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EPIGENETIC TARGETING IN BREAST CANCER: THERAPEUTIC IMPACT AND FUTURE DIRECTION

M.B. Lustberg and **B. Ramaswamy***
Division of Hematology and Oncology, Comprehensive Cancer Center, The Ohio State University Medical Center, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, Columbus, OH, USA.

SUMMARY

Breast carcinogenesis is a multistep process involving both genetic and epigenetic changes. Epigenetics is defined as a reversible and heritable change in gene expression that is not accompanied by alteration in gene sequence. DNA methylation and histone modifications are the two major epigenetic changes that influence gene expression in cancer. The interaction between methylation and histone modification is intricately orchestrated by the formation of repressor complexes. Several genes involved in proliferation, antiapoptosis, invasion and metastasis have been shown to be methylated in various malignant and premalignant breast neoplasms. The histone deacetylase inhibitors (HDi) have emerged as an important class of drugs to be used synergistically with other systemic therapies in the treatment of breast cancer. Since epigenetic changes are potentially reversible processes, much effort has been directed toward understanding this mechanism with the goal of finding novel therapies as well as more refined diagnostic and prognostic tools in breast cancer.

PRINCIPLES OF EPIGENETICS

Introduction

Growing evidence in the last several decades has documented the pivotal role of epigenetics in the development and progression of various malignancies including breast cancer. It is now widely accepted that genetic changes alone do not fully account for the initiation and propagation of malignancy. Carcinogenesis is a multistep process involving genetic and epigenetic changes. Epigenetics was classically defined as the heritable changes in gene expression by mechanisms that do not cause changes in DNA sequences.[1,2] A new definition proposed by Adrian Bird questions whether heritability should be mandatory in a contemporary view of epigenetics.[3] He argues that some histone modifications are transient and under current definition would not be considered an epigenetic change and hence puts forth an alternative unifying definition of epigenetics as a structural adaptation of chromosomal regions, so as to register, signal or perpetuate altered activity states. Such epigenetic changes would include modification of the DNA, noncoding RNA and proteins such as histones.[2] Since epigenetic changes are potentially reversible processes, much effort

*Correspondence B. Ramaswamy, Division of Hematology and Oncology, Comprehensive Cancer Center, The Ohio State University Medical Center, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, Columbus, OH, USA. bhuvaneswari.ramaswamy@osumc.edu.

Since epigenetic changes are potentially reversible processes, efforts are directed toward understanding this mechanism in order to find novel therapies, as well as diagnostic and prognostic tools in breast cancer.

DISCLOSURE
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has been directed toward understanding this epigenetic mechanism with the goal of finding novel therapies that can relieve transcriptional repression of key genes and improve outcomes. Furthermore, epigenetic alterations may serve as bio-markers for diagnosis, and for predicting prognosis and response to therapies.

DNA methylation and DNA methyltransferases

The role of DNA methylation in epigenetic regulation of human breast cancer has been well characterized. CpG dinucleotide-rich regions located in the 5′-end region of the genes are called CpG islands. Methylation of CpG islands, predominantly present in promoter regions, is the most commonly studied epigenetic change in human cancer.[4] Most CpG islands are unmethylated in noncancerous cells, which promotes active gene transcription. In cancer cells, CpG islands become hypermethylated, leading to inactivation of tumor suppressor genes.[2] Poorly differentiated breast tumors have more methylated CpG islands than moderately or well-differentiated breast tumors.[5]

The methylation of CpG islands is mediated by the action of DNA methyltransferases (Dnmt). There are three active DNA methyltransferases: Dnmt1, Dnmt3a and Dnmt3b. Dnmt1 is the most common and active DNA methyltransferase and functions as a "maintenance" Dnmt by replicating the methylation pattern from the parental to daughter strand during cell division. Dnmt3a and 3b are "de novo methyltransferases" and are upregulated in aging cells.[6] Although Dnmt1 seems to be the key regulator maintaining abnormal promoter methylation in cancer, recent studies suggest that the cooperation between Dnmt1 and Dnmt3b is vital to this process and the removal of both is needed to disrupt methylation.[7] Data also suggest that Dnmt1 levels may be upregulated in poorly differentiated tumors irrespective of the methylation status of the genes studied and were indeed shown to be an independent prognostic factor in non-small cell lung cancer.[8] In addition, Nass et al. showed that high Dnmt levels in estrogen receptor (ER)-positive breast cancer cell lines were associated with increased S-phase and estrogen-independent growth, without methylation-mediated silencing of ERα, suggesting that high Dnmt levels may be a marker for proliferation.[9,10] The exact role of a fourth Dnmt member, Dnmt2, has yet to be determined but it may be involved in RNA methylation.[11]

There is an overall increase in Dnmt activity in malignant cells[12] and, in particular, both Dnmt1 and 3b show increased expression in breast cancer.[6] Dnmts contribute to gene silencing through routes other than promoter methylation. This includes direct interaction with and recruitment of histone deacetylases (HDs) and the methyl-CpG-binding domain (MBD) family of proteins to the promoter regions to form a repressive transcription complex. Dnmt1 through its N-terminus binds to HD2 and a Dnmt1-associated protein called DNMAP1 to form a complex at replication foci.[13] This complex is important in the conversion of acetylated histones to the deacetylated inactive form and highlights the interplay between methylation and acetylation processes in epigenetic regulation.

