Epigenomics of Breast Cancer

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Abstract

Breast cancer is the second most common malignant cancer and accounts for 1.38 million of the total new cancer cases and 458,400 of the total cancer deaths reported in 2008. Breast cancer with several subtypes is an extremely heterogeneous disease caused by interaction of both genetic and environmental risk factors. In order to understand the etiology of this heterogeneity, new perspectives like epigenetics are needed.

The term *epigenetics* was coined by Conrad Hal Waddington in the early 1940s. It refers to the study of gene function and regulation alterations without changes in the DNA sequence of the genome. The main epigenetic modifications are DNA methylation, histone modifications, and small noncoding RNAs (miRNAs). DNA methylation is the first to be associated with cancer and the most widely studied among epigenetic modifications. It regulates the gene expression by modifying the accessibility of DNA to the transcriptional machinery.

The importance of histone modification has been realized during the last 10 years, after identification of the coexistence of histone modifications. From the dynamically changing pattern of histone modification has emerged a new concept termed "histone cross talk." The epigenetic modifications are faster and reversible than mutation and easily affected by

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aging, environmental stimuli, and food in heritable manner. These characteristics provide a vital position in the etiology of diseases. After several investigations, it is well understood that the epigenetic modifications are involved in not only many biological processes such as X-chromosome inactivation, genomic imprinting, RNA interference, and programming of the genome but also several disease like breast cancer. Today we realize that the accumulation of epigenetic modifications occurs in the development of breast cancer. In addition, the epigenetic modifications improve our knowledge about the biology and heterogeneity of breast cancer by large-scale methods. Therefore, the researchers focused on epigenetic alterations-based breast cancer therapy, and it is speculated that epigenetic modifications may be markers for breast cancer. It is likely that epigenetics-based therapy will become a reality in the near future.

Keywords
Epigenetics • Breast cancer • DNA methylation • Histone modification • miRNA

Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. It is estimated that about 12.7 million cancer cases and 7.6 million cancer deaths occurred in 2008. Among them, 56 % of the cases and 64 % of the deaths occurred in economically developing countries [1]. In order to effectively combat it, we must understand the basic principles and processes of cancer. At the cellular level, we must understand the complex circuitries that dictate the cell-division cycle, survival, migration, and invasion. At the tissue level, the susceptible target cell population and the interactions between cancer cells and the microenvironment must be understood. Finally, the complex features that establish cancer "organ" at primary and distant sites, including metabolic and physiological effects and the establishment of a blood nutrient supply (angiogenesis), must also be clarified [2]. It had traditionally been considered that the underlying foundation of the mechanism of cancer development is the accumulation of genetic mutations. However, this paradigm has now been expanded to incorporate the distribution of epigenetic regulatory mechanisms that are prevalent in cancer [3–7].

Breast cancer is the second most common malignant cancer and the leading cause of cancer death in women, with increasing age bringing a sharp rise in incidence [1, 8]. Breast cancer constituted 23 % (1.38 million) of the total new cancer cases and 14 % (458,400) of the total cancer deaths reported in 2008. About half the breast cancer cases and 60 % of the deaths are estimated to occur in economically developing countries. In general, incidence rates are high in Western and Northern Europe, Australia/New Zealand, and North America; intermediate in South America, the Caribbean, and Northern Africa; and low in sub-Saharan Africa and Asia [9]. The American Cancer Society estimates that approximately 230,480 new cases of invasive breast cancer and 39,520 breast cancer deaths occurred among US women in 2011 [10].

Breast cancer is an extremely heterogeneous disease with molecular, histological, and phenotypic diversity caused by interaction of both inherited and environmental risk factors (age, obesity, alcohol intake, lifetime estrogen exposure, and mammographic density). Breast cancer can be classified into five major subtypes that differ significantly with regard to both molecular and clinical features. These subtypes are luminal A, luminal B, triple negative/basal like, HER2

enriched, and normal like [11, 12]. However, the main classification of breast cancer is based on the presence or absence of the estrogen receptor (ER), and investigations have been done regarding these subtypes [13]. Estrogens, sex steroid hormones, are responsible for the development of sex characteristics like breasts. Estrogens have also been recognized as the major factor in the development of breast cancers. The activity of estrogens is mediated by the two main isoforms of intracellular estrogen receptors (ERs): ERα and ERβ. The major breast cancer subtypes are ER-positive and ER-negative tumors [14, 15]. Our knowledge about breast cancer subtypes has increased since developing new high-throughput molecular techniques, such as microarray, next-generation sequencing, etc. [16, 17], and new perspectives like epigenetics [18–20].

Applications of Epigenomics of Breast Cancer

In the early 1940s, Conrad Hal Waddington coined the term epigenetics as "the causal interactions between genes and their products, which bring the phenotype into being." Nowadays, epigenetics refers to the study of gene function and regulation alterations in heritable manner. Unlike the genotoxic mechanism involving changes in genomic DNA sequences leading to mutations, the epigenetic modifications modulate the gene expression directly without changes in the DNA sequence of the genome. Epigenetic mechanisms coordinate biological processes such as X-chromosome inactivation, genomic imprinting, RNA interference, and programming of the genome during differentiation and development leading to gene silencing. Both the genetic and epigenetic events change the function and regulation of the gene products or lead to gain/loss of function of genes.

It is now acknowledged that genetic alterations are not the only path to gene disruption; reversible epigenetic modifications are increasingly being considered in cancer [18, 21–23]. In cancer cells, oncogenes are activated by mutations or overexpression, whereas tumor-suppressor genes become silenced. Accumulation of epigenetic modifications is also associated with oncogenesis. The epigenetic modifications occur early during carcinogenesis as potentially initiating events for cancer development, they have been identified as promising new targets for cancer prevention strategies [24]. Nowadays, the epigenetic mechanisms are known to be involved in several cancer types and diseases [25–34]. The epigenetic mechanisms also explain how two identical genotypes can give rise to different phenotypes in response to the same environmental stimulus [35].

