DNA methylation in breast cancer

X Yang, L Yan and N E Davidson
Breast Cancer Program, The Johns Hopkins Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, USA

(Requests for offprints should be addressed to N E Davidson, Department of Oncology, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB416, Baltimore, Maryland 21231, USA; Email: davidna@jhmi.edu)

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 in many cancer types. 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 diagnosis, prognosis, progression and therapy.

Introduction

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). This epigenetic alteration in DNA is heritable but does not alter nucleotide sequence, in contrast to genetic changes (Feinberg 2001). Thus, unlike genetic changes, epigenetic modifications are potentially reversible (Baylin et al. 2001). About 3–6% of cytosines are methylated in mammals. Approximately 70–80% of CpG sites in the human genome are methylated (Vanyushin et al. 1970, Antequera & Bird 1993, Bird 1995). Cytosine residues in newly synthesized DNA are methylated by DNA-cytosine methyltransferase-1 (DNMT1) (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). DNA methylation plays a role in such diverse functions as gene imprinting (Forme 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 (Keshet et al. 1985, Cedar 1988, Chan et al. 2000), and the suppression of parasitic DNA sequences (Yoder et al. 1997). It is believed to exercise its critical role in gene expression via several routes. First, methylation of a CpG island is associated with loss of transcription of the target gene. Secondly, 5-methylcytosine (5meC) residues are susceptible to deamination to thymine, resulting in a transition mutation (Schmutte & Jones 1998). Thirdly, it is possible that inappropriate hypomethylation could be linked to dysregulated gene induction (Muller et al. 2001).

DNMTs

To date, three members of the Dnmt gene family have been identified. Direct sequence analysis has revealed that the Dnmt gene family is highly conserved among eukaryotes, suggesting a central role of these proteins for development (Bestor 2000).

DNMT1 is the best known and studied member of the DNMT family. 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 vitro systems
The observation that embryonic stem (ES) cells from DNMT1 knockout mice are still capable of de novo maintained (Rhee 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 contains a CXXC motif that interacts directly with histone deacetylases (HDACs), which act to remove acetyl tails from histones in the nucleosome to generate a transcriptionally inactive chromatin structure (Fukas et al. 2000). Secondly, through its first 120 amino acids, it binds to a transcriptional co-repressor, DMAP1, that represses transcription independence of HDAC activity (Rountree et al. 2000). Lastly, amino acids 416–931 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), tumorogenesis (De Marzo et al. 1999) and tumor progression (Issa et al. 1993). For example, overexpression 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 the malignant phenotype (Ramchandani et al. 1997). In addition, fos-mediated transformation of normal fibroblasts 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 DNMTs (Li et al. 1992, Lei et al. 1996).

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

Two isoforms of the DNMT3 enzyme family, de novo DNMTs 3a and 3b (Dnmt3a and 3b) were recently isolated in the 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 ES 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 an 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 the syndrome of immunodeficiency, centromeric instability and facial anomalies 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 in testis, thyroid and bone marrow. Overexpression of both 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.

**Altered CpG island methylation, chromatin organization and 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 been 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 NFκB 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, Wade et al. 1998, Ng et al. 1999, 2000, Snape 2000). 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, TIMP-3, CDKN2b and CDKN2a, could be robustly reactivated by a combination of DNMT1 and HDAC inhibition, suggesting that DNMT1 and HDAC 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 complexes with HDAC as well as other co-repressors (Rountree et al. 2000).

DNA methylation patterns in normal tissues and cancer

Cell type and tissue-specific methylation patterns are established during early development, in part through the action of the de novo Dnmt3a 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 the H19 gene (Tremblay et al. 1995). Conversely, most neoplastic tissues demonstrate whole genomic hypomethylation and local promoter hypermethylation in certain critical tumor suppressor and growth regulatory genes (Baylin et al. 1998). The mechanism responsible for this type of pattern remains largely unclear. It is believed that the cell cycle checkpoint gene, p21WAF1/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, these observations are beginning to shed light on the paradox of global hypomethylation, increased CpG island hypermethylation, and increased DNMT1 activity in tumor cells.

DNA methylation and genetic instability

DNA methylation changes may ultimately lead to the genetic instability characteristic of cancer in several ways. First, 5meCs serve as sites of transition mutations by the hydrolytic deamination of 5meC to thymine. For example, such mutations frequently occur in the well-known p53 tumor suppressor gene (Magewu & Jones 1994). Similar point mutations characterize the mutations found in several other important genes like Rb, and c-H-ras-1 (Ghazi et al. 1990).

