Ovarian cancer remains a major threat to women's health in the United States. It was estimated that, in 2004, ~25,580 women would be diagnosed with and 16,090 would die from ovarian cancer (1). Because of the inability to detect ovarian cancer at an early, more curable stage, its survival rate has been essentially unchanged over the past 20 years. Early detection and prevention, therefore, depend on the ability to identify genetic and epigenetic events that underlie the development and progression of ovarian cancer. Epigenetic events, such as de novo cytosine-DNA methylation at CpG sites in the promoter region, can alter mRNA expression, one of the phenotypic characteristics of tumor development and progression (2). Mounting evidence suggests that aberrant methylation of CpG islands is one of the major pathways involved in the inactivation of tumor suppressor genes and the development of cancer (3). Indeed, several types of cancer, including ovarian cancer, exhibit a methylation phenotype (4, 5). In particular, certain types of tumors, including ovarian cancer, show aberrant methylation of CpG islands in the promoter regions of tumor suppressor genes, including the p15INK4b (6) and p16INK4a (7, 8). Both p15INK4b and p16INK4a proteins are inhibitors of cyclin-dependent kinases that prevent the cell from going through the G1-S phase transition; therefore, inactivation of p15INK4b and p16INK4a is thought to be an important step in cancer development (6–9). p15INK4b and p16INK4a promoter methylation has been reported in several types of primary tumors and cancer cell lines, including acute myeloid leukemia (6, 10, 11) and cancer of the lung (4, 6, 12), breast (4, 6, 13), bladder (4, 14), head and neck (6, 15, 16), prostate (6), colon (6), liver (17), kidney (18), and stomach (19).

Several studies of the methylation status of p15INK4b and p16INK4a in ovarian cancer have been published (20–23). Whether either gene is aberrantly methylated in ovarian cancer is still controversial, particularly in the most common form of

Abstract

**Purpose:** The purpose of this research was to compare methylation status and mRNA expression of p15INK4b and p16INK4a in serous epithelial ovarian cancer tissues and normal ovarian tissues.

**Experimental Design:** We analyzed the DNA methylation status and mRNA expression of p15INK4b and p16INK4a in 52 ovarian cancer specimens and 40 normal ovarian specimens by using methylation-specific PCR and real-time reverse transcription-PCR, respectively.

**Results:** Although the p15INK4b and p16INK4a mRNA expression levels were highly correlated with each other (P < 0.001), the methylation status did not seem to be linked with levels of mRNA expression, as no association between the two events was found for either gene. Promoter hypermethylation of p15INK4b was more common in ovarian cancer (30.8% for the 52 cases) than in normal ovaries (5% for the 40 controls without ovarian cancer; P = 0.005) but not methylation of p16INK4a (25% for cancer versus 37.5% for normal; P = 0.288). The relative mRNA expression levels of p15INK4b were significantly lower in ovarian cancer (12.9%) than in normal ovaries (41.7%; P = 0.008) but not those of p16INK4a (27% for cases versus 32.8% for controls; P = 0.754). Only high methylation rate and low mRNA expression of p15INK4b were independent risk factors for ovarian cancer (adjusted odds ratio, 5.67; 95% confidence interval, 0.85-37.9 for high methylation rate and odds ratio, 8.98, 95% confidence interval, 1.58-50.9 for low mRNA expression, respectively).

**Conclusions:** Our results suggest that epigenetic alterations in p15INK4b but not p16INK4a have an important role in ovarian carcinogenesis and that mechanisms other than methylation may exist to reduce gene expression of p15INK4b in ovarian cancer.
serous epithelial ovarian cancers. However, at least one study reported an association between methylation of p16^{INK4a} and disease progression, suggesting that an understanding of the regulation of methylation and the expression of p16^{INK4a} is critical to future management strategies for ovarian cancer (8). Thus, although it is clear that the expression of p15^{INK4b} or p16^{INK4a} is aberrant in ovarian cancer, it is not clear whether methylation contributes to the alterations in gene expression or whether these two events are independent in ovarian carcinogenesis. Therefore, the role of de novo DNA methylation in ovarian cancer remains uncertain.

