

## Exhibit 4

# Epigenetic Factors Controlling the *BRCA1* and *BRCA2* Genes in Sporadic Ovarian Cancer<sup>1</sup>

Kelvin Y. K. Chan, Hilmi Ozcelik, Annie N. Y. Cheung, Hextan Y. S. Ngan, and Ui-Soon Khoo<sup>2</sup>

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 Pathobiology, 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 cytosine 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  $\mu$ g) was reverse-transcribed to cDNA by standard procedures.

**Real-time Quantitative RT-PCR<sup>3</sup> and PCR.** Two  $\mu$ l of the synthesized cDNA and genomic DNA (10 ng/ $\mu$ l) 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  $\mu$ l that contained 1X FastStart DNA Master Hybridization Probes reaction mix (Roche Molecular Biochemicals), 5 mM MgCl<sub>2</sub>, 0.5  $\mu$ M each forward and reverse primers (Table 1), and 0.16  $\mu$ M 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.

<sup>1</sup> 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.

<sup>2</sup> 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.

<sup>3</sup> The abbreviations used are: RT-PCR, reverse-transcription PCR; *PBGD*, porphobilinogen deaminase; *TBP*, TATA box-binding protein; Bis-PCR, bisulfite PCR; UTR, untranslated.

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

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

Gene	Primer direction	Sequences (5' to 3')	Location
BRCA1	Forward-1	GGCTATCCTCTCAGAGTGACATT	Exon 10
	Reverse-1	CTGATGTGCTTTGTTCTGGA	Exon 11
	TaqMan Probe	TGTGTTAGAACAGCATGGGAGC	Exon 11
BRCA2	Forward-1	CTAATTAAGTTCAGCCAGT	Exon 10
	Reverse-1	CTAGAACATTTCCCTCAGAATTGTC	Exon 11
	TaqMan Probe	TATTGCATCTCTGTGAAAAGAAGCT	Exon 11
PBGD	Forward-2	AGTGAATGTGATTGATGGTACTT	Intron 10
	Forward-1	AGTGTGGTGGGAACCAGC	Exon 9
	Reverse-1	CAGGATGATGGCACTGAACTC	Exon 10
TBP	TaqMan Probe	CCTCAACACCCGGCTTCGGAAGA	Exon 10
	Forward-2	GCCAGCTAGAGAGGAAAGAC	Intron 9
	Forward-1	ACGAACCACGGCACTGAT	Exon 4
TBP	Reverse-1	AACCCAACCTTCTGTACAACCTAGCA	Exon 5
	TaqMan Probe	ACAGTCCAGACTGGCAGCAAGAAAATA	Exon 5
	Forward-2	AGTATTTAGCTGGCTCTGAGTATGAAT	Intron 4

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 harbor 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*: D17S0855 (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, Foster City, CA), using GeneScan and Genotyper (version 3.1; Applied Biosystems) to analyze the allelic-loss status of each case. The results were confirmed by visual inspection. In informative cases, allelic loss on the marker location was defined as a decrease of 50% of the signal intensity of one of the alleles compared with that of another allele. The allelic-loss analysis was repeated twice and even three times for some marginal cases.

**Bis-PCR and Enzyme Restriction Analysis.** The following method, as described previously by Clark *et al.* (18), with modifications was used. Genomic DNA (500 ng) was denatured with 0.3 M NaOH and treated with 3.6 M sodium bisulfite solution (0.5 mM hydroquinone, adjusted to pH 5.0) at 55°C for 15 h in the dark, purified by Wizard DNA Cleanup System (Promega, Madison, WI), desulfonated with 0.3 M NaOH at room temperature, and neutralized by ammonium acetate followed by ethanol precipitation. The bisulfite-treated DNA was suspended in 30  $\mu$ l of Tris-EDTA buffer [10 mM Tris (pH 8)-1 mM EDTA], and 2  $\mu$ l of the DNA were subjected to PCR. Primers were specifically designed to target the bisulfite-treated DNA sequence of *BRCA1* and *BRCA2*. They flanked the transcriptional initiation sites, -256 to +66 and -135 to +210, respectively, of these two genes: for *BRCA1*, 5'-GTAATTGGAAGAGTAGAGGTTAGAG-3' and 5'-AAAAC-CCCACAACCTATCCC-3'; for *BRCA2*, 5'-GGTTGGGATGTTGATAAG-3'

