High-throughput DNA hypermethylation profiling in different ovarian epithelial cancer subtypes using universal bead array

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Abstract. DNA hypermethylation is common and plays a critical role in the regulation of gene expression. It is considered a major cause of carcinogenesis. High-throughput profiling method has been developed to analyze the methylation status of hundreds of pre-selected genes simultaneously. The aim of this study was to analyze promoter hypermethylation profiles of each subtype of ovarian epithelial cancer (OEC), to improve the understanding of the role of epigenetic silencing in carcinogenesis. DNA hypermethylation profiles on fresh frozen tissue samples of 5 serous, 3 mucinous, 5 endometrioid and 4 clear cell types of OEC, as well as 5 normal ovarian tissue samples as control. We used a high-throughput method for analyzing the hypermethylation status of 1,505 CpG loci selected from 871 genes simultaneously by GoldenGate Methylation Cancer Panel I (Illumina Human-6 v2 Expression BeadChip). Methylation status of seven genes was verified by methylation specific PCR (MSP). We identified 20, 37, 15 and 56 hypermethylated CpG locations in serous, mucinous, endometrioid and clear cell type OEC compared to control. Only 6 CpG loci were commonly hypermethylated across all subtypes of OEC. Hypermethylated loci of serous 17 (81.0%) and endometrioid type 10 (71.4%) were identical to that of clear cell type. However, mucinous type showed 17 peculiar loci (43.6%) out of 39 hypermethylated loci. The unique DNA hypermethylation patterns identified in different OEC subtypes suggest that their cause may involve different epigenetic mechanisms and the Bead chip used in this study is a useful tool to analyze DNA hypermethylation.

Introduction

Ovarian cancer is the fourth leading cause of cancer death in women (1). Patients (70%) have advanced disease (stage III or IV) upon presentation, with a 5-year survival between 15 and 20% at best with aggressive treatment. Ovarian epithelial cancer (OEC) accounts for over 90% of all cases and includes the following major histological subtypes: serous, mucinous, endometrioid and clear cell carcinomas. Cytogenetic and molecular analyses indicated that multiple genetic alterations were involved in the pathogenesis of ovarian cancer. BRCA1 and BRCA2 mutations are associated with increased ovarian cancer risks (2). Some studies have showed that p16 loss was associated with ovarian cancer prognosis (3). However, it remains unclear how genetic alterations lead to development and subsequent progression of ovarian cancer. Better understanding of the molecular mechanisms responsible for ovarian cancer development and progression will improve diagnosis and treatment of this disease.

DNA methylation is an epigenetic alteration that plays an important role in carcinogenesis (4). The importance of aberrant CpG island methylation as an alternative mechanism to inactivate tumor suppressor genes has been recognized recently. Addition of a methyl group to the cytosine residues of CpG dinucleotide clusters in the 5' regulatory regions of genes occurs frequently in cancer cells, but seldom in non-malignant cells. Aberrant DNA methylation occurs at the cytosines of CpG dinucleotides, which often exist in clusters called CpG islands. When methylation of these sites occurs in the promoter region of a gene, it can result in gene silencing. Silencing of functionally important genes leads to a state of high cellular proliferation. Hypermethylated CpG islands play a causal role in promoting cancer development and are useful molecular markers for diagnosis and prognosis.

Aberrant promoter methylation has been associated with loss of expression of a growing number of tumor-related genes in a variety of human cancers (5-8). For example, aberrant DNA methylation is a frequent epigenetic event in ovarian cancer. The importance of the role of aberrant methylation in ovarian cancer has become increasingly apparent with a growing list of genes, such as p16 (2,9), BRCA1, HIC1, MLH1 and RASSF1A (2,10-15). Methylation frequencies were higher in OEC than in borderline ovarian tumors (9). Currently, relatively little is known about the specific patterns of CpG island hypermethylation in different subtypes of OEC (16).

A number of recent methodological advances in the investigation of DNA methylation have enhanced the analysis of the role methylation plays in cancer. The application of DNA
microarray technology has enabled the study of a large number of gene expression profiles from numerous tissue samples. It has provided an opportunity to classify different neoplasms based on characteristic expression patterns (17).

