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PRINCIPAL INVESTIGATOR: Lan Yan, M.D., Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
School of Medicine
Baltimore, Maryland 21205

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13. ABSTRACT (Maximum 200 Words)

Estrogen and progesterone and their receptors play major roles in the development and function of the mammary gland as well as in breast cancer biology and treatment. However, 25-30% of human breast cancers lack ER α and PR expression and respond poorly to hormonal treatment. Absence of ER α and PR expression is associated with lack of transcript, methylation of the CpG islands in the promoter regions of these genes, and increased DMT activity and DMT1 protein. The purpose of this proposal is to address the hypothesis that specific inhibition of DMT1 by antisense oligonucleotides (DMT1 ASO) is sufficient to reexpress ER and PR genes in ER-/PR- human breast cancer cell lines. Our results demonstrated that after DMT1 ASO treatment, human breast cancer cell lines used in this study showed significant growth inhibition, DMT1 expression was blocked at protein level as well as at mRNA level. ER expression was observed after DMT1 ASO multiple treatment. The re-expression of PR and other breast cancer suppressing genes are currently under investigation.

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Introduction

One-quarter of all human breast cancers lack both estrogen receptor α (ER α) and progesterone receptors (PR). In general, these tumors are associated with poorer differentiation, higher growth fraction, and worse clinical outcome than ER+/PR+ breast cancer. Also, these cancers are estrogen-independent and rarely respond to hormonal therapy with agents like the antiestrogen tamoxifen. Recent findings suggest that epigenetic changes may be important for tumor initiation and progression. Abnormal methylation of CpG islands has been associated with inhibition of expression for a variety of tumor suppressor genes, including estrogen receptor α gene. A CpG island in the promoter region of the ER gene is extensively methylated in ER-negative breast cancer cells, but is unmethylated in normal breast cells. Furthermore, expression of the major enzyme which catalyzes cytosine methylation, DNA methyltransferase 1 (DNMT1) is significantly elevated in ER-negative breast cancer cell lines compared to ER-positive lines. Thus, DNMT1 may play a role in blocking ER expression during progression to an aggressive, hormone insensitive phenotype.

Our study tests the hypothesis that specific inhibition of DNMT1 is sufficient to re-express ER α as well as other critical breast cancer genes in ER-/PR- human breast cancer cell lines. Our results demonstrated that a specific DNMT1 inhibitor, DNMT1 antisense oligo 98 (ASO98), could dramatically inhibit DNMT1 expression at mRNA level as well as at protein level. We also observed ER α re-expression after ASO98 treatment in two ER-negative cell lines, MDA-MB-231 and Hs578t. Further investigation of the methylation events associated with estrogen receptor gene regulation

will enhance our understanding of hormone resistance in breast cancer as well as search for new therapeutic approaches for this the most common cancer in American women.

Body of Report

The work on this project is continuing on schedule. On January 1, 2000, Lan Yan, MD, Ph.D, became the principal investigator for this grant with departure of the former principal investigator, Sharyl Nass, Ph.D, to take a scientific position at the Institute of Medicine. The transition was smooth, and progress has been good.

Two ER-negative human breast cancer cell lines, MDA-MB-231 and Hs578t, were selected as model systems to test our hypothesis that specific inhibition of DNMT1 could block the synthesis of DNMT1, demethylate ER α promoter CpG island, and re-express ER α and other breast cancer suppress genes. A DNMT1 antisense oligonucleotide (ASO98), an 18 mer targeting the 3'-untranslated region (UTR) of DNMT1 mRNA, as well as its missense control (ASO207) were synthesized by The Midland Certified Reagent Company. Then effects on cell growth were first assessed. Cells were grown in the presence of no treatment; vehicle only (Sham); DNMT1 ASO98 (100nM x 3 days for MDA-MB-231 cells or 150 nM x 3 days for Hs578t cells carried by lipofectin (Gibco)), or ASO207 (same treatment as ASO98). Significant growth reduction was observed for both ASO98-treated cell lines after 48 hr, and this inhibition was more obvious after 72 hr or 96 hr. Some growth inhibition was also observed for both mutated ASO207-treated cell lines, possibly due to the non-specific effects of ASO. DNMT1 expression was blocked within 48 hr -96 hr of exposure to DNMT1 ASO98 as detected by Western blot whereas mutated DNMT1 ASO207 had no effect. RT-PCR analysis showed DNMT1 mRNA was blocked after ASO98 treatment but not the mutant ASO207, and that reexpression of ER α , PR, Retinoic Acid Receptor β (RAR β), and Cyclin D2 (CD2) mRNA was observed in both cell lines as detected by RT-PCR. ER α

promoter CpG methylation status was assessed by methylation specific PCR (MSP) – a technique designed to analyze methylation of CpG dinucleotides across the entire CpG island. Four sets of primers (ER1, 3,4 and 5) were used to amplify overlapping fragments of CpG island in ER α promoter region. ER α -positive MCF-7 cells were used as unmethylated control. Distilled water instead of bisulfite-treated genomic DNA was used as MSP-negative control. Cells treated with ASO98 showed the possibility of partial demethylation pattern in ER5 primer set. These results suggested that the re-expression of ER α by specific DNMT1 inhibition might not requiring CpG demethylation. Recent studies indicated that besides maintaining CpG methylation, DNMT1 can also form a repressive transcription complex at replication foci with histone deacetylases (HDACs) and a newly identified protein, DMAP1 (DNMT1 associated protein), at the non-catalytic amino terminus of DNMT1. Whether the re-expression of ER α by ASO98 is through the inhibition of DNMT1 expression with resulting disruption of the repressive transcription complex at ER promoter region needs future investigation.

Appendix 1

Key Research accomplishments

The following tasks have been completed:

1. A DNMT1 antisense oligonucleotide, ASO98, and its mutant control for non-specific effects of the antisense oligo, ASO207, were synthesized by Midland Certified Reagent Company.
2. These ASOs were introduced into ER α -negative human breast cancer cell lines, MDA-MB-231 and Hs578t, mediated by lipofectin using a repetitive treatment strategy (24hr, 48hr and 96hr) because of the short duration of antisense effects.
3. Growth studies indicated that Untreated and Sham samples showed little alteration in cell growth. However, ASO98 samples were substantially growth inhibited whereas ASO207 samples were partially growth suppressed, presumably because of non-specific growth effects associated with the oligos.
4. Proteins were isolated from cells without any treatment (Untreated), treated with lipofectin alone (Sham), ASO98 treatment and ASO207 treatment. Western analysis showed substantial inhibition of DNMT1 protein expression by ASO98 but not ASO207. Measurement of DNMT1 enzyme activity is in progress.
5. RNAs were isolated from the same samples as above using Trizol reagent. First strand cDNA was synthesized. RT-PCR was performed and demonstrated reexpression of ER α , PR, CD2, and RAR β mRNA in ASO98 treated cells but not ASO 207 treated cells.
6. DNAs were isolated from those samples. ER α gene methylation status was assessed using methylation specific PCR (MSP). Specifically four sets primers (ER1, 3,4 and

5) were used to amplify overlapping fragments of CpG island in ER α promoter region.

Appendix 2

Reportable outcomes:

1. Yan L, Nass JS, and Davidson NE. Effects of DNMT1 antisense oligodeoxynucleotide on steroid receptor expression in breast cancer cell lines. Proc. Am. Assoc. Cancer Res. 42:849, 2001.

2. One manuscript was published.

Nass SJ, JG Herman, E. Gabrielson, PW Iversen, FF Parl, SB Baylin, NE Davidson, and JR Graff. Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. Cancer Res. 60: 4346-4348, 2000.

3. Two manuscripts were accepted for publication.

Yan L., Yang X, and Davidson NE. Role of DNA methylation and histone acetylation in steroid receptor expression in breast cancer. J. Mammary Grand Biol. Neoplasia 6(2): 183-192, 2001.

Yang X, Yan L, and Davidson NE. DNA methylation in breast cancer. Endocrine-Related Reviews. (In press).

AACR 2001 Online Abstract Submitter52nd Annual Meeting
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New Orleans, LA**Abstract ID: 8656****Instructions**[Tips and Frequently Asked Questions](#)[Submission Guidelines](#)**[Abstract Checklist](#)**[Authors](#)[Title](#)[Body](#)[Keywords/Indexing](#)[Abstract Category and Subclassification](#)[Sponsor](#)[Awards/Support for Attendance](#)[Copyright Transfer](#)[Payment Information](#)**Other Options**[Presenting Author](#)[Contact Information](#)[Logout](#)**EFFECTS OF DMT1 ANTISENSE OLIGODEOXYNUCLEOTIDE ON STEROID RECEPTOR EXPRESSION IN BREAST CANCER CELL LINES**Yan, Lan; Nass, Sharyl J; Davidson, Nancy E
Johns Hopkins Oncology Center, Baltimore MD

Estrogen and progesterone and their receptors (ER α and PR) play major roles in the development and function of the mammary gland as well as in breast cancer biology and treatment. However, 25-30% of human breast cancers lack ER α and PR expression and respond poorly to hormonal treatment. Absence of ER α and PR expression is associated with lack of transcript, methylation of the CpG islands in the promoter regions of these genes, and increased DMT activity and DMT1 protein. Treatment of ER-negative human breast cancer cell lines with a non-specific DMT inhibitor 5-azacytidine led to reactivation of functional ER protein. This study addresses the hypothesis that specific inhibition of DMT1 by antisense oligonucleotides (DMT1 ASO) is sufficient to reexpress ER and PR genes in ER-/PR- human breast cancer cell lines. MDA-MB-231 and Hs578t cells were transfected with 100 nM and 200 nM DMT1ASO respectively. Significant growth reduction was observed after 48 hr, and this inhibition was more obvious after 72 hr or 96 hr. DMT1 expression was blocked within 48 hr of exposure to DMT1 ASO as detected by Western blot whereas mutated DMT1 ASO had no effect. However, methylation specific PCR indicated that ER promoter CpG methylation was preserved and RT-PCR did not show reexpression of ER or PR mRNA after 48 hr. Prolonged exposure to DMT1 ASO for 72 and 96 hr was associated with DMT1 reexpression, possibly due to the short half-life of ASO. These results suggest that short-term inhibition of DMT1 is not sufficient to reactivate ER or PR expression in receptor-negative human breast cancer cells. The effects of more prolonged exposure, expression of other methylated target genes, and role of other members of the DMT family is under study. Supported by DAMD 17-98-1-8116 and NIH R01 CA78352.

Aberrant Methylation of the Estrogen Receptor and E-Cadherin 5' CpG Islands Increases with Malignant Progression in Human Breast Cancer¹

Sharyl J. Nass, James G. Herman, Edward Gabrielson, Philip W. Iversen, Fritz F. Parl, Nancy E. Davidson,² and Jeremy R. Graff

Oncology Center [S. J. N., J. G. H., N. E. D., J. R. G.] and Department of Pathology [E. G.], The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231; Department of Pathology, Vanderbilt University, Nashville, Tennessee 37232 [F. F. P.]; Lilly Research Labs, Eli Lilly and Company, Indianapolis, Indiana 46285 [J. R. G., P. W. I.]; and the Institute of Medicine, National Academy of Sciences, Washington, DC 20418 [S. J. N.]

