Chapter 10
Epigenetic Signatures of Breast Cancer Genes

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Abstract Breast cancer is a leading malignancy among women with higher western countries, suggesting significant role for environmental factors in developing breast cancer. Recently, epigenetic modifications such as aberrant methylation and acetylation of genes and histones have been shown to play a critical role in breast cancer development. There are several articles published in the recent years with the major epigenetic signatures of breast cancer genes. Therefore compiling these information could lead to a greater understanding of the development of breast cancer and novel approaches for chemoprevention. Here we have provided different modes of epigenetic regulation including DNA methylation, histone modification, polycomb group of proteins, and non-coding RNAs. In addition, we have provided information on chemotherapeutic drugs that act through regulation of epigenetics and have progressed to clinical trials. Most importantly, we have analyzed the epigenetic regulation in the chemotherapy resistant breast cancer stem cell population. Furthermore, the epigenetic regulatory mechanisms of various breast cancer related genes are discussed in detail. Taken together, in this review we have discussed the current understanding of the modes of epigenetic regulation, and the epigenetic signatures seen in breast cancer.

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10.1 Introduction

Breast cancer is a leading malignancy among women, and has long evaded attempts at prevention. In spite of early detection and improved treatment, every year nearly 250,000 women discover that they have invasive breast cancer [1]. Roughly 58,000 more will be diagnosed with early cases of the disease and about 40,000 will die [2]. The incidence of breast cancers is much higher in western countries than that in other parts of the world, suggesting a significant role for environmental factors in developing breast cancer. Genetic and epigenetic alterations have both been shown to play an important role in breast carcinogenesis. The former is irreversible while the epigenetic changes are reversible. Epigenetic malfunctions are manifested through aberrant methylation and acetylation of genes and histones involved in normal tissue development, leading to activation or silencing gene expression. Thus numerous molecular events could go awry because of epigenetic malfunctions. The advancement in the science of epigenetics has led to a greater understanding of how breast cancer forms, resulting in the discovery of novel approaches for chemoprevention. Specifically, identifying DNA methyltransferases and histone deacetylases that control epigenetic modifications has resulted in utilizing these enzymes as primary targets for epigenetic therapy. In this review, we will discuss the current understanding of the modes of epigenetic regulation, and the epigenetic signatures seen in breast cancer.

10.2 History of Epigenetics

Epigenetics is the study of changes in phenotype or gene expression caused by mechanisms other than those that alter the DNA sequence. In 1942, Conrad Waddington coined the term epigenetics; he derived the name from a Greek word epigenesis, a theory of development [3]. In 1969, Griffith and Mahler were the first to suggest that DNA methylation might have an important biological role on gene expression, and that changes in DNA methylation might explain how genes are turned on and off [4]. A few years later, in 1975, Sager and Kitchin proposed that there are enzymes in eukaryotic organisms that restrict unmodified DNA [5]. Since then it has become apparent that changes in DNA methylation might play important role during carcinogenesis [6]. More recently, this has expanded to other types of modification, including histone modifications [7].

10.3 Types of Epigenetic Regulation in Breast Cancer

The interesting aspect of epigenetic alteration of DNA is that such changes are heritable but does not alter nucleotide sequence. This is in contrast to a genetic change where the nucleotide changes. Furthermore, unlike genetic changes, epigenetic modifications are potentially reversible; this aspect gives the potential for therapy against cancer. The science of epigenetics has explained how nutrients and drugs can change the cancer cell cycle [8, 9]. Epigenetics causes the organism's genes to behave differently, such as the changes seen when cells differentiate or become malignant.

Different modes of epigenetic regulation have been observed in breast cancers including (1) DNA methylation (stable and long term repression), (2) histone modification (dynamic and can be changed upon stimulation), (3) polycomb group of proteins (maintain the silenced state of developmental regulators), and (4) Non-coding RNAs (microRNA, small nucleolar RNA, repeat-associated small interfering RNA) (Fig. 10.1). In this chapter, we will describe each one of these regulatory mechanisms (Table 10.1).

10.3.1 DNA Methylation

DNA methylation, a major mode of epigenetic regulation occurs on cytosine residues of CpG dinucleotides [10]. DNA methylation affects the packing of chromatin and the architecture of the nucleus thereby critically regulating gene expression.

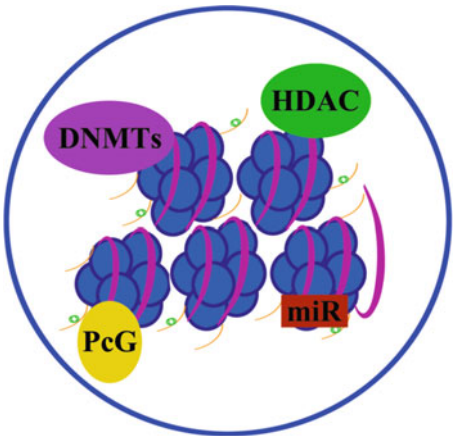

Fig. 10.1 Major modes of epigenetic regulations include DNA methylation, histone modifications, Polycomb Group proteins, and microRNA. DNA Methyltransferases (*DNMTs*) adds methyl group to the cytosine and inhibits transcription, while Histone deacetylases (*HDAC*) along with Polycomb Group proteins and microRNA forms a repressive complex to inhibit gene transcription

Table 10.1 Modes of epigenetic regulation

Modification	Mechanism
DNA methylation	Occurs on cytosine residues of the CpG dinucleotides in DNA
Histone modification	Covalent modification of the N-terminal of certain amino acids on the histone tails
Poly Group Proteins	Forms repressive complex for inhibition of transcription
MicroRNA	Gene silencing, and by triggering transcriptional silencing via chromatin remodeling

CpG islands are regions of at least 500 bp and have more than 55 % GC content [11]. CpG islands have mostly been identified within promoter regions, and methylation within this region makes the DNA inaccessible and no longer recognizable by the transcriptional machinery resulting in gene silencing. A methyl group is added to the cytosine ring by DNA methyltransferases by an enzymatic transfer from the methyl donor S-adenosylmethionine to the carbon-5 position of cytosine [12]. Surprisingly, according to Antequera and Bird, approximately half of all genes in the human (~45,000 genes) contain CpG islands [13]. Various studies have clearly shown that about 70–80 % of CpG sites in the human genome are methylated. DNA-cytosine methyltransferase-1 (DNMT1) was the first methyltransferase to be identified [14]. Subsequent studies have suggested that the DNA methyltransferase 1 (DNMT1) plays a key role in the maintenance and restoration of methylation after DNA replication, while DNMT3A and DNMT3B initiate *de novo* methylation and establish new DNA methylation patterns [15].

In general, normal cells are hypomethylated, while in cancer cells hypermethylation is frequent leading to silencing of tumor suppressor genes. Retinoblastoma gene was characterized to be the first tumor suppressor gene and also they are the first to be identified as hypermethylated. The importance of promoter hypermethylation has been characterized using demethylating agents 5-aza-2′-deoxycytidine to reactivate the genes in cancer cell lines. DNA methylation has been shown to play important role in normal development e.g. development of fertilized egg into an embryo. Apart from this, nearly 45 % of the human genome has repetitive sequences and loss of methylation of these sequences is thought to account for most of the global hypomethylation observed in all human cancers. Hypermethylation of the CpG promoter regions, in the tumor suppressor genes, results in gene silencing and therefore resulting in the oncogenic transformation. These changes have been shown to account for various cancers including breast cancer development. Methylation inactivates the transcription; demethylation may result in transcriptional interference and dysregulation of normal gene expression, leading to destabilization and chromosomal translocations.

There are several genes that are silenced by promoter methylation during breast cancer development. Here, we have attempted to comprehensively provided the list of genes that are hypermethylated at the promoter region during breast cancer development.