Methyl-CpG-binding domain family of proteins

Along with the Dnmts, the MBD family of proteins plays an important role in epigenetic silencing of tumor suppressor genes.[14] The MBD family of proteins has five members: MeCP2 and MBD1, MBD2, MBD3 and MBD4. They all share a methyl-binding domain that allows them to bind to hypermethylated promoters of CpG islands of tumor suppressor genes and mediate the interaction between histone modification and methylation. Ballestar et al. showed that MBDs were present in hypermethylated promoters in breast cancer cell lines MCF7 and MDA-MB-231. The presence of MBDs was associated with histone deacetylation and lysine 9 histone H3 methylation. MBD2 binds to the largest number of CpG islands in breast cancer cells.[15] MBD2 also mediates glutathione S-transferase P

(GSTP1) silencing associated with hypermethylated CpG islands in MCF7 cells.[16] There is ongoing research regarding the role of MBDs in the formation of various transcription repression complexes.[17–19] The extent of the involvement of MBDs in breast carcinogenesis is still under investigation. Higher expression of MeCP2 RNA has been found in neoplastic breast tissue compared to normal tissue and higher MeCP2 expression was also shown in ER-positive tumors.[20]

Histone modification and repressor complexes

Epigenetic regulation of gene transcription relies on the cooperative mechanisms of DNA methylation and histone acetylation. Two universal markers of histone modification in malignancy are loss of acetylation of lysine 16 and trimethylation of lysine 20 in histone H4.[21] Histone modification and overall chromatin structure are governed by balanced counteractivity of histone acetyltransferases (HAT) and HDs. Acetylation places an acetyl group on the NH_2-terminal lysine residue on histones leading to neutralization of positive charges resulting in a looser, less compact, transcriptionally active chromatin. In contrast, deacetylated histones have a condensed, inactive chromatin structure. There is a dynamic relationship between acetylation of histones and methylation patterns; methylated inactive genes are associated with underacetylated histones, whereas unmethylated active genes are associated with acetylated histones and a more open chromatin.[22]

The mechanistic interaction between methylation and acetylation is intricately orchestrated by the formation of repressor complexes. Dnmt1 forms a repressive transcription complex with HD2 and DNMAP1. Dnmt1 can also form a complex with retinoblastoma-associated protein (Rb), transcription factor E2F1 and HD1 and repress transcription from E2F–responsive promoters.[13,23] There are three class I HD-containing co-repressor complexes. These are HD1/HD2-containing Sin3, Mi2/NuRD and HD3/N-CoR complexes.[24–26] The NuRD complex contains metastasis-associated proteins (MTAs) and the N-CoR complex interacts with the silencing mediator of thyroid hormone and retinoic acid receptor (SMRT)-modulating ER activity.[27,28] The tamoxifen-bound ER interacts with N-CoR complexes leading to chromatin condensation and gene silencing.[29]

Post-translational modification of histones resulting in gene silencing is not restricted to acetylation alone but also involves methylation of the histone residues. Two major histone methylation patterns have been identified: methylation of lysine residue 9 of the core histone protein H3 results in gene silencing, whereas methylation of lysine 4, and recently lysine 27, of H3 are signs of activated genes.[30,31] In breast cancer, methylation of lysine residue 9 is evident in the regulation of the MUC1 promoter[32] as well as in the transforming growth factor β signaling pathway genes.[33] Recently, the loss of trimethylation of lysine 27 of H3 (H3K27) has been shown to be a negative prognostic indicator in several cancers including breast cancer.[31]

Epigenetic modification of microRNAs in breast cancer

Epigenetic changes are not limited to CpG islands and histones but also encompass the regulation of microRNAs (miRNAs). miRNAs are small noncoding RNAs involved in the regulation of gene expression and can be either oncogenes or tumor suppressors in breast cancer.[34,35] Hypermethylation has been described as one of the ways by which the miRNA regulatory mechanism is disrupted in malignant cells. MIR126 was the first miRNA that was shown to be deregulated in breast cancer resulting in increased proliferation of MCF7 breast cancer cell lines.[36,37] Subsequent studies have shown aberrant hypermethylation of miRNA classes of MIR9-1, MIR124-3, MIR148, MIR152 and MIR663 in 71 primary breast tumor specimens.[38] Treatment with 5-azacytidine (5-aza, a DNA-demethylating agent), resulted in increased expression of MIR9-1 with corresponding gene demethylation.[38]

Hypermethylation of MIR9-1 was seen in preinvasive intraductal lesions in addition to the primary tumors, suggesting that hypermethylation may be an early step in breast carcinogenesis. MIR335 and MIR126 have recently been identified as metastasis suppressor miRNAs in human breast cancer and the loss of their expression is associated with poor metastasis-free survival.[39] Furthermore, recent studies have examined the role of miRNAs in the hormonal regulation of breast cancer which will be paramount for improving the current understanding of endocrine resistance in breast cancer.[40,41] Whether epigenetic mechanisms may be involved in the suppression or overexpression of these miRNAs in breast cancer will need to be further investigated.