Abstract

Loss of expression for both the *estrogen receptor-α* and *E-cadherin* genes has been linked to disease progression in human ductal breast carcinomas and has been associated with aberrant 5' CpG island methylation. To assess when, during malignant progression, such methylation begins and whether such methylation increases with advancing disease, we have surveyed 111 ductal carcinomas of the breast for aberrant methylation of the estrogen receptor- α and E-cadherin 5' CpG islands. Hypermethylation of either CpG island was evident prior to invasion in ~30% of ductal carcinoma *in situ* lesions and increased significantly to nearly 60% in metastatic lesions. Coincident methylation of both CpG islands also increased significantly from ~20% in ductal carcinoma *in situ* to nearly 50% in metastatic lesions. Furthermore, in all cases, the pattern of methylation displayed substantial heterogeneity, reflecting the well-established, heterogeneous loss of expression for these genes in ductal carcinomas of the breast.

Introduction

Human breast carcinomas most frequently evolve from the epithelial lining of the terminal mammary ducts as DCIS³ that may progressively become invasive and ultimately metastatic (1). The transformation of normal mammary epithelial cells into a carcinoma and the subsequent progression to invasion and metastasis involve the accumulation of numerous genetic "hits," including the activation or amplification of dominant oncogenes and the deletion or inactivating mutation of key tumor suppressor genes (2). It has recently become evident that tumor suppressor genes may also be transcriptionally silenced in association with aberrant promoter-region CpG island methylation (3, 4).

The *ER α* gene and the *E-cad* gene have been implicated frequently in the initiation and/or progression of human breast cancer. Loss of expression of either gene has been associated with poorly differentiated tumors and poorer prognosis (5-10). Furthermore, several studies have reported an association between E-cad and ER expression in breast tumors (7, 9, 10). In the case of E-cad, classical mutations and deletions may play a role in loss of gene expression (11, 12). However, loss of E-cad expression, as well as loss of ER expression, has also been associated with aberrant 5' CpG island methylation in breast

cancer cell lines and primary human breast tumors (13-18). It is currently unclear when, during malignant progression of ductal breast carcinoma, aberrant methylation of these CpG islands begins and whether the incidence of such methylation tracks with advancing disease for either or both genes. Therefore, we have evaluated a total of 111 ductal breast carcinomas for the incidence of CpG island methylation for these two key suppressor genes in *in situ*, invasive, and metastatic lesions. Our results indicate that the aberrant methylation of either CpG island begins before invasion and increases with metastatic progression. Coincident methylation of both CpG islands also increases with progression, suggesting that the malignant progression of ductal breast carcinoma involves the accumulation of multiple epigenetic "hits."

Materials and Methods

Tissue Samples. A total of 111 human breast tumor samples identified as DCIS, IDC, and LA/MDC were obtained from the Department of Pathology at Johns Hopkins University School of Medicine and from the Department of Pathology at Vanderbilt University Hospital. Seventy-five % of the LA/MDC samples were derived from lymph nodes, whereas the remaining 25% consisted of samples from a variety of sites including the chest wall, bone, and lung. Two cases of recurrent breast cancer after lumpectomy were also included. In the case of DCIS, samples were carefully microdissected prior to DNA isolation to avoid sample contamination with other cells. A portion of these tumors had been analyzed previously for E-cad methylation (18). The preliminary results of that study prompted us to expand the tumor sample pool and to include analysis of a second gene (ER). None of the results for ER methylation in this tumor set have been reported previously.

Cell Lines. Two human breast cancer cell lines were used as controls for methylation assays. MCF-7 cells express both ER and E-cad, and the CpG islands of both genes are unmethylated in this cell line. The MDA-MB-231 cell line exhibits extensive methylation of the *ER* and *E-cad* gene CpG islands, and the cells lack expression of the two genes at both the mRNA and protein level (13, 14, 16, 17). The cell lines were routinely maintained as described previously (13).

DNA Isolation. DNA was isolated from the tissues and cell lines as described previously (14, 16). DNA samples were labeled with a coded identification number so that MSP analysis could be performed and analyzed without knowledge of the sample origin.

MSP. ER and E-cad 5' CpG island MSP was performed on sodium bisulfite-treated DNA as described previously (15, 17). The ER primers (primer set #5; Ref. 15) target a region of the gene about 400 bp downstream from the transcription start site near a *NotI* site. MSP primers spanning the transcription start site of E-cad were described previously as Island 3 (17). Earlier studies showed that methylation in the regions targeted by these primer sets correlated best with loss of gene expression (15, 17). A fraction of the tumor samples in the current study were also analyzed with additional MSP primer sets for the two genes to verify the density of CpG island methylation in these tumors. For many samples, the methylation status of ER and E-cad was assessed concurrently by including primers for both genes in the same reaction (termed duplex PCR).

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² To whom requests for reprints should be addressed, at Johns Hopkins Oncology Center, Breast Cancer Research Program, 1650 Orleans Street, Room 409, Baltimore, MD 21231.

³ The abbreviations used are: DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; E-cad, E-cadherin; MSP, methylation-specific PCR; IDC, invasive ductal carcinoma; MDC, metastatic ductal carcinoma; LA, locally advanced.

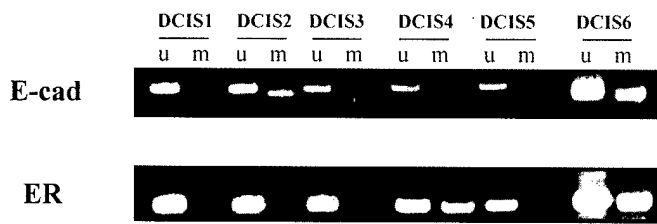


Fig. 1. MSP analysis of the E-cad and ER CpG islands in human breast cancers (DCIS). MSP was used to assess the methylation status of each CpG island. Representative results from six DCIS lesions are shown. The two genes were analyzed concurrently by performing duplex PCR reactions that contained primers for both islands. *u*, primers specific for unmethylated DNA; *m*, primers specific for methylated DNA.

Statistical Analysis. Any tumor sample that reliably yielded a PCR product in the methylated reaction visible by ethidium bromide staining was considered positive for CpG methylation. The Mantel-Haenszel χ^2 test for trend was applied to 3-by-2 tables of tumor type *versus* methylation (yes/no) to assess the change in percentage of methylation with increasing tumor progression. Then each pair of tumor types was compared using logistic regression. Significance was set at $P < 0.05$.

Results and Discussion

The ER and the homotypic cell:cell adhesion molecule, E-cad, both play a role in maintaining the normal differentiated state of the mammary gland epithelium (6, 19). Loss of the ER during breast cancer progression is associated with poorer histological differentiation, higher growth fraction, and poorer clinical outcome and may represent a key mechanism facilitating hormone resistance (5, 20). Similarly, loss of E-cad expression has been repeatedly associated with loss of differentiation, increased invasive and metastatic potential, and decreased patient survival (6, 9, 11, 21). The transcriptional silencing of both ER and E-cad in human breast cancer has been associated with aberrant promoter-region CpG island hypermethylation. In addition, treatment of human breast cancer cell lines lacking ER and/or E-cad with DNA methyltransferase inhibitor (5-deoxyazacytidine) elicits CpG island demethylation and re-expression of E-cad and ER protein, thereby indicating that aberrant methylation of these CpG islands plays a substantial role in suppressing transcription of these two key suppressor genes in breast cancer cells (16, 22).

Because expression of both ER and E-cad is lost in association with aberrant 5' CpG island methylation during breast tumorigenesis, we sought to define the stage of breast tumor progression at which the hypermethylation of these two CpG islands begins and whether such methylation tracks with advancing disease. We analyzed a total of 111

ductal breast carcinomas comprised of *in situ* lesions (DCIS), invasive, and metastatic cancers by MSP (23).

The Incidence of CpG Island Methylation Increases with Tumor Progression. MSP has been used previously to detect aberrant DNA methylation of several genes, including *ER* and *E-cad*, in human cancers (15, 17, 22). Neither gene is methylated in normal breast epithelia (13–17). However, methylation of the two CpG islands was evident in all tumor stages and showed remarkably similar increases during progression from DCIS to metastatic tumors. Methylation of the *ER* gene was evident in 34% (12 of 35) of DCIS lesions, whereas E-cad methylation was evident in 31% (11 of 35). Coincident methylation was present in only 21% of these DCIS lesions. (Fig. 1 and summarized in Table 1). In invasive and metastatic ductal carcinomas (IDC or MDC), the incidence of methylation markedly increased relative to the DCIS lesions. Twenty-five of 48 (52%) IDC samples showed methylation of the ER or E-cad 5' CpG island (Fig. 2; Table 1). Of these 48 samples, 18 (38%) showed distinct, coincident methylation of both CpG islands. Of the locally advanced and metastatic tumor samples, nearly 60% exhibited methylation for each of the CpG islands (Fig. 2; Table 1), whereas coincident methylation of both CpG islands was apparent in 50% (14 of 28) of these samples.

These data indicate that the epigenetic inactivation of either gene may occur early, prior to invasion, but increases as cells acquire invasiveness and metastatic potential. The Mantel-Haenszel χ^2 test for trend demonstrated that the trend toward increased methylation during progression was statistically significant for each gene ($P < 0.05$; Table 1). Furthermore, pair-wise comparison of the three tumor stages demonstrated that the incidence of methylation in metastatic tumors was significantly higher than in DCIS for both ER (odds ratio, 2.96; $P = 0.039$) and E-cad (odds ratio, 3.37; $P = 0.022$). The incidence of methylation in IDC samples was not statistically different from the other two categories, however.

The trend toward increasing coincident methylation of the two genes during progression was also statistically significant ($P = 0.013$;

Table 1. Incidence of CpG island methylation for ER and E-cad genes in human breast tumors

Tumor type	% ER methylation	% E-cad methylation	% ER and E-cad methylation	% ER or E-cad methylation
All	49% (54/111)	48% (53/111)	35% (39/111)	61% (68/111)
DCIS	34% (12/35)	31% (11/35)	21% (7/35)	46% (16/35)
IDC	52% (25/48)	52% (25/48)	38% (18/48)	67% (32/48)
LA/MDC	61% (17/28)	61% (17/28)	50% (14/28)	71% (20/28)
<i>P</i> (trend) M-H ^a χ^2	0.034	0.019	0.013	0.032

^a M-H, Mantel-Haenszel.

