# Promoter Hypermethylation Profile of Ovarian Epithelial Neoplasms

Prakash B. Makarla, M. Hossein Saboorian, Raheela Ashfaq, Kiyomi O. Toyooka, Shinichi Toyooka, John D. Minna, Adi F. Gazdar, and John O. Schorge

## Abstract

**Purpose:** Ovarian carcinomas are believed to arise *de novo* from surface epithelium, but the actual molecular pathogenesis is unknown. The aim of this study was to compare the promoter hypermethylation profiles of ovarian epithelial neoplasms to better understand the role of epigenetic silencing in carcinogenesis.

**Experimental Design:** We analyzed the DNA promoter methylation status of eight tumor suppressor and cancer-related genes (*p16, RARβ, E-cadherin, H-cadherin, APC, GSTP1, MGMT, RASSF1A*) in 23 benign cystadenomas, 23 low malignant potential (LMP) tumors, and 23 invasive carcinomas by methylation-specific PCR.

**Results:** Benign cystadenomas exhibited promoter hypermethylation in only two genes, *p16* (13%) and *E-cadherin* (13%). LMP tumors also showed *p16* (22%) and *E-cadherin* (17%) methylation, in addition to *RARβ* (9%) and *H-cadherin* (4%). All eight genes were hypermethylated in invasive cancers at a frequency of 9% to 30%. The mean methylation index was highest in invasive tumors (0.20 versus 0.065; *P* = 0.001). Promoter methylation of at least one gene was most commonly observed among invasive cancers (78% versus 44% in LMP; *P* = 0.003) and 26% (cystadenomas; *P* = 0.0009). Three genes exhibited higher methylation frequencies in invasive tumors: *RASSF1A* (30% versus 0%; *P* = 0.0002), *H-cadherin* (22% versus 2%; *P* = 0.013), and *APC* (22% versus 0%; *P* = 0.003).

**Conclusions:** Promoter hypermethylation is a frequent epigenetic event that occurs most commonly in invasive epithelial ovarian carcinomas. The profile of aberrant methylation suggests that an accumulation of events at specific genes may trigger malignant transformation of some benign cystadenomas and LMP tumors.

## Ovarian Cancer

Ovarian cancer is expected to account for 16,210 deaths in the United States in 2005, more than all other gynecologic malignancies combined (1). Three quarters of patients present with stage III to IV disease, chiefly because there is no reliable screening technique for early detection (2). The surface epithelium gives rise to >80% of ovarian cancers and a spectrum of other lesions ranging from benign cystadenomas to low malignant potential (LMP) tumors (3, 4). The majority of invasive ovarian epithelial carcinomas are believed to arise *de novo* from surface epithelium, but the actual molecular pathogenesis is unknown. Loss of tumor suppressor gene (TSG) function by allelic loss and somatic mutation has been reported in ovarian carcinomas, but does not seem to be the main mechanism of inactivation (5, 6).

Epigenetic silencing by DNA methylation is the major alternative to accomplish TSG inactivation (7). CpG aberrant methylation has been associated with loss of expression of a growing number of tumor-related genes in a variety of human cancers and has been proposed as the most common mechanism for gene regulation in cancer (8, 9). Methylation of numerous cancer-associated genes has been reported in ovarian cancers (10–14). However, few studies have compared the epigenetic aberrations of benign cystadenomas, LMP tumors, and invasive epithelial ovarian carcinomas (15). The profile of aberrant genetic alterations that accumulate in ovarian epithelial neoplasms might aid in determining whether some ovarian cancers originate from noninvasive precursor lesions.

Our study profiled the promoter hypermethylation status of eight genes by methylation-specific PCR in benign cystadenomas, LMP tumors, and invasive ovarian epithelial cancers. These genes are frequently hypermethylated and silenced in several cancer types. They include TSGs, a detoxification gene, and other genes involved in development, differentiation, and tumor invasion (16). The aim of this study was to compare the methylation profiles of the major forms of ovarian epithelial neoplasms to better understand the role of epigenetic silencing in ovarian tumorigenesis.

