Methylation of Estrogen and Progesterone Receptor Gene 5' CpG Islands Correlates with Lack of Estrogen and Progesterone Receptor Gene Expression in Breast Tumors

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Abstract

Hormonal factors have a profound influence on the development, treatment, and outcome of breast cancer. The absence of steroid hormone receptors is highly correlated with resistance to antihormonal treatments. Work in cultured human breast cancer cell lines has shown that the absence of estrogen receptor (ER) gene expression in ER- cells is associated with extensive methylation of the ER gene 5' CpG island, and treatment with agents that demethylate the ER gene CpG island results in the production of functional ER protein. The current study shows that CpG islands in the 5' region of the ER and progesterone receptor (PR) genes are methylated in a significant fraction of primary human breast cancer tissues. The ER CpG island is methylated at the methylation-sensitive NotI restriction site in 9 of 39 (25%) of primary ER- breast cancers but remains unmethylated in 53 ER+ breast cancers and 9 normal breast specimens. Three methylation-sensitive restriction sites in the PR gene CpG island are not methylated in normal breast specimens and PR+ human breast cancers but are hypermethylated in 40% of PR- human breast tumors. These data demonstrate that methylation of the ER and PR gene CpG islands is associated with the lack of ER and PR gene expression in a significant fraction of human breast cancers.

Introduction

The treatment and outcome of breast cancer are significantly affected by hormonal factors. One-third of breast cancers lack ER and PR, whereas another one-third are ER+ but PR-. Since the expression of PR requires ER as a transcriptional activator (2), the presence of PR in ER+ tumors is a better predictor for antiestrogen sensitivity than ER alone (1). Because antiestrogens are a first line approach in the treatment of hormone-responsive breast cancer and antiprogestins are currently under investigation, mechanisms of ER and PR gene regulation are critical areas of study.

ER- tumors lack ER gene expression, but this is not due to mutations within the ER gene (3, 4). PR tumors lack PR transcription, but no mutations within the DNA-binding domain of the ER gene or major polymorphisms or deletions within the PR gene have been identified (5, 6). Acquired loss of transcription of these hormone receptor genes is a potential mechanism of resistance to hormonal therapies. One mechanism by which gene expression can be silenced is aberrant methylation of cytosine-guanine-rich areas termed CpG islands in the 5' regulatory region and first exon of genes (7). CpG islands are unmethylated in normal adult tissue with the exception of transcriptionally silent genes on the inactive X chromosome and some imprinted genes (8, 9). Methylation of these islands has been shown to inhibit transcription directly or stabilize chromatin in a conformation that prevents transcription (10).

Work in cultured human breast cancer cells has shown that the absence of ER gene expression in ER- cells is associated with increased capacity to methylate DNA and extensive methylation of the CpG island of the ER gene (11). Treatment of two human ER- breast cancer cell lines with the demethylating agent, 5-aza-2'-deoxycytidine, results in the demethylation of the ER CpG island and the production of functional ER protein (12, 13). In this study, we determine whether ER CpG island methylation is also observed in primary breast cancers and whether a CpG island identified in the 5' region of the PR gene could also be altered by DNA methylation.

Materials and Methods

Cell Lines and Tissue Samples. DNAs from ER+/PR+, ER-/PR-, and ER+/PR- breast cancer specimens were obtained from the Department of Pathology at Vanderbilt University Hospital. Hormone receptor status was analyzed by both biochemical hormone-binding assay and ER immunohistochemical assay as described previously (4, 14). Additional primary breast cancers and reduction mammoplasty specimens were obtained from the Geraldine Brush Cancer Center and the Department of Medicine at Northwestern University Medical

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3 The abbreviations used are: ER, estrogen receptor; ER+ and ER-, ER positive and negative, respectively; PR, progesterone receptor; PR+ and PR-, PR positive and negative, respectively.
School. ER+/PR+ status was defined as greater than 3 fmol/mg protein for each steroid receptor. Tissues derived from reduction mammoplasty specimens and some cancers were enriched for epithelial cells by digestion with collagenase and hyaluronidase, followed by filtration and centrifugation to obtain an organoid preparation (15). DNA was isolated from the various tissues by solubilization in a SDS/proteinase K solution, followed by phenol/chloroform extractions and ethanol precipitation. DNA concentration was quantified by determination of OD260, and the integrity of DNA was verified on a 1% agarose gel.