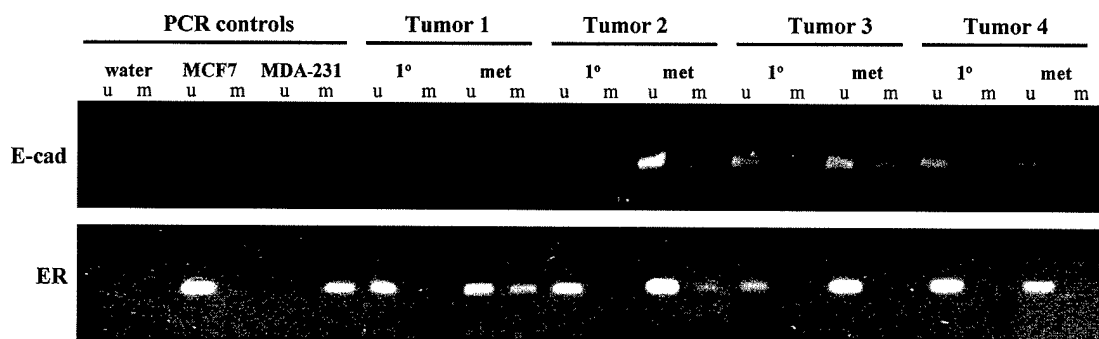


Fig. 2. MSP analysis of the E-cad and ER CpG islands in human breast cancers (IDC and MDC). Representative results from four primary (*1°*)-metastatic (*met*) pairs are shown. MSP reactions for E-cad and ER were run and analyzed separately. Metastatic sites were as follows: 1, bone; 2, chest wall; 3, axillary lymph node; 4, supraclavicular lymph node. *u*, primers specific for unmethylated DNA; *m*, primers specific for methylated DNA. Water served as a negative control, and DNA from MCF-7 and MDA-MB-231 cells served as positive controls for the unmethylated and methylated reactions, respectively.

Table 1). Thus, the frequency of coincident methylation of both CpG islands increases with advancing disease, suggesting that malignant progression of ductal breast carcinoma involves the accumulation of multiple epigenetic "hits." However, it is important to note that the similarity in the trends for ER and E-cad methylation was not attributable to complete coincidence of methylation for the two genes. At every stage of progression, the rate of coincident methylation was lower than the incidence of methylation for each individual gene (Table 1). Overall, ~25% of the samples analyzed showed methylation of either ER or E-cad, but not both. These results imply that aberrant methylation of these CpG islands does not simply reflect a generalized increase in CpG island methylation but may reflect a more specific selection process targeting key suppressor genes.

CpG Island Methylation Is Heterogeneous in Breast Tumors.

In all samples harboring methylation, unmethylated alleles were invariably also evident (Figs. 1 and 2). For the IDC and LA/MDC samples, which were not microdissected, these unmethylated alleles may reflect the contribution from normal cells in the sample. Alternatively, these alleles may be derived from cancer cells that harbored only unmethylated copies of the E-cad and ER CpG islands. However, this same heterogeneous pattern was evident in the methylated DCIS samples, which were carefully microdissected, suggesting that methylation of these CpG islands in these tumors is heterogeneous. Interestingly, expression studies have routinely revealed that the loss of both E-cad and ER exhibits distinct heterogeneity in ductal breast carcinomas (6, 9, 10, 24). In addition our earlier studies have demonstrated that heterogeneity of both ER (15) and E-cad (18) methylation is associated with heterogeneity of protein expression. Limitations in our ability to recover the tissue specimens associated with these DNA samples (especially those derived from *in situ* lesions) precluded a simultaneous evaluation in this study. However, it seems likely that the heterogeneous patterns of CpG island methylation parallel the heterogeneous loss of E-cad and ER expression in these tumors.

In summary, these data indicate that the malignant progression of human ductal breast carcinomas involves a heterogeneous pattern of methylation for both the ER and E-cad 5' CpG islands that begins prior to the acquisition of invasiveness and increases for each CpG island with advancing disease. In the case of E-cad, these results are particularly striking because loss of E-cad expression is generally associated with the acquisition of invasive or metastatic potential rather than the earlier stages of tumorigenesis. Finally, the increase in the coincident methylation of both CpG islands suggests that malignant progression of human breast cancer involves not only the well-documented accumulation of genetic "hits" but also an accumulation of epigenetic "hits" that contribute to the diminished expression of key tumor suppressor genes like *ER* and *E-cad*.

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Role of DNA Methylation and Histone Acetylation in Steroid Receptor Expression in Breast Cancer¹

Lan Yan,² Xiaowei Yang,² and Nancy E. Davidson^{2,3}

DNA methylation is an epigenetic modification that is associated with transcriptional silencing of gene expression in mammalian cells. Hypermethylation of the promoter CpG islands contributes to the loss of gene function of several tumor related genes, including estrogen receptor α (ER α) and progesterone receptor (PR). Gene expression patterns are also heavily influenced by changes in chromatin structure during transcription. Indeed both the predominant mammalian DNA methyltransferase (DNMT1), and the histone deacetylases (HDACs) play crucial roles in maintaining transcriptionally repressive chromatin by forming suppressive complexes at replication foci. These new findings suggest that epigenetic changes might play a crucial role in gene inactivation in breast cancer. Further, inhibition of DNA methylation and histone deacetylation might be a therapeutic strategy in breast cancer, especially for those cancers with ER and PR negative phenotypes.

← change all
DNMT1 "to
DNMT1"

KEY WORDS: Breast cancer; DNA methylation; histone acetylation; steroid receptor.

INTRODUCTION

DNA Methylation and Cancer-Related Gene Expression

Approximately 3–5% of the cytosine residues in the human genome are methylated (1). Seventy to eighty percent of these 5-methylcytosines are located in clusters of CpG dinucleotides, termed CpG islands, typically found in the 5' promoter region and first exon of certain genes (2). This methylation modification is

essential not only in mammalian development, but also in epigenetic regulation of gene expression, including genomic imprinting and X chromosome inactivation (3–5). Methylation of a CpG island is frequently associated with loss of expression of the target gene. Recent studies have provoked increasing interest in the role of DNA hypermethylation in tumorigenesis through its ability to alter the expression of tumor suppressor genes (6).

The DNA methylation reaction is catalyzed by a family of DNA methyltransferases (DNMTs) by use of the universal methyl donor, S-adenosyl-methionine. Three distinct DNMTs have been identified in mammalian cells so far, namely DNMT1, DNMT2 and DNMT3. DNMT1 is constitutively expressed in mammalian cells and its function is to maintain the identical methylation pattern after DNA replication (7). That this function is critical is suggested by the finding that Dnmt1 gene knockout mice demonstrated global DNA demethylation and embryonic lethality (8, 9). On the other hand, somatic knockout of the DNMT1 gene in human colorectal carcinoma cells led to markedly decreased cellular DNMT activity, but only a 20% decrease in overall genomic methylation was observed,

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² Johns Hopkins Oncology Center, 1650 Orleans Street, Baltimore, Maryland 21231.

³ To whom correspondence should be addressed. e-mail: davidna@jhmi.edu

⁴ Abbreviations: estrogen receptor α (ER); progesterone receptor (PR); DNA methyltransferase (DNMT); histone deacetylase (HDAC); histone acetyltransferase (HAT); methylation-specific polymerase chain reaction (MSP); 5-azacytidine (5aza); 5-aza-2'-deoxycytidine (deoxyC); trichostatin (TSA); retinoic acid (RA); acute promyelocytic leukemia (APL).

mainly at juxtacentromeric satellites. Most of the gene-specific CpG islands analyzed remained fully methylated and silenced (10). These results suggest that DMT1 might not be the only critical enzyme in maintaining human genome methylation status, and its exact role in mammalian development and gene transcription regulation needs to be further elucidated. DMT2 is expressed at low levels in most adult tissues examined but recombinant mouse DMT2 has no DNA methyltransferase activity (11–13). Thus the importance of DMT2 is not clear. DMT3 does have *de novo* methyltransferase activity and is highly expressed in embryonic stem cells. It has two isoforms, namely DMT3A and DMT3B. Unlike DMT2, recombinant mouse DMT3A and 3B can methylate cytosine residues in various native and synthetic DNA templates (14). DMT3A expression is ubiquitous. It can be readily detected in most adult tissues, whereas DMT3B expression is highly elevated in several tumor cell lines, including leukemia, melanoma, and colorectal cancer cell lines, to a level comparable to DMT1 in these cell lines (15). How other DMT family members, such as DMT2, DMT3A, DMT3B, or other novel methylating proteins contribute to CpG methylation during normal development or tumorigenesis needs to be addressed in future study.

Methylation patterns in tumors are perplexing. In general, the level of 5-methylcytosine in tumor cells is lower than that in normal cells (16, 17). However, this global hypomethylation is observed in conjunction with regional hypermethylation at CpG islands, in turn associated with transcriptional inactivation of an increasing number of cancer-related genes. Thus far, a variety of genes, including tumor suppressor genes, DNA mismatch repair genes, cell cycle related genes, hormone receptors and tissue or cell adhesion molecules have been reported to be regulated by promoter CpG methylation. The inactivation of gene expression by aberrant CpG island hypermethylation is supported by studies using the demethylating agents, 5-azacytidine (5aza) and 5-aza-2'-deoxycytidine (deoxyC). Application of these agents to several cancer cell lines has been shown to demethylate CpG islands and reactivate expression of the previously silenced genes. In addition, recent studies suggest that hypermethylation of a CpG island not only can silence the gene it regulates, but also facilitate genetic alterations in tumor progression. P16^{INK4a} (18–21), MLH1 (22, 23), and GSTP1 (24, 25) are the best examples to support this "facilitation hypothesis." Accumulating evidence suggests that hypermethylation of these three genes occurs in the early stages

of tumorigenesis, predisposing cells to later genetic instability, which then contributes to tumor progression. Thus it appears that both epigenetic and genetic changes can contribute to the carcinogenic process.

Histone Acetylation, Chromatin Stability and Gene Expression

Chromatin structure and gene transcription are regulated partially by histone acetylation. During the S phase of the cell cycle, histone acetyltransferases (HAT) transfer an acetyl moiety to the ϵ -amino group of the amino acid, lysine, on histones, leading to neutralization of the positive charge and reduced affinity of histone for DNA. The ultimate consequence of this acetylation modification is the transformation of a tight-coiled inactive chromatin structure into a loose, transcriptionally active one (26). This process is reversed by histone deacetylation mediated by histone deacetylases (HDAC) during the G2 phase of the cell cycle. Deacetylated histones expose their positive charges to negatively charged DNA, leading to a condensed inactive chromatin structure. HDAC seems to play a role in gene silencing as well as in transcriptional activation (27, 28). In addition, HDACs are also involved in cell differentiation, cell-cycle arrest, apoptosis, chemosensitization, radiosensitization, antitumor effects and up-regulation of MHC class I (29). Three families of HDAC have been characterized so far, *S. cerevisiae* RPD3p (mammalian equivalents HDACs 1, 2 and 3), *S. cerevisiae* HDA1p (mammalian equivalents HDACs 4, 5 and 6), and *Zea mays* HD2 (29). The understanding of these deacetylases, especially the roles of HDAC1 and 2 in transcription regulation, is expanding.

Like DNA methylation, histone acetylation has recently been associated with tumorigenesis. For example, HAT CBP (a CREB-binding protein) is fused to the MLL gene in cases of acute leukemia or myelodysplasia secondary to therapy with drugs targeting DNA topoisomerase II (30, 31). This novel fusion protein leads to dysregulated histone acetylation that might contribute to the leukemogenic process (31). Histone acetylation may also play a role in breast cancer. The carboxy-terminal domains of proteins encoded by the breast cancer susceptibility genes, BRCA1 and BRCA2, can interact with Rb and Rb binding proteins as well as HDAC1 and HDAC2 (32). It has been proposed that the recruitment of HDACs by BRCA proteins could cause gene deregulation in the progression of hereditary breast

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cancer (33, 34). For additional discussion of BRCA1 and BRCA2, see in this issue the article by Mielnicki *et al.* (35).