## Materials and Methods

**Specimen collection and DNA extraction.** Institutional Review Board approval was obtained to retrieve tumor tissue from 69 patients...
undergoing oophorectomy for an adnexal mass at the University of Texas Southwestern Medical Center. All of the samples were stored at -70°C. Ovarian LMP tumors and invasive epithelial cancers were staged according to the International Federation of Gynecology and Obstetrics (17). The DNA was extracted from at least 100 mg of frozen tissue specimens. Surgically resected tumor tissues were macroscopically dissected to separate them from nonmalignant tissues. DNA was extracted from at least 100 mg of frozen tissue specimens. Surgically resected tumor tissues were macroscopically dissected to separate them from nonmalignant tissues. DNA was prepared using a standard technique of digestion with 200 μg/mL proteinase K in the presence of SDS at 50°C overnight, followed by phenol/chloroform, and precipitation with 100% ethanol (18).

**Methylation-specific PCR.** DNA was treated with sodium bisulfite as previously described (19). Treated DNA was purified by use of Wizard DNA Purification System (Promega Corp., Madison, WI), desulfonated with 0.3 mol/L NaOH, precipitated with ethanol, and resuspended in water. Aberrant methylation of p16, RARβ, E-cadherin, H-cadherin, APC, GSTP1, MGMT, and RASSF1A was determined by using primers specific for the methylated and unmethylated alleles of each gene after treatment of the DNA with sodium bisulfite. These genes were selected based on their frequent methylation in a variety of tumors as reported previously (15, 20–26).

**Data analysis.** Frequencies of methylation of the three histologic groups were compared using Fisher's exact test. The methylation index (MI) is a reflection of the methylation status of all of the genes tested. The MI was determined for each tumor as the total number of genes methylated divided by the total number of genes analyzed. To compare the extent of methylation for the panel of genes examined, we calculated the MIs for each case (27) and then determined the mean for the different groups. Statistical analysis of MI between different groups was compared using the Student's t test and Mann-Whitney nonparametric U test. For all tests, probability values of P < 0.05 were regarded as statistically significant. All statistical tests were two sided.

**Results**

Mean patient ages were 41.7 years (range, 24-72) for benign cystadenomas, 45.6 years (range, 19-87) for ovarian LMP tumors, and 51.5 years (range 20-86) for invasive carcinomas. Twenty-one LMP tumors were stage I, one was stage II, and one was stage III. Early stage (I-II) invasive ovarian carcinomas more commonly had mucinous or endometrioid adenocarcinoma histology (Table 1; P = 0.006); advanced stage (III-IV) lesions were more commonly serous or undifferentiated (P = 0.01).

The hypermethylation status of a panel of eight normally unmethylated tumor suppressor or cancer genes in 69 ovarian epithelial neoplasms was examined using the methylation-specific PCR assay (Fig. 1). Benign cystadenomas exhibited promoter hypermethylation in only two genes: p16 (13%) and E-cadherin (13%). Ovarian LMP tumors showed methylation in four genes: p16 (22%), E-cadherin (17%), RARβ (9%), and H-cadherin (4%). Invasive lesions were more likely to have methylation at these four genes compared with benign cystadenomas (23% versus 7%; P = 0.003), but only slightly more than LMP tumors (13%; P = 0.12). Early stage and advanced invasive ovarian carcinomas had similar rates of methylation at these four genes (P = 0.65, 0.56, 1.0, and 0.62, respectively). Overall, promoter hypermethylation was observed in 9% to 30% of invasive carcinomas at all eight genes (Table 2). RASSF1A, APC, GSTP1, and MGMT showed aberrant methylation exclusively in invasive ovarian carcinomas.