Epigenomic Markers for Breast Cancer Diagnosis

The geographical variation of the incidence of breast cancer ratio indicates a significant role of factors affecting the epigenetic mechanism for the breast cancer risk [36]. Because epigenetic modifications are significant factors in the development of breast cancers, the assessment of the breast cancer in terms of the epigenetics could strongly improve our understanding of the biology and heterogeneity of breast cancer [37, 38]. The best-known epigenetic markers are DNA methylation, histone modifications and chromatin remodeling, and miRNAs.

DNA Methylation and Breast Cancer

The first and most widely studied epigenetic modification in mammals is DNA (cytosine) methylation [39, 40]. DNA methylation plays a crucial role in modulating the expression of the genetic information by modifying the accessibility of DNA to the transcriptional machinery, generally resulting in transcriptional gene silencing (activation of the gene is also possible in some cases). It involves several mechanisms like imprinting, X-chromosome inactivation, and inhibition of repeat elements and transposons transcription [41, 42]. DNA methyltransferases (DNMTs) catalyze the addition of the methyl group ($-CH_3$) using S-adenosylmethionine (SAM) as a methyl donor to dC residues in DNA.

Although most cytosine methylation occurs in the sequence CpG dinucleotides, the cytosine nucleotide of the CpA and CpT dinucleotides may also be methylated in some cases. CpG dinucleotides are found throughout the genome, but largely concentrated in small regions termed "CpG islands" [43, 44]. CpG islands are short sequences (length of 0.5 kilobases to several kilobases) of genomic DNA with a G+C content of at least 50 % and a ratio of observed to statistically expected CpG frequencies of at least 0.6. It is found in approximately 60–70 % of gene promoters commonly 5′-regulatory (promoter) regions of many "housekeeping" genes (which are essential for general cell function) and some tissue-specific genes [45–47]. CpG islands also can be found in the 3′-region of the gene and within the body of the genes (referring to exonic CpG island) [48]. Recent studies showed that not only methylation of CpGs in promoter but also methylation of CpGs within the gene bodies associate with transcriptional activation [49]. In contrast to expectation (the methylation level negatively correlates with the gene expression level), there is a positive correlation between gene-body methylation and gene activity in humans. It is proposed that the gene-body methylation may repress transcriptional noise, inhibit antisense transcription, and relate to replication timing [21, 50]. Intragenic methylation is also found at repetitive sequences in human DNA [51]. To date, several DNMTs (DNMT1p, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMTB3a, DNMT3b, and DNMT3L) have been identified. Among them only DNMT1, DNMTB3a, and DNMT3b have catalytic methyltransferase activity [52]. DNMT1 recognizes established hemimethylated DNA (the one strand of the CpG dinucleotides methylated, the other one not) and then methylates newly synthesized CpG dinucleotide whose partners on the parental strand are already methylated [53, 54]. Besides the capability of methylating hemimethylated DNA, the primary function of DNMT3a and DNMT3b is capable of de novo methylation patterns (both strands of the CpG dinucleotides are not methylated) during embryogenesis [55, 56]. Several methyl-binding proteins such as MBD1, MBD2, MBD3, and MeCP2 provide a platform for the DNA methylation [57], and it has already been determined that the mutations in DNMTs and MBDs contribute to diseases like acute myeloid leukemia (AML) [58, 59].

There are three types of DNA methylation: hypermethylation, hypomethylation, and loss of imprint. The CpG islands in the promoter region are commonly unmethylated (genes active) in normal tissues. In hypermethylation the CpG islands in the promoter region are aberrantly methylated, leading to gene silencing through the inhibition of transcription via recruitment of chromatin remodeling corepressor complexes. The loss of DNA methylation occurs in many gene-poor genomic areas including repetitive elements, retrotransposons, and introns at hypomethylation. It causes genomic instability and leads to reactivation of the genes. Loss of imprinting could be explained as the loss of specific monoallelic expression of genes in a parent-origin-specific manner [12, 35, 37].

DNA methylation is the first epigenetic mechanism to be associated with cancer after demonstration of global DNA hypomethylation and CpG-island hypermethylation in cancer tissues compared to normal tissues [60]. Global and gene-specific DNA hypomethylation and site-specific hypermethylation are common features in tumorigenesis [61]. The most extensively studied epigenetic alteration in cancer is DNA methylation of CpG islands. When the CpG islands of important genes like tumor-suppressor genes are hypermethylated, the tumor-suppressor genes become inactive and cancer emerges [62–64]. Nowadays, the next-generation sequencing (NGS) platform gives us enormous data relating genome-wide maps of CpG methylation. It is demonstrated that 5–10 % of normally unmethylated CpG promoter islands become abnormally methylated in various cancer genomes, and hypermethylation of promoter region also affects expression of various noncoding RNAs, some of which have a role in malignant transformation [5, 64]. DNA hypomethylation is observed in several tumor types, such as colorectal and gastric cancers, melanomas, etc. [65]. Decreased DNA methylation is thought to promote chromosomal instability, eventually leading

to carcinogenesis. Genome-wide DNA hypomethylation also affects transcription through loss of imprinting and upregulation of silent genes, all of which might induce tumor development [66]. During tumor progression, the degree of hypomethylation of genomic DNA increases as the lesion derives from a benign proliferation of cells to an invasive cancer [67].