We hypothesized that cytosine-DNA methylation differentially regulates the expression of p15^{INK4b} and p16^{INK4a}, and these differences may play a differential role in the processes contributing to ovarian tumorigenesis. Here, we report the methylation status and mRNA expression of both p15^{INK4b} and p16^{INK4a} in serous epithelial ovarian cancer tissue specimens from 52 patients compared with 40 apparently normal ovarian tissue samples from different individuals without ovarian cancer. Methylated CpG islands in the promoters of p15^{INK4b} and p16^{INK4a} were detected by methylation-specific PCR (MSP) and compared with levels of mRNA expression in the same individuals measured by real-time reverse transcription-PCR.

**Materials and Methods**

**Tissue samples.** Specimens from surgically resected ovarian tumors and from unaffected ovaries were used in this study. Briefly, between January 2000 and March 2003 at The University of Texas M.D. Anderson Cancer Center, tumor tissues were obtained from 52 patients with newly diagnosed primary serous ovarian carcinoma. Control specimens came from two sources: from apparently normal contralateral ovaries of 8 patients with unilateral ovarian cancer and from unaffected ovaries were used in this study. Briefly, between January 2000 and March 2003 at The University of Texas M.D. Anderson Cancer Center, tumor tissues were obtained from 52 patients with newly diagnosed primary serous ovarian carcinoma. Control specimens came from two sources: from apparently normal contralateral ovaries of 8 patients with unilateral ovarian cancer and from 40 other patients, of whom 27 underwent surgery for nonovarian cancer (9 endometrial cancer, 11 with cervical cancer, 6 with uterine cancer, and 1 appendix mucinous tumor) and 13 for benign conditions (9 endometriosis, 2 cervical hyperplasia, 1 fallopian paratubal cysts, and 1 unknown). All samples were snap frozen after surgical removal, stored at −80°C, and tested after pathologic examination. DNA and RNA were extracted from −200 mg of fresh frozen tissue specimens. Informed consent was obtained from each patient, and the study was approved by M.D. Anderson Cancer Center institutional review board.

**Methylation-specific PCR.** DNA was prepared by overnight digestion with 200 μg/mL proteinase K (Life Technologies, Inc., Rockville, MD) at 42°C followed by phenol/chloroform (1:1) extraction and ethanol precipitation. We used MSP to examine the promoter methylation status of the p15^{INK4b} and p16^{INK4a} genes. Briefly, genomic DNA (1 μg) was modified with sodium bisulfite by using a CpGenome DNA Modification kit according to the manufacturer’s instructions (Sero- logicals Corp., Norcross, GA). The bisulfite-modified DNA (100 ng) was separately amplified by using primers specific for methylated p15^{INK4b} (forward GCCGTGCATTITTTGCGGTT and reverse CGTACAA- TAACCCGAACGGCAGCAGA) and for unmethylated p15^{INK4b} (forward TGTGATGTGTATTTTGCGGTT and reverse GACCGCGAAACGGCAGCAGA) and for unmethylated p16^{INK4a} (forward TTA- TTAGAGGCTGGGGCGGATGCG and reverse GACCGCGAAACGGCAGCAGA) and for unmethylated p16^{INK4a} (forward TTA- TTAGAGGCTGGGGCGGATGCG and reverse GACCGCGAAACGGCAGCAGA). For methylation detection, CpGenome Universal Methylated DNA (Serologicals) was used as the positive control for amplification of methylated alleles, and water blanks without added DNA were included as the negative PCR controls in each assay. DNA amplification was carried out by using reagents supplied in a HotStarTaq DNA Polymerase kit (Qiagen, Inc., Valencia, CA). The PCR mixture contained PCR buffer [16.6 mmol/L ammonium sulfate (pH 8.6), 67 mmol/L Tris (pH 8.8), 1.5 mmol/L MgCl₂, 10 mmol/L 2-mercaptoethanol], deoxynucleoside triphosphates (each at 1.25 mmol/L), and primers (300 ng each per reaction) in a final volume of 50 μL. Reactions were hot started at 95°C for 15 minutes, and amplification was carried out in a PTC-200 Peltier thermal cycler (MJ Research, Inc. Waltham, MA) for 40 cycles (30 seconds at 95°C, 1 minute at the annealing temperature 60-65°C, and 1 minute at 72°C) followed by a final 10-minute extension at 72°C. PCR products were analyzed on 2% agarose gels containing ethidium bromide (Fig. 1). The results were evaluated independently by two researchers, and samples with questionable results were retested to achieve agreement between observers.