Molecular Mechanisms for the Epigenetic Regulation of Gene Expression

It appears that both DNA methylation and histone acetylation are involved in epigenetic regulation of gene expression in normal mammalian development as well as in tumorigenesis through their ability to modify chromatin structure during transcription. It also has been demonstrated that methylated inactive genes are associated with underacetylated histones whereas unmethylated active genes are linked to hyperacetylated histones (36, 37). These observations raise the following questions: How do DNA methylation and histone acetylation inhibit transcription? Do these processes interact with each other or act separately during this process? Are there any other factors involved?

Recent studies suggested that DMT1 can form a repressive transcription complex at replication foci with HDAC2 and a newly identified protein, DMAP1 (DMT1 associated protein), at the noncatalytic amino terminus of DMT1. DMAP1 has intrinsic transcription repressive activity and interacts with DMT1 throughout S phase, whereas HDAC2 joins the complex only during late S phase. These findings indicate that there is a connection between DMT1 and HDAC, and histone deacetylation mediated by HDAC occurs after DNA replication (38). In another study, DMT1 has been shown to form a transcription repressive complex with Rb, E2F1 and HDAC1 (39). Therefore, DMT1, in addition to its function of maintaining CpG methylation, has direct inhibitory effects on transcription by formation of a repressive complex during DNA replication.

DNA METHYLATION AND HISTONE ACETYLATION IN REGULATION OF ER AND PR EXPRESSION IN BREAST CANCER

ER and PR Expression in Normal Mammary Gland and Breast Cancer

Estrogen and progesterone and their receptors play important roles in the development and function of the mammary gland as well as other female reproductive organs. At puberty, the hypothalamus and pituitary gland stimulate the ovary to secrete estrogen

that is responsible for the proliferation of the mammary epithelial cells and elongation and branching of mammary ducts. Progesterone is synthesized by the corpus luteum and placenta to promote the growth of mammary lobuloalveolar structures, especially during pregnancy in preparation for milk secretion after parturition (40). Although estrogen and progesterone receptors are expressed at very low basal levels in normal human mammary epithelial cells after a woman reaches sexual maturity (41, 42), they seem to play an important role in the initiation, progression and prognosis of breast cancer. They are also good predictors for endocrine therapy (43). Approximately two-thirds of breast cancers express ER transcript and synthesize ER protein at levels higher than in normal breast tissues and half of these ER-positive tumors express both ER and PR protein (ER+/PR+) (43). These tumors tend to be more differentiated and are more responsive to hormonal therapies. One-quarter of all breast cancers lack both ER and PR (ER-/PR-). In general, these tumors are associated with poorer differentiation, higher growth fraction, and worse clinical outcome than ER+/PR+ breast cancer (43). These cancers are estrogen-independent and rarely respond to hormonal therapies. Of note, up to one-third of initially ER+ tumors can evolve to an ER- status during tumor progression and become resistant to hormone therapy (44).

ER Methylation and DMT Expression in Breast Cancer

The human ER cDNA and gene were cloned in 1986 (45-47). This 140 kb ER gene has eight exons and is located on chromosome 6q25.1. Since then, efforts have been devoted to the possible molecular genetic mechanisms for the loss of ER expression in breast cancers. Genetic changes, such as insertions, deletions, rearrangements, or point mutations of the ER gene were extensively sought in breast cancer cells since these in-frame or out-of-frame sequence alterations would lead to either absence of ER expression or expression of an unstable mutant that might be degraded shortly after protein translation by the ubiquitin protease system. Although several sequence mutations for the ER gene were identified and shown to be related to decreased ER expression and estradiol binding, they are uncommon and cannot explain the loss of ER expression in a significant fraction of human breast cancers (40). This finding suggests that mechanisms other than genetic changes might

Table I. Frequency of ER Gene Methylation of Primary Breast Human Tissues by Use of Southern Blot Analysis^a

Genotype	ER gene methylation at the <i>NotI</i> site
ER+/PR+ tumor	0/29 (0%)
ER+/PR- tumor	0/24 (0%)
ER-/PR- tumor	9/39 (25%)
ER-/PR- metastases	2/2 (100%)
Normal breast	0/9 (0%)

^aAdapted from Ref. (51).

also contribute to the loss of ER expression in breast cancer.

One extensively studied epigenetic mechanism that might lead to loss of ER expression is CpG methylation. Interestingly, the ER gene has a CpG island in its promoter and first exon regions marked by a clustering of sites for methylation-sensitive restriction endonucleases (46, 48) (Fig. 1). In addition, absence of ER protein expression in human breast cancer specimens is frequently associated with loss of ER transcript (49). Those findings raise the possibility that absence of ER expression is associated with ER gene hypermethylation.

We have addressed this hypothesis using human breast cancer cell lines as a model system. Ottaviano *et al.* (50) reported that ER- human breast cancer cells displayed extensive methylation of the CpG island in the 5' promoter region of the estrogen

receptor gene, in association with silencing of ER gene expression. The 6.3 kb ER mRNA transcript was detected easily in three ER+ cell lines (MCF-7, T47D and ZR75-1), but none was detected in three ER- cell lines (Hs578t, MDA-MB-231 and MDA-MB-468). Southern blot analysis using methylation-sensitive restriction enzymes showed that the ER CpG island was methylated at a critical *NotI* site in multiple ER-negative breast cancer cell lines. As expected for an autosomal gene, the ER gene was unmethylated at the *NotI* site in the CpG island in all normal tissues studied including breast epithelium. In addition, the ER gene was unmethylated at multiple other restriction sites in its CpG island in all ER+ cell lines studied, but these sites were frequently methylated in ER-negative cell lines. Of note, the major DNA methyltransferase, DMT1, was highly expressed in ER- cell lines at the mRNA level as well as the protein and enzymatic activity levels. Careful assessment of the relationship between ER expression and DMT1 showed that DMT1 protein expression was correlated with S-phase fraction in ER-positive cell lines, but not in ER-negative cell lines. Thus DMT1 expression was elevated in ER-negative cell lines and was unlinked from cell cycle regulation (51).

A key question is whether these tissue culture findings have any relevance to human breast cancer. Using Southern blot analysis, Lapidus *et al.* (52) reported that the ER CpG island was methylated at the *NotI* restriction site in 9 of 39 (25%) of

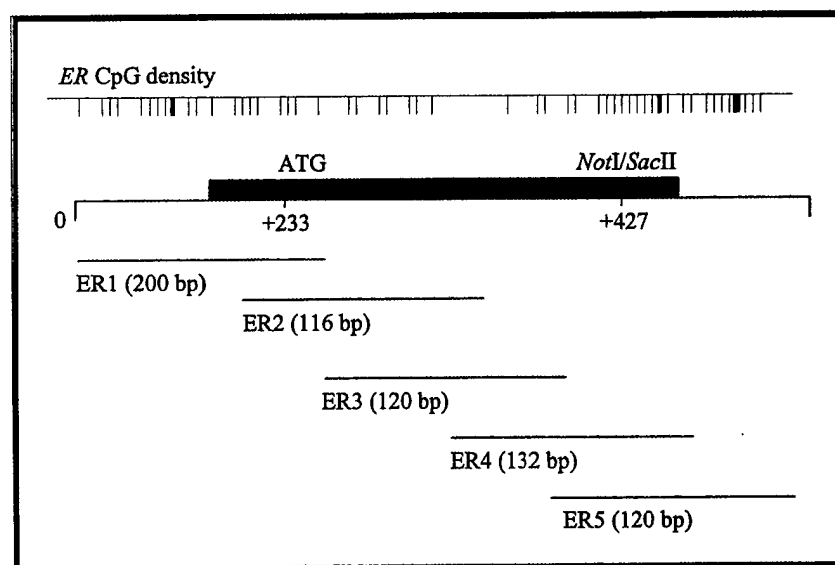


Fig. 1. Map of ER CpG island and locations of PCR products generated by use of specific ER MSP primers. Box represents exon 1 of ER gene.

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primary ER- breast cancers but remained unmethylated in 53 ER+ breast cancers and 9 normal breast specimens (Table I). The possible explanations for the different frequency of ER methylation in cell lines and tissues include the presence of normal cells in primary cancers, heterogeneity of ER expression within breast cancers, methylation of other sites within the CpG island which were not examined, and the relative insensitivity of Southern blot analysis. These possibilities have been partially addressed through the development of a PCR-based assay termed methylation-specific PCR or MSP, a technique designed to analyze methylation of CpG dinucleotides across the entire ER CpG island (Fig. 1). By use of MSP, normal breast tissues and ER+ cell lines shown only an unmethylated product across all 5 primer sets used, whereas ER- cell lines shown methylated products across much of the island (52) (Table II). To confirm that ER CpG methylation could also be detected by MSP in primary human breast cancers, a panel of 33 primary breast cancers of known receptor status was studied. All 33 tumors showed at least some degree of methylation at one or more primer sites. By use of a semiquantitative scoring system for elevated CpG density, the percentage of tumors displaying substantial methylation can be calculated for each tumor set defined by receptor status. As shown in Table III, about one-third of ER+/PR+ tumors showed methylation, whereas the percentage increased to 100% in ER-/PR- tumors (53). Taken together, data derived from both our study and others (54) support a correlation between absence of ER

Table III. ER Gene Methylation of Primary Human Primary Breast Cancers by Use of MSP

Receptor status	No. of tumors	No. of methylated tumors (percentage)
ER+/PR+	11	4 (36%)
ER+/PR-	11	8 (72%)
ER-/PR-	11	11 (100%)

^aAdapted from Ref. (53).

expression and aberrant CpG island methylation of ER gene. Whether methylation status actually acts solely or partially to silence ER transcription is a key question.

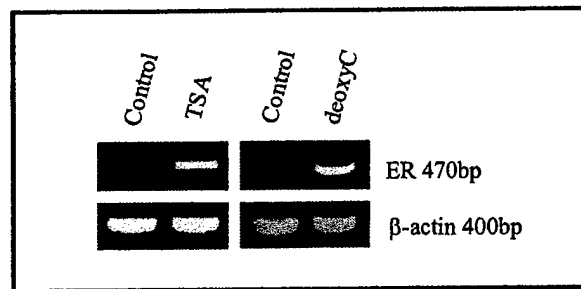
Demethylation of the ER Gene Results in Re-Expression of ER

silencing of is
 If ER expression is a function of CpG island methylation of ER gene, it is possible that demethylation should result in the re-expression of ER in ER- breast cancer cells. In accord with this prediction, treatment of the ER- human breast cancer cell line, MDA-MB-231, with the demethylating agents, 5-aza and deoxyC, led to re-expression of ER (55). After treatment with either drug, the DNA from these cells became partially demethylated at several methylation-sensitive restriction enzyme sites, including HhaI, NotI, and SacII, within the ER promoter CpG island. Demethylation correlated with re-expression of the ER gene as detected by reverse transcriptase-PCR (RT-PCR) as demonstrated in Fig. 2 and Western Blot analysis. Functional activity of this ER protein was examined in two ways. First, its ability to activate expression of an endogenous estrogen-inducible gene, PR, was assessed. PR mRNA and protein were induced by estrogen treatment of deoxyC treated MDA-MB-231 or Hs578t cells. Second, the ability of the deoxyC-induced ER to transduce expression of a luciferase reporter gene linked to an estrogen response element (ERE) was assessed. MDA-MB-231 cells were stably transfected with an ERE-tk-luciferase construct with the expectation that deoxyC-induced ER expression would increase luciferase expression. As expected, increased luciferase activity (1.5-5.6 fold in 10 independent clones) over the background level of the untreated cells was observed (54). These results provide powerful evidence that DNA methylation of the ER CpG island plays a functional role in suppression of ER expression in ER- breast cancer cells.