Global genomic and promoter hypomethylation

Although hypermethylation has been presented thus far as the predominant mode of epigenetic modification, it is important to point out that global genomic hypomethylation has also been noted outside of promoter regions in the genome of cancer cells. Genomes of MCF7 breast cancer cell lines have mega-base pair regions of hypomethylated zones.[42] The impact of hypomethylation on carcinogenesis has been attributed to chromosomal instability, reactivation of transposable elements and loss of imprinting.[43] Furthermore, although a certain pattern of hypermethylated promoters is generally observed in most tumors including breast cancer, some genes are actually activated by hypomethylation. One such example of hypomethylation includes the γ synuclein gene CpG island (SNCG gene). This oncogene's promoter is activated by hypomethylation and overexpression of SNCG promotes breast cancer proliferation and metastasis.[44] The intricate epigenetic regulatory mechanisms in breast cancer are only partially understood to date and our current understanding will be summarized in the next section.

KNOWN EPIGENETIC CHANGES IN BREAST CANCER

Epigenetic targets

DNA methylation with corresponding silencing of several key genes involved in breast carcinogenesis has been reported. Investigators have analyzed breast cancer methylation patterns in various ways. Some have reported methylation patterns of a panel of genes associated with certain biological features of the tumor such as HER2/NEU overexpression[45] or hormone receptor status.[46] Yet others have tried to associate methylation of certain specific genes such as ESR1, BRCA1, RARB, CCND2, etc., with clinical outcomes. Hypermethylation has also been used as a diagnostic tool to distinguish between normal and malignant tissue and more recently epigenotyping of the peripheral blood cell DNA has been evaluated as a measure of breast cancer risk.[47–49] To discuss all of the reported alterations of genes in breast cancer would be too exhaustive for this review but a summary of the currently characterized breast cancer epigenetic changes is presented in Table 1. The epigenetic targets affect all six capabilities that malignant cells acquire as described by Hanahan: 1) limitless replicative potential; 2) self-sufficiency in growth signals; 3) insensitivity to growth-inhibitory signal; 4) evasion of programmed cell death; 5) sustained angiogenesis; and 6) tissue invasion and metastasis.[50–51] Furthermore, cancer cells and stem cells share these characteristics of self-renewal, migration and division suggesting that all types of cancer originate in the organ- or tissue-specific stem cells present in a particular organ. Another hallmark is genetic instability in relation to aneuploidy, and telomere erosion may occur in tumor stem cells.[52,53] Such genetic instability may be associated with epigenetic modifications.[54] Recently, renewed efforts have delineated the role of a shift in glucose metabolism from oxidative phosphorylation to glycolysis as one of the biochemical hallmarks of tumor cells. Mitochondrial DNA (mtDNA) instability may play a key role in this metabolic shift. In the past decade, point mutations and deletions of the mtDNA has been reported, but thus far no definitive epigenetic modifications of mtDNA

in humans have been found.[55] We will focus our review on the epigenetic changes of some of the key genes involved in breast cancer and their clinical implications.

Early epigenetic events in breast cancer

Local hypermethylation of key promoter regions, leading to silencing of tumor suppressors, is one of the key processes regulating early disease. Aberrant methylation has been identified in over 100 CpG islands in ductal carcinoma in situ (DCIS) and stage I breast tumors.[56] High levels of certain hypermethylated genes can be detected in ductal lavage and nipple aspirates of patients with DCIS, mammary epithelial hyperplasia and intraductal papillomas.[57,58] Increased methylation of the CpG islands of the tumor suppressor $p16^{INK4a}$ and a corresponding increase in cyclin-D1 were seen in intraductal proliferative lesions of the breast, with highest levels in DCIS.[59] Other promoter hypermethylation patterns have been described including increased hypermethylation of the promoter of the tumor suppressor RUNX3 in DCIS, as well as invasive ductal carcinomas, compared to normal breast tissues.[60] A higher frequency of RUNX3 hypermethylation is seen with estrogen and progesterone expression.[60,61] The significance of this association is not currently well understood. NES1 is another tumor suppressor that is hypermethylated in DCIS and may serve as a predictive marker for invasive carcinoma at the time of definitive surgery.[62–64]

The tumor microenvironment may also undergo epigenetic changes that regulate the transition of in situ to invasive breast cancer. Myoepithelial cells and stromal fibroblasts undergo epigenetic modifications in early breast cancer carcinogenesis.[65,66] Increasing evidence suggests that signals from the stromal microenvironment, including the breast cancer-associated fibroblasts, may serve as triggers for the initiation of epigenetic modifications which promote early disease progression.[67–69] Distinct epigenetic changes are present in the stromal cells of breast cancers.[66] Promoter hypermethylation of the cystatin-M (CST6) tumor suppressor due to aberrant AKT activation is one of the epigenetic changes that occur when breast epithelial cells are co-cultured with cancer fibroblasts.[69] Additional studies are ongoing to investigate other triggers of epigenetic silencing in the tumor microenvironment.