The GoldenGate genotyping assay was implemented on a BeadArray platform, a high throughput tool for studying methylation alterations, to determine the role of aberrant methylation in different OEC subtypes. The methylation status of 1,505 CpG sites in 871 genes was investigated in this study (18).

Materials and methods

Tissue and preparation of DNA samples. Normal and malignant ovarian tissue samples were obtained from the Pusan National University Hospital. Samples were stored at -70°C. They included samples of fresh frozen tissue of 5 serous, 3 mucinous, 5 endometrioid and 4 clear cell type OEC. Histologic subtypes were determined according to World Health Organization (WHO) standards. Five normal ovarian tissue samples were used as control. Tissue samples were obtained from patients with advanced stage OEC (stage III, 9; stage IV, 8). Staging was performed according to the FIGO staging system. The mean age of patients was 55 years, and patient age ranged from 43 to 68 years. Genomic DNA was extracted from these specimens using the QIAamp tissue kit (Qiagen). Bisulfite-converted DNA samples were prepared using the Zymo EZ DNA Methylation kit (Zymo Research). Bisulfite-converted DNA (5 μl) was mixed with 5 μl of photobiotin (MSI; Illumina) and incubated at 95°C for 30 min.

Assay oligo extension and ligation. Biotinylated bisulfite-converted DNA was precipitated to remove free biotin, and subsequently dissolved biotinylated in solution (RS1; Illumina) bound to allele-specific oligonucleotides (ASOs) and locus-specific oligonucleotides (LSOs). Extension was carried out at 30°C overnight. Master mix (37 μl) for extension and ligation (MEL; Illumina) was added to the extension products, and incubated for 15 min at 45°C.

Polymerase chain reaction (PCR) amplification and PCR product preparation. After extension and ligation, beads were washed with universal buffer 1 (UB1; Illumina), re-suspended in 35 μl of elution buffer (IP1; Illumina) and heated at 95°C for on 1 min to release ligated products. The supernatant was then used in a 60-μl PCR. PCR reactions were thermocycled as follows: 10 min at 37°C, 3 min at 95°C; 34 cycles (35 sec at 95°C, 35 sec at 56°C, 2 min at 72°C); 10 min at 72°C then cooled to 4°C for 5 min. Three universal PCR primers (P1, P2 and P3) were respectively labeled with Cy3, Cy5 and biotin.

Double-stranded PCR products were immobilized on to paramagnetic particles by adding 20 μl of Paramagnetic Particle B Reagent (MPB; Illumina) to each 60 μl PCR, and incubated at room temperature for a minimum of 60 min. Bound PCR products were washed with universal buffer 2 (UB2; Illumina) and denatured by adding 30 μl of 0.1 N NaOH. After spending 1 min at room temperature, released single-stranded (ss) DNAs was neutralized with 30 μl of hybridization reagent (MH1; Illumina) and hybridized to arrays.

Array hybridization and imaging. Arrays were exposed to labeled ssDNA samples described above. Hybridization was performed under a controlled temperature gradient, from 60 to 45°C over 12 h. Hybridization was held at 45°C until the arrays were processed. After hybridization, the arrays were rinsed twice in UB2 and once with WC1 (WC1; Illumina) at room temperature, dried for 20 min, and then imaged at 0.8 μm resolution using a BeadArray Reader. Cy3 and Cy5 dyes were excited by laser emitted at 532 and 635 nm, respectively.

Methylation status was determine by calculating β, defined as the ratio of fluorescent signals of both methylated allele to the sum of the fluorescent signals of both methylated and unmethylated alleles. β value ranged from 0 in the case of completely unmethylated sites to 1 in completely methylated sites. To identify hypermethylated sites, we applied an additional filter that required a minimum difference of 0.15 in β between malignant and control samples.

Methylation specific polymerase chain reaction (MSP). The methylation status of 7 CpG island loci in malignant ovarian tissue samples and controls was determined by MSP. DBC1, HOXA9, SCGB3A1, SPARC, SOX1, TWIST1 and THY1 were selected for validation by MSP. DNA was extracted from 50 fresh frozen tissue samples of serous, mucinous, endometrioid, clear cell adenocarcinoma and normal ovarian tissue samples, using QIAamp DNA Micro kit (Qiagen, Hilden, Germany). Bisulfite treatment was carried out with 2 μg genomic DNA using EZ DNA Methylation Cold kit (Zymo Research) according to the manufacturer’s protocol. DNA samples were then purified by the Wizard DNA Clean-Up system (Promega, Madison, WI), then treated again with NaOH, ethanol-precipitated, and re-suspended in water.

