Epigenetic Factors Controlling the BRCA1 and BRCA2 Genes in Sporadic Ovarian Cancer

Kelvin Y. K. Chan, Hilmi Ozcelik, Annie N. Y. Cheung, Hextan Y. S. Ngan, and Ui-Soon Khoo

Departments of Pathology [K. Y. K. C., A. N. Y. C., U.-S. K.], Obstetrics and Gynecology [H. Y. S. N.], Queen Mary Hospital and the University of Hong Kong, Hong Kong; Department of Laboratory Medicine and Pathology, University of Toronto, and Samuel Lunenfeld Research Institute/Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5 Canada [H. O.]

ABSTRACT

Hypermethylation of the BRCA1 promoter has previously been shown to cause reduced mRNA expression in both breast and ovarian cancers. Nothing is yet known of the expression pattern or methylation status of the promoter region of BRCA2 in sporadic ovarian cancer. Whereas our analysis of 30 sporadic ovarian carcinomas showed a statistically significant reduction of BRCA1 mRNA expression (P = 0.001), it also showed, in contrast, overexpression of BRCA2 mRNA (P = 0.002) in tumor compared with nontumor. Hypermethylation of the BRCA1 promoter highly correlated with decreased BRCA1 expression (P = 0.017). Methylated CpGs at the BRCA2 promoter were either absent or at very low levels in tumor DNA, whereas they were present at high levels in nontumor DNA. Such hypomethylation also correlated with elevated levels of BRCA2 mRNA (P = 0.043) and showed a statistically significant correlation with tumor stage (P = 0.037). This supports the role of methylation in BRCA2 contributing to the pathogenesis of sporadic ovarian cancer. Furthermore, 14 (58.2%) and 9 (56.3%) of all of the cases with aberrant BRCA mRNA expression and methylation patterns, respectively, demonstrated opposing mRNA expression and methylation patterns of the BRCA1 and BRCA2 genes within the same cases. Our findings suggest that both genes may be involved in the development of sporadic ovarian cancer.

INTRODUCTION

Germline mutations of the BRCA1 and BRCA2 genes, located on chromosome 17q12-21 and 13q12-13, respectively, predispose to the development of breast and ovarian cancer (1, 2). The majority of germ-line mutations found in ovarian cancer cases usually lead to a truncated protein that disrupts the function of the encoded proteins (3, 4). Somatic mutations in BRCA1 and BRCA2 are rare and do not seem to play a significant role in the etiology of sporadic ovarian cancers (3, 5). Allelic deletions that include the BRCA1 and BRCA2 region occur in high frequency in both familial, as well as sporadic forms, of breast and ovarian carcinomas (6, 7). This implies, by Knudson’s two-hit hypothesis, that BRCA1 and BRCA2 might play roles as tumor-suppressor genes in the development of sporadic breast and ovarian cancers. Allelic instability or loss at the DNA level might also affect the expression of the genes. Decreased BRCA1 mRNA levels or allele-preferential expression have been reported in sporadic breast and sporadic ovarian cancer, respectively (8–10). Bieche et al. (11), on the other hand, demonstrated elevated BRCA2 mRNA expression levels in a series of sporadic breast tumors. These aberrant expressions may be attributable to epigenetic factors such as aberrant cytotoxic methylation of the CpG dinucleotides in the gene-promoter region that alter the transcription level of these two genes. A correlation between the aberrant level of gene transcript and methylation status of the promoter has been demonstrated in some putative tumor-suppressor genes including RB (12), p16 (13), and hMlh1 (14). Hypermethylation of the BRCA1 promoter in breast and ovarian carcinomas has been shown to reduce BRCA1 mRNA expression in several studies (15, 16). To date, only one study has shown the absence of methylation in the promoter region of BRCA2 in breast cancers cell lines and other normal human breast, bladder, colon, and liver tissues (17). The study (17), however, was unable to demonstrate any correlation with BRCA2 mRNA expression patterns. BRCA2 mRNA and methylation status of the promoter region has not yet been investigated in ovarian carcinoma.

In the present study, both BRCA1 and BRCA2 expression were evaluated in epithelial invasive ovarian carcinoma and their normal counterparts using real-time PCR. Statistically significant reduction and elevation of BRCA1 and BRCA2 mRNA expression respectively, were found in tumor specimens with respect to their nontumor counterpart. To understand the mechanism of alteration of expression in these genes, we investigated for possible allelic loss, gene amplification, and methylation in these tumors.