**Real-time reverse transcription-PCR for mRNA expression.** Total RNA was extracted with Tri-Reagent according to the manufacturer’s protocol (Molecular Research Center, Cincinnati, OH). We assessed the quality of the extracted total RNA on 1% agarose gels after electrophoresis by visualizing the 18S and 28S RNA bands under UV light (Fig. 2), and RNA concentration was determined with the GeneQuant Pro RNA/DNA Calculator (Amersham Pharmacia Biotech, Cambridge, United Kingdom). We assessed the quality of the extracted total RNA on 1% agarose gels after electrophoresis by visualizing the 18S and 28S RNA bands under UV light (Fig. 2), and RNA concentration was determined with the GeneQuant Pro RNA/DNA Calculator (Amersham Pharmacia Biotech, Cambridge, United Kingdom).

![Fig. 1. MSP analysis of the methylation status of p15^{INK4b} (A) and p16^{INK4a} (B). Representative products of the promoter region of the p15^{INK4b} and p16^{INK4a} genes amplified by the MSP method. P, positive control (CpGenome Universal Methylated DNA); Ca, ovarian cancer tissues; Cn, normal ovarian tissues; N, negative control (water blank); M, methylated; and U, unmethylated.](image-url)
Kingdom) before it was used for assessment of mRNA levels. The primers and probes for detecting p15INK4b, p16INK4a, and GAPDH cDNA sequences (Genbank accession nos. XM_027626, XM_027621, and AK026525, respectively) were designed by using Primers Express software (Perkin-Elmer Applied Biosystems, Foster City, CA). They were forward CGTGGGAAAGAAGGGAAGGTT, reverse CCCCGACGGGCAGC, and probe FAM-CGGCCAACGGTGGATTATCCGGA-TAMRA for p15INK4b; forward CTCAGCTGCTCAGAGGC, reverse AGTCGACAGCTTCCGAGG, and probe FAM-TCATGTGGG-CATTTCTTGCGAGG-C TAMRA for p16INK4a; and forward GAAGGTGGAAGGTCGGAGTC, reverse GAAGATGGTGATGGGATTTC, and probe FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA for GAPDH (used as an internal control for relative quantification). The fluorogenic probes contained a reporter dye [FAM (6-carboxyfluorescein)] covalently attached at the 5’ end and a quencher dye [TAMRA (6-carboxytetramethylrhodamine)] covalently attached at the 3’ end. Total RNA from each sample (50 ng) was used to quantify the cDNA copy number of p15INK4b, p16INK4a, and GAPDH. Reverse transcription-PCR was done by using the TaqMan One-Step Reverse Transcriptase-PCR Master Mix Reagents with an ABI PRISM 7700 Sequence Detection System according to the protocol of the manufacturer (Perkin-Elmer Applied Biosystems). The system detects the fluorescence emitted from the fluorogenic oligonucleotide probes, and the signal is directly proportional to the number of template molecules in the reaction mixture after they have crossed a fluorescence detection threshold (25). The ABI PRISM 7700 Sequence Detector is a thermal cycler designed to monitor multiple fluorescent signals in a 96-well format. Samples were amplified in 96-well optical PCR trays and caps, and the data were directly collected during the cycling procedure. After activation of the AmpliTaq Gold at 48°C for 30 minutes and 95°C for 10 minutes, the samples were subjected to 40 cycles, each consisting of 95°C for 15 seconds and 60°C for 1 minute. The GAPDH signal was used as the internal control. The expression level of the target genes was calculated based on a standard curve established with a standard human RNA sample that contained a large number of copies to ensure the detection of GAPDH mRNA (1 × 10^5 copies/ng, Perkin-Elmer Applied Biosystems). The standard curve was constructed with a continuation of six data points equivalent to 50,000, 5,000, 500, 50, 5, and 0.5 pg of human RNA. Each sample was measured in triplicate and the means of the three values were calculated for statistical analysis. About 10% of the samples were retested using the same RNA samples, and the measurements were found to be consistent between repeats.