10.3.2 Cell Signaling Genes That Are Hypermethylated During Breast Cancer

10.3.2.1 Secreted Frizzled-Related Proteins (SFRPs)

Wnt signaling plays a significant role in development of various types of cancer and inhibiting the pathway hinders the progression of tumorigenesis [16]. Secreted frizzled-related proteins (SFRPs), a family of proteins that include SFRP1 to SFRP5, are extracellular antagonists for Wnt signaling [17]. These proteins are shown to sequester Wnt molecules at the cell surface membrane and thereby regulate the Wnt signaling pathway during breast cancer development [18]. Several reports have also suggested that increased nuclear and cytoplasmic accumulation of β-catenin is due to disruption of SFRPs and Wnt equilibrium in the breast tumor tissues [19]. Subsequent studies have demonstrated that SFRP1 and SFRP5 genes are targets for the promoter hypermethylation leading to inactivation [20, 21]. Moreover, promoter hypermethylation is associated with unfavorable prognosis in breast cancer.

10.3.2.2 Estrogen Receptor (ER)

Estrogens are hormones that play a key role in the growth and development of breast cancers. Estrogens mediate its action through intracellular estrogen receptors (ER) [22]. There are two estrogen receptors ER-α and ER-β, which are encoded by ESR1 and 2 genes, respectively [23]. Estrogen receptors can also function as transcription factors to regulate the expression of target genes. In breast cancers, ER-α methylation is a predictive marker for response to hormone therapy [24]. Importantly, up to a third of the breast cancers lack ER-α at the time of diagnosis and a significant proportion of cancer patients who are initially ER-α positive become ER-α negative during tumor progression [25]. Initially it is thought that these changes are due to genetic alterations such as deletion or mutation; however, recent studies have demonstrated that there is DNA methylation in the promoter region [26]. The ER-α gene, located at chromosome 6q25.1, has CpG rich regions in both its promoter and the first exon [27]. While these CpG rich regions are unmethylated in normal breast tissues and in ER-positive breast cancer, it is methylated in ~50 % of the unselected primary breast cancers and most of the ER-negative breast cancer cell lines [28]. However, treatment with methyltransferase inhibitors such as 5-aza-cytidine (5-aza-C) and 5-aza-deoxycytidine (5-aza-dC) results in partial demethylation and restoration of ER mRNA expression and protein [29]. This has been further confirmed by various studies where it was observed that DNMT1 expression at both RNA and protein levels in ER-negative breast cancer cell lines are significantly increased compared with their ER-positive counterparts.

10.3.2.3 Retinoic Acid Receptor β2 (RARβ2)

RARβ2 gene is a member of the nuclear receptor superfamily [30]. There are six receptors (RARα, -β and -γ and retinoid X receptors-α, -β and -γ) in this family and all of them are ligand activated transcription factors [31]. RARβ gene, located at chromosome 3p24, has been implicated in playing an important role in limiting the growth of various tumors, including breast cancer [32]. Methylation of CpG islands in the RARβ2 promoter region is one of the factors linked to downregulation of its expression in breast cancer [33]. Furthermore, methylation of the RARβ promoter has been shown in several RARβ2-negative human breast cancer cell lines and in about one-third of unselected primary breast cancer specimens, a result of which is decreased or complete loss of RARβ2 expression [34]. However, treatment with 5-aza-dC can partially restore RARβ2 expression further confirming the promoter methylation mediated suppression of its transcription [35].

10.3.2.4 Aplasia Ras Homolog Member I (ARHI)

The Ras-related novel tumor suppressor gene aplasia Ras homolog member I (ARHI; also known as DIRAS3) encodes a small GTPase with 60 % homology to Ras and Rap. The expression of this tumor suppressor is down regulated in 40 % of DCIS and 70 % of invasive breast cancers [36]. Furthermore, reexpression of ARHI protein suppresses clonogenic growth of breast cancer cells, inhibits their invasiveness, and induces apoptosis. There are three CpG islands in the ARHI gene. The first two CpG islands are located in the promoter region, while the third CpG island is located in the coding region [37]. Furthermore, hypermethylation of the second CpG island within the promoter region results in the complete loss of ARHI expression in breast cancer cells [38].

10.3.3 DNA Damage Response Genes That Are Methylated During Breast Cancer

10.3.3.1 BRCA1

The BRCA1 gene located at chromosome 17q21, is a well-known breast cancer susceptibility gene [39]. Inhibition of BRCA1 expression through antisense oligonucleotides increases the proliferation of normal and malignant mammary cells while overexpression of wild-type BRCA1 suppresses MCF-7 breast cancer cell tumorigenesis in mice [40]. Inherited mutations in the BRCA1 gene account for one-half of inherited breast carcinomas. However, in contrast to other tumor suppressor genes, somatic mutations in this gene have not been reported, despite the high degree of LOH at the locus in breast cancers [41]. Subsequent studies of the BRCA1 promoter region demonstrated increased methylation in 11 % of sporadic breast cancer cases, which was inversely correlated with expression of both ER and

progesterone receptor [42]. Additionally, BRCA1 promoter methylation is associated with medullary and mucinous subtypes [43]. Moreover, while BRCA1 was unmethylated in normal tissues and in various breast cancer cell lines, two xenograft studies from two of these cell lines demonstrated increased promoter methylation and the concomitant loss of transcript [44]. In addition, the study demonstrated that loss of heterozygosity could happen through the aberrant methylation of the second allele. Finally, BRCA1 methylation is observed only in breast and ovarian cancers but not in tumors of colon or liver or leukemia, supporting a tissue-specific event for the process.

10.3.3.2 ANKRD11

The ankryin repeat domain-containing protein ANKRD11, a co-activator for p53 is a putative tumor suppressor gene in breast cancer. Downregulation of ANKRD11 is associated with breast tumorigenesis [45]. The promoter region of ANKRD11 is loaded with CpG-rich regions, and analysis of this region demonstrated that high levels of methylation in 40 % of breast tumors but not in normal breast tissues or normal blood samples. Moreover, treatment of breast cancer cell lines with DNA demethylating agents results in upregulation of ANKRD11 expression suggesting that promoter DNA methylation plays a key role in downregulating the protein expression.

10.3.3.3 Mismatch Repair Genes MLH1 and MSH2

Loss of genomic stability is associated with a variety of diseases, particularly cancer. Of the many proteins that maintain genomic integrity, two important ones are the mismatch repair genes MLH1 and MSH2 [46]. Analysis of these mismatch repair genes in locally advanced breast cancers demonstrated that hypermethylation of the MLH1 gene in 43.5 % of patients with primary breast cancer, of whom 66.9 % had locally advanced breast cancer (stages IIIA to IIIC) [47]. Similarly, there was hypermethylation of the MSH 2 gene in 16 % of primary breast cancer cases. Of these patients, 21.3 % had locally advanced breast cancer [48].