Epigenetic regulation of estrogen receptor

The mechanisms surrounding the epigenetic regulation of the ER have been a focus of considerable research in breast cancer. Approximately one-third of primary breast cancers are ER-negative and genetic changes in DNA sequence alone cannot explain loss of ER expression in these breast cancers.[70] ER expression is controlled by transcription in ER-negative cells and not by other mechanisms such as degradation or abnormal nuclear transport.[71] Response to hormonal therapy is largely determined by ER expression. Many ER-positive tumors lose ER expression as breast cancer progresses. Therefore, understanding the mechanisms governing the regulation of the ER is of crucial importance both for prognosis and treatment decisions. There are two distinct ER receptors, α and β. Most research to date has centered on ERα and our understanding of the role of ERβ in the pathogenesis of breast cancer is evolving.

The epigenetic regulation of ERα is mediated though the recruitment of multimolecular complexes containing HD1 and Dnmt1 that are recruited to the promoter region, as well as other members including N-CoR and SMRT.[28,72,73] Higher expression levels of DNMT1 exist in ER-negative cells.[9] Treatment of ER-negative human breast cancer cells with the methyltransferase inhibitor 5-aza led to partial demethylation of the ER CpG island, re-expression of ER messenger RNA and synthesis of functional ER protein.[74,75] Inhibition of DNMT1 by antisense, oligo or short interfering RNA (siRNA) may increase estrogen responsiveness in ER-negative breast cancer cells and increase responsiveness to 5-aza.[75,77]

Studies determining the levels of Dnmt in estrogen-dependent ER-positive cell lines, estrogen-independent ER-positive cell lines and ER-negative cell lines show that the Dnmt levels correlated tightly with the S-phase of ER-positive cell lines, while ER-negative cells expressed DNMT throughout the cell cycle.[9] This study also showed that the estrogen-independent phenotype of MCF7 cells, even in conjunction with increased Dnmt levels, was not accompanied by aberrant methylation of the ER gene, suggesting that multiple steps are required for de novo methylation of the ER gene. Subsequent studies have shown that the interaction of deacetylated histones with the methylated ER gene promoter is a prerequisite for its inactivation.[75,78] The role of the pRb/p130 complex with HD1 and Dnmt1 in the regulation of ERα transcription has been shown.[72]

Preclinical research on whether ER-negative breast cancer cells can be sensitized to respond to estrogen or to selective estrogen receptor modulators (SERMs) has been investigated by many different groups. The HD inhibitor trichostatin sensitizes ER-negative breast cancer cells to tamoxifen treatment[78,79] and may upregulate ERβ activity leading to sensitization of ERα-negative breast cancer cells to tamoxifen.[80] Transcriptional activation of ERα by trichostatin was achieved without alteration in CpG island methylation, suggesting that histone deacetylation was a key component of ER gene silencing in human breast cancer.[75] After sensitizing ER-negative cell lines to tamoxifen via 5-aza and trichostatin, ER-responsive genes are repressed by the tamoxifen-bound, reactivated ER. This suggests that the reactivated ER is indeed functional. The association of ERα with HD1 resulted in suppression of its transcriptional activity. Thus, HD1 may also be a therapeutic target for some ER-negative breast cancers to induce sensitivity to endocrine therapy.[75,81] Selective inhibition of HD2 by siRNA downregulated both ER and progesterone receptor (PR) expression and potentiated the effects of tamoxifen in ER-positive breast tumor cells.[82] Collectively, these preclinical data give proof of principle that the inhibition of histone acetylation may induce sensitivity to SERMs and has led to the clinical testing of these strategies by combining tamoxifen and HD inhibitors.

A recent study by Subramanian et al. has shown that ERα is regulated by SETD7 histone-lysine *N*-methyltransferase in human breast cancer cells.[83] SETD7 is a histone methyltransferase that is known to monomethylate H3K4 resulting in transcriptional activation. It was demonstrated to induce K302 methylation, resulting in stabilization of the ERα protein, which in turn resulting in increased sensitivity to estrogens, enhancing the transcription of estrogen response elements.[83] This reinforces other studies suggesting that post-translational modification of ERα may play a role in hormone response.

Loss of ER signaling also leads to epigenetic silencing of downstream ERα targets.[84] A study by Leu et al. showed that abrogation of ER signaling resulted in recruitment of polycomb repressors and HDs to the promoters of the target genes and was then followed by DNA methylation of the promoters.[84] This study demonstrated that epigenetic silencing of ER target genes is crucial to the development of ERα-independent growth.