Because of the high histological and molecular heterogeneity, the assessment of breast cancer in terms of the epigenetic aspects, especially DNA methylation, helps us to clarify the breast cancer mechanism. It is speculated that changed DNA methylation pattern of global or specific genes, such as RASSF1A, GHSR, etc., may be markers for breast cancer, after their appearance in several studies [68–71]. To find a reliable biomarker, several changed methylation pattern genes in breast cancer have been reported during the last decade, based on the tumor's clinicopathological characteristics, such as hormonal receptor status [72–76]. The methylated RASSF1A, CCND2, GSTP1, and TWIST genes for ER-positive breast cancers and PGR, TFF1, and CDH13 genes, predominantly for ER-negative breast cancers, have been linked [12, 37].

The involvement ERα in breast cancer is already known, and ERα is expressed approximately in 65–75 % of diagnosed breast tumors. ERα is encoded by the estrogen receptor 1 (ESR1) gene. The promoter region and first exon of the ESR1 gene contain five CpG islands [77, 78]. Several mechanisms relating the lack of ERα expression in ER-negative breast cancer have been proposed to date. Among them, the suppression of the ESR1 gene by hypermethylation of CpG islands has been investigated [79]. DNMTs are responsible for this methylation, and it has been demonstrated that the re-expression of the ER gene is possible by a DNMT1 inhibitor (5-aza-2′-deoxycytidine) or antisense oligonucleotide for inhibiting DNMT1 specifically [80, 81]. A recent study also showed that ER promotes genomic methylation through upregulation of DNMT1 in ER-positive breast cancer cells [82]. Another important molecule in breast cancer, E-cadherin, is responsible for maintaining the normal differentiated state of the mammary gland epithelium. Similarly, the loss of E-cadherin expression in all tumor stages of breast cancer has been observed due to hypermethylation of CpG islands. Therefore, epigenetic suppression of ERα and E-cadherin may occur prior to invasion and then increases as cells acquire invasiveness and metastatic potential [18, 78].

Cancer is a disease characterized by uncontrolled cell division due to checkpoints damaged by several factors such as chemical, UV, etc. [83, 84]. Despite the fact that the exact role of the BRCA1 protein is not clarified in detail, BRCA1 protein is known to be a tumor-suppressor gene. It involves several important biological processes, such as DNA repair damage, induction of apoptosis, etc. [85–87]. The mutations on BRCA1 and BRCA2 genes increase the development of familial breast cancers [88, 89]. The other mechanism of suppression of BRCA1 expression is hypermethylation of promoter region of genes. Recent studies have shown that suppression of BRCA1 expression by hypermethylation is involved not only in breast and ovarian cancer but also lung and oral cancers [90, 91].

Hypermethylation of CpG islands resulting from overactivity of DNMTs occurs in many cancers. Several studies reported that DNMTs are also overexpressed in breast cancer [92, 93]. A recent study in Tunisian breast cancer showed overexpression of three hypermethylating enzymes (DNMT1, DNMT3a, and DNMT3b) by immunohistochemistry. They found that overexpression of various DNA methyltransferases might be involved in epigenetic inactivation of multiple tumor-suppressor genes, leading to the development of aggressive forms of sporadic breast cancer [94]. However, re-expression of promoter-methylated genes can be achieved after DNMT inhibitor treatment, such as 5-aza-2′-deoxycytidine treatment [95, 96].

The other epigenetic mechanism, hypomethylation, also is involved in activating genes in breast cancer. The promoter region of the MDR1 gene is always highly methylated in normal conditions, while its hypomethylation occurs during tumorigenesis, and it might be a putative implication in biological aggressiveness of tumors [97]. Several hypermethylated and hypomethylated

genes are involved in biological functions linked to breast cancer. The demonstrated genes are in listed Table 5.1.

Moreover, global hypomethylation can be seen in breast cancer. It is widely assumed that global hypomethylation activates the gene expression. However, it might decrease the gene expression when accompanied by a gain of repressive chromatin. Taken together, it has been found that the global hypomethylation silences tumor-suppressor genes via repressive chromatin domains in breast cancer [98].

Male breast cancers often differ from female breast cancers in several respects. Kornegoor et al. studied the comparison of male and female breast cancers in terms of the DNA methylation patterns. The methylation patterns of the most frequently methylated genes (MSH6, WT1, PAX5, CDH13, GATA5, and PAX6) were found to be similar in male and female breast cancer. On the other hand, methylation occurred less often in male breast cancer when compared to female breast cancer [99].

Histone Modifications in Breast Cancer

The chromatin is a highly organized structure of DNA and protein. The organization of DNA in chromatin (euchromatin, active; heterochromatin, inactive) has many functions, such as packaging DNA into smaller volume, preventing DNA damage, and controlling DNA replication, transcription, and repair [100]. The fundamental unit of chromatin is the nucleosome, an octomeric structure containing two copies each of histones (H3, H4, H2A, and H2B) around which 147 base pairs of DNA are wrapped [101]. The states of chromatin are controlled by chemical modification of histone tail (N-terminus) via posttranscriptional including acetylation, methylation, phosphorylation, sumoylation, poly(ADP)-ribosylation, and ubiquitination and histone composition in conjunction with other nonhistone proteins [102, 103].