**Southern Analysis.** For the analysis of DNA methylation status, 7.5 µg of DNA was digested with 10 units/µg EcoRI (methylation-insensitive enzyme used as a flank digest) and one of the following methylation-sensitive enzymes, 25 units/µg NotI, 15 units/µg SacII, 15 units/µg EagI or 15 units/µg BssH2 and subjected to electrophoresis on a 1% agarose gel. DNA was transferred to Zeta-probe membrane (Bio-Rad), and filters were hybridized as described by Ottaviano et al. (11) with a radiolabeled 300-bp PvuII/EcoRI cDNA fragment from the plasmid, pOR3 (obtained from American Type Tissue Collection, Rockville MD) or a 1.5-kb BamHI fragment from the plasmid php1 containing human PR genomic sequence (provided by Dr. P. Chambon, Institut de Chimie Biologique, Strasbourg, France). DNA probes were oligolabeled using a Redi-Prime kit (Amersham). To control for partial enzymatic digestion, all filters were rehybridized with a radiolabeled 1.3-kb NotI fragment from the plasmid c-myc (16) or a 658-bp PCR product of the c-abl gene.

**Results**

**Methylation Status of ER Gene.** The methylation status of the NotI site within the 5′ Cpg island of the ER gene was evaluated in a series of ER+ and ER− breast tumors and organoids by restriction endonuclease analysis and Southern hybridization (Fig. 1). Digestion of DNA with the methylation-insensitive enzyme, EcoRI, produces a 3.1-kb DNA fragment (Fig. 1). If the DNA is unmethylated, simultaneous digestion with EcoRI and the methylation-sensitive enzyme NotI produces 1.9- and 1.2-kb DNA fragments (Fig. 1). As expected, the NotI site in the ER gene Cpg island was unmethylated in DNA from all nine normal breast organoids tested (Table 1). In addition, DNAs from 53 ER+ primary cancers tested were all unmethylated at the NotI site (Table 1). Results for one normal breast organoid specimen, three representative ER+/PR+ tumors, and one ER+/PR− tumor demonstrating the expected 1.2- and 1.9-kb DNA fragments are shown in Fig. 1B. In contrast, DNAs from 9 of 39 (25%) of ER− breast tumors were partially methylated at the NotI site; two examples are shown in Fig. 1B. The methylation of the NotI site within ER− tumor cells is demonstrated by the presence of the 3.1-kb DNA fragment, whereas the unmethylated DNA, possibly derived from nonmalignant cells that infiltrate the breast tumors, is digested into 1.2- and 1.9-kb DNA fragments. Finally, DNA derived from two ER− metastases to bone and ovary were also partially methylated at the NotI site (Fig. 1B).

To exclude the possibility that the pattern of partial DNA methylation observed in ER− tumors was due to incomplete restriction endonuclease digestion, all filters were rehybridized with a genomic DNA fragment from the ubiquitously expressed c-myc gene, the corresponding Cpg island of which should be unmethylated. Results showed complete restriction endonuclease digestion for all samples, thus eliminating partial digestion as a cause of these findings (data not shown). In summary, the DNA from a significant fraction (25%) of ER− tumors is methylated at the NotI site in the ER Cpg island, whereas this site was unmethylated in DNAs from all 53 ER+ cancers and 9 normal breast tissues studied. This indicates that DNA methylation is associated with the lack of ER gene expression in human breast cancer in vivo as it is in vitro.

