Epigenetic Modifications of the Estrogen Receptor β Gene in Epithelial Ovarian Cancer Cells

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Abstract. Background: The mechanisms of estrogen insensitivity or estrogen resistance in ovarian cancer cells are not known. Studies on regulation of the estrogen receptor (ER) gene have suggested a role for epigenetics in silencing ER expression. Materials and Methods: Cells from insensitive ovarian cancer cells, SKOV3 and HEY, were cultured with and without the DNA methyltransferase (DNMT) inhibitor, 5-aza-2’-deoxycytidine (AzaC) and the histone deacetylase (HDAC) inhibitor, trichostatin (TSA). ERβ promoter methylation was examined using bisulfite sequencing. RNA was collected for oligonucleotide array studies. Results: Cell type-specific ERβ promoter methylation was found as well as relative hypomethylation of the ERβ promoter in SKOV3 compared to HEY cells. Preferential demethylation of specific CpGs by different treatments was found. AzaC and TSA resulted in significant tumor growth inhibition and alterations in expression of numerous genes. Conclusion: The ERβ promoter is differentially methylated in ovarian cancer cells. Moreover, AzaC and TSA can inhibit ovarian cancer cell growth.

Ovarian cancer is the most lethal malignancy of the female reproductive tract in the United States, with about 15,000 deaths annually (1). While the 5-year relative survival rates improved from 37% in 1973 to 43% in 1997, the ovarian cancer survival rate remains much worse than the 61.5% overall cancer survival for women (2). Up to 70% of women with epithelial ovarian cancer (EOC) suffer recurrences despite initial complete clinical response with surgical tumor debulking and chemotherapy (3). Hence, novel approaches to understanding the etiology of EOC and improving outcomes of women diagnosed with this disease are warranted.

One such approach seeks to understand the role of hormones in the pathogenesis of ovarian cancer. Appreciable epidemiological data implicate hormones, but the specific mechanisms involved are not well understood (4). Some evidence suggests that estrogen via its receptors, ERα and ERβ, can affect ovarian epithelial proliferation and tumor progression (5), and may play an important role in the genesis of EOC (4, 5). Of particular interest is the observation that as many as 60% of EOCs express ERs (5). Research has also proven the efficacy of tamoxifen use in chemotherapy-refractory ovarian cancer, albeit in a small number of patients and for a variable duration of response (0-56% response rate with a median duration of 4.4 months) (6-8).

While the exact mechanisms of estrogen insensitivity or estrogen resistance in ovarian cancer cells are not known, it is likely that responses to estrogen are dependent on the expression levels of ERs in the target tissue. Indeed, studies in human ovarian tissues have reported a differential ER isoform expression in cancer versus benign tumors or normal ovarian surface epithilum. In particular, a markedly increased ERα/ERβ mRNA ratio has been observed in ovarian cancer (9-14). ERα mRNA appears to be the main form expressed in malignant tumors (11, 13, 14), whereas decreasing ERβ expression is inversely correlated with tumor progression and metastatic disease (12-14). Recent work also suggested that ERβ appears to regulate ovarian cancer cell proliferation, motility and apoptosis (14). These findings lend support to the hypothesis that perhaps ERβ signaling might play a protective role in tumorigenesis and possibly counter the proliferative effects of ERα in EOC.

Furthermore, studies on transcriptional regulation of the ER gene have revealed a role for epigenetic events, particularly aberrant DNA methylation and histone acetylation in silencing its expression. It has been documented that the absence of ER gene expression in various tumors, including breast (15),

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endometrial (16), prostate (16), and colorectal (17), is associated with an inactive chromatin structure characterized by deacetylated histones and cytosine methylation of the ER CpG island. Interestingly, DNA methyltransferase (DNMT) inhibitors such as 5-aza-2’-deoxycytidine (AzaC) and histone deacetylase (HDAC) inhibitors such as trichostatin A (TSA) have been successfully used to induce ER expression in ER-negative breast cancer cells (15, 18), and sensitize hormone-resistant ER-negative breast cancer cells to tamoxifen (19). It is, therefore, increasingly recognized that epigenetic gene silencing plays a major role in tumor initiation and progression (20). Indeed, studies in ovarian cancer have confirmed a prominent role for epigenetic aberrations in disease progression, chemotherapy resistance, and outcomes (21, 22).

