HRD)

Homologous DNA recombination repair (HRR) is a key mechanism for the repair of double-stranded DNA damage (Li and Heyer 2008); the inability to repair double-strand breaks is known as homologous recombination deficiency (HRD). Several studies have shown that HRD is associated with an increased risk of developing different types of cancers, including breast, ovarian, and prostate cancer (Wagener-Ryczek et al. 2021). It is estimated that approximately 40% of hereditary or sporadic BC are HRD positive (den Brok et al. 2017; Ali et al. 2021).

The tumor suppressor genes *BRCA1* and *BRCA2* are critical at different stages of HRR and are often mutated in hereditary breast or ovarian cancers (Prakash et al. 2015). *BRCA1/2* mutation status is still the main genetic biomarker of HRD in clinical use (Nguyen et al. 2020). Besides *BRCA* genes, several other genes are involved in the HRR pathway including *RAD51*, *ATM*, and *MRE11* (Gou et al. 2020). Therefore, *BRCA1/2* mutations, *BRCA1* promoter methylation, mutations in other HRR genes, and other undetermined factors contribute to HRD status (Takaya et al. 2020).

Determining HRD tumor status also involves the evaluation of a set of changes called "genomic scars" through the genomic scar score (GSS), also called the genomic instability score (GIS), calculated using three different signatures related to genomic instability, namely, loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large-scale state transitions (LST) in tumor tissue. Thus, to identify HRD tumor status, it is necessary to use high-throughput techniques such as

Table 20.2 Commercial and custom NGS panels currently marketed for breast cancer

Type of panel	Company	Panel name	Number of genes	Testing category		Type of test		Changes detected					
				Cancer-specific	Pan-cancer	Germline	Somatic	SNV	CNV	Indel	Fusion	MSI	TMB
Commercial	Ambry Genetics	BRCANext™	18	X		X		X	X	X			
		CancerNext™	36		X	X		X	X	X			
	Centogene	CentoBreast®	30	X		X		X	X	X			
		CentoCancer®	70		X	X		X	X	X			
		Solid Tumor Panel	149		X		X	X		X			
	Foundation Medicine	FoundationOne®CDx	324		X		X	X	X	X	X	X	X
	GeneDx	Breast Cancer Management Panel	10	X		X		X	X	X			
		Breast/Gyn Cancer Panel	25		X	X		X	X	X			
	Illumina	TruSight Cancer	94		X	X		X	X	X			
		TruSight Oncology 500	523		X		X	X	X	X	X	X	X
	Invitae	Invitae Breast Cancer STAT Panel	9	X		X		X	X	X			
		Invitae Multi-Cancer Panel	84		X	X		X	X	X			
	Myriad Genetics	myRisk™ Hereditary Cancer	35		X	X		X	X	X			
	Quest Diagnostic	Hereditary Breast Cancer Panel	16	X		X		X	X	X			
		Comprehensive Hereditary Cancer Panel	66		X	X		X	X	X			
		Solid Tumor Core Panel	49		X		X	X	X	X	X	X	X

	Thermo Fisher Scientific	Oncomine BRCA Expanded Panel	15	X			X	X	X	X			
		Oncomine Comprehensive Assay Plus	Over 500		X		X	X	X	X	X	X	X
Custom	Ambry Genetics	CustomNext-Cancer®	Up to 91	X	X	X		X	X	X			
	GeneDx	OncoGeneDx Custom Panel	Up to 83	X	X	X		X	X	X	X		
	Illumina	AmpliSeq for Illumina Custom	Undefined	X	X	X	X	X	X	X	X		
	Sophia Genetics	Hereditary Cancer Solutions	Up to 143	X	X	X		X	X	X			
	Thermo Fisher Scientific	Ion AmpliSeq Custom DNA Panels	Undefined	X	X	X	X	X	X	X	X		
	Twist Bioscience	Twist Custom Panels	Undefined	X	X	X	X	X	X	X	X		

NGS, which evaluate multiple loci simultaneously (Watkins et al. 2014; Wagener-Ryczek et al. 2021).

HRD-positive patients show the highest responses to PARP inhibitors. PARP plays a key role in single-stranded DNA repair and PARP inhibitors can block single-strand repair. During replication, unrepaired single-strand breaks are converted to double-strand breaks. Thus, the occurrence of double-stranded level damage coupled with the inability to repair by HRD-positive tumor cells creates a synthetic lethal effect, leading to tumor cell death (Ashworth and Lord 2018). Thus, HRD has been considered the Achilles' heel of tumors and has been used as a sensitive biomarker for the use of PARP inhibitors in clinical oncology (Noordermeer and van Attikum 2019; Wagener-Ryczek et al. 2021).

Recent clinical studies have revealed that patients with both germline and somatic mutations can benefit from PARP inhibitory therapies, such as olaparib, which represents an improvement in treatment options for sporadic BC patients (Konstantinopoulos et al. 2020; Tung et al. 2020; Han et al. 2020). Olaparib is currently approved in the United States, Japan, and other countries for germline *BRCA*-mutated *HER2*-negative metastatic BC previously treated with chemotherapy (Tutt et al. 2021).

The results of HRD tumor status and responses to PARP are not always concordant, as some tumors HRD positive can restore the homologous recombination function by somatic reversion mutations (Hoppe et al. 2018). Therefore, understanding the details of the tests and their limitations is critical for ensuring personalized clinical application (Stover et al. 2020). In general, HRD tests look for one or more of the following genomic alterations: (1) germline and somatic mutations or copy number variations in HRR-related genes, such as *BRCA1*, *BRCA2*, *PALB2*, *RAD51*, *RAD51C*, and *RAD51D*; (2) gene expression silencing caused by promoter methylation; (3) GIS; (4) mutational signatures highly prevalent in tumors with *BRCA*ness and distinct mutational signatures found in BC (Keung et al. 2019).

The HRD Focus Panel (AmoyDx), myChoice (Myriad Genetics), BRACAnalysis (Myriad Genetics), Foundation Medicine (Foundation One), and TumorNext-HRD (Ambry Genetics) are examples of commonly used tests with specific pipelines. A major challenge for wider HRD pipelines is the interpretation of clinical VUS variants (e.g., rare missense and intronic variants that may alter RNA splicing), leading to partial or leaky splicing. To define best practices for these tests, the European Society for Medical Oncology (ESMO) Translational Research and Precision Medicine Working Group launched a systematic review (Miller et al. 2020).

20.2.3 Copy Number Variation (CNV)

CNV can be described as a structural change that involves an increase or decrease in the number of copies of a gene (or certain regions) (Redon et al. 2006; Shao et al. 2019). Some authors use the term CNV when alterations occur in germline cells, while the term copy number alteration (CNA) is used for somatic cells (Zhang et al.

2018; Fernandes et al. 2021). However, in this chapter, we will adopt the term CNV to describe all changes in the copy number of a gene, regardless of cell origin.

Pathological roles such as the initiation and progression of BC have been associated with CNVs in many genes, such as *BRCA1*, *BRCA2*, *CHEK2*, *MTUS1*, *TP53*, and *hTERT* (Walker et al. 2017; Pan et al. 2019). Besides, the amplification or overexpression of *HER2* (*ERBB2*) occurs in almost 20% of BC and is an important biomarker for targeted therapy (van Bockstal et al. 2020; Niu et al. 2020). Compared to techniques such as fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC), NGS-based *HER2* evaluation is reliable owing to lower rates of erroneous results (Dumbrava et al. 2019; Niu et al. 2020).

Although the gold standard methods for CNV detection include multiplex ligation-dependent probe amplification (MLPA) and array comparative genomic hybridization (aCGH), NGS has reduced the number of tests and costs in hereditary genetic disease screening. The evaluation of CNVs by NGS has been shown to be more stable and robust than gene expression evaluations, reinforcing the importance of CNV as a biomarker in BC (Pan et al. 2019). Therefore, CNVs have proved to be of great clinical value, having different applications in oncology, including BC risk assessment and prognosis (Kuiper et al. 2010; Kumaran et al. 2017; Zhang et al. 2018; Pan et al. 2019; Tao et al. 2019).

