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 LMP and 0.033 cystadenomas; P = 0.001). Promoter methylation of at least one gene was most commonly observed among invasive cancers (78% versus 44% LMP; P = 0.03) 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.

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 staged according to the International Federation of Gynecology and Obstetrics.

**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.

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%), \( RAR\beta \) (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 serous
Table 2. Summary of gene hypermethylation in ovarian epithelial neoplasms

<table>
<thead>
<tr>
<th>Type of ovarian specimen</th>
<th>p16</th>
<th>RARβ</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>
</tr>
</thead>
<tbody>
<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>
<td>0.20</td>
</tr>
<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>
<td>0.27</td>
</tr>
<tr>
<td>Clear cell</td>
<td>0% (0/5)</td>
<td>20% (1/5)</td>
<td>0% (0/5)</td>
<td>0% (0/5)</td>
<td>20% (1/5)</td>
<td>20% (1/5)</td>
<td>20% (1/5)</td>
<td>20% (1/5)</td>
<td>0.10</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>50% (1/2)</td>
<td>0% (0/2)</td>
<td>50% (1/2)</td>
<td>50% (1/2)</td>
<td>50% (1/2)</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
<td>0.25</td>
</tr>
<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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<tr>
<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>
<td>0% (0/23)</td>
<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>
<td>0% (0/12)</td>
<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>
<td>0% (0/11)</td>
<td>0% (0/11)</td>
<td>0% (0/11)</td>
<td>0.068</td>
</tr>
<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>
<td>0% (0/23)</td>
<td>0% (0/23)</td>
<td>0% (0/23)</td>
<td>0.033</td>
</tr>
<tr>
<td>Serous</td>
<td>8% (1/13)</td>
<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>
<td>0.028</td>
</tr>
<tr>
<td>Mucinous</td>
<td>20% (2/10)</td>
<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>
</tr>
<tr>
<td>Normal control</td>
<td>0% (0/16)</td>
<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>

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 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
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 epithelial 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

Promoter Hypermethylation Profile of Ovarian Epithelial Neoplasms

Prakash B. Makarla, M. Hossein Saboorian, Raheela Ashfaq, et al.


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