It was first proposed in 1964 that histone modifications may affect the regulation of gene expression, after demonstrating acetylation of the ε-amino group of lysine residues on histones

Table 5.1 Hypermethylated and hypomethylated genes in human breast cancer

Gene (description)	Function	Sample obtained	Case #	Methy. status	Marker	Reference
14-3-3-σ/stratifin (SFN)	Cell cycle regulation	Cell lines, tissue	20	Hyper	Therapeutic	Ferguson et al. [202]
14-3-3-σ/stratifin (SFN)å	Cell cycle regulation	Serum	100	Hyper	Diagnostic, prognostic	Mirza et al. [203]
ESR1 (estrogen receptor 1) or 14-3-3-σ/stratifin (SFN)	Cell cycle regulation	Serum	106	Hyper	Diagnostic	Martínez-Galán et al. [204]
RASSF1A (ras association domain family protein1)	Cell cycle regulation	Cell lines, tissue	45	Hyper	Therapeutic	Dammann et al. [205]
APC (adenomatous polyposis of the colon)	Inhibitor of β-catenin	Tissue	50	Hyper	Therapeutic	Jin et al. [206]
RASSF1, APC, DAPK1		Serum	34	Hyper	Diagnostic	Dulaimi et al. [207]
RARβ (retinoic acid receptor β)	Cell cycle regulation	Cell lines, tissue	24	Hyper	Therapeutic	Sirchia et al. [208]
RASSF1A and RARβ	Cell cycle regulation	Serum	20	Hyper	Diagnostic, prognostic	Shukla et al. [209]
RASSF1A or ATM	Cell cycle regulation	Plasma	50	Hyper	Diagnostic	Papadopoulou et al. [210]
RASSF1, RARB, MGMT, APC		Serum, tissue	33	Hyper	Prognostic	Taback et al. [211]
TMS1 (target of methylation-induced silencing-1)	Involved in apoptosis	Cell lines, tissue	27		Therapeutic	Conway et al. [212]

Table 5.1 (continued)

Gene (description)	Function	Sample obtained	Case #	Methy. status	Marker	Reference
TMS1, BRCA1, ERα, and PRB		Serum	50	Hyper	Diagnostic	Mirza et al. [173]
CCND2 (cyclin D2)	Cell cycle regulation	Tissue	106	Hyper	Diagnostic, prognostic	Evron et al. [213]
CCND2, CDKN2A, and SLIT2		Serum, tissue	36	Hyper	Diagnostic, prognostic	Sharma et al. [214]
CDH1 (E-Kadherin)	Cell adhesion and invasion	Tissue	151	Hyper	Prognostic	Shinozaki et al. [215]
CDH1 (E-Kadherin)	Cell adhesion and invasion	Tissue	79	Hyper	Prognostic	Caldeira et al. [216]
CDKN2A (cyclin-dependent kinase inhibitors)	Cell cycle regulation	Plasma	35	Hyper	Diagnostic	Silva et al. [217]
CDKN2A or CDH1		Serum	36	Hyper	Diagnostic, prognostic	Hu et al. [218]
CDH 13 (H-Kadherin)	Cell adhesion and invasion	Cell lines, tissue	55	Hyper	Therapeutic	Toyooka et al. [219]
BRCA1 (breast cancer 1)	DNA repair and recombination	Tissue	143	Hyper	Diagnostic	Birgisdottir et al. [220]
BRCA1, CDKN2A, or 14-3-3σ		Serum	38	Hyper	Diagnostic	Jing et al. [221]
APC, RASSF1, or ESR1		Serum	79	Hyper	Prognostic	Van der Auwera et al. [222]
GSTP1 (glutathione-S-transferase P1)	Carcinogen detoxification	Tissue	77	Hyper	Prognostic	Esteller et al. [223]
GSTP1, RARB, RASSF1, or APC		Plasma	47	Hyper	Diagnostic	Hoque et al. [224]
TWIST (TWIST homology of drosophila)	Involved in cell death	Mammary ducts' fluid	72	Hyper	Therapeutic	Vesuna et al. [225]
CCND2, RARB, TWIST1, or SCGB3A1		Plasma	34	Hyper	Diagnostic	Bae et al. [226]
RUNX3 (run-related transcription factor 3)	Transcriptional regulation	Cell lines, tissue	44	Hyper	Diagnostic	Lau et al. [227]
RUNX3, CDKN2A, RASSF1, or CDH1		Serum	19	Hyper	Diagnostic, prognostic	Tan et al. [228]
MDR1 (multidrug resistance 1)	Transmembrane efflux pump	Serum, tissue	100	Hypo	Prognostic	Sharma et al. [97]
CAV1 (Caveolin 1)	Cell invasion, metastasis	Cell line	30	Hypo	Prognostic	Rao et al. [229]
NAT1 (N-acetyltransferase type 1)	Cell invasion, metastasis	Tissue	103	Hypo	Prognostic	Kim et al. [230]
UPA (Urokinase)	Cell invasion, metastasis	Cell line	1	Hypo	Therapeutic	Pakneshan et al. [231]

[104]. After nearly half a century, it is has been elucidated that the posttranscriptional modifications of histone tails determine not only transcriptional activity but also all DNA-templated processes. The identification of the coexistence of histone modifications associated with activation or repression led to the proposal that the modification constitutes a code that could be recognized by transcription factors to determine the transcriptional state of a gene 10 years before [105]. However, these patterns appear to be not static, and a dynamically changing and

complex landscape via the chromatin signaling pathway led to the new concept termed "histone cross talk." This term represents the influence one or more coexisting histone modifications have on the deposition, interpretation, or erasure of other histone modifications [5, 106]. The recent investigations showed that histone cross talk mechanisms commonly seen and have a great importance for biological processes in organism [107, 108].