Epigenetic regulation of progesterone receptor

The regulatory mechanisms of ERα transcription may impact the regulation of the PR. The majority of breast tumors that express the ER also express the PR. A CpG island is located in the first exon of the PR gene and is hypermethylated in 40% of tumors that are PR expression-negative.[85] Demethylation of the progesterone CpG island is not required for PR expression.[74] However, demethylation of the CpG island is required for ERα binding to this regulatory sequence.[86] DNA demethylation is also necessary for re-expression of ERα target genes. In ER-negative tumors, silencing of the ERα gene also affects downstream estrogen-regulated gene expression including PR expression.[86] Epigenetic regulation of PR

may also be controlled in part by the interaction with retinoic acid receptors, which are discussed in the next section.[87]

Epigenetic regulation of the retinoid receptor

Retinoic acid resistance in breast cancer has been documented in association with down-regulation of retinoic acid receptor β (RARβ) expression.[87–89] Retinoic acid-induced apoptosis and growth inhibition has been characterized in human breast cancer cells and the loss of this tumor suppressor gene, RARβ, in breast cancers is well established.[89] Estrogen can reverse RARβ downregulation in ER-positive breast cancer cell lines but not in ER-negative cell lines.[90] RARB expression is controlled by two promoters in the regulatory region. The transcription of the RARB2 promoter is a multistep process which involves the recruitment of RARα and RARβ and formation of a transcription complex with HATs and HDs.[91]

Epigenetic modifications play a role in the transcription of the RARB2 promoter. Hypermethylation of the promoter has been shown in both breast cancer cell lines and many primary breast tumors.[92] Treatment with the Dnmt inhibitor 5-aza or the HD inhibitor trichostatin led to re-expression of RARB[92] and reactivation of the epigenetically silenced receptor by retinoic acid.[93] Additional studies with the combination of HD inhibitors and retinoic acid metabolism-blocking agents (RAMBA) are under way.[94] VN/14-1 is one of the novel RAMBA that has shown promising activity in vitro in letrozole-insensitive breast cancer cells.[95] Additional understanding of the epigenetic regulation of the retinoic receptor may elucidate additional therapeutic targets for overcoming resistance.

Epigenetic regulation of BRCA genes

There is evidence for epigenetic regulation of BRCA1 and BRCA2 genes in both sporadic and hereditary breast cancers. Promoter hypermethylation of BRCA1 and resulting gene inactivation occurs in a minority of sporadic cancers but is more common with loss of heterozygosity and in certain tumor subtypes such as medullary and mucinous carcinomas.[96,97] BRCA2 promoter methylation has been shown in 4% of sporadic ovarian cancers.[98] Conflicting data are reported regarding promoter methylation of BRCA2 in breast cancer.[99] The more predominant mode of epigenetic regulation of BRCA2 is via formation of repressor complexes and chromatin remodeling.[84] The Tudor domain ‘Royal Family’, epigenetic repressor family of chromatin remodeling factors has been shown to interact with BRCA2 in sporadic cancers.[85]

Hereditary breast and ovarian cancers have variable penetrance even in family members with identical mutations.[100–102] Modifications of risk by single nucleotide polymorphisms[103], as well as by epigenetic alterations, including hypermethylation of the promoter CpG island, have been reported.[104] In a recent study, BRCA1 promoter hypermethylation was present at a low frequency of 5% in BRCA1 and BRCA2 carriers with and without breast cancer as well in healthy controls in an Israeli Jewish study population.[104] The results of this study, if confirmed by others, would suggest that BRCA promoter methylation is not a definitive risk modifier in hereditary breast cancers and a more complex epigenetic cascade may yet need to be elucidated. Epigenetic and genomic changes that impact the X chromosome stability in BRCA1 and basal-like tumors have been investigated.[105–109] The role of X inactive-specific transcript RNA coating, or XIST RNA, in BRCA1 tumors and its contribution to epigenetic instability have been debated. Earlier work on small numbers of BRCA1 tumors showed decreased XIST in these tumors,[105–107] although a more recent study showed that XIST RNA coating of the unaffected X chromosome is not affected by the BRCA1 mutation.[109] Additional studies are needed to

further clarify the epigenetic regulation and sequelae of BRCA mutations in hereditary cancer.

Epigenetic silencing in (Wnt) pathway

The wingless and integration site growth factor (Wnt) pathway is one of the deregulated pathways in breast cancer.[110] Hypermethylation is the predominant mechanism of Wnt pathway aberrancy in breast cancer. Epigenetic silencing of Wnt antagonist genes including SFRP has been shown in breast cancer with a corresponding loss of function resulting in activation of Wnt signaling in breast carcinogenesis[111], and is associated with an unfavorable prognosis.[112] Larger studies are needed to confirm if activation of this pathway could be used as a prognostic marker and perhaps could serve as a target for epigenetic therapies.