When benign cystadenomas, LMP tumors, and invasive lesions were considered together, aberrant methylation at one or more of the eight genes was detected in 49% (34 of 69). Promoter methylation of at least one gene was found in 78% of invasive ovarian carcinomas (18 of 23), compared with 44% (10 of 23; P = 0.03) of LMP tumors, 26% (6 of 23; P = 0.0009) of benign cystadenomas, and 31% (5 of 16; P = 0.007) of normal controls. Three genes exhibited higher frequencies of promoter hypermethylation in invasive lesions compared with noninvasive tumors: RASSF1A (30% versus 0%; P = 0.0002), H-cadherin (22% versus 2%; P = 0.013), and APC (22% versus 0%; P = 0.003).

The overall mean MI was higher (0.20) in invasive ovarian cancers, compared with the noninvasive tumors (LMP: 0.065 and benign cystadenomas: 0.033; P < 0.0001) and normal controls (0.039; P = 0.002). The mean MI was similar for early stage and advanced invasive carcinomas (0.22 versus 0.18; P = 0.57). Serous ovarian adenocarcinomas had a higher mean MI than serous LMP tumors (P = 0.019) or benign serious

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**Table 1. Clinical features and methylation indices of invasive epithelial ovarian cancers**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>8 (35)</td>
<td>0.22</td>
</tr>
<tr>
<td>II</td>
<td>2 (9)</td>
<td>0.25</td>
</tr>
<tr>
<td>III</td>
<td>11 (47)</td>
<td>0.17</td>
</tr>
<tr>
<td>IV</td>
<td>2 (9)</td>
<td>0.25</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2 (9)</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>3 (13)</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>17 (78)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not determined; FIGO, International Federation of Gynecology and Obstetrics.

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**Fig. 1. Methylation-specific PCR in ovarian epithelial neoplasms.** Bands indicate amplification for the corresponding genes: p16 U (unmethylated), p16 M (methylated), RARβ, E-cadherin, and RASSF1A in representative samples from invasive (I) epithelial cancers, borderline (B) ovarian LMP tumors, and benign cystadenomas (C). The methylation specificity was 100%, 100%, 94%, and 87%, respectively.
cystadenomas \( (P = 0.0004; \text{Fig. 2A}). \) Mucinous ovarian adenocarcinomas also had a higher mean MI compared with either type of noninvasive tumor \( (P = 0.042 \text{ and } P = 0.023, \text{respectively}; \text{Fig. 2B}) \) and approached significance \( (P = 0.06) \) in having a higher mean MI compared with clear cell adenocarcinomas.

**Discussion**

Promoter hypermethylation is a frequent epigenetic event that occurs more commonly in invasive epithelial ovarian carcinomas than LMP tumors or benign cystadenomas. Numerous TSGs and other cancer-related genes are methylated and thereby inactivated in a variety of human malignancies (7). Reexpression has been shown to suppress tumor growth and sensitize cells to chemotherapy (28). This novel approach to cancer treatment involves compounds that can readily reverse epigenetic silencing, such as the DNA methyltransferase inhibitor 2'-deoxy-5-azacytidine (Decitabine, SuperGen, Inc., Dublin, CA; ref. 29). Clinical trials utilizing compounds that reverse epigenetic inactivation are currently under way in patients with a variety of gynecologic cancers (30). Because DNA methylation patterns seem to be tumor-type specific and normal cells are unmethylated, tumor specificity should be enhanced (31). Currently, women with recurrent ovarian cancer are considered incurable and palliative treatment is directed at improving their quality of life. Biological agent therapy is an especially attractive option for these patients. We observed promoter hypermethylation of at least one gene in 78% of epithelial ovarian carcinomas and the overall mean MI was 0.20. Our findings support the biological plausibility of pursuing demethylating agent therapy in the treatment of ovarian cancer (28, 29).

