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

Yvonne L. Ottaviano, Jean-Pierre Issa, Fritz F. Parl, Helene S. Smith, Stephen B. Baylin, and Nancy E. Davidson

Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231 (Y. L. O., J-P. I., S. B. B., N. E. D.); Department of Pathology, Vanderbilt University, Nashville, Tennessee 37232 (F. F. P.); and Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, California 94115 (H. S. S.)

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 ER3 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 PvuII fragment of pOR3 containing the 3’ portion of exon 1; and a 1.1-kilobase PvuII fragment of AGHER1, 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 [32P]dCTP and a multiprime labeling kit (Amersham). A human γ-actin cDNA probe, LK220, was nick-translated using [32P]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 70°C and posthybridization washes at 70°C. Restriction enzymes obtained from New...
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). These results suggest that the loss of ER gene transcription could account for the loss of ER 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 NotI site, 3 HhaI sites, 4 HpaII sites, and 2 SacII 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). Of note, the ER-negative cell line, MDA-MB-468, which was partially methylated at the NotI site, was completely methylated at two SacII sites (Fig. 3C).

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.

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 (E), ER probes (B) and predicted sizes in kilobases (kb) of restriction digestion fragments are shown. EcoRI and BsmI are methylation-sensitive and were used for flanking cuts. NotI, SacII, HpaII, and HhaI cut only if their target sequences were unmethylated.
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 (pOR3) 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, pOMER1, 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 (BamHI) 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/AdR 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/AdR cell 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.
ESTROGEN RECEPTOR GENE METHYLATION IN BREAST CANCER CELLS

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

Acknowledgments

We thank Drs. M. Abeloff, E. Barrack, J. Isaacs, T. Kensler, M. Makos, B. Nelkin, and P. Vertino for helpful discussions and review of the manuscript and B. Lee, S. Lund, and T. Hess for administrative assistance.

References


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


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/10/2552

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.