**Statistical analysis.** Student’s t test was used to compare the differences in the relative mRNA expression levels, which were analyzed as a continuous variable between groups. The χ^2 test was used to test associations between methylation status and risk for ovarian cancer, and the Pearson correlation coefficients were calculated for correlation analysis. For calculation of crude odds ratios (OR) and 95% confidence intervals (95% CI), the median relative expression level of p15INK4b and p16INK4a mRNA in the controls was used as the cutoff point for defining lower versus higher expression level. Adjusted ORs were calculated by fitting logistic regression models with adjustment for patient age and ethnicity and other variables of interest. All statistical analyses were done with SAS software version 8.0e (SAS Institute, Inc., Cary, NC).

**Results**

**p15INK4b and p16INK4a methylation and expression in ovarian tumor and noncancerous ovarian tissues.** Raw data on p15INK4b and p16INK4a promoter methylation and relative mRNA expression levels in ovarian tumor specimens (the cases) are presented in Table 1 and those in normal ovary specimens (the controls) in Table 2. The promoter methylation results are presented as either “U” for unmethylated or “M” for methylated. Overall, cases were older than controls (61.5 ± 9.4 versus 50 ± 11.5 years; P = 0.001), but no difference was found in the distribution of ethnic groups between cases (45 whites, 25 blacks, and 16 Hispanics).

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**Fig. 2.** Real-time reverse transcription-PCR to detect mRNA expression in ovarian tumors and normal tissues. A, construction of a standard curve; B, standard curve for expression quantification; C, test results of a batch of samples; D, quality check of total RNA for the 18S and 28S bands visualized by UV light on an agarose gel.
Table 1. Methylation status and relative mRNA expression levels of p15^{INK4b} and p16^{INK4a} genes in 52 serous epithelial ovarian cancer

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</table>

Abbreviations: W, White; B, Black; H, Hispanic; A, Asian; U, unmethylated; M, methylated.
3 non-White Hispanics, 2 African Americans, and 2 Asians) and controls (36 whites, 6 non-white Hispanics, 5 African Americans, and 1 Asian; \( P = 0.256 \)). The methylation status of \( p15^{INK4b} \) and that of \( p16^{INK4a} \) were not associated among either the 52 cases (\( \chi^2 = 1.9259; P = 0.165 \)) or the 48 controls (\( \chi^2 = 0.140; P = 0.708 \)), nor was methylation status associated with the relative mRNA expression levels (\( P = 0.172 \) for \( p15^{INK4b} \) and \( P = 0.365 \) for \( p16^{INK4a} \)) among the cases; \( P = 0.561 \).

### Table 2. Methylation status and relative mRNA expression levels of the \( p15^{INK4b} \) and \( p16^{INK4a} \) in normal ovaries from 48 control subjects

<table>
<thead>
<tr>
<th>TB_ID</th>
<th>Primary</th>
<th>Status</th>
<th>Age (y)</th>
<th>Race</th>
<th>p15-DNA</th>
<th>p16-DNA</th>
<th>p15-mRNA</th>
<th>p16-mRNA</th>
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<td>M</td>
<td>107.2</td>
<td>58.9</td>
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</table>

Abbreviations: B, benign; M, tumor mass other than ovarian cancer; T, tumors.
for p15INK4b and $P = 0.716$ for p16INK4a among the controls). However, the relative mRNA expression levels of p15INK4b and p16INK4a were highly correlated with each other ($r = 0.5$ and $P < 0.001$ for the cases and $r = 0.7$ and $P < 0.001$ for the controls). When the 48 controls were stratified by their primary cancer status, the mean age was 49.3 ± 11.9 years for those with ovarian cancer ($n = 8$), 52.2 ± 12.5 years for those with cancers other than ovarian cancer ($n = 27$), and 46.1 ± 8.4 years for those without any cancer ($n = 13$; Table 3).

The p15INK4b methylation rate was higher among the 52 cases (31%) than among all 48 controls (6.3%; $P = 0.004$) and also higher than any of the control subgroups [8 with primary ovarian cancer (12.5%; $P = 0.510$), 27 with primary cancer other than ovarian (3.7%; $P = 0.012$), and 13 without cancer (7.8%; $P = 0.178$)]. In contrast, the p16INK4a methylation rate seemed to be lower among the cases (25%) than among the controls (37.5% for all 48 controls, 37.5% for the 8 with unilateral ovarian cancer, 29.6% for the 27 with other cancers, and 53.9% for the 13 with no cancer), but none of the apparent differences was statistically significant (Table 3).