For MSP, bisulfite-treated DNA samples were then used as PCR template with primers designed specifically for the CpG regions of each tested gene. Primer sequences of the methylated and unmethylated reaction were described in Table I. PCR mixture contained 2.5 μl of 10X PCR buffer, 1.0 μl of each primer, 2.5 μl of 2.5 mM dNTP and 0.2 μl (1 U) hot start Taq polymerase (Takara) in a final reaction volume of 25 μl. PCR products were electrophoresed on 2.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

A methylation-positive DNA control was prepared in vitro using SssI methylase (New England Biolabs, Beverly, MA), which methylated every cytosine of CpG dinucleotide in the DNA. Ten samples of non-malignant ovarian tissue were selected as control.

Results

Global methylation profiles in OECs. We measured the methylation status of the 1,505 CpG sites from 871 genes in different types of OECs, including serous, mucinous, endometrioid, and clear cell adenocarcinoma, and normal ovarian tissue samples as control. From this study, we obtained a list of differentially methylated markers that distinguished malignant from normal tissue samples. We identified 20, 37, 15 and 56 hypermethylated CpG sites from 19, 33, 14 and 46 genes in respectively serous, mucinous, endometrioid and
clear cell OEC subtype, which showed higher $\beta$ values (≥0.6) compared to that of normal ovarian tissue (≤0.4). Only 6 CpG loci were commonly hypermethylated across all types of OEC; \textit{ALOX12}, \textit{DAB2IP}, \textit{HOXA9}, \textit{HOXA11}, \textit{MOS} and \textit{SPARC}. Hypermethylated loci in serous 17 (81.0%) and endometrioid subtypes 10 (71.4%) were in common with that of clear cell subtype. However, the mucinous subtype showed 17 peculiar loci (43.6%) out of 39 hypermethylated loci. Selected genes fall into various classes, including tumor suppressor genes and genes involved in apoptosis, DNA repair, cell cycle, cell proliferation, differentiation, development, cell signaling, cell adhesion, transcription regulation and angiogenesis. Figs. 1 and 2 illustrate differential methylation profiles in malignant and control tissue samples, as well as specific methylation signatures obtained for individual OEC subtype.

### Table I. MSP primer sequence, product size and annealing temperature.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
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<tbody>
<tr>
<td></td>
<td>Mf</td>
<td>Mr</td>
<td>Uf</td>
</tr>
<tr>
<td>\textit{DBC1}</td>
<td>5'-TAGAGAGATGTTGATATAAATGG-3'</td>
<td>5'-CCAAAATAAACTAAACTAACCATA-3'</td>
<td>5'-ATAGAGAGACGCTAGATATAAACCAG-3'</td>
</tr>
<tr>
<td></td>
<td>\textit{HOXA9}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{SCGB3A1}</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>\textit{SPARC}</td>
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<td></td>
<td>\textit{SOX1}</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>\textit{TWIST1}</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>\textit{THY1}</td>
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Validation of array results by MSP. We used MSP to confirm methylation status of the CpG sites of 7 genes identified by our microarray analysis; HOXA9, SPARC, SOX1, DBC1, TWIST1, SCGB3A1 and THY1 (Fig. 3).

HOXA9 and SPARC were hypermethylated in all OEC subtypes and were selected for validation. They were hypermethylated in relatively high frequencies (22/40, 55% and 11/40, 27.5%). SOX1 promoter hypermethylation observed in all OECs except the endometrioid subtype on microarray was detected in 12 OEC tissue samples (30%). Only one endometrioid adenocarcinoma tissue sample was confirmed SOX1 hypermethylation on MSP. Hypermethylated DBC1 and TWIST1 demonstrated only in mucinous adenocarcinoma were evaluated by MSP. Mucinous adenocarcinoma showed more promoter hypermethylation in two genes compared to other OEC subtypes. Promoter hypermethylation in DBC1 and TWIST1 were detected in respectively 4 (40%) and 2 (20%) mucinous adenocarcinoma samples. In contrast, DBC1 hypermethylation was detected in only 13.3% (4/30) of the other OEC subtypes. Particularly, no hypermethylation was observed in other OEC subtypes for TWIST1. Four clear cell adenocarcinomas samples (40%) showed SCGB3A1 hypermethylation, and only one serous adenocarcinoma (10%) showed SCGB3A1 hypermethylation. MSP identified THY1 hypermethylation in 1 serous (10%), 1 mucinous (10%) and 3 clear cell adenocarcinomas (30%) tissue samples, but not in mucinous adenocarcinoma.