**Methylation Status of PR Gene.** The possibility that methylation of the PR gene is correlated with the lack of PR gene expression was next explored. The PR gene has a typical Cpg island in its first exon, which is 1-kb in size and has 70% GC content (Fig. 2A). It contains a wide array of methylation-sensitive restriction sites in the first exon. The methylation status of the PR gene in normal breast organoids and ER+/PR+, ER+/PR− and ER−/PR− human breast tumors is shown in Fig. 2, B and C. Digestion of DNA with the methylation-insensitive enzyme, EcoRI, produces a 5.6-kb DNA fragment. If the Cpg island is unmethylated, simultaneous restriction with EcoRI and any of the three methylation-sensitive restriction

![Fig. 1. Methylation status of ER gene Cpg island in ER+/PR+, ER+/PR−, and ER−/PR− human primary breast cancers.](image-url)
enzymes will produce the smaller fragments overlapping the probe as described in Fig. 2A. If the PR gene CpG island is methylated, larger fragments ranging in size to 5.6-kb will be observed. As a consequence of the large percentage of normal infiltrating cells within breast cancers, the smaller fragments corresponding to unmethylated DNA are also detected.

The PR gene was unmethylated at the EagI sites in all normal tissues tested including lymphocytes, whole lung, endometrium, bone marrow, kidney, liver, and colon (data not shown). These two EagI sites were also unmethylated in 6 normal breast organoid specimens (Fig. 2B; Table 1) and 13 ER+/PR+ primary breast tumors (two representative tumors are shown in Fig. 2B; Table 1). In contrast, 40% (8 of 20) of DNA from ER+/PR− tumors and 46% (11 of 24) ER−/PR− tumors were partially methylated at one or both of the EagI sites (Table 1). Examples of partially methylated tumor DNAs are shown in Lanes 6, 8, 9, and 10 in Fig. 2B. Also, DNA from one of two ER−/PR− metastases was partially methylated at the EagI sites (Table 1).

The methylation status of the three SacII sites within the PR gene CpG island was also examined. Methylation of one or more SacII sites is demonstrated by 3.0-, 3.4-, and 5.6-kb DNA fragments (Fig. 2A). The upstream SacII site (marked with * in Fig. 2A) was partially methylated in every normal tissue tested including lymphocytes, whole lung, endometrium, bone marrow, kidney, liver, and colon (data not shown). It was also partially methylated in 6 of 6 normal breast organoid preparations, 15 of 15 ER+/PR+ tumors and 19 of 19 ER+/PR−, and 23 of 23 ER−/PR− breast tumors, as indicated by the 3.0-kb band in Fig. 2C. Thus, methylation of the upstream SacII* site was universal and did not correlate with PR expression.

In contrast, the two downstream SacII sites (Fig. 2A) were unmethylated in every normal tissue tested, in 6 of 6 normal breast organoids, and in 15 of 15 ER+/PR+ tumors (Fig. 2C; Table 1). These two downstream SacII sites were partially methylated in DNAs from 4 of 19 (21%) of ER+/PR− tumors and 9 of 23 (39%) of ER−/PR− tumors (Table 1); results from representative cancers are shown in Fig. 2C. Thus, similar to the EagI sites, methylation of the two downstream SacII sites, which lie in the most dense part of the CpG island, is highly correlated with PR gene expression.

To confirm that methylation of the PR CpG island in PR− tumors occurred in the significant, CG-rich part of the island, two BssH2 sites were examined (Fig. 2A). These two sites were unmethylated in 3 of 3 breast organoids and in 5 of 5 ER+/PR+ breast tumors tested (data not shown; Table 1). DNAs from 2 of 6 ER+/PR− tumors, 4 of 9 ER−/PR− tumors, and 1 of 2 ER−/PR− metastases were partially methylated at the BssH2 sites, which is depicted by the presence of 3.6- and 5.6-kb DNA fragments (data not shown; Table 1). The unmethylated DNA fragment at 3.4 kb derived from the nonmalignant cells was present as expected in all PR− specimens tested. Within a DNA sample, each time methylation of the BssH2 sites and downstream SacII sites was observed, we also detected methylation at the EagI sites, verifying extensive methylation of the CpG island in PR− tumors.