The profile of aberrant genetic alterations suggests that some invasive epithelial ovarian cancers may originate from noninvasive precursor lesions by an accumulation of methylation events at specific genes. \( p16 \) and \( E-cadherin \) were hypermethylated in benign cystadenomas; all six other genes in this study were unmethylated. LMP tumors exhibited methylation at these two genes, while accumulating \( RARb \) and \( H-cadherin \) methylation. Ovarian carcinomas were also hypermethylated at these four genes, while accumulating \( RASSF1A, APC, GSTP1, \) and \( MGMT \) methylation (10, 11, 15). Some molecular genetic analyses support a continuum of events in a subset of cystadenomas that leads to the development of LMP tumors and ultimately invasive epithelial ovarian cancer (32). However, the vast majority of epithelial ovarian carcinomas are believed to arise de novo by transformation of the surface epithelium via an unknown cascade of molecular events (33). Further

<table>
<thead>
<tr>
<th>Type of ovarian specimen</th>
<th>( p16 )</th>
<th>( RARb )</th>
<th>( E-cadherin )</th>
<th>( H-cadherin )</th>
<th>( RASSF1A )</th>
<th>( APC )</th>
<th>( GSTP1 )</th>
<th>( MGMT )</th>
<th>MI (mean)</th>
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<tr>
<td>Invasive cancer</td>
<td>30% (7/23)</td>
<td>13% (3/23)</td>
<td>26% (6/23)</td>
<td>22% (5/23)</td>
<td>30% (7/23)</td>
<td>22% (5/23)</td>
<td>9% (2/23)</td>
<td>9% (2/23)</td>
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<tr>
<td>Serous</td>
<td>22% (2/9)</td>
<td>11% (1/9)</td>
<td>22% (2/9)</td>
<td>22% (2/9)</td>
<td>33% (3/9)</td>
<td>11% (1/9)</td>
<td>22% (2/9)</td>
<td>0% (0/9)</td>
<td>0.18</td>
</tr>
<tr>
<td>Mucinous</td>
<td>50% (3/6)</td>
<td>17% (1/6)</td>
<td>33% (2/6)</td>
<td>33% (2/6)</td>
<td>17% (1/6)</td>
<td>50% (3/6)</td>
<td>0% (0/6)</td>
<td>17% (1/6)</td>
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<tr>
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<td>20% (1/5)</td>
<td>20% (1/5)</td>
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<td>20% (1/5)</td>
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<td>50% (1/2)</td>
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<td>0% (0/2)</td>
<td>0% (0/2)</td>
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<tr>
<td>Undifferentiated</td>
<td>100% (1/1)</td>
<td>0% (0/1)</td>
<td>100% (1/1)</td>
<td>0% (0/1)</td>
<td>100% (1/1)</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
<td>0.38</td>
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<td>Low malignant potential</td>
<td>22% (5/23)</td>
<td>9% (2/23)</td>
<td>17% (4/23)</td>
<td>4% (1/23)</td>
<td>0% (0/23)</td>
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<td>0% (0/23)</td>
<td>0% (0/23)</td>
<td>0.065</td>
</tr>
<tr>
<td>Serous</td>
<td>17% (2/12)</td>
<td>8% (1/12)</td>
<td>17% (2/12)</td>
<td>8% (1/12)</td>
<td>0% (0/12)</td>
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<td>0% (0/12)</td>
<td>0% (0/12)</td>
<td>0.063</td>
</tr>
<tr>
<td>Mucinous</td>
<td>27% (3/11)</td>
<td>9% (1/11)</td>
<td>18% (2/11)</td>
<td>0% (0/11)</td>
<td>0% (0/11)</td>
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<td>0% (0/11)</td>
<td>0% (0/11)</td>
<td>0.068</td>
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<tr>
<td>Benign cystadenoma</td>
<td>13% (3/23)</td>
<td>0% (0/23)</td>
<td>13% (3/23)</td>
<td>0% (0/23)</td>
<td>0% (0/23)</td>
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<td>0% (0/23)</td>
<td>0% (0/23)</td>
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<tr>
<td>Serous</td>
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<td>0% (0/13)</td>
<td>15% (2/13)</td>
<td>0% (0/13)</td>
<td>0% (0/13)</td>
<td>0% (0/13)</td>
<td>0% (0/13)</td>
<td>0% (0/13)</td>
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</tr>
<tr>
<td>Mucinous</td>
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<td>0% (0/10)</td>
<td>10% (1/10)</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
<td>0.038</td>
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<td>Normal control</td>
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<td>0% (0/16)</td>
<td>6% (1/16)</td>
<td>13% (2/16)</td>
<td>13% (2/16)</td>
<td>0% (0/16)</td>
<td>0% (0/16)</td>
<td>0% (0/16)</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Table 2. Summary of gene hypermethylation in ovarian epithelial neoplasms.

