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Epigenetic Regulation in Estrogen Receptor Positive Breast Cancer—Role in Treatment Response

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Abstract

Recent advances in breast cancer treatment have allowed increasing numbers of patients with estrogen receptor (ER) positive (+) breast cancer to receive various forms of endocrine therapy. Unfortunately, de novo and acquired resistance to endocrine therapy remains a major challenge in the clinic. A number of possible mechanisms for drug resistance have been described, which include activation of growth factor receptor pathways, overexpression of ER coactivators, and metabolic resistance due to polymorphisms in metabolizing enzymes. While many of these changes are caused by genetic alterations, there is also increasing evidence to implicate epigenetic gene regulatory mechanisms in the development of endocrine resistance. Since epigenetic modifications are easier to reverse than genetic mutations, they are appealing therapeutic targets, and thus future improvements in medical care for breast cancer patients will depend upon a better understanding of the roles epigenetic modifications play in endocrine resistance. In this review we will focus on recent advances made in the understanding of epigenetic gene regulation in estrogen response and endocrine resistance in breast cancer. We will also summarize current clinical-translational advances in epigenetic therapy, and discuss potential future clinical use of epigenetic changes as therapeutic targets, especially with respect to endocrine treatment.

Keywords

Epigenetics; Breast cancer; Estrogen; Antiestrogen; Tamoxifen

Background—Endocrine Therapy

Estrogen regulates a variety of physiological responses in many target tissues, and is well known to be involved in breast cancer development and progression. Estrogen exerts its biological action through binding to ER α and ER β , which are members of the nuclear receptor superfamily of transcription factors. In contrast to ER α , little is known about the role of ER β in breast cancer due to the relative scarcity of studies that focus on it, but current data suggest

that ER α , and not ER β , is the critical functional ER in breast cells. (For simplicity, we will use the term “ER” for “ER α ” throughout this review).

Approximately 70% of breast tumors are positive for ER expression at diagnosis. Patients with ER+ tumors are suitable candidates for endocrine therapy, including selective estrogen receptor modulators (SERMs) such as tamoxifen, which competes with estradiol for binding to ER, selective ER down-regulators (SERDs) such as fulvestrant, which induces destabilization and degradation of ER, and aromatase inhibitors (AI), which reduce the production of estrogen in peripheral tissues and within the tumor through the inhibition of the enzyme aromatase, an enzyme which synthesizes estrogens from testosterone and androstenedione. Tamoxifen has been the mainstay of endocrine therapy in both early and advanced breast cancer patients for almost three decades, and remains to date the most successful targeted cancer therapy. However, it is now clear that AIs offer advantages over tamoxifen as adjuvant treatment for postmenopausal women [1]; the results of the Arimidex, Tamoxifen Alone or in Combination (ATAC) trial [2], and the International Breast Cancer Study Group's Breast International Group (BIG) 1–98 trial (comparing the efficacy and safety of letrozole vs tamoxifen) [3] showed small but significant increased efficacy of AIs, and in general fewer side effects.

Despite the well documented benefits of endocrine therapy in breast cancer, not all patients with ER+ tumors respond to endocrine manipulation (de novo resistance), and unfortunately a substantial number of ER+ tumors which do initially respond will later become refractory to therapy (acquired resistance) [4,5]. For example, it is estimated that one third of women treated with tamoxifen for 5 years will have recurrent disease within 15 years, and therefore that endocrine resistant ER+ tumors represent approximately one fourth of all breast cancers [6]. While a significant body of literature has identified possible mechanisms of resistance to tamoxifen, there is less insight into mechanisms of resistance to SERDs and AIs. It is conceivable that there will be shared mechanisms of resistance between the different endocrine treatments, but the lack of clinical cross-resistance [7,8] indicates that there may also be mechanisms which are unique to one or other treatment.

The main mechanism of de novo resistance to endocrine treatment is lack of ER expression. In addition, progesterone receptor (PR) status significantly improves prediction of outcome over ER status alone for adjuvant endocrine therapy [9]. In particular, tamoxifen-treated patients with ER+/PR⁻ tumors showed a significantly higher relative risk of recurrence and death as compared with patients with ER+/PR+ tumors. Recent studies have shown that activation of growth factor receptor (GFR) pathways can lead to decreased PR expression, providing evidence for a role of GFR signaling in endocrine treatment response [10]. Indeed, the bidirectional crosstalk between steroid receptors and receptor tyrosine kinases is a major determinant of endocrine resistance—overexpression of EGFR family members (EGFR and HER2) and IGF1R has been associated with tamoxifen resistance. For example, a tamoxifen resistant model derived from MCF-7 cells showed increased expression of EGFR and HER2 [11], associated with increased activation of EGFR/HER2 heterodimers, and increased phosphorylation of MAPK, AKT, and ER [12,13]. Conversely, in HER2-overexpressing MCF-7 cells, tamoxifen acts as an agonist—it activates EGFR/HER2 signaling, leading to activation of both MAPK and PI3K/AKT signal transduction pathways [14]. Activation of GFR signaling has also been described as a mechanism for resistance to estrogen deprivation. Long-term estrogen-deprived (LTED) MCF-7, which have adapted to grow in very low levels of estrogen [15], have higher levels of nuclear ER, but also increased levels of membrane ER, resulting in increased non-genomic ER functions, and activated Src, ras/raf/MEK/MAPK, and PI3K signaling pathways [16]. A second LTED model also showed enhanced transcriptional activity of ER associated with an increase in the activation of GFR pathways, which in turn trans-activate ER [17]. In addition to activation of GFRs and/or therein downstream signaling intermediates, Erk and PI3K pathways, endocrine resistance has been associated with

deregulation of estrogen signaling, such as overexpression of coactivators or loss of corepressors. For example, the coactivators SRC1 [18] and SRC3/AIB1 [19] are overexpressed in tamoxifen-resistant breast tumors, whereas the corepressor NCoR is lost [20].