Building on this research, our hypothesis is that ERs may play an important role in the pathogenesis of EOC, such that the reactivation of ERβ expression by epigenetic modifications may inhibit tumorigenesis. In the present study, we begin to test this hypothesis by assessing the methylation status of the ERβ promoter region and ERβ gene expression in response to treatments with chromatin remodeling agents AzaC and TSA. We do this using two human ovarian cancer cell lines that are reported to be estrogen-insensitive: SKOV3, which is ER-positive (ERα>ERβ) but estrogen-nonresponsive, and HEY, which is ER-negative (9). We provide evidence that AzaC and TSA treatments are associated with partial demethylation of ERβ CpG islands and inhibition of cell proliferation.

Materials and Methods

Cell culture and treatments. Human ovarian carcinoma cell lines SKOV3 and HEY were generously provided by Drs. R. Alvarez and D. Curiel (University of Alabama at Birmingham, Birmingham, AL, USA). Cells were grown in culture at 37°C, 5% CO₂ in RPMI medium 1640 (Gibco BRL, Gaithersburg, MD, USA) supplemented with penicillin, streptomycin, L-glutamine (Gibco BRL), and 10% fetal calf serum (Gibco BRL). TSA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in absolute ethanol at a stock concentration of 1 mg/ml (3.30 mM), and stored at –20°C. AzaC (Sigma-Aldrich) was prepared in acetic acid at a stock concentration of 25 mg/ml (1.1 mM) and stored at –20°C. Cells were plated at 500,000 cells/10 cm plate 24 h prior to treatment. Cell treatments included AzaC (5 μM) for 96 h, and stored at –20°C. Cells were plated at 500,000 cells/10 cm plate 24 h prior to treatment. Cell treatments included AzaC (5 μM) for 96 h, and TSA (100 ng/ml) for 48 h, or a combination of AzaC (96 h at 5 μM) and TSA (added only during the last 48 h at 100 ng/ml).

Cell growth assays. SKOV3 and HEY cells were plated at a cell density of 5,000 cells/well in 12-well plates and treated as described above. Cells were counted daily using a Coulter counter and the percentage growth inhibition was determined by a comparison of the treated versus control (treated with diluent ethanol and/or acetic acid) cells. Cell growth was also measured by a Cell Titer assay (CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay, Promega, Madison, WI, USA). Briefly, 1x10⁴ cells were seeded in triplicates in 96-well plates. MTS and PMS detection reagents were mixed at a ratio of 20:1 immediately prior to addition to the cell culture. A volume of 20 μl of the MTS/PMS solution was added to the cells which were incubated for 3 h at 37°C, 5% CO₂, following the manufacturer’s recommendations. A microplate reader was used to read the absorbance at 492 nm. Statistical analysis was performed using Student’s t-test. Significance was defined as p<0.05.

DNA methylation analyses. Genomic DNA was isolated using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s recommendations. DNA quality was evaluated by measuring the 260/280 nm optical density ratio prior to bisulfite modification using the MethylEasy DNA Bisulphite Modification kit (Human Genetic Signatures, Macquarie Park, NSW, Australia). PCR amplification was then performed using the Qiagen PCR Purification kit (Qiagen, Valencia, CA, USA), examined by agarose gel electrophoresis and then used for sequencing to determine site-specific methylation changes in the amplified regions. The ERβ primer sequences were: 5'-GGATTGGTTTATTTTTTATTTTATTTTTTT-3' and 5'-CACCTCCTACAACTCAACTCC-3' (GenBank accession no. AF051427, nucleotide location 18-43 and 192-213).

Real-time PCR. Expression of ERβ was measured using quantitative real-time PCR in conjunction with SYBR Green I reagents (Bio-Rad, Hercules, CA, USA). Briefly, intact total RNA was extracted using the RNaseasy Mini kit (Qiagen), according to the manufacturer's instructions. RNA quality was evaluated by measuring the 260/280 nm optical density ratio, denaturing agarose gel and with an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Reverse transcriptase reactions were performed using the iScript cDNA kit (Bio-Rad). cDNA was amplified using the Bio-Rad IQ Supermix SYBR reaction buffer containing Taq DNA polymerase at an annealing temperature of 60°C for 40 cycles. The ERβ primers were: forward 5'-AAGGTTGGCCGACAAAG-3' and reverse 5'-ACAGGCTGAGCTCCA-3'. The comparative threshold cycle (Ct) method was used to calculate amplification fold, as specified by the manufacturer. Values for 18S ribosomal RNA (rRNA) were also determined and used to normalize the quantity of cDNA used in the experiments.