MATERIALS AND METHODS

Sample Collection. All tissue samples were collected from a consecutive series of surgical excision specimens (from 1995 to 1999) of patients diagnosed to have sporadic epithelial ovarian carcinoma in Queen Mary Hospital, Hong Kong. The samples were snap-frozen at least 30 min after surgical removal. All samples were verified by histology. Tumor samples were used only if they contained >70% tumor cells within the sample. Forty cases with available ovarian tumor tissue and nontumor counterparts (from the fallopian tube) were selected. Because epithelial ovarian carcinoma is known to be of Mullerian cell origin, fallopian tube tissue is the most appropriate source of a nontumor counterpart of Mullerian origin.

DNA and RNA Extraction. Genomic DNA was isolated by phenol/chloroform preceding proteinase K treatment. Total cellular RNA was extracted by using TRizol reagent (Life Technologies, Inc., Tokyo, Japan). The extracted nucleic acid was examined by electrophoresis, and the yield was measured spectrophotometrically before use. High-quality genomic DNA and total cellular RNA were obtained successfully from both tumor and nontumor samples of 30 cases. Total RNA (1 μg) was reverse-transcribed to cDNA by standard procedures.

Real-time Quantitative RT-PCR and PCR. Two μl of the synthesized cDNA and genomic DNA (10 ng/μl) were used in the real-time PCR for the RNA expression and gene amplification studies, respectively. A real-time PCR reaction was carried out with the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) in a total volume of 20 μl that contained 1X FastStart DNA Master Hybridization Probes reaction mix (Roche Molecular Biochemicals), 5 mM MgCl2, 0.5 μM each forward and reverse primers (Table 1), and 0.16 μl of TaqMan probe (Table 1). The reaction was performed by the two-step thermal cycling method: 5 min at 95°C to activate the FastStart polymerase enzyme, 10 s at 94°C, and 30 s at 60°C for 40 cycles; and a cooling step of 30 s at 30°C. PBGD (GenBank accession no. M95623) and human TBP (GenBank accession no. NM_003194) were chosen as housekeeping genes because they are known not to harbor pseudogenes that might interfere with

Received 1/15/02; accepted 5/7/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This study was supported by grants from the Committee on Research and Conference Grants from the University of Hong Kong (10203373/14285/21200/323/01), and Ontario Cancer Genetics Network, Cancer Care Ontario and the Pacific Bridge Project.

2 To whom requests for reprints should be addressed, at Department of Pathology, Queen Mary Hospital, The University of Hong Kong, Pokfulam Road, Hong Kong. Phone: 852-2855-4410; Fax: 852-2872-5197; E-mail: uskhoo@pathology.hku.hk.

3 The abbreviations used are: RT-PCR, reverse-transcription PCR; PBGD, porphobilinogen deaminase; TBP, TATA box-binding protein; Bis-PCR, bisulfite PCR; UTR, untranslated.
neutralized by ammonium acetate followed by ethanol precipitation. The Tris (pH 8)-1 mM EDTA, and 2
PBGD quantitative measurement.

Serial-diluted DNA templates with known concentrations and one replicate for endogenous controls. The calibration standard curve was set up using three and one exon of the specific DNA sequence. The housekeeping genes acted as for the gene amplification study (Table 1) were designed to harbor one intron gene to avoid amplification of minute DNA contaminants. Similarly, primers in ovarian cancer. The primers (Table 1) spanned at least two exons on each