10.3.4 Cell Cycle and Apoptosis Genes That Methylated During Breast Cancer

10.3.4.1 Ras Association Domain Family 1A Gene (RASSF1A)

RASSF1A gene, located within the 120 kb 3p21.3 minimal homozygous deletion region is epigenetically inactivated in cancers, suggesting a tumor suppressor function for the protein [49]. The protein is 55 % homologous to Nore1, a noncatalytic protein identified by its ability to bind selectively to active Ras [50]. Forced ectopic

expression of RASSF1A in cancer cells reduced colony formation, suppressed anchorage-independent growth and inhibited tumor formation in nude mice [51]. More importantly, methylation in the RASSF1A gene is highly correlated with breast cancer risk, atypical cytology and benign breast disease requiring biopsy [52]. With respect to age, RASSF1A gene methylation has been noted to increase linearly between the ages of 32 and 55 [53]. In microdissected breast tissue, Lehmann et al. showed that the RASSF1A promoter was methylated in all epithelial hyperplasia and papilloma samples and in 83 % of ductal carcinoma in situ, suggesting methylation of RASSF1A promoter as a new marker for nonphysiological epithelial proliferation in the breast [54]. The study also found that in most cases of progression to invasive growth, epigenetic inactivation takes place before invasive growth develops, an observation confirmed by Pasquali et al., who observed a progressive gain of methylation for RASSF1A from normal to hyperplasia acquiring statistical significance at CIS and invasive carcinoma [55]. Functionally, one thing that RASSF1A does is it influences G1-S cell cycle checkpoint by regulating the levels of cyclin D1 protein. Reduced RASSF1A expression due to epigenetic silencing leads to accumulation of cyclin D1, which may represent an important mechanism for overriding cell cycle control under conditions of increased cell cycle pressure [56].

10.3.4.2 Cyclin D2 (*CCND2*)

CCND2 is a member of the D-type cyclins, implicated in cell cycle regulation, differentiation, and malignant transformation [57]. It should be noted that cyclin D2 is not expressed in the majority of breast cancer cell lines, whereas abundant expression can be detected human mammary epithelial cells with a finite lifespan [58]. Also, loss of CCND2 expression caused by methylation is an early event in breast cancer tumorigenesis. Hypermethylation of the CpG island in the promoter can be detected by methylation-specific PCR in most breast cancers, and this has been associated with silencing of cyclin D2 gene expression. Promoter hypermethylation was also detected in ductal carcinoma *in situ*, suggesting that loss of cyclin D2 expression is an early event in tumorigenesis [59]. Furthermore, methylation of CCND2 has been correlated with poor prognosis, implying that CCND2 has a tumor suppressor function.

10.3.4.3 Cyclin D/CDK4 Complex Inhibitor (CDKN2/p16)

Frequent LOH and homozygous deletion has suggested the presence of tumor suppressor genes in chromosome 9p21 [60]. One such gene in this locus is inhibitor of the cyclin D/CDK4 complex (CDKN2/p16), which is frequently deleted in human cancer cell lines. The protein binds to CDK4 and CDK6, and blocks Gl to S transition by inhibiting cyclin-D-dependent phosphorylation of the Rb protein and

maintains its binding to the E2F transcriptional factor [61]. Since the initial reports of homozygous deletion, numerous studies have shown varying, but in general much less frequent, abnormalities of p16 in primary tumors of these cancers. For example, although the rate of homozygous deletions ranged from 40 to 60 % of breast cancer cell lines, neither homozygous deletion nor point mutations were observed frequently in primary breast carcinomas [62]. Furthermore, certain neoplasms, such as prostate and colon cancer, have not been found to harbor homozygous deletions in established cell lines. However, *de novo* methylation of the 5′ CpG lsland is a frequent mode of suppressing CDKN2/p16 expression, and also firmly demonstrate that CDKN2/p16 is one of the most frequently altered genes in human neoplasia.

10.3.4.4 Retinoblastoma Protein-Interacting Zinc Finger Gene RIZ1

RIZ was first isolated in a functional screening for Rb-binding proteins, and was also independently isolated as DNA-binding protein MTB-Zf, GATA3 transcription factor-binding protein G3B, and a coactivator of estrogen receptor. RIZ contains the canonical Rb-binding motif LXCXE and the nuclear hormone receptor-binding motif LXXLL [63]. In addition, RIZ contains a novel protein methyltransferase domain, called the PR domain or SET domain, which is present in ~50 human genes [64]. Two products of the gene exist: (a) RIZ1, which contains the PR domain; and (b) RIZ2, which lacks the domain. RIZ1 but not RIZ2 has tumor-suppressive properties [65]. The *RIZ1* gene maps to chromosome 1p36, a region commonly deleted in more than a dozen different types of human cancers. RIZ1 expression, but not RIZ2 expression, is commonly silenced in many types of human tumors, including breast cancer, liver cancer, colon cancer, neuroblastoma, melanoma, lung cancer, and osteosarcoma through DNA methylation. Further studies are required to determine specific sites of methylation in the gene.

10.3.4.5 TMS1

TMS1 belongs to a growing family of apoptotic signaling molecules that contain a CARD domain [66]. This domain is found within the prodomain of a number of caspases, and oligomerization with upstream CARD-containing regulatory proteins mediates their cleavage and activation. Other CARD-containing proteins with known roles in apoptosis include the Caenorhabditis elegans CED-3 and CED-4, the human homologue of CED-4, apoptotic protease activating factor-1, the cellular and viral inhibitors of apoptosis, the cellular homologue of herpes virus EHV2 E10 protein, BCL10, and several proteins involved in the activation of NF-kB. The protein was found to act during the initiation phase of an apoptotic pathway, coupling death receptors at the cell surface or intrinsic death signals to the activation of the caspase cascade. Earlier studies suggest that the gene encoding TMS1 is silenced by DNA methylation [67].

10.3.4.6 14-3-3-σ

14-3-3-σ is a member of a superfamily of proteins that are responsible for instituting cell cycle checkpoint in response to DNA damage [68]. Several lines of evidence have demonstrated that hypermethylation of the 14-3-3-σ gene occurs in 91 % of primary breast cancers and is strongly associated with loss of 14-3-3-σ gene expression. Hypermethylation of the 14-3-3-σ gene occurs in a CpG-rich region that extends from the transcriptional initiation site to the middle of the coding region. Bisulfite genomic sequencing of the 500-bp region showed that it is consistently and densely methylated in cell lines and primary breast tumors. This dense methylation just downstream of its transcriptional start site is strongly associated with gene silencing. Further proof of methylation silencing was obtained when treatment with 5-aza-dC resulted in demethylation of the CpG island and reactivation of gene expression [69]. Moreover, 14-3-3-σ expression is undetectable in 94 % (45/48) of breast tumors. Subsequent studies have clearly demonstrated that CpG island methylation is the epigenetic event that is largely responsible for silencing of the 14-3-3-σ gene and occurs in a majority of breast cancers.

10.3.5 Other Genes That Are Hypermethylated in Breast Cancers

10.3.5.1 Death-Associated Protein Kinase (DAP-Kinase)

DAP-kinase is a 160-kDa serine/threonine, microfilament-bound kinase recently shown to be involved in γ-interferon-induced apoptosis [70]. Tumor invasiveness and aggressiveness has been associated with hypermethylation of a CpG island in the promoter region of the gene. In a small series of B-cell malignancies, a similar finding of methylation of the DAP-kinase CpG was also described, suggesting that hypermethylation of the DAP-kinase gene and loss of γ-interferon-mediated apoptosis may be important in the development of B-cell malignancies [71]. Cell lines derived from these cancers also demonstrate reduced or DAP-kinase gene expression, and reexpression can be seen following treatment with a DNA demethylating drug such as 5-aza-dC [72].