A number of studies have shown that the above described deregulation of GFR or estrogen signaling occur as a consequence of *genetic* changes, such as amplification of *AIB1* [21] and *ERBB2* [22], and mutation in the PI3K pathways [23,24]. There is however increasing evidence that *epigenetic* regulation of GFR and ER pathways is involved in endocrine treatment response, which will be the focus of this review.

Epigenetic Mechanisms of Resistance to Endocrine Treatment

Introduction to Epigenetics

The field of epigenetics is rapidly expanding, and has changed and revised traditional paradigms of inheritance. Epigenetics, literally “beyond genetics” or “in addition to genetics”, is defined as the study of heritable changes in gene expression that occur without a change in DNA sequence. There is some discussion about what should be considered as “epigenetic marks”, but in this review we will focus on modifications of DNA and histones, which are critical regulators of the dynamic state of chromatin.

DNA methylation takes place in the 5' position of cytosine (5mC) in CpG dinucleotides, mediated by DNA methyltransferases (DNMTs). In humans, the de novo methyltransferases DNMT3a and DNMT3b methylate the embryonic genome, and the maintenance methyltransferase DNMT1 methylates hemimethylated DNA after mitosis. Hypermethylation of a promoter generally silences the associated gene unless the methylation signal can be overridden by alterations in factors that modulate chromatin, such as removal of methylated cytosine binding proteins or the deacetylase SIRT1 [25,26]. In addition, a number of candidate biochemical pathways may mediate DNA demethylation, such as direct removal of the methyl group in the C5 position of the cytidine ring (bona fide demethylation) [27] or the entire cytidine base (or nucleoside or nucleotide; indirect demethylation), but this activity is controversial. DNA methylation can be inhibited by cytosine analogs, 5-azacytidine (AZA) and 5-aza-2-deoxycytidine (DAC), which are incorporated into DNA, ultimately resulting in degradation of DNMT. DNA synthesis in the absence of these enzymes results in hypomethylation in the daughter cells and eventual reactivation of silenced gene expression.

Histones, small proteins which serve as spools for DNA wrapped in nucleosomes, are subjected to numerous posttranslational modifications, including acetylation, methylation, ubiquitylation, and phosphorylation. The best characterized modifications are acetylations and methylations. Increased acetylation of histones by histone acetyltransferases (HATs) is associated with gene activation, in part due to weakened charge attraction between DNA and histone proteins; histone deacetylases (HDACs) in contrast remove acetyl groups leading to gene inactivation. Histone methylation mediated by histone methyltransferases (HMTs) has either positive effects (e.g. at H3 lysine 4) or negative effects (e.g. at H3 lysine 9 and H3 lysine 27) on gene expression depending on location and association of other protein complexes. In general, it is difficult to predict gene regulation based on the study of a single histone modification, since it is the combination of these modifications, also called the histone code, which ultimately controls gene expression [28]. Also, the position of histones, and hence their activity, is controlled by other DNA interacting proteins, such as members of the nucleosome remodeling complex, including the SWI/SNF and ISWI proteins [29]. DNA methylation, histone modification, and nucleosomal remodeling are intimately linked in the permanent silencing of genes [26]. For example, DNA methylation may repress genes by recruiting methyl-CpG-binding domain (MBD) proteins to the methylated CpG dinucleotides—these

proteins then act as a bridge between DNA methylation and repressive histone modifications by binding to HDACs and reinforcing the repressive chromatin state [30,31].

Deregulation of Epigenetic Control in Cancer

Epigenetics is a prominent theme in many fundamental developmental processes such as embryonic development, fertilization, sexual differentiation, and aging, through mechanisms such as imprinting, and X chromosome inactivation [26]. It has also become clear that deregulation of epigenetic processes occurs in many diseases, including cancer. In neoplastic cells, simultaneous genome-wide DNA hypomethylation, CpG island hypermethylation, and increased DNA methyltransferase activity have been observed [32]. Aberrant promoter CpG island hypermethylation is associated with transcriptional silencing, for example, of tumor suppressor genes [33]. In breast cancer, promoter hypermethylation and silencing occur in a variety of genes including cell cycle inhibitor genes (e.g. *p16 RASSF1A*), DNA repair genes (e.g. *BRCA1*), proapoptotic genes (e.g. *HOXA5*, *TMS1*), metabolic enzymes (e.g. *GSTP1*), and genes involved in cell adherence and the metastatic process (e.g. *CDH1*, *CDH13*) [34]. Recent advances in techniques used to study methylation, especially the use of whole-genome and high-throughput approaches, have allowed more comprehensive studies. For example, a recent study using high-throughput MALDI-TOF mass array analyzed 42,528 CpG dinucleotides on 22 genes in 96 different paraffin-embedded tissues (48 breast cancerous tissues and 48 paired normal tissues), led to the identification of hypermethylated CpG islands located near the consensus sequences of the transcription factor binding sites in 10 genes (*APC*, *BIN1*, *BMP6*, *BRCA1*, *CST6*, *ESRb*, *GSTP1*, *P16*, *P21* and *TIMP3*) which distinguished cancer and normal breast tissue [35]. Moreover, it is possible that early epigenetic lesions may indicate future risk of breast cancer; for example, hypermethylation of p16INK4a in normal mammary epithelial cells seems to precede the clonal outgrowth of premalignant lesions [36,37].