The mRNA and genomic sequence of these genes were obtained from GenBank

Table 1 Sequences of the primers and TaqMan probes used in real-time RT-PCR and PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer direction</th>
<th>Sequences (5′ to 3′)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1</td>
<td>Forward-1</td>
<td>GCCATCTCCTCTAGAGTGAAT</td>
<td>Exon 10</td>
</tr>
<tr>
<td></td>
<td>Reverse-1</td>
<td>TGGTTTAGAAACGATGGAGGC</td>
<td>Exon 11</td>
</tr>
<tr>
<td></td>
<td>TagMan Probe</td>
<td>TGGTTTAGAAACGATGGAGGC</td>
<td>Exon 11</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Forward-1</td>
<td>CTAAAGAGACTGTCGACGGCT</td>
<td>Exon 10</td>
</tr>
<tr>
<td></td>
<td>Reverse-1</td>
<td>CTGAGACTCCCTAGAGTGGT</td>
<td>Exon 11</td>
</tr>
<tr>
<td></td>
<td>TagMan Probe</td>
<td>TGGTTTAGAAACGATGGAGGC</td>
<td>Exon 11</td>
</tr>
<tr>
<td></td>
<td>Forward-2</td>
<td>AGTAGTTGATTAAGTGTGATTC</td>
<td>Intron 10</td>
</tr>
<tr>
<td>PBGD</td>
<td>Forward-1</td>
<td>AATGTTGCGGACAGCGCAGC</td>
<td>Exon 9</td>
</tr>
<tr>
<td></td>
<td>Reverse-1</td>
<td>CAGAGTAGTCAGTCAACTGAC</td>
<td>Exon 10</td>
</tr>
<tr>
<td></td>
<td>TagMan Probe</td>
<td>AATGTTGCGGACAGCGCAGC</td>
<td>Exon 9</td>
</tr>
<tr>
<td></td>
<td>Forward-2</td>
<td>GCCAGCTTCTAGGAGGAAAGAC</td>
<td>Intron 9</td>
</tr>
<tr>
<td>TBP</td>
<td>Forward-1</td>
<td>AGCAAGAGCAGCAGTATGAT</td>
<td>Exon 4</td>
</tr>
<tr>
<td></td>
<td>Reverse-1</td>
<td>AACCCCACTCTGGCAGAATCTCACTCA</td>
<td>Exon 5</td>
</tr>
<tr>
<td></td>
<td>TagMan Probe</td>
<td>AGCAAGAGCAGCAGTATGAT</td>
<td>Exon 4</td>
</tr>
<tr>
<td></td>
<td>Forward-2</td>
<td>AGTATTCTTCTCGTCTGATGATGA</td>
<td>Intron 4</td>
</tr>
</tbody>
</table>

quantitative measurement. PBGD and TBP are located at 11q23.3 and 6q27, respectively, and deletions in these chromosomal regions are seldom reported in ovarian cancer. The primers (Table 1) spanned at least two exons on each gene to avoid amplification of minute DNA contaminants. Similarly, primers for the gene amplification study (Table 1) were designed to harborne one intron and one exon of the specific DNA sequence. The housekeeping genes acted as endogenous controls. The calibration standard curve was set up using three serial-diluted DNA templates with known concentrations and one replicate for each concentration. These DNA templates are linearized plasmids that contained a BRCA1, BRCA2, PBGD, or TBP cDNA insert (or DNA insert for BRCA2 gene amplification study). Reproducibility of the measurements was assessed by conducting duplicate reactions.

Allelic Loss Analysis. Four (fluorescent labeled) microsatellite markers were used for BRCA1: D17S1085 (6-carboxyfluorescein), D17S1185 (6-carboxytetramethylrhodamine), D17S1322 (6-carboxytetramethylrhodamine), and D17S1325 (hexachloro-6-carboxyfluorescein). Amplified microsatellite fragments were run on an ABI PRISM 377 automated sequencer (Applied Biosystems), a positive methylated DNA control was included in each sequencing experiment, which ensured adequate bisulfite chemical reaction of the cases being analyzed. Bisulfite Direct Sequencing. Direct sequencing on bisulfite-treated DNA was performed using the ABI 377 automated sequencer (Applied Biosystems). A protein truncation test (29:1) PAGE and stained by 1

Table 2 Results of all type of tumors analyzed for BRCA mRNA expression, allelic loss, and methylation status

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. of cases</th>
<th>Reduced expression</th>
<th>Allelic loss</th>
<th>Hypermethylation</th>
<th>Elevated expression</th>
<th>Amplification</th>
<th>Hypomethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>12</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>MC</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>EC</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>CCC</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>20</td>
<td>12</td>
<td>15</td>
<td>24</td>
<td>3</td>
<td>16</td>
</tr>
</tbody>
</table>

SC, serous carcinoma; MC, mucinous carcinoma; EC, endometroid carcinoma; CCC, clear cell carcinoma.
Eight of 10 informative cases showed preferential allelic expression. All of these cases were confirmed to have allelic loss and were found to have a significant 2-fold reduction of \( BRCA1 \) mRNA expression as well.

**Gene Amplification.** Quantitative real-time PCR and TaqMan assay using the LightCycler were also used to evaluate possible \( BRCA2 \) gene amplification. The quantitative values of each case were measured against the standard curve that was included in each run. The linear coefficient value of the standard curve was 0.99 to 1.00. The value for \( BRCA2 \) was normalized against that of \( TBP \) and \( PBGD \) reference genes. The cutoff point for amplification was set as \( >2.0 \). Only three cases were shown to have a ratio of \( \sim 2.0 \). These three cases were found to have \( >5 \)-fold of \( BRCA2 \) mRNA expression. Overall statistical analysis did not show statistical significance (\( P > 0.05 \); Wilcoxon test) to support \( BRCA2 \) gene amplification as the cause for mRNA overexpression.