Histone modifications affect the chromosome function via several mechanisms. Generally it is believed that histone modifications cause structural changes in histone. This structural change may act as specific binding sites for protein domains (e.g., bromodomains, chromodomains, tudor domains) [109, 110]. Among the epigenetic mechanisms, histone modifications have further grown over the last decade with the discovery and characterization of a large number of histone-modifying molecules and protein complexes. The deregulation of these molecules or complexes may lead to deregulation of the control of chromatin-based processes by changing histone modifications and may have been associated with a large number of human malignancies. Genome-wide studies revealed that the histone modifications of malignant cells patterns disrupted when compared to healthy cells [111]. The posttranslational modification at amino acid tail of histone protein may result in changed transcription of important genes such as tumor suppressors. Changed patterns of histone modifications are a hallmark of cancer, and great amount of histone modifications have been linked to several cancer types to date [112]. The most well-known histone modifications types are acetylation/deacetylation and methylation/demethylation [113].

Histone Acetylation/Deacetylation in Breast Cancer

Histone acetylation/deacetylation status regulates several important regulatory proteins and transcription factors and is controlled by the interplay of histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. HATs transfer acetyl groups from acetyl-CoA to the amino group of lysine residues in histone tail. It removes the positive charges, thereby reducing the affinity between histones and DNA. This makes RNA polymerase and transcription factors easier to access the promoter region. So histone acetylation facilitates gene expression by allowing transcription factors to access the DNA. In contrast, the HDACs remove the acetyl group from histones to coenzyme A (CoA), resulting in coiling of chromatin, which inhibits transcription [22, 103].

At least 25 HATs and 18 HDACs have been identified in humans [114]. HATs were the first enzymes shown to modify histones [115]. There are two major classes of HATs: type A and type B. The type A HATs are nuclear proteins and can be grouped into at least three families—Gcn5/PCAF, MYST, and p300/CBP—depending on amino acid sequence homology [116]. In contrast to type A HAT, the type B HATs are predominantly cytoplasmic and show similar highly conserved primary structures, with acetylate-free histones but not those already deposited into chromatin, and newly synthesized histones H4 at K5 and K12. This pattern of acetylation is important for deposition of the histones [117]. The HDACs also have critical importance in the regulation of expression of genes involving cell survival, proliferation, differentiation, and apoptosis and can be divided into four major groups depending on sequence homology and target both histone and nonhistone proteins. Class I includes HDACs 1, 2, 3, and 8; class II includes HDACs 4, 5, 6, 7, 9, and 10; and class IV includes HDAC 11. In contrast to other HDACs, class III HDACs consist of NAD^+-dependent sirtuin family 1–7 [5]. HDACs also regulate the expression of tumor-suppressor and specific cell cycle regulatory genes. It has been observed that high HDAC expression level and hypoacetylation can be seen in several cancers. So HDAC inhibitors have been targeted for cancer therapy [118, 119]. The mechanism of the antiproliferative effects of HDAC inhibitors is complex. The target of HDAC inhibitors is the zinc cofactor at the active site of the HDACs to change chromatin structure and cause re-expression of aberrantly silenced genes [120].

Histone Methylation/Demethylation in Breast Cancer

Besides the gene promoter regions, the methylation/demethylation can occur on histone protein residues. DNA methylation at CpG islands of

promoter regions generates long-term gene silencing and makes the majority chromatin inaccessible for transcription, but histone methylation results in short-term inhibition of gene expression. Methylation, unlike acetylation and phosphorylation, does not alter the overall charge of the molecule [5, 18]. Histone methylation takes place at lysine and arginine residues by histone methyltransferases (HMTs). HMTs transfer a methyl group from the cofactor S-adenosyl methionine to lysine or arginine residues on histone tails, which play important roles in chromatin remodeling and transcriptional activity. The methylation at arginine residue of histone tails usually activates the gene transcription, although it may be involved in transcriptional repression in some cases. The methylation at lysine residue of histone tails can contribute to either activation or repression of transcription, depending on the position of methylation, and adjacent modifications [121, 122]. Some lysine methylases (like H3K4, H3K36, H3K79) often activate genes in euchromatin, while others (like H3K9, H3K27, and H3K20) are associated with heterochromatin regions of the genome. The methylation status (mono-, di-, or trimethylation) also alters gene expression. For example, the monomethylations of H3K27, H3K9, H4K20, H3K79, and H2BK5 are all linked to gene activation, whereas trimethylations of H3K27, H3K9, and H3K79 are linked to repression [123]. Histone demethylases (HDMs), discovered nearly 7 years ago, have been classified into two groups depending on their mechanism of action [124].

Several HMTs and HDTs relevant to cancer development have been identified to date [125]. The EZH2 one of the HMTs acts mainly as a gene silencer; it is the major enzyme that methylates lysine-27 of histone H3 (H3K27). EZH2 can add up three methyl groups to the ε-amino group of the lysine side chain, leading to chromatin condensation [126, 127, 128]. The overexpression of EZH2 is seen in many cancer types, including prostate and melanoma [129, 130]. The elevated EZH2 levels are associated with breast cancer [131]. It also correlates with tumor aggressiveness and poor prognosis, which suggests that EZH2 was an oncogene [132, 133]. However, loss-of-function mutations in EZH2 gene have described several malignancies, suggesting EZH2 was tumor-suppressor gene [134, 135]. In addition, some chemicals like diethylstilbestrol (DES) or bisphenol A (BPA) contribute to the formation of breast cancer by increasing EZH2 expression [136].

Another study relating EZH2 to breast cancer concluded that the overexpression of EZH2 regulates BRACA1 gene expression and genomic instability mediated by PI3K/Akt-1 pathway [137]. These investigations suggest that EZH2 histone methyltransferase is involved in breast cancer etiology.