Table 3. Comparisons of methylation status and relative mRNA expression of p15INK4b/p16INK4a between ovarian cancer and normal ovarian tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (y)</th>
<th>Methylation status of p15INK4b</th>
<th>Relative mRNA expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>p16INK4a/ p15INK4b (% methylated)</td>
<td>p15INK4b/p16INK4a (mean ± SD)</td>
</tr>
<tr>
<td>All cases</td>
<td>52</td>
<td>61.5 ± 9.4</td>
<td>30.8/25.0</td>
<td>12.9 ± 41.2/270 ± 114.0</td>
</tr>
<tr>
<td>All controls</td>
<td>48</td>
<td>50.0 ± 11.5</td>
<td>6.3/37.5</td>
<td>40.4 ± 56.9/319 ± 670</td>
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<tr>
<td>Controls with ovarian cancer</td>
<td>8</td>
<td>49.3 ± 11.9</td>
<td>12.5/37.5</td>
<td>33.8 ± 351/276 ± 23.5</td>
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<tr>
<td>Controls with cancer other than ovarian</td>
<td>27</td>
<td>52.2 ± 12.5</td>
<td>3.7/29.6</td>
<td>32.2 ± 474/207 ± 32.3</td>
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<tr>
<td>Controls with no cancer</td>
<td>13</td>
<td>46.1 ± 8.4</td>
<td>7.8/53.9</td>
<td>61.6 ± 80.2/57.9 ± 118.2</td>
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<tr>
<td>Controls without ovarian cancer</td>
<td>40</td>
<td>49.3 ± 11.9</td>
<td>5.0/37.5</td>
<td>41.7 ± 61.1/32.8 ± 27.0</td>
</tr>
</tbody>
</table>

*P < 0.01 versus ovaries from cases.
*Subgroup of women with unilateral ovarian cancer.
*P < 0.05 versus ovaries from cases.
*Tissues obtained from unaffected ovaries.

We found that ovarian tumor tissues were more likely to have methylated p15INK4b than normal ovarian tissues (age- and ethnicity-adjusted OR, 5.68; 95% CI, 1.14-28.2), but no such association was evident for p16INK4a (adjusted OR, 0.47; 95% CI, 0.16-1.37; Table 4). Furthermore, low mRNA expression levels of p15INK4b were associated with a 3-fold increased risk of ovarian cancer (adjusted OR, 9.04; 95% CI, 2.51-32.5) and, low mRNA expression levels of p16INK4a were associated with a 7-fold increased risk of ovarian cancer (adjusted OR, 2.81; 95% CI, 1.04-7.62; Table 4). Although the 52 cases were substantially older than the 40 controls (a 10-year difference in the mean age), the ORs with and without adjustment for age and ethnicity (Table 4) did not change the results substantially, suggesting that age may not have a major effect on either methylation or mRNA expression levels in this study population.

