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Advances in Brief

Methylation of the Estrogen Receptor Gene CpG Island Marks Loss of Estrogen Receptor Expression in Human Breast Cancer Cells[1]

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Abstract

Breast cancer is the most common malignancy in women and hormone resistance is a challenging problem in its treatment. Loss of estrogen receptor expression is an important means of hormone resistance, but the mechanisms involved are poorly understood. We now demonstrate a potential role for abnormal DNA methylation in transcriptional inactivation of the estrogen receptor gene. Estrogen receptor-negative human breast cancer cells growing in culture lack estrogen receptor mRNA, have a higher capacity to methylate DNA, and display extensive methylation of the CpG island in the 5′ promoter region of the estrogen receptor gene, which would correlate with silencing of expression. These results suggest that abnormal methylation could account for transcriptional inactivation of the estrogen receptor gene and subsequent hormone resistance in some human breast cancers.

Introduction

Both the initiation and progression of breast carcinoma are profoundly influenced by hormonal factors. Breast tumors that express the ER[3] are slower growing, associated with a better long-term disease-free survival, and amenable to endocrine therapy with agents such as the antiestrogen tamoxifen. However, only two-thirds of breast cancers are ER positive by hormone-binding or immunocytochemical assays at the time of diagnosis and, of these, only 60% respond to endocrine manipulation (1). In addition, many ER-positive cancers that initially respond to endocrine therapy ultimately progress to a more aggressive hormone-independent phenotype.

The molecular mechanisms underlying the clinical problem of hormone resistance are poorly understood. Hormone resistance clearly can develop in some breast cancers, occasionally through mutant receptors (2, 3) or more commonly through changes distal to the ER (4). However, one-third of breast cancers are ER negative, and *de novo* or acquired loss of ER gene expression at the transcriptional level is a likely mechanism for hormone resistance. Analysis of one ER-negative human breast cancer cell line shows direct evidence of loss of ER gene transcription by nuclear run-on assays (5). Dot blot (6), Northern (6, 7), reverse transcription-polymerase chain reaction analysis (2), and RNase protection assay (3) indicate that ER- and progesterone receptor-negative human breast cancers also lack ER mRNA. The absence of ER expression in these tumors is not due to detectable deletions or other gross structural alterations in the ER gene (8).

One mechanism that could block transcription of the ER gene in ER-negative breast cancers without structural alteration in the gene is methylation of cytosine-rich areas, termed "CpG islands," in the 5′ regulatory region of the gene (9). CpG islands are always unmethylated in normal adult tissues, with the exception of transcriptionally silent genes on the inactive X chromosome (9) and selected genes which are parentally imprinted to silence expression of one allele. The importance of CpG island methylation in silencing such imprinted genes has been recently shown in mutant mice that are deficient in DNA methyltransferase activity (10). Also, methylation in these islands *in vitro* can block transcription of downstream sequences (9, 11).

Such abnormal CpG island methylation has been observed for several genes in immortalized and transformed cells (9, 11, 12). However, it has not been described as a consistent finding for a gene known to play a central role in the progression of a common human cancer. In the present study, we report that the absence of ER gene expression in ER-negative breast cancer cells is associated with extensive abnormal methylation in the CpG island of the 5′ region of the ER gene. Furthermore, these cells have an increased capacity to methylate DNA, another property of neoplastic cells. This abnormal methylation pattern could account for transcriptional inactivation of the ER gene and subsequent hormone resistance in some human breast carcinomas.

Materials and Methods

Cell Lines and Tissue Samples. The acquisition and routine maintenance of MCF-7, ZR-75-1, T47D, Hs578t, MDA-MB-231, MCF-7WT, and MCF-7/AdrR human breast cancer cell lines have been described previously (13, 14). The MDA-MB-468 cell line was obtained from the American Type Culture Collection (Rockville, MD), and the MDA-MB-435 cell line was obtained from Dr. J. Price (M. D. Anderson Cancer Center, Houston, TX). Normal breast, thyroid, whole lung, bronchial epithelium, and cervix tissues and circulating mononuclear cells were obtained from the Department of Pathology, Johns Hopkins University School of Medicine (Baltimore, MD).