In 2011, Chattopadhyay and colleagues published the first multi-ethnic database for identifying CNVs associated with cancer called CNVIntegrate (Chattopadhyay et al. 2021). This CNV catalog and other databases with data of healthy individuals, such as PanSNPdb (Ngamphiw et al. 2011) and Korean Variant Archive (KOVA) (Lee et al. 2017b), improved the baseline construction and CNV annotation (Table 20.1). Subsequently, several CNV detection tools were developed for NGS analysis, making it possible to recognize variants as short as 50 bp (Zhang et al. 2019). Two algorithms, namely, Read Depth (RD) or Read Count (RC) and Pair-End Mapping (PEM) or Read Pair (RP), are popular for CNV detection. Both algorithms use statistical and clustering approaches. However, RD/RC is better at detecting exact copy numbers, large insertions, and copy number variations in complex genomic regions, while PEM/RP is more suitable for detecting tandem duplications and mobile elements (insertions and inversions). Some authors have recently evaluated the performance of such tools in terms of sensitivity and specificity (Zhao et al. 2020; Moreno-Cabrera et al. 2020). They highlighted tools that performed poorly in detecting multi-exon alterations, but well in detecting large CNVs. Thus, Truty and colleagues emphasized the importance of a good baseline for identifying CNVs, mainly intragenic variations (Truty et al. 2019).

20.2.4 Fusion

Acquired chromosome rearrangements are a characteristic of neoplasia and have been associated with carcinogenesis in diverse types of human tissues (Mitelman et al. 2007). Fusion genes originate from chromosomal rearrangements, such as translocation, inversion, duplication, or deletion, resulting in the joining of two

unrelated genes (Edwards and Howarth 2012). Fusion genes may drive cancer progression through (1) generating constitutively active kinases or transcription factors, (2) amplifying growth factor signaling, (3) inactivating apoptotic factors, or (4) promoting uncontrollable cell growth (Veeraraghavan et al. 2016). Gene fusions have long been described only in leukemias and sarcomas (Edwards and Howarth 2012). However, with the advent of NGS, genomic rearrangements are being discovered in diverse types of tumors, including BC (Kim et al. 2018).

The first recurrent fusion described for BC was *SEC16A-NOTCH1*. Robinson and colleagues sequenced cDNA from 41 BC cell lines and 38 breast tumors and reported many other gene fusions, beyond *SEC16A-NOTCH1* (Robinson et al. 2011). A well-studied gene fusion in BC is *ETV6-NTRK3*, whose expression in mammary tissues results in the development of fully penetrant multifocal BC. Moreover, studies have established that the ETV6-NTRK3 fusion protein is sufficient to initiate mammary tumorigenesis (Li et al. 2007; Veeraraghavan et al. 2016).

Several studies have identified gene fusions having clinical impacts for the development of new targeted therapies (Koivunen et al. 2008; Laetsch and Hawkins 2019; Li et al. 2020b). For example, the FDA has approved the use of tyrosine kinase (TRK) inhibitors entrectinib and larotrectinib for the treatment of TRK fusion-positive cancers regardless of tumor histology. *NTRK* gene fusions are genetic abnormalities that can occur at a low frequency in most solid tumors, but they are present at higher frequencies in rare tumors such as secretory BC (Bebb et al. 2021; Stenzinger et al. 2021).

Gene fusions also play a key role in therapy resistance mechanisms, which represent an important cause of mortality in BC (Schram et al. 2017; Fultang et al. 2020). For instance, evidence suggests that *ESR1* mutations or fusions altering its ligand-binding domain are an important driving mechanism of endocrine therapy resistance. According to the RNA-sequencing (RNA-seq) analysis of 990 primary luminal breast samples, almost 2% contained recurrent fusion transcripts involving the first two non-coding exons of *ESR1* fused to the *CCDC170* gene (Veeraraghavan et al. 2014). Therefore, although endocrine therapy is an effective treatment for estrogen receptor (ER)-positive BC, its effectiveness can be impaired by the development of resistance to therapies (Hartmaier et al. 2018; Lei et al. 2019; Li et al. 2020b). Christie and colleagues detected multiple fusions involving the *ABCB1* gene that encodes a glycoprotein involved in the cellular efflux of chemotherapeutic drugs. The fusions described in *ABCB1* were associated with drug resistance mechanisms in high-grade serous ovarian and BC samples from chemotherapy-treated patients (Christie et al. 2019).

RNA-seq is the most commonly used method for fusion transcript detection because it focuses only on the expressed regions in the genome. In addition to being relatively less expensive with a quick turnaround time, it can also detect multiple fusion variants simultaneously (Beadling et al. 2016; Veeraraghavan et al. 2016). RNA-seq also allows the identification of novel fusions, which, through computational algorithms are discovered in public databases, and it has become an effective method to detect fusion transcripts in precision medicine pipelines

(Sakharkar et al. 2009). Some cancer fusion databases include TumorFusions/TCGA Fusion Portal (Hu et al. 2018), FusionHub (Panigrahi et al. 2018), and FusionCancer (Wang et al. 2015) (Table 20.1).

Many studies have sought to evaluate bioinformatics tools for identifying gene fusions (Liu et al. 2016; Kumar et al. 2016; Haas et al. 2019; Uhrig et al. 2021). The most common strategies used in RNA-seq analyses are: (1) aligning reads to references to identify discordant mapping that are indicative of rearrangements, and/or (2) assembling reads into longer transcripts, identifying chimeric transcripts compatible with chromosomal rearrangements. The reliability of predicted fusions can be measured by the coverage of chimeric reads (split or junction) that directly overlap the fusion transcript, or as discordant read pairs, where each pair of reads maps to opposite sides of the junction (Haas et al. 2019). To avoid the presence of false-positive events, fusion genes can be detected by the combination of two or more algorithms. Because of short read size, reliable detection is computationally difficult, and paired sequences need to be used to overlap the fusion junction (Heyer et al. 2019).

Besides NGS, several methods are available to test for gene fusions, such as reverse transcription polymerase chain reaction (RT-PCR) and FISH. Compared to FISH and RT-PCR methods, in a cohort of clinical samples, RNA-seq increases the diagnostic rate from 63% to 76% (Heyer et al. 2019). Using the same application, Edgren and colleagues identified 28 promising fusion gene candidates for BC (Edgren et al. 2011). IHC is also used for gene fusion detection. It is a widely implemented technique with a lower turnaround time and cost than molecular methods. For *TRK* fusions, however, a positive IHC test alone is insufficient to confirm diagnosis and indicate the need for TRK inhibitor therapy because of the low sensitivity and specificity of the currently available IHC assay. Molecular testing is necessary to confirm or exclude *NTRK* gene fusion (Perreault et al. 2021).

20.2.5 Tumor Mutation Burden (TMB)

TMB is defined as the number of somatic mutations normalized by the length of the coding genomic region (muts/Mb). Tumor mutations can generate neoantigens on the cell surface, and a high TMB is generally associated with increased neoantigens and a better response to immunotherapy (Hellmann et al. 2018; Allgäuer et al. 2018; Meléndez et al. 2018). Krasniqi and colleagues demonstrated that there is a large variability in tumor burden between BC subtypes, especially between hormone receptor (HR) tumors—HR-negative tumors usually have a higher TMB than HR-positive tumors (Allgäuer et al. 2018).

Although therapies based on immune checkpoint inhibitors PD-1/PDL-1 benefit patients with several types of cancer, studies have shown that for breast tumors, only a small fraction of patients, including patients with metastatic triple-negative breast cancer (mTNBC) are eligible for immunotherapy (Krasniqi et al. 2019). In 2021, the FDA-approved pembrolizumab for high-risk, early-stage triple-negative BC (TNBC), and the NCCN guidelines (version 1.2022) (Daly et al. 2021) recommend

pembrolizumab for patients with TMB >10 muts/Mb. Therefore, TMB measurement may increase the chances of more patients benefiting from these therapies.

WES allows direct and accurate measurement of TMB; however, it has high costs (Fancello et al. 2019). Target NGS panels, in turn, are robust, affordable, and faster alternatives for TMB assessment (Damodaran et al. 2015; Chalmers et al. 2017). Chalmers and colleagues suggested that approximately 1.1 Mb of exonic binding regions are sufficient to reliably assess TMB (Chalmers et al. 2017). Foundation One CDx (1.8 Mb), MSK-IMPACT (1.5 Mb), TruSight Oncology 500 (1.94 Mb), and Oncomine Tumor Mutation Load Assay (1.7 Mb) are currently available NGS panels for TMB analysis.