The HMT G9a methylates at the ε-amino group of lysine 9 residues of histone 3. It has also been proven that G9a is involved in Snail-mediated E-cadherin repression by interacting with Snail in human breast cancer [138]. Another study proposed that G9a contributes to the estradiol (E2)-dependent induction of some endogenous target genes of estrogen receptor (ER)α in MCF-7 breast cancer cells [139]. Other lysine HMTs (NSD1, NSD3L, and SMYD3) are overexpressed in several cancers [125, 140]. Unlike lysine HMT, arginine HMTs have not been as well characterized. Arginine HMTs catalyze methylation of nitrogen of arginine residues, called protein arginine methyltransferases (PRMT). The 10 PRMTs are nearly identified and categorized into two groups based on the type's methylarginine products they produced [141]. Among PRMTs, the altered PRMT1 gene expression has been investigated in breast cancer [142].

Several types of histone lysine demethylases (HDMs) have been identified, but the pathological roles of their dysfunction in human disease have not been clarified. Among them, lysine-specific demethylase (LSD1) is the first identified histone lysine demethylase. LSD1 specifically demethylates histone H3 lysine 4, which is linked to active transcription [143]. After discovery of LSD1, the concept of histone methylation changed, and it is understood that histone methylation is a dynamically regulated process under enzymatic control rather than chromatin marks that could only be changed by histone replacement [19]. It has been reported that the expression level of LSD1 is elevated in human bladder [144],

small cell lung, colorectal, and neuroblastoma cancers, and the mutation of LSD1 gene causes prostate cancer [145].

In breast cancer, LSD1 expression has been found to be strongly upregulated in ER-negative breast cancer; it makes LSD1 a putative biomarker for aggressive tumor biology and a novel attractive therapeutic target for treatment of ER-negative breast cancer [146]. It is also demonstrated that LSD1 inhibits the invasion of breast cancer cells in vitro and suppresses breast cancer metastatic potential in vivo [147]. Other histone demethylase genes GASC1, PLU-1, and JMJD2B are involved in human breast cancers. The GASC1 gene may be linked to the stem cell phenotypes and show oncogene properties in human breast cancer [148]. PLU-1 is an H3K4 demethylase and plays an important role in the proliferative capacity of breast cancer cells through repression of tumor-suppressor genes, including BRCA1 [149]. The methylation status of histone H3 lysine 4 (H3K4) and of H3K9 is mutually exclusive, and H3K9 trimethyl demethylase JMJD2B is an integral component of the H3K4-specific methyltransferase MLL2. It has been demonstrated that the JMJD2B/MLL2 complex interacts to define the methylation status of H3K4 and H3K9 in ERα-activated transcription, and JMJD2B itself is transcriptionally targeted by ERα and may thus form a feed-forward regulatory loop in promoting hormonally responsive breast carcinogenesis [150]. JMJD2B also functions as coregulator of ERα signaling in breast cancer growth and mammary gland development [151]. And the histone protein LSD1 is able to demethylate nonhistone proteins, such as p53 and DNMT1 [152, 153].

miRNA in Breast Cancer

Scientists have long been aware of the existence of noncoding RNAs (ncRNAs). In spite of the great amount of knowledge about the function and types of ncRNAs, we are still far from fully knowing the role of large fractions of the transcriptome that do not encode for proteins [154]. Among ncRNAs, microRNAs are 18–25 nucleotides-long RNA molecules encoded in the genome that are transcribed by RNA polymerase II and important regulators of protein of gene expression that control both physiological and pathological processes, such as DNA methylation, development, differentiation, apoptosis, and proliferation [155, 156]. miRNAs are synthesized and processed in the nucleus, exported to the cytoplasm, and then bind to the target mRNA. The regulation of RNA transformation by miRNA is accomplished through RNA-induced silencing complex (RISC). miRNAs can inhibit mRNA translation or degrade mRNA [157, 158]. Major mechanisms of miRNA deregulation include genetic and epigenetic alterations as well as defects in the miRNA processing machinery. Each miRNA regulates multiple mRNAs and, conversely, each mRNA may be targeted by multiple RNAs (several hundreds). They can act as oncogenes or tumor suppressors and have been implicated in cancer initiation and progression, and the profiles of miRNA expression differ between normal and tumor tissues and between tumor types [159–161]. To date, several investigations relating to miRNA profiling has led to the identification of miRNAs' changed expression level in human breast cancer [162, 163]. The expression level of these miRNAs was correlated with specific breast cancer biopathological features, such as estrogen and progesterone receptor expression, tumor stage, vascular invasion, or proliferation [164]. miRNAs act as tumor suppressors and are oncogenic in breast cancer like other cancer types. So, tumor formation may arise from the overexpression (or amplification) of oncogenic miRNA and/or reduction (or deletion) of a tumor-suppressor miRNA [165].

miRNA-21 is overexpressed in breast cancer like in other cancer types [164, 166]. p53 and programmed cell death 4 (PDCD4) are tumor-suppressor proteins, and the deregulation of them may lead to cancer development. miRNA have been linked to breast cancer by targeting these proteins in breast cancer cells [167].

Epigenomic Markers for Breast Cancer Prognosis

Despite the extreme heterogeneity of breast cancer, global breast cancer survival rates have increased during the past decades due to advances

in the central role of genetic alterations in the diagnosis, treatment, prevention of breast cancer, and prognosis [2, 168]. Survival rates should be further improved by finding epigenetic molecular markers associated with risk assessment and/or prognosis of breast cancer. The knowledge about epigenetic alterations profiles in detail might prove vital in many respects. First, it might help us to estimate breast cancer risk and take precautions before breast cancer develops. In addition, there are several subtypes of breast cancer and corresponding therapies currently used. Each subtype, even each individual, has unique molecular epigenetic characteristics. The elucidating of epigenetic characteristic might contribute to a better estimation of breast cancer prognosis and lead to the choice of the most useful therapy [169]. In this way, patients will not be exposed to ineffective toxins associated with expensive therapy. Several reports have proposed that hypermethylation or hypomethylation of specific genes and global methylation status might be useful epigenetic markers for breast cancer prognosis. The recent studies also included miRNAs' expression profiles into putative epigenetic markers of breast cancer.