Secondly, epigenetic inactivation of certain critical genes in cancer by promoter methylation 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. 1999a). 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 p-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 (Lee et al. 1994, Henderson et al. 1998, Matsui et al. 2000). Further, a p53-inducible gene, 14–3–3σ is methylated and inactivated in many breast cancers. Loss of its expression may also facilitate the accumulation of genetic damages (Ferguson et al. 2000).

Apart from regional hypermethylation of some critical tumor suppressor genes, genome-wide hypomethylation is an important feature in cancer. This also could contribute to genetic instability (Schmutte & Fishel 1999).

Methylation of critical tumor suppressor and growth regulatory genes in breast cancer

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, carcinogen
Methylation of cell cycle-related genes in breast cancer

p16/p16\textsuperscript{INK4A}/CDKN2A/MTS methylation and breast cancer

The p16 gene is located on chromosome 9p21. It encodes a cyclin-dependent kinase inhibitor, p16\textsuperscript{INK4A}, that regulates the transition from G1- to S-phase via its effect on Rb phosphorylation (Liggett & Sidransky 1998). The transcription of the p16\textsuperscript{INK4A} gene can yield two distinct transcripts (\(\alpha\) or \(\beta\)) that code for two functionally distinct proteins, p16\textsuperscript{INK4A} and p16\textsuperscript{SMT}. These two transcripts share identical second and third exons but have distinct first exons (Sharpless & DePinho 1999). Loss of p16\textsuperscript{INK4A} resulting from homozygous deletion, methylation of p16 promoter or point mutation is a common feature of many cancers. Methylation of the 5′ promoter and exon 1 regions is observed in both human breast cancer cell lines (Table 2) and 20–30% of primary breast cancers (Herman et al. 1995, Woodcock et al. 1999). The methylation phenotype is associated with loss of expression at both mRNA and protein levels although it does not correlate with some important clinical parameters in some relatively small cohort studies. For example, a study of 97 patients with breast cancer showed no association between p16 methylation and overall or disease-free survival (Hui et al. 2000).

Finally, the stepwise inactivation of cyclin D-dependent kinase inhibitor p16\textsuperscript{INK4A} in human mammary epithelial cells (HMEC) is associated with progressive methylation of the p16 promoter CpG island. This allows HMECs 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).

14–3–3\(\sigma\) gene inactivation by methylation

The 14–3–3\(\sigma\) gene (also known as \(HMET\)), 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 \(\sigma\) is induced in response to DNA damage, and it causes cells to arrest in G2. However, \(\sigma\) 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 exon 1 region of the gene, instead of genetic alterations such as loss of heterozygosity (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 methylation specific PCR (MSP) analyses. The use of 5-aza-2′-deoxycytidine (5-aza-dC) to treat the methylated non-expressing lines \textit{in vitro} led to induction of transcription, further supporting the role of CpG island methylation in its repression. In addition, six DNA samples from microdissected 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.

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 (ER) \(\alpha\), progesterone receptor (PR) and retinoic acid receptor-\(\beta\) (RAR\(\beta\)).

ER\(\alpha\) methylation and hormone resistance

Steroid hormones, particularly estrogen, have long been linked to mammmary 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). That 17\(\beta\)-estradiol stimulates the growth of certain breast cancers via functional ER is well recognized, and endocrine therapy is an established and important part of breast cancer management (Ruiz-Cabello et al. 1999).
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, 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 the methyltransferase inhibitors 5-aza-cytidine (5-aza-C) and 5-aza-dC, led to partial demethylation of the PR CpG island (Yang et al. 2000). A related question is whether an inactive chromatin structure mediated by HDAC is involved in ER gene silencing. In fact, inhibition of HDAC by HDAC inhibitor (A, trichostatin 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 was associated with increased sensitivity to DNaseI at the 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 (Yang et al. 2000). Our findings identified a role for both DNA methylation and histone acetylation in the regulation of ER gene transcription.

## PR gene methylation

The 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\(_{\alpha}\) (79 kDa) and hPR\(_{\beta}\) (109 kDa), which differ in both their N-terminal sequences and biological activities. The hPR\(_{\alpha}\) transcript is preferentially transcriptionally silenced. In fact, inactivation in ER-negative breast cancer cells (Nass et al. 1999). The data also showed that DNMT1 and p21 expression are inversely correlated in the breast cancer cell lines examined. Studies using DNMT1 antisense constructs showed that decreased expression of DNMT1 protein 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).