**Methylation Analysis.** Methylation status of the tumor and non-tumor specimens were investigated in 15 and 31 CpG dinucleotides within the \( BRCA1 \) and \( BRCA2 \) promoter and 5’-UTR regions, respectively. All 30 cases were analyzed using the enzyme-digested Bis-PCR method. Bisulfite direct sequencing for both genes was completed successfully in 23 cases. For \( BRCA1 \), 15 of the 23 (65%) cases were shown to have more methylated CpG dinucleotides in tumor than in nontumor DNA. In contrast, for \( BRCA2 \), 16 cases were found to have fewer methylated CpG dinucleotides in tumor than in nontumor DNA (Table 2). Nine of the 16 (56.3%) cases showing aberrant methylation were found to have both \( BRCA1 \) hypermethylation and \( BRCA2 \) hypomethylation at the same time.

Cases K20 (Figs. 2I and 3I) and K37 (Fig. 3I) illustrate hypermethylation in the \( BRCA1 \) promoter and 5’-UTR regions. These cases were found to have \( >80\% \) of CpG dinucleotides methylated within the studied region. The highest frequency of methylation was noted at the +1 and +8 CpG dinucleotides upstream to the transcription initiation site of \( BRCA1 \). Hypermethylation in the \( BRCA1 \) promoter region showed a statistically significant correlation with decreased \( BRCA1 \) mRNA expression (\( P = 0.017; \chi^2 \) test).

Cases K27 and K11 (Fig. 2H) demonstrate hypomethylation in the \( BRCA2 \) promoter and 5’-UTR regions. These cases were found to have less frequent methylation of the CpG dinucleotides in tumor compared with nontumor DNA. This finding was confirmed by bisulfite sequencing (Fig. 3I). In contrast, case K23 (Fig. 2H) had equivalent \( BRCA2 \) mRNA expression in tumor and nontumor. Statistical analysis also showed a significant correlation between hypomethylation and overexpression of \( BRCA2 \) in cases having \( >3 \)-fold of overexpression (\( P = 0.043; \chi^2 \) test).

The presence of methylated CpG dinucleotides was found in 26 of the 30 fallopian tube samples (nontumor tissue) studied by Bis-PCR restriction-enzyme analysis. We investigated whether methylation could also be detected in other normal tissues of the body (two cases of each tissue type). Results showed that methylated CpG dinucleotides in the \( BRCA2 \) promoter appeared to be found in normal tissues of Mullerian origin: from the cervix (two cases), endometrium (two cases), ovary (two cases) as well as fallopian tube (two cases). One case of breast and thyroid tissue also showed this finding. Methylation of \( BRCA2 \) promoter was however absent in tissues like spleen, bladder, and colon.

**Correlation with Clinical Data.** Results of \( \chi^2 \) analysis showed a statistically significant correlation (\( P = 0.037; \chi^2 \) test) between \( BRCA2 \) hypomethylation with disease stage. However, there was no significant correlation with survival (\( P > 0.05 \); log rank test). No significant correlation was found for all \( BRCA1 \) data.
Methylation status of the CpG dinucleotides in the promoter and/or transcriptional regulatory region of certain cancer susceptibility genes have been studied in various types of cancers. In our study, hypermethylation in the BRCA1 promoter region showed a statistically significant correlation with a decrease of BRCA1 mRNA expression ($P = 0.017; \chi^2$ test). In fact, 12 and 10 of 16 cases with significantly reduced BRCA1 expression were noted to be methylated at the upstream +1 and +8 CpG dinucleotides of BRCA1, respectively. Mancini et al. (20) suggested previously that the presence of specific methylated sites may effectively alter gene expression. A further search of the promoter sequence upstream to the transcriptional initiation site in the BRCA1 promoter, using TFSEARCH, revealed the presence of +1 and +2 CpG dinucleotides, which harbor a putative transcriptional factor, POU, binding site. Methylation of these two sites may affect the binding affinity of this transcriptional regulatory element to activate the transcription of the BRCA1 gene. CpG methylation within the binding region of another transcription factor, cAMP-responsive element binding, has also been demonstrated to abolish cAMP-responsive element binding and transcriptional activation to BRCA1 (20). Further investigation of the POU motif sequence should be performed to establish whether the binding of this putative transcriptional factor to the target promoter sequence might be affected by the methylated CpG dinucleotides.