Oligonucleotide array analyses. RNA samples were labeled and hybridized to the Agilent Whole Human Genome Oligo Array, according to standard Agilent protocols. In brief, complementary RNA (cRNA) was generated and fluorescently labeled from 1 μg of total RNA in each reaction using the Agilent Low RNA Input Fluorescent Linear Amplification Kit. For each sample pair, the control human reference RNA (Stratagene) was labeled with cyanine 3 and experimental RNA samples with cyanine 5. Hybridization was performed following the Agilent oligonucleotide microarray hybridization user’s manual and the Agilent in situ Hybridization Plus kit with 750 ng of labeled cRNA. The arrays were scanned using the Agilent dual laser DNA microarray scanner with SureScan technology. The relative fluorescent intensity values in the Cy3 and Cy5 channels were then extracted from images using the Agilent Feature Extraction software 8.1 and the gene expression data were analyzed using GeneSpring software. An average of three replicate samples were used for each experimental condition and genes were defined as being differentially expressed if the fold change was equal or greater than 2.0, and the p-value was less than 0.05.
Results

Previous studies revealed an association between the absence of ER gene expression and methylation of the ER gene promoter region in ER-negative cancer cell lines. We therefore conducted analyses of two ovarian cancer cell lines that lack ER activity: SKOV3, an ER (ERα>ERβ)-positive but estrogen-nonresponsive cell line and HEY, an ER-negative cell line.

We first examined whether DNMT and HDAC inhibitors affect ovarian cancer cell growth. The IC50 for AzaC was determined to be 2.5-5 μM in SKOV3 and HEY cells after 72 h of treatment, whereas the IC50 for TSA was 50-100 μg/ml after 24 h of treatment (data not shown). Based on these results, concentrations of 5 μM of AzaC and 100 μg/ml of TSA were chosen for the studies. Cells were treated with vehicle or AzaC for 24, 48, 72 or 96 h and TSA for 12, 18, 24, or 48 h. To test the hypothesis that combination treatment might have additive or synergistic effects, a strategy employing 5 μM of AzaC for 96 h with the addition of 100 ng/ml of TSA for the last 48 h of culture was also used. As shown in Figure 1, treatment with AzaC alone did not inhibit SKOV3 cell proliferation (Figure 1A); however, AzaC led to significant growth inhibition of HEY cells within 48 h and this inhibition was further increased at 96 h (Figure 1B). Inhibitory effects of TSA treatments were observed in both
SKOV3 and HEY cells after 12 h of treatment (Figure 1C, D). Cell proliferation in both cell lines was also significantly inhibited by the combined treatment after 48 h (Figure 1E, F). The MTS cell proliferation assay confirmed the above findings (data not shown).

We next investigated the methylation status of the ERβ promoter region. Thirteen CpG dinucleotide sites were detected within the promoter (Table I). In the absence of any treatment, the ERβ promoter region in SKOV3 cells was relatively hypomethylated as compared to the same region in HEY cells. We observed 7 unmethylated sites in SKOV3 cells as compared to 3 unmethylated CpG dinucleotides in HEY cells. In an attempt to demethylate the CpG island and alter chromatin configuration, cells were treated with AzaC and TSA as single agents and in combination. Exposure of SKOV3 and HEY cells to these drugs resulted in preferential demethylation of several CpG sites by the different treatments (Table I). Bisulfite sequencing PCR analysis showed that treatment with AzaC produced demethylation of 4 additional CpG sites in SKOV3 cells and 7 new CpG sites in HEY cells. Similar results were noted for the combination treatment with one new CpG dinucleotide demethylated in SKOV3 cells and 6 additional demethylated CpG sites in HEY cells. Interestingly, treatment of both cell lines with TSA alone, a specific histone deacetylase inhibitor, led to alterations in the methylation status of the ERβ CpG island, as well. Taken together, these results demonstrate that AzaC and TSA as single agents and in combination lead to demethylation at several CpG dinucleotide sites within the ERβ promoter sequence.