Although at this point less studied compared to DNA methylation, increasing evidence suggests that histone modifications are also perturbed in cancer. A classical example is the recruitment of HDACs and MBDs to the PML-RAR α fusion protein causing transcriptional repression and ultimately blocking hematopoietic differentiation and leading to acute promyelocytic leukemia [38]. Histone H3K27 trimethylation is increased in prostate cancer cells compared to normal prostate, and down-regulation of EZH2, the H3K27me3 methyltransferase, restored expression of many silenced genes [39]. Similar findings have been made in breast cancer: Intriguingly, EZH2 is known to be overexpressed in many cancers including breast cancers [40,41]. This might be an early event, since EZH2 (and other polycomb proteins such as SUZ12) were shown to be upregulated in models of early breast tumorigenesis, marking some target genes (e.g. *HoxA9*) for subsequent DNA methylation [42]. These findings are supported by studies in mouse models, where overexpression of EZH2 in mouse mammary glands disrupts morphogenesis and causes hyperplasias [43].

Another study shows that global histone modifications in breast cancer correlates with tumor phenotypes, prognostic factors, and patient outcome. Briefly, high relative levels of global histone acetylation and methylation were associated with favorable prognosis and detected almost exclusively in luminal-like tumors whereas moderate to low levels were observed in carcinomas of less favourable outcomes, including basal carcinomas and HER-2 positive tumors [44]. Another example is the frequent downregulation of the histone acetyltransferase hMOF in primary breast carcinoma (and medulloblastoma where the downregulation was associated with worse survival rates) [45]. The analysis of global and gene-specific alterations of histone modifications in breast cancer is a topic of intense study in many groups, and we can certainly expect many exciting findings in the near future.

Epigenetic Regulation of Hormone Receptor Expression in Breast Cancer

There are a number of studies showing that the 5' region of the *ESR1* gene is methylated in ER⁻ breast cancer cell lines [46]. Conversely, cells with increased ER levels, such as LTED cells, contain hypomethylated CpG islands in the *ESR1* promoter [47]. Methylation of the *ESR1* promoter is causatively associated with lack of expression since treatment with a DNA methyltransferase inhibitor, DAC, or specific inhibition of DNMT1 by antisense oligonucleotides leads to reactivation of functional ER protein in ER⁻ human breast cancer cells [48]. Recent data indicate that there is interplay between DNA methylation and chromatin inactivation mediated by histone modifications such as deacetylation in *ESR1* silencing, for example through the interaction of DNMT1 with either HDAC1 or HDAC2 [49]. As a result, co-treatment with the DNMT1 inhibitor AZA and the HDAC inhibitor Trichostatin A (TSA) or the novel HDAC inhibitor scriptaid resulted in synergistic induction of *ESR1* gene expression in ER⁻ breast cancer cells [50,51]. Interestingly, a recent finding suggests that HDAC inhibitors alone can restore expression of the silenced *ESR1* gene by reorganizing the heterochromatin-associated proteins without alteration in promoter DNA hypermethylation [52].

Similarly, the expression of PR can be epigenetically regulated, and the elimination of epigenetic barriers by treatment with AZA and TSA induces re-expression of the *PR* gene in PR⁻ breast cancer cell lines [50,53]. Also, treatment of MDA-MB-231 and MCF-7 breast cancer cells with the novel DNMT inhibitor zebularine resulted in induction of PR (and ER) mRNA, and the combination of zebularine with decitabine or vorinostat significantly inhibited cell proliferation and colony formation in MDA-MB-231 cells [54]. The analysis of *PR* methylation is complicated by the fact that the two PR receptors, PRA and PRB, are transcribed from two adjacent alternative promoters, and translated at two different AUG codons. Structurally, PRB differs from PRA only in that PRB contains an additional stretch of 164 amino acids at the N-terminus of the protein [55]. It has been shown that patients with PR⁺ tumors but high PRA:PRB ratios, were more likely to relapse than patients with lower ratios, indicating resistance to tamoxifen [56]. This clinical finding, together with laboratory studies showing some diverse functions and target genes of PRA and PRB suggest that promoter-specific methylation needs to be analyzed to cover the apparent complexity of PR biology.