Table II. ER Gene Methylation of Human Breast Cancer Lines by Use of MSP^a

Source	ER1	ER2	ER3	ER4	ER5
Normal breast	-	-	-	-	-
Epithelial cells					
ER+ cell lines					
MCF-7	-	-	-	-	-
T47D	-	-	-	-	-
ZR-75-1	-	-	-	-	-
ER- cell lines					
MDA-MB-231	+	+	+	+	+
Hs578t	+	+	±	+	+
MDA-MB-435	±	-	±	+	±
MDA-MB-468	+	±	±	±	±
MCF-7/Adr	+	+	±	±	±

^aThe results of normal breast epithelial cells are representative of five normal breast epithelial samples. "+": methylated; "-": unmethylated; "±": heterogeneous, both methylated and unmethylated PCR products. Adapted from Ref. (53).



Author: please check?
Fig. 2. RT-PCR analysis of ER mRNA re-expression by TSA (100 ng/ml \times 2 days) or deoxyC (2.5 μ M \times 4 days) treatment in ER- MB-MDA-231 cells. β -actin RT-PCR product was used as a control for the amount of RNA used.

DNA Methylation and PR Gene Expression

As discussed in the Introduction, one quarter to one-third of breast cancers lack both ER and PR, whereas another one-third are ER+ but PR-. It also has been demonstrated that the expression of PR requires ER as a transcriptional activator (56). Therefore, the presence of PR in ER+ tumors may be a better predictor of hormone responsiveness than ER alone (43). Interestingly, the PR gene also has a typical CpG island in its first exon. The PR CpG island is 1 kb in size, has 70% GC content, and contains a wide array of methylation-sensitive restriction sites. Southern analyses targeted at three methylation-sensitive restriction sites in the PR CpG island showed that these sites are not methylated in normal breast tissue specimens but are hypermethylated in 40% of primary tumors that lack PR protein expression (52). Thus the question of why the PR hypermethylation could be responsible for a lack of PR expression was studied in MDA-MB-231 cells which lack both ER and PR (57). Treatment of these cells with the demethylating agent, deoxyC, led to partial demethylation of the ER and PR CpG islands and was associated with induction of PR protein expression as demonstrated by Western blot analysis. However, Ferguson *et al.* (57) Using MDA-MB-231 cells stably transfected with an inducible expression vector for ER, found that the induction of PR gene expression by ligand-bound ER did not require demethylation of the PR CpG island. In addition, induction of PR transcription was inhibited by blocking the interaction of ER with SRC-1A, a coactivator of ER function. These results suggested that a transcription factor with the potential to remodel heterochromatin (ER) could activate PR gene expression without altering the methylation status of

the CpG island in the PR gene. These results raise the possibility that demethylation and histone acetylation are distinct but complementary mechanisms for destabilizing heterochromatin and activating gene transcription (58).

Histone Acetylation and ER Expression

As discussed earlier, histone acetylation and deacetylation are intimately involved in chromatin structure changes during transcription. In addition, new models of transcriptional suppressive complexes suggest that HDACs and DMT1 are in direct contact. Recent studies indicate that silencing of a gene by methylation involves the generation of an inactive chromatin structure in which methyl CpG-binding protein (MeCP2) and the adapter protein, mSin3A, recruit histone deacetylase (59, 60). The deacetylation of lysine groups of histones H3 and H4 allows ionic interactions between positively charged lysines and negatively charged DNA, resulting in a more compact nucleosome structure that limits gene activation. The question emerges of whether epigenetic modification of histone acetylation alone could result in re-expression of genes that are inhibited by the transcriptional suppressive complex, or whether both histone acetylation and CpG demethylation are required for this event.

The answers to this question have been variable. For example, HDAC inhibitors like trichostatin (TSA) or phenylbutyrate alone restored retinoic acid receptor α (RAR α) expression in retinoic acid (RA) resistant acute promyelocytic leukemia (APL) cell lines as demonstrated by the differentiation of APL cells in the presence of RA (61–63). However, hypermethylated genes such as MLH1, TIMP3, INK4B (p15) and INK4a (p16) could not be transcriptionally reactivated by TSA alone, but could be re-expressed in colon cancer cells with a combination of TSA and the demethylating agent, 5-aza (58).

To study the role of histone acetylation in ER expression, ER- MDA-MB-231 cells were treated with HDAC inhibitor, TSA. A time- and dose-dependent reactivation of ER mRNA expression was observed (64). As shown in Fig. 2, TSA alone at 100 ng/ml for 2 days could reactivate ER expression as well as deoxyC in ER- MDA-MB-231 breast cancer cell line. By use of quantitative competitive PCR assay, an increase of 5.5 fold of ER transcript expression was reported (64). MSP analysis of the ER CpG island showed no

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change in its methylation after TSA treatment, suggesting that TSA's effects on ER re-expression were not associated with a change in methylation status. This finding was confirmed via a more rigorous assay, bisulfite genomic sequencing of the ER promoter region, a technique which permits the direct examination of the methylation status of each CpG dinucleotide within the promoter region. Multiple clones of control and TSA-treated cells were examined and did not show any change in the methylation status of the ER gene with TSA. A logical extension of these studies will be to examine the effect of combinations of demethylating agents and HDAC inhibitors on expression of ER as well as other methylated genes in breast cancer cells.

DNA Methylation and HDAC Inhibitors as Therapeutic Strategies in Breast Cancer

Abundant evidence indicates that epigenetic mechanisms play pivotal roles in the pathogenesis of cancer. Both DNA methylation and histone acetylation have been shown to be involved in the regulation of expression of cancer-related genes. The studies in breast cancer summarized earlier demonstrated that both demethylating agents and HDAC inhibitors could re-activate expression of a number of important growth regulatory genes in several breast cancer cell lines, thus raising the possibility of using these types of agents as therapeutic strategies for breast cancer.

Two ~~HDAC inhibitors~~ demethylating agents, 5aza and deoxyC, have been studied clinically, particularly in patients with hemoglobinopathies. For example, 5aza has been successfully used to treat patients with β -thalassemia. It has been reported that loss of expression of the γ -globin gene is associated with methylation of its promoter (65). Infusion of 5aza into β -thalassemia patients led to an increase of γ -globin expression and amelioration of anemia (66). Similarly, 5aza can increase HbF production and reduce anemia in sickle cell disease (67). In addition, in an ongoing trial, the efficacy of treatment of nasopharyngeal tumors with 5aza is being evaluated. The promoter of one of the Epstein Barr Virus-related proteins is heavily methylated in nasopharyngeal carcinoma. It has been proposed that demethylation of this promoter could lead to reactivation of an EBV latency protein that could then become the target for a secondary immune therapy (R. Ambinder, Johns Hopkins Oncology Center, personal communication). Another possible

approach is to use antisense oligonucleotides against DNA methyltransferase to inhibit DNA methylation. It has been shown that antisense oligonucleotides have *in vitro* and *in vivo* antitumor activity and a clinical trial of this approach is in progress (68, 69).

Several HDAC inhibitors have the potential to be used as anti-cancer agents (29). The most plausible and extensively studied are butyrate, trichostatin (TSA), and trapoxin (TPX) and their relatives. The IC_{50} of butyrate for HDAC is in the millimolar range, whereas TSA and TPX act at low nanomolar concentrations. One triglyceride analogue of the short-chain fatty acid butyrate, tributyrin, has been reported to induce transcription of p21, arrest of cells in G2/M and apoptosis in MCF-7 human breast cancer cell lines. (70). Two butyrate derivatives, phenylbutyrate (PB) and phenylacetate (PA), have powerful growth inhibitory effects on several cell types including ovarian and prostate cancers (71). A clinical trial with sodium phenylacetate in patients with thalassemia showed an increase of fetal hemoglobin in some patients (72). Other HDAC inhibitors under study include FR901228, oxamflatin, depudecin, suberoylanilide hydroxamic acid (SAHA), m-carboxycinnamic acid bishydroxamide (CBHA) and apicidin.

A potential strategy is to combine demethylating agents and HDAC inhibitors. From a clinical point-of-view, the potential benefits of this combination could include lowering of drug concentration requirements, shorter periods of drug exposure, and lower toxicity, as well as the possibility of synergy. For example, synergistic effects were observed in a combination study with both a demethylating agent and a HDAC inhibitor in colon cancer cells. If cancer cells were treated with 100 nM 5aza (a dose that had little effect on gene expression if used alone) for 24 hours followed by 300 nM TSA for 24 hours, (again a treatment that was ineffective alone), re-expression of several genes was obvious (58). A key question in development of this strategy will be the specificity and selectivity of gene re-expression patterns and how they might be exploited clinically.

Finally, it should be noted that demethylation and histone acetylation are only two mechanisms for epigenetic regulation of target genes like ER. Other transcriptional modulators might also contribute to the epigenetic regulation of gene expression. For example, it was reported that AP2 transcription factor can trans-activate the cloned human ER α promoter in MDA-MB-231 cells (73).

demethylating agents

CONCLUSIONS

The evidence for epigenetic regulation of gene expression in tumorigenesis is accumulating. Two components, demethylation of C5-methylcytosines at the CpG island within the promoter region of target genes and histone acetylation of chromatin, have been studied extensively by use of demethylating agents and HDAC inhibitors. *ER* and *PR*, two critical genes in breast cancer development and treatment, have been reported to be densely methylated in ER- but not ER+ human breast cancer cell lines. Treatment of ER- human breast cancer cells with a demethylating agent or a HDAC inhibitor can lead to reactivation of ER expression in these cells. However, demethylation of *PR* CpG island is not required for *PR* expression. These data in combination with similar results observed with other critical genes in breast cancer and other types of cancer support the potential for compounds that can modulate epigenetic regulation in the treatment of human cancers.

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DNA Methylation in Breast Cancer¹

Xiaowei Yang, Lan Yan and Nancy E. Davidson²

Breast Cancer Program, The Johns Hopkins Oncology Center, Johns Hopkins University School of Medicine, Baltimore, MD 21231

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² To whom requests for reprints should be addressed, at Department of Oncology, Johns Hopkins School of Medicine, 1650 Orleans Street, CRB416, Baltimore, MD 21231. E-mail: davidna@jhmi.edu

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1. Abstract

Like all cancers, breast cancer is considered to result in part from the accumulation of multiple genetic alterations leading to oncogene overexpression and tumor suppressor loss. More recently, the role of epigenetic change as a distinct and crucial mechanism to silence a variety of methylated tissue-specific and imprinted genes has emerged. This review will briefly discuss basic aspects of DNA methylation, recent advances in DNA methyltransferases, the role of altered chromatin organization and the concept of gene transcriptional regulation built on methylated CpGs. In particular, we discuss epigenetic regulation of certain critical tumor suppressor and growth regulatory genes

implicated in breast cancer, and its relevance to breast cancer progression, diagnosis and therapy.