and 5'-CAACCCACTACCACCACCCTAA-3'. A methylation-positive DNA control prepared *in vitro* was made using SssI methylase (New England Biolabs, Beverly, MA), which methylated every cytosine of CpG dinucleotide in the DNA. Five microliters of Bis-PCR product of each case, as well as that of the positive control, were used for digestion by *HhaI* and *AcI* (New England Biolabs) for *BRCA1* and by *BstUI* and *AcI* (New England Biolabs) for *BRCA2*. These two enzymes cleaved the Bis-PCR products, which contained the unmodified methylated cytosine CpG dinucleotides. The digested products were separated by 8% (29:1) PAGE and stained by 1 $\times$  SYBR Gold (Molecular Probes, Eugene, OR) fluorescent dye and visualized under UV transilluminator. The band pattern was compared between the tumor and nontumor of each case.

**Bisulfite Direct Sequencing.** Direct sequencing on bisulfite-treated DNA was performed using the ABI 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.

**Screening for BRCA1 and BRCA2 Mutations.** A protein truncation test and single-stranded conformation polymorphism analysis, as described previously (3, 19), were performed on all tumor samples to screen for mutations throughout the entire *BRCA1* and *BRCA2* coding sequences and the poly(A)-signal sequence of the *BRCA1* gene. Any case in which mutation had been found was excluded from our study.

**Statistical Analysis.** GraphPad PRISM 3.02 for Windows software (GraphPad Software, San Diego, CA) was used to carry out the statistical analysis of experimental data;  $P < 0.05$  with two-tailed *t* test was considered statistically significant. Correlation with clinical data, namely that of disease stage and survival, was also performed with SPSS 10.1 for Windows (SPSS Inc., Chicago, IL).

## RESULTS

**RNA Expression Analysis.** The thirty ovarian cancer cases included four histological subtypes (Table 2): 12 serous, 6 mucinous, 6 endometrioid, and 6 clear cell carcinoma cases. The mRNA level of the *BRCA1* and *BRCA2* genes was measured using real-time RT-PCR. TaqMan probes were used on a LightCycler for this quantitative study. The standard curve of each run was generated with a high linear coefficient value of 0.99 to 1.00. For validation purposes, the experiments were repeated in 80% of all cases. The results showed high concordance with no more than a 5–10% difference in each test sample. The mRNA expression of *BRCA1* and *BRCA2* was calculated by normalizing the *BRCA* gene values against that of the housekeeping genes. The normalized values were then compared between the tumor and nontumor in each case. Fig. 1 shows the overall distribution of expression of the *BRCA* genes in tumor and nontumor specimens. In a comparison between results for each tumor with its nontumor counterpart, 20 of 30 (67%) cases were shown to have at least a 2-fold reduction of *BRCA1* mRNA level. In contrast, 24 of 30 (80%) cases were shown to have at least a 2-fold higher elevation of *BRCA2* mRNA levels (Table 2). Among these 24 cases, 15 (63%) of them even showed to have an elevation of *BRCA2* expression that was >3-fold. Interestingly, 14 of 24 (58.2%) cases were observed to have aberrant mRNA expression of reduced *BRCA1* mRNA expression, but simultaneously elevated *BRCA2* mRNA expression. Overall, the mRNA expression results for both *BRCA1* and *BRCA2* showed a

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

Sample type	No. of cases	BRCA1			BRCA2		
		Reduced expression	Allelic loss	Hypermethylation	Elevated expression	Amplification	Hypomethylation
SC <sup>a</sup>	12	9	8	7	9	1	8
MC	6	4	2	3	5	1	2
EC	6	3	1	3	6	1	3
CCC	6	4	1	2	4	0	3
Total	30	20	12	15	24	3	16

<sup>a</sup> SC, serous carcinoma; MC, mucinous carcinoma; EC, endometrioid carcinoma; CCC, clear cell carcinoma.