Given that genetic changes in *ESR1*, such as mutations, deletions, or polymorphisms, have very rarely been found and have not consistently been described to be associated with endocrine response, one could conclude that epigenetic inactivation of ER (and possibly PR) maybe a main mechanism of endocrine resistance. It is therefore no surprise that numerous studies have analyzed *ESR1* and *PR* methylation in breast tumors, summarized in Table 1. It can be immediately noted that the percentage of methylation is highly variable, which is at least in part a result of the different sensitivities of the methods used to measure methylation. This finding is not specific for ER and PR, since similar wide ranges of methylation rates have been observed for other frequently methylated genes such as *CDH1* [57–59], highlighting the need for standardized assays in the measurement of gene methylation. For example, MSP is a very sensitive method which can result in artificially high methylation rates, due to its ability to amplify methylation in a very small subset of the starting material. Truly quantitative methods, such as bisulfite pyrosequencing or use of MethyLight assays, are necessary to compare results between different groups. Despite the inconsistencies, the *ESR1* methylation studies do show that there is a consistently higher degree of methylation of its promoter in ER⁻ vs ER⁺ tumors. Interestingly, one study showed that *ESR1* methylation outperformed hormone receptor status itself as a predictor of clinical response in tamoxifen-treated patients, possibly reflecting an association between *ESR1* methylation and other events independent of ER expression which are associated with hormone response [60]. This is not a consistent finding, however, since other studies using tumors from tamoxifen-treated breast cancer patients failed to detect an

association between *ESR1* methylation and treatment response [61,62], indicating the need for additional studies, using standardized assays, sufficiently large sample sizes, and preferably tumors from homogeneously treated patients (i.e. mono-therapy).

Importantly, recent studies have shown that response to tamoxifen treatment could be restored in ER⁻ breast cancer cells by combination treatment with both an HDAC inhibitor and a DNMT inhibitor [63,64]. A recent xenograft study provided promising evidence that epigenetic drugs might also restore sensitivity to aromatase inhibitors: combination treatment with the HDAC inhibitor entinostat (SNDX-275) plus letrozole resulted in improved inhibition of MDA-MB-231 tumor growth in vivo, compared to letrozole or entinostat only [65]. Thus, epigenetic regulation of *ESR1* represents an important treatment target in endocrine resistant disease (please see also Section 4, “Epigenetics in the Clinic”, in this review).

Similarly, the current data suggest that epigenetic regulation of *PR* may play a key role in developing resistance to endocrine therapy. *PR* methylation is higher in PR⁻ vs PR⁺ tumors, and a higher prevalence of methylation of *PR* was found in HER2⁺ cancers in comparison to HER2⁻ tumors [66]. In vitro, it has been shown that long-term treatment of breast cancer cells with antiestrogens such as tamoxifen can irreversibly inhibit the expression PR through chromatin remodeling and epigenetic mechanisms [67]. Such treatments recruit heterochromatin proteins including HP1 α , resulting in irreversible transcriptional inactivation [68]. Furthermore, C4-12 cells, which are MCF-7 clones isolated after long-term estrogen deprivation, have lost expression of ER as well as PR, but interestingly, re-expression of ER did not restore estrogen induction of PR expression [69]. Subsequent studies indicate that the *PR* promoter is hypermethylated and stably silenced in C4-12 cells [70].

A few studies have studied *PR* methylation in breast tumors (Table 1). Similar to the *ESR1* studies, methylation rates varied widely, but there were consistently higher rates of methylation in PR-negative tumors compared to PR⁺ tumors. Unfortunately, none of the studies published to date has analyzed, and compared and contrasted methylation of PRA and PRB promoter. Given the differences between the two receptor isoforms in biology, and potentially in clinical significance, future studies should address promoter specific methylation in breast tumor samples, and association with endocrine treatment response.

Epigenetic Regulation of Other Pathways with Potential Importance in Resistance to Endocrine Treatment

Huang's group has shown that disruption of ER signaling in MCF-7 breast cancer cells by small interfering RNA causes the recruitment of polycomb repressors and histone deacetylases, resulting in stable repression of the ER target genes [70]. Since this event was accompanied by acquired DNA methylation, they then went on to study genome-wide DNA methylation in MCF-7 cells resistant to tamoxifen (MCF7-T) and resistant to fulvestrant (MCF7-F) [71]. Intriguingly, the dominant epigenetic change was hypomethylation (in contrast to hypermethylation) of a large number of genes. Specifically, fulvestrant resistance was characterized by significant hypomethylation and induction of multiple growth stimulatory pathways, such as EGFR/ERBB2, Wnt/ β -catenin, Notch, IFN, and cytokine pathways, resulting in ER-independent autocrine-regulated proliferation. Conversely, acquired resistance to tamoxifen correlated with maintenance of the ER⁺ phenotype, although receptor-mediated gene regulation was altered. Gene families most prominently affected were a) PKA pathway, b) caveolins, c) annexins and S100 calcium binding proteins, d) MAPK phosphatases, and e) ID (inhibitor of differentiation) proteins. Several genes which showed increased basal expression levels in MCF7-T or MCF7-F cells are known to have oncogenic properties and are known poor prognostic markers in breast cancer, such as CDH2, ID4, BRAF, CTNNB1, and Wnt11. This finding reflects a general finding that many genes or even gene signatures associated with treatment response are also correlated with outcome irrespective of treatment,

and highlights the need for a “control” group (i.e. no adjuvant treatment but surgery only) in order to differentiate truly predictive markers from general prognostic markers.