The major breast cancer subtype is ER-positive, and it has generally had a more favorable prognosis than ER-negative tumors. It is well established that ERα and E-cadherin are frequently involved in pathogenesis of breast cancer. The aberrant methylation of these genes is associated with malignant progression in human breast cancer [170]. ERα expression level is also regulated by miRNAs in the context of breast cancer. miRNA-206 [171] and miRNA-221/222 [77] target and regulate human ERα. miRNA-206 was upregulated in ERα-negative breast cancer. Another study found that miRNA-206 inhibits the expression of ESR1 mRNA through two binding sites in the ESR1 3′-untranslated region (3′-UTR). The researchers also found other miRNAs (miRNA-18a, miRNA-18b, miRNA-193b, and miRNA-302c) targeting to ESR1 mRNA in breast cancer cells [172]. Therefore, the aberrant methylation of the ESR1 gene and certain miRNAs altering the ESR1 gene expression might be putative epigenetic markers for human breast cancer prognosis.

BRCA1-associated breast cancer, hereditary or nonhereditary, occurs at early age due to involvement of the cellular DNA repair machinery. The inactivation of the BRCA1 by hypermethylation has been suggested to be the putative prognostic marker in breast cancer [173]. Besides the methylation, BRCA1 expression level could be regulated by miRNA-335. Overexpression of miR-335 resulted in an upregulation of BRCA1 mRNA expression, suggesting a functional dominance of ID4 signaling [174].

RASSF1A (Ras association domain family 1 isoform A) is a recently discovered tumor-suppressor gene. The protein encoded by RASSF1A interact is involved in the regulation of the cell cycle, apoptosis, and genetic instability. Thus, loss or altered expression level of the RASSF1A gene has been associated with several cancers. After illustrating the association between inactivation of the RASSF1A gene and the hypermethylation of its CpG-island promoter region, the RASSF1A gene has become the attractive biomarker for early cancer detection, diagnosis, and prognosis in many cancer types [175, 176]. The increased methylation level of the RASSF1A gene was observed in tumor size and lymph node status in breast cancer [177]. Similar results have been obtained by a meta-analysis of published data conducted with 1795 breast cancer patients. They concluded that RASSF1A promoter hypermethylation associates with worse survival in breast cancer patients [178]. These findings have indicated the great potential for the methylation of the RASSF1A gene in terms of the prognostic value of the breast cancer.

EZH2, histone-lysine N-methyltransferase acts as gene silencer by methylation and is related to several cancers. The overexpression of EZH2 is associated with aggressive breast cancer because of the enhanced cancer cell proliferation and a marker of poor prognosis in many solid tumor carcinomas including breast [179–181].

It has been investigated that several miRNAs are involved in breast cancer pathogenesis like cell regulation, and it has been proposed to be a prognostic factor for breast cancer. The miRNA-17-5p and miRNA-17/20 have been reported to be involved in breast cancer cell proliferation [182, 183]. miRNA-21 also could be a molecular

prognostic marker for breast cancer and disease progression because of its association with advanced clinical stage, lymph node metastasis, and patient poor prognosis [184].

Another strategy to clarify the role of miRNA in breast cancer is the analysis of DNA methylation and expression miRNAs in combination. Alteration of methylation in the promoters of miRNAs has also been linked to transcriptional changes in cancers. Morita et al. found that DNA methylation in the proximal promoter of miRNAs is tightly linked to transcriptional silencing [185].

Applications of Epigenomics in Breast Cancer Therapy

Cancer emerges not only because of the accumulation of genetic mutations, but also because of the reversible epigenetic changes. The dynamic alterations of the epigenetic mechanisms offer us a new field for developing novel cancer drugs that can react to epigenetically silenced tumor-suppressor genes [186]. So histone deacetylases and DNA methyltransferases have become the main targets for cancer therapy. In breast cancer, epigenetic silencing of tumor-suppressor genes due to alteration in both HATs and HDACs (histone modification) in combination with DNA hypermethylation is commonly observed [187]. The clarification of the epigenetic dysregulation mechanism in breast tumorigenesis has great importance in terms of the development of new therapies for breast cancer patients.

Aberrant HDAC activity has been investigated in several cancer types, especially in breast cancer. HDAC-1 expression and HDAC-3 protein expressions were analyzed immunohistochemically on a tissue microarray containing 600 core biopsies from 200 patients by Krusche et al. They found that moderate or strong nuclear immunoreactivity for HDAC-1 was observed in 39.8 % and for HDAC-3 in 43.9 % of breast carcinomas. HDAC-1 and HDAC-3 expressions correlated significantly with estrogen and progesterone receptor expression [188]. Another study concentrated on HDAC-6 expression levels in breast cancer has been done by Zhang et al. They also found that HDAC-6 mRNA expression is at significantly high levels in breast cancer patients with small tumors measuring less than 2 cm, with low histological grade, and in estrogen receptor α- and progesterone receptor-positive tumors. However, multivariate analysis concluded that the mRNA and protein of HDAC-6 were not independent prognostic factors for both overall survival and disease-free survival [189]. These studies led to the development of new therapies for breast cancer by finding suitable HDAC inhibitors. To date, a number of HDAC inhibitors have been designed and synthesized based on their chemical structure and are generally divided into four groups including hydroxamic acids, benzamides, cyclic peptide, and aliphatic acids (small chain fatty acids). The potential use of these inhibitors for breast cancer therapy has been investigated, as shown in Table 5.2.

