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Epigenomics and breast cancer

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Abstract

Breast carcinogenesis involves genetic and epigenetic alterations that cause aberrant gene function. Recent progress in the knowledge of epigenomics has had a profound impact on the understanding of mechanisms leading to breast cancer, and consequently the development of new strategies for diagnosis and treatment of breast cancer. Epigenetic regulation has been known to involve three mutually interacting events – DNA methylation, histone modifications and nucleosomal remodeling. These processes modulate chromatin structure to form euchromatin or heterochromatin, and in turn activate or silence gene expression. Alteration in expression of key genes through aberrant epigenetic regulation in breast cells can lead to initiation, promotion and maintenance of carcinogenesis, and is even implicated in the generation of drug resistance. We currently review known roles of the epigenetic machinery in the development and recurrence of breast cancer. Furthermore, we highlight the significance of epigenetic alterations as predictive biomarkers and as new targets of anticancer therapy.

Keywords

breast cancer; CpG islands; demethylating agents; DNA methylation; epigenetics; histone deacetylase inhibitors; histone modifications; nucleosomal rem odeling

Breast cancer is among the most frequently diagnosed neoplasias and the second leading cause of cancer death among American women. Generally, cancer has been viewed as a disease that is driven by progressive genetic abnormalities, involving mutations in oncogenes and tumor suppressor genes, and chromosomal abnormalities [1,2]. However, it has been shown that breast cancer, similar to other types of cancer, is also a disease that is driven by epigenetic alterations, which do not affect the primary DNA sequence [3,4]. The outcome of these alterations is aberrant transcriptional regulation that results in a change in expression patterns of genes implicated in cellular proliferation, survival and differentiation [3,5,6]. Epigenetic alterations occur at the chromosomal level in transformed cells. These involve changes in DNA methylation and histone modifications, and altered expression and function of factors implicated in regulating assembly and remodeling of nucleosomes [5-9]. Alterations in DNA methylation include global hypomethyation and focal hypermethylation. Global hypomethylation has been found to increase with age and is linked to genomic instability and activation of oncogene expression [10-12]. By contrast, gene-locus-specific hypermethylation can lead to the transcriptional silencing of tumor suppressor genes [3,5-9]. In addition to DNA methylation, post-translational histone modification is another epigenetically regulated mechanism that can modulate chromatin structure to regulate gene expression [5-8,13]. DNA methylation is often associated with some specific types of histone modifications that can

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cooperatively affect chromatin structure to silence gene expression [6,8,14,15]. In addition, current work on identifying and studying regulators that control nucleosomal remodeling has deciphered that some of them are also involved in regulation of DNA methylation and histone modifications [5,7-9,13,15]. Therefore, three epigenetic events, DNA methylation, histone modifications and nucleosomal remodeling, mutually interact with each other to regulate gene-expression. The effort to elucidate molecular events in chromatin regulation that initiate and maintain epigenetic gene silencing and oncogene activation in cancer cells, one can envision, is very important for the translation of epigenomics to clinical application. Here, we provide an overview of the current understanding of the contribution of epigenetic alterations to breast tumorigenesis, recent advances in genome-scale technologies aimed at revealing epigenetic alterations in breast cancer, and the current progress in translating this profiling knowledge into diagnosis, prognosis and therapy of breast cancer.

The epigenome in breast cancer

■ DNA methylation

DNA methylation is one of the three known layers of epigenetic control of germline- and tissue-specific gene expression. Hypermethylation plays an integral role in genomic imprinting wherein one of the two parental alleles of a gene is silenced in order to establish monoallelic expression; X-chromosome inactivation in females occurs through a similar imprinting mechanism [16,17]. Stated simply, DNA methylation is a heritable, epigenetic change that alters gene expression, and is confined to the addition of a methyl group to the 5-carbon position of cytosine in a CpG dinucleotide. In the vertebrate genome, CpG dinucleotide sequences have been severely depleted to approximately 20% of the predicted frequency during evolution, and among the remaining CpG dinucleotides, over 70% are methylated [3]. A study of the human genome revealed that the distribution of CpG dinucleotides is not random, and some of them cluster together to form CpG-rich DNA regions called CpG islands. CpG islands are mostly located in the upstream promoter and exon 1 region of over half of human genes [18]. In normal cells, CpG islands in actively expressed genes are unmethylated. However, during neoplastic transformation, DNA methylation in cancer cells exhibits inverse profiles compared with normal cells; focal hypermethylation of CpG islands of 5′-end regions of many genes is observed [5-8]. Thus, a change in DNA methylation profile is a hallmark of almost all human cancers, including breast cancer.

DNA methylation is mediated by DNA methyltransferases (DNMTs) that catalyze the transfer of the methyl group from *S*-adenosyl L-methionine (SAM) to the cytosine in CpG dinucleotide [19]. To date, the known DNMTs are DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. The maintenance of established methylation patterns in hemimethylated genes is mediated by DNMT1 that copies methylation patterns from the parent strand to the daughter [20]. *De novo* DNA methylation is catalyzed by DNMT3a, DNMT3b and DNMT3L [21,22]. DNMT3L lacks the ability to bind to SAM, and is responsible for increasing the binding of DNMT3a to SAM [22,23]. DNMT2, a small 391-amino-acid protein, is reported to possess weak DNA methyltransferase activity, but its biological function is not yet elucidated [24]. Very recent studies have shown that Dicer-mediated microRNA biogenesis is involved in modulation of DNA methylation by indirectly regulating the expression of *DNMT3* genes [25,26]. Dicer belongs to the RNase III family enzymes and is implicated in processing the biosynthesis of small interfering RNAs (siRNAs) and microRNAs (miRNAs) [27]. In Dicer$^{-/-}$ cells, the microRNAs of the miR-290 cluster are depleted and expression levels of their target Rbl2 protein (retinoblastoma-like protein) are increased, leading to downregulation of *DNMT3* gene expression through Rbl2-mediated transcriptional repression, and in turn causing the DNA methyl ation defect (global hypomethylation) [25,26]. Regarding the role of DNMTs in breast tumori genesis, it has been reported that DNMT3b mRNA is overexpressed in breast cancer,

a finding that correlates well with the hypermethylator phenotype and poor prognosis in breast tumors [28,29].

In normal cells, repetitive genomic sequences (e.g., centromeric satellite α-DNA and juxtacentromeric satellite DNA) are heavily methylated [6,7]. The maintenance of methylation in this repetitive DNA could be important for the protection of chromosomal integrity by preventing chromosomal rearrangements, translocations and gene disruption through the reactivation of transposable elements [7,11,30]. Besides hypermethylation of gene-associated CpG islands, hypomethylation of repetitive genomic DNA has also been identified as a specific feature in human cancers [7,31,32]. Although less well studied than DNA hypermethylation, several lines of investigation indicate that the global DNA hypomethylation identified in cancer cells might contribute to structural changes in chromosomes, loss of imprinting (LOI), micro satellite and chromosome instability through aberrant DNA recombination, aberrant activation of proto-oncogene expression and increased mutagenesis [7,11,33,34]. Global genomic hypomethylation in breast cancer has been known to correlate with some clinical features such as disease stage, tumor size and histological grade [35]. Some proto-oncogenes implicated in proliferation and metastasis (e.g., *s*ynuclein γ and urokinase genes) or drug resistance to endocrine therapy (e.g., *N-cadherin*, *ID4*, annexin *A4*, β-catenin and *WNT11* genes) have been found to be upregulated in breast cancer through the hypomethylation of their promoters [36-38].

CpG-island-containing gene promoters are usually unmethylated in normal cells to maintain euchromatic structure, which is the transcriptionally active conformation allowing gene expression. However, during cancer development, many of these genes are hypermethylated at their CpG-island-containing promoters to inactivate their expression by changing open euchromatic structure to compact heterochromatic structure (Figure 1) [5-7,9,14,15]. These genes are selectively hypermethylated in tumorigenesis for inactivation owing to their functional involvement in various cellular pathways that prevent cancer formation. Some of the methylated genes identified in human cancers are classic tumor suppressor genes in which one mutationally inactivated allele is inherited. According to Knudson's two-hit model, complete inactivation of a tumor suppressor gene requires loss-of-function of both gene copies [39]. Epigenetic silencing of the remaining wild-type allele of the tumor suppressor gene, thus, can be considered as the second hit in this model. For example, some well-known tumor suppressor genes, such as *p16INK4a*, *APC* and *BRCA1*, that are mutationally inactivated in the germline occasionally lose function of the remaining functional allele in breast epithelial cells through DNA hypermethylation [40-42]. Since the consequence of aberrant DNA methylation is transcriptional silencing, novel tumor suppressor genes can be identified using methylated CpG islands as a marker. Hypermethylated genes identified from breast neoplasms now form a long list (summarized in Table 1). Their biological functions encompass cell cycle regulation (*p16INK4a*, *p14ARF*, *14−3−3*σ, cyclin *D2*, *p57KIP2*), apoptosis (*APC*, *DAPK1*, *HIC1*, *HOXA5*, *TWIST*, *TMS1*), DNA repair (*GSTP1*, *MGMT*, *BRCA1*), hormone regulation (*ERα*, *PR*), cell adhesion and invasion (*CDH1*, *APC*, *TIMP3*), angiogenesis (maspin, *THBS1*), cellular growth-inhibitory signaling (*RAR*β, *RASSF1A*, *SYK*, *TGF*β*RII*, *HIN1*, *NES1*, *SOCS1*, *SFRP1*, *WIF1*), and so on. In addition to protein-coding genes, recent studies showed that microRNAs with tumor-suppressor function could be silenced in breast cancer cells through DNA methylation [43]. These breast-genome methylation patterns have been developed as biomarkers for early detection and the classification of subtype of breast tumors, as predictors for risk assessment and for monitoring prognosis, and as indicators of susceptibility or response to therapy [3]. These advances in the knowledge of the breast methylome strongly indicate that DNA hypermethylation plays a crucial role in initiation, promotion and maintenance of breast carcinogenesis, which cooperatively and synergistically interact with other genetic alterations to promote the development of breast cancer. For example, human mammary epithelial cells (HMECs) that gained the ability to emerge from the first transient growth plateau lost