TMB implementation into clinical routine remains a challenge because of several pre-analytical, analytical, and post-analytical caveats, such as sample quality, deamination artifacts, TMB panel size, sequencing platforms, bioinformatics pipeline, type of mutations (synonymous and/or non-synonymous), and cut-offs definition (Damodaran et al. 2015; Chalmers et al. 2017; Chang et al. 2018). Bioinformatics solutions usually involve (1) quality control of sequences, (2) alignment of sequenced data against a reference genome and sorting, (3) combination of variant calling with different algorithms (e.g., GATK and Mutect2), (4) variant annotation and filtration, and (5) TMB calculation by accounting for all variants within the tumor and dividing by the total size of the sequenced genome (Heydt et al. 2020).

Thus, standardized approaches for TMB measurement are essential for comparing results and ensuring test applicability (Chang et al. 2018). Despite the lack of consensus, some authors have shown that results from different pipelines are highly correlated, suggesting that it is possible to reliably assess TMB in different clinical laboratories (van der Velden et al. 2017; Büttner et al. 2019).

20.2.6 Microsatellite Instability (MSI)

Microsatellites (MS), also known as short tandem repeats (STRs) or simple sequence repeats (SSRs), are simple repetitive sequences ranging from 1 to 6 nucleotides that typically repeat 10 to 60 times and are distributed throughout the genome (Cortes-Ciriano et al. 2017; Li et al. 2020a). They account for approximately 3% of the human genome and are located mainly in the non-coding region and approximately only 8% are in the coding region (Hause et al. 2016; Lee et al. 2021).

The highly repetitive nature of MS region makes it difficult to faithfully replicate the sequence by DNA polymerase during DNA synthesis (Siah et al. 2000). Thus, MS tends to suffer a mismatch due to slippage during DNA replication, resulting in the insertion or deletion of one or more repeating units (Li et al. 2020a; Lee et al. 2021). In normal tissues, these errors are detected and corrected by a DNA mismatch repair system (MMR). However, in tumor cells, the genes involved in MMR may be defective, allowing alteration of the number of microsatellites repeats in a process called microsatellite instability (MSI) (Siah et al. 2000).

MSI has important biological and clinical significance. MSI analysis allows detection of deficiency in the DNA MMR, which helps understand cancer

pathogenesis and prevention. Moreover, MSI can be a useful diagnostic tool for some types of cancer; it can be predictive of tumor responsiveness to certain chemotherapy agents and has prognostic significance (Anbazhagan et al. 1999; Hause et al. 2016; Marabelle et al. 2020).

MSI was initially described in colorectal cancer (CRC) and has been extensively studied; 15–20% of CRCs have an MSI phenotype (Rodriguez-Bigas et al. 1997; Ribic et al. 2003; Umar et al. 2004; Sargent et al. 2010; Vilar and Tabernero 2013; Yang et al. 2018; Li et al. 2020a). The MSI phenotype has been well characterized in some colon, gastric, pancreatic, and endometrial cancers. However, it is yet to be well understood in BC (Hause et al. 2016; Cortes-Ciriano et al. 2017; Waalkes et al. 2018).

Most reports have described MSI to be infrequent in BC patients and have shown inconsistent results regarding the prognostic value of MSI status (Paulson et al. 1996; Anbazhagan et al. 1999; Özer et al. 2002; Trabucco et al. 2019; Ren et al. 2021). However, the available data on the incidence of MSI for BC is still restricted to a specific origin, subtype, ethnicity, or population, which have been shown to affect the frequency of MSI in other cancers. In addition, these studies, have been conducted using the same microsatellite markers that were standardized for CRC, which may not reflect the actual status of the MSI phenotype, as most microsatellites suggestive of MSI are specific to particular cancer types and are especially challenging in BC (Siah et al. 2000; Hause et al. 2016; Cortes-Ciriano et al. 2017; Long et al. 2020). Therefore, further investigations using other criteria are needed before conclusions can be drawn.

Different approaches have been applied to MSI detection in cancer, including PCR, IHC, and NGS. MSI-PCR is considered the gold standard method. Traditionally, a paired analysis is performed on DNA samples extracted from normal and tumoral tissues using fluorescent multiplex PCR followed by capillary electrophoresis to compare the amplification of five microsatellite markers, namely, BAT25, BAT26, D2S123, D5S346, and D17S250 (MSI-PCR). The mutational status of markers allows classification of the tumor as high microsatellite instability (MSI-H), low microsatellite instability (MSI-L), or microsatellite stable (MSS). MSI-H shows instability in two or more of the five markers, MSI-L shows instability at only one of the five markers, and MSS does not show instability in any markers. However, it is limited in that it can simultaneously evaluate relatively few microsatellite loci (Rodriguez-Bigas et al. 1997; Umar et al. 2004; Li et al. 2020a).

In this sense, NGS has shown great potential in MSI studies, and several bioinformatics approaches based on NGS data have been used to detect MSI (MSI-NGS). MSI-NGS show results comparable to MSI-PCR, presenting a limit of detection of 1% of MSI in an MSS background, and with the advantage of allowing the simultaneous analysis of a large number of microsatellite markers, ranging from five to hundreds of loci, depending on the protocol applied (Salipante et al. 2014; Waalkes et al. 2018; Trabucco et al. 2019). However, they also have the disadvantage of being affected by factors such as sequencing depth and panel size (Zhou et al. 2021).

MSI algorithms for NGS are constantly being developed, and some of the most widely used algorithms include MSIFinder (Zhou et al. 2021), MSIpred (Wang and Liang 2018), MANTIS (Kautto et al. 2017), MSIseq (Huang et al. 2015), mSINGS (Salipante et al. 2014), MSISensor (Niu et al. 2014), and MOSAIC (Hause et al. 2016). Each MSI region is evaluated by comparing with pre-established models and categorized as stable or unstable. To lower the classification bias, the models must contain a sufficient amount of data on the cancer under study. In addition, the choice of microsatellite markers may impact the MSI detection rate in each cancer type and the selection of inappropriate makers can increase the rate of inconclusive results. Some algorithms are also based on changes in the mutation burden of microsatellite locus repeats and mutation type, requiring a large panel size suitable for WES. A second approach measures the stability of MSI by comparing data from normal and tumor samples, thereby increasing costs (Bonneville et al. 2020).

20.3 Genetic Counseling

NGS tests have changed the dynamics of genetic counseling, and genetic counselors face various challenges. With many commercially available genetic tests and the use of larger panels with clinically relevant genes and genes with suspected or limited/conflicting evidence (moderate to low-penetrance genes), multigene tests should be chosen carefully. Panels can be grouped in different ways, for example, in disease-specific panels, guideline-based panels, or comprehensive cancer panels, each of which has its pros and cons (Pilarski 2021).

The NCCN guidelines (Version 1.2022) (Daly et al. 2021) recommend multigene testing for genes having strong or moderate evidence of actionability and do not include *CDKN2A* screening for BC. *CDKN2A* is a well-known susceptibility gene for melanoma and pancreatic cancer and is potentially associated with other cancer types (Chan et al. 2021). LaDuca and colleagues demonstrated increased BC risk for germline *CDKN2A* pathogenic variants not previously reported (LaDuca et al. 2020). *CDKN2A* pathogenic variants carriers do not meet the NCCN criteria and their absence for cancer screening may result in the underdiagnosis of hereditary BC. In contrast, many pathogenic variants identified still do not change medical management, even for well-understood genes and expanded panel testing increases the rate of detection of VUS. Therefore, the inclusion of more genes and/or more patients, even healthy women, remains controversial (Beitsch et al. 2019; Copur et al. 2019; Pilarski 2021; Abdel-Razeq 2021).

As mentioned before (see Sect. 20.1.2), genetic and environmental factors also add to cancer risks. An increasing number of companies offer direct-to-consumer (DTC) genetic testing that usually account for only a small portion of cancer risk. These tests are marketed directly to consumers without the direct involvement of a healthcare provider and are not recommended for clinical use. Polygenic risk score (PRS) testing, in turn, may represent a new tool for patient management. BC PRS is being offered by two diagnostic laboratories despite insufficient evidence and the

absence of clinical guidelines. The NCCN guidelines (Version 1.2022) (Daly et al. 2021) do not recommend PRS testing to genetic counselors.

