Frequent Methylation of Estrogen Receptor in Prostate Cancer: Correlation with Tumor Progression

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ABSTRACT

Prior studies have shown that the estrogen receptor (ER) gene is down-regulated in prostate cancer, but the mechanism of its inactivation is not known. We hypothesize that inactivation of the ER gene in prostate cancer is through promoter methylation. To test this hypothesis, we investigated the methylation status of the ER gene in prostate cancer cell lines, prostate cancer, and benign prostatic hyperplasia (BPH) tissues. By using the bisulfite genomic sequencing methods, we examined a 447-bp region of the ER gene promoter. The results show that the ER gene promoter was methylated in 100% (six of six) of the prostate cancer cell lines tested and all were accompanied by loss of ER mRNA expression. Treatment of these cell lines with demethylating agent 5-aza-2'-deoxycytidine restored ER mRNA expression in all of the ER-negative cell lines. In addition, elevated expression of DNA methyltransferase mRNA was found in all of the prostate cancer cell lines. Of the prostate tissue samples analyzed, 60% (6 of 10) in the BPH samples, 80% (8 of 10) in the low-grade cancer samples (grades I and II), and 95% (20 of 21) in the high-grade cancer samples (grades III-V) exhibited promoter methylation of the ER gene. The overall methylation levels in the cancer samples were higher than that in the BPH samples. Differences between the high-grade cancer samples and BPH samples were significant at all CpG sites. Only at three CpG sites were the differences significant between the low-grade cancer samples and BPH samples. This study presents the first evidence that ER gene is transcriptionally inactivated by DNA methylation in prostate cancer. Our data suggest that ER expression may be involved in the pathogenesis of prostate cancer, as well as BPH.

INTRODUCTION

During the past 50 years, estrogen has been used for the treatment of prostate cancer. Palliative, although not curative, effects have been widely acknowledged (1). It is generally believed that the favorable response to estrogen therapy in hormone-sensitive prostate cancer is mediated primarily via suppression of the hypothalmo-hypophysal axis, thus reducing the circulating androgens. Mangan et al. (2) have proposed a direct action of estrogen on prostate, presumably via the ER.

This hypothesis is also supported by several other investigators (3, 4); however, the functional role of estrogenic steroids and ER in the prostate is not clear. In recent years, with the demonstration of ER in normal, hyperplastic, and cancerous prostate tissue (5, 6) and of the in vitro growth of prostate cancer cells (7, 8), evidence began to accumulate that estrogen may exert direct effects on prostate via its own receptor. The ER gene is located on chromosome 6q25.1 (9) and belongs to a superfamily of transcription activators (10). Its protein product is a transcription factor that regulates the expression of estrogen-responsive genes by binding to a specific DNA sequence found in their regulatory regions. As a mediator of estrogen hormone action, the ER is involved in many important physiological processes. Loss or down-regulation of ER expression in prostate cancer has been frequently documented (5, 11, 12). In addition, an inverse correlation was found between ER expression and histological grade or pathological stage by Nativ et al. (13) and others (14, 15). Low ER expression was also associated with poor prognosis for effective endocrine therapy (14).

However, the precise role of ER in neoplastic transformation of prostate has not been established.

No mutation or other gross structural alterations of the ER gene in prostate cancer has been reported thus far to be responsible for ER down-regulation. One mechanism that could block transcription of the ER gene in ER-negative prostate cancer, without structural alteration in the gene, is the de novo methylation of cytosine-rich areas, termed “ CpG islands,” in the 5’ regulatory region of the gene (16). To date, the DNA Mtase encoded by Dnmt 1 is the only enzyme that has been shown to cause increased CpG dinucleotide methylation (17) and to trigger transformation (18). ER gene methylation has been observed in several human cancers, such as breast cancer (19), lung cancer, colorectal cancer, (20) and hematopoietic neoplasms (21), and has been related to inactivation of ER expression. The purpose of this investigation was to determine whether ER methylation is involved in inactivation of the ER gene in prostate cancer. Using the bisulfite genomic sequencing methods, we examined a 447-bp region of the ER promoter located immediately upstream from the transcribed sequence of the human ER gene.