Several studies have reported that a high expression ratio of HOXB13/IL17BR predicts tumor recurrence in node-negative, ER+ breast cancer patients treated with tamoxifen [72,73]. While the details of deregulation of HOXB13 and IL17BR are still under investigation, there is evidence that expression of HOXB13 is under epigenetic control. In breast cancer cells, its promoter region is frequently hypermethylated, which was associated with lower expression, and accordingly, treatment with DAC restored its expression [74]. The same study also showed increased promoter hypermethylation of HOXB13 in ER+ clinical breast tumors. Intriguingly, methylation was associated with poor prognostic markers, such as increased lymph node metastasis, large tumor sizes, and shorter disease-free survival in breast cancer patients [74]. Since treatment and nodal status were very different in the study groups utilized for analysis of methylation and the HOXB13/IL17BR expression ratio, it is difficult to compare the results; in any case, methylation of HOXB13, and its relation to endocrine resistance deserve to be studied in more detail.

Another recently identified hypermethylated gene with a potential role in endocrine resistance is CDK10. Using an shRNA screen, Ashworth's group identified CDK10 as an important determinant of resistance to tamoxifen and estrogen deprivation, and showed that CDK10 silencing increased ETS2 driven transcription of c-RAF, resulting in MAPK pathway activation and loss of tumor cell reliance upon estrogen signaling [75]. They also showed that tamoxifen-treated patients with ER+ tumors that express low levels of CDK10 have shorter disease free survival, demonstrating its clinical significance. Of importance in the context of this review, the authors showed that low levels of CDK10 expression in tumors were associated with methylation of the CDK10 promoter; however, a recent study was unable to detect hypermethylation of *CDK10*, using MSP in formalin-fixed, paraffin-embedded surgical specimens of 96 breast carcinoma patients [76], suggesting that additional analyses are necessary to fully understand the role of cdk10 methylation in breast tumorigenesis.

So far, at least three studies have used high-throughput DNA methylation profiling to associate DNA methylation of candidate genes with outcome of breast cancer patients after tamoxifen therapy. Laird's group generated DNA methylation profiles of 148 tumors from patients who had received adjuvant tamoxifen therapy, using MethyLight technology [60]. They found that promoter methylation of *ESR1* and *CYP1B1* (which encodes a tamoxifen and estradiol metabolizing cytochrome p450 enzyme), predicted response in tamoxifen-treated patients. Moreover, Foeken's group analyzed promoter DNA methylation status of candidate genes in 200 HR+ tumors of patients who received tamoxifen as first-line treatment for recurrent breast cancer [62]. They amplified CpG sites from regulatory regions of 117 candidate genes from bisulfite-treated DNA and then hybridized to an array of immobilized oligonucleotides reflecting the methylated (CG) and non-methylated (TG) status of each CpG position. Promoter DNA methylation status of 10 genes including *PSAT1*, *STMN1*, *S100A2*, *SFN*, *PRKCD*, *SYK*, *VTN*, *GRIN2D*, *TGFBR2*, and *COX7A2L* was significantly associated with clinical outcome of tamoxifen therapy, with *PSAT1* being most significant. Harbeck's group also used a microarray-based technology to study DNA methylation profiles of tumors from 109 patients who received adjuvant tamoxifen, and showed that methylation of the paired-like homeodomain transcription factor-2 (PITX2) was predictive of response to tamoxifen [77, 78]. This finding was subsequently validated in an independent cohort ($n=427$) [77,78]. Altogether, these studies identified a number of novel DNA methylation markers which might be clinically useful predictors of response to hormonal breast cancer therapy, although with very few exceptions (e.g. PITX2) there is little consistency in the identified markers, or markers have yet to be tested in a second independent cohort, clearly highlighting the need for additional studies.

We would like to note that an advantage of using DNA methylation as a biomarker is the fact that it can be easily measured on small biopsy samples obtained during the routine diagnostic work-up of patients, on archived frozen or paraffin embedded tissues, or potentially even on blood specimens. Increased concentrations of free DNA have been detected in the blood of many cancer patients in comparison to healthy individuals [79]. The serum of breast cancer patients contains, on average, approximately four times more free DNA compared with that of healthy individuals [80,81]. Tumor cell specific promoter hypermethylation of *APC*, *RASSF1A*, and *DAP-kinase* was detected in serum DNA from patients with preinvasive and early-stage breast cancer [82], and hypermethylation of *RASSF1A* and/or *APC* in serum DNA from breast cancer patients was associated with a worse outcome [83]. Similarly, methylation of *ESR1* and *14-3-3-sigma* gene promoters, detected in serum, was suggested to be a biomarker for diagnosis of breast cancer [84]. Finally, there is some evidence that the measurement of DNA methylation in serum could be a good biomarker for predicting response to adjuvant therapy—loss of *RASSF1A* DNA methylation in serum throughout tamoxifen treatment was associated with a response, whereas persistence or new appearance was associated with resistance to adjuvant tamoxifen treatment [85]. Another area of interest is the identification of serum biomarkers to measure efficacy of epigenetic therapy. Some progress has been made, an example being LINE-1 methylation, which was shown to be a biomarker of activity for DNMT inhibition in solid tumors, including breast tumors [86]. The confirmation of promising epigenetic markers in the serum, such as *RASSF1A* methylation, should be a priority in our efforts to identify markers which might have clinical utility in the near future.