Healthcare providers and genetic counselors are indispensable for facilitating appropriate genetic testing and interpretation of results. However, there exists no optimal testing strategy. Genetic testing and counseling are evolving fields, and guidelines need to be constantly revised and updated to identify more at-risk patients. Besides, psychosocial support must also be expanded.

20.4 Future Perspectives

New technologies continue to be developed including third-generation sequencing that proposes sequencing genetic material from a simple DNA molecule in real-time, which may solve the problems related to sample quantity and errors by polymerase during the PCR. Furthermore, third-generation sequencing produces reads up to 30,000 bases in length, allowing for a more accurate analysis of pseudogenes, inversions, deletions, and other gene rearrangements. Despite these benefits, its use in clinical settings is still very limited owing to the high error rate in sequencing (5–20%); however, some research groups use it in conjunction with massive parallel or second-generation sequencing. The main representatives of this methodology are Oxford Nanopore Technologies' MinION sequencers and Pacific Bioscience's single-molecule real-time (SMRT) technology (Xiao and Zhou 2020). Other applications of NGS, although not yet incorporated into clinical practice, have a strong scientific basis and are under development by various research groups. The following are some promising approaches.

20.4.1 Personalized Cancer Vaccine

In addition to MSI and TMB, cancer immunotherapy vaccines can also benefit from NGS approaches. Personalized cancer vaccines (PCVs) induce a specific immune response through patient exposure to tumor antigens (TAA, tumor-associated antigens or TSA, tumor-specific antigens) (Pardi et al. 2018; Pastor et al. 2018). The benefits of PCV include (1) high specificity, prevention of off-target damage to non-cancer cells and tissues; (2) potent humoral and cellular immune responses; (3) safety and stability; and (4) protection against disease recurrence (post-treatment immunological memory). While the limitations of PCV include high costs and time delays for vaccine production, among others (Blass and Ott 2021).

Tumor antigens can occur from genetic (somatic mutations) or epigenetic alterations, transcriptome-based aberrations (e.g., alternative splicing and gene overexpression), and post-translational protein changes (e.g., methylation and phosphorylation). Tumor WGS, WES, and RNA-seq gene expression analyses provide useful information about mutated genes and their expression, contributing to the identification of tumor antigens in cancer patients and the determination of the complete landscape of tumor neoantigens (Roudko et al. 2020).

Bioinformatics algorithms are essential for immunogenic neoepitope prediction, and many computational methods have been developed in this respect. Tumor Neoantigen Selection Alliance (TESLA), a global bioinformatics consortium for neoantigen research, aims to develop the correct algorithms for targeting neoantigens through predictive pipelines and machine learning. Key characteristics that strongly indicate tumor epitope immunogenicity have been proposed: strong MHC binding affinity, high binding stability, high tumor abundance/expression, and T cell peptide recognition (low peptide agretopicity or high peptide foreignness). Besides, cancer type, TMB, and NGS approaches could also influence neoepitope immunogenicity prediction (Wells et al. 2020). For example, tumors with high TMB are likely to have several neoantigen candidates for vaccine formulation. The advantages and limitations of available methods for prediction and validation of neoantigens have been discussed by Roudko and colleagues (2020).

A large number of preclinical and clinical studies on cancer vaccines are underway, and none has been approved by the FDA. For example, Moderna and Merck developed mRNA-4157 associated with KEYTRUDA (pembrolizumab) for the treatment of different cancers, and KEYNOTE-603 is evaluating its safety, tolerability, and immunogenicity (Clinical Trial information: NCT03313778). As pembrolizumab is an FDA-approved drug for high-risk, early-stage TNBC, they can benefit from mRNA-4157 research.

TNBC patients are also candidates for other clinical trials because of the lack of established therapeutic targets. The Mutanome Engineered RNA Immunotherapy (MERIT) consortium is validating an innovative individualized mRNA-based vaccine for TNBC treatment (Clinical Trial Information: NCT02316457). This study introduces a novel concept for an individualized vaccine against cancer (IVAC®) MUTANOME. Mutanome refers to the unique repertoire of mutations in a patient's tumor, and IVAC MUTANOME is a poly neoepitopic coding RNA-based vaccine (Clinical Trial information: NCT02035956). Li and Bu have reviewed some BC vaccine therapies in progress (Li and Bu 2017). Blass and Ott have summarized the current status of PCV clinical studies (Blass and Ott 2021).

20.4.2 Liquid Biopsy

The search for minimally invasive methods that allow the early and accurate diagnosis of cancer, essential for improving the survival rate of patients, has been one of the central objectives of oncology research (Mattox et al. 2019; Bai et al. 2020). Liquid biopsy is the use of fluid samples such as blood, urine, saliva, feces, cerebrospinal fluid, and other minimally invasive biological samples to determine the status of a disease or condition (Chen et al. 2020).

Liquid biopsy is less invasive and cheaper than conventional biopsy and imaging techniques and is more sensitive and specific than conventional techniques to detect biomarkers in blood (e.g., electrochemiluminescence). Furthermore, sample for liquid biopsy can be collected again and used to (1) assess tumor progression at different stages in the disease, (2) describe the molecular profile of the tumor (which

can change prognosis), (3) estimate the risk of recurrence or metastatic progression, (4) stratify in real-time and monitor therapies, (5) identify therapeutic targets and resistance mechanisms (predictive markers), and (6) understand the development of metastases in patients with different types of cancers (Olsson et al. 2015; Pérez-Callejo et al. 2016; Siravegna et al. 2017; Mastoraki et al. 2018; Hiemcke-Jiwa et al. 2018; Rolfo et al. 2018).

Several studies have analyzed the communication between tumor cells and their microenvironment and identified tumor cells or biomolecules (e.g., circulating tumor cells (CTCs), circulating DNA (cDNA), microRNAs (miRNAs), cell-free DNA (cfDNA), proteins, microvesicles, and exosomes) in body fluids such as blood, cerebrospinal fluid, saliva, and urine, indicating great potential for clinical application of these biomarkers for diagnosis and monitoring in different types of cancers (Chen et al. 2013; Crowley et al. 2013; Warton et al. 2016; Phallen et al. 2017; Qi et al. 2018; Marrugo-Ramírez et al. 2018; Forman and Sotelo 2020). The analysis of cfDNA from blood samples shows promise for a new generation of diagnostic approaches (Phallen et al. 2017). Thus, liquid biopsy has gained increasing prominence and is being extensively discussed in regard to its clinical value and associated advantages such as minimal invasiveness, low reagent consumption, and ease of use (Qi et al. 2018; Marrugo-Ramírez et al. 2018; Bai et al. 2020).

According to Fribbens and colleagues, recent research has shown that ctDNA is detected in the plasma of cancer patients and can provide a robust, noninvasive method for detecting *ESR1* mutations (Fribbens et al. 2016). Therefore, monitoring the evolution of BC using minimally invasive techniques, such as liquid biopsy, in patients undergoing treatment is strategically important to choose the most appropriate treatment, which may increase patient survival.

20.5 Conclusions

The versatility of NGS methods has contributed to the spread of this technique in oncology studies and to the complementary diagnosis of cancer, including BC. Allowing evaluation of point mutations and gene fusions in DNA, up to group of genes and total genome studies, the NGS became one of the powerful tools in the field of precision medicine. The huge data generation from sequencing initiatives around the world contributed to the creation of databases that promoted a collaborative network in the identification of cancer molecular signatures. Then, several established panels, involving germline and somatic mutations research, are currently used both in the molecular classification of BC, as in choosing target therapies and even as prognostic tools, including recommendations by guidelines such as the NCCN. Since its beginning, NGS has proven to be a technique in continuous improvement, which opens promising doors for future applications, such as DNA vaccines and liquid biopsy. Although the NGS has some limitations, such as cost, availability of the technique and analysis methods, this technique has proven to be an important ally to decipher the marks associated with BC heterogeneity, allowing for great advances in the area of precision medicine.