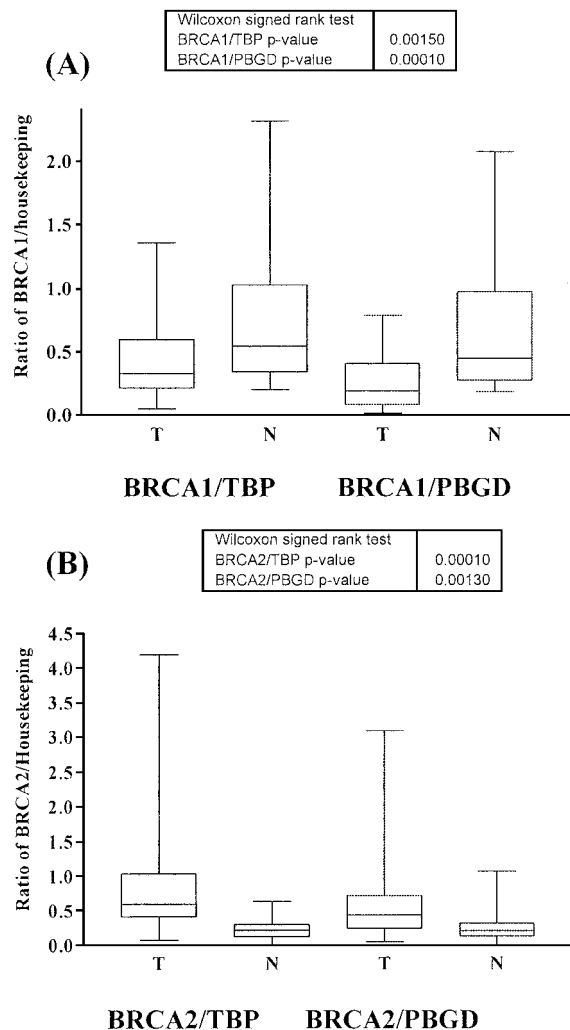


Fig. 1. Overall distribution of real-time quantitative RT-PCR results of *BRCA1* and *BRCA2*. *BRCA1* and *BRCA2* each were normalized by the endogenous housekeeping gene controls (*PBGD* and *TBP*) in tumor and nontumor RNA. The housekeeping genes showed consistent results in each *BRCA* gene mRNA expression study. The results showed that a differential mRNA expression pattern of *BRCA* genes in ovarian tumor compared with the nontumor counterparts: reduced *BRCA1* mRNA expression (A); elevated *BRCA2* mRNA expression (B) in tumor versus nontumor.

statistically significant reduction and elevation, respectively, with  $P < 0.05$ , respectively (Wilcoxon test; Fig. 1). The mRNA calculated expression results of *BRCA1* and *BRCA2* normalized against either of the two housekeeping genes also showed statistically significant concordance ( $\chi^2$  test) with  $P = 0.0015$  and  $P = 0.02$ , respectively.

**Allelic Loss.** The markers D17S855 and D17S1322 are intragenic, whereas D17S1325 and D17S1185 are 5'- and 3'-flanking the *BRCA1* locus, respectively. The heterozygosity rate, investigated previously in the Southern Chinese population (19), are as follows: 90% for D17S1325, 81% for D17S855, 81% for D17S1322, and 82% for D17S1185. Cases showing loss in at least one intragenic marker of *BRCA1* were considered to have allelic loss. Allelic loss of *BRCA1* was found in 40% (12 of 30) of the tumor cases (Table 2). Among the 12 allelic loss cases, 8 cases (66.7%) were found to have a significant 2-fold reduction of *BRCA1* mRNA expression in tumor samples when compared with nontumor samples.