PRECLINICAL STUDIES WITH HD INHIBITORS AND DEMETHYLATING AGENTS IN BREAST CANCER

Mechanism of action of HD inhibitors

HD inhibitors (HDi) have been shown to have antiproliferative and proapoptotic effects in many different cell lines. There are four main groups of HDi which are classified based on their structure. These are hydroxamic acids, cyclic peptides, aliphatic acids and benzamides. The most well-studied HDi include trichostatin and suberoylanilide hydroxamic acid (SAHA, vorinostat), both of which belong to the hydroxamate group.[4] For a more complete review of HDi, please see a recent review by Marchion and Munster.[113]

HDi promote hyperacetylation of histones which leads to the transcriptional upregulation of proapoptotic genes and cyclin-dependent kinase inhibitors such as p21 and p27.[114] The net result is cell cycle growth arrest and apoptosis of cancer cells along with in vivo regression of tumors.[115] In addition, HDi increase acetylation of the chaperone complex of heat shock protein 90 (Hsp90) leading to the destabilization of the complex with corresponding inhibition of chaperone function.[116] In preclinical models, the estrogen receptor is one of the Hsp90 client proteins that is most sensitive to Hsp90 inhibition.[117] Hsp90 chaperone activity is regulated by HD6 and inhibition of HD6 leads to Hsp90 hyperacetylation causing separation from p23 and the corresponding loss of chaperone activity.[118,119] As a result, proteosome targeting and degradation of multiple client proteins including HER2, ER, AKT and cRaf are increased.[117,120] Downstream effects of client protein degradation affect a myriad of pathways including dose-dependent increase of proapoptotic proteins BAK (Bcl-2 homologous antagonist/killer) and Bcl-2-like protein 11 and attenuation of antiapoptic proteins Bcl-2 and Bcl-x_L.[119] HDi also target angiogenesis pathways.[121,122]

Preclinical studies of HDi, SAHA and LAQ-824

By attenuating these downstream signaling pathways, HDi may sensitize breast cancer cells to endocrine and cytototoxic therapy. LAQ-824 (dacinostat) and SAHA are part of the hydroxamic acid family of HDi. These drugs have in vitro antiproliferative activity.[123,124] Munster et al. showed that vorinostat inhibits the proliferation of both ER-positive and -negative human breast cancer cell lines and also promotes differentiation as evidenced by expression of the MFGM protein and milk fat globulin protein.[125] Exposure to these drugs resulted in downregulation of p-AKT, AKT and cRaf, and sensitized ERα-positive breast cancer cells to tamoxifen.[126] The combination of vorinostat with trastuzumab or docetaxel is synergistic in breast cancer cells with attenuated levels of cRaf and AKT[119] Synergistic interaction of vorinostat with either trastuzumab or docetaxel has been shown in human breast cancer cell lines that amplify HER2[119] and prolonged exposure to vorinostat sensitized breast cancer cells to topoisomerase inhibitors.[127] Similarly, LAQ-824 sensitizes

human breast cancer cells to trastuzumab and various chemotherapeutic agents.[124] Based on the promising preclinical data, there are multiple clinical trials in breast cancer investigating the effects of vorinostat in combination with endocrine therapy and cytotoxic agents.

Preclinical studies with valproic acid

Valproic acid, a commonly used antiepileptic drug, is in the aliphatic acid family of HDi and has been shown to target histone deacetylase in a variety of cancer lines.[128,129] The combination of valproic acid with *all-trans* retinoic acid and 5-aza restores expression of RARB2 in breast cancer cells.[130] Interestingly, valproic acid alone, without concurrent 5-aza, did not restore the expression of RARB2. Histone acetylation was increased as a result of this combination in the RARB2 promoter of MCF7 breast cancer cells. This preclinical study was one of the earliest to show that valproic acid may be an effective drug in combination therapy in breast cancer.

Preclinical data with demethylating agent 5-aza in breast cancer

Multiple studies have utilized the methyltransferase inhibitor 5-aza to investigate epigenetic regulation in breast cancer. Treatment of ER-negative breast cancer cell lines with 5-aza causes partial demethylation of the ER CpG island and leads to expression of functional ER.[78] The activity of 5-aza is enhanced when coupled with antisense oligonucleotide targeting of DNMT1.[77] Studies have shown that combination therapy of 5-aza with HDi (trichostatin and scriptaid) was more effective than either agent alone in inducing ER expression in ER-negative breast cancer cell lines.[78,131] Additional studies in retinoid-resistant cell lines have shown that 5-aza promotes reexpression of RARβ[92] and reactivation of the epigenetically silenced receptor by retinoic acid.[93] A phase I multicenter trial of 5-aza in patients with advanced solid tumor malignancies including breast cancer who had failed standard therapy was recently completed.[132] Results of the trial are not yet available.

CLINICAL TRIALS

HDi trials

In a phase I trial, vorinostat administered intravenously in patients with solid tumor and hematological malignancies was well tolerated. Post-therapy tumor biopsies showed accumulation of acetylated histones.[133] No partial or complete responses were seen but there was radiographic evidence of tumor regression in two patients with lymphoma and two patients with bladder cancer. A subsequent phase I trial using oral vorinostat was completed showing responses in six patients.[134] Histone acetylation was seen in peripheral blood mononuclear cells and lasted beyond the half-life of vorinostat. Vorinostat was relatively well tolerated with the most common toxicities being nausea, vomiting and diarrhea. Based on these two trials, studies with vorinostat as a single agent and in combination with other chemotherapeutic agents are ongoing.