p16INK4A expression concurrently with hypermethylation of *p16INK4A* promoter, indicating that loss of tumor-suppressor function of p16INK4A is required for HMECs to gain growth competency by successfully bypassing the stage of cell senescence [3,44]. This finding is consistent with other studies where the life span of stem cells could be extended by germline loss of this gene [45]. Deregulation of cell cycle control by inhibiting the function of the cyclin-dependent kinase inhibitor, p16INK4A, could create a context for facilitating early abnormal clonal expansion of cells at risk for cancer. It is believed that loss of *p16INK4A* gene is permissive for enabling such expanding cells to develop genomic instability [46] and further epigenetic gene-silencing events [47]. In addition to cell-cycle regulatory genes, DNA methylation-mediated silencing of DNA repair genes, such as *BRCA1* and *MGMT*, could result in further inactivation of tumor suppressor genes or activation of oncogenes, which further drive breast tumorigenesis [48]. More recently, the genes that function as inhibitors of WNT oncogenic pathway, such as *SFRP1* and *WIF1*, have been found to be frequently hypermethylated in primary breast tumors [49,50]. Thus, in addition to the genetic mutation-mediated mechanism, epigenetic gene silencing is another mechanism that fosters malignant transformation of the mammary gland by aberrantly activating oncogenic signaling pathways.

■ Connections between DNA methylation, histone modifications & nucleosomal remodeling

Gene-silencing through DNA methylation is tightly associated with chromatin modifications mediating the packaging of DNA. Thus, knowledge of how chromatin structure is organized and maintained is key to understanding the origins of epigenetic alterations in cancer. The basic building unit of chromatin is the nucleosome, a protein–DNA complex structure composed of an octamer of four core histone proteins (H2A, H2B, H3 and H4) that is wrapped around by a 147-bp stretch of DNA [51]. Core histones are subject to a variety of covalent modifications including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation [52-54], which are critically implicated in regulation of chromatin structure and gene expression. Each histone modification is a unique mark to show the status of chromatin structure (active or repressive). Acetylation of histone lysines has been known to be associated with open chromatin structure and active transcription; methylation of these residues is associated with either active or repressive states of chromatin architecture and transcription depending on the modified site [13,53]. For example, the promoters of transcription-active genes are associated with active histone marks, such as acetylation at lysine 9 (K9) of H3 as well as K5, K8, K12 and K16 of H4 and methylation at K4 of H3 (H3K4me), which are involved in a loosening of chromatin structure (euchromatic state) [6,53]. By contrast, when genes are silenced in cancer cells through hypermethylation of their promoters, these active histone marks are replaced by repressive histone marks, including mono-, di- and tri-methylation of histone H3 lysine 9 (H3K9), H3K27 and H4K20, that are implicated in initiating and maintaining closed chromatin structure (heterochromatic state) [5,6,8,13,14]. The mechanisms underlying histone-modification-mediated chromatin remodeling and control of gene transcription have been known to involve the binding of chromatin-associated proteins to these histone modified sites, including bromodomain-containing proteins that recognize acetylated lysine residues and chromodomain-containing proteins that recognize methylated lysine residues [13,55,56].

The dynamics of histone acetylation is balanced by enzymatic actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs), and histone methylation by histone methyltransferases (HMTs) and histone demethylases [6,13,57-59]. Histone modifications are further characterized by the presence of mono-, di-, and tri- forms of lysine methylation, which are catalyzed by various HMTs [57,60]. Some of these histone modification enzymes have been recognized as components of nucleosomal remodeling complexes, which work together to regulate chromatin structure and gene expression [8,13,60].

In humans, 18 HDACs have been identified that are subdivided into four classes (summarized in Table 2) based on their homology to yeast HDACs, their subcellular localization, and their enzymatic activities [61-63]. Class I HDACs with homology to yeast Rpd3 (reduced potassium deficiency 3), including HDAC-1, -2, -3 and -8, are generally localized in nucleus and associate with various transcriptional repressors and cofactors to form protein complexes. It has been found that HDAC1 and HDAC2 can associate with corepressor complexes such as nucleosome remodeling and histone deacetylase (NuRD), Sin3A and CoREST [64-66]; whereas another class 1 HDAC enzyme, HDAC3, is found to associate with nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) corepressor complexes [67]. Importantly, it has been found that DNMTs can be recruited by class I HDACs and function as corepressors [68-70]. Class II HDACs are larger proteins homologous to the yeast histone deacetylase 1 (Hda1) and shuttle between the cytoplasm and the nucleus. Based on the protein structure, class II HDACs are further subdivided into class IIa with one catalytic domain (HDAC-4, -5, -7 and -9) and class IIb with two catalytic domains (HDAC-6 and -10). The HDAC proteins of class I and II contain a hydrophobic catalytic pocket domain that requires a pivotal zinc ion to stabilize its domain structure to allow the insertion of a lysine side chain. Class III HDACs are composed of homologs of yeast silencing information regulator 2 (Sir2) and their enzymatic activities require nicotinamide adenine dinucleotide (NAD^+) as a cofactor. The newly identified HDAC11 is the sole enzyme categorized as the class IV HDACs. HDAC11 has no significant similarity to both class I and II HDACs except for its catalytic core domain [71]. For the role of HDACs in carcinogenesis, it has been shown that global hypoacetylation of histone H4 is a common hallmark of human cancers. Aberrant alterations in H4 acetylation, preferentially at K16, occur early in the tumorigenic process, indicating that HDACs may be engaged in abnormal post-translational modification of H4 [72]. In addition to histones, many nonhistone proteins have been identified to be the substrates of HDACs, such as proteins involved in transcription (p53, p73, E2F1, STAT1, STAT3, GATA1, YY1, HMGB1, and NF-κB), hormone response (AR, ERα, GR), nuclear transport (importin-α7), cytoskeletal structure (α-tubulin), DNA repair (Ku70), DNA architecture (WRN helicase), WNT signaling (β-catenin) and heat shock/chaperone response (HSP90) [61,63, 73-79]. The broad spectrum of HDAC substrates indicates the complexity of HDAC functions to regulate the functions of substrate proteins and gene expression.

For histone methylation, the modifications are regulated in an even more complicated manner than histone acetylation, involving a large number of chromosomal remodeling regulatory complexes. The dimethyl- and trimethyl-H3K4 modifications (an active histone mark) have been reported to be catalyzed by the Trithorax group of histone methyltransferases, such as SET1 and MLL [13,80,81]. It has been shown recently that Chd1, a component of Spt-Ada-Gcn5-acetyltransferase (SAGA) complex with HAT activity, interacts specifically and directly with methylated H3K4 to modulate chromatin structure to the euchromatic state [82]. Trithorax group factors have long been known to be implicated in the transcriptional activation of developmental regulatory genes and their actions are balanced by the opposing effects of the Polycomb group (PcG) factors [83]. In an analogous manner, PcG proteins also participate in gene silencing in normal and cancer cells by modulating methylation modifications of H3K27 (a repressive histone mark) [13,84,85]. Increasing evidence from cancer epigenomic studies suggests a critical role for PcG factors in abnormal epigenetic silencing of tumor suppressor genes in cancer cells [5,8,9,86,87]. There are at least four different PcG complexes identified in mammalian, including the maintenance complex, PRC1, composed of RING, HPC, HPH, and BMI1, and three different initiation complexes, PRC2 through PRC4, which are formed by enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 (SUZ12), and different isoforms of embryonic ectoderm development (EED) [5,8,88,89]. In particular, PRC4 exists in embryonic, stem, progenitor and cancer cells and associates with a class III HDAC called SIRT1 [5,8]. The crucial function of PRC complexes in H3K27 methylation is mediated by EZH2, a histone lysine methyltransferase, that catalyzes this lysine methylation [13,84,85].

Methylation of H3K27 possibly stabilizes the binding of PcG complexes to this histone mark to facilitate long-term gene silencing [13,90]. Importantly, H3K27me is often present at the promoters of the DNA hypermethylated and silenced cancer genes investigated thus far [91], indicating that PcG proteins play an essential role in aberrant gene silencing in cancer cells. A recent study also showed that PcG-targeted genes in normal cells are closely associated with *de novo* DNA methylation in cancer cells, suggesting that PcG may preprogram its targeted genes as targets of subsequent DNA methylation in cancer cells [92,93]. In addition, several studies have shown that expression of PcG proteins such as EZH2, SUZ12 and BMI1 is aberrantly elevated in breast cancer and other cancers [94,95], suggesting deregulation of components of nucleosomal remodeling complexes can also be a mechanism resulting in gene silencing in cancer cells. In the case of another repressive histone mark, H3K9me2 (me3), this lysine methylation is catalyzed by several histone lysine methyltransferases, including SUV39H, SETDB1, G9a and GLP among others [13,96-98]. Although the defined role of H3K9 methylation in epigenetic gene silencing remains elusive, one possible mechanism is that this mark can serve as a binding site for heterochromatin protein HP1, which has an intrinsic ability to recruit DNA methyltransferases to the silenced genes [99,100].