10.3.5.2 ID4

ID4 is the most recently discovered member of the Inhibitor of DNA binding/Inhibitor of differentiation family of transcription factors [73]. ID proteins contain a helix-loop- helix (HLH) domain enabling interaction with other basic helix-loop-helix (bHLH)-proteins. Following heterodimerization with those transcription factors, ID proteins act as dominant negative inhibitors of gene transcription [74]. In addition, ID proteins can bind to other important non-bHLH transcription factors such as the retinoblastoma protein (RB) or the paired box (PAX)-proteins, thereby regulating

important pathways in cell proliferation and differentiation [75]. Furthermore, ID4 is an important factor for the development of the nervous system. In this regard, the ID4 gene is highly expressed in migrating postmitotic neurons, in Purkinje cells, as well as in the adult cerebellum. Since ID proteins regulate fundamental cellular processes, a link of ID dysregulation with human carcinogenesis has been recently postulated. ID1 and ID2 are overexpressed in several human tumor cancers including pancreatic cancer and colorectal adenocarcinomas, suggesting a putative oncogeneic function for these two proteins [76]. However, ID3 and ID4 expression is reduced in several tumor types such as ovarian adenocarcinomas. In human breast tissue ID4 mRNA was found to be constitutively expressed in normal mammary epithelial cells, but suppressed in estrogen receptor ER-positive breast carcinomas and pre-neoplastic lesions. A human ribozyme library-based inverse genomics approach revealed that ID4 might act as a negative regulator of the common tumor suppressor gene BRCA1. Moreover, ID4 expression levels were decreased in BRCA1/ER-positive breast cancer specimens, suggesting that ID4 participates in molecular events regulating ER and BRCA1 expression. Recently, it has become very evident that promoter methylation plays a decisive role in the expression of these genes in cancer. Aberrant hypermethylation of the ID4 gene promoter was associated with an increased risk for lymph node metastasis [77].

10.3.5.3 Paired-Like Homeodomain Transcription Factor 2 (PITX2)

PITX2 acts as a transcription factor and are shown to regulate procollagen lysyl hydroxylase gene expression. Furthermore, this protein is involved in the development of organs [78]. Hypermethylation of the PITX2 promoter is associated with a high risk of recurrence in node-negative, steroid hormone receptor-positive breast cancer following tamoxifen adjuvant therapy [79]. In addition, DNA hypermethylation of eight candidate genes (BMP4, LMX1A, BARX1, FGF4, NR5A1, LHX4, ZNF1A1, and CCND2) linked to the PITX2 signal transduction pathway was found to be significantly associated with patient outcome [80].

10.3.5.4 Adenomatosis Polyposis Coli (APC)

The APC gene product modulates β-catenin function by binding to the protein and driving it to ubiquitin-proteosomal degradation [81]. Genetic and epigenetic alterations in APC, a tumor suppressor gene originally implicated in colon cancer have been reported in many malignancies including breast cancers. A study of 76 breast cancer patients by Liu and colleagues has demonstrated that APC gene methylation correlated positively with TNM staging and negatively with protein expression [82]. Lee et al. reported that methylation occurs in the APC promoter in 42 % of breast cancer aspiration fluid samples. However, the gene was unmethylated in the aspiration fluids from normal breast tissue in patients with breast cancer and all benign breast disease patients in their cohort [83].

10.3.5.5 GSTP1

Glutathione (GSH) and its corresponding cytosolic GSTs are involved in the detoxification pathway of xenobiotics and chemotherapeutic agents [84]. They catalyze intracellular detoxification reactions by conjugating chemically reactive electrophiles to GSH, inactivating electrophilic carcinogens. The GSTs, encoded by several different genes at different loci, have been classified into α, μ, π and θ families. The π-class GST, encoded by the GSTP1 gene, on chromosome 11, is of particular importance in breast cancer [85, 86]. In cultured breast cancer cell lines an inverse relationship between GSTP1 and ER gene expression has been reported, i.e. GSTP1 was expressed in ER-negative but not in ER-positive lines, although the underlying mechanism is unclear. Treatment of the GSTP1-negative cell line MCF-7 with 5-aza-dC induces *de novo* synthesis of π-class protein. In addition, GSTP1 promoter methylation has been associated with gene inactivation in about 30 % of primary breast carcinomas, and this correlates with PR expression. It is postulated that methylation-associated inactivation of GSTP1 can result in adenine or guanine mutation by estrogen metabolites-DNA adduct formation and lead to genetic instability [87].

10.3.5.6 TIMP3

TIMP3 belongs to a family of molecules that inhibit the proteolytic activity of matrix metalloproteinases [88]. The protein can suppress primary tumor growth *via* its effects on tumor development, angiogenesis, invasion and metastasis. TIMP3 is methylated in ~30 % of human breast cancer cell lines and also in ~30 % of primary breast tumors [89]. Hypermethylation of 5′CpG island in TIMP3 promoter has been observed in normal and benign (ALH) lesions and in DCIS and tumor lesions, implicating TIMP3 as an early event. Furthermore, 5-aza-dC treatment has been shown to induce TIMP3 expression supporting a role for epigenetic mechanism in TIMP3 gene regulation [90].

10.3.5.7 Progesterone Receptor (PR)

Progesterone receptor (PR) is a classical estrogen-regulated gene [91]. Receptor status is important in classification of breast cancers. The PR gene encodes two isoforms, hPRA (79 kDa) and hPRB (109 kDa), which differ in both their N-terminal sequences and biological activities. The PR gene, located at chromosome 11q13, also has a CpG island in its first exon [92]. PR gene methylation has been demonstrated in ~40 % of PR-negative breast tumors and PR-negative breast cancer cell lines [93]. Additional proof of the functional role for CpG island methylation was obtained when treatment of PR-negative MDA-MB-231 cells with 5-aza-dC in the presence of estrogen led to partial demethylation of the PR CpG island and re-expression of PR gene [94].

10.3.5.8 E-Cadherin

The E-cadherin gene, located at chromosome 16q22.1, encodes a cell-surface adhesion protein that is important in maintaining cell–cell adhesion in epithelial tissues [95]. Considerable evidence shows that loss of expression and function of E-cadherin protein contributes to increased proliferation, invasion and metastasis in breast cancer. This also correlates with decreased patient survival. While mutations and deletions clearly play a role in loss of the E-cadherin expression and function, several studies have also demonstrated that epigenetic silencing of the E-cadherin gene by 5′CpG methylation occurs in human breast cancer cell lines as well as about 50 % of unselected primary breast cancers [96]. Recent studies have demonstrated that hyper methylation of the E-cadherin CpG island was evident in about 30 % of ductal carcinomas *in situ* and increased significantly in nearly 60 % of metastatic lesions [97].

10.3.5.9 LAMA3

Interaction between epithelial cells and extracellular matrix is important for the structural integrity and specialized function of breast epithelium. Two way signaling occurs *via* extracellular proteins (laminins) and their transmembrane receptors, the integrins. Hemidesmosomes are structures used by normal epithelia to adhere to basement membrane. The major structural proteins of the hemidesmosomes are the integrins and its ligand laminin 5 (LN5). In lobular and ductal structures of the breast, both myoepithelial and luminal cells has hemidesmosomes. LN5 is specific to epithelium, and it is a heterotrimeric protein member of the laminin family and consists of three polypeptide chains α3, β3, and γ2, which are the products of three different genes LAMA3, LAMB3, and LAMC2. The chains are assembled in a coiled cruciate-like structure, which is deposited in the basement membrane [98]. Silencing of LAMA3 gene by methylation plays an important role in pathogenesis of breast cancers [99].

10.3.5.10 Klotho

Klotho is a single pass transmembrane protein, associated with premature aging and acts as a potent inhibitor of insulin receptor [100]. Its expression is reduced associated during breast cancer development. Methylation of its promoter region and silencing of its expression has been reported to occur at the early stages of breast cancer [101].

10.3.5.11 Slit2

The Slit family comprises large ECM 3 secreted and membrane-associated glycoproteins [102]. Human Slits (SLIT1, SLIT2, and SLIT3) are candidate ligands for

the repulsive guidance receptors, the ROBO gene family. The SLIT2 gene has been mapped to chromosome 4p15.2, and studies have shown that the putative SLIT2 receptor, is methylated in some breast tumors [103]. In addition, Slit2 promoter hypermethylation is detected in tissue and serum samples from breast cancer patients [104] Furthermore, ectopic expression of SLIT2 in several breast cancer cell lines suppressed growth and reduced colony formation abilities.

