Methylation Profiles of Sporadic Ovarian Tumors and nonmalignant Ovaries from High-Risk Women

Asha Rathi, Arvind K. Virmani, John O. Schorge, Keren J. Elias, Riichiroh Maruyama, John D. Minna, Samuel C. Mok, Luc Girard, David A. Fishman, and Adi F. Gazdar

Hamon Center for Therapeutic Oncology Research [A. R., A. K. V., J. O. S., K. J. E., R. M., J. D. H., L. G., A. F. G.], and Departments of Pathology [A. K. V., A. F. G.] and Obstetrics and Gynecology [J. O. S., K. J. E.], University of Texas Southwestern Medical Center, Dallas, Texas 85930; Laboratory of Gynecologic Oncology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115 [S. C. M.]; and Department of Obstetrics and Gynecology, Northwestern University Hospital, Chicago, Illinois 60611 [D. A. F.]

Abstract

Purpose: The purpose of this research was to examine the DNA methylation profiles of primary sporadic ovarian cancers and ovarian tissues from high-risk women.

Experimental Design: We analyzed the DNA methylation status of nine cancer-related genes in 49 primary ovarian tumors, 39 nonmalignant ovarian tissues obtained from 16 women with no known risk and from 23 high-risk women with a strong family history of breast and/or ovarian cancer or BRCA1 germ-line mutations, and 11 ovarian cancer cell lines, by methylation-specific PCR.

Results: Our findings are as follows: (a) methylation rates of four of nine genes, RASSF1A (41%), HIC1 (35%), E-cadherin (29%), and APC (18%) were significantly higher in tumors compared with controls. At least one of the four genes was methylated in 76% of the tumors; (b) a low frequency of methylation was present in nonmalignant tissues; (c) no significant differences in methylation frequencies were seen between the nonmalignant ovarian tissues from women at high-risk and those with no known risk of developing ovarian cancer; (d) methylation of the BRCA1 gene was found in 10% of sporadic tumors but in none of the samples from women with a germ-line BRCA1 mutation; and (e) ovarian cancer cell lines showed a similar frequency of methylation to ovarian tumors except for the HIC1 gene.

Conclusions: Our results suggest that aberrant methylation of specific genes, including two not described previously, may be important in ovarian cancer pathogenesis but not in ovaries at risk for cancer development.

Introduction

Ovarian cancer is the most lethal tumor of the female genital tract and the second most common gynecological cancer (1). Sixty percent of those diagnosed eventually die of the disease. The most significant clinical prognostic factors are tumor stage and extent of disease at diagnosis (2). A family history of breast and/or ovarian cancer increases the lifetime risk by 40% and mutations in the BRCA1 gene increase risk by 63% in hereditary forms of the disease (3, 4). Germ-line mutations of BRCA1 or BRCA2 genes are believed to account for 5–10% of all ovarian cancers (5). There has been a slight increase of 1.4% to 1.8% in ovarian cancer incidence over the past 5 decades, and survival is disappointingly low because of advanced-stage (stages III and IV) disease at diagnosis in 75% of cases (6). A poor understanding of the biology of sporadic forms of the disease, imperfect screening markers (such as serum CA125 antigen concentrations), and very few symptoms or warning signs of early stage disease (7) contribute to failure of early detection.

Acquired genetic changes such as high frequencies of loss of heterozygosity (loci of putative tumor suppressor genes) have been reported in ovarian cancer on several chromosome arms (8, 9). However, a lack of somatic mutations in tumor suppressor genes suggests that other mechanisms of inactivation may be involved.

DNA methylation is an epigenetic alteration that plays an important role in tumorigenesis (10, 11). Addition of a methyl group to cytosine residues of CpG dinucleotide clusters (islands) in the 5’ regulatory regions of genes occurs frequently in cancer cells but seldom in nonmalignant cells. The importance of CpG island aberrant methylation as an alternative mechanism for the inactivation of tumor suppressor genes has been recognized recently and may be the most common mechanism for gene regulation in cancer (12). Aberrant promoter methylation has been associated with loss of expression of a growing number of tumor-related genes in a variety of human cancers (13). Costello et al. (14) have shown that DNA methylation patterns are tumor-type specific.

Accumulating evidence suggests that aberrant promoter methylation of genes may be used successfully to detect neoplastic DNA in body fluids such as urine (15). Whereas methylation of some cancer-associated genes e.g., p16 (16), RASSF1A (17), BRCA1 (18), and hMLH1 (19) has been reported in ovarian cancers, only a few studies have examined concurrent methylation of multiple genes (20, 21). Additional knowledge of DNA methylation changes may improve our understanding of the role of DNA methylation in ovarian cancer.