In breast cancer, several trials are ongoing including a multicenter phase I/II trial with the addition of vorinostat to paclitaxel and bevacizumab in metastatic breast cancer.[135] The results from this trial are very encouraging and will lead to testing of additional combinations with vorinostat in breast cancer. Vorinostat has been shown to downregulate Hsp90 and its client protein HER2 and ERα. Hence the combination with trastuzumab[136] and endocrine therapies is also being tested in the metastatic and neoadjuvant settings.[137] A phase I trial of valproic acid followed by the topoisomerase II inhibitor epirubicin was conducted in advanced heavily pretreated solid tumors. This combination was well tolerated and had antitumor activity.[138] Currently, sponsored by the National Cancer Institute (NCI), a phase II trial of valproic acid with FEC100 (5-fluorouracil, epirubicin and cyclophosphamide) in advanced breast cancer is ongoing.[139]

DIAGNOSTIC AND PROGNOSTIC IMPLICATIONS IN EPIGENETIC ALTERATIONS IN BREAST CANCER

Epigenetic changes in breast cancer have been investigated to improve diagnosis and prognostication. Identification of DNA hypermethylation in breast biopsy samples from BRCA carriers may be useful when there is diagnostic uncertainty.[47] Epigenetic changes have also been investigated as prognostic markers in breast cancer. For example, DNA hypermethylation of PITX2 is a marker of poor prognosis in node-negative hormone receptor-positive breast cancer after adjuvant tamoxifen therapy.[140] The role of epigenetic changes as potential markers for chemotherapy sensitivity has also been investigated.[141] Promoter methylation of NEUROD1, a member of the stem cell polycom group target genes, is a marker for chemosensitivity in the neoadjuvant setting.[142]

Diagnostic and prognostic implications of hypermethylated genes in breast ductal lavage and random periareolar fine needle aspiration (RPFNA) have been investigated. As a diagnostic tool in high-risk asymptomatic women, ductal lavage cytology has low sensitivity and high specificity in cancer detection,[143] although novel biomarker identification with proteomic analysis can improve sensitivity.[144] Identification of epigenetic alterations in ductal fluid may also improve diagnostic yield of ductal lavage.[48] Utilization of quantitative multiplex methylation-specific PCR on a nine-gene panel including RASSF1, TWIST and HIN1 on ductal lavage from women undergoing mastectomy doubled sensitivity compared to cytology.[48] Similarly, epigenetic changes in RPFNA have included RARB2 promoter methylation.[145] Determination of epigenetic changes using these techniques remains investigational and is not routinely used.

Epigenetic changes in serum DNA from breast cancer patients is currently under investigation as a tool for earlier detection of cancer. Paired analysis of serum and tumor DNA for RASSF1 and RARB from patients with invasive ductal carcinoma showed high sensitivity and specify.[146] Most recently, investigators from Europe reported on the first large-scale epigenotyping study of peripheral blood DNA and demonstrated that DNA methylation provides a good prediction of breast cancer risk, which was not completely independent of traditional risk factors for breast cancer.[49] Lack of peripheral blood DNA methylation of the estrogen target gene ZNF217 was significantly associated with ERα bioreactivity in a functional assay, suggesting that methylation was a marker of estrogen exposure.[49] This is a proof-of-principle trial and will lead to further studies that may establish DNA methylation patterns as a link between the environment and the genome.

FUTURE DIRECTION

Despite the exhaustive data regarding the extent of epigenetic changes in breast cancer, many uncertainties remain. These uncertainties revolve around the following key questions: 1) Does the epigenetic change actually initiate the gene silencing leading to tumor initiation or is it a downstream change or a response pattern resulting from a more fundamental problem? (a chicken and egg question); 2) Demethylating agents and HDi are likely to induce expression of many genes while we only focus on the key target genes. Would this lead to reactivation of genes that could potentially promote tumor growth?; and 3) Does the methylation pattern change as the tumor progresses and could the methylation of a specific gene in the primary tumor therefore be an irrelevant target in the metastatic setting? Moreover, in the clinical setting, questions that still need to be resolved include those regarding dose, duration and treatment schedules for these agents that will result in an ideal pharmaco-dynamic endpoint, as we know that the epigenetic changes induced by these agents are only transient and reversible. Furthermore, the biomarkers that can be used to confirm the biological endpoint require adequate tissue sampling which is not readily

available in patients with solid tumors. However, great strides have been made in all these areas as evidenced by the fact that there are currently at least 10 NCI-sponsored clinical trials using HDi in breast cancer including one in noninvasive breast cancer. Considering the plethora of preclinical data of epigenetic changes in breast cancer, the early clinical data using epigenetic modulators in breast cancer and the exciting new era of identification of various target miRNAs, combination strategies of these agents with other targeted therapies should be further evaluated in breast cancer.