### Table 2 Methylation status of critical tumor suppressor and growth regulatory genes in human breast cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gene</th>
<th>p16</th>
<th>14–3–3a</th>
<th>ER</th>
<th>PR</th>
<th>RAR-(\beta)2</th>
<th>BRCA1</th>
<th>GSTP1</th>
<th>E-cadherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMECs</td>
<td>U/M</td>
<td>U</td>
<td>U</td>
<td>ND</td>
<td>U</td>
<td>U</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>HBL-100</td>
<td>U</td>
<td>U</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>U</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>MCF-7</td>
<td>D</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
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<tr>
<td>T47-D</td>
<td>M</td>
<td>ND</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>ZR75-1</td>
<td>U/M</td>
<td>ND</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>D</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>ND</td>
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<tr>
<td>MDA-MB-468</td>
<td>U</td>
<td>ND</td>
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<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>ND</td>
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<tr>
<td>MDA-MB-435</td>
<td>ND</td>
<td>M</td>
<td>M</td>
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<td>M</td>
<td>U</td>
<td>U</td>
<td>ND</td>
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<tr>
<td>Hs578t</td>
<td>U</td>
<td>M</td>
<td>M</td>
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<td>U</td>
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<td>MCF7/Adr</td>
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<td>ND</td>
<td>U</td>
<td>ND</td>
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</tbody>
</table>

U: unmethylated; M: methylated; U/M: partially methylated; D: biallelic deletion; ND: not done.

### References


and re-expression of PR gene. Co-treatment with both 5-aza-dC and a pure anti-estrogen, ICI 182,780, prevented PR reactivation, suggesting that demethylation alone 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).

RARβ2 gene methylation
RARα, -β and -γ and retinoid X receptors-α, -β and -γ are also members of the nuclear receptor superfamily (Minucci & Pelicic 1999). All six of these receptors are ligand-activated transcription factors (Chambon 1996). The RARβ gene, located at chromosome 3p24, appears to play an important role in limiting the growth of certain tumor types, including breast, lung and others. RARβ2 expression is often reduced or lost in breast cancer cells and they become resistant to induction by all-trans-retinoic acid (ATRA) (Swisshelm et al. 1994, Jing et al. 1996, Widschwendter et al. 1997). DNA methylation of the RARβ promoter is believed to be one of the factors linked to RARβ2 downregulation in breast cancer (Widschwendter et al. 2000). RARβ promoter methylation has been demonstrated by Southern and methylation-specific PCR analyses in several RARβ2-negative human breast cancer cell lines and about one-third of unselected primary breast cancer specimens (Sirchia et al. 2000). 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 RARβ2-negative cell lines with 5-aza-dC can partially re-induce RARβ2 transcripts. Of note, the HDAC inhibitor TSA can also reactivate RARβ2 expression in the presence of a methylated promoter, implicating inactive chromatin conformation as another possible regulatory process.

**GSTP1 inactivation by methylation and its predisposition to genetic instability**

Glutathione (GSH) and its corresponding cytosolic GSTs are involved in the detoxification pathway of xenobiotics and chemotherapeutic agents (Daniel 1993). They catalyze intracellular detoxification reactions by conjugating chemically reactive electrophiles to GSH, inactivating electrophilic carcinogens (Mannervik et al. 1985). 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 (Cairns et al. 1992, Gilbert et al. 1993). 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 (Jhaveri & Morrow 1998).

Treatment of the 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 (Esteller et al. 1998). The detection of *GSTP1* methylation correlates with PR expression but there was no correlation with other clinical parameters such as the age at onset, histological type and grade, tumor size, nodal metastasis, DNA ploidy or ER status (Esteller et al. 1998).

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 genetic instability (Cavalieri et al. 1997).

**BRCA1 methylation in sporadic breast cancer**

The *BRCA1* gene, located at chromosome 17q21, is a well-known breast cancer susceptibility gene (Miki et al. 1994). 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 (Friedman et al. 1994). However, in contrast to other tumor suppressor genes, somatic mutations in this gene have not been reported, despite the high degree of LOH at the *BRCA1* locus in sporadic breast and ovarian cancer (Merajver et al. 1995).

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 *BRCA1* (Dobrovic & Simpfendorfer 1997, Magdinier et al. 2000). 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 (Catteau et al. 1999). A study with 194 primary breast carcinomas demonstrated that the *BRCA1* promoter is methylated in 13% of unselected primary breast tumors (Esteller et al. 2000). 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 or liver or leukemia, supporting a tissue-specific event for the process. Using chromatin immunoprecipitation and endonuclease chromatin accessibility assays, transcriptional repression of *BRCA1* by cytosine methylation is also mechanistically linked to histone deacetylation and inactive chromatin structure.
**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 (Ilyas & Tomlinson 1997). Considerable evidence shows that loss of expression and function of E-cadherin protein contributes to increased proliferation, invasion and metastasis in breast cancer (Oka et al. 1992). Classical mutations and deletions clearly play a role in loss of the E-cadherin expression and function (Cleton-Jansen et al. 1994, Risinger et al. 1994). 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% of unselected primary breast cancers (Graff et al. 1995, Hiraguri et al. 1998). Its loss of expression is associated with tumor metastatic progression and decreased patient survival (Bringuier et al. 1993). Our recent work demonstrated that hypermethylation of the *E-cadherin* CpG island was evident in about 30% of ductal carcinomas in situ and increased significantly to nearly 60% of metastatic lesions (Nass et al. 2000), suggesting a role for this process in tumor progression.