To ensure that the DNA methylation observed was not due to partial restriction enzyme digestion, all filters were rehybridized with a genomic fragment from the c-abl gene, which should be unmethylated. Digestion was complete in all cases (data not shown). In conclusion, a significant fraction of PR− tumor DNAs is methylated at a number of methylation-sensitive restriction sites in the PR CpG island, which indicates that aberrant DNA methylation may play a role in silencing of the PR gene in human mammary carcinoma.

**Discussion**

Resistance to endocrine therapy is a major problem in the treatment of breast cancer and, in some cases, may be mediated by loss of ER and PR gene expression. One potential mechanism for loss of gene expression is methylation within 5′ CpG islands of target genes. Such aberrant DNA methylation in tumors has been associated with loss of expression of several tumor suppressor genes, including the p16 gene in a variety of tumor types, the von Hippel-Lindau gene in renal cancers, and the metastasis suppressor gene, E-cadherin, in breast and prostate cancers (21–23). Our laboratory has demonstrated that methylation of the ER gene CpG island is correlated with loss of ER gene expression in human breast cancer cell lines (11), and treatment of the ER−/PR− MDA-MB-231 and Hs578t cell lines with a demethylating agent results in reexpression of a functional ER protein (13). The goal of the current study was to investigate the methylation status of the ER and PR gene CpG islands in primary human breast cancers to validate the biological significance of our in vitro findings.

Our present study focused on the methylation status of the NotI site in the ER CpG island because our in vitro studies demonstrated an excellent correlation between methylation at the NotI site and lack of ER expression. We have shown that methylation of this site occurs in 25% of the ER− primary human breast cancers examined but is not observed in normal breast tissue or ER+ breast tumors. To date, there are rare examples of deletions, mutations, or polymorphisms within the

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**Table 1** ER and PR CpG island methylation status of primary breast tumors

<table>
<thead>
<tr>
<th>Gene phenotype</th>
<th>ER gene methylation</th>
<th>PR gene methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NotI site</td>
<td>EagI site</td>
</tr>
<tr>
<td>ER+/PR+</td>
<td>0/29 methylated (0%)</td>
<td>0/13 methylated (0%)</td>
</tr>
<tr>
<td>ER+/PR−</td>
<td>0/24 methylated (0%)</td>
<td>8/20 methylated (40%)</td>
</tr>
<tr>
<td>ER−/PR−</td>
<td>9/39 methylated (25%)</td>
<td>11/24 methylated (46%)</td>
</tr>
<tr>
<td>ER−/PR− metastases</td>
<td>2/2 methylated</td>
<td>1/2 methylated</td>
</tr>
<tr>
<td>Normal breast</td>
<td>0/9 methylated</td>
<td>0/6 methylated</td>
</tr>
</tbody>
</table>

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Fig. 2. Methylation status of the PR gene CpG island in ER+/PR+, ER+/PR−, and ER−/PR− primary human breast cancers. A, map of restriction sites in the 5′ region of the human PR gene adapted from sequences described in Kastner et al. (19) and Misrahi et al. (20). Exon 1, hgp1 PR probe, and predicted sizes in kilobases of restriction digestion fragments are shown. EcoRI (E) is the methylation-insensitive flank digest. *, EagI; ∆, SacII; ⊞, NruI; ★, BssH2; ⊙, SmaI; vertical lines, HhaI; *, upstream SacII site. The CpG dinucleotide frequency for the PR gene CpG island is shown. Included are 0, 6, and 12% CpG (CG) dinucleotide frequencies. B, Southern analysis of the PR gene CpG island EagI site in ER+/PR+, ER+/PR−, and ER−/PR− breast tumors. DNA (7.5 μg) was digested with EcoRI alone (Lane 1) or EcoRI and the methylation-sensitive restriction enzyme EagI (Lanes 2–10). Lanes 1 and 2, DNA from normal breast organoid specimens; Lanes 3 and 4, ER+/PR+ primary breast cancers; Lanes 5 and 6, ER+/PR− primary breast cancers; Lanes 7–10, ER−/PR− primary breast cancers. Lanes 6, 8, 9, and 10 show evidence of partial methylation. C, Southern analysis of the SacII sites within the PR gene CpG island. DNA (7.5 μg) was digested with EcoRI alone (Lanes 1 and 8) or EcoRI and SacII (Lanes 2–7, 9, and 10). Lanes 1–3 and 8, DNA from normal breast organoid; Lanes 4 and 5, ER+/PR+ primary breast cancers; Lanes 9 and 10, ER+/PR− primary breast cancers; Lanes 6 and 7, ER−/PR− primary breast cancers. Lanes 6 and 9 show evidence of partial methylation of the downstream SacII sites.
ER gene or other transcriptional mechanism to explain the lack of ER in breast cancer. Our findings are the first to suggest a mechanism, CpG island methylation, that could account for loss of ER expression in a significant fraction of primary human breast cancers.