Finally, to assess whether the risk for ovarian cancer associated with low mRNA expression of both p15INK4b and p16INK4a was independent, we fit all variables (age, ethnicity, and methylation status and expression levels of both p15INK4b and p16INK4a) in one logistic regression model. We found that the risk for ovarian cancer was associated with both high methylation status (adjusted OR, 5.67; 95% CI, 0.85-37.9) and low mRNA expression levels (adjusted OR, 8.98; 95% CI, 1.58-50.9) of p15INK4b but not of p16INK4a (adjusted OR, 0.30; 95% CI, 0.09-1.07 for high methylation status and OR, 0.94; 95% CI, 0.21-4.29 for low mRNA expression levels), suggesting that p15INK4b may play a major role in ovarian carcinogenesis but that other unknown factors may have caused changes in both genes.
similar biochemical characteristics, their proteins have dis-
p16INK4a
risk of ovarian cancer. Our results of
p15INK4b
with other ovarian cancer studies, but our findings on
methylation in the promoter region of these genes that
other mechanisms may differentially regulate the expression
p16INK4a
that of
p15INK4b
, and ectopic expression of
in vitro
growth
chromosome 9p21. The p15 INK4b protein binds to one or
methylation rate and low expression of
correlated. In multivariate analysis, however, both hyper-
methylation status and mRNA levels for either
p15INK4b
expression of
p16INK4a
were more common in
ovarian cancer tissues than in normal ovarian tissues and that
both changes were associated with increased risk of ovarian
cancer. Although no association was found between the
methylation status and mRNA expression levels for either
p15INK4b
or
p16INK4a
, their expression levels were highly
correlated. In multivariate analysis, however, both hyper-
methylation rate and low expression of
p15INK4b
but not
p16INK4a
were independent risk factors for ovarian cancer. To
the best of our knowledge, no reported studies have simulta-
neously investigated the association between promoter meth-
ylation status and mRNA levels of
p15INK4b
and
p16INK4a
and risk of ovarian cancer. Our results of
p16INK4a
are consistent
with other ovarian cancer studies, but our findings on
p15INK4b
are novel.
The
p15INK4b
and
p16INK4a
genes are colocalized on
chromosome 9p21. The
p15INK4b
protein binds to one or
more cyclin-dependent kinases and inhibits its functions
in vitro, and ectopic expression of
p15INK4b
inhibits cell
growth in vitro (26). Although
p15INK4b
and
p16INK4a
have similar biochemical characteristics, their proteins have
distinct functions in vitro. The expression of
p15INK4b
but not
that of
p16INK4a
can be induced by transforming growth
factor-β (27). The prevalence of point mutations in
p16INK4a
vary in different tumor lineages, but point mutations are
extremely rare in
p15INK4b
(26), supporting the notion that
other mechanisms may differentially regulate the expression
of these two genes. Nevertheless, aberrant cytosine-DNA
methylation in the promoter region of these genes that
could disrupt the function of
p15INK4b
and
p16INK4a
has been
found in numerous tumors, including ovarian cancer (4, 6, 9, 20, 28–31).
Although hypermethylation of
p16INK4a
is common in
breast, renal cell, colon, and prostate carcinomas (4, 28), the
reported rates of
p16INK4a
methylation in ovarian cancer tissues
have ranged from 0% to 40% (21, 23, 32, 33). One study
indicated that methylation of
p16INK4a
was present in 40% of
ovarian cancers and was associated with disease progression
(8), whereas other studies showed methylation of
p16INK4a
to be a rare event (21, 32–34). The discrepancies among these
results may reflect different experimental designs, inclusion
of ovarian cancer of different histologic types, and different
methylation detection methods. By including only serous
tumors and using a well-tested and sensitive MSP method
(35), we found
p16INK4a
to be methylated in 13 of 52 cases of
serous epithelial ovarian cancers (25%), consistent with the
most recent published findings (8). Interestingly, we also
detected methylation of
p16INK4a
in 15 of 40 normal ovarian
tissues (37.5%), a finding that has not been reported elsewhere,
and we found that methylation status did not differ substan-
tially between normal and tumor tissues, suggesting that
methylation of
p16INK4a
may not play a major role in ovarian
carcinogenesis.