All of OEC tissue samples were found to have hypermethylation in one or more of the 7 genes of interest. Normal ovarian samples were unmethylated at these sites (Table II).

Discussion

This study utilized a high-throughput methodology to analyze the methylation status of hundreds of genes simultaneously, in order to elucidate methylation signatures that distinguish malignant from normal ovarian tissue samples. We used universal bead array methylation profiles (GoldenGate Methylation Cancer Panel I; Illumina Human-6 v2 Expression BeadChip) of 1,505 CpG sites from 871 genes in a panel of different types of OEC. Bead array-based platform has recently been adapted to detect DNA methylation (18). The GoldenGate Methylation assay on a bead array platform retains the high sample throughput (up to 96 samples) provided by bisulfate-based techniques, but greatly expands the number of loci (up to 1,505 CpG sites) that can be interrogated simultaneously. This method can detect changes in methylation status with only 200 ng of genomic DNA. It does not simply measure DNA methylation qualitatively (positive vs. negative methylation), but a quantitative measure of DNA methylation levels. In this study, 22 OEC and normal ovarian tissue samples were simultaneously analyzed. We showed that CpG island hypermethylation is widespread in OEC genomes, and various hypermethylation patterns present in the different OEC subtypes.

Differential methylation was independently confirmed by MSP, yielding a select group of CpG loci that have previously been reported in OEC and other types of cancer, which may be useful as epigenetic markers for OEC. We also observed high concordance between results obtained by our microarray-based methylation analyses and that by MSP. These results led us to conclude that bead array methylation assay is appropriate for large-scale analyses of hypermethylated loci throughout the genome, whereas MSP is more sensitive in identifying a hypermethylated CpG locus at a time. As such, bead array methylation assay and MSP data did not completely correlate. This microarray-based technology can be considered a powerful method to simultaneously assess the methylation status of hundreds of genes in large populations.

In this study, numerous CpG loci were identified and the involving genes fall into various classes, including tumor suppressor genes and genes involved in apoptosis, DNA repair, cell cycle, cell proliferation, differentiation, development, cell signaling, cell adhesion, transcription regulation and angiogenesis. Some have previously been reported to be hypermethylated in OEC, including APC (11), HOXA9 (19), HOXAI1 (20), MYOD1 (21), RASSF1 (22) and SCGB3A (19).
Although homeobox genes have been studied extensively and their expression profiles determined in a number of human tissues, little is known about their methylation patterns, in both normal and malignant tissues. In this study, the promoter of HOXA9 was frequently hypermethylated in all types of OEC. High HOXA9 methylation frequency has been reported in all types of OEC (19). Therefore, aberrant gene expression of HOXA9 may be involved in ovarian carcinogenesis.

Secreted protein, acidic and rich in cysteine (SPARC) is a 35-kDa calcium binding glycoprotein involved in cell adhesion, motility and interactions with extracellular matrix components. SPARC expression and SPARC gene functional analysis in malignant tissues have been widely studied. Recent
reports highlighted the role of this molecule as positive and negative modulators in the pathogenesis of different malignancies (23). In many cancers, up-regulation of SPARC has been reported in the peri-tumoral stromal cells of prostate,
breast and esophageal cancer, as well as glioma (23,24). However, SPARC was down-regulated in some cancers, such as colorectal carcinomas (25). Down-regulation of SPARC is related to aberrant methylation of CpG islands in the promoter region. Ovarian cancer cells treated with SPARC showed inhibition of cell proliferation and underwent apoptosis (26). SPARC promoter hypermethylation was found in certain OEC subtypes in this study.