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Emerging Paradigm of Breast Cancer Resistance and Treatment

21

Saima Shakil Malik and Nosheen Masood

Abstract

We are at earlier stages of developing methods to use liquid biopsy, machine learning, and artificial intelligence for the betterment of patients. Advancement in technology will surely provide better, cheaper, and efficient therapeutic options that would be simpler enough to be used and learned by clinicians in daily clinical settings. Development of liquid biopsy-related biomarkers will assist the conventional methods of cancer diagnostics but for advanced stage disease, therapeutic strategies will be modified on the basis of disease biology involving the concept of personalized medicine. Numerous works are in process to establish the use of liquid biopsy into a standard of care tool for regulating cancer patients treatment across the globe. Still a lot more is required in terms of clinical trials and studies from academia and industry to speed up the pace of liquid biopsy-related research so that we can find ways to improve patients health outcomes across different tumor stages and types along with in-depth understanding of underlying therapeutic resistance mechanisms.

Keywords

Cancer genomics · Cancer evolution · Artificial intelligence · Machine learning

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21.1 Introduction

Clonal evolution and selection paves the way towards the development of tumor's resistance to different treatments over a period of time (Greaves and Maley 2012). This happens when cancerous cells find ways to survive in opposition to available treatments. Cells that survive against both of the preoperative (neoadjuvant) and postoperative (adjuvant) systemic therapies develop resistance and lead to metastasis and disease recurrence (Siravegna et al. 2015). In the last two decades, science has made major contributions towards breast cancer treatment, and survival rate has been improved largely but still many patients develop disease relapse and resistance to standard/conventional therapies (Tang et al. 2016). Next-generation sequencing (NGS) has emerged as a powerful technique to analyze tumor evolution almost upto precision and contributed towards better understanding of mechanistic insights of tumor initiation and development. Regardless of these advancements, comprehension of clonal evolution, intra-tumor heterogeneity, and aptitude for viable release of resistant clones is rarely pondered in the clinical setting (LeBlanc and Marra 2015; Soto et al. 2016). Consequently, understanding the insights of all the mechanisms that can cause treatment resistance via clonal evolution is mandatory to introduce unique diagnostic and therapeutic interventions and improve overall breast cancer survival rate (Eccles et al. 2013). NGS comes up with a novel application of matching primary and metastatic samples aiding in recognizing sample connection, arrays of private and shared mutations, and predicting fairly accurate evolutionary relationship (Schweiger et al. 2011). On the basis of primary tumor biology, clinical management of breast cancer chiefly counts on three predictive/prognostic clinical markers including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Malik et al. 2020).

Considering the dynamic nature of cancer evolution, tumor biopsies provide limited information about intra-tumor heterogeneity which normally subsitutes cancer clonal evolution (Masood and Malik 2020). In the recent times, liquid biopsy has emerged as a novel source of tumor DNA and RNA (Pinzani et al. 2021). Blood serves as a hub for storing a variety of circulating biomarkers, for example, circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), tumor-associated macrophages (TAMs), tumor-educated platelets (TEPs), and circulating RNA that extend the potential to explore and transfer current therapeutic paradigm in appraising tumor biology (Schwarzenbach et al. 2014). Many clinical studies have explored the potential of CTCs as prognostic and response predictor marker in breast cancer. Although their limited quantity is obtained in various experiments, they provide a clear picture of different events occuring at replication (DNA), transcription (RNA), and translational (protein) levels (Gradilone et al. 2011; Jauch et al. 2019; Cayrefourcq and Alix-Panabières 2020). Using NGS, molecular alterations in ctDNA such as point mutations, amplifications, rearrangements, and gene copy number variations can be identified. Very few cancer genomic centers have initiated the use of CTCs and ctDNA for monitoring genetic events and disease response that arise and influence drug resistance around the globe (Low et al. 2018; Castro-Giner and Aceto 2020). Therefore, in this chapter we are going to discuss the role of

understanding cancer clonal evolution using intricate knowledge about cancer genomics of ctDNA and integrating artificial intelligence. It may help in identifying novel therapeutic resistance targets other than the usual FGFR, EGFR, and HER2 suspects.

21.2 Cancer Genomics

Breast cancer arises as a group of abnormal cells in the areas regarded as breast and its surrounding tissues. It is recognized as a lump or other observable physical abnormality in the breast tissue and molecularly identified by scanning of estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2) using immunohistochemical techniques. These three receptors are the basis of classification of breast cancer into different subtypes like claudin low, basal-like, normal-like, HER2-enriched, luminal A or B (Prat et al. 2010). Epidermal growth factor receptor (EGFR) and CK 5 or 6 expression along with Ki 67 protein levels are also used for classification of breast cancer. HER2-positive patients are considered to have a worse prognosis and speedy growth of tumor compared with HER2 negative luminal A type of tissue. The subtype luminal B is HER2-positive and less aggressive; HER2-enriched category is also HER2-positive, but ER- and PR-negative has the worst prognosis and the fastest growth rate compared with luminal type A and B. Similarly, normal-like breast cancer cells are almost normal with very few cancerous cells and reduced gene expression of proliferating proteins. Mutation in BRCA1 gene is very common in women, and most of them are triple negative for ER, PR, and HER2 expression and are mostly known as basal-like breast cancer (Reis-Filho and Tutt 2008). Claudin low cancer show reduced levels of Ki 67 and marker genes compared with other subtypes. Most common type of breast cancer patients are triple-negative. HER2-enriched has a success rate higher than others when treated with Trastuzumab. Triple-negative breast cancer (TNBC) patients are least responsive towards hormone therapy or targeted treatment options. Therefore, they are mostly administered with combination of chemotherapeutic drugs. Basal-like breast cancer is treated mostly with neoadjuvant therapy, whereas luminal type A patients are given endocrine therapy (Carey et al. 2007). Before administering any type of treatment option or drug detailed analysis of genetic as well as physiological analysis is made. Many different types of tests are performed that include molecular profiling using PCR, RT PCR, immunohistochemistry, western analysis, etc. as many molecular subtypes may have different genetic profiles; therefore, no precise drug can be attributed to any subtype without detailed genetic analysis (Fig. 21.1). Studies revealed that although TNBC is a molecular subtype of breast cancer, it consists of variations based on molecular variations (Yin et al. 2020).

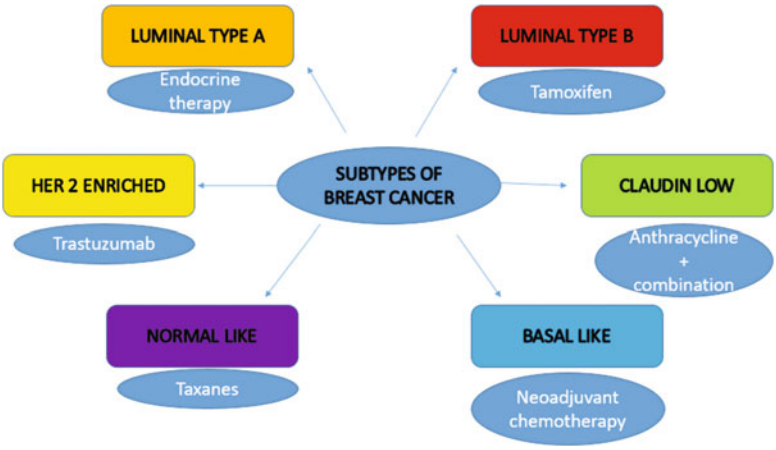

Fig. 21.1 Subtypes of breast cancer along with information about possible treatments given to them

21.3 Cancer Evolution

The main concern while studying cancer is how cancer cells evolved over time. How have they become resistant? What makes them change over time? Originally, the term cancer evolution means that the cells that initiate malignant tumor undergo changes and acquire the characteristics that were never a part of them. They learn to move from one place to another, and ultimately they become drug resistant. The main concern of present-day oncologists is to encounter this evolution, but first we need to know the reasons. The first one being heterogeneity of tumor cells, i.e., the cells are not of the same type and their rate of cell division and sensitivity to external stimulus also varies resulting in phenotypic and genotypic differences within the tumor (Fidler et al. 2007). Tumor cells are mainly clone of cells with similar genotypic and phenotypic properties, or they may be a subclone of cells where different types of cells arise due to mutations from single tumor cell. The number of subclone cells is directly proportional to the severity of disease with metastasis and higher recurrence rate (Espiritu et al. 2018). Heterogeneity of tumor not only occurs between the tumor cell population but also between different individuals. Each person is unique and carries his own type of malignant cells different from other person.