Hypermethylated gene promoter regions demonstrating significant correlation with the aberrant mRNA expression in genes, such as RB (12), p16 (13), and hMsi1 (14), suggest gene silencing or inactivation by this epigenetic factor. Similarly, our findings of reduced expression of BRCA1 showed a high correlation with the epigenetic factor of hypermethylation at the promoter region. Allelic loss and/or hypermethylation of the BRCA1 promoter support the Knudson’s two-hit hypothesis that BRCA1 is a tumor suppressor gene. Both events occurring simultaneously could lead to mono- or biallelic inactivation, which might lead to partial or complete lack of function of the BRCA1 gene. This association was clearly demonstrated in this study. In the preferential allelic expression, 8 of 10 informative cases showed preferential allelic expression that were subsequently confirmed to have allelic loss. These cases had reduced mRNA expression and were also hypermethylated.

In contrast, BRCA2 promoter had relatively few or no methylated CpG dinucleotides in the tumor DNA compared with that of nontumor DNA. This suggests that hypomethylation of the BRCA2 promoter and 5'-UTR regions might lead to overexpression of BRCA2 mRNA in these cases. Statistical analysis showed a significant correlation between hypomethylation and those cases showing a >3-fold overexpression of BRCA2 ($P = 0.043; \chi^2$ test).

Interestingly, similar to the observed opposing patterns of mRNA expression of the BRCA1 and BRCA2 genes, 9 of 16 cases (56.3%) showing aberrant methylation, demonstrated opposing patterns of BRCA1 and BRCA2 methylation within the same cases. Correlating experimental results with the clinical data of disease stage and survival of the cases studied, hypomethylation of BRCA2, but not overexpression, was found to show a statistically significant correlation with tumor stage ($P = 0.037; \chi^2$ test). Cases demonstrating BRCA2 hypomethylation had a higher tumor stage. No correlation, however, was found with survival. Correlation of hypomethylation, rather than overexpression, may suggest the importance of the role of methylation in BRCA2 as a factor contributing to the pathogenesis of sporadic ovarian carcinoma.

The scenario of hypomethylation in tumor suggests the possibility of the loss of gene imprinting. Imprinted genes, such as IGF2 and

Internet address: http://www.cbrc.jp/research/db/TFSEARCH.html.
H19, have been shown to be involved in the development of cervical, breast, and ovarian cancers by loss of imprinting (21). Imprinting of one of the parental gene alleles by methylation will cause partial silencing or inactivation of that gene. Loss or relaxation of imprinting could theoretically cause biallelic gene expression; in other words, doubling of the active gene dosage. As a result, this might cause mRNA overexpression of the gene in tumor. Interestingly, the presence of methylated CpG dinucleotides in the promoter region of BRCA2 was demonstrated in tissues of Mullerian origin. These findings are in contrast with that reported by Collins et al. (17). However, their study only demonstrated the absence of methylation of CpG dinucleotides in the 5' UTR region of BRCA2, whereas we have investigated the methylation status of a much larger region, from −135 to +210 relative to the transcription initiation site of BRCA2 gene. This region contains 31 CpG dinucleotides among which 13 are in the promoter region, with the rest in the 5' UTR sequence of BRCA2. A recent report has demonstrated that the region we are studying harbors strong basal activity to promote transcription of BRCA2 (22).

Gene amplification of BRCA2 appears unlikely to cause elevated mRNA expression in our ovarian cancer samples. Nevertheless, there were three cases that did have an almost 2-fold increase of BRCA2 gene amplification and that were found to have very high levels (>5-fold) of mRNA expression in tumor relative to that in nontumor samples. Thus, the expression of BRCA2 is likely to be regulated by other gene-regulatory factors. A recent report has shown nuclear factor-κB to be bound to the BRCA2 promoter, thus causing transcription up-regulation (23). Nuclear factor-κB has been shown to regulate expression of several genes that play critical roles in apoptosis, tumorigenesis, and inflammation. An Alu-repeat transcriptional silencer (24) has also been identified previously and characterized upstream to the promoter of BRCA2. Mutation or loss of this silencer might contribute to the aberrant BRCA2 expression in the tumor cases. Although promoter methylation may be a significant epigenetic factor affecting the BRCA2 mRNA expression, functional regulation of BRCA2 by other transcriptional controlling factors and elements would also need to be investigated further.

ACKNOWLEDGMENTS
We thank Dr. W. C. Yam, Department of Microbiology, The University of Hong Kong, for the use of the LightCycler system.

REFERENCES
EPIGENETIC FACTORS AND THE \textit{BRCA} GENES IN OVARIAN CANCER