Most of the studies described above were designed to analyze single genes, pathways, or a limited number of genes. As DNA sequencing becomes more affordable, whole genome sequencing of bisulfite-converted DNA to detect genome-wide methylation profiles will be feasible, and necessary. The use of tumor material from mono-therapy (i.e. patients treated with endocrine treatment only) will help to identify truly predictive markers. Together with the fact that many methylation events are cancer specific, and are chemically stable, one could expect that the DNA methylation profile could become a widely used biomarker for endocrine treatment response in breast cancer.

Epigenetics in the Clinic

For many years, the search for new cancer therapy targets focused on genetic changes associated with the transformation of normal cells into malignant cells, and with resistance to therapy. However, it is now clear that epigenetic mechanisms are also essential targets in cancer therapy. Epigenetic changes are frequent events, and unlike genetic mutations, epigenetic modifications are reversible events; thus, the inhibition of these mechanisms could be a potential therapeutic strategy for treatment of breast cancer, either through direct effects on epigenetic changes, or by modulating known targets of other therapies (e.g. ER).

Several inhibitors of enzymes controlling epigenetic modifications, specifically DNMT and HDACs, have been developed, and show promising anti-tumorigenic effects. The DNMT inhibitors include Vidaza (AZA), Decitabine (DAC), and zebularine. A number of HDAC inhibitors including hydroxamates (e.g. TSA, SAHA, LBH-589), cyclic peptides (e.g. Depsipeptides), aliphatic acids (e.g. phenylbutyrate, valproic acid), and benzamides (e.g. SNDX-275, p-N-acetyl dinalin, MGC0103) have been clinically investigated [87]. The DNMT inhibitors Vidaza (AZA) and Decitabine (DAC), and the HDAC inhibitor Vorinostat (SAHA) have been FDA-approved in hematological malignancies, where the in general the greatest success has been achieved. For example, treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients with Vidaza and Decitabine resulted in very encouraging response rates and improved survival outcomes compared with cytotoxic chemotherapy [88,89]. Whether these epigenetic drugs could be used in other forms of cancer,

and in particular in solid malignancies, is being evaluated. In breast cancer, there are only a few studies which have investigated the use of demethylating or HDAC inhibitors alone, or in combination with other agents such as endocrine therapies, cytotoxic agents, or novel targeted therapies. Early reports suggest that there may be stabilization of disease or some response; however, thus far these agents have shown a less impressive clinical track record in solid tumors (including breast cancer) compared to hematological malignancies. One problem is decreased drug efficacy in solid tumors, due to a pool of low replicating cells [88] limiting the use of current demethylating agents in non-hematological malignancies.

Several phase I and II clinical trials are ongoing using DNA methylation and/or HDAC inhibitors in breast cancer patients (summarized in Table 2). Decitabine and Vidaza are currently in clinical trials in breast cancer patients with advanced and metastatic disease. The HDAC inhibitors Vorinostat, Panobinostat, and Entinostat are being tested in trials with patients with ductal carcinoma in situ, as well as advanced and metastatic tumors. In a phase II study, 14 patients received oral vorinostat, 200 mg twice daily for 14 out of 21 day cycles. Although stable disease was observed in 4 patients (time to progression of 4, 8, 9, and 14 months), the study was terminated due to lack of complete or partial responses in the first stage [90]. In a phase I study of Decitabine and Vorinostat in patients with advanced solid tumors or relapsed/refractory non-Hodgkin's lymphoma, Canadian investigators studied two different schedules and reported results from a sequential schedule. The investigators reported disease stabilization for 4 or more cycles in 7 of 22 (31.8%) evaluable patients, which included two patients with breast cancer [91]. Dose-limiting toxicities included mainly myelosuppression, constitutional and gastrointestinal symptoms in 7 of 27 (26%) participants. In the HDAC inhibitor trial, the most common adverse events included fatigue, nausea, diarrhea, and lymphopenia [90]. These side effects were expected, since they have been previously reported to be associated with targeting HDACs. In general, various HDAC inhibitors differ in their toxicity profile when comparing the side effects described in the available clinical studies of HDAC inhibition in the treatment of cancer. There are potentially other, more serious side effects including cardiotoxicity, and effects on chromosomal stability. Finally, these drugs may also affect normal hematopoiesis; hematologic toxicity is common to many drugs but stimulation of hematopoiesis seems to occur for others. The observed side effects might result from effects on epigenetic regulation of various target genes, but more likely they are caused by inhibition of other targets of acetylases—acetylation is an important posttranslational modulation of several proteins (in addition to histone) involved in the regulation of cell proliferation, differentiation and apoptosis in normal as well as cancer cells.