**Methylation and inactivation of TIMP-3 gene**

TIMP-3 belongs to a family of molecules that inhibit the proteolytic activity of the MMPs (Gomez et al. 1997, 1999). This protein can suppress primary tumor growth via its effects on tumor development, angiogenesis, invasion and metastasis (Uria et al. 1994). Methylation of its 5′CpG island has been associated with the loss of TIMP-3 expression at both transcript and protein levels in several tumor types (Bachman et al. 1999). The TIMP-3 promoter region is methylated in ∼30% of human breast cancer cell lines as well as ∼30% of primary breast tumors (Bachman et al. 1999). TIMP-3 gene methylation has been associated with its loss of expression as its expression could be restored by 5-aza-dC treatment, again supporting a role for epigenetic mechanism in TIMP-3 gene regulation.

**Clinical implications of epigenetic regulation in breast cancer**

Recognition of the important roles that DNA methylation and histone deacetylation play in gene expression in malignancy including breast cancer has led to consideration of how these findings can be exploited clinically. Two areas for exploration are the use of methylated markers for detection and prognosis, and the application of DNMT and HDAC inhibitors therapeutically to re-express silenced tumor suppressor and growth inhibitory genes.

**Methylated markers for detection and prognosis**

One of the tenets of epigenetic regulation is that CpG islands are generally unmethylated in normal adult tissues with rare exceptions. In contrast, as noted earlier, hypermethylation of various gene promoters is a common feature of malignant cells and these changes can occur early in the progression process. For example, about one-third of ductal carcinoma in situ lesions demonstrated methylation of the *E-cadherin* or ER promoter CpG islands (Nass et al. 2000). The availability of PCR-based strategies to assess methylation changes in minute quantities of biological materials raises the possibility that identification of methylated alleles might serve as a means of molecular risk assessment or detection (Herman et al. 1996). Indeed the ability to detect methylated genes in serum, urine, bronchoalveolar lavage fluid and lymph nodes derived from individuals with various types of malignancies has been described (Esteller et al. 1999b). In some cases methylated changes were also detected in samples banked well before the diagnosis of malignancy, raising the possibility that this type of analysis could facilitate risk assessment or early diagnosis strategies. One potential application in breast cancer would be the assessment of methylated markers using nipple duct lavage fluids or fine needle aspirates of the breast, as it could be hypothesized that their presence would predict a higher likelihood of subsequent breast cancer development. Other applications could include molecular staging of sentinel lymph nodes or bone marrow aspirates. Such studies will first require the development of a panel of methylated markers that would together identify virtually all breast cancers but that are unmethylated in normal tissues (Evron et al. 2001).

Finally, another possible clinical application for gene-specific methylation is prediction of prognosis or treatment outcome in certain cancers (Weinstein 2000). For example, the DNA-repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) inhibits the ability of alkylating agents to kill tumor cells; loss of its expression by methylation might be expected to sensitize tumor cells to this class of agents. Indeed, methylation of MGMT is correlated with increased overall and disease-free survival and improved response to the alkylating agent carmustine in glioblastoma patients (Esteller et al. 2000a). Therefore, MGMT promoter methylation is a marker of good prognosis and predicts response to chemotherapy for glioblastoma patients.

**DNMT and HDAC inhibitors for therapy of breast cancer**

Although heritable, epigenetic changes are potentially reversible. Therefore, the prospect of intervening to reverse these changes as a possible means of reverting the malignant
phenotype is an attractive one. The availability of DNMT and HDAC inhibitors makes this a testable strategy.