Before the allelic loss study, analysis of preferential allelic expression of *BRCA1* was performed (data not shown) using the *EaeI* restriction enzyme to digest the polymorphism, which was contained within the fragment amplified from conventional RT-PCR reaction.

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 nontumor 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. 2II) 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. 3II). In contrast, case K23 (Fig. 2II) 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.



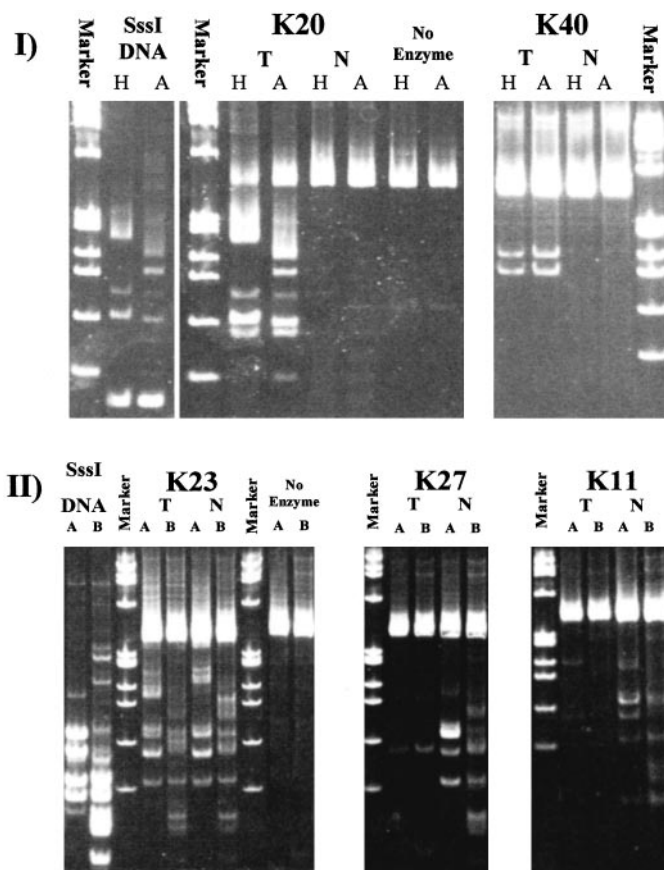


Fig. 2. The restriction enzyme digestion band pattern of *BRCA1* (I) and *BRCA2* (II) promoter after the bisulfite PCR reaction. The marker is  $\phi$ 174/HaeIII. SssI DNA was the methylation positive control DNA for which placenta DNA was *in vitro* methylated at the CpG islands by SssI methylase. H, HhaI; A, AclI; No enzyme, nonenzyme digested products; B, *Bsr*UI. I, digested band patterns showed that both K20 and K40 had hypermethylated *BRCA1* promoter in tumor when compared with that in nontumor. II, in K23 the digested band patterns between tumor and nontumor are similar. This demonstrates similar methylation status of *BRCA2* promoter and 5'-UTR regions in tumor and nontumor, whereas, in the case of K27 and K11, the band patterns suggested the hypomethylation of the studied region found in tumor DNA with respect to that in nontumor DNA.

## DISCUSSION

This study investigated *BRCA1* and *BRCA2* mRNA expression levels and possible allelic loss, gene amplification, and methylation status in a series of tumor and nontumor tissue from sporadic ovarian cancer patients. We found that a significant proportion of our sporadic ovarian carcinomas expressed relatively reduced levels of *BRCA1* mRNA when compared with that in their nontumor counterpart. In contrast to the *BRCA1* findings, our study found statistically significant overexpression of *BRCA2* mRNA in