Few studies incorporated HDAC inhibitors with cytotoxic agents. In a proof-of-principle study, the demethylating agent hydralazine and the HDAC inhibitor magnesium valproate were given in combination with neoadjuvant doxorubicin and cyclophosphamide to locally advanced breast cancer patients to assess their safety and biological efficacy [92]. It was found that hydralazine and valproate exerted their proposed molecular effects by significantly decreasing global 5mC content and HDAC activity. There was an up-regulation of 1,091 and down-regulation of 89 genes in primary tumors. Importantly, this treatment was safe and well-tolerated, and appeared to increase the efficacy of doxorubicin and cyclophosphamide. Also, a recent phase II single-arm study of hydralazine and magnesium valproate added to the same schedule of chemotherapy on which patients were progressing, suggesting that hydralazine and valproate overcome epigenetic changes, mediating chemotherapy resistance regardless of chemotherapy drug and tumor type [93]. In another phase I/II trial, women with metastatic breast cancer received first line chemotherapy with paclitaxel 90 mg/m² on days 1, 8, 15, bevacizumab 10 mg/kg on days 1, 15 every 28 days, and oral vorinostat 200 mg (*N*=3) or 300 mg (*N*=41) twice daily for 3 days given the day before, day of, and day after each paclitaxel dose. The dose level recommended phase II dose of vorinostat was 300 mg twice a day, and a sufficient number of responses occurred in the first stage of the phase II trial (15/28) to continue

accrual to the second stage. Toxicity was not different than expected for paclitaxel and bevacizumab therapy. Preliminarily, at least 25 of 43 evaluable patients at both dose levels have had either a confirmed ($N=22$) or unconfirmed ($N=3$) objective response (ORR 58%, 95% C.I. 42%, 73%). Two patients had tumor biopsies and peripheral blood mononuclear cells collected before and 4 h after the third dose of Vorinostat, which revealed increased acetylation of the lysine residue K69 of the chaperone protein Hsp 90, as well as up-regulation of Hsp70, and down-regulation of AKT [94].

Another promising approach is the combination of epigenetic and endocrine therapy. In a recent phase II trial, the HDAC inhibitor Vorinostat and tamoxifen were given in combination to patients with ER+ metastatic breast cancer that had progressed on prior hormonal and chemotherapy regimens [95]. Of 29 participants, 6 (21%) patients had an objective response, and 3 (10%) had disease stabilization for 6 or more months. Since most of the patients had received prior adjuvant tamoxifen or up to two aromatase inhibitors, these results support the hypothesis that adding an HDAC inhibitor to tamoxifen may restore hormone sensitivity. This might be a result of re-expression of silenced ER, a main rationale for combining epigenetic and endocrine treatment. It is however very likely that HDAC inhibitors will not only alter the “histone code” (i.e. affecting both histone acetylation and methylation) at the *ESR1* (and other) genes, but also impact a host of transcription factors, and thus might act via other pathways. Measurement of target gene expression, and identification and measurement of other potential biomarkers is a critical task for ongoing and future trials with epigenetic drugs.

In summary, early reports of combining HDAC inhibitors and other standard agents suggest that there is a role for further investigation in breast cancer and in other solid malignancies. Challenges include agent selection as well as dose, schedule, and identification of biomarkers.

Future Directions

“Pharmaco-epigenomics” promises a new era of personalized cancer therapy, and in this review we have summarized compelling evidence that epigenetic marks may be useful predictive markers for hormone therapy in breast cancer patients. In the past, the use of cell line models and/or only a small number of clinical samples in a single gene candidate approach were limitations of some studies. Another hurdle in the epigenetic field is the use of assays with enormous differences in sensitivities, which have made it difficult to compare results between different studies. Future biomarker studies would clearly benefit from more standardized assays and approaches. We should also look forward to additional comprehensive and unbiased studies with genome-wide DNA methylation and histone modification analyses using microarrays and next-generation sequencing aimed at the identification of novel determinants of resistance.

Epigenetic changes are not only biomarkers but also represent an exciting area for targeted therapy. While their reversibility makes them ideal candidates for drug development, many limitations remain. For example, the gene re-expression and other changes induced by DNMT and HDAC inhibitors appear to be reversible upon drug discontinuation, suggesting a requirement for prolonged continuous treatment with epigenetic drugs. Since epigenetic drugs may be associated with side effects (for example, through re-expression of genes that should remain repressed), such prolonged treatments could lead to decreased quality of life. Therefore, there is an urgent need to identify optimal dose, schedule, and duration of therapy for future clinical trials. An equally critical task is the identification of biomarkers predicting responses to epigenetic therapy.

In addition, further studies to understand HDAC class and enzyme specificity, and the mechanism of action of individual HDAC inhibitors, are warranted. For example, there is a good rationale for combining HDAC inhibitors and ER-targeted therapies such as tamoxifen,

since epigenetic therapy results in re-expression of functional ER which renders the cells susceptible to tamoxifen (or other ER-targeted) therapy. However, it is also known that HDACs are necessary for tamoxifen-mediated inactivation of ER. A deeper knowledge of which HDAC isoforms are involved in repression of *ESR1* promoter activity and which ones are critical for turning off tamoxifen-bound ER, and subsequent development of HDAC isoform-specific inhibitors, might result in increased efficacies of combined HDAC inhibition and tamoxifen therapy. Thus, there is no doubt that further understanding of mechanisms of epigenetic regulation will enhance our understanding of breast cancer biology, and shift our current knowledge into new paradigms of endocrine resistance.

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Abbreviations

ER	estrogen receptor
PR	progesterone receptor
HDAC	histone deacetylase
SERM	selective estrogen receptor modulators
AI	aromatase inhibitor

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Table 1

A summary of *ESR1* and *PR* methylation data in breast tumors.