10.3.5.12 Caveolin-1 (Cav1)

Cav1 is a ubiquitous scaffolding protein that coats plasma membrane invaginations termed caveolae in various cell types [105]. The Cav1 gene is located in the locus D7S522 of human chromosome 7q31.1, a region that is frequently deleted in human cancers, implicating Caveolin-1 as a tumor suppressor [106]. Aberrant promoter methylation of Cav1 gene is associated with reduced of expression, and occurs at the precancerous stage [107].

10.3.5.13 Lost-On-Transformation 1 (LOT1)

LOT1 is a growth suppressor gene localized on chromosome 6 at band q24–25, which is a frequent site for loss of heterozygosity in many solid tumors [108]. The gene encodes a nuclear transcription factor and is strongly regulated by the activation of the epidermal growth factor receptor-signaling pathway [109]. Earlier studies have identified CpG islands in the upstream sequences of exon 1 and in the promoter region, suggesting the potential for DNA methylation [110]. In addition, recent reports have demonstrated that the gene is located within a maternally imprinted chromosomal region, and the gene is methylated. Moreover, breast cancer cell lines have high levels of CpG methylation in its promoter region.

10.3.5.14 Sulfotransferase 1A1 (SULT1A1)

SULT1A subfamily of sulfotransferases is an important phase II xenobiotic metabolizing enzyme that mediates the sulfonation of drugs, carcinogens, and steroids [111]. Specifically, SULT1A1 plays a significant role in the sulfoconjugation of xenobiotics, such as p-nitrophenol, N hydroxy-heterocyclic and -aromatic amines, and endogenous compounds such as di-iodothyronine and estrogens. The CpG methylation rate of the SULT1A1 gene in breast cancer was shown to be denser than in normal and benign tissues [112].

10.3.5.15 Cystatin E/M (CST6)

Cystatin M or E/M (encoded by the CST6 gene) is an endogenous inhibitor of lysosomal cysteine proteases that functions to protect cells against uncontrolled

proteolysis. CST6 has been shown to be involved in the degradation of components of connective tissues and basement membranes *in vitro*, and aberrant expression and activity of these proteases accompany cancer invasion and metastasis *in vivo* [113]. Absence of CST6 expression is believed to result in increased proteolysis of tissue architecture, facilitating the spread of cancer cells. Studies have established a strong link between CST6 promoter hypermethylation and loss of CST6 expression in breast cancer [114].

10.3.5.16 WW Domain Containing Oxidoreductase (WWOX)

WWOX is a tumor suppressor gene spanning a genomic region of f1 Mb located at chromosome 16q23.3-24.1, a region with a high incidence of loss of heterozygosity (LOH) in breast, prostate, and other cancers [115]. The area is highly methylated in breast cancers, resulting in reduced gene expression. Similarly, a significantly greatly reduced levels of WWOX gene expression was observed in MDA-MB-231 cells, which was determined to be partly due to the methylation of the CpG islands [116].

10.3.5.17 Dickkopf-3 (DKK3)

Dickkopf genes (DKK) encode a class of extracellular signaling molecules. Together, they control cell fate during embryonic development and regulate tissue homeostasis in adults. There are four DKK members, DKK1–DKK4. While DKK1, DKK2 and DKK4 antagonize canonical Wnt/β-catenin signaling at the membrane, DKK3 functions in antagonizing nuclear β-catenin levels. DKK3 expression is frequently lost in human cancer tissues because of aberrant 5′-cytosine methylation within its promoter region including in breast cancers [117].

10.3.5.18 CCAAT/Enhancer Binding Protein D (CEBPD)

C/EBPs are a highly conserved family of leucine zipper proteins that regulate cell growth and differentiation in multiple organs [118]. Specifically, C/EBPδ functions to initiate and maintain growth arrest of mammary epithelial cells [119]. Loss of function alterations in C/EBPδ gene expression has been reported in human breast cancer and in rodent carcinogen-induced mammary tumors [120]. This has been associated with C/EBPdelta gene promoter hyper- and site specific-methylation [121].

10.3.5.19 Deleted in U Twenty Twenty (DUTT1)

DUTT1is a member of NCM family of receptors was mapped at human 3p12 [122]. DUTT1 protein consists of an ectodomain of five Ig domains and three-fibronectin type III repeats. It also contains a single transmembrane segment and a long cytoplasmic domain that does not contain any recognizable motifs except proline-rich

repeat and sequences of low compositional complexities as predicted by the SMART program. The gene is frequently deleted in breast cancer. In addition, the gene can be hypermethylated in breast cancer and its expression reactivated by 5-aza-dC treatment [123].

10.4 Histone Modification

Histones are proteins that assemble and have DNA wrapped around them. There are four histones, with the core histones present as octamers (2 each of 4 core histones) as well as two linker histones H1 and H5. For many years it has been known that post-translational modifications of histone tails determine, in part, which regions of the genome are an open and thus transcriptionally active conformation, and which are closed and thus transcriptionally inactive. Histone tails determines whether the region will be in an actively transcribed state or in an inactive state. Histone modifications can result in alteration of the chromatin structure for transcription machinery or recruiting regulatory proteins. Histones can be modified by methylation, acetylation, ribosylation, ubiquitination, sumoylation, and phosphorylation, which can result in either an increase in transcription or in gene silencing [124]. There are several reports confirming that cancers have altered patterns of histone modifications, chief among them being histone acetylation, histone methylation and histone phosphorylation. Recently, the aberrant histone methylation has been shown to result in cancer-specific loss of expression of surrounding genes. In breast cancer, abnormal histone modification in combination with DNA hypermethylation is frequently associated with epigenetic silencing of tumor suppressor genes and genomic instability [125]. Understanding the mechanisms of dysregulation of histone tail post-translational modifications and their contribution to breast tumorigenesis is critically important in the development of novel targeted therapy for breast cancer patients.

10.4.1 Histone Acetyltransferases

The acetylation of lysine residues on the N-terminus of histones is generally associated with active gene transcription. The HATs can be grouped into three main families based on their sequence similarities: Gcn5/PCAF, p300/CBP and the MYST family of HAT proteins [126]. Most HATs are present as part of large protein complexes and act as transcriptional coactivators. Many of them have also been shown to acetylate proteins other than histones.

10.4.2 Histone Deacetylases

HDACs promote gene repression through removal of acetyl groups from lysine residues in histone tails. At least 18 HDAC genes have been recognized in the

human genome, grouped into three main classes based on sequence homology to the yeast counterparts Rpd3, Hda1 and Sir2/Hst [127, 128]. HDACs act mostly as part of large multiprotein complexes that function as transcriptional corepressors. HDAC family is divided into zinc-dependent enzymes (classes I, IIa, IIb, and IV, of which there are 11 subtype enzymes) and zinc-independent enzymes (class III, also called sirtuins), which require NAD+ for their catalytic activity [129]. Pruitt and colleagues demonstrated that inhibition of class III HDAC SIRT1 using a pharmacologic inhibitor, splitomicin, or siRNA reactivates epigenetically silenced SFRP1, SFRP2, E- cadherin, and CRBP1 genes in human breast cancer cells [130].

HDACs remove the acetyl groups from histone lysine tails and are thought to facilitate transcriptional repression by decreasing the level of histone acetylation. Like HATs, HDACs also have non-histone targets. Several HDACs have been found to be involved in breast cancer. HDAC1 and HDAC4 are overexpressed in breast cancers [131]. In ER-positive MCF-7 cells, expression of HDAC6 was increased after being treated by estradiol, and the elevated HDAC6 could deacetylate alpha-tubulin and increase cell motility [132]. In contrast, ER antagonist tamoxifen (TAM) or ICI 182,780 prevents estradiol-induced HDAC6 upregulation, and then reduces cell motility [133]. Moreover, patients with high levels of HDAC6 tend to be more responsive to endocrine treatment than those with low levels, indicating that the levels of HDAC6 expression might be used both as a marker of endocrine responsiveness and also as a prognostic indicator in breast cancer. Studies have also suggested that sirtuins SIRT3 and SIRT7 are overexpressed in breast cancer [134].