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Table I

Epigenetic targets in breast cancer[50]

Gene	Function	Clinical implications	Ref.
CDKN2A (cyclin-dependent kinase inhibitor 2A)	Cell cycle regulation, tumor suppressor	Prognostic	59, 147–149
NOEY2 (ras homolog gene family member 1)	Cell cycle regulation	Prognostic	150
TGFBR2 (transforming growth factor β receptor II)	Cell cycle regulation	ER-positive breast cells insensitive to TGFβRII become sensitized after treatment with 5-azacytidine (5-aza)	151
RARB (retinoic acid receptor β)	Differentiation and regulation of cell growth	Chemoprevention potential of retinoids	92,152 153
BRCA1	DNA repair, cell cycle regulation	Potential target to identify chemosensitivity	96
SRBC (serum deprivation response factor [sdr]-related gene product that binds to c-kinase)	Interacts with BRCA1	Breast carcinogenesis? Prognostic	154
CCND2 (cyclin-D2)	Cell cycle regulator	Promoter hypermethylation seen in DCIS suggesting early event in carcinogenesis	155, 156
SFN (stratifin/14-3-3 protein σ)	Cell cycle regulator	Hypermethylation detected in ductal carcinoma in situ and atypical hyperplasia in addition to invasive carcinomas	157, 158
Wnt Pathway (inhibitory factor1 and promoter)	Cell growth and regulation	Aberrant in cancer stem cells. Potential target	111, 159
ESR1 (estrogen receptor)	Growth signal	Restoration of ER expression with demethylating agents or HDAC inhibitors	78, 85, 160
PGR (progesterone receptor)	Growth signal	Response to endocrine therapy	74, 85
HIN1	Tumor suppressor	Hypermethylation present in situ lesions in addition to invasive lesions	161
NES1	Tumor suppressor	PrognosticLoss in DCIS increases probability of invasive component	62–64
RUNX3	Tumor suppressor	Breast carcinogenesis Early event DCIS? Prognostic	60, 61, 162
RIZ (retinoblastoma protein-interacting zinc finger protein)	Tumor suppressor	5-aza treatment activated RIZ expression; may be a downstream modulator of ER	163, 164
FHIT (fragile histidine triad protein)	Tumor suppressor	Prognostic	165
LATS1 and LATS2	Tumor suppressor	Prognostic	166
CST6	Tumor suppressor	Breast carcinogenesis? Prognostic	69, 167
BRMS1 (breast cancer metastasis-suppressor 1)	Tumor suppressor	Prognostic	168
Human SRBC	Tumor suppressor	Breast carcinogenesis	154
PTPRO	Candidate tumor suppressor	Estrogen responsive gene? Related to tamoxifen resistance	169
PROX1	Candidate tumor suppressor	Prognostic	170
RASSF1 (ras association domain-containing protein 1)	Apoptosis; growth inhibition	Breast carcinogenesis prognosis	171, 172
SYK (spleen tyrosine kinase)	Growth inhibition	Prognostic	173
HOXA5 (homeobox protein Hox-A5)	Apoptosis; p53 upregulation	HOXA5 has a role in PR regulation;	174, 175

Gene	Function	Clinical implications	Ref.
		Dnmt inhibitors can restore expression of HOXA5	
TMS1 (target of methylation-induced silencing 1)	Apoptosis	Breast carcinogenesis	176
MUC1 (mucin-1)	Apoptosis	Triple negative breast cancers and response to therapy including vaccines	32
TWIST	Transcription factor growth and invasion	Involved in epithelial mesenchymal transition in breast cancer	177
ZAC (pleomorphic adenoma-like protein)	Apoptosis/tumor suppressor	Treatment with 5-aza induced ZAC expression in breast cancer cell lines	178
SERPINB5 (protease inhibitor 5, maspin)	Inhibitor of angiogenesis	Reactivated in by treatment with demethylating agent	179
THBS1 (thrombospondin-1)	Inhibition of angiogenesis	Promotes tumor cell migration/proangiogenic	180
CDH1 (e-cadherin)	Suppresses invasion and metastasis	Treatment with 5-aza increases expression	10, 181
CDH13 (h-cadherin)	Suppresses invasion	Treatment with 5-aza increases expression	182
BCSG1 (breast cancer-specific gene 1 protein)	Invasion	Prognostic	183
APC	Invasion, apoptosis, regulation of cell cycle	Treatment with 5-aza increases APC expression	184, 185
TIMP3 (tissue inhibitor of metalloproteinases 3)	Suppresses invasion and angiogenesis	Treatment with 5-aza increases TIMP3 expression	186
SATB1	Tumor growth and metastasis	Prognostic? Potential target	187
Metastasis-associated genes (MTAs)	Invasion	Prognostic	188
microRNAs 148a, 34b/c and 9	Invasion and metastasis	Prognostic? Potential target	189
TERT (telomere reverse transcriptase)	Synthesis of telomere ends (cell immortalization)	Estrogen-activated telomerase by interactions with TERT promoter	190, 191
GSTP1 (glutathione S-transferase P)	Carcinogen detoxification	GSTP1 is hypermethylated in ER-positive GSTP1 nonexpressing cell lines but undermethylated in ER-negative GSTP1 expressing cell lines	192, 193
TGM2 (transglutaminase-2)	Function in chemosensitivity	Marker for chemosensitivity	141