Discussion

We found here that promoter hypermethylation and low
expression of
p15INK4b
but not
p16INK4a
were more common in
ovarian cancer tissues than in normal ovarian tissues and that
both changes were associated with increased risk of ovarian
cancer. Although no association was found between the
methylation status and mRNA expression levels for either
p15INK4b
or
p16INK4a
, their expression levels were highly
correlated. In multivariate analysis, however, both hyper-
methylation rate and low expression of
p15INK4b
but not
p16INK4a
were independent risk factors for ovarian cancer. To
the best of our knowledge, no reported studies have simulta-
neously investigated the association between promoter meth-
ylation status and mRNA levels of
p15INK4b
and
p16INK4a
and risk of ovarian cancer. Our results of
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are consistent
with other ovarian cancer studies, but our findings on
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are novel.
The
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and
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genes are colocalized on
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protein binds to one or
more cyclin-dependent kinases and inhibits its functions
in vitro, and ectopic expression of
p15INK4b
inhibits cell
growth in vitro (26). Although
p15INK4b
and
p16INK4a
have similar biochemical characteristics, their proteins have
distinct functions in vitro. The expression of
p15INK4b
but not
that of
p16INK4a
can be induced by transforming growth
factor-β (27). The prevalence of point mutations in
p16INK4a
vary in different tumor lineages, but point mutations are
extremely rare in
p15INK4b
(26), supporting the notion that
other mechanisms may differentially regulate the expression
of these two genes. Nevertheless, aberrant cytosine-DNA
methylation in the promoter region of these genes that
could disrupt the function of
p15INK4b
and
p16INK4a
has been
found in numerous tumors, including ovarian cancer (4, 6, 9, 20, 28–31).
Although hypermethylation of
p16INK4a
is common in
breast, renal cell, colon, and prostate carcinomas (4, 28), the
reported rates of
p16INK4a
methylation in ovarian cancer tissues
have ranged from 0% to 40% (21, 23, 32, 33). One study
indicated that methylation of
p16INK4a
was present in 40% of
ovarian cancers and was associated with disease progression
(8), whereas other studies showed methylation of
p16INK4a
to be a rare event (21, 32–34). The discrepancies among these
results may reflect different experimental designs, inclusion
of ovarian cancer of different histologic types, and different
methylation detection methods. By including only serous
tumors and using a well-tested and sensitive MSP method
(35), we found
p16INK4a
to be methylated in 13 of 52 cases of
serous epithelial ovarian cancers (25%), consistent with the
most recent published findings (8). Interestingly, we also
detected methylation of
p16INK4a
in 15 of 40 normal ovarian
tissues (37.5%), a finding that has not been reported elsewhere,
and we found that methylation status did not differ substan-
tially between normal and tumor tissues, suggesting that
methylation of
p16INK4a
may not play a major role in ovarian
carcinogenesis.

However, previous studies of
p16INK4a
expression in ovarian
cancer are less discrepant. One study showed that 26% of 42
ovarian cancer samples did not express
p16INK4a
protein, and
this finding was unrelated to DNA methylation (36). In another
study, low
p16
protein expression was found in 22 of 60
ovarian epithelial tumors (37%) and correlated significantly
with low
p16
mRNA expression but was unrelated to gene
deletion or point mutation (20). However, two later studies of

| Table 4. Crude and adjusted ORs and 95% CIs for the methylation status and relative mRNA expression of
p15INK4b/p16INK4a in ovarian cancer patients and controls |
<table>
<thead>
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<tbody>
<tr>
<td>Patients</td>
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<tr>
<td>(n = 52)</td>
</tr>
<tr>
<td>n (%)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Methylation status</td>
</tr>
<tr>
<td>p15INK4b</td>
</tr>
<tr>
<td>Unmethylated</td>
</tr>
<tr>
<td>Methylated</td>
</tr>
<tr>
<td>p16INK4a</td>
</tr>
<tr>
<td>Unmethylated</td>
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<tr>
<td>Methylated</td>
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<tr>
<td>Expression level1</td>
</tr>
<tr>
<td>p15INK4b</td>
</tr>
<tr>
<td>High</td>
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<tr>
<td>Low</td>
</tr>
<tr>
<td>p16INK4a</td>
</tr>
<tr>
<td>High</td>
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<tr>
<td>Low</td>
</tr>
</tbody>
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1Two-sided χ2 test.
1Adjusted for age (in years) and ethnicity (non-Hispanic whites versus others) in a logistic regression model.
1Adjusted for age (in years), ethnicity (non-Hispanic whites versus others), and each other by including all methylation and expression variables in the same logistic regression model.
1The median relative mRNA expression level in the controls was used as the cutoff point for each gene.
that p15INK4b but not p16INK4a in Ovarian Cancer

References

12. Furonaka O, Takeshima Y, Aways H, Ishida H, Kobna N, Inai K. Aberrant methylation of p14(ARF), p15(INK4b) and p16(INK4a) genes and location of different histologic subtypes of primary ovarian carcinoma showed that loss of p16 protein expression could be caused by hypermethylation of regions other than that of the promoter (37, 38). Although we did not measure p16INK4a protein expression, we found that serous epithelial ovarian cancer tissue expression had similar levels of p16INK4a mRNA to those of normal ovarian tissues, that mRNA expression of p16INK4a was not associated with methylation of p16INK4a, and that low mRNA expression of p16INK4a was not an independent risk factor for ovarian cancer. However, the expression of p16INK4a correlated highly with the expression of p15INK4b, suggesting that p15INK4b may have a role in ovarian carcinogenesis.