The epigenetic mechanisms for gene silencing involve the interplay between DNA methylation, histone modifications and nucleosomal remodeling (Figure 1). The families of methyl-CpG binding proteins (MBD and Kaiso families) have been identified to play a key role in this interplay. The molecular functions of methyl-CpG binding proteins are dependent on their ability to recognize and bind methylated DNA [8,101,102]. Accumulating evidence suggests that methyl-CpG binding proteins can associate directly or indirectly with DNMTs, HDACs and HMTs and cooperate with them to modify chromatin structure and suppress initiation of gene transcription [102-106]. The associated partners of methyl-CpG binding proteins have also been found to include many nucleosomal remodeling complexes such as NuRD, CoREST, NCoR/SMRT, Sin3A, SUV39H and SWI/SNF [64,102,106-111]. The significant role of methyl-CpG binding proteins in cancer epigenetics is supported by the findings that they are localized to DNA hypermethylated and aberrantly silenced cancer genes [14,112,113]. Thus, it has been postulated that methyl-CpG binding proteins initially recognize and bind to methylated DNA, and then bring down nucleosomal remodeling complexes to modify chromatin to the repressive compact heterochromatin structure, which causes gene silencing. Inversely, the results from some other studies show that chromatin remodeling activities can further facilitate binding of methyl-CpG binding proteins to methylated DNA sites [110,114], suggesting interaction between methyl-CpG binding proteins and nucleosomal remodeling complexes results in mutual stimulation of each others' activity. Taken together, methyl-CpG binding proteins represent an important class of chromosomal proteins that associate with multiple protein partners to modify surrounding chromatin and silence transcription, providing a functional link between DNA methylation and chromatin modification and remodeling. Needless to say, this is an emerging field and much remains to be understood regarding the components of the complex intersecting pathways that together engage in epigenetic regulation of gene expression.

■ The role of estrogen signaling-regulated epigenetic processes in the development of breast cancer & drug resistance

Much of the research effort to date has concentrated on the identification of silenced genes implicated in breast tumorigenesis. However, it is also critical to understand the factors leading to epigenetic alterations in cancer. Findings in animal models and supported by epidemiological studies, have shown that prolonged exposure of undifferentiated (immature) breast cells to estrogen or estrogen-mimetic compounds during early development increases breast cancer risk in adult life. This phenomenon is called estrogen imprinting [115]. These studies can explain why, in addition to genetic factors, the risk of breast cancer is affected by

pregnancy, lifestyle in terms of intake of food and drink, and environment. Although the tumorigenic mechanism underlying this phenomenon and its connection with epigenetic regulation are still largely unknown, recently published findings provide insight into this mechanism. One line of evidence is from the study of DNA methylation patterns in several subtypes of breast cells. Bloushtain-Qimron *et al.* found that several transcription factor genes involved in stem cell function were hypomethylated and highly expressed in breast progenitor/stem (undifferentiated) cells compared with differentiated breast epithelial cells [116], suggesting the epigenetic programs define mammary epithelial cell phenotypes. Since breast progenitor/stem cells possess self-renewal and proliferating ability and more sensitively respond to estrogenic action, this subtype of cells has been thought to be potent targets of malignant transformation [117]. The second line of evidence is from the study of the effects of estrogen exposure on breast progenitor/stem cells, using a primary culture system to decipher the phenomenon of estrogen imprinting. Cheng *et al.* compared DNA methylation profiles of epithelial progeny of estrogen-exposed breast progenitor cells with those of epithelial progeny of nonestrogen-exposed progenitor cells. They found that estrogen exposure caused epithelial progeny to exhibit a cancer-like methylome, leading to silencing of some tumor suppressor genes [118]. Although the dose of estradiol (E2) used in their study was higher than normal physiological levels, their findings suggest abnormal exposure to estrogen or estrogenic chemicals induces epigenetic alterations in breast progenitor cells, which have been previously implicated in breast cancer.

Although aberrant activation of estrogen signaling can lead to tumor-associated alterations in the epigenome of breast progenitor cells, approximately 30% of diagnosed breast cancer cases lack estrogen signaling due to loss or downregulation of estrogen receptor (ER)-α, also subject to epigenetic silencing [119,120]. ER-negative breast cancers exhibit more aggressive characteristics than ER-positive breast cancers and are resistant to anti-estrogen therapy. How ER-negative breast cancer cells acquire more aggressive properties after loss of estrogen signaling is a very important issue in the field of breast cancer research. The study by Leu *et al.* provides evidence to link loss of ER signaling to epigenetic silencing of ERα downstream target genes [121]. Their study showed that abrogation of ERα signaling by small interfering RNA-mediated knockdown of ERα expression resulted in epigenetic inactivation of ERα targets, which began from recruiting PcG repressors and HDACs to their promoters and was then progressively followed by DNA methylation of their promoters [121]. Their results suggest that epigenetic regulation on ERα target genes is required for establishing ERα-independent growth and other characteristics of ER-negative breast cancer cells.

Another important issue is whether epigenetic regulation plays a role in the development of drug resistance in breast cancer. Fan *et al.* performed a genome-wide analysis of gene expression and DNA methylation profiles in breast cancer cells after adapting the cells to the anti-estrogens, tamoxifen and fulvestrant [38]. Their results demonstrate that the development of anti estrogen resistance was associated with the changes in methylation profiles of multiple genes [38]. Their findings imply that aberrantly epigenetic regulation is involved in adaptation of breast cancer cells to anti-estrogens. This proposed concept is further supported by the recent finding that epigenetic silencing of cyclin-dependent kinase 10 (CDK10) is implicated in the development of tamoxifen resistance [122]. These several lines of evidence, taken together, point out the significance of epigenetic regulation in initiating breast carcinogenesis, promoting tumorigenic phenotypes, and assisting in the development of drug resistance.

Current technological approaches to breast cancer epigenomics

To facilitate understanding of the scope of epigenetic modifications that occur in normal and cancer cells, a range of gene-specific or genome-wide technological approaches have been

developed. We present an overview of recent technological developments and discuss the merits and the limitations of these approaches with respect to studies on cancer cells.

■ Technologies for detection of DNA methylation

DNA methylation is the first identified epigenetic mechanism of gene regulation. The initial methods of detection of methylation were restricted to the quantitation of total 5-methylcytosine content by high-performance liquid chromatography (HPLC) or high-performance capillary electrophoresis (HPCE) [123], and the study of DNA methylation of selected sequences using restriction enzymes that can distinguish between methylated and unmethylated recognition sites in genes of interest [124]. For restriction-enzyme-based methods, incomplete restriction-enzyme digestion and the limitation of enzyme-recognition sites restricted their extensive application. These technical limitations caused delay in advances in the cancer epigenetic field in contrast to the rapid development in cancer genetics field.

The development of bisulfite-conversion technique that reproducibly changes unmethylated cytosines to uracil but leaves methylated cytosines unchanged [125] was a key development that drastically speeded up progress in the field. Several sensitive DNA methylation detection techniques (summarized in Table 3) were developed upon the basis of bisulfite conversion, including bisulfite sequencing, methylation-specific PCR (MSP), combined bisulfite restriction analysis (COBRA) and so on [125-127]. Among these technologies, MSP is the most popular and powerful method for DNA methylation detection, which needs limited amounts of DNA material [126]. Since MSP is a gel-based assay, it cannot provide quantitative information and is subjective, several real-time methylation-specific PCR methods, such as bisulfite treatment in combination with MethyLight™ [128], quantitative multiplex-MSP (QM-MSP) [129,130], or pyrosequencing [131], have been developed and used in DNA methylation studies, which have improved features to detect minimal amounts of aberrant DNA methylation in a quantitative manner.

In addition to these gene-locus specific DNA methylation detection methods mentioned above, several recently developed genome-wide techniques have been applied to the study of global DNA methylation profiles in normal and cancer cells (summarized in Table 3). Restriction landmark genomic scanning (RLGS) is one of the earliest methods to be applied to genome-wide methylation analysis [132]. RLGS has an ability to globally analyze the methylation status of approximately 1000 unselected CpG islands. Other important techniques for analy zing altered DNA methylation patterns across the genome have relied on an arbitrary primed PCR technique (e.g., AIMS) [133,134]. Further advances in this field are derived from the application of the DNA microarray technology. A widely used example is differential methylation hydridization (DMH) that uses CpG-island and promoter sequence microarrays, which enables the simultaneous analysis of the methylation levels of a large number of CpG-islands in the genome [135]. In addition, a recently developed related technique called HpaII tiny fragment enrichment by ligation-mediated PCR assay (HELP) uses a modified approach to globally analyze DNA methylation patterns [136]. Methods (e.g., Methylated DNA immunoprecipitation [methyl-DIP]) based on chromatin immunoprecipitation (ChIP) using the ChIP-on-chip technology are other seminal recent advances in the epigenomic profiling of cancer cells [92,137,138]. Finally, gene-expression profiling using microarrays is another powerful and widely used technique for assessing genome-wide DNA methylation patterns. This approach is used to compare gene expression levels from cancer cell lines before and after treatment with a demethylating drug, a HDAC inhibitor, or a combination of both drugs [139-141]. As with all microarray-based technologies, the identified candidate genes are further verified, in this case, by quantitative RT-PCR and promoter methylation analyses. In addition to microarray-based techniques, a serial analysis of gene expression (SAGE)-technology-based method, known as methylation-specific digital karyotyping (MSDK), has been developed

recently for the genome-wide analysis of methylation profiles [142]. The advantage of this new technique is that there is no need of prior sequence information for analysis and the obtained data can be used to map the methylated gene loci and to determine their methylation levels.