Among them, some HDAC inhibitors like vorinostat (SAHA) and romidepsin (FK-228) have already been approved by the US Food and Drug Administration for clinical treatment of cutaneous T-cell lymphoma. Vorinostat is the first HDAC inhibitor and currently under evaluation in several phase II trials in breast cancer. It is already shown that vorinostat has profoundly antiproliferative activity and inhibits proliferation of both ER-positive and ER-negative breast cancer cell lines [190]. Entinostat (MS-275) and panobinostat (LBH-589) HDAC inhibitors are in phase I and II studies in combination with endocrine therapies, chemotherapeutic agents, or novel targeted therapy in women with breast cancer [12, 120]. A recent phase II study relating to the HDAC inhibitor vorinostat combined with tamoxifen for the treatment of patients with ER-positive metastatic breast cancer using 43 patients has been done. Even though the number of patients was small, they concluded that the combination of vorinostat and tamoxifen is well tolerated and exhibits encouraging activity in reversing hormone resistance. HDAC inhibitor with tamoxifen may restore hormone sensitivity by causing re-expression of a silenced ER gene [191].

In addition to phase trials, preclinical investigations have been widely done. The other idea for treatment of ER-negative breast cancer cells is using the synergistic effects of a combination

Table 5.2 The investigations of HDAC inhibitors in breast cancer

Agent(s)	Alternative name	Class	Study design	Samples	Case #	Reference
Vorinostat	SAHA, suberoylanilide hydroxamic acid	Hydroxamic acid	Preclinic	Human breast cancer cells		Munster et al. [190]
Vorinostat			Phase II	Metastatic breast cancer	14	Luu et al. [232]
Vorinostat + tamoxifen			Phase II	ER-positive metastatic breast cancer	43	Munster et al. [191]
Vorinostat + paclitaxel + bevacizumab			Phase I–II	Metastatic breast cancer	54	Ramaswamy et al. [233]
Panobinostat	LBH-589	Hydroxamic acid	Preclinic	Human breast cancer cells		Chen et al. [234]
Panobinostat			Preclinic	ER-negative human breast cancer cells		Zhou et al. [194]
Panobinostat			Preclinic	Human breast cancer cells		Rao et al. [235]
Panobinostat			Preclinic	Triple-negative breast cancer cells		Tate et al. [236]
Entinostat	MS-275, SNDX-275	Benzamide	Preclinic	Human breast cancer cells		Lee et al. [237]
Entinostat			Preclinic	Human breast cancer cells		Huang et al. [238]
Entinostat			Preclinic	ERα-negative human breast cancer cells		Sabnis et al. [239]
Entinostat + trastuzumab			Preclinic	Human breast cancer cells		Huang et al. [120]
Romidepsin	Depsipeptide (FK-228), FR901228	Cyclic peptide	Preclinic	Human breast cancer cells		Hirokawa et al. [240]
Valproic acid	–	Aliphatic acids	Preclinic	Human breast cancer cells		Jawed et al. [241]
Valproic acid + tamoxifen			Preclinic	Human breast cancer cells		Hodges-Gallagher et al. [242]
Valproic acid + trichostatin A			Preclinic	Human breast cancer cells		Reid et al. [243]
Valproic acid + retinoic acid + 5-aza-2′-deoxycytidine			Preclinic	Human breast cancer cells		Mongan et al. [244]
Phenylbutyrate	–	Aliphatic acids	Preclinic	Human breast cancer cells		Dyer et al. [245]

treatment of HDAC inhibitors and DNMT inhibitors (demethylating agents). Fan et al. and Sharma et al. used 5-aza-2′-deoxycytidine (AZA) as a DNMT1 inhibitor and trichostatin A (TSA) as a HDAC inhibitor to investigate this synergistic effect. Both studies have shown the reactivate ERα and PR gene expression in ER-negative breast cancer cell lines, which are known to be aberrantly silenced in breast cancer [192, 193]. Other studies have shown that the HDAC

inhibitors lead to reactive of ERα and PR expression by inhibition of the HDAC activity in breast cancer cells [194–196].

The other enzyme families to target for cancer therapy are HMTs and HDMs, previously implicated in cancer, inflammation, and diabetes [197]. The gene expressions level of the histone-modifying enzymes (HDMs and HTMs) are specific to cell types and highly correlated with target gene expression [198]. A recent study examined the expression profiles of 16 different histone-modifier genes including HATs, HDACs, and HDMs in breast cancer. They found that significantly different expression levels of histone-modifier genes exist between breast tumors and normal tissue, and their findings were significantly associated with conventional pathological parameters and clinical outcomes. So, it appears that histone-modifier enzymes offer utility as biomarkers and potential for targeted therapeutic strategies [199].

After these recent findings, miRNAs also have become the target for developing therapies for breast cancer. The miRNA-based treatments, in combination with traditional chemotherapy, may be a new strategy for the clinical management of drug-resistant breast cancers in the near future [200]. One of the initial studies has concluded that miRNA-221/222 confers breast cancer fulvestrant resistance by regulating multiple signaling pathways [201].

Conclusion and Future Perspective

A new field has been opened to developing effective clinical therapies now that we understand the importance of epigenetic alterations. In contrast to genetic code, the epigenetic codes may be easily affected by aging, environmental stimuli, and food in heritable manner. Breast cancer is a multifactorial disease with molecular, histological, and phenotypic diversity caused by the interaction of both inherited and environmental risk factors. The importance of epigenomics for breast cancer development has been realized after gaining of great amount of knowledge by large-scale methods. Epigenetics-based therapy for breast cancer will most likely become a reality in the near future.

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