SCGB3A1, also named HIN1 (high in normal-1), encodes a small secreted protein, secretoglobin 3A1 and belongs to the secretoglobin family. It is reported to be a potent inhibitor of anchorage-dependent and anchorage-independent cell growth, cell migration and invasion (27). Hypermethylation-induced down-regulation of this gene has been found in several cancer types, such as breast, lung, colorectal and testicular cancer, suggestive of tumor suppressor function (28). SCGB3A1 hypermethylation has been reported in mucinous and clear cell adenocarcinoma subtypes (19). This study has also demonstrated that SCGB3A1 promoter hypermethylation occurs in OECs, suggesting that this event plays a role in the development of certain OEC subgroup.

SOX1 is crucial for neuronal development. It has been reported that SOX1 expression attenuates carcinogenic potential of neuronal precursors after neural stem cell transplantation (29). A recent study of DNA methylation using microarray in cervical cancer showed that SOX1 was frequently hypermethylated in squamous cell carcinoma (30). We found that SOX1 hypermethylation might be associated with OEC.

THY1 is a 25- to 28-kDa surface glycoprotein, which is expressed on the cytoplasmic membrane in different cell types (31). THY1 triggers a variety of cellular functions, including proliferation, lymphokine release, differentiation and apoptosis. Despite extensive investigations, the exact function and physiologic role of THY1 in the cell remains unknown (32). THY1 is associated with tumor suppression in human ovarian cancer. However, there is a lack of direct evidence in support of THY1 as a candidate tumor suppressor gene in ovarian cancer (32). A recent study showed that THY1 was a good candidate tumor suppressor gene and the mechanism of THY1 gene inactivation was attributed to hypermethylation in nasopharyngeal carcinoma (33). We suggest that THY1 may be a candidate marker in ovarian carcinogenesis.

The deleted in bladder cancer 1 gene (DBC1) has been identified as a potential tumor suppressor gene commonly hypermethylated or deleted in bladder cancer (34). Abnormal methylation or deletion of DBC1 has also been described in other cancers, such as oral squamous cell carcinoma and non-small cell lung cancer (35,36). Twist belongs to the basic-helix-loop-helix family of transcription factors and is implicated in lineage-specific cellular differentiation and survival (37). TWIST1, an anti-apoptotic and pro-metastatic transcription factor, is overexpressed in many epithelial cancers such as breast cancer (37). Recently, human breast carcinomas have been reported to exhibit TWIST1 promoter hyper-

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**Table II. Summary of gene hypermethylation validated by MSP in EOCs.**

<table>
<thead>
<tr>
<th>Type of OEC</th>
<th>HoxA9 (%)</th>
<th>SPARC (%)</th>
<th>SOX1 (%)</th>
<th>DBC1 (%)</th>
<th>TWIST1 (%)</th>
<th>SCGB3A1 (%)</th>
<th>THY1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous (n=10)</td>
<td>7 (70)</td>
<td>3 (30)</td>
<td>4 (40)</td>
<td>1 (10)</td>
<td>0 (0)</td>
<td>1 (10)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Mucinous (n=10)</td>
<td>5 (50)</td>
<td>2 (20)</td>
<td>3 (30)</td>
<td>4 (40)</td>
<td>2 (20)</td>
<td>0 (0)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Endometrioid (n=10)</td>
<td>4 (40)</td>
<td>3 (30)</td>
<td>1 (10)</td>
<td>1 (10)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Clear cell (n=10)</td>
<td>6 (60)</td>
<td>3 (30)</td>
<td>4 (40)</td>
<td>2 (20)</td>
<td>0 (0)</td>
<td>4 (40)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>Total (n=40)</td>
<td>22 (55)</td>
<td>11 (27.5)</td>
<td>12 (30)</td>
<td>8 (20)</td>
<td>2 (5)</td>
<td>5 (12.5)</td>
<td>5 (12.5)</td>
</tr>
</tbody>
</table>

Figure 3. Representative methylation-specific polymerase chain reaction (MSP) results are shown for the 7 analyzed genes.
methylation at high frequency, and methylation of the TWIST1 promoter is a good predictor of human breast cancer (38). DBC1 and TWIST1 were hypermethylated predominantly in ovarian mucinous adenocarcinoma in this study, and therefore potentially a candidate marker of this OEC subtype. This study has elucidated the importance of epigenetic regulation in the development of different OEC subtypes. We identified several epigenetically dysregulated gene targets in OEC through global screening, using a microarray-based assay and a subsequent validation method.

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