MATERIALS AND METHODS

Cell Lines and Treatment. Human prostatic cancer cell lines LNCaP, PC3, and DU145 were obtained from American Type Culture Collection (Manassas, VA). NDI (human primary prostate cancer) and BPH1 (human benign prostate epithelium) cell lines were developed in our laboratory (22, 23). DU145 and NDI1 cells were cultured in DMEM. PC3 cells were cultured in F-12 Ham’s. LNCaP, TSUPr1, DUPro, and BPH1 cells were maintained in RPMI 1640. All media were supplemented with 10% (v/v) fetal bovine serum and 2 mm l-glutamine. All media and supplements were obtained from the University of California–San Francisco Cell Culture Facility. For drug treatment, exponentially growing cells were seeded at a density of 2 × 10⁶ cells/83 cm² flask (day 0). Cells were allowed to attach overnight before the addition of freshly prepared 5-azaC (Sigma Chemical Co., St. Louis, MO). On day 1, a final concentration of 2 μg/ml 5-azaC in PBS was added to the flask. The next day, the medium was changed. On day 3 and day 5, the cells were treated two more times as on day 1. On day 6, the cells were harvested.

Tissue Samples and Microdissection. Archived prostate cancer and BPH samples were obtained from the Veterans Affairs Medical Center in San Francisco and the University of California–San Francisco. Sections were cut 5 μm thick from formalin-fixed, paraffin-embedded tissues and mounted on microscope slide and diagnosed according to the Gleason scoring system. To collect cancer tissues for DNA extraction, microdissection was carried out as described previously (24).

Nucleic Acid Extraction. DNA from cell lines and microdissected tissues was extracted using QIAamp Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. Total RNA was extracted by guanidium thiocyanate-phenol-chloroform extraction using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH).

Reverse Transcription-PCR. Total cellular RNA (1–5 μg) was reverse transcribed using random hexamers primer and Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in a 40-μl reaction.
cDNA was amplified by differential PCR using primers specific for the ER gene (GGAGACATGAGAGCTGCCA, sense; CCAGCAGCATGTCGAGATC, antisense) and the β-actin gene (TCTACAATGAGCTGCGTGTG, sense; ATCTCCTTCTGCATCCTGTC, antisense). For amplification of Dnmt1 gene, primers TTCCATCCTTCTGCACAGG (sense) and TCTCCATCTTCGTCCTCGTCAG (antisense) were used, and the β-actin gene was also amplified as an interior control using primer TCTACAATGAGCTGCGTGTG (sense) and primer AATGTCAGGCACGATTTCCC (antisense). PCR reactions were performed in a PTC-200 thermal cycler (MJ Research, Watertown, MA) at 94°C for 1 min, 30 cycles at 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s, followed by an extension step at 72°C for 5 min. The PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide and were visualized by UV rays.

Bisulfite Genomic Sequencing. Bisulfite modification of genomic DNA was carried out according to reported methods (25). Modified DNA was amplified by two rounds of PCR with primers S1 (AAAGTGGTTAAGAGGTGGATTTA, upstream, sequence position 464 to 487; GenBank accession number X68051) and S2 (TCAAATTTACAAAATAAAACATAAA, downstream, sequence position 912 to 887; Fig. 1). The PCR conditions were 94°C

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**Fig. 1.** Schematic representation of ER gene promoter structure. TATA, ATG signal, and CACC box are indicated. Primer S1 and primer S2 were used to amplify a 447-bp fragment for direct sequencing. The position of primers is indicated (GenBank accession number X68051). ● CpG sites amplified by PCR.

**Fig. 2.** ER methylation in prostate cell lines and tissue samples. DNA was bisulfite-modified and amplified by two rounds of PCR. The resulting PCR products were direct sequenced. A, ER methylation in prostate cell lines. Methylation percentage of the individual CpG site is presented as the ratio of methylated cytosine to the total cytosine (methylated plus unmethylated) in each cell line. B, ER methylation in prostate cancer and BPH tissues. The methylation percentage of the individual CpG site is presented as the average ratio of methylated cytosine to the total cytosine (methylated plus unmethylated) of all samples in each group. *, sites with significant difference in methylation between cancer and BPH samples. Numbers on X axis are the position of CpG sites (GenBank accession number X68051).

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was amplified by PCR from bisulfite-modified DNA. The ER gene promoter structure is depicted in Fig. 1. Sequencing results revealed that all cytosines were converted to thymines, except those that existed as CpG doublet, and were methylated.