2.1. Cytosine methylation and CpG islands in mammals

In vertebrate genomes, methylation of DNA occurs on cytosine residues of the CpG dinucleotides in DNA (Bird 1980). Mammalian genome contains about 3-6% of methylated cytosine residues in the 5 position of the CpG sequences, approximately 70 to 80% of these CpG sites in human are methylated (Antequera & Bird 1993, Bird 1995, Vanyushin *et al.* 1970). Cytosine residues in newly synthesized DNA are methylated by a DNA-cytosine methyltransferase1, DNMT 1 (Bestor 1988, Bestor & Verdine 1994). This enzyme transfers a methyl group from the methyl donor, S-adenosylmethionine, to nascent DNA using a hemimethylated DNA template in order to maintain DNA methylation patterns during cell division in mammals. CpG dinucleotides are not randomly distributed throughout the genome. Rather they are frequently clustered into CpG islands, regions that are rich in CpG sites. These islands extend about 0.5-3 kb, occur on average every 100 kb in the genome and are often found in the promoter area of genes (Cross & Bird 1995). Indeed, approximately half of all genes in the human (~45,000 genes) contain CpG islands (Antequera & Bird 1993). CpG island methylation plays a role in such diverse functions as gene imprinting (Forné *et al.* 1997, Reik & Walter 1998), X-chromosome inactivation (Heard & Avner 1994, Heard *et al.* 1997), normal development (Li *et al.* 1993, Weiss & Cedar 1997), repression of gene transcription (Cedar 1988, Keshet *et al.* 1985) (Chan *et al.* 2000) and the suppression of parasitic DNA sequences (Yoder *et al.* 1997).

2.2 DNA methyltransferases

Mammalian DNA methylation is carried out by members of DNA cytosine-methyltransferase (Dnmt) family. Three members of the Dnmt gene family have been identified to date. Direct sequence analysis has revealed that the *Dnmt* gene family is highly conserved among eukaryotes, suggesting a central role of these proteins for mammalian development (Bestor 2000).

DNMT1 is the best known and studied DNMT family member. It is primarily a maintenance methylase, that is, it reproduces DNA methylation patterns from hemimethylated DNA during cell division (Bestor 1988). However, there is some evidence that DNMT1 may also have *de novo* methylase activity, at least in *in vitro* system (Laayoun & Smith 1995, Pradhan *et al.* 1997). *Dnmt1* gene knockout mice die in mid-gestation with reduced levels of DNA methylation (Li *et al.* 1992), disrupted imprinting and ectopic X chromosome activation (Li *et al.* 1993), indicating that maintenance of DNA methylation is pivotal for development.

The human *DNMT1* gene is located at human chromosome 19p13.2 (Yen *et al.* 1992) and encodes a 200 kDa protein whose methyltransferase catalytic domain is located at the C terminus of the protein. The large N terminal portion of DNMT1 targets to replication foci through proliferating cell nuclear antigen (PCNA) (Chuang *et al.* 1997). Recent studies have identified new functions for this domain. First, its

amino acid (653-730) sequence that contains CXXC motif interacts directly with histone deacetylases, which act to remove acetyl tails from histones in the nucleosome to generate a transcriptionally inactive chromatin structure (Fuks *et al.* 2000). Secondly, through its first 120 amino acids, it binds to a transcriptional co-repressor, DMAP1, that represses transcription independent of histone deacetylase activity (Rountree *et al.* 2000). Lastly, amino acids 416-913 of the N terminus of DNMT1 interact with the retinoblastoma protein, Rb (Robertson *et al.* 2000). Thus, the N-terminal portion of DNMT1 alone or in collaboration with other co-repressors and recruited HDACs significantly suppresses transcription *in vitro*.

A large body of data demonstrates that DNMT1 activity is elevated in neoplastic cells and this increased activity is associated with increased cell proliferation (el-Deiry *et al.* 1991), tumorigenesis (De Marzo *et al.* 1999), and tumor progression (Issa *et al.* 1993). For example, over-expression of DNMT1 can transform NIH-3T3 mouse fibroblast cells (Wu *et al.* 1993), and inhibition of this enzyme by antisense constructs can induce global DNA demethylation and revert malignant phenotype (Ramchandani *et al.* 1997). In addition, *fos*-mediated transformation of normal fibroblast is associated with increased DNMT1 expression and total methylation content in the genome (Bakin & Curran 1999). Finally, it is also reported that elevation of DNMT1 is an essential component of transformation induced by SV40 large T antigen via the Rb pathway (Slack *et al.* 1999).

However, increased DNMT1 expression is apparently not an obligatory feature of malignant cells (Eads *et al.* 1999). Somatic knockout of *DNMT1* expression in human colon cancer cells is not a lethal event. Further, total genomic methylated CpG content was reduced by only about 20% and certain gene-specific CpG island methylation patterns were maintained (Rhee *et al.* 2000). These findings, together with the observation that embryonic stem (ES) cells from *DNMT1* knockout mice are still capable of *de novo* methylation, suggest the possible existence of other cytosine DNA methyltransferases (Lei *et al.* 1996, Li *et al.* 1992).

One such methyltransferase, Dnmt2, was isolated by several groups (Okano *et al.* 1998). However, its catalytic domain lacks methyltransferase activity in human and it is not discussed further.

Two isoforms of DNMT3 family enzyme, *de novo* DNA methyltransferases 3a and 3b (Dnmt3a and 3b) were recently isolated in mouse (Okano *et al.* 1999). They methylate CpG dinucleotides of unmethylated and hemimethylated DNA *in vitro*. The two genes are expressed at high levels in embryonic stem cells and relatively low levels in adult somatic tissues. Human *DNMT3a* has been mapped to chromosome 2p23 whereas *DNMT3b* maps to chromosome 20q11.2 (Robertson *et al.* 1999, Xie *et al.* 1999).

Disruption of both Dnmt3a and Dnmt3b in mice by gene targeting blocks *de novo* methylation in ES cells and early embryos, but has no effect on maintenance of imprinted methylation pattern (Okano *et al.* 1999). However, methylation capability is retained after inactivation of either Dnmt3a or 3b, indicating some redundancy in the function of these two *de novo* methylases. Dnmt3b appears to be critical for the methylation of a particular compartment of the genome; loss of DNMT3b catalytic activity by gene mutation in ICF syndrome causes demethylation of only specific

families of repeated sequences and CpG islands on the inactive X chromosome (Hansen *et al.* 1999). Human DNMT3A is ubiquitously expressed but DNMT3B is expressed at low levels except testis, thyroid and bone marrow. Both over-expression of *DNMT3b* and *DNMT3a* appears to characterize multiple types of human tumors (Xie *et al.* 1999). Four spliced forms of DNMT3b with altered enzymatic activity were expressed in a tissue-specific manner (Robertson *et al.* 1999). Future study will be needed to elucidate the possible roles of *DNMT3* family members in tumorigenesis, *de novo* tissue-specific gene methylation and transcriptional regulation in somatic tissues.

2.3. DNA methylation patterns in normal and cancer tissues

Cell type and tissue-specific methylation patterns are established during early development in part through the action of the *de novo* methyltransferases 3a and 3b (Okano *et al.* 1999). The sperm genome is extensively methylated while the oocyte genome is not. After fertilization, genes are demethylated and then remethylated before implantation. As the embryoblast differentiates, tissue-specific genes are demethylated in a tissue-specific fashion while housekeeping genes remain demethylated from fertilization through organogenesis (Bestor 1998).

CpG islands are generally unmethylated in normal adult tissues with the exception of transcriptionally silent genes on the inactive X chromosome and imprinted genes like *H19* gene (Tremblay *et al.* 1995). Conversely, most neoplastic tissues demonstrated whole genomic hypomethylation and local promoter hypermethylation in certain critical tumor suppressor and growth regulatory genes. The mechanism responsible for this type of pattern remains largely unclear. It is believed that the cell cycle checkpoint gene, p21^{WAF1/CIP1} may play a role in methylation regulation (Baylin *et al.* 1998). Since p21 competes with DNMT1 binding to PCNA, loss of p21 function may increase DNMT1 at replication sites (Chuang *et al.* 1997). In addition, mutation of another cell cycle gene, Rb, may play a role as Rb mutation in its A/B pocket domain might disrupt the function of the transcriptionally repressive protein complex that involves Rb, DNMT1 and HDAC. Mistargeting of DNMT1 could then result (Robertson *et al.* 2000). Together those observations are beginning to shed light on the paradox of global hypomethylation, increased CpG island hypermethylation, and increased DNMT1 activity in tumor cells.

2.4 DNA methylation and genetic instability

DNA methylation changes may ultimately lead to the genetic instability characteristic of cancer. For example, mutation in the well-known p53 tumor suppressor gene frequently occurs at CpG dinucleotides as a consequence of a transition from methylated cytosine to thymine (Magewu & Jones 1994). Similar transitions characterize the mutation found in several other tumor suppressor genes (Ghazi *et al.* 1990). Further, a p53-inducible gene, 14-3-3 σ is methylated and inactivated in many breast cancers. Loss of its expression may also facilitate the accumulation of genetic damages and immortalization of the cell (Ferguson *et al.* 2000).

Also, inactivation of certain other genes that are involved in DNA repair may predispose to genetic instability (Herman & Baylin 2000). For instance, methylation of *MLH1*, a gene involved in mismatch repair, precedes the MIN⁺ phenotype in sporadic colon, gastric, and endometrial cancers (Esteller *et al.* 1999). Further, there is a striking correlation between mismatch repair, genetic instability and methylation capacity in colon cancer cell models (Lengauer *et al.* 1997, 1998). In addition, promoter CpG island methylation and resulting inactivation of the detoxifying π -class glutathione S transferase (GST) can lead to accumulation of oxygen radicals and subsequent DNA damage. The resulting adenine or guanine mutations are implicated in carcinogenesis in prostate, breast and other tissues (Henderson *et al.* 1998, Lee *et al.* 1994, Matsui *et al.* 2000).