Molecular Probes. ER probes used included pOR3, a 1.3-kilobase cDNA probe obtained from the American Type Culture Collection; a 0.3-kilobase *Pvu*II fragment of pOR3 containing the 3′ portion of exon 1; and a 1.1-kilobase *Pvu*II fragment of λGHER1, kindly provided by Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, France) (15). A previously described 2.5-kilobase human cDNA probe was used to detect DNA methyltransferase transcripts (16). The above inserts were oligo-labeled using [^{32}P]dCTP and a multiprime labeling kit (Amersham). A human γ-actin cDNA probe, LK220, was obtained from Dr. L. Kedes (Stanford University, Palo Alto, CA) and nick-translated using [^{32}P]dCTP and a kit from GIBCO-BRL.

DNA Methyltransferase Activity Assay. Enzyme activity was determined as described previously (17).

Northern and Southern Hybridization Analyses. Northern analysis using total cellular RNA and nitrocellulose filters was performed using standard methods. For Southern analysis, 7.5–10 μg of DNA were fractionated on 2% agarose gels after restriction endonuclease digestion. Southern hybridizations were performed using Zetaprobe filters. Hybridizations were done at 65°C and posthybridization washes at 70°C. Restriction enzymes obtained from New

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[3] The abbreviations used are: ER, estrogen receptor; cDNA, complementary DNA; MT, methyltransferase.

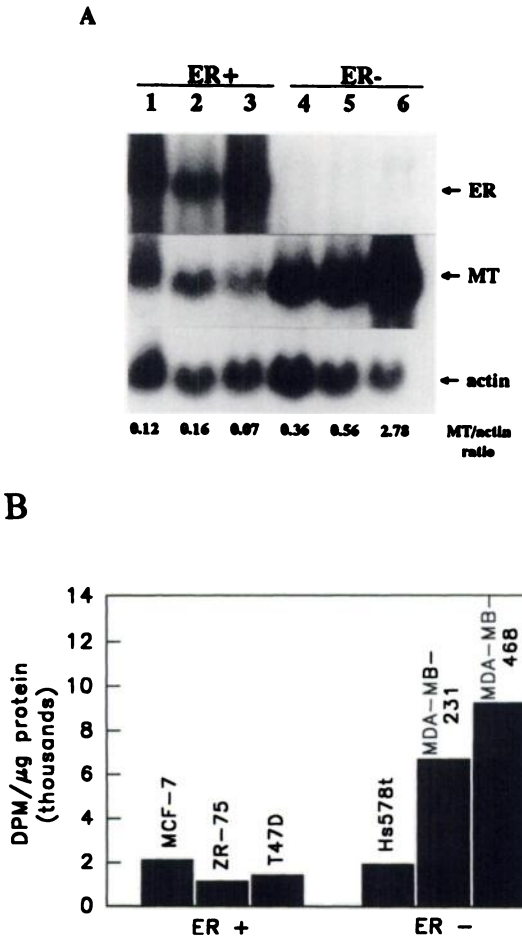

Fig. 1. ER and DNA MT mRNA expression and DNA MT activity in breast cancer cell lines. (*A*) Northern analysis using total cellular RNA (40 μg) isolated from 3 ER-positive (*ER+*) cell lines (*Lane 1*, MCF-7; *Lane 2*, T47D; *Lane 3*, ZR-75-1) and 3 ER-negative (*ER−*) cell lines (*Lane 4*, Hs578t; *Lane 5*, MDA-MB-231; *Lane 6*, MDA-MB-468) was performed with the indicated probes. MT:actin ratios were determined with a Tobias optical densitometer. (*B*) MT activity was determined as described previously (17) on the same panel of breast cancer cell lines. An average of duplicate determinations on confluent cells is shown.

England Biolabs (Beverly, MA) were used at the following concentrations: *Eco*RI, 15 units/μg DNA; *Bsm*I, 10 units/μg; *Not*I, 15–30 units/μg; *Hpa*II, 10 units/μg; *Hha*I, 10 units/μg; *Sac*II, 20 units/μg; and *Msp*I, 10 units/μg.

Results and Discussion

A panel of ER-positive and ER-negative human breast cancer cell lines was examined for steady state ER mRNA expression by Northern analysis (Fig. 1). The expected 6.3-kilobase ER mRNA transcript was easily detected in all three ER-positive cell lines (MCF-7, ZR-75-1, and T47D) but in none of the ER-negative cell lines (MDA-MB-231, MDA-MB-468, or Hs578t), supporting the hypothesis that the loss of ER gene transcription could account for the loss of ER expression in some breast cancer cell lines.