In this study, we analyzed a cohort of primary sporadic ovarian tumors and nonmalignant ovarian tissues from women
with no known risk and women at high-risk of developing ovarian cancer, for concurrent methylation of nine genes by MSP.\(^3\) The genes we chose in this study are frequently hypermethylated and silenced in several cancer types. Several of these genes are mentioned in a recent review of methylation (22), and they include tumor suppressor genes, a detoxification gene, and genes involved in development and differentiation, as well as tumor invasion.

**Materials and Methods**

**Tissue Samples and DNA Extraction.** Forty-nine tumor samples consisted of 45 surgically resected invasive ovarian tumor tissues and 4 peritoneal fluid samples of metastatic primary ovarian cancers. Twenty-nine of the ovarian tumor samples were collected from patients at the University of Texas Southwestern Medical Center, and 20 were collected from Brigham and Women’s Hospital. All of the samples were stored at \(-70^\circ\text{C}\). The mean age of the patients was 56 years of age (range 40–79 years of age). Histological classification was assessed according to the WHO criteria, and tumor stage was established according to the Federation International Gynecological Oncologists classification (23). Thirty-nine nonmalignant ovarian tissues served as controls. Of these, 16 samples ("no known risk") with a mean age of 47 years (range 36–60 years of age) were collected from patients without cancer undergoing bilateral salpingo-oophorectomy at the time of surgery for benign gynecological disease at University of Texas Southwestern Hospitals. Twenty-three nonmalignant ovarian tissues (mean age, 47 years; range, 36–66 years) were collected at the time of prophylactic oophorectomy at the Northwestern University Hospital, Chicago, IL, from patients at "high-risk" of developing ovarian cancers because of the presence of germ-line \(BRCA1\) mutations (\(n = 14\)) or strong family histories for breast or ovarian cancer (\(n = 9\)). Of these 23, 2 had epithelial hyperplasia, and 4 had benign cystic changes. The other 17 had normal or atrophic histologies. These women were enrolled in a National Ovarian Cancer Early Detection Program. All of the samples were collected after obtaining appropriate Institution Review Board approved informed consent.

DNA was extracted from 100 mg (or more) of frozen tissue specimens. Surgically resected tumor tissues were macroscopically dissected to separate them from nonmalignant tissues. A generous wedge of tissue was removed from apparently normal ovaries at the time of hysterectomies performed on noncancerous cases, and included surface epithelium and underlying ovarian tissue. DNA was prepared by overnight digestion with 200 \(\mu\text{g/ml}\) proteinase K (Life Technologies, Inc.) at 50 \(^\circ\text{C}\). Treated DNA was purified by use of a Wizard DNA Purification System (Promega Corp., Madison, WI). Modified DNA was resuspended in 20 \(\mu\text{l}\) of sterile water and stored at \(-70^\circ\text{C}\) until used. Ten-nineteenth bisulfite-treated DNA was used for PCR with specific primers for methylated sequences as described previously for the following genes: \(p16\), (24), \(RAR\) (25), \(GSTP1\) (26), \(RASSF1A\) (27), \(APC\) (28), \(H-cadherin\) (29), \(E-cadherin\) (30), \(BRCA1\) (18), and \(HIC1\) (31). The unmethylated form of the \(p16\) (\(p16\)) gene was examined as a control for DNA integrity in all of the samples. Normal lymphocyte DNA was methylated in vitro with Sss1 methyltransferase (New England Biolabs, Inc., Beverly, MA), subjected to bisulfite modification, and used as positive control for amplification of methylated alleles. Water blanks without added DNA were included as negative PCR controls in each assay. PCR products were analyzed by 2% agarose gels containing ethidium bromide. When sufficient DNA was available, the bisulfite reaction was repeated for analysis.

**Sequencing Analysis.** Direct sequencing of methylated \(HIC1\) PCR products was performed using an automated ABI sequencing system with the primers used for MSP. PCR amplification was performed as described above in a 100-\(\mu\text{l}\) reaction. Ten \(\mu\text{l}\) was run on a 2% agarose gel and stained with ethidium bromide to check for the presence of desired product, and the remaining was ethanol precipitated. One-hundred ng of DNA were used for each sequencing reaction.