All of the six prostate cancer cell lines examined showed extensive methylation of the ER gene promoter (Fig. 2A). Of them, DU145, T5UPr1, DuPro, and ND1 exhibited 100% methylation at most of the CpG sites. PC3 and LNCaP were less methylated but still had sites that were 100% methylated. However, the normal cell line BPH1 did not show methylation at any CpG sites (Fig. 2A). We further treated ER-negative cell lines with the demethylating agent 5-azaC for 3 nonsuccessive days. After treatment, DNA was extracted and modified again, and the ER gene promoter was amplified using the same primers. Sequencing results of the PCR products from 5-azaC-treated cell lines revealed that most of the previously methylated CpG sites were demethylated completely with no methylated cytosine existing at the same CpG sites compared with that before treatment. Although the demethylation was not complete in some of the CpG sites, a lesser degree of methylation was observed when compared with the degree of methylation in nontreated cells (data not shown).

**ER Methylation in Human Prostate Cancer Tissues.** A total of 31 prostate cancer and 10 BPH tissue samples were analyzed for ER gene methylation. For cancer samples, we used microdissection techniques to collect tumor cells for DNA extraction and subsequent PCR amplification. Of the 31 prostate cancer tissue samples, 10 were low grade (including grades I and II) and 21 were high grade (including grades III-V). Overall, the percentages of samples that showed ER methylation were 60% (6 of 10) in BPH, 80% (8 of 10) in the low-grade cancer, and 96 (20 of 21) in the high-grade cancer. In contrast to methylation found in cell lines, no CpG site with complete methylation was found in tissue samples. To correlate methylation levels with tumor grades, we calculated the average methylation levels of individual CpG sites among each group. The resulting data from the cancer samples were compared with that from BPH samples using Student’s t test. DNA from cancer exhibited a universally higher average of methylation at every CpG site than DNA from BPH. The differences between the high-grade samples and BPH samples were significant at every CpG site (P < 0.01; Fig. 2B). Only three sites (positions 493, 516, and 528) showed significantly higher methylation in the low-grade samples compared with the BPH samples (P < 0.01; Fig. 2B). Examples of sequencing chromatograms are displayed in Fig. 3.

**Inactivation and Reactivation of the ER Gene in Prostate Cancer.** To understand whether mRNA expression of ER is inactivated by ER promoter methylation, we performed RT-PCR analysis. No ER mRNA expression was found in all of the prostate cancer cell lines examined, whereas BPH1, the normal prostate epithelial cell line, showed normal levels of ER mRNA expression (Fig. 4A). The mRNA expression in cell lines correlated well with their methylation status, as revealed by sequencing study (Fig. 2A). Treatment of cell lines with 5-azaC for 3 nonsuccessive days restored ER mRNA expression in all cell lines.

**RESULTS**

**ER Methylation in Human Prostate Cancer Cell Lines.** Using direct bisulfite genome sequencing techniques, we examined seven prostate cell lines (six cancerous and one normal) for ER gene promoter methylation. A 447-bp region (sequence position 464–912) of the proximal promoter of the ER gene, encompassing eight CpG sites, for 2 min, 35 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The first PCR products (1 µl) were subjected to a second round PCR cycled for 30 times. The resulting products were sequenced on an ABI automated sequencer with Dye terminators (Perkin-Elmer Corp.). Upper sequence represents GenBank sequence of the ER promoter (number X68051) from position 681–689. A CpG site is underlined, and the cytosine (C) or thymine (T) peaks are indicated by asterisks. A, complete methylation as found in prostate cancer cell lines. B, partial methylation as found in prostate cancer tissue. C, no methylation as found in BPH1 cell line.
of the cancer cell lines that showed no ER expression before treatment (Fig. 4B).

**Dnmt1 mRNA Expression** To understand the possible mechanism of ER methylation, we also examined the mRNA expression of the Dnmt1 gene in the cell lines and found all of the cancer cell lines exhibited elevated levels of Dnmt1 expression, whereas, interestingly, BPH1 showed only minimal expression (Fig. 5).

**DISCUSSION**

The results of our study demonstrate that the ER gene is extensively methylated both in prostate cancer cell lines and prostate cancer tissues. The methylation levels correlate with tumor pathological grades and reversely correlate with ER gene expression. We also analyzed ER gene methylation in BPH tissues as a control. Surprisingly, the ER gene is also methylated in BPH tissue, although at much lower levels when compared with cancer. In human colorectal mucosa, physiological aging has been associated with de novo methylation in the ER gene (20). BPH is an age-related benign condition seen in the majority of men over 50 years and is characterized by stromal and/or glandular (epithelial) hyperplastic changes of the prostate. It is not clear whether ER methylation in BPH is an age-related event because data on ER methylation in normal prostate is lacking. Another possible explanation for ER methylation found in BPH is that it represents a disease-specific epigenetic mechanism that may contribute to pathogenesis of BPH. Although no link has been established between BPH and prostate cancer, it has been suggested that BPH and malignant prostate growth share a common origin because they commonly coexist and demonstrate androgen dependency (27). Therefore, BPH may be a premalignant lesion in prostate cancer development. The methylation of the ER gene in BPH could be one of the earliest events that predispose to prostate cancer.