■ Technologies for detection of histone modifications

Characterization of post-translational histone modifications is a greater challenge than analysis of DNA methylation and needs special technology. Currently, the gold standard for accurately assessing global levels of histone modifications is mass spectrometry [6]. Since antibodies specifically recognizing the amino acid modifications of histone proteins are available, a simple Western blot analysis is also used for detecting histone modifications.

In addition to determining the types and relative levels of histone modification in cells, characterization of distribution of each type of histone modification on chromosomes also provides very important information. The current techniques for genome-wide analysis all adopt the ChIP technology with antibodies against specifically modified histones (summarized in Table 3). The first-developed and widely used technique is ChIP-on-chip [143]. In contrast to the ChIP-on-chip method, another new technique for profiling histone modifications at a genomic scale is ChIP-SAGE, which combines ChIP experiments with the SAGE technology [144]. The merit of ChIP-SAGE is that, unlike ChIP-on-chip that requires sequence information of preselected genomic regions to manufacture genomic microarrays, no prior genomic sequence information is required in this assay. However, the cost for ChIP-SAGE is higher than ChIP-on-chip due to the use of the more expensive traditional sequencing methods. Besides, the fact that not every region of chromosomes contains restriction-enzyme recognition sites used to cleave the ChIP DNA limits the capacity of ChIP-SAGE to study the entire genome. More recently, a new technique, chromatin immunoprecipitation combined with high-throughput sequencing techniques (ChIP-Seq), has been developed for analyzing ChIP DNA using a high-throughput massively parallel signature sequencing-like technique developed by Solexa [145,146]; this technique is more powerful and cost-effective than the ChIP-SAGE technique. There are several advantages in this new technique, including the use of less PCR amplification of ChIP DNA after ligation to adaptors and a highly efficient sequencing procedure (sequencing-by-synthesis). In contrast to ChIP-on-chip, more quantitative data of histone modification levels at different chromosomal regions can be obtained in ChIP-Seq experiments. In addition to mapping the genome-wide histone-DNA binding patterns, it can be envisioned that this new technique has great potential for globally defining the methylome of a particular cell type. With rapid and striking technological advancements, global analysis of DNA methylation and histone modification mapping on chromosomes has become eminently practical.

Translational application of epigenomics in breast cancer

■ Epigenomic profiles as breast cancer biomarkers for diagnosis & prognosis

In addition to cancer-specific genetic and gene-expression signatures, epigenetic signatures have emerged as potential biomarkers for cancer detection. The sensitivity and specificity of this type of diagnostic assay relies on the use of those DNA-methylation markers that are frequently hypermethylated in cancer cells, but are always unmethylated in normal cells. For breast cancer, as for other cancers, early detection is the most efficient way to decrease mortality. The current widely used method for early diagnostic screening is mammography. However, while mammography is highly sensitive, its lack of specificity has created the need for other highly sensitive diagnostic methods for early detection of breast cancer. As a result of this need, cancer-specific hypermethylated genes have been considered as potential and promising biomarkers for early detection of breast cancer.

The use of a panel of cancer-specific methylation markers in conjunction with MSP, MethyLight or QM-MSP has proven to be promising for breast cancer detection. For example, Evron *et al.* successfully used a three-gene panel (Cyclin *D2*, *RAR*β and *TWIST*) to detect malignant breast cancer cells in ductal fluid from routine operative breast endoscopy (ROBE) and ductal lavage [147]. Fackler *et al.* improved this method and tested a four-gene panel (*RASSF1A*, *TWIST*, *HIN1* and Cyclin *D2*) using the QM-MSP assay to examine clinical tissue samples [129]. The cumulative methylation of these four genes is commonly observed to be higher in primary invasive breast cancers compared with reduction mammoplasty specimens from healthy women [129]. Fackler *et al.* further used the same technique but adopted a nine-gene panel (*RASSF1A*, *TWIST*, *HIN1*, Cyclin *D2*, *RAR*β, *APC*, *BRCA1*, *BRCA2* and *p16*) to examine ductal lavage samples from women with or without breast cancer. This trial demonstrated that methylation-marker detection was twice as sensitive as cytological diagnosis of ductal lavage cells [148]. In addition to biopsied tissue sections and ductal fluid, methylated DNA is also detected in blood since the blood of patients with manifest breast cancer contains detectable amounts of circulating methylated DNA [149]. The blood detection of tumor-specific methylated DNA has been pursued for its potential for prognostic prediction and monitoring relapse of breast cancer after therapy [149-151].

In addition to cancer detection, it has been reported that the DNA methylation assay might be used for risk assessment and prognosis of breast cancer. Lewis *et al.* studied five frequently methylated genes, including *RASSF1A*, *APC*, H-cadherin, *RAR*β, and cyclin *D2*, and found a higher methylation frequency of both *RASSF1A* and *APC* genes in unaffected women at high risk for breast cancer compared with those at low or intermediate risk based on the Gail model analysis. This suggests that promoter hypermethylation of these genes is associated with epidemiologic markers of increased breast cancer risk [152]. This finding needs confirmation that such alterations do indeed occur earlier than abnormal histological findings, and by follow-up studies to examine whether these changes are associated with subsequent development of breast cancer. The prognostic significance of aberrant DNA methylation has been investigated by Muller *et al.* After screening 39 genes in DNA from serum of normal control patients and patients with primary or metastatic breast cancer, they identified two genes, *RASSF1A* and *APC*, whose methylation has a statistically significant association with poor outcome [151]. Other methylated genes, such as *GSTP1*, *SFRP1*, have also been identified to be associated with poor prognosis [153,154].

■ Pharmacoepigenomics as a predictor of response to chemotherapy of breast cancer

When the functions of genes are involved in mediating the therapeutic effects of certain chemotherapeutic agents, silencing of this class of genes through epigenetic mechanisms results in abrogating or blunting the drug's effects. Therefore, they can be used as a predictor to evaluate the response of patients to chemotherapy. The prototypic case is that of the expression of *ER*α gene, which is recognized as an important predictor of response to endocrine therapy of breast cancer. As mentioned above, approximately 30% of breast tumors lack expression of ERα and are resistant to endocrine therapy. Previous studies have shown that loss of ERα expression is often associated with promoter hypermethylation of *ER*α gene [119,120]. Therefore, in addition to the immunohistochemical detection of ERα protein expression, analysis of *ER*α methylation status presents an alternative to determine whether endocrine therapy will be effective in breast cancer patients [155]. More recently, through an RNAi screen, Iorns *et al.* identified downregulation of cyclin-dependent kinase 10 (CDK10) expression leading to tamoxifen resistance [122], which has been addressed previously. Their study showed that patients with ERα-positive breast tumors that expressed low levels of CDK10 relapsed early under tamoxifen treatment. More importantly, downregulated CDK10 expression is associated with methylation of CDK10 promoter [122], suggesting that CDK10 gene methylation can serve as a novel marker for determining response to endocrine therapy.

Besides ERα, inactivation of retinoic acid receptor β (RARβ) by epigenetic mechanisms is another important predictor for determining the response to retinoic acid-based chemo prevention and therapy of breast cancer as well as other types of cancer [3]. Binding of retinoids to RARβ triggers receptor dimerization and transactivation of retinoid-responsive genes, which in turn induce cellular differentiation and apop tosis [156]. Therefore, the expression and functional status of RARβ can critically affect the anti cancer activity of retinoids. Loss or down regulation of RARβ has been known to occur in many kinds of cancers, including breast cancer, and correlate with the malignant phenotype [157,158]. Therefore, RARβ promoter hypermethylation in cancer is an important mechanism causing abrogation of the RARβ2 signaling. The epigenetic mark that shows the silencing of RARβ2, therefore, appears to be a potential determinant of retinoic acid response.

■ DNA methylation & histone deacetylation as therapeutic targets in breast cancer

The frequent occurrence of epigenetic alterations in the pathogenesis of cancer has created targets for the development of novel anticancer therapeutics. Unlike genetic alterations that are irreversible processes, epigenetic alterations in cancer are potentially reversible. This feature of epigenetics has promoted the development of pharmacologic inhibitors of DNA methylation and histone deacetylation [61-63,159]. These inhibitor compounds can induce DNA demethyl ation and inhibit histone deacetylation to reverse epigenetic silencing of tumor suppressor genes, leading to re-expression of these genes in cancer cells and reactivation of pivotal cellular tumor-suppression pathways.

DNA methyltransferase inhibitors—Since DNMTs are the core enzymes that catalyze DNA methylation, inhibitors of DNMTs are promising anticancer agents. 5-azacytidine (Vidaza™) and 5-aza-2′-deoxycytidine (Decitabine) are the most extensively studied DNMT inhibitors. The pharmacological action of these two compounds is mediated by their incorporation into DNA in the place of the natural base, cytosine, during DNA replication, leading to covalent trapping of DNMTs [160]. This causes the depletion of active DNMT enzymes and demethylation of genomic DNA. The inhibitory effect of 5-aza-2′-deoxycytidine on DNMTs is stronger than 5-azacytidine since 5-aza-2′-deoxycytidine incorporates only into DNA, unlike 5-azacytidine that incorporates into both DNA and RNA [159]. Both compounds have been approved by the US FDA as elective therapeutic agents for treatment of a pre-leukemic disease, myelodysplastic syndrome. A major disadvantage of these two compounds is their instability in neutral aqueous solution. This has resulted in a search for more stable cytosine analog-based demethylating agents, such as zebularine. Zebularine is a novel DNMT inhibitor with several merits, such as very stable chemical property suitable for oral administration, low toxicity and a high selectivity for tumor cells. [161]. However, the requirement of high levels of drug to achieve efficacy of zebularine may negatively affect its potential in clinical application. In addition, non-nucleotide DNMT inhibitors have been developed recently for avoiding the toxicity inherent in nucleotide analogs. These small-molecule demethylating agents include antiarrhythmic agents such as procainamide and procaine, the antihypertensive agent such as hydralazine, epigallocathechin-3--gallate derived from green tea, and the novel compound RG108 [162-165]. They all have the ability to bind to the active sites of all DNMTs and perturb inter actions between the enzymes and their target sites. The therapeutic agents that specifically target one type of DNMTs have also been developed, such as MG98, an antisense oligonucleotide that specifically inhibits DNMT1 function [166]. It has been demonstrated that the major anticancer mechanism of demethylating agents is attributable to reactivation of silenced tumor suppressor genes in cancer cells.