**Statistical Analysis.** Statistical differences between groups were examined by the use of Fisher’s two-tailed test. Values of \(P < 0.05\) were considered to be statistically significant. To compare the extent of methylation for the panel of genes examined, we calculated the MI for each case by determining the total number of genes methylated/total number of genes analyzed. Statistical comparison of MIs between nonmalignant and tumor samples was performed. To determine coordination of methylation at multiple loci, we compared the frequency with which other loci were methylated when a particular

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\(^3\) The abbreviations used are: MSP, methylation-specific PCR; MI, methylation index.
Materials and Methods

The cohort consisted of 49 ovarian tumors, 23 nonmalignant ovaries from women with no known cancer risk and 23 tissue samples obtained from women without cancer, and included 16 samples from women with cancer. The mean age of the women with cancer was 56 years. Nonmalignant ovaries were obtained from women with no known risk and those at high risk of developing ovarian cancer (i.e., those with BRCA1 mutations or family histories of breast and/or ovarian cancer). Hence, the two nonmalignant control groups were combined for analysis with tumors. The methylation of gene loci observed in the high-risk cases did not associate with any particular type of BRCA1 mutation (data not shown).

Four loci corresponding to the gene promoter regions of APC, E-cadherin, RASSF1A, and HIC1 genes showed significant methylation ranging from 18 to 41% in the tumors, whereas methylation frequencies of GSTP1, p16, H-cadherin, and RARB genes were absent or very low. The BRCA1 gene was methylated in 10% of sporadic tumors but in none of the nonmalignant ovaries carrying germ-line BRCA1 gene mutations. Methylation frequencies in ovarian cancer cell lines were not significantly different from those of the tumors except for the HIC1 gene, which was positive in 73% (8 of 11) of tumor cell lines (Fig. 1). Because Strathdee et al. (20) found a lower incidence of HIC1 methylation in ovarian cancers we confirmed HIC1 methylation by direct sequencing of the HIC1 MSP product from 6 samples (4 cell lines and 2 tissue samples). All of the samples were methylated at the 12 CpG sites contained in the amplicon (data not shown). No methylation of BRCA1 was observed in any of the ovarian cancer cell lines tested.

Methylation of at least one of four loci mentioned above was found in most (37 of 49, 76%) of the tumor samples, and methylation of two or more loci (among the four genes that were significantly higher in tumors) was seen in 14 of 37 (38%) cases. Only 6 of the 49 cases (5 papillary serous and 1 endometrioid tumor) did not show methylation for any gene. Methylation at any one locus was not significantly concordant with methylation at other loci.

The MI was calculated (see “Materials and Methods”) for the panel of nine genes (Table 1). Methylation indices were significantly higher in tumors compared with each of the nonmalignant groups (with no known risk and high risk), as well as to all of the nonmalignant samples (P < 0.0001). The MI was higher in clear cell tumors compared with other histological types. MIs of stage III or IV tumors were similar. Association with grade or stage was not determined because most cases were grade 3 and high-stage (stage III and IV) tumors.

Discussion

Epithelial ovarian tumors constitute 90% of all of the cases in women. Although ovarian cancer susceptibility genes such as BRCA1 have been identified, most cases (90%) are sporadic and lack mutations in these genes. The cause of epithelial ovarian carcinoma is unknown, and diagnosis is confounded by the lack of symptoms in early stage disease. Considering the lethal nature of this tumor, a better understanding of the molecular aberrations in this cancer may be clinically relevant and potentially provide early diagnostic markers. In this regard, changes in the status of DNA methylation are a common molecular alteration in human neoplasia (32). Genes that are frequently aberrantly methylated in specific tumors have been used as molecular targets for the detection of neoplastic cells in body fluids (15). Thus, an important result of our study is the demonstration that DNA methylation of four genes occurs frequently in ovarian tumors. Whereas we did not study the ex-