There is a lower frequency and lower level of DNA methylation observed in primary ER− human breast cancers compared to that of cultured ER− breast cancer cell lines. Several explanations for these findings must be considered: (a) breast carcinomas are often diffusely infiltrated with normal tissue elements. Therefore, hypermethylation in the subpopulation of tumor cells within the tissue specimen may be more difficult to detect because it is obscured by unmethylated normal DNA. This may lead to an underestimation of the number of samples that are designated positive for DNA methylation at the ER gene CpG island as well as underestimation of the amount of methylation in each tumor sample. In support of our observations, Herman et al. (22) and Graff et al. (23) also had more difficulty characterizing methylation patterns for the 5′ region of the p16 and E-cadherin genes in breast cancers as compared to other tumor types; (b) immunohistochemical staining of breast cancer specimens demonstrates some cellular ER expression, even within tumors classified as ER− by biochemical assay. Consequently, these ER+ cells will also contribute DNA that is unmethylated at the ER gene CpG island; (c) Southern blot analysis is relatively insensitive and may not detect relatively small populations of ER− cells that are methylated at the ER gene CpG island; and (d) it is unlikely that ER gene methylation is the only mechanism by which breast cancer cells lose ER gene expression. We are currently developing an assay to monitor methylation in microdissected tissues to address the issue of tissue heterogeneity.

In addition, we have identified a CpG island within the 5′ region of the PR gene that includes several methylation-sensitive restriction sites. As expected, the two Eagl sites were unmethylated in DNA from all normal tissues and PR+ cancers tested but were partially methylated in greater than 40% of PR− tumors tested. Similar results were obtained using the methylation-sensitive enzymes, SacII and BssH2. PR gene expression is clearly associated with the methylation status of the two downstream SacII sites and BssH2 sites that are located in the most CG-rich portion of the island, as shown in Fig. 2A. Methylation of the Eagl site, BssH2, and the downstream SacII sites were consistently in accordance among samples showing extensive methylation within the body of the island, and methylation of this area correlates best with lack of PR gene expression in PR− breast cancers. Our data demonstrating that methylation at the upstream SacII site is found in DNA from all tissues examined, including those that express the PR gene, are consistent with recent results suggesting that gene expression can occur despite methylation of certain CpG nucleotides within or flanking the CpG island (24). The proximity of methylation-sensitive sites to key cis-acting promoter and enhancer sequences (25), methylation-induced conformational changes (26), promoter strength (24, 27), and the CG density within the island (24, 27) all play crucial roles in determining whether a particular gene is silenced by DNA methylation at a specific site.

In summary, we have demonstrated a significant correlation between aberrant methylation at the ER and PR CpG islands and lack of gene expression. These results suggest that this mechanism of gene silencing could account for loss of ER and PR gene expression and subsequent hormone resistance in a significant fraction of human breast cancers. Future studies should focus on designing new therapeutic strategies to target breast tumor cells that exhibit this phenotype.

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References


Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors.

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