Histone deacetylase inhibitors—Abnormal recruitment of HDACs to the promoters of tumor suppressor genes, which leads to cancer, has provided a strong mechanistic rationale for the use of HDAC inhibitors (HDACIs) in cancer therapy. It has been demonstrated that

transcriptional repression of multiple genes implicated in cell cycle progression, differentiation and apoptosis could be reversed by inhibition of HDAC function [6,61,63]. Furthermore, HDACIs have proven potent anti-tumor activities in human cancer cells and xenograft models [61,63,167]. More importantly, transformed cancer cells have been shown to be at least ten-times more sensitive to growth inhibition by HDACIs compared with nontransformed cells, demonstrating that HDACIs have a tumor-selective feature [168]. The tumor-selective characteristics of HDACIs may be attributable to the fundamental epigenetic differences between normal and tumor cells. Taken together, these findings support the notion that HDACIs can be used as potential anticancer agents. Currently, a large number of structurally diverse HDACIs have been synthetically developed or purified from natural sources. According to their chemical characteristics and pharmacological mechanism for HDAC inhibition, HDACIs are classified into the following groups: hydroxamates, short-chain fatty acids, cyclic peptide, benzamides, and anilides [61,63,159,169] (summarized in Table 4). Several of them have progressed to clinical trials and suberoylanilide hydroxamic acid (SAHA) has been approved by FDA for treatment of cutaneous T-cell lymphoma (CTCL) [169].

Multiple anticancer activities of HDACIs involve cell cycle arrest, promotion of apoptosis, induction of differentiation and suppression of angiogenesis [61-63,159]. Although the mechanisms underlying the pleiotropic cellular effects of HDACIs are still not completely deciphered, a growing body of evidence has shown that HDACI-mediated anti-tumor effects involve both transcriptional and nontranscriptional mechanisms [61,63]. Treatment with HDACIs causes hyperacetylation of histones and nonhistone transcription factors such as p53, p73, E2F1, STAT1, STAT3 and NF-κB, which lead to transcriptional activation or repression of their target genes [61,170-173]. Many HDACI-induced genes have been reported (summarized in Table 5), including cell cycle regulatory genes, tumor suppressor genes, apoptotic genes, differentiation regulatory genes and immune response genes. [61,63]. For example, HDACIs can induce $p21^{WAF1/CIP1}$ expression through hyperacetylated histone-mediated transactivation in a p53-dependent or p53-independent manner, leading to cell cycle arrest [174]. HDACIs can also upregulate many apoptotic genes (e.g., *CD95*, *TRAIL*, *DR4*, *DR5*, *Bax*, *Bak*, *Bim*, *Bmf* and *Apaf1*) involved in the extrinsic death-receptor and intrinsic mitochondrial death pathways [61]. In addition, treatment with HDACIs has been found to repress the expression of genes required for cell cycle progression (cyclin *D1*, cyclin *A*), antiapoptosis (*Bcl-2*) and angiogenesis (*VEGF*, *HIF-1α*) [63].

In addition to transcriptional mechanisms for anticancer effects of HDACIs, many lines of evidence point to nontranscriptional mechanisms as mediators of the anti-tumor effect of HDACIs. [61-63]. For example, it has been reported that HDACIs could induce defective mitoses in tumor cells and in turn trigger cell death. This effect may be attributed to changes in chromatin conformation caused by hyperacetylation of centromeric histones, resulting in abnormal chromosomal segregation or impaired mitotic progression [175]. For the apoptotic effect, it has been found that acetylation of K70 in response to HDACIs results in the release of its binding partner Bax, causing translocation of Bax to the mitochondrial outer membrane to stimulate apoptosis [78,176]. Besides these examples, HDACIs deplete protein levels of many oncoproteins whose stability is regulated by chaperonic heat-shock proteins (HSPs) [61,63]. HSP90 has been found to be hyperacetylated after treatment with HDACIs, which leads to the proteasomal degradation of HSP90 client proteins such as HER2/neu, Akt, c-Raf, ERα [61,63,77,177]. Taken together, these examples demonstrate that the interplay between transcriptional effects on the regulation of gene expression and nontranscriptional effects on mitosis and nonhistone targets contributes to anticancer activity of HDACIs. These attractive combinational anti-tumor activities enable HDACIs to be potentially very effective anticancer agents.

Clinical application of epigenetic drugs in breast cancer therapy—As single-agent anticancer drugs, epigenetic modulators such as demethylating agents and HDACIs have shown the promising efficacy against multiple types of cancers in the laboratory studies and clinical trials [61,63,159]. Few of these agents (e.g., Vidaza, Decitabine and SAHA) have been approved by FDA and applied to hematological malignancies. For breast cancer, epigenetic drugs still remain in the stage of preclinical and clinical trials and await more promising clinical data for application to patients. Here we provide a review of advances in the preclinical and clinical testing of epigenetic therapeutic agents in breast cancer therapy.

Since abnormal epigenetic gene silencing mediated by aberrant DNA methylation and chromosomal remodeling (including histone modifications) is one of the major mechanisms leading to breast tumorigenesis, it is reasonable to argue that the combination of both demethylating agents and HDACIs can synergistically transactivate a set of epigenetically silenced tumor-suppressor genes and more effectively eliminate cancer cells than treatment with a single agent [178]. This rationale has been tested in ER-negative breast cancer cells, MDA-MB-231, which are resistant to traditional anti-estrogen therapy. Yang *et al.* have demonstrated that cotreatment of these cells with DNA methyltransferase and HDAC inhibitors led to synergistic demethylation of ERα promoter and re-expression of functional ERα [179]. Subsequent studies by Sharma *et al.* showed that restoration of expression and function of ERα resensitized ER-negative breast cancer cells to tamoxifen treatment [180]. Their findings, taken together, suggest that the approach of combining demethylating agents and HDACIs is a very promising regimen for treatment of tamoxifen-resistant breast cancer patients. In addition to ERα restoration, this two-drug-combination regimen has also been demonstrated to induce RARβ2 re-expression in breast cancer cells, which caused growth inhibition of breast cancer cells regardless of ER status by cotreatment with retinoic acids [158,181]. A very recent study also introduces a new strategy that the therapeutic regimen of combining two chromatin remodeling drugs (5-aza-2′-deoxycytidine and SAHA) can be used to synergize with artificial transcription factors for the reactivation of the tumor suppressor gene maspin in breast cancer cells [182]. This novel therapeutic approach that takes advantages of both epigenetic and genetic strategies to reactivate silenced tumor suppressor genes will be an effective regimen if the expression constructs of artificial transcription factors can be designed to be specifically delivered to breast tumors.

Given that HDACIs have been clinically tested with minimal toxicity to normal cells and can lower the apoptotic threshold in cancer cells due to their abilities to stimulate extrinsic and intrinsic cell death pathways as described above, the effect from cotreatment with HDACIs and conventional chemotherapeutic drugs has been examined. Recent studies that used this cotreatment approach have shown that HDACIs have synergistic effects with other chemotherapeutic agents. For example, it has been demonstrated that treatment with HDACIs such as SAHA and LAQ824 can further sensitize breast cancer cells with amplification of HER2/neu gene to trastuzumab (Herceptin®) and other chemotherapeutic agents such as gemcitabine (antimetabolite), docetaxel and epothilone B (antimicrotubule) [77,183,184]. This synergistic anti-tumor effect might result from HDACI-induced hyperacetylation of HSP90 that further causes degradation of its client HER2/neu protein [61,63,77,177], implying that inhibition of the growth-promoting and survival HER2/neu signaling pathway lowers the apoptotic threshold and renders tumor cells more vulnerable to chemotherapeutic drugs. Moreover, it has been recently reported that treatment of EGFR-overexpressing, ER-negative breast cancer cells with SAHA could decrease the EGFR levels through destabilizing EGFR mRNA [185], suggesting that combining anti-EGFR agents and HDACIs may provide a promising strategy for dual targeted therapy. Since ERα has also been found to be one of the HSP90 clients, several lines of evidence demonstrate that HDACIs can trigger depletion of ERα protein in the ERα-positive breast cancer cells through ubiquitin-mediated degradation [177,186,187]. These findings are clinically significant based on the further supportive studies

that HDACIs can sensitize ERα-positive breast cancer cells to anti-estrogenic agents [177]. Taken together, these preclinical findings support the development of HDACIs in combination with conventional chemotherapeutic drugs to treat both ER-positive and -negative breast tumors. A recent clinical study of a small group of patients with locally advanced breast cancer has shown that the therapeutic regimen with a combination of demethylating hydralazine and HDAC inhibitor magnesium valproate could cause a statistically significant decrease in global 5-methylcytosine content and HDAC activity, and this cotreatment in combination with neoadjuvant doxorubicin and cyclophosphamide appeared to increase the chemotherapeutic efficacy [188]. Although the result from this trial still needs to be further extensively evaluated, it is a first proof-of-concept study to support the efficacy of epigenetic therapy for breast cancer.