In contrast to p16INK4a, methylation and mRNA expression of p15INK4b in ovarian cancer have not been extensively investigated. Inactivation of p15INK4b by CpG island hypermethylation has been reported to occur selectively in leukemias and gliomas but not in colon, breast, or lung carcinomas (4, 28). In one study, a p15INK4b mutation occurred in only 1 of 70 ovarian tumors and homozygous deletion of p15INK4b was observed in only 1 additional case (20), leading the authors to conclude that p15INK4b did not have an important role in ovarian tumorigenesis (20). However, we found more hypermethylation and less mRNA expression of p15INK4b in ovarian cancer than in normal ovarian tissues, and both were independent risk factors for ovarian cancer, further suggesting that inactivation of the p15INK4b gene through epigenetic regulation could be one of the major events during ovarian carcinogenesis.

In the present study, we found that only 5% of normal ovarian tissues (2 of 40) had p15INK4b methylation, similar to that 8% rate in healthy nonsmokers/nondrinkers found in a previous study (16). However, we found methylated p15INK4b in 31% of ovarian cancers (16 of 52), a rate comparable with the 33% (15 of 45 cases) in a Japanese study (39). Studies of other cancers showed rates that were similar, such as 29% of 271 patients with acute lymphoblastic leukemia (40), or higher, such as 65% of 20 patients with head and neck squamous cell carcinoma (16) and 49% of 51 patients with hepatocellular carcinoma (41); however, in another study, no methylated p15INK4b was detected in 44 medulloblastomas (42). Thus, methylation of p15INK4b may be tumor specific and may be affected by environmental factors involved in the etiology of each type of tumor.

The fresh human tumor samples that we used may have contained both normal and tumor tissue, making detection of tumor-specific changes difficult. However, the sensitivity of MSP makes it useful for evaluating primary tumors, because it allows aberrantly methylated alleles to be detected even if they contribute relatively little to the overall DNA in a sample (24), thereby overcoming false-negative results due to contamination of normal tissues. The tissue samples obtained from unaffected ovaries in the controls in our study should not have had such problems, and it is unlikely that they would have yielded false-positive results. Although we cannot rule out the possibility that some cross-contamination might have occurred during experimentation, the consistency of our results with published data makes this possibility unlikely.

Overall, in the present study, abnormal methylation of both p15INK4b and p16INK4a in ovarian cancer and normal ovarian tissues did not predict mRNA expression levels, suggesting that other molecular mechanisms may have caused the changes in p15INK4b and p16INK4a mRNA expression. However, when methylation status and mRNA expression of both p15INK4b and p16INK4a were considered in the same multivariate logistic regressional model, both methylation status and mRNA expression of p15INK4b but not p16INK4a remained independent risk factors for ovarian cancer, suggesting that inactivation of the p15INK4b gene through epigenetic regulation could be a key event during ovarian carcinogenesis. However, only ~25% of epithelial ovarian cancers in general have methylated p15INK4b, suggesting that other mechanisms, such as aberrations in the p14ARF-MDM2-p53 pathway (43) or in the hMLH and PTEN (44) or SOCS genes (45), may contribute to the development of epithelial ovarian cancer in addition to aberrant p15INK4b. Whether these epigenetic events play a role in the prognosis of epithelial ovarian cancer warrants further investigations (4, 25).

In summary, we found in this case-control analysis that both methylation and mRNA levels of p15INK4b were independent risk factors for ovarian carcinogenesis and that, compared with p15INK4b methylation, p15INK4b mRNA levels were a strong predictor for the risk of ovarian carcinogenesis. However, no such associations were observed for p16INK4a. These results support the hypothesis that cytokine-DNA methylation may differentially regulate the expression of p15INK4b in ovarian cancer. It is likely that other regulating mechanisms, in addition to hypermethylation of the p15INK4b promoter, may cause lower levels of p15INK4b mRNA in patients with epithelial ovarian cancer. However, these findings need to be verified in larger studies with unaffected ovaries from cancer-free individuals.

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Methylation and Messenger RNA Expression of $p15^{INK4b}$ but Not $p16^{INK4a}$ Are Independent Risk Factors for Ovarian Cancer

Zhensheng Liu, Li-E Wang, Luo Wang, et al.


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