Fig. 2. Comparison of frequencies of aberrant promoter hypermethylation in (A) serous and (B) mucinous ovarian neoplasms.
defining the frequency and types of molecular aberrations across a spectrum of ovarian epithelial neoplasms might clarify this controversy and identify which genes are integral to tumorigenesis.

Ovarian LMP tumors had a methylation profile that was intermediate between that of benign cystadenomas and invasive ovarian carcinomas (34). Our study represents the largest description of ovarian LMP methylation data to our knowledge (15, 35). Clinically, these tumors have a histologic appearance and biological behavior that are intermediate between benign and frankly malignant ovarian neoplasms (3, 36). Surgically staged patients do not benefit from adjuvant therapy due to the indolent nature of growth and propensity for late recurrences. Overall, the prognosis is excellent and even women with stage III disease have a 96% survival rate at 10 years (37). Most studies have not detected a common pattern of genetic alterations between invasive epithelial ovarian carcinomas and LMP tumors (38, 39). However, Edelson et al. (40) hypothesized that some invasive lesions had progressed through an earlier, clinically inapparent tumor based on their identification of a common region of allelic loss. This hypothesis is also supported by the identification of a high percentage of K-ras mutations in both LMP tumors and invasive epithelial ovarian carcinomas (41). Our findings also suggest that a minority of LMP tumors represent an intermediate step from transformation of benign cystadenomas to frankly malignant ovarian cancer.

Invasive ovarian epithelial carcinomas were the only neoplasms to exhibit methylation of RASSF1A (30%), APC (22%), GSTP1 (9%), and MGMT (9%). Methylation of the RASSF1A (40%) gene has been previously reported in epithelial ovarian cancers at a comparable frequency (11). In a prior study of 49 ovarian carcinomas, we observed promoter methylation in RASSF1A (41%), APC (18%), and GSTP1 (2%; ref. 10). MGMT has not been previously studied. Epigenetic silencing of any of these genes might support the conventional view whereby invasive lesions arise de novo without any precursor lesion. Alternatively, these changes may represent a pathway whereby additional genetic alterations are accumulated to initiate a clinically more aggressive behavior.

TSG silencing is important for the development of ovarian carcinomas (15, 42). P16 gene silencing by methylation or other mechanisms greatly exceeds gene inactivation by mutation or allelic loss (15). Loss of RARβ expression contributes to the tumorigenicity of human ovarian cancer cells and reinduction leads to apoptosis (42). Loss of E-cadherin expression is associated with poorer prognosis in patients with ovarian cancer (43). However, reexpression in nude mice prolongs survival (44). Promoter hypermethylation of H-cadherin and RASSF1A has also been associated with gene inactivation in ovarian cancer (11, 45). APC methylation is a proposed mechanism behind its down-regulation in ovarian cancer (10, 46). Invasive lesions were more likely to have methylation at p16, E-cadherin, RARβ, and H-cadherin compared with benign cystadenomas in this study. In addition, the TSGs RASSF1A and APC were only methylated in invasive lesions. Targeted reexpression of TSG epigenetic inactivation may provide an effective means for novel treatment strategies in ovarian cancer.

Our study represents the first comprehensive comparison of the profile of the three major forms of ovarian epithelial neoplasms. We have shown that aberrant promoter hypermethylation of TSGs and cancer genes is frequent, histologically widespread, and can occur relatively early in ovarian tumorigenesis. Our data support the dualistic model for ovarian carcinogenesis: one pathway involves a stepwise progression from LMP tumors and the other is characterized by de novo transformation of the ovarian surface epithelium or inclusion cysts (47).

References


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