Transformed human cells have increased DNA MT activity, and this activity has been shown to increase further during progression of tumors like colon cancer (17). The expression of DNA MT mRNA and activity in the same breast cancer cell lines was therefore examined. Northern analysis showed 2–10-fold higher levels of DNA MT mRNA in ER-negative lines compared with ER-positive cell lines (Fig. 1*A*). DNA MT activity was similarly elevated in 2 of 3 ER-negative cell lines (Fig. 1*B*). These results suggest there is an increased overall capacity to methylate DNA in ER-negative breast cancer cell lines. The possibility that the ER CpG island might be a target for abnormal methylation in ER-negative breast cancer cells was thus studied.

The ER gene has a CpG island in its promoter and first exon regions marked by the clustering of sites for methylation-sensitive restriction endonucleases (Fig. 2) (15, 18). Thus, the ER gene is a candidate for transcriptional inactivation by methylation. The methylation status of the island was examined directly by Southern analysis of DNA digested with methylation-sensitive enzymes that cut within a fragment produced by a flanking cut with a methylation-insensitive enzyme, *Eco*RI or *Bsm*I. Digestion of unmethylated DNA with the methylation-sensitive enzyme, *Not*I, would be expected to generate 1.2- and 1.9-kilobase fragments from a 5′ *Eco*RI 3.1-kilobase fragment in the ER CpG island (Fig. 2).

As expected for an autosomal gene, the ER gene was unmethylated at the *Not*I site CpG island in all normal tissues examined, including thyroid, whole lung, bronchial epithelium, cervix, and circulating mononuclear cells (data not shown). This was also the case in the 3 ER-positive breast cancer cell lines, as well as in normal breast tissue (Fig. 3*A*). *Not*I digestion uniformly produced two lower molecular weight fragments from the 3.1-kilobase *Eco*RI fragment of the ER gene. However, the 3.1-kilobase *Eco*RI fragment was completely resistant to *Not*I digestion in each of three ER-negative cell lines and partially resistant in a fourth (MDA-MB-468), indicating that methylation at this locus is unique to ER-negative cell lines (Fig. 3*A*).

In addition, the *Not*I site was methylated in an ER-negative cell line, MCF-7/AdrR, derived from the ER-positive unmethylated MCF-7WT cell line by chronic treatment with the chemotherapeutic agent, doxorubicin (Fig. 3*A*, *Lanes 3* and *10*). Coincident with doxorubicin resistance and methylation at this site, MCF-7/AdrR cells have developed hormone resistance and no longer express ER or progesterone receptor as determined by hormone-binding assays (14).

Methylation of the CpG island of the ER gene in cultured ER-negative breast cancer cells is extensive and involves multiple methylation-sensitive restriction enzyme sites. In addition to the *Not*I site, 3 *Hha*I sites, 4 *Hpa*II sites, and 2 *Sac*II sites within the island were completely unmethylated in all ER-positive lines studied but were methylated to varying degrees in all ER-negative lines studied (Fig. 3, *B* and *C*). Of note, the ER-negative cell line, MDA-MB-468, which was partially methylated at the *Not*I site, was completely methylated at two *Sac*II sites (Fig. 3*C*).

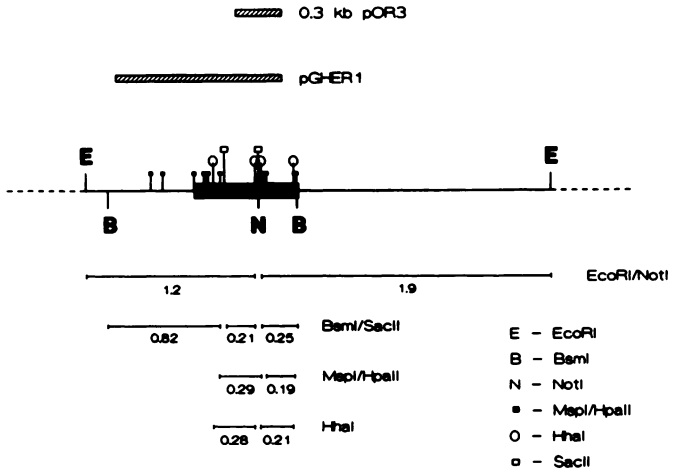

Fig. 2. Map of restriction sites in the 5′ region of the human ER gene adapted from the works of Green *et al.* (15) and Piva *et al.* (7). Exon 1 (■), ER probes (■) and predicted sizes in kilobases (*kb*) of restriction digestion fragments are shown. *Eco*R1 and *Bsm*I are methylation insensitive and were used for flanking cuts. *Not*I, *Sac*II, *Hpa*II, and *Hha*I cut only if their target sequences were unmethylated.