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
<th>MI</th>
</tr>
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<tbody>
<tr>
<td>All Tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary serous</td>
<td>32 (65)</td>
<td>0.18</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>7 (14)</td>
<td>0.14</td>
</tr>
<tr>
<td>Mucinous</td>
<td>2 (4)</td>
<td>0.27</td>
</tr>
<tr>
<td>Clear cell</td>
<td>2 (4)</td>
<td>0.44</td>
</tr>
<tr>
<td>Adenocarcinoma other</td>
<td>6 (12)</td>
<td>0.13</td>
</tr>
<tr>
<td>Stage (FIGO)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3 (6)</td>
<td>0.11</td>
</tr>
<tr>
<td>II</td>
<td>3 (6)</td>
<td>0.26</td>
</tr>
<tr>
<td>III</td>
<td>31 (63)</td>
<td>0.18</td>
</tr>
<tr>
<td>IV</td>
<td>12 (24)</td>
<td>0.17</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3 (6)</td>
<td>ND*</td>
</tr>
<tr>
<td>2</td>
<td>6 (12)</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>40 (82)</td>
<td>ND</td>
</tr>
<tr>
<td>Mean age</td>
<td>56 (40–79) yrs.</td>
<td></td>
</tr>
<tr>
<td>All Nonmalignant ovaries</td>
<td>47 (36–66) yrs.</td>
<td>39</td>
</tr>
<tr>
<td>No known risk mean age</td>
<td>47 (36–60) yrs.</td>
<td>16</td>
</tr>
<tr>
<td>High-risk mean age</td>
<td>47 (36–66) yrs.</td>
<td>23</td>
</tr>
<tr>
<td>Ovarian cancer cell lines</td>
<td>11 (82)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Adenocarcinoma other includes three not specified, one undifferentiated, and two transitional types.
* ND, not determined.
* Statistical significance P < 0.0001 compared with tumors. Values were analyzed by the Mann-Whitney U test.
pression of these genes, previous studies have demonstrated that inactivation of expression of these genes was highly correlated with DNA promoter methylation (12). Thus, frequent methylation of four of the nine genes examined i.e., APC, RASSF1A, HIC1, and E-cadherin, has not been reported previously in ovarian tumors despite loss of E-cadherin expression in malignant ovarian tumors (33). Frequent methylation of these genes has been reported in other tumors including breast cancer (28, 34).

Many ovarian tumors (76%) were methylated at one or more of the four frequently methylated loci. Thirty-eight percent showed methylation at more than one locus. In contrast to the report by Strathdee et al. (20), we did not observe a coordination or concurrent methylation at the multiple loci. Tumor samples had a higher frequency of DNA methylation than the nonmalignant tissues for the panel of genes analyzed in this study, and the MI for all of the tumors was significantly higher than the nonmalignant tissues (P < 0.0001). Clear-cell tumors, generally regarded as the most aggressive histological type of epithelial tumors, showed a high index. Because of the very small number of these tumors, a larger series needs to be examined to evaluate prognostic significance of the extent of methylation (measured as MI) with histological subtype, tumor stage, and grade.

Although conflicting reports of methylation for the RASSF1A gene in ovarian tumors have been reported, our results are in agreement with the 40% incidence observed by Yoon et al. (17). Inactivation of RASSF1A was highly correlated with methylation of the CpG sites in the promoter region in breast and lung cancer (27, 35).

A high incidence of HIC1 methylation was reported recently in cervical tumors (31). aberrant methylation of HIC1 and associated loss of expression is also common in breast cancers (36). We found methylation for the HIC1 gene in 35% of ovarian cancers. Our incidence of HIC1 methylation in tumors was about two-fold higher than the report by Strathdee et al. (20). Although the primer sets used by us (31) and by Strathdee et al. (20) are in the 5' CpG island and share the same reverse primer, the product sizes are 95 bp and 246 bp, respectively. It is possible that the lower incidence in the study by Strathdee et al. (20) may be because their primers amplify a larger product. Nevertheless, we confirmed the methylation status of the HIC1 MSP products by sequence analysis in six samples and found them to be methylated at all of the CpG sites.

Ahluwalia et al. (21) reported that epigenetic signatures determined by differential methylation hybridization of ovarian tumors included several CpG island tags that were aberrantly methylated in human breast cancers. It was interesting to note that genes such as p16, GSTP1, RARβ, and H-cadherin that are aberrantly methylated in breast cancer showed low or no significant methylation in ovarian tumors. The 5' CpG island methylation of p16 is important in a subset of human cancers such as lung, head and neck, pancreas, and squamous cell carcinomas (37), and in epithelial tumors of the cervix (38) but not in epithelial ovarian tumors as reported by Shih et al. (39) and as observed in our study. McCluskey et al. (16) reported that p16 gene silencing may be important for the development of ovarian carcinomas because of the high frequency of loss of expression. However, p16 methylation was observed in only 5% of carcinomas but was higher (33%) in a subset of low malignant potential ovarian tumors. RARβ and H-cadherin are methylated frequently in breast cancer (25, 40) but not in ovarian cancers. Methylation rates of H-cadherin reported in ovarian tumors by Kawakami et al. (41) were similar to our incidence. A differential susceptibility of these critical CpG island loci to DNA methylation in various tumor types may influence tumor development.