**DNMT inhibitors**

The classic DNMT inhibitors, 5-aza-C and 5-aza-dC are cytosine analogs that are incorporated into replicating DNA (Jones & Taylor 1980, Jones 1985). The subsequent formation of covalent adducts between DNMT and 5-aza-C-substituted DNA irreversibly inactivates DNMT (Santi et al. 1984, Ferguson et al. 1997). One potential consequence then is the reactivation of previously methylated genes that have been transcriptionally silent. These analogs have been used clinically for treatment of patients with hemoglobinopathies, myelodysplasia and leukemia (Rivard et al. 1981, Ley et al. 1983). The clinical benefit observed has been associated with reactivation of previously silenced genes as, for example, the observation that 5-aza-treated sickle cell patients have improved hemoglobin and increased fetal hemoglobin (Nienhuis et al. 1985). Such strategies could potentially be applied to epithelial malignancies as well. For example, a gene microarray analysis of the effect of 5-aza-dC on HT29 colon cancer cells identified members of the interferon (IFN) response pathway as re-expressed genes (Karpf et al. 1999). Follow-up studies showed that 5-aza-dC treatment of these cells in vitro sensitized them to growth inhibition by exogenous IFN-α2a. Thus unmasking of new therapeutic targets may be enhanced.

The elevated level of DNMT activity in cancer has also prompted development of more specific agents like antisense oligonucleotides to DNMT1. In vitro studies have shown that antisense treatment of human T24 bladder cancer cells and A549 non-small lung carcinoma cells can cause reduced DNMT1 levels, demethylation of the p16\(^{\text{ink4a}}\) gene promoter, re-expression of p16\(^{\text{ink4a}}\) gene, accumulation of the hypophosphorylated form of the Rb protein, increase in p21\(^{\text{WAF1/CIP1}}\), and cell growth arrest (Fournel et al. 1999). Thus, this specific targeting of DNMT1 can activate silent tumor suppressors.

**HDAC inhibitors**

Several structural classes of HDAC inhibitors have been identified. Phenybutyrate, a short chain fatty acid that inhibits HDAC at millimolar concentrations and is approved for clinical use by the US Food and Drugs Administration (FDA), is an example of the first class (Carducci et al. 1996). The second class includes the hydroxamic acids, trichostatin A (Yoshida et al. 1990) and suberoylanalide hydroxamic acid (SAHA) (Richon et al. 1998). Nanomolar concentrations of TSA have been used widely in in vitro systems to inhibit HDAC activity and modify gene transcription. SAHA is also active in in vitro models at micromolar concentrations. It induces terminal cell differentiation with milk protein synthesis in MCF-7 cells (Richon et al. 1998). Further, its administration reduced the incidence of N-methyl-nitrosourea-induced mammary carcinoma in rats without apparent toxicity (Desai et al. 1999). Phase I trials of SAHA are in progress (Marks et al. 2000). The third class of HDAC inhibitor, benzamide derivatives like MS-27–275, also showed marked activity against human tumor xenografts in mice (Saito et al. 1999). The fourth class includes cyclic tetrapeptides containing a 2-amino-8-oxo-9,10-epoxy-decanoyl (AOE) moiety such as trapoxin A (Kijima et al. 1993), while FR 901228 and apicidin are representative of the fifth class of cyclic peptides that lack an AOE moiety (Nakajima et al. 1998).

**Combination strategies**

Combined therapies may ultimately offer the best antineoplastic approach. If successful, such strategies could utilize agents that alone are ineffective but together result in the desired biological outcome. For example, sequential treatment of colon cancer cells with a demethylating agent followed by an HDAC inhibitor led to reactivation of multiple target genes whose expression was not affected by either agent alone (Cameron et al. 1999). Our own studies show that combined therapy of MDA-MB-231 cells with 5-aza followed by TSA results in re-expression of ER at lower doses than those needed with either single agent (Yang et al. 2001). Thus, it may be possible to use lower doses or shorter exposure durations to achieve the appropriate molecular effects, thereby increasing therapeutic effects, minimizing toxicity and improving ease of administration.

Alternatively, use of an ‘epigenetic modifier’ may enhance the response to another type of agent. The use of 5-aza and IFN in colon cancer cells was mentioned above. A human proof of principle for this general strategy is seen in the report of a patient with acute promyelocytic leukemia resistant to ATRA who developed a sustained remission after concomitant treatment with phenylbutyrate and ATRA (Warrell et al. 1998).

**Conclusions**

Taken together, substantial evidence demonstrates the importance of epigenetic mechanisms in the transcriptional regulation of critical tumor suppressor and growth regulatory genes in breast cancer. These genes include those that play crucial roles in DNA repair, cell cycle regulation, cell growth and cell–cell adhesion. These changes, along with the intrinsic ability of 5meC to function as a mutagen and the negative effects of dysregulated DNMT1 activity, can all contribute to breast cancer tumorigenesis and/or progression.

A better understanding of epigenetic regulation of gene expression in a gene-specific and tissue-specific fashion will help efforts to modulate gene expression selectively with the ultimate goal of improved breast cancer prevention and therapy.
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