There are two possible mechanisms of inactivation of the ER gene in prostate cancer. First, activators responsible for ER transcription are not available or transcriptional repressors predominate. Alternatively, the CpG island within the promoter region of the ER gene may be methylated, which leads to transcriptional inactivation of the ER gene through several undefined mechanisms. In the prostate cancer cell lines analyzed in our study, mRNA expression of the ER gene was well correlated with methylation status in those cell lines. Furthermore, ER mRNA expression was restored by treatment with demethylating agent 5-azaC, a cytosine analogue that acts as a suicide substrate for DNA methyltransferase when incorporated into DNA at the target site for DNA methylation, CpG dinucleotides (28). These results clearly indicate that the promoter methylation is the mechanism of ER inactivation in prostate cancer.

Promoter methylation is an epigenetic mechanism by which the gene is silenced. Unlike germline mutation, which may affect the whole cell population of the body, DNA methylation may be tissue-specific or cell-specific. For methylation analysis of tumor DNA, contamination by DNA from normal cells may present a major concern because different methylation patterns may exist in these two cell populations. In the present study, we isolated pure population of cancer cells using microdissection technique for methylation study. Our data should have reflected the methylation status of a pure cancer cell population rather than a mixture of malignant and nonmalignant cells.

In normal adult tissues, CpG islands are unmethylated, with the exception of transcriptionally silent genes on the inactive X chromosome and some imprinted genes (29, 30). An imbalance of DNA methylation, involving widespread hypomethylation, regional hypermethylation, and increased cellular capacity for methylation, is characteristic of human neoplasm. This imbalance begins in preneoplastic cells and becomes more extensive throughout subsequent stages of tumor progression (31). The data from our work showed a distinct trend of ER methylation in prostate cancer. With tumor progression, the ER is gradually methylated, leading to transcriptional inactivation. The most extensive methylation is observed in cultured cancer cell lines, which may be explained as the result of the fact that most of them were derived from metastatic prostate cancer.

The function of estrogen in the prostate has remained unclear. The hypothesis is proposed that estrogen interact with androgen in setting up the pace of prostatic growth and function. Estrogen not only directs stromal proliferation and secretion but also, through insulin-like growth factor I, conditions the response of the epithelium to androgen (32). The action of estrogen requires presence of its receptor, ER, in its target cells. The ER gene itself has metastasis suppressor properties in breast cancer cells (33) and suppresses the growth of many different cell types in vitro (34). Therefore, a tumor-suppressor role has been suggested (20). The altered methylation patterns observed during prostate cancer progression may possess wide implications in our understanding of the role of estrogen and its receptor in the pathogenesis and endocrinal manipulation of prostate cancer. Our study provides a clue that estrogen and its receptor may be involved in the initiation and progression of prostate cancer, as well as BPH.

To understand the potential mechanism of ER methylation, we examined in cell lines the mRNA expression of Dnmt1, the enzyme that methylates cytosines that are 5 ‘ to guanosines and responsible for generating and maintaining DNA methylation patterns. Dnmt1 expression was elevated in all of the cancer cell lines examined. Our results are consistent with the current concept that the level of Dnmt1 expression is elevated significantly in neoplastic cells compared with normal cells (33). Recent evidence showed that increased DNA Mtase activity is an early event in carcinogenesis (34). One possible molecular mechanism of this elevation of DNA Mtase in cancer cells is that the expression of the Dnmt1 gene is regulated by oncogenic signaling pathways, such as the Ras-Jun signaling pathways (35, 36).

In conclusion, we demonstrate for the first time that ER gene methylation is a common event in prostate cancer, as well as BPH, can lead to inactivation of ER transcription, and is markedly associated with tumor progression. Our data offer insight into the mechanism by which ER is down-regulated in prostate cancer and may support the hypothesis that estrogen can have direct effects on prostate via its own receptor.

**REFERENCES**


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