2.5 CpG island methylation, chromatin organization and gene transcriptional regulation

Much experimental evidence has documented the association of CpG island methylation and gene transcriptional inactivity but only recently have the underlying mechanisms of transcriptional silencing by methylation partially clarified. One possible mechanism of transcriptional repression is the direct interference by methylation with the binding of sequence-specific transcription factors, such as AP-2, E2F and NF κ B to DNA (Hermann & Doerfler 1991). A second possibility is that methylated CpG sequences recruit transcriptional co-repressors like mSin3A, DMAP1, TSG101 or Mi2, thereby contributing to transcriptional repression. Finally, chromatin structure is emerging as an important and more generalized mechanism to silence a variety of methylated tissue-specific and imprinted genes by HDAC family members. The deacetylation of lysine groups of histones H3 and H4 allows ionic interactions between positively charged lysines and negatively charged DNA, resulting in a more compact nucleosome structure that limits gene activity. The discovery of the family of methyl-CpG-binding proteins provides a mechanistic linkage between DNA methylation and histone deacetylation as mediators of gene transcription. To date, six methyl-CpG-binding proteins including MeCP2, MBD1, MBD2a, MBD2b and MBD3 have been identified in vertebrates (Nan *et al.* 1998, Ng *et al.* 2000, Ng *et al.* 1999, Snape 2000, Wade *et al.* 1998). The common functional features for these proteins are that they bind to methyl-CpGs in DNA and frequently associate with members of the HDAC family, which currently includes eight distinct members (Ng & Bird 2000). That these processes might collaborate to regulate gene expression is demonstrated by a recent study, showing that multiple hypermethylated genes, such as *MLH1*, *TIMP3*, *CDKN2B* and *CDKN2A*, could be robustly reactivated by a combination of DNMT1 and HDAC inhibition, suggesting that DNMT1 and histone deacetylase are both essential in the silencing process in these colon cancer cells (Cameron *et al.* 1999). The above observation was confirmed by a very recent study that the known DNA methylation machinery protein, DNMT1 itself, is implicated in forming transcriptionally repressive complex with histone deacetylase as well as other co-repressors (Rountree *et al.* 2000).

A large body of evidence has demonstrated that CpG island hypermethylation is implicated in loss of expression of a variety of critical genes in breast cancer. Some important genes inactivated by methylation in breast cancer are summarized in Table 1 and described below. They fall into several broad categories including cell cycle regulating, steroid receptor, tumor susceptibility, cell detoxifying, cell adhesion and inhibitors of MMPs genes.

3. Methylation of cell cycle-related genes in breast cancer

3.1 *p16/p16^{INK4A}/CDKN2A/MTS* methylation and breast cancer

The *p16* gene is located on chromosome 9p21. It encodes a cyclin-dependent kinase inhibitor, $p16^{\text{INK4A}}$, that regulates the transition from G1 to S-phase via its effect on Rb phosphorylation (Liggett & Sidransky 1998). The transcription of the *p16^{INK4A}* gene can yield two distinct transcripts (α or β) that code for two functionally distinct proteins, $p16^{\text{INK4A}}$ and $p19^{\text{ARF}}$. These two transcripts share identical second and third exons but have a distinct first exons (Sharpless & DePinho 1999). Loss of $p16^{\text{INK4A}}$ is a common feature of many cancers resulting from homozygous deletion, methylation of *p16* promoter or point mutation. The first two mechanisms are responsible for the majority of the gene inactivation in multiple cancers. Methylation of the 5' promoter and exon1 regions is observed in both human breast cancer cell lines (Table 2) and 20-30% of primary breast cancers (Herman *et al.* 1995). DNAs from normal breast tissues are unmethylated whereas from some breast carcinomas are methylated, supporting altered methylation pattern in cancer (Woodcock *et al.* 1999). The methylation phenotype is associated with loss of expression at both mRNA and protein levels although lack of correlation with some important clinical parameters in some relatively small cohort studies. A study of analyzing 97 patient showed no association between *p16* methylation and overall or disease-free survival (Hui *et al.* 2000).

The stepwise inactivation of cyclin D-dependent kinase inhibitor $p16^{\text{INK4A}}$ in human mammary epithelial cells (HMEC) is associated with progressive methylation of the *p16* promoter CpG island. This allows HMEC cells to escape from M0 proliferation block, thereby identifying CpG methylation together with *p16* silencing as a possible contributor to breast tumorigenesis (Foster *et al.* 1998).

3.2 *14-3-3 sigma* (σ) gene inactivation by methylation

The *14-3-3 σ* gene (also known as *HME1*), is localized at chromosome 1p35, and is a member of a gene family responsible for instituting the G₂ cell cycle checkpoint in response to DNA damage in human (Chan *et al.* 1999). Normally expression of σ is induced in response to DNA damage, and it causes cells to arrest in G₂. However, σ protein expression was downregulated in a significant fraction of primary bladder, colon and breast tumors (Celis *et al.* 1999). Studies of the molecular mechanisms responsible for the reduced expression have implicated hypermethylation of the CpG-rich exon1 region of the gene (91%), instead of genetic alterations such as LOH and

intragenic mutations in breast cancer (Ferguson *et al.* 2000). DNA from HMECs, immortal MCF-10A and HBL100 cells and two breast cancer cell lines, MCF-7 and MDA-MB-231, were unmethylated at the sigma locus. In contrast, Hs578t and MDA-MB-435 cells were fully methylated as demonstrated by bisulfite genomic sequencing and MSP analyses. The use of 5-aza-dC to treat the methylated non-expressing lines *in vitro* led to induction of σ transcription, further supporting the role of CpG island methylation in its repression. In addition, six DNA samples from micro-dissected normal mammary epithelial cells demonstrated an unmethylated pattern while 32 samples from microdissected breast carcinomas were methylated. Together these cell line- and tissue-based studies support a role for methylation in the loss of 14-3-3 sigma expression in breast cancer.

4. Methylation of steroid receptor genes in breast cancer

The methylation of three members of the steroid hormone superfamily has been extensively studied in breast cancer models. These include estrogen receptor α (ER), progesterone receptor (PR) and retinoid receptor β (RAR β).

4.1 Estrogen receptor α (ER) methylation and hormone resistance

Steroid hormones, particularly estrogen, have long been linked to mammary carcinogenesis (Fishman *et al.* 1995). The role of estrogen and its catechol metabolite in breast cancer initiation and promotion is a continuing area of controversy (Yager 2000). But that 17 β -estradiol stimulates the growth of certain breast cancers via functional ER is well recognized, and endocrine therapy like antiestrogens is an established and important part of breast cancer management (Davidson 2000, Ruiz-Cabello *et al.* 1995). The presence of ER in breast tumors is a predictive marker for response to hormone therapy. However, up to one third of breast carcinomas lack ER at the time of diagnosis and a proportion of cancers that are initially ER-positive lose ER during tumor progression (Hortobagyi 1998). Genetic alterations, such as homozygous deletion, loss of heterozygosity (LOH), or ER gene mutation have not been reported to play a major role in loss of ER expression.

However, recent studies have shown that epigenetic alteration appears to play a role in inactivation of the gene (Ottaviano *et al.* 1994). The ER gene, located at chromosome 6q25.1, has a CpG island in its promoter and first exon regions. As demonstrated by Southern and methylation-specific PCR analyses, the ER CpG island is unmethylated in normal breast tissue and ER-positive tumor cell lines, such as MCF-7, T47-D and ZR75-1; it is methylated in ~50% of unselected primary breast cancers and most ER-negative cancer cell lines e.g., MDA-MB-231, MDA-MB-435, MDA-MB-468, Hs578t, and MCF-7/Adr (Table 2) (Lapidus *et al.* 1998). The ER CpG island methylation is associated with reduced or absent ER mRNA expression. The use of methyltransferase inhibitor 5-aza-C and 5-aza-dC led to partial demethylation and restoration of ER mRNA expression, and synthesis of functional ER protein (Ferguson *et al.* 1995). Restored ER function was documented by eliciting ERE-driven promoter

activity from an exogenous plasmid as well as expression of the ER-responsive gene, PR.

In order to study *de novo* ER gene methylation *in vitro*, DNMT1 activity levels were measured in a panel of breast cancer cell lines. Expression of DNMT1 at both RNA and protein levels in ER-negative breast cancer cell lines is significantly elevated in ER-negative breast cancer cell lines compared with their ER-positive counterparts (Ottaviano *et al.* 1994). Detailed studies showed that DNMT1 expression was tightly correlated with S phase fraction in ER-positive cells, while ER-negative cells expressed DNMT1 throughout the cell cycle, suggesting its expression is dysregulated in ER-negative breast cancer cells (Nass *et al.* 1999). The data also showed that DNMT1 and p21 expressions are inversely correlated in breast cancer cell lines examined. Studies using DNMT1 antisense constructs showed that decreased expression is linked to increased p21 protein expression. Since p21 competes with DNMT1 for targeting to PCNA, the increased p21 may lead to inhibition of DNA replication and methylation in these tumor cells (Chuang *et al.* 1997).

A related question is whether an inactive chromatin structure mediated by histone deacetylase is involved in *ER* gene silencing. In fact, inhibition of histone deacetylase by TSA induced *ER* transcript by 5-fold in a panel of well-characterized ER-negative MDA-MB 231, Hs578t and MCF-7/Adr cell lines. This transcriptional reactivation is associated with increased sensitivity to DNaseI at *ER* locus without alteration of the methylated CpG sites, suggesting that open chromatin structure is associated with *ER* expression even in the presence of *ER* CpG island methylation (). Our findings identified a role for both DNA methylation and histone acetylation in the regulation of *ER* gene transcription.

4.2 Progesterone receptor (PR) gene methylation

The *progesterone receptor (PR)* gene, located at chromosome 11q13, also has a CpG island in its first exon (Lapidus *et al.* 1996). The *PR* gene encodes two isoforms, hPR_A (79 kDa) and hPR_B (109 kDa), which differ in both their amino terminal sequences and biological activities. The hPR_B transcript is preferentially induced by ER while the hPR_A is not. Since ligand-bound ER is a major transcriptional activator of hPR_B gene expression, the presence of PR is indicative of functional ER. PR gene methylation has been demonstrated by Southern analysis in ~40% of PR-negative breast tumors and several PR-negative breast cancer cell lines (Table 2). A possible functional role for PR CpG island methylation is suggested by the observation that treatment of PR-negative MDA-MB-231 cells with 5-aza-dC in the presence of estrogen led to partial demethylation of PR CpG island and reexpression of PR gene. Co-treatment with both 5-aza-dC and a pure anti-estrogen, ICI182,780 prevented PR reactivation, suggesting that demethylation is not sufficient to reactivate PR expression. Rather it appears that ER-mediated chromatin remodeling is essential and sufficient to activate PR gene expression even in the presence of a methylated PR CpG island (Ferguson *et al.* 1998).

4.3 *RARβ2* promoter methylation and refractory to differential therapy

The retinoic acid receptors (RAR- α , - β , and - γ) and retinoid X receptors (RXR- α , β and - γ) are also members of the nuclear receptor superfamily (Minucci & Pelicci 1999). All six of these receptors are ligand-activated transcription factors. The *RAR β* gene, located at chromosome 3p24, appears to play an important role in limiting the growth of certain tumor types, including breast, lung and others. *RAR β 2* expression is often reduced or lost in breast cancer cells and they become resistant to induction by all-*trans*-retinoic acid (ATRA). DNA methylation of *RAR β* promoter is believed to be one of the factors linked to RAR β 2 down-regulation in breast cancer. *RAR β* promoter methylation has been demonstrated by Southern and methylation-specific PCR analyses in several RAR β 2-negative human breast cancer cell lines and about one third of unselected primary breast cancer specimens. It is not observed in normal breast tissue or HMECs. There is no apparent correlation with ER status. As with other methylated genes, treatment of RAR β 2-negative cell lines with 5-aza-dC can partially re-induce RAR β 2 transcripts. Of note, the HDAC inhibitor TSA can also reactivate RAR β 2 expression in the presence of a methylated promoter, implicating inactive chromatin conformation as another possible regulatory process.