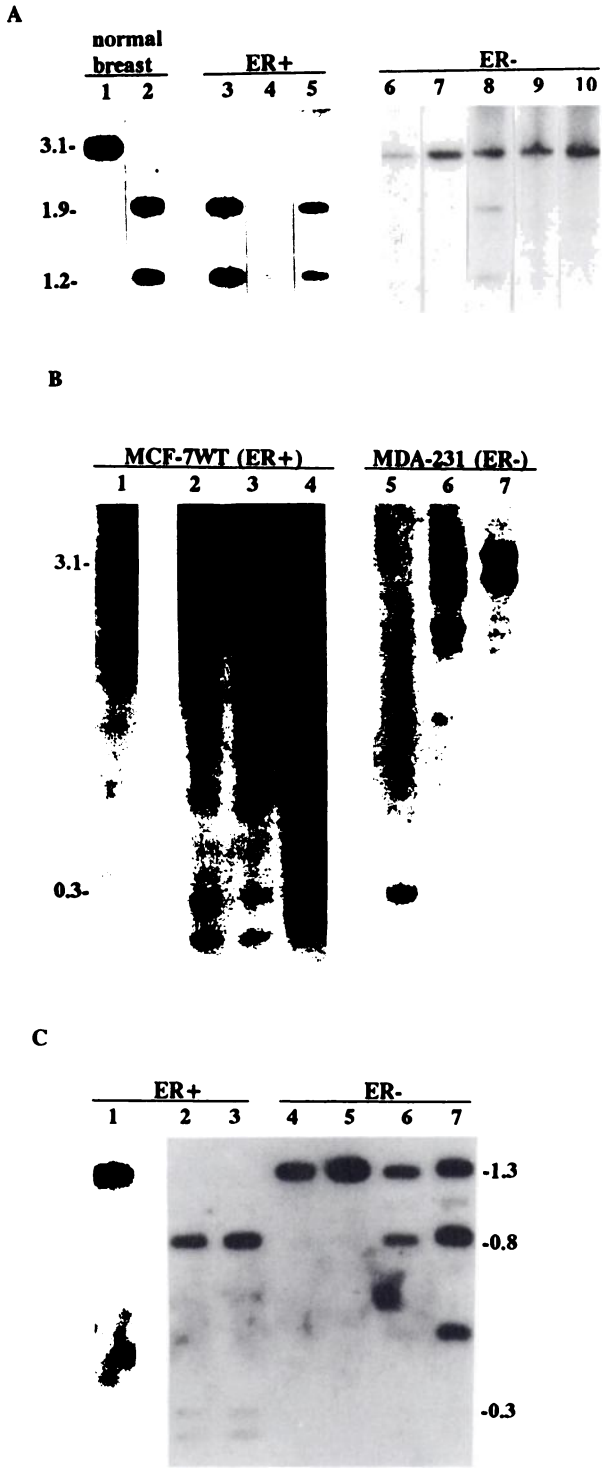

Fig. 3. Southern analysis demonstrating different methylation patterns in the ER CpG island in ER-positive (*ER+*) *versus* ER-negative (*ER−*) human breast cancer cell lines. (*A*) *Eco*RI flanking cut in normal breast tissue (*Lane 1*) is shown, followed by *Eco*RI/*Not*I digestions of remaining samples (*Lane 2*, normal breast; *Lane 3*, MCF-7WT; *Lane 4*, ZR-75; *Lane 5*, T47D; *Lane 6*, Hs578t; *Lane 7*, MDA-MB-231; *Lane 8*, MDA-MB-468; *Lane 9*, MDA-MB-435; *Lane 10*, MCF-7/AdrR). A 0.3-kilobase *Pvu*II fragment of the pOR3 ER cDNA probe (Fig. 2) was used. *Ordinate*, sizes in kilobases. (*B*) Results of restriction digestions with *Eco*RI alone (*Lane 1*), *Eco*RI/*Msp*I (*Lanes 2* and *5*), *Eco*RI/*Hpa*II (*Lanes 3* and *6*), and *Eco*RI/*Hha*I (*Lanes 4* and *7*) are shown for a representative ER-positive cell line, MCF-7WT (*Lanes 1–4*) and an ER-negative line, MDA-MB-231 (*Lanes 5–7*). *Msp*I recognizes the same sequence as *Hpa*II but cuts regardless of methylation status. The 0.3-kilobase ER cDNA was used as above. (*C*) *Bsm*I flanking cut is shown for MCF-7WT cells (*Lane 1*), followed by *Bsm*I/*Sac*II digestions of remaining samples (*Lane 2*, MCF-7WT; *Lane 3*, ZR-75; *Lane 4*, MDA-MB-231; *Lane 5*, MDA-MB-468; *Lane 6*, MDA-MB-435; *Lane 7*, Hs578t). A 1.1-kilobase *Pvu*II fragment of the genomic ER probe, λGHER1, was used (Fig. 2). Similar results were obtained for the ER-positive cell line, T47D, and the ER-negative cell lines, MDA-MB-435 and MCF-7WT/AdrR (data not shown).