Aberrant methylation of BRCA1 was reported in 5–13% of breast and ovarian cancers but not in any normal tissues (18, 42, 43). BRCA1 methylation was not found in patients with germline mutations (44). Our observations are consistent with these reports. In our sporadic tumors, cases that were methylated for BRCA1 lacked gene mutations, whereas the high-risk cases with BRCA1 germ-line gene mutations lacked methylation. Epi-

\[ Table 2 \]

<table>
<thead>
<tr>
<th>Gene Loci</th>
<th>RASSF1A</th>
<th>HIC1</th>
<th>E-cad</th>
<th>APC</th>
<th>H-cad</th>
<th>P16M</th>
<th>BRCA1</th>
<th>RARβ</th>
<th>GSTP1</th>
<th>P16U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor (n = 49)</td>
<td>No.Pos (%)</td>
<td>20 (41)</td>
<td>17 (35)</td>
<td>14 (29)</td>
<td>9 (18)</td>
<td>9 (18)</td>
<td>5 (10)</td>
<td>5 (10)</td>
<td>1 (2)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Nonmalignant ovarian tissues (no known risk) (n = 16)</td>
<td>No.Pos (%)</td>
<td>2 (13)</td>
<td>3 (19)</td>
<td>1 (6)</td>
<td>0 (0)</td>
<td>2 (13)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Nonmalignant ovarian tissues (high-risk) (n = 23)</td>
<td>No.Pos (%)</td>
<td>4 (17)</td>
<td>0 (0)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>3 (13)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>23 (100)</td>
</tr>
<tr>
<td>All nonmalignant tissues (n = 39)</td>
<td>No.Pos (%)</td>
<td>0.06</td>
<td>0.001</td>
<td>0.03</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tumor cell lines (n = 11)</td>
<td>No.Pos (%)</td>
<td>4 (36)</td>
<td>8 (73)</td>
<td>1 (9)</td>
<td>2 (18)</td>
<td>2 (18)</td>
<td>4 (36)</td>
<td>0 (0)</td>
<td>1 (9)</td>
<td>1 (9)</td>
</tr>
</tbody>
</table>

*No.Pos, number positive; ns, not significant. *P between the two control groups were not significant for any gene loci.

*p for data in this row compared with tumors. *P < 0.05 are statistically significant.
genetic inactivation of the \textit{BRCA2} gene does not appear to play a role in sporadic ovarian tumors (45).

Women with mutations of the \textit{BRCA1} gene are at an increased risk for the development of ovarian cancer at a relatively early age (46, 47). We examined ovaries obtained at the time of prophylactic oophorectomy from 23 women with known \textit{BRCA1} mutations or with strong family histories of breast/ovarian cancer. The average age of these women was 47 years, similar to the average age at which ovarian cancer develops in women with germ-line \textit{BRCA1} mutations (47). Whereas most ovaries showed normal or atrophic histology, 4 had benign cysts and 2 had epithelial hyperplasia. Scully (48) has suggested that these changes are preneoplastic, and similar changes have been described frequently by some (49) but not all of the investigators in ovaries removed prophylactically from women with \textit{BRCA1} mutations (50, 51). However, we found no differences in the methylation profiles of ovaries removed from high-risk women compared with those from women without increased risk. The reasons for infrequent methylation in pathologically normal tissue could be because of undetected cancer or evidence of low

\textbf{Fig. 1} Methylation profile of ovarian tumors, ovarian cancer cell lines, and nonmalignant ovarian tissues for \textit{RASSF1A, HIC1, E-cadherin (Ecad), APC, H-cadherin (Hcad), p16, BRCA1, RARb, and GSTP1} genes. Dark gray boxes represent samples that are methylated; light gray boxes represent samples that are not methylated.
levels of methylation in normal cells. Of interest, methylation of certain genes in inflammatory conditions or as an aging change has been described (52, 53). The low frequency of gene methylation in both groups of nonmalignant ovarian samples is an important finding with respect to developing these markers as early detection aids for ovarian cancer arising in women at increased risk.

Ueki et al. (54) have shown that aberrant methylation in tumor cell lines often reflects that of primary tumors. Whereas the methylation frequencies of other markers in ovarian cancer cell lines were comparable with tumors, the reason for a higher incidence of HIC1 methylation in ovarian cancer cell lines is not clear but could reflect increased methylation of this gene in the tumor subtype from which they originated and/or an expansion in CpG methylation during cell culture.

The genes examined herein represent only a fraction of the total methylated genes involved in tumorigenesis. No differential methylation was observed between nonmalignant control tissues from women with no known risk and those at high risk. However, significant methylation of only four of nine specific gene loci in invasive tumors compared with control tissues indicates that methylation of these genes may play a role in ovarian carcinogenesis. These genes may be suitable candidates for exploring ovarian tumor progression, and may represent additional targets for molecular detection of invasive ovarian tumors and for development of antimethylation therapeutic strategies in future studies.

References