Besides chemotherapy, radiation therapy is one of therapeutic modalities for breast cancer treatment, especially for advanced metastatic disease. Although radiation has well-documented palliative effects on metastatic disease of brain and meninges, the survival rate may vary from a few months to a couple of years. A further improvement in the standard radiotherapy, therefore, might greatly benefit many metastatic patients [189]. Since HDACIs can alter chromatin structure by promoting hyperacetylation of histones, it is possible that changes in chromosomal remodeling can modulate DNA damage response. Indeed, it has recently been shown that treatment with trichostatin A could activate ATM-p53 DNA damage signaling pathway and had the synergistic effect on enhancing ionizing-radiation-induced ATM activation [190]. This raises the possibility that HDACIs are able to augment the effects of DNA damaging agents. Furthermore, HDACIs have been found to radiosensitize human melanoma cells by inhibiting DNA repair activity, concomitantly correlating with HDACI-induced decreased expression of DNA repair-related genes such as *Ku70*, *Ku86* and DNA protein kinase catalytic subunit (*DNA-PKcs*) [191]. These data suggest that HDACIs may override the DNA damage defense response and facilitate radiation-induced mitotic cell death. Recently, Nome *et al.* have tried a similar strategy using a breast carcinoma cell line with an ability to metastasize to the brain and found that following pretreatment with the HDACI (trichostatin A), the clonogenic regrowth of breast cancer cells after ionizing radiation was significantly reduced [192]. Their finding is in accordance with the notion that the disruption of chromatin structure increases the probability of mitotic cell death. These studies support the possibility that HDACIs may have the therapeutic potential to serve as radiosensitizers.

Conclusion

Dramatic advances in powerful technologies (e.g., ChIP-seq) for epigenomic studies have enabled us to move forward in big steps towards understanding the role of epigenetics in normal and cancerous cells. The comprehension of the interplay between DNA methylation, histone modifications and nucleosomal remodeling, and epigenetic mechanisms regulating chromatin structure, gene transcription and diverse cellular responses has provided a strong basis for the development of epigenetics-based therapeutic biomarkers and drugs for cancer diagnosis and treatment. In breast cancer, the discovery of cancer-specific methylated genes has led to the development of potential DNA methylation markers for early detection, risk assessment, prognosis and prediction of drug response. Discovery and development of epigenetic drugs (e.g., demethylating agents and HDACIs) have also shed new light on the development of new therapeutic regimens for the treatment of breast cancer.

Future perspective

Genome-wide profiling of mutated genes in breast and colorectal tumors has been recently catalogued [193]. This advance in the human cancer genetic field has urged epigenetic researchers to undertake national and international human epigenome projects, with the ultimate aim to map all epigenetic modifications in both normal and diseased cells [194]. It is

plausible that the implementation of these projects will enable us to more completely define the progressive changes in epigenomic patterns during the development of a particular cancer type such as breast cancer and elucidate the pathological roles of these epigenetic alterations. The outcome of the human epigenome projects is essential for the mature development of epigenetic markers and therapeutics, which will be very important for application of these advances to diagnosis, prognosis and therapy of breast cancer patients. Given that the roles of epigenetic alterations in development of resistance to anticancer drugs during breast cancer therapy remain elusive, exploration of drug-resistance mechanisms by the genome-wide epigenetic study is also very important. Furthermore, although diagnostic screening using epigenetic markers has been demonstrated to be a promising prospect, several issues still need to be solved, including development of specific and sensitive screening panels, and the necessity of improving and optimizing assay protocols for screening and final testing in large prospective clinical studies. The clinical significance of epigenetic markers also needs to be evaluated by comparing the efficacy of these panels with classic screening methods and other developing screening procedures derived from proteomics, mRNA expression, or microRNA arrays.

Although the current development of epigenetic therapeutics is promising for the treatment of breast cancer, there are many issues that need to be addressed before moving forward. The more detailed comprehension of the molecular mechanisms underlying the anticancer activities of demethylating agents and HDACIs is essential. Further translational studies of epigenetic drugs are also required to find the correlation between reversion of epigenetic changes, gene re-expression and therapeutic response of breast tumor. For clinical use of epigenetic agents in breast cancer

Executive summary

The epigenome in breast cancer

Epigenetic alterations are one of main driving mechanisms leading to breast cancer. Epigenetic alterations are heritable and reversible, and do not involve any change in the primary DNA sequence itself.

Epigenetics involves three molecular events including DNA methylation, histone modifications and chromatin remodeling. A sophisticated interplay between these three epigenetic events modulates chromatin conformation and gene expression.

DNA methylation is mediated by the catalytic activity of DNA methyltransferases. During tumorigenesis, alterations in DNA methylation involve global hypomethylation and locus-specific hypermethylation, resulting in genomic instability and rearrangement, activation of oncogenes and inactivation of tumor suppressor genes.

Histone modifications mediated by various histone tail-modifying enzymes collaborate with or without DNA methylation to communicate with chromatin remodeling factors, which adapt chromatin structure to the open euchromatin (active transcription) or the closed heterochromatin (repressive transcription) states.

Epigenetic regulation controls the expression program of stem-cell-function genes and defines mammary cell phenotypes.

In vitro cultured breast progenitor-cell model shows that abnormal exposure to estrogen causes epigenetic alterations in epithelial progeny, leading to silencing of tumor suppressor genes.

Epigenetically regulatory mechanisms are implicated in the development of estrogen receptor (ER)-negative breast cancer phenotypes and drug resistance.

Current technological approaches to breast cancer epigenomics

The development of bisulfite-conversion-based techniques facilitated the detection of methylated genes and made a revolutionary advance in the epigenetic field; a combination with real-time PCR made quantitation of gene methylation possible.

The development of chromatin immunoprecipitation (ChIP)-based techniques promoted progress in profiling of genome-wide methylome and histone-modification maps on chromosomes from normal and diseased cells.

Translational application of epigenomics in breast cancer

Epigenetic signatures specific to breast cancer have emerged as innovative biomarkers for early detection, risk assessment, prognostic prediction and the evaluation of drug response.

The reversibility of epigenetic modifications provides the rationale for developing epigenetic agents to reverse the epigenetic silencing of tumor suppressor genes.

To reduce side effects, maximize selective anticancer effects and prevent drug resistance, the developed epigenetic agents, such as demethylating agents and histone deacetylase inhibitors, can be administered in combination with other epigenetic agents, conventional chemotherapeutic agents, differentiation agents or radiation.

Conclusion

The advances in comprehension of epigenetic mechanisms implicated in modulating chromatin conformation, gene transcription and diverse cellular signaling pathways have provided the essential basis for the current development of epigenetics-based biomarkers and drugs for the diagnosis and treatment of breast cancer.

therapy, it will be important to determine their optimal therapeutic doses, timing and mode of administration. To obtain synergistic therapeutic efficacy and lower toxicity for breast cancer patients, the epigenetic anti-tumor drugs should be tested and analyzed in preferential combination with other currently used anti-breast-cancer drugs such as anti-estrogen agents, conventional chemotherapeutic agents, kinase inhibitors and differentiation agents. Another way to reduce the side effects of epigenetic drugs is to identify specific epigenetic modulators (e.g., some specific HDACs) involved in breast tumori genesis and to develop the specific drugs to target these molecules. Since the entire picture of the upstream regulatory pathways and downstream events of epigenetic modifications that are specific to breast cancer are not yet completely deciphered, the continuation of efforts to define these molecular events is still very important for the development of the next new generation of specific anti-tumor agents against breast cancer. In addition, relapse of breast cancer has been assumed to involve the failure of cancer stem cells to respond to current cancer therapy. Given that it has been shown that epigenetic modulation is required for the maintenance of cancer stem-cell features, whether epigenetic agents have effects against breast cancer stem-cells is worthy of investigation.

From the tremendous progress we have witnessed in both basic and clinical studies with regard to epigenetics, we have clearly entered a remarkable era of the epigenome. A greater understanding of the molecular events for the initiation and maintenance of epigenetic gene silencing during the process of breast tumorigenesis could promote the development of promising epigenetic-marker strategies for early detection, risk assessment, prognosis and drug-response prediction, and could also speed up the progress in developing potential clinical strategies for breast cancer prevention and therapy.