Analysis of gene expression following treatment with AzaC, TSA, and AzaC + TSA was performed using the Agilent Whole Human Genome Oligo Array chip that encodes 41,000 genes and transcripts. mRNA expression levels from the treatment groups were compared to control samples and analyzed using the GeneSpring global error model and volcano plot analyses. As summarized in Table II, a total of 265 genes were up-regulated following AzaC treatment, 180 following TSA treatment, and 35 genes following AzaC + TSA treatment in SKOV3 cells. In the same cell line, 247 genes were down-regulated with AzaC, 128 with TSA, and 10 with AzaC + TSA treatments. Similarly, in HEY cells, 7 genes were up-regulated following AzaC, 46 following TSA, and 44 following AzaC + TSA treatments; while 64 genes were down-regulated with AzaC, 126 with TSA, and 93 with AzaC + TSA treatments. Few genes were significantly differentially regulated in common between the two cell lines: 6 following AzaC treatment, 34 following TSA treatment, and 7 following AzaC + TSA treatment. The observed induction of ERβ expression by RT-PCR was also visualized in these array experiments. ERβ gene expression was induced 3.2-fold in SKOV3 cells and 3.0-fold in HEY cells by AzaC; 4.3-fold in SKOV3 and 4.5-fold in HEY cells by TSA; and 5.2-fold in SKOV3 and 6.1-fold in HEY cells by the combination of AzaC and TSA (Figure 2).

**Discussion**

Recent studies have suggested that loss of ERβ expression is an important event in ovarian tumorigenesis, metastasis, and hormone-unresponsiveness (13, 14). ERβ expression appears to be protective, since adenoviral delivery of the gene of this
receptor subtype into ovarian cancer cells strongly inhibits estradiol-mediated cell proliferation and motility (14). On the basis of these data and the information regarding epigenetic regulation of ER expression in cancer (15-17), we hypothesized that loss of ERβ expression in epithelial ovarian cancer might be due to hypermethylation and/or histone acetylation of the ERβ promoter region.

Our studies suggest that DNA methylation of the ERβ promoter sequences is associated with transcriptional inactivation of the ERβ gene in SKOV3 and HEY cells. While the methylation patterns of the CpG sites within the ERβ promoter were cell-type specific, we observed preferential partial demethylation of specific CpG dinucleotides following treatment with AzaC and TSA as single agents and in combination. The finding that TSA, a HDAC inhibitor, demethylated the ERβ CpG island was unexpected but perhaps inhibition of histone deacetylases disrupts a repressive transcription complex that involves suppression of DNMT activity.

We also found that AzaC and TSA treatments were associated with inhibition of cell growth. While single agent AzaC treatment did not inhibit SKOV3 cell proliferation, TSA and the combination of AzaC and TSA did significantly inhibit cell growth in both cell lines. To define potential signaling pathways associated with induction of ERβ expression in ovarian cancer cells, we used oligonucleotide microarrays to globally analyze gene expression. We found that the expression of many genes was altered with exposure to AzaC, TSA, and the combination of AzaC and TSA. The gene networks that have been impacted by these treatments are currently under investigation. However, several genes that have been previously demonstrated to be epigenetically regulated in epithelial ovarian cancer cells, such as FANCF, DAPK, and hMLH1 (21) did not appear to be differentially expressed following treatments with chromatin-remodeling agents. This discordance could reflect cell line specificity, since we used immortalized human ovarian cancer cells as opposed to primary human ovarian cancer cells, and limitations of the current analysis.

In conclusion, our observations from this study suggest that DNA methylation of the ERβ promoter region may play a role in suppressing ER gene expression in estrogen non-responsive ovarian cancer cells. These results are similar to those reported by groups studying the role of ERs in other hormonally sensitive tumors, such as breast, endometrial, prostate and colorectal cancer. We showed that the chromatin-remodeling agents, AzaC and TSA, are associated with inhibition of cell growth.

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References


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