10.4.3 Histone Methyltransferases

Methylation of arginine and lysine residues in histones is involved in the regulation of a wide range of processes including gene activity, chromatin structure and epigenetic memory. Arginine can be either mono- or dimethylated, and in symmetric or asymmetric configurations. Lysine can be in mono-, di- or trimethylated forms. Commonly, lysine methylation at H3K9, H3K27 and H4K20 is associated with gene silencing, whereas methylation at H3K4, H3K36 and H3K79 is associated with gene activation [135].

10.4.3.1 Histone Lysine Methyltransferase (HKMTs)

Histone lysine methylation is a reversible process, dynamically regulated by both lysine methyltransferases and demethylases. Methylation occurs on histone H3 at ε-amino group of lysines 4, 9, 14, 27, 36, and 79 and on histone H4 at lysines 20 and 59 [136]. In general, methylation at H3K4 or H3K36, mono-methylations of H3K27, H3K9, H4K20, H3K79, and H2BK5 are associated with transcriptional activation, whereas trimethylations of H3K27, H3K9 H3K79, and H4K20 are linked to transcriptional repression [137].

Histone methylation is regulated in breast cancer in an even more complicated manner than histone acetylation *via* a large number of chromosomal remodeling regulatory complexes. Modification of H3K4 methylation is catalyzed by the Trithorax group of histone methyltransferases, including SET1 and MLL [138]. The activity of Trithorax proteins is balanced by the opposing effects of the Polycomb group factors, another important histone methyltransferase family that mediates methylation usually associated with epigenetic gene silencing [139].

10.4.3.2 Histone Arginine Methyltransferase (HRMTs)

The protein arginine methyltransferase (PRMT) family is the main HRMTs that act on histones. They are classified into four groups depending on the type of methylarginine they generate: Type I PRMTs (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8) catalyze the formation of ω-NG, monomethylarginines (MMA) and NG-asymmetric dimethylarginines (aDMA); Type II PRMTs (PRMT5, PRMT7 and PRMT9) catalyze the formation of MMA and ω-NG, N′G-symmetric dimethylarginines (sDMA); Type III PRMTs catalyze only the monomethylation of arginine residues in proteins; Type IV PRMTs catalyze the methylation at delta (Δ) nitrogen atom of arginine residues [140, 141]. Similar to HKMTs, evidence for the involvement of HRMTs in human cancers is weak. Underexpression of PRMT1 has been reported in breast cancer [142]. PRMT4, also known as coactivator-associated arginine methyltransferase-1 (CARM1), is a coactivator for nuclear receptors and is overexpressed in breast cancers [143]. PRMT4 plays an essential role in estrogen induced cell cycle progression in the MCF-7 breast cancer cells. When stimulated with estrogen, the E2F1 promoter is subjected to PRMT4-dependent dimethylation on H3R17, and this recruitment of PRMT4 by ERα are dependent on the presence of the NCOA3 [144].

10.4.4 Histone Demethylases

It used to be thought that histone methylation was a permanent and irreversible histone modification. However, more recent studies have identified enzymes with the ability to demethylate the methylated histone lysine/arginine residues *via* amine oxidation, hydroxylation or deamination. The histone demethylases could be divided into three distinct classes. The first class (petidylarginine deaminase 4, PADI4) converts a methyl-lysine to citrulline. The second class (lysine-specific demethylase 1, LSD1) reverses histone H3K4 and H3K9 modifications by an oxidative demethylation reaction. The third class of demethylases is the family of Jumonji C (JmjC)-domain containing histone demethylases (JHDMs). In contrast to LSD1, JHDMs can demethylate all three methylated states (mono- di- and tri-methylated lysine). Until now, JHDMs have been found to demethylate H3K36 (JHDM1),

H3K9 (JHDM2A) and H3K9/K27 (JHDM3 and JMJD2A-D) [145]. Histone demethylase JARID1B (PLU-1) is overexpressed in breast cancers but expressed very low in normal adult tissues, and it is essential for the proliferation of the MCF-7 cells and for the nude mice tumor growth of mammary carcinoma cells. Several target genes of JARID1B have also been identified to be associated with proliferation of breast cancer, such as 14–3–3σ, BRCA1, CAV1, and HOXA5 [146]. LSD1 could be a coactivator in the ER signaling. JMJD1C expression is decreased in breast cancer tissues compared with normal breast tissues, suggesting that it might be a tumor suppressor [147].

10.4.5 Histone Phosphorylation in Breast Cancer

Phosphorylation event of histone is thought to have a role in chromatin remodeling and in transcription, and therefore could potentially be associated with the human cancer development. Phosphorylation of H3 on S10 and S28 is essential not only during mitotic chromosome condensation but also in transcriptional activation of immediate early genes. When MCF7 cells were treated with phorbol ester, the number of H3 pS10 foci was increased, and were positioned next to actively transcribed regions in the nucleus. Seemingly, these nuclear sites represent the nuclear location of genes that are induced or in a competent state. Therefore, growth factors stimulating the Ras/MAPK and increasing H3 pS10 at transcriptionally active loci may contribute to aberrant gene expression and breast cancer progression [148].

10.4.6 Other Histone Modifications in Breast Cancer

Apart from acetylation, methylation and phosphorylation, there are some other modifications of histones occur. These epigenetic changes include ubiquitination/sumoylation, ADP-ribosylation, deamination, and proline isomerization. However, their function and mechanism is not clear, some studies have showed that they are also associated with breast cancer and other human cancers.

Regulation of the expression of genes by phosphorylated and undersumoylated PRs is a novel form of hormone independent PR action that is predicted to contribute to breast cancer cell growth and survival [149]. Recently, E3 ubiquitin ligase has been shown to play important role in breast carcinogenesis. Ubiquitin-mediated protein degradation plays an important role in many cancer-related cellular processes. E3s play crucial role because they control the specificity of the substrate. Gathering evidence suggests that genetic and expression alteration of E3s plays important role in breast carcinogenesis [150]. Sumoylation of histone also seem to govern chromatin structure and function to mediate transcriptional repression and gene silencing [151].

10.5 Polycomb Group (PcG) Proteins

The Polycomb gene was discovered about 60 years ago as a mutation inducing a particular homeotic phenotype. Later studies indicated that Polycomb is a general repressor of homeotic genes [152]. Genes with similar functions were identified and grouped under the name Polycomb group (PcG) genes. These genes have demonstrated epigenetic regulation of genes during development and differentiation. These proteins function to maintain a silenced state of developmental regulators. The PcG genes encode subunits, which are part of the polycomb repressive complex. Furthermore, these PcG genes are closely associated with coordinated regulation of histone modification and methylation, thus inter connecting the various epigenetic mechanisms. The Polycomb Repressive Complex 1 (PRC1) plays a crucial role in reading histone methylation marks and silencing target genes [153]. PRC1 can be recruited to chromatin by the PC chromodomain-mediated recognition of the H3K27me3 mark, which is deposited by PRC2. PRC1 components can also repress transcription without directly contacting transcription factors. Isolation of a core PRC1 complex, called PCC, that contains PC, PH, PSC and dRING1, revealed that these four PcG proteins are sufficient to inhibit ATP-dependent chromatin remodeling by the SWI/SNF complex, a homolog of the Drosophila BRM complex.