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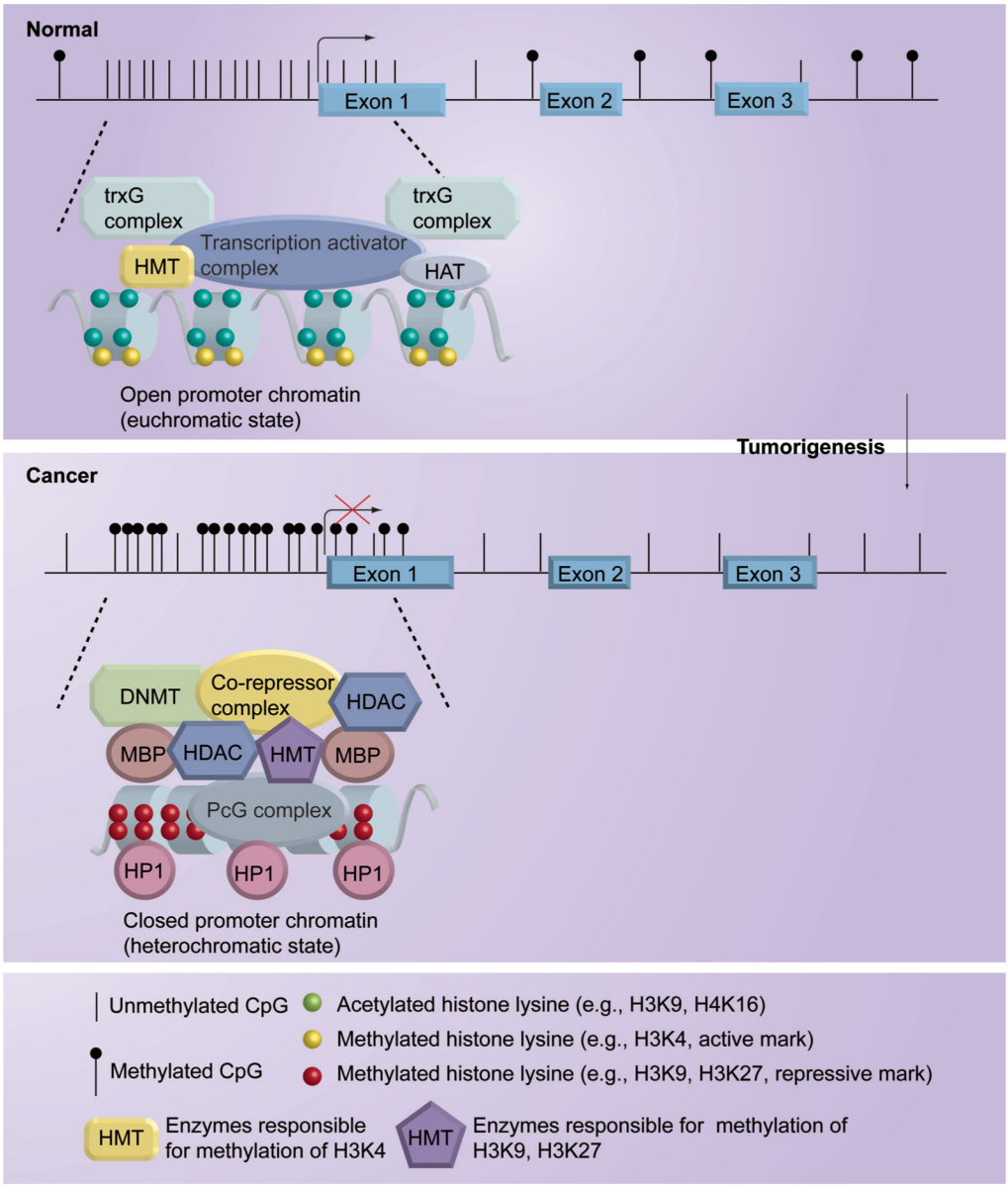

Figure 1. DNA methylation, histone modifications and chromatin remodeling in normal and cancer cells

DNMT: DNA methyltransferases; HAT: Histone acetyltransferases; HDAC: Histone deacetylases; HMT: histone methyltransferases; HP1: Heterochromatin protein 1; MBP: Methyl-CpG binding proteins; PcG: Polycomb group; trxG: Trithorax group.

Table 1

Genes hypermethylated in breast cancer.

Gene	Description	Function	Ref.
14−3−3σ	Stratifin	Cell cycle regulation	[195,196]
APC	Adenomatous polyposis of the colon	Inhibitor of β-catenin	[41,197]
B4GALT1	β-1,4-galactosyltransferase I	An enzyme involved in glycoconjugate biosynthesis	[141]
BRCA1	Breast cancer 1	DNA repair and recombination, transcriptional regulation	[42]
CCND2	Cyclin D2	Cell cycle regulation	[198]
CDH1	E-cadherin	Cell adhesion	[199]
CDH13	H-cadherin	Cell adhension	[200]
CDK10	Cyclin-dependent kinase 10	Regulates cell cycle progression and signaling transduction	[122]
DAPK1	Death-associated protein kinase 1	Involved in induction of apoptosis	[201]
ER	Estrogen receptor α and β	Involved in transduction of estrogen signaling	[3,119,120]
FHIT	Fragile histidine triad gene	Modulates cell proliferation and apoptosis, tumor suppression	[202]
GPC3	Glypican 3	Involved in control of cell division and growth regulation	[203]
GSTP1	Glutathione-*S*-tranferase P1	Carcinogen detoxification	[204]
HIC-1	Hypermethylated in cancer 1	Transcriptional regulation	[205]
HIN1	Cytokine high in normal-1; secretoglobin	Suppresses cell growth	[206]
KIF1A	Kinesin family member 1A	An anterograde motor protein that transports membranous organelles along axonal microtubules	[141]
MGMT	O-6-methylguanine-DNA methyltransferase	DNA repair of O6-alkyl-guanine	[48]
NES1	Kallikrein-related peptidase 10	Tumor suppression	[207]
NISCH	Nischarin; imidazoline receptor candidate	Candidate for the I1-imidazoline receptor	[141]
Nm23-H1	Metastasis inhibition factor NM23	Suppressor of metastasis	[208]
NOEY2	Ras homolog gene family member I	Suppresses clonogenic growth and regulates cell cycle	[209]
NORE1	Ras association (RalGDS/AF-6) domain family member 5	Regulation of lymphocyte adhesion and suppression of cell growth in response to activated Rap1 or Ras	[210]

Gene	Description	Function	Ref.
OGDHL	Oxoglutarate dehydrogenase-like	Conversion of 2-oxoglutarate (α-ketoglutarate) to succinyl-Coenzyme A and CO_2 during the Krebs cycle	[141]
p16INK4a	Cyclin-dependent kinase inhibitor 2A	Cell cycle regulation, involved in senescence	[3]
PAK3	p21 protein (Cdc42/Rac)-activated kinase 3	Cytoskeleton reorganization and nuclear signaling	[141]
PR	Progesterone receptor	Growth regulation	[211]
*RAR*β	Retinoic acid receptor β	Regulates apoptosis, proliferation and differentiation	[157,158]
RASSF1A	Ras association domain family protein 1	Functions as tumor suppressor and inhibits tumor formation	[212]
RIZ1	Retinoblastoma protein-binding zinc finger protein	Transcriptional regulation	[213]
RUNX3	Runt-related transcription factor 3	Transcriptional regulation	[214]
SERPINB5	Serpin peptidase inhibitor; Maspin	Inhibitor of angiogenesis	[215]
SFRP1	Secreted frizzled-related protein 1	Inhibitor of Wnt signaling	[49]
SOCS1	Suppressor of cytokine signaling 1	Inhibitor of JAK-STAT signaling pathway	[3]
SRBC	Sdr-related gene product that binds to c-kinase	BRCA1-binding protein	[216]
SYK	Spleen tyrosine kinase	Inhibits tumor growth and metastasis	[217]
TGFβ*RII*	Transforming growth factor β receptor II	Cell cycle regulation	[3]
TIMP3	Tissue inhibitor of metalloproteinase 3	Suppresses tumor growth, angiogenesis and metastasis	[218]
TMS1	Target of methylation-induced silencing-1	Involved in apoptosis	[219]
TWIST	TWIST homolog of Drosophila	Involved in p53-mediated cell death	[147]
WIF1	WNT inhibitory factor 1	Inhibitor of Wnt signaling	[50]

Table 2

Classification and characteristics of histone deacetylases.

Class type	Class I	Class IIa	Class IIb	Class III	Class IV
Human HDAC	HDAC-1, -2, -3, -8	HDAC-4, -5, -7, -9	HDAC-6, -10	SIRT1−7	HDAC11
Yeast homolog	RPD3	HDA1	HDA1	SIR2	RPD3
Subcellular localization	Nucleus	Nucleus/cytoplasm	Mostly cytoplasm	Nucleus	Nucleus/cytoplasm
Tissue distribution	Ubiquitous	Brain, heart, skeletal muscle	Liver, Kidney, spleen, pancreas	Unknown	Brain, heart, skeletal muscle, kidney
Substrates (partial list)	Histones, p53, E2F1, STAT1, STAT3, NF-κB, GATA1, YY1, MyoD, SHP, RelA, MEF2D, AR, GR	Histones, GATA1, HP-1, SMAD7, PLAG1, PLAG2, GCMa	Histones, tubulin, HSP90, SHP, SMAD7	Histones, tubulin, p53	Unknown
Associated protein complexes (partial list)	NuRD, SIN3, CoREST, NCoR, SMRT	NCoR, SMRT		PcG (PRC4)	
Binding partners (partial list)	RB, BRCA1, DNMT1, DNMT3a, DNMT3b, MBD2−3, MeCP2, ATM	HIF-1α, BCL-6, actinin, AR, ER, MEF2	RUNX2		
Cofactor	Zn	Zn	Zn	NAD^+	Zn

AR: Androgen receptor; ATM: Ataxia telangiectasia mutated; BCL-6: B-cell lymphoma 6; BRCA1: Breast cancer 1, early onset; CoREST: Corepressor for element-1-silencing transcription factor; DNMT1: DNA methyltransferase 1; ER: Estrogen receptor; GATA1: GATA binding protein 1; GCMa: Glial cells missing homolog α; GR: Glucocorticoid receptor; HDAC1: Histone deacetylase 1; HIF-1a: Hypoxia-inducible factor-1α; HP-1: Heterochromatin protein 1; HSP90: Heat shock protein 90; MBD2−3: Methyl-CpG binding domain 2−3; MeCP2: Methyl-CpG binding protein 2; MEF2: Myelin expression factor 2; MEF2D: Myocyte enhancer factor 2D; MyoD: Myogenic differentiation 1; NCoR: Nuclear receptor corepressor; NuRD: Nucleosome remodelling and histone deacetylase; PcG: Polycomb group; PLAG1−2: Pleiomorphic adenoma gene 1−2; RB: Retinoblastoma; RelA: v-rel reticuloendotheliosis viral oncogene homolog A; RPD3: Reduced potassium deficiency 3; RUNX2: Runt-related transcription factor 2; SHP: Small heterodimer partner; SIN3: SWI-independent 3; SIR2: Silencing information regulator 2; SMAD7: Mothers against decapentaplegic homolog 7; SMRT: Silencing mediator for retinoid and thyroid hormone receptors; STAT-1 and -3: Signal transducers and activators of transcription-1 and -3; YY1: YIN-YANG-1 transcription factor.