Our work in cultured human breast cancer cells shows that there is a tight relationship between the absence of ER gene transcripts, increased cellular capacity for overall DNA methylation, and extensive abnormal methylation of the ER gene CpG island. In both *in vitro* test systems and immortalized transformed cells, such abnormal gene methylation either completely blocks gene transcription or marks chromatin changes which are incompatible with transcription (9, 11, 12). Thus, the abnormal ER gene hypermethylation observed may either mark or account for the ER-negative hormone-resistant phenotype in some breast cancers.

These findings are in contrast to interpretations drawn from previous studies of ER gene methylation status in breast cancer. However, it is essential to recognize that our studies are the first to examine directly methylation changes within the CpG island of the ER gene rather than potential methylation sites surrounding the island. Numerous studies have suggested that methylation in CpG islands, but not in surrounding sites, can inhibit transcription or stabilize structural changes in chromatin that do not allow transcription (9).

In earlier studies, other investigators found no relationship between ER gene methylation patterns and ER expression in a total of 87 breast cancers and 6 breast cancer cell lines (19, 20). These studies used a large ER gene probe (pOR8) that corresponded to internal ER gene sequences rather than the 5′ CpG island examined in the data presented here. Thus, their negative findings are not surprising since methylation of CpG sites in the body of genes generally has a variable correlation with gene expression (9).

In another study, the genomic 5′ probe, pGHER1, that does span the CpG island was used (7). However, the methylation-sensitive restriction sites examined were outside the CpG island region on the ER gene (*i.e.*, they fell well outside the ER gene map shown in Fig. 2). Also, the methylation-insensitive enzyme used for the flanking cut (*Bam*HI) actually cuts near the middle of the CpG island, making it difficult to draw any interpretation about the methylation status of the island. Using this strategy, the investigators found no association between methylation status and ER expression in human breast cancer cell lines in contrast to our own data, which show an excellent correlation between the methylation status of the CpG island and ER expression. In addition, they noted hypermethylation at the methylation-sensitive sites studied (which lie outside the CpG island) in all normal tissues examined, including WBC, endometrium, and normal breast. This result strongly suggests that the investigators did not examine the ER gene CpG island because CpG islands in autosomal genes are generally unmethylated with rare exceptions (9). Taken together, these data suggest that the methylation sites in the 5′ region of the ER gene examined in this study were not within the critical CpG island region and are therefore not likely to have any transcriptional significance.

Our finding of acquired methylation in the MCF-7/AdrR cell line is of particular interest in view of the observation that doxorubicin has been shown to induce methylation in lung adenocarcinoma and rhabdomyosarcoma cell lines (21). Doxorubicin-induced methylation of the ER gene in the MCF-7/AdrR line could conceivably account for the loss of ER transcription and subsequent hormone resistance. This raises the interesting possibility that doxorubicin treatment of ER-positive breast tumors might actually promote development of hormone-resistant, ER-negative clonal subpopulations associated with a worse prognosis from within the ER-positive tumors.

In summary, our work in cultured human breast cancer cells shows that the absence of ER gene expression in ER-negative cells is associated with an increased capacity to methylate DNA, as well as extensive methylation in the CpG island of the 5′ region of the ER gene. These *in vitro* findings suggest that abnormal methylation could account for transcriptional inactivation of the estrogen receptor gene

and subsequent hormone resistance in some human breast cancers, a possibility that should be studied using primary human breast cancer specimens.

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