The Polycomb group (PcG) comprises several proteins that form multiprotein complexes, 2–5 MDa in size, that regulate gene activity at the chromatin level. The first components of Drosophila PcG were identified in 1980s and after several years it was recognized in mammalian cells, indicating strong evolutionary conservation [154]. PcG proteins, along with the counteracting Trithorax group (TrxG) proteins, were initially recognized as part of the memory system that transmission of cell identities throughout cell division [155]. PcG protein expression appears to be tightly regulated during normal cell proliferation and differentiation. While the expression, of PcG is frequently dysregulated in several cancer types [156]. Several PcG genes regulate self-renewal of specific stem cells, suggesting a link between the maintenance of cell homeostasis and carcinogenesis [157]. Bmi-1 was initially identified as an oncogene that cooperated with c-Myc in the generation of mouse pre-B-cell lymphomas [158]. It is also considered to be the first functional mammalian PcG protooncogene, and has been implicated in axial patterning, hematopoiesis, cell cycle regulation, and senescence. Data obtained in mice and *in vitro* studies have indicated that Bmi-1 protein regulates the INK4a/ARF locus, which encodes two unrelated tumor suppressors, p16INK4a and p19ARF (p14ARF in humans), which act in the two main cell cycle control pathways (pRb and p53, respectively) [159]. However, the effect of Bmi-1 overexpression on the inactivation of the INK4a/ARF transcripts in human tumorigenesis is unclear. One study demonstrated that although high levels of Bmi-1 were frequently observed in tumors, they did not correlate with downregulation of p16INK4a or p14ARF. A correlation between c-Myc and Bmi-1 expression levels has been shown; however, tumors showing elevated expression of both genes were not associated to a worse prognosis.

10.6 Non-coding RNAs

Many non-coding RNAs such as microRNA, small nucleolar RNAs, and repeat-associated small interfering RNA have been shown to alter transcription. MicroRNAs are small non-coding RNAs of approximately 22 nucleotides [160]. Several genes that are responsible for breast cancer progression are shown to be targeted by microRNAs, for example, miR-199b-5p was shown to inhibit HER2 expression by directly targeting its 3′-untranslated region (3′UTR) in breast cancer cells [161].

10.6.1 MicroRNAs Inhibit Target mRNA Translation

Initial studies on miRNAs suggested that these small non-coding RNAs inhibit mRNA translation, with perfect or near perfect complimentarity inducing mRNA degradation, while imperfect binding resulting in inhibition of translation [162]. Oncogenic microRNAs are also called as OncomiRs and they have been shown to promote breast cancer. These include miR-10 family, which regulate Hox transcripts [163]. In case of breast cancer, miR-10 family is reported to be involved both in the development and metastasis through miR-10a and miR-10b, respectively. Expression of miR-21 is negatively correlated with expression of PTEN in breast cancer and also correlates with advanced stage and metastasis and poor survival [164]. Finally, miR-17 ~ 92 cluster is a polycistron and is located in a region of DNA that is amplified in various cancer. The expression of miR-17–5p is increased in invasive MDA-MB-231 cells but not in non-invasive MCF-7 breast cancer cells [165]. Ectopic expression of this miRNA in MCF-7 cells can lead to more invasive and migratory phenotypes by targeting HBP1/β-catenin pathway. Similarly, down regulation of miR-17–5p suppresses the migration and invasion of MDA-MB-231 cells in vitro.

There are also tumor suppressor microRNAs. The Let-7 family includes members that affect muscle formation, cell adhesion and regulation of gene expression and development [166]. Let-7 expression is lost in breast cancer at an early stage of disease progression in breast cancer. Similarly, the miR-200 family is lost in invasive breast cancer cell lines with mesenchymal phenotypes and also in regions of metaplastic breast cancer specimens lacking E-cadherin. The miR-200 family has been shown to regulate PLCG1, Bmi1, TGF-β2, FAP-1, ZEB and Suz12, hence acting as tumors suppressor [167, 168]. The expression of miR-205 is restricted to basal epithelium of normal mammary ducts and lobules, but its expression is reduced or lost in tumor [169]. Ectopic expression of miR-205 in breast cancer cells inhibits invasion, proliferation and anchorage independent growth, in part through direct targeting of Her3 and VEGF-A [170]. In addition, miR-145, is significantly down-regulated in breast cancer specimen compared with normal breast tissue [171]. Furthermore, miR-145 can directly target estrogen receptor-α (ER-α) protein expression through direct interaction and promotes apoptosis in both ER-α positive and wild type TP53-expressing breast cancer cells.

Breast cancer metastatic related microRNAs, there are miRNAs that promote the metastasis in breast cancer include miR-9, miR-10b, miR-21, miR-29a, miR-155 and miR-373/520 family. On the other hand there are microRNAs reported to suppress metastasis and they are, miR-7, miR-17/20, miR-22, miR-30, miR-31, miR-126, miR-145, miR-146, miR-193b, miR-205, miR-206, miR-335, miR-448, miR-661 and let-7 [172].

10.6.2 MicroRNA Control of Epigenetic Mechanisms

Both miRNAs, and small interfering RNAs (siRNAs) are involved in both DNA methylation and histone modifications. Maison et al. showed that RNAse treatment can abolish the localization of methylated H3 lysine 9 and HP1 to pericentromeric chromatin [173]. Fukagawa et al. demonstrated that Dicer-related RNAi machinery is necessary for the formation of heterochromatin structure [174]. Furthermore, miR22, miR206, and miR-221/222 regulate ER-α expression in breast cancer [175]. MicroRNAs can target genes coding for enzymes responsible for histone modification (EZH2) and DNA methylation (DNMT3A and DNMT3B) [176]. miR-101 and miR-26a target the EZH2 mRNA 3′UTR and inhibits its translation [177].

Studies from Shimono et al. reported that 37 miRNAs are differentially expressed in CD44+/CD24-/low breast cancer stem cells as compared with non-tumorigenic cancer cells. In particular, three clusters, miR-200c-141, miR-200b-200a-429 and miR-183–96–182, are significantly down regulated. Furthermore, loss of p53 leads to a decreased level of miR-200c and an increase in the expression of EMT and stemness markers, leading to the development of a high tumor grade [178]. In addition, Han and colleagues isolated ALDH1+ and CD44+/CD24-/low cells from MCF-7 parental cells and found that HIF-1α and miR-21 are upregulated in the stem-like cells [179]. Moreover, reduction in miR-21 expression by antagomir leads to reversal of EMT, downregulation of HIF-1α, as well as suppression of invasion and migration. This indicates that miR-21 regulates EMT transition in breast cancer stem cells as well as HIF-1α overexpression.

10.6.3 Epigenetic Control of MicroRNA Expression

While microRNAs regulate epigenetic mechanisms, microRNA expression itself can be regulated in a similar manner. Interestingly, CpG island methylations in miRNA regions influence miRNA function, thereby altering the processes of tumorigenesis [180]. Nearly half of all identified miRNAs are associated with CpG sites, and studies revealed methylation levels at several miRNA loci across normal and malignant cell lines [181]. Silencing of miRNA gene expression due to hypermethylation is also a feature of several cancers. MicroRNAs can trigger transcriptional silencing *via* chromatin remodeling [182]. Tumor suppressor miRNAs could undergo aberrant DNA methylation accompanied by histone modifications associated

with transcriptional inactivation. Interestingly, miR-124a silencing by DNA methylation was accompanied by the absence of active histone markers, such as acetylation of histone H3, acetylation of histone H4, trimethylation of histone H3-lysine 4, and occupancy by MBDs such as MeCP2 and MBD2 [183]. The epigenetic regulation of miR-196a-2 in breast cancer development has also been studied. Hypermethylation of a CpG island 700 bp upstream of the miR-196a-2 precursor promoter was associated with reduced breast cancer risk [181]. Putative targets for the microRNA include HOXD10, LSP1 and TOX3. HOXD10 is a target for initiation of breast cancer invasion and metastasis, *LSP1* and *TOX3* (TNRC9) were identified as novel breast cancer susceptibility markers in large-scale whole-genome association studies [184]. Furthermore, miR335 locus on 7q32.2 is a selective metastasis suppressor and tumor initiation suppressor locus in human breast cancer. In fact, miR-335 regulates a set of genes that regulate metastasis [185]. This locus also undergoes epigenetic hypermethylation in every patient metastatic cell population. In addition, genetic deletion of miR-335 is a common event in human breast cancer [185]. There are several other miRNAs that are regulated by hypermethylation of the promoter region including miR-9-1 (targets transcription factor REST and its partner CoREST), miR-124a3, miR-148, and miR663 [186].