Table 3

Technologies for assessing and profiling DNA methylation and histone modifications.

Technology	Description	Ref.
Gene-specific detection of DNA methylation		
Bisulfite sequencing	Bisulfite-converted DNA is PCR-amplified to enrich the target templates. The purified DNA templates are subjected to sequencing analysis directly or after cloning into plasmid.	[125]
Methylation-specific PCR (MSP)	This technique takes advantage of the altered sequence of bisulfite-converted unmethyated and methylated DNA for designing primers, which can amplify DNA in a methylation-state-specific manner.	[126]
Combined bisulfite restriction analysis (COBRA)	The qualitative and quantitative detection of methylated alleles is achieved by restriction enzyme-mediated digestion of PCR amplified target amplicons from bisulfite-modified DNA.	[127]
MethyLight™	By including the fluorescent probe technology (TaqMan®), this method is able to quantitatively and sensitively detect methylated alleles.	[128]
Quantitative multiplex MSP (QM-MSP)	This technology is a modified version of fluorogenic probe-based quantitative MSP assay. This method includes the multiplex PCR step that allows amplification of multiple target alleles. The diluted multiple PCR products are subjected to quantitative MSP assay for multiple gene detection.	[129,130]
Pyrosequencing	This method is based on sequencing-by-synthesis technology to quantitative detect methylation levels of individual CpG site by monitoring the real-time incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a bioluminometric signal.	[131]
Genome-wide profiling of DNA methylation		
Restriction landmark genomic scanning (RLGS)	In the RLGS technique, restriction-enzyme digestion, radioactive labeling and two-dimensional electrophoresis combine to quantitatively display DNA methylation levels of thousands of CpG islands.	[132]
Ampliferation of intermethylated sites (AIMS)	The AIMS method combines methylation-sensitive restriction enzyme digestion with the display of methylation fingerprint of PCR amplified DNA fragments, which can then be isolated and characterized individually by sequencing.	[134]
Differential methylation hydrization (DMH)	DMH is a promoter-sequence-microarray-basis method that combines methylation-sensitive restriction-enzyme digestion with linker-basis PCR labeling to serve as probes for array hybridization, capable of globally displaying methylated CpG islands.	[135]
Hpall tiny fragment enrichment by ligation-mediated PCR assay (HELP)	The HELP method adopts differential digestion with a methylation-sensitive restriction enzyme or its methylation-insensitive isoschizomer and ligation-mediated PCR amplification of digested templates for cohybridization to a genomic microarray, enabling the display of genome-wide methylated CpG islands.	[136]
Methylated DNA immunoprecipitation (methy-DIP)	The methyl-DIP technique uses antibodies against methyl-CpG-binding domain proteins (MBDs) to immunoprecipitate sheared genomic DNA for isolation of	[92,137,138]

Technology	Description	Ref.
	methylated DNA fragments. Methyl-DIP has been combined with tiling microarrays or with high-density promoter arrays to map the human methylome.	
Microarray-based gene expression profiling	Gene-expression microarray has been applied to display expression-profile changes in cells treated with epigenetic inhibitors for identification of methylation-targeted genes.	[139-141]
Methylation-specific digital karyotyping (MSDK)	The MSDK method is a modified serial analysis of gene expression (SAGE) assay that combines a methylation-sensitive restriction enzyme and a fragmenting restriction enzyme to generate short sequence tags for providing information on gene loci and their methylation levels.	[142]
Genome-wide profiling of histone modifications		
Chromatin immunoprecipitation (ChIP)-on-chip	This method combines ChIP technology with high-density microarrays for measuring and mapping histone-binding genomic loci.	[143]
ChIP-SAGE	This method adopts a combination of ChIP and SAGE technologies to globally quantify and map genomic binding sites for specifically modified histones.	[144]
ChIP-Seq	This new method employs a high-throughput sequencing technique to analyze ChIP DNA for genome-wide mapping of histone-DNA binding patterns.	[145,146]

Table 4

Histone deacetylase inhibitors.

Class	Compound	HDAC targets (potency)	Effects on cancer cells	Stage of development (company/sponsor)
Hydroxamate	Suberoylanilide hydroxamic acid (SAHA, Vorinostat)	Class I and II (μM)	Differentiation, growth arrest, apoptosis, mitotic failure, senescence, polyploidy	US FDA approval for CTCL (Merck)
	PXD101 (Bellinostat)	Class I and II (μM)	Growth arrest, apoptosis	Phase I, II (CuraGen, TopoTarget)
	LAQ824	Class I and II (nM)	Growth arrest, apoptosis	Phase I (Novartis)
	LBH589 (Panobinostat)	Class I and II (nM)	Growth arrest, apoptosis	Phase I (Novartis)
	PCI-24781	Class I and II (NA)	Apoptosis	Phase I (Pharmacyclics)
	Pyroxamide	Class I and II (μM)	Differentiation, growth arrest, apoptosis	Phase I (NCI)
	ITF2357	Class I and II (nM)	Growth arrest, apoptosis	Phase I (Italfarmaco)
	SK-7041	HDACs 1 and 2 (nM)	Growth arrest, apoptosis	NA
	SK-7068	HDACs 1 and 2 (nM)	Growth arrest, apoptosis	NA
	TSA	Class I and II (nM)	Differentiation, growth arrest, apoptosis	NA
	Tubacin	Class IIb (μM)	Inhibition of HDAC6-mediated cell motility	NA
	CBHA	NA (μM)	Apoptosis	NA (Merck)
	Oxamflatin	NA (μM)	Differentiation, growth arrest	NA
	Scriptaid	NA (μM)	NA	NA
Short-chain fatty acid	Phenylbutyrate	Class I and IIa (mM)	Differentiation, growth arrest, apoptosis	Phase I, II
	Valproic acid (VPA)	Class I and IIa (mM)	Differentiation, growth arrest, apoptosis, senescence	Phase I, II (Abbot)
	AN-9 (prodrug)	NA (μM)	Differentiation, growth arrest, apoptosis	Phase I, II (Titan Pharmaceuticals)
	Baceca	Class I (NA)	NA	Phase I, II (Topotarget)
	Savicol	NA (NA)	NA	Phase I, II (Topotarget)
Cyclic peptide	Depsipeptide (Romidepsin, FK228)	Class I (nM)	Differentiation, growth arrest, apoptosis, mitotic failure	Phase IIb for CTCL and PTCL (Gloucester Pharmaceuticals)
	Trapoxin A	Class I and IIa (nM)	Differentiation, growth arrest	NA
	Apicidin	HDACs 1 and 3, not HDAC8 (nM)	Differentiation, growth arrest	NA
	CHAPs	Class I (nM)	NA	NA
Benzamide	MS-275	Class I (μM)	Differentiation, growth arrest, apoptosis	Phase I, II (Schering AG)
	CI-994 (Tacedinaline)	NA (μM)	Growth arrest, apoptosis	Phase I, II, III (Warner-Lambert)
Anilide	MGCD0103	Class I (NA)	Differentiation, growth arrest, apoptosis	Phase I, II (Methylgene)

CBHA: m-carboxycinnamic acid bis-hydroxamide; CHAPs: cyclic-hydroxamic-acid-containing peptides; CTCL: Cutaneous T-cell lymphoma; HDAC: Histone deacetylase; NA: Not available; NCI: National Cancer Institute; PTCL: Peripheral T-cell lymphoma; TSA: Trichostatin A.

Table 5

Genes whose mRNA or protein expression is altered by treatment with histone deacetylase inhibitors.

Class	Gene list
Histone deacetylase inhibitor-upregulated gene expression	
Cell cycle inhibitory gene	*p21WAF1, p16INK4A, p27KIP1, GADD45*
Tumor suppressor gene	*p53, VHL, p107, gelsolin, IGFBP3*
Proapoptotic gene	*CD95, TRAIL, DR4, DR5, Bax, Bak, Bim, Bmf, Apaf1*
Differentiation gene	*RARβ2, TGFβ1*
Antimetastatic gene	*TIMP1, TIMP2, RECK*
Immune response gene	*MHC-I, MHC-II, CD40, CD80, CD86, ICAM1*
Histone deacetylase inhibitor-downregulated gene expression	
Cell cycle regulatory gene	Cyclin D1, Cyclin A, thymidylate synthetase, CTP synthase
Antiapoptotic gene	*Bcl-2, Bcl-XL, Bcl-w, MCL1, XIAP*
DNA repair gene	*Ku70, Ku86, DNA-PKcs*
Hormone receptor gene	*EGFR, ERα, HER2/neu*
Metastatic gene	*MMP2, MMP9*
Signal transduction gene	*Akt, Flt-3, Raf-1, Abl*
Angiogenic factor gene	*HIF1α, VEGF, IL2, IL10, bEGF, TIE2, eNOS, CXCR4*