5. Glutathione S transferase (*GSTP1*) inactivation by methylation and its predisposition to genetic instability

Glutathione (GSH) and its corresponding cytosolic glutathione S-transferases (GSTs) are involved in the detoxification pathway of xenobiotics and chemotherapeutic agents (). They catalyze intracellular detoxification reactions by conjugating chemically reactive electrophiles to glutathione, inactivating electrophilic carcinogens (). The GSTs, encoded by several different genes at different loci, have been classified into α , μ , π and θ families (). The π -class GST, encoded by the *GSTP1* gene, on chromosome 11, is of particular importance in breast cancer (). In cultured breast cancer cell lines an inverse relationship between *GSTP1* and *ER* gene expression has been reported, i.e., *GSTP1* was expressed in ER-negative but not in ER-positive lines (Table 2) although the underlying mechanism is unclear (). Treatment of *GSTP1*-negative cell line MCF-7 with 5-aza-dC could induce mRNA expression and *de novo* synthesis of π -class protein (). MSP-based studies of human tissues demonstrated that *GSTP1* promoter methylation is associated with gene inactivation in about 30% of primary breast carcinomas (). The detection of *GSTP1* methylation correlates with PR expression but there was no correlation with other clinical parameters such as the age of onset, histological type and grade, tumor size, nodal metastasis, DNA ploidy, or ER status (). It is postulated that methylation-associated inactivation of *GSTP1* can result in adenine or guanine mutation by estrogen metabolites-DNA adduct formation () and lead to a drug-resistant phenotype and genetic instability ().

6. *BRC1* methylation in sporadic breast cancer

The *BRCA1* gene, located at chromosome 17q21, is a well-known breast cancer susceptibility gene. Inhibition of *BRCA1* expression through antisense oligonucleotides increases the proliferation of normal and malignant mammary cells while overexpression of wild-type *BRCA1* suppresses MCF-7 breast cancer cell tumorigenesis in mice (). Inherited mutations in the *BRCA1* gene account for one-half of inherited breast carcinomas. However, in contrast to other tumor suppressor genes, somatic mutations in this gene have not been reported, despite the high degree of loss of heterozygosity at the *BRCA1* locus in sporadic breast and ovarian cancer (). Since *BRCA1* transcript and protein are either absent or reduced in sporadic breast cancer, DNA methylation has been proposed as an alternative mechanism to inactivate the *BRCA1* (). By Southern analysis of the *BRCA1* promoter region, methylation was detected in 11% of sporadic breast cancer cases and was inversely correlated with expression of both ER and PR (). A study with 194 primary breast carcinomas demonstrated that the *BRCA1* promoter is methylated in 13% of unselected primary breast tumors. *BRCA1* methylation was especially associated with medullary and mucinous subtypes. As expected, *BRCA1* was unmethylated in all normal tissues examined as well as 21 breast cancer cell lines (Table 2). The methylation was present in two breast cancer xenografts with concomitant loss of gene transcript. In this study one allele is lost by LOH and the other is inactivated by aberrant methylation, thereby resulting in biallelic inactivation and loss of functional *BRCA1* gene product. Finally, *BRCA1* methylation is only observed in breast and ovary cancers but not in tumors of colon, liver or leukemia, supporting a tissue-specific event for the process. Using chromatin immuno-precipitation and endonuclease chromatin accessibility assays, transcriptional repression of *BRCA1* by cytosine methylation is also mechanistically linked to histone deacetylation and inactive chromatin structure.

7. *E-cadherin* gene methylation and breast tumor progression

The *E-cadherin* gene, located at chromosome 16q22.1, encodes a cell-surface adhesion protein that is important in maintaining homophilic cell-cell adhesion in epithelial tissues. Considerable evidence shows that loss of expression and function in *E-cadherin* protein contributes to increased proliferation, invasion, and metastasis in breast cancer (). Classical mutations and deletions clearly play a role in loss of the *E-cadherin* expression and function (). However, Several studies demonstrate that epigenetic silencing of the *E-cadherin* gene by 5'-CpG methylation occurs in some human breast cancer cell lines (Table 2) as well as about 50% unselected primary breast cancers (). Its loss of expression is associated with tumor metastatic progression and decreased patient survival (). Our recent work demonstrated that hypermethylation of the *E-cadherin* CpG island was evident in about 30% of ductal carcinoma *in situ* and increased significantly to nearly 60% of metastatic lesions (), suggesting a role for this process in tumor progression.

8. Methylation and inactivation of *TIMP3* gene

TIMP-3 belongs to a family of molecules that inhibit the proteolytic activity of the matrix metalloproteinases (MMPs). This protein can suppress primary tumor growth via their effects on tumor development, angiogenesis, invasion and metastasis. Methylation of this 5'CpG island has been associated with the loss of *TIMP-3* expression at both transcription and protein levels in several tumor types. The *TIMP-3* promoter region is methylated in ~30% of human breast cancer cell lines as well as ~30% of primary breast tumors. The gene methylation has been associated with its loss of expression. Expression could be restored by 5-aza-dC treatment, again supporting a role for epigenetic mechanism in *TIMP3* gene regulation.

9. Clinical implications of DNMT and HDAC inhibitors

The classic DNA methyltransferase inhibitor, 5-aza-cytidine (5-aza-C) and 5-aza-2'-deoxycytidine (2-aza-dC) are cytosine analogs, which inhibits the enzyme by formation of covalent adducts between DNA methyltransferase and 5-aza-dC-substituted DNA to irreversibly inactivates DNA methyltransferase (). These cytosine analogs have a remarkable ability to induce heritable changes in gene expression (reviewed by Haaf 1995). They have been, for more than 20 years, broadly used as valuable tools to reactivate the methylated gene expression *in vitro* (). These cellular processes are associated with demethylation, in a replication-dependent manner, of specific DNA sequences although genes may be activated by other effects of 5-aza-C analogs, such as on chromatin structure. By gene expression microarray approach to examine the effect of 5-aza-dC treatment in HT29 colon adenocarcinoma cells, induction of the IFN-response pathway as a major cellular response was identified (). This study found that 5-aza-dC treatment sensitized these colon cancer cells to growth inhibition by exogenous IFN- α 2a, suggesting 5-aza-dC can be investigated as a potentiator of IFN responsiveness in certain IFN-resistant tumors.

The elevated levels of DNA methyltransferase activity in cancer have prompted targeting of DNMT1 as an anticancer strategy. The two analogs have been used clinically for the treatment of patients with myelodysplasia, hemoglobinopathies and leukemia (). In an effort to investigate the role of DNA methyltransferase1 in oncogenesis, specific potent DNMT1 anti-sense inhibitors have been developed. These anti-sense oligonucleotide or oligodeoxynucleotide can form a stable complex with DNMT1 and inhibit its activity (). With this approach specific reduction of cellular DNMT1 levels in human bladder and A549 human non-small lung cancer cells was achieved (). At the meantime, the anti-sense inhibitor causes demethylation of p16^{INK4A} gene promoter, re-expression of p16^{INK4A} protein, accumulation of the hypophosphorylated form of retinoblastoma protein (pRB) and cell growth arrest (). In the same study it is found that a rapid increase in the cell cycle regulator p21^{WAF1/CIP1} protein follows the stepwise reduction of cellular Dnmt1 levels by anti-sense oligonucleotide treatment. All these findings demonstrated that specific targeting of DNMT1 could activate silenced tumor suppressors.

Several structural classes of HDAC inhibitors have been identified. First of all, short-chain fatty acids such as sodium phenylbutyrate (PB), with an active HDAC inhibiting dose at millimolar levels, is currently clinically approved HDAC inhibitor.

The second class of HDAC inhibitor are hydroxamic acids, such as trichostatin A (TSA), a potent HDAC inhibitor that works at nonomolar levels, and suberoylanilide hydroxamic acid (SAHA), which is effective at micromolar levels. TSA has been widely used to inhibit HDAC activity to study gene transcriptional regulation (). SAHA, recently, has been demonstrated its antitumor activity *in vivo* in addition to its activity *in vitro*. With the use of SAHA one study showed that N-methylnitrosourea-induced mammary carcinoma in rats has been dramatically reduced, without apparent toxicity, in compared to control rats (). Similar results also obtained through other experiments with animals using SAHA (). Thereby, SAHA has currently entered in several phase I clinical trials (). The third class of HDAC inhibitor is benzamide derivative, e.g., MS-27-275, which also showed a marked *in vivo* antitumor activity against human tumor in xenograft mice (), demonstrating its potential utility in clinical settings. The fourth class is cyclic tetrapeptides containing a 2-amino-8-oxo-9, 10-epoxy-decanoyl (AOE) moiety such as trapoxin A (). And the fifth one is cyclic peptide do not contain the AOE moiety such as FR901228 and apicidin (). In common, these HDAC inhibitors could acetylate the core histones and induce growth arrest, differentiation in cultured transformed and neoplastic cells (). For example, SAHA induces terminal cell differentiation (milk protein synthesis) in MCF-7 human breast cancer cells () and in malignant cells of other types (). Virtually all the five classes of HDAC inhibitors (e.g., PB, TSA, SAHA, MS-27-275) demonstrated the ability to induce G1 arrest by upregulation of *p21/Waf1/Cip1*. Increased p21 expression led to growth arrest in cancer cells by blocking cyclin-dependent kinase and proliferating cell nuclear antigen.

Inhibition of histone deacetylation strategy demonstrated its clinical utility, especially if combined with other types of therapy. For example, a patient with acute promyelocytic leukemia resistant to all trans-retinoic acid developed a sustained remission after concomitant treatment with PB and all trans-retinoic acid (). In animal experiment the combined use of tamoxifen or raloxifene with retinoic acid prevented mammary carcinogenesis induced by N-nitroso-N-methylurea in Sprague-Dawley rats (). Other potential combinations, such as DNMT1 and HDAC, or DNMT1 and all trans-retinoic acid, or tamoxifen, even multiple combinations such as DNMT, HDAC and tamoxifen, may also benefit some tumor patient who are refractory to hormonal therapy. These combinations may increase therapeutic effects, minimize toxicity and increase target specificity.

10. Conclusions and future directions

Taken together, substantial evidence demonstrated that epigenetic mechanism in transcriptional regulation of critical tumor suppressor and growth-regulatory genes in breast cancer. These genes play crucial roles in DNA repair, cell cycle regulation, cell growth, cell-cell adhesion, and metabolism. Therefore, it is not surprising that loss of these critical genes by methylation could lead to mutations in DNA, abnormal cell cycle control, formation and metastasis of tumors as well as other cell dysfunctions. Furthermore, 5-methyl cytosine as intrinsic mutagen, plus dysregulated DNA methyltransferase1 activity all possibly contribute to tumorigenesis and/or progression

of breast cancer, and resistance to chemoprevention, chemotherapy, hormonal, and differential therapies. A better understanding of epigenetic regulation of gene expression in a gene-specific and tissue-specific fashion, will help to selectively modulate gene expression, eventually, lead to improved breast cancer prevention and therapy.

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