10.7 Epigenetics and Cancer Stem Cells

Cancer stem cells (CDCs) are relatively rare subpopulation of cells within a tumor that has the ability to initiate new tumor growth and have the capacity to self-renewal, the use of key regulatory pathways and establishment of dynamic epigenetic profiles. Compared to normal adult stem cells, CSCs will have higher proliferative rates and less dependence to stem cell niche. They may give rise to cellular heterogeneity in the tumor by initiating epigenetic reprogramming, this is because most of the currently known markers including CD44, Bmi1, ALDH1, CD133, and EPCAM have been shown to be regulated by either DNA methylation or histone modifications [187]. Furthermore, analysis of DNA methylation profile of CSCs, suggest that epigenetic markers of stemness of CSCs resemble embryonic stem cells rather than the adult normal stem cells pattern. The major problem with cancer chemotherapy is the development of resistance to the drugs, especially in CSCs; therefore understanding the epigenetic profile of these CSCs will provide us new avenues for developing new therapeutics for breast cancer.

10.8 Epigenetic Modifier Drugs Against Breast Cancer

There are many drugs in clinical trials for breast cancer that are epigenetic modifiers (Table 10.2), most of which come under the classification of HDAC inhibitors. Vorinostat a hydroxamic acid is being used as a single agent or in combination with tamoxifen, aromatase inhibitor, paclitaxel, carboplatin, trastuzumab, ixabepilone,

Table 10.2 Epigenetic drugs in clinical trials

Classification	Drugs in Pre-clinical studies	Drugs in clinical trial
DNMT inhibitors		
Nucleoside analogues	RG108, MG98, NVP-LAQ824, Decitabine, SGI-110	5-azacytidine, 5-aza-2′-deoxycytidine, 5′-fluro-2′-deoxycytidine, Zebularine, Epigallocatechin-3 gallate, Hydrazine
HDAC inhibitors		
Short chain fatty acids		Valproic acid and phenylbutyrate
Hydroxamic acids		Suberoylanilide hydroxamic acid (SAHA), Panobinostat, Belinostat, CHR-3996, Tefinostat, JNJ-26481585
Cyclic tetrapeptides	Trapoxin, Despsipeptide, cyclic hydroxamic acid-containing peptide (CHAP), and Apicidin	
Benzamides		Entinostat (MS-275), N-acetly-dinaline (CI-994), Mocetinostat (MGCD-0103)

lapatinib [188]. Another HDAC inhibitor in the breast cancer clinical trial is Entinostat, which comes under Benzamide sub class, this agent is being combined with exemestane, Anastrozole and lapatinib [188].

10.8.1 Nucleoside Analogs

There are several nucleoside analogue methylation inhibitors such as 5-azacytidine, 5-aza-2′-deoxycytidine, 5′-fluro-2′-deoxycytidine and Zebularine. These inhibitors affect DNA synthesis and are implicated to regulate DNA methylation. The formation of covalent complexes with DNMTs results in depletion of the enzyme and finally, a reversal of the methylation pattern. The non-nucleoside analogues are shown to inhibit DNA methylation by binding directly to the catalytic region of the DNMT without incorporating into DNA. RG108, was shown to inhibit DNA methyltransferase [189], however, it has not entered clinical trials. A non-toxic antisense oligonucleotide, MG98 has been shown to prevent translation of DNMT1 mRNA by hybridizing to the 3′UTR of the DNMT1 mRNA [190]. In addition, NVP-LAQ824, a derivative of a compound derived from the sponge *Psseudoceratina purpurea* called Psammapalin, inhibits both DNMTs and HDACs [191]. Furthermore, there are several other agents such as Decitabine and SGI-110 that are nucleoside analogues that inhibit DNMT [188, 192]. The green tea compound epigallocatechin-3 gallate (EGCG) has also been shown to reduce DNA methylation and increase transcription of tumor suppressor genes [193]. Currently, EGCG is being tested in clinical trial [194].

10.8.2 Histone Deacetylation Inhibitors

These are agents that inhibit histone deacetylase enzymes leading to increased acetylation in histones and resulting in altered cellular processes that have become defective in cancerous cells. The compounds are divided into short chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides. Currently, there are several HDAC inhibitors in clinical development for treatment of variety of cancers.

10.8.3 Short Chain Fatty Acids (SCFA)

HDAC inhibition by SCFAs was first demonstrated using butyrate resulting in hyperacetylate of histones H3 and H4 [195]. Valproic acid and phenylbutyrate is now used clinically as histone deacetylase inhibitor for breast cancer [196]. They induce proteosomal degradation of HDAC2 by inhibiting the catalytic activity of class I HDACs.

10.8.4 Hydroxamic Acids

Trichostatin A is an HDAC inhibitor that can inhibit the viability of breast cancer cells [197]. Suberoylanilide hydroxamic acid (SAHA) is another molecule that has been shown to inhibit both class I and II HDAC enzymes [198]. SAHA has FDA approval for treating cutaneous manifestations of lymphoma patients. Furthermore, there are other drugs such as Panobinostat, Belinostat, CHR-3996, Tefinostat, JNJ-26481585 that have entered the clinical studies [188].

10.8.5 Cyclic Tetrapeptides

Trapoxin accumulates highly acetylated core histones [199]. Even low concentrations of trapoxin can bind to histone deacetylase through epoxide moiety and inhibit deacetylation of acetylated histones [200]. Despsipeptide, a bicyclic peptide increases p53 expression in breast cancer cells [201]. In addition, CHAP, cyclic hydroxamic acid-containing peptide has been shown to inhibit HDAC [202]. Apicidin, a cyclic tetrapeptide increases the levels of acetylated histone H3 and H4 in breast cancer cells [203].

10.8.6 Benzamides

There are many benzamides that are either in clinical trials or are in preclinical testing. Entinostat (MS-275) treatment results in hyperacetylation of nuclear histones in

various tumor cells [204]. This class I HDAC selective inhibitor is currently being used in the phase II clinical trials for triple negative breast cancers. In addition, Mocetinostat (MGCD-0103), a class-selective HDAC inhibitor with IC50s in the submicromolar range has entered clinical trials, although not for breast cancers [188]. Finally, N-acetyldinaline (CI-994), another histone deacetylase inhibitor with a substituted benzamide derivative causes accumulation of acetylated histones [205]. The combination of current therapy with these novel inhibitors can result in successful treatment of breast cancer.

10.9 Conclusion

Over the last decade our knowledge on hypermethylation of DNA sequences and histone modifications along with Polycomb group proteins and microRNAs, and their epigenetic role in regulation of breast cancer gene expression has been increased tremendously. However, much needs to be done to understand the exact mechanisms initiating these changes during tumor development and progression. There may be a critical cross talk between the genetics and epigenetics, which has to be studied thoroughly to enhance our understanding of breast cancer initiation and progression. Identifying the epigenetic signature of breast cancer and the molecular mechanisms will definitely enhance the treatment opportunity.

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