Heterogeneity may be spatial or temporal and both types lead to cancer evolution (Burrell et al. 2013; de Bruin et al. 2014). The main cause of heterogeneity is different, but mainly genetic instability causes high mutation rate forming bulk of subclone cells. It was found that BCL11A, FOXA1, and CDK4 are associated with cancer causing intra-tumoral heterogeneity (Jamal-Hanjani et al. 2017; Jamal-Hanjani 2021). Another cause attributed to this concept of heterogeneity is the epigenetic inheritance rate. It is believed that promotors and enhancers are known

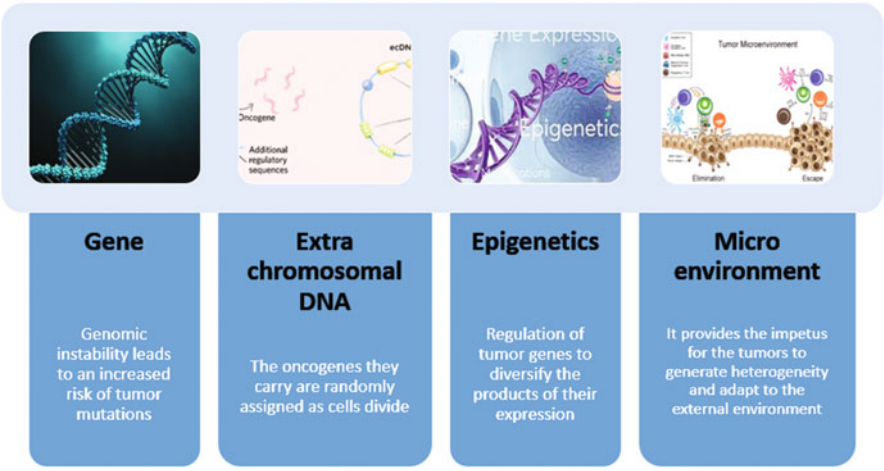

Fig. 21.2 Mode of action of different factors that lead to heterogeneity causing cancer cell evolution

to change the expression of protein and it results in altered clone of cells as seen in bacteria that exhibit drug-resistant and drug-sensitive states. When a drug is continuously administered, the promotors and enhancers no longer identify the target site and lead to constitutive expression rather than controlled by the drug, but if the drug is withdrawn and again administered after a gap the promotors identify and respond to treatment; this is due to heterogeneity. Heterogeneity in epigenetics can be attributed to histone modifications, DNA methylation, and enhancers in addition to many other phenomenon (Jamal-Hanjani 2021).

Another promising new discovery is the extra chromosomal DNA found in tumor cells but not in normal cells (Verhaak et al. 2019). Since they are extra chromosomal, they are not transferred between generations in a Mendelian fashion rather their exact physiology is still unknown. HLAI expressional variation is one reason for immune system weakening leading to cancer proliferation (Fig. 21.2).

Evolutionists have divided the process of evolution into theories like linear, branch, convergent, and parallel evolution (Venkatramana et al. 2019). Most of the cancerous cells have survival advantage that allow them to live for longer times and rest of the cells die and diminish. This is known as linear evolution model; breast cancer cells show this type of cellular organization. Most of the cancer cells have unstable genes that mutate and multiply at a higher rate making them the predominant type of cells; this theory is known as branch evolution theory, for example, colorectal cancer. Convergent evolution states that tumor cells in different areas of same patients mutate in a similar pattern provided the external environment is the same; renal cancer cells proved this theory in studies by different researchers. Sometimes different population of cells mutate that originated from same cancer and due to same pressure of external environment and are consistent in evolutionary direction; such tumors are known to show parallel evolution.

21.4 Circulating Tumor DNA

21.4.1 Applications, Methods, and Clinical Trials

Most of the DNA is enclosed in the cell, but sometimes the DNA is found circulating in the blood. This DNA can be used as a very useful tool for identification of many diseases including cancer. Tumor cells if present in the body also cause circulating cell-free DNA in the blood and that can be detected easily through liquid biopsy. Liquid biopsy is a noninvasive method and is satisfying for the patient and many diagnosis can be made through this method (Han et al. 2020; Gorgannezhad et al. 2018). These circulating tumor DNAs can be detected through real-time PCR or next-generation sequencing. The biggest advantage is the identification of type and severity along with personalized medicine treatment can be administered to the patients leading to better survival and least recurrent rates. Mutational analysis reflects as one of the use of circulating tumor DNA, it can also be utilized for other detections. Size of tumor DNA that is circulating in the blood can also determine the tissue of origin with an accuracy level of more than 60% (Chin et al. 2019). Sometimes methylation and other epigenetic variations are also found in this tumor DNA which can differentiate between early and late progressive cancer of pancreas. Aneuploidy is another detected mutation in circulating tumor DNA that can also determine the type as well as progression of cancer (Campos-Carrillo et al. 2020; Chin et al. 2019).

Personalized anticancer therapy is one of the main target areas for analyzing circulating tumor DNA. Researchers working on non-small cell lung cancer identified cancer clones as well as their mutations and then targeted that pathway. Most of the mutations were in EGFR that can be overcome by using tyrosine inhibitors like gefitinib and erlotinib (Murtuza et al. 2019; Singh and Jadhav 2018). Unfortunately, patients develop resistance to this therapy due to T790M point mutation in the EGFR kinase domain. This mutation can be detected in circulating tumor DNA as well.

Metastatic cancer patients are nowadays treated with a new therapy called immune checkpoint inhibitor (ICI). Imaging of patients undergoing ICI therapy shows pseudo progression of disease, and this therapy can be toxic for patients. These are the most important drawbacks (Basler et al. 2020). With the use of circulating tumor DNA, the immune response can be predicted and toxicity level can be measured in vitro. Circulating tumor DNA levels also correlate with radiological imaging and in case of HNC (head and neck cancer) pre-treatment of circulating tumor DNA represented tumor volume expected by oncologist while planning computed tomography as a part of radiotherapy (Eun et al. 2020). Most of the common uses of circulating tumor DNA have been outlined in Fig. 21.3.

After surgery, the testing of circulating tumor DNA in plasma can be used to monitor minimal residual disease. This concept was approved for pilot study in December 2020 and the results will be available by 2024 (https://clinicaltrials.gov/ct2/show/NCT04726800). Similarly, another study titled as “Prognostic value of circulating tumour DNA in metastatic pancreatic cancer patients: post-hoc analyses

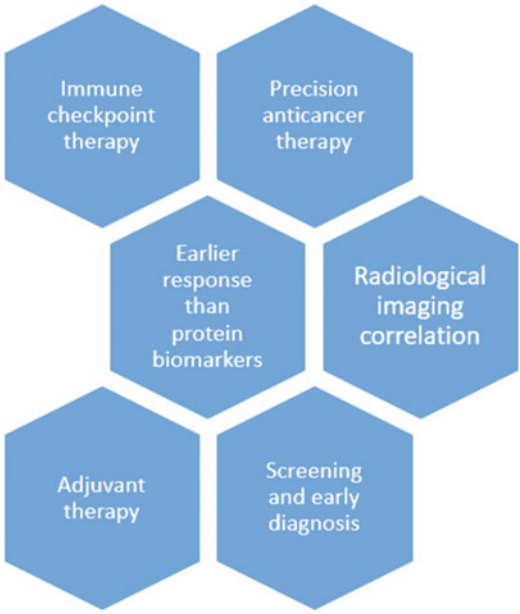

Fig. 21.3 Applications of circulating tumor DNA in different field of oncology

of two clinical trials" reported that nearly 57% patients showed circulating tumor DNA in metastatic pancreatic adenocarcinoma and confirmed that these can be used as an independent marker for prognosis (Pietrasz et al. 2021).

21.5 Artificial Intelligence and Machine Learning

In artificial intelligence, machine learning is a model that uses computers to predict and classify different models from the information available. In 1950, the term was coined first by A. Samuel, who was an employee of IBM (Samuel 1950). Since then, machine learning has transformed the field of health care remarkably. Computational tools have benefitted the population health by analyzing patterns of disease, diagnosis, and taking care of sick along with personalized drug administration. All these features have helped reducing errors by clinicians, detecting sepsis, and decision-making in therapeutics.