Gene	Authors	Method—methylation	Breast tumors	Method—ER and PR	% Me	Ref
<i>ESR1</i>	Lapidus, R.G. et al	Methylation sensitive enzyme digestion	ER- <i>n</i> =39 ER+ <i>n</i> =53	IHC	25% 0%	[96]
	Falette N.S. et al	Methylation sensitive enzyme digestion	ER- <i>n</i> =26 ER+ <i>n</i> =24	Ligand binding assay	20% 20%	[97]
	Lapidus, R.G. et al	MSP	ER- <i>n</i> =13 ER+ <i>n</i> =22	IHC	100% 52%	[98]
	Omoto, H.I. et al	MSP	ER- <i>n</i> =27 ER+ <i>n</i> =29	Enzyme immuno assay	81% 31%	[99]
	Miza S. et al	MSP	ER- <i>n</i> =14 ER+ <i>n</i> =22	IHC	78% 59%	[100]
	Widschwendter, M. et al	MethylLight	ER- <i>n</i> =50 ER+ <i>n</i> =98	IHC	100% ^a	[60]
	Feng W, et al	Bisulfite pyrosequencing	<i>n</i> =40 ^b	IHC	5%	[101]
	Wei M, et al	MSP	ER- <i>n</i> =59 ER+ <i>n</i> =59	d	78% 22%	[102]
	Li S, et al	MSP	ER- <i>n</i> =54 ER+ <i>n</i> =134	d	91% 81%	[103]
	Archev W.B. et al	Bisulfite sequencing	ER- <i>n</i> =40 ^b <i>n</i> =111 ^b	IHC d	32% 49%	[104] [105]
<i>PR</i>	Nass S.J. et al	MSP	ER+ <i>n</i> =8	RT-PCR	90.9%	[61]
	Chang, H.G. et al	Bisulfite sequencing	ER- <i>n</i> =22		12.5%	
	Lapidus, R.G. et al	Methylation sensitive enzyme digestion	PR- <i>n</i> =65 PR+ <i>n</i> =29	IHC	40% 0%	[96]
	Cornack M. et al	MSP	PR- <i>n</i> =66 PR+ <i>n</i> =28	IHC	84% ^c 53% ^c	[106]
	Feng W, et al	Bisulfite pyrosequencing	<i>n</i> =48 ^b	IHC	0% ^c	[101]
	Miza S. et al	MSP	PR- <i>n</i> =20	IHC	80% ^c	[100]

Gene	Authors	Method—methylation	Breast tumors	Method—ER and PR	% Me	Ref
	Widschwendter, M. et al	MethylLight	PR+ <i>n</i> =16	IHC	37% ^c	[60]
			PR- <i>n</i> =53		100% ^d	
			PR+ <i>n</i> =95			

MSP Methylation Specific PCR, % Me % Methylation of number of breast tumors, IHC Immunohistochemistry

^aIt should be noted that the level of methylation at the locus was low in this study

^bUnsufficient details on number of ER- (or PR-) and ER+ (or PR+) tumors used for the methylation analysis

^cPRB methylation was measured

^dUnknown

Table 2

Epigenetics clinical trials in breast cancer.

Category	Epigenetic drug	Combination drugs	Type of breast cancer	Phase	Ref
DNMT inhibitors	Decitabine (DAC)	None	Advanced	Phase I	[107]
	Vidaza (AZA)	Abiraxane	Advanced or metastatic	Phase I/II	[107]
HDAC inhibitors	Vorinostat (SAHA)	None	Metastatic	Phase II	[90]
	Vorinostat (SAHA)	Capecitabine	Advanced	Phase II	[107]
	Vorinostat (SAHA)	Paclitaxel, Bevacizumab	Locally advanced, metastatic, HER2-	Phase I/II	[94]
	Vorinostat (SAHA)	Trastuzumab	Locally advanced, metastatic, HER2+	Phase I/II	[107]
	Vorinostat (SAHA)	Paclitaxel Trastuzumab Doxorubicin Cyclophosphamide	Locally advanced HER2+	Phase I/II	[107]
	Vorinostat (SAHA)	Paclitaxel Trastuzumab Doxorubicin Cyclophosphamide	Locally advanced HER2+	Phase I/II	[107]
	Vorinostat (SAHA)	Carboplatin Nab-paclitaxel	Neoadjuvant, HER2-	Phase II	[107]
	Vorinostat (SAHA)	Tamoxifen	Advanced	Phase II	[95]
	Vorinostat (SAHA)	None	Ductal carcinoma in situ	Phase I/II Neoadjuvant, biomarker	[107]
	Vorinostat (SAHA)	None	Invasive	Neoadjuvant, biomarker	[108]
DNMT inhibitors + HDAC inhibitors	Panobinostat (LBH589)	Trastuzumab Paclitaxel	Metastatic HER2+	Phase I/II	[107]
	Panobinostat (LBH589)	None	Metastatic HER2+	Phase I/II	[107]
	Entinostat (SNDX-275)	Aromatase inhibitor	Advanced	Phase II	[107]
	Hydralazine	Doxorubicine	Locally advanced	Phase I	[92]
	Magnesium valproate	Cyclophosphamide	Advanced	Phase II	[93]
	Hydralazine Magnesium valproate	Chemotherapy	Advanced	Phase II	[93]
	Decitabine (DAC)	Vorinostat (SAHA)	Solid tumors or non-Hodgkin's lymphoma	Phase I	[91]
		None			