Incredible revolutions in NGS expertise are explored to produce omics data which has precipitously generated an insightful effect on the expense of genome analysis and led to the creation of huge amounts of data. The task of how to exploit the vast amount of clinical genomic facts, and from it the subsequent targetable therapeutics, has been pushed to the lead of radical and innovative analytics. Researchers have been anticipating that this vast genomics data itself will expect advanced computing means in the upcoming decades (Stephens et al. 2015). It is a big challenge for the bioinformaticians to explore and test novel options of advanced algorithms so that scientific information can be coded and stored leading to the

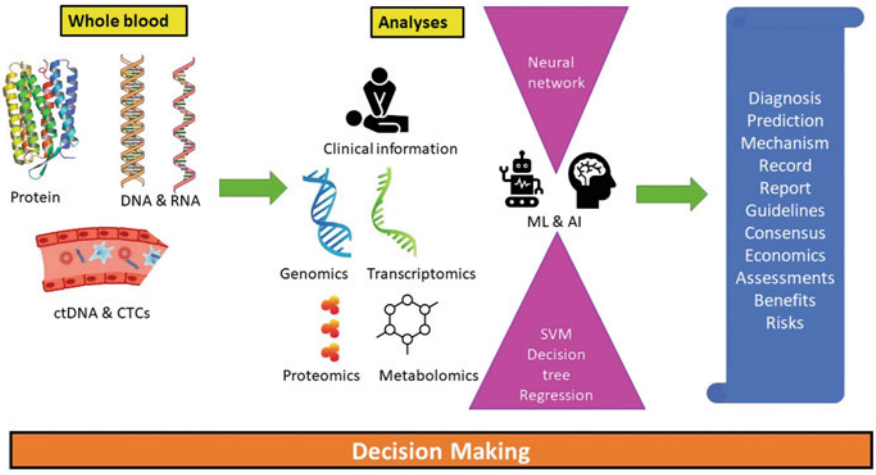

Fig. 21.4 Schematic representation of steps involved in efficient healthcare decision-making

sharing of accurate genomic and transcriptomic data into progressive and innovative understanding of disease pathogenesis and pharmacogenomic-driven treatment response (Huang et al. 2007).

NGS technologies generate tons of data ranging from mutations to variations to gene expression levels to methylation status and many more genomic alterations. All of this data is a part of intricate cellular and molecular biological systems developing complex pathways, networks, and natural biological systems. Therefore, computational tools with the highest accuracy and efficiency are required to use this biological information for the betterment of patients in the future (Fig. 21.4). As we have mentioned that NGS is producing huge amounts omics data and it will keep on generating even more data, the challenging part is the storage, transformation, computational capacity, and generation of reliable results with advanced analytical tools which can ultimately lead to decision-making and therapeutic betterments for patients (Costa et al. 2013; Mutz et al. 2013; Servant et al. 2014; Tomczak et al. 2015). Therefore, following technical aspects and key strategies could be taken into consideration to combat with present and future challenges. Based on the nature, complexity, and novelty of biological information, various machine learning algorithms can produce varied performance leads. Therefore, it is required to select the algorithm that can answer all the underlying questions. It would be of great help if the findings of all these intricate analyses are presented in an intuitive and interpretable way, for example, survival curves, graphical representations, or Manhattan plots. Algorithms should be designed in a way that they must be computationally scalable and capable of integrating variable components of complex clinical/biological data to successfully interpret the meaning, function, and structure of the genetic/transcriptomic information so that it can be translated into operational therapeutics (Camacho et al. 2018; Zitnik et al. 2019).

A long time ago, clinicians have started using the concept of precision/personalized medicine for better treatment or survival outcomes (Collins and Varmus 2015). So, basically bioinformaticians have come up with the idea of providing real-time feedback to clinicians and surgeons using machine learning and artificial intelligence technologies so that the value of care provided to the patients and quality of life can be improved. Cancer involves a complex interplay of many genetic, epigenetic, environmental, and epidemiological factors and is a heterogeneous disease making its genome more difficult to understand (Mubarik et al. 2019). Genomic data depends on numerous cellular and molecular mechanisms along with interacting pathways; therefore, researchers around the globe are working to develop machine learning technologies that can interpret this much large and complex data. Computational, biological, clinical, and mathematical researchers/scientists are encouraging the need to construct more accurate and trustworthy machine learning techniques that can manage the huge information (Ledford 2015). The concept behind making machine learning technologies is that the models would aid in infering, interpreting, predicting, and clarifying the observed data. Once the machine is taught to work with a known data set, it can use the model to make interpretations of the future data (Sarker 2021).

The genotype and phenotype have complex underlying relationship that is enormously complicated in tumor's milieu with continuous evolutionary transformation through complex and interrelated biochemical and biophysical processes and unknown factors such as association of the genome with various food forms that individual consumes and their socio-environmental/economic surroundings where they live in makes it far more hard to accomplish the near-human predictions via machine learning technologies. Thus, believing the "variables" that best fit in the training models might adopt human-like circumstances and handle with continuously evolving data (Kim and Przytycka 2013).

While dealing with prediction modeling applications, an important issue is the missing/lacking some information like sometimes we don't have proper clinical record of all the diagnostic tests of a patient that would be of great help if available in terms of inferring treatment effectiveness with reference to respective diagnosis. Hence, to overcome this issue of missing/lacking data in multiple logistic regression, researchers have developed a substitutive model called alternative decision tree to precisely predict the diagnostic along with therapeutic outcomes among primary breast carcinoma patients (Sugimoto et al. 2013). One of the recent studies on breast cancer cases has used principal component analysis and showed expression as an effective indicator of paclitaxel sensitivity with 82% accuracy via support vector machines. They have also reported the importance of expression and copy number for gemcitabine sensitivity with an accuracy of 85%. For this copy number, profiles of three genes (*NT5C*, *ABCC10*, and *TYMS*) were factored in, accompanied by the expression of seven other genes including *RRM1*, *ABCB1*, *RRM2B*, *ABCC10*, *DCTD*, *CMPK1*, and *NME1*. But considering the integrity of nucleic acids for tumor blocks, gemcitabine support vector machines showed 62% prediction accuracy only (Dorman et al. 2016). Therefore, it has been proposed that determining the isoform abundance and nucleic acid integrity can help in improving the model,

prediction accuracy, and sensitivity, too. So, it is required to include the factors/variables that can affect the aptitude to get high-quality gene expression data from old tumor tissue blocks and might aid in improving the performance of support vector machines. Researchers have used variable machine learning techniques and RNA sequencing among different cancer types and control group (healthy individuals) considering the relationship of tumor cells and platelets. They reported an accuracy of 71% and 96% to locate primary tumor and distinguishing localized or metastasized tumors in patients from healthy ones, respectively. While reporting this accuracy, they have found some limitations like heterogeneous nature of the cohort, impact of other indicators on platelets, and mRNA degradation (Best et al. 2015).

Researchers from MIT and Harvard have constructed a patch-level prediction for distinguishing between tumor and normal/healthy patches using a trained convolutional neural network by tutoring millions of patches. During the classification and tumor localization of the full slide image in metastatic breast cancer sentinel lymph node biopsies, 85% reduced human error rate was reported with the combination of pathologist's diagnosis and predictions by machine learning technologies. Natural language processing algorithms aid in automated retrieval of pathological and mamographic results from pathology reports and free text mammogram (Patel et al. 2017). Therefore, researchers have used these algorithms to find that ER-positive tumors were plausible to speculated margins and the ones having HER2 overexpression were expected to have pleomorphic and heterogeneous calcifications. These findings lead us to the conclusion that machine learning technologies can significantly contribute towards the accurate disease diagnosis (Wang et al. 2016).

With rapidly increasing and evident need of machine learning technologies in clinical setups, considering the variables that might affect patient information, health risks, lifestyle modifications, tumor cell interactions, its microenvironment, genetic and epigenetic impact, quality of somatic and tumor cell genomes, liquid biopsy timing, therapy, medications, and dosage schedules, treatment responses, adverse effects, numerous sequencing platform limitations, and impartial aptitude to deal with various variables while working with machine learning technologies may aid in achieving therapeutic options that could bring betterment in patients' overall health. While using machine learning or single cell genome technologies, it is vital to select a technique that provides accurate isolation of single cells; otherwise, results can be messed up during the analyses. Therefore, biotechnology-based companies are working hard to introduce easy, quick, highly accurate, greater throughput, and smoothly reproducible single cell isolation methods/kits (Blainey 2013; Navin 2014). During the development of whole-genome amplification techniques, many other options have been considered such as false-positives, PCR artifacts, amplification biasness, chimeras, and genome loss. In order to get clinically relevant information, one must have to carefully look into the type of genomic information, i.e., DNA/RNA as well (Sung et al. 2012).

Although data scientists are constructing evolutionary trees with circulating tumor cells (Nagrath et al. 2007) or DNA (Snyder et al. 2016), still a lot more research is required to predict the response biomarkers and prognostics providing a

range of best therapeutic options to the oncologists (Aceto et al. 2014). This needs to be done through a joint collaboration of biologist, data scientist, mathematician, and a clinician. In addition, it is required to test the model and repeat it in such a way that it is easier for a biologist and clinician to understand so that they reproduce the findings and double check every possibility before implenting it to therapeutics. It will help in building clinician and patient trust by using machine learning and respective models in disease diagnosis, prognosis, and treatment instead of just relying on the previously published literature. Another important aspect that needs to be considered is conservation. Most of the presently available models lay emphasis on evolutionary conservation, but it is the hour of need to consider functional conservation while designing models because all evolutionary conserved sequences/motifs/domains are not functionally conserved in the genome (Kellis et al. 2014; Reva et al. 2011). It is crucial to check the flexibility of algorithm while opting for machine learning and artificial intelligence. Algorithms should be capable of accommodating conventional outcomes in clinical trials such as binary, continuous, and specifically time to event and prepare enough to deal with covariates. Also, they should be developed in order to handle diverse genetic and clinical heterogeneity as clinical trials comprising varied ancestories (Welch et al. 2014). Therefore, to acknowledge the importance of machine learning and artificial intelligence, MIT, Harvard, and IBM have initiated a 5-year research collaboration with a funding of $50 million to study the underlying mechanisms of drug resistance pattern in cancers. They are using IBM Watson's computational and machine learning methods to explore drug resistance in all tumor types. They are trying to generate bulks of tumor genome sequence data of patients who had responded to different treatment or adjuvant therapies but developed drug resistance at later stages (Cancer Discovery 2016). Researchers are using Watson for data analyses and identification of genomic patterns that might be helpful to clinicians/oncologists to foresee drug resistance and sensitivity as well. Therefore, at first it is required to learn the drug resistance patterns in different cancers, stage/grade and metastatic involvement, timing and factors leading to resistance to develop respective algorithms that would be useful/helpful in the near future.

Eventually, scientists are trying to link the data generated by NGS and analyzed by artificial intelligence to come up with widely available open networks such as ICGC and TCGA which can educate, appraise, and support cancer research and treatment (Tomczak et al. 2015; Jennings and Hudson 2016; Liu et al. 2018). One of the wonderful ongoing projects is the development of CancerLinQ by the American Society of Clinical Oncology and shaping the future of cancer care. It is a non-profit-based health-related technology company aiming to provide patient care, quality of life, and improving treatment practices using evidence-based research and medicine. They are collecting data from all the cancer patients throughout the USA and making large-scale data repository to regulate clinical workflows and increase productivity (Sledge et al. 2013). But still a lot more research is needed to understand cancer evolution and underlying mechanisms. Many researchers have explored drug resistance mechanisms and the use of variable of technologies in different cancers that

could help clinicians in taking timely decisions to manage patient's health (Osborne and Schiff 2011; André et al. 2014; Yang et al. 2017; Gao et al. 2016).

21.6 Harnessing the Immune System Using Liquid Biopsy, Machine Learning, and Artificial Intelligence

With every passing day, Steven Rosenberg's dream of fighting against cancer by harnessing the immune system is getting real existence (Rosenberg and Restifo 2015). Gradually, many breakthrough and encouraging stories are coming up with tumor regression and complete cure gaining researchers and funding bodies interest in rapidly emerging field of immunotherapy. A number of clinical trials are in progress across the globe implicating either immunotherapy alone or in combination with other treatments. Our body is blessed with a network of cells, tissues, systems, and pathways or mechanisms that build immunity against foreign particles. But in certain circumstances, cancer still finds the ways to hide or overcome this immunity and damaging individual's health (Coico 2021).

Cancer immunotherapy has made significant contribution in the treatment of melanoma patients (Prieto et al. 2012; Di Giacomo et al. 2013); moreover, vigorous efforts have been made to discover prognostic predictors in other cancer types (Herbst et al. 2014; Tumeh et al. 2014; Angelova et al. 2015; Smid et al. 2016). Researchers have analyzed whole genome of a large breast cancer cohort and suggested a substitution that might be more effective in triggering an immune response (Smid et al. 2016). Studies using various tumor types verified immunoediting and presented genetic amplifications and immuno-suppressive factors like PDL1/2 in tumor-intrinsic resistance to cytolytic activity (Rooney et al. 2015). However, still a lot more to explore regarding the potential of immunoediting and its impact on patients' well-being (Schreiber et al. 2011). The role of cytotoxic T cells (CTLs) and tumor-associated macrophages is well developed in some cancers, but the clinical relevance of other immune cells still remains unrecognized (Li et al. 2018; Pathria et al. 2019; Cheng et al. 2021). Therefore, in-depth genomic, transcriptomic, and translational analyses are required to increase the understanding of anti-tumor response and select effective immunotherapies for breast cancers. The basic purpose of harnessing the immune system is to develop such an antibody that makes immune system efficient enough to recognize and fight cancer cells. So, basically two different immunotherapy approaches are under consideration. First is the concept of personalized medicine or treatment for every person by taking some of the cells from the patient, modifying them to stop the growth of cancerous cells and infusing them (cancer-free cells) back to the patient's blood stream so that they can fight with metastasis. The other way is the large-scale production of non-personalized drugs that work with immunity to fight against cancer via checkpoint control mechanism. It can be combined with other treatment options to overcome resistance (Finn 2018). Radio- and chemotherapy can modulate and boost the sensitivity of tumor cells to immunotherapy. In the past few years, DNA of various tumor types comes with a huge number of mutations and the

neoantigens encoded by mutated genes have significant contribution in current cancer immunotherapeutics (Schumacher and Schreiber 2015). Isolation and keeping the growth cultures of tumor infiltrating lymphocytes is a tough job; therefore, availability of fresh tumor samples can ease this process and would serve as a shortcut to retrieve neoantigen-specifc T cells from patients (Schumacher and Schreiber 2015). The reason behind the identification of peripheral blood's tumor-reactive T cell population is to spotlight cytotoxic CD8+ T cell population, responsible for expressing surface molecule programmed cell death 1 (a well-known biomarker for exhausted/activated T cells). One of the studies with four melanoma patients was lucky enough to extract mutation-specifc T cells from the blood of three of them (Gros et al. 2016). This basically inspired the scientists to develop and explore reliable liquid biopsy extraction and detection methods with greater sensitivity where breast cancer cases are provided with immunotherapy (Brown et al. 2014). With advancement in NGS, machine learning algorithms, and artificial intelligence, scientists believe that advancing in liquid biopsy research will help in developing tumor-specific T cells along with their products and reach better understanding of breast cancer resistance mechanisms.

21.7 Conclusion

Breast cancer has increased at an alarming rate throughout the globe, and its genetic aspects are still under study. Researchers have identified many genes like BRCA1, BRCA2, HER2, NEU, etc., but still there is a need to identify many more patterns of genetic involvement that initiate breast cancer. Cancer evolves rapidly from type to type as well as within the same type. Cancerous cells keep on mutating and producing new cells that are better adopted to survive, replicate, and take control of host. To overcome the issues related to breast cancer prognosis and disease severity as well as personalized medicine and treatment, nowadays many new tools are in use that require machine learning. Artificial intelligence is a field that helps identify the gaps in already available techniques like liquid biospy and circulating tumor DNA identification. This is a relatively new field and is promising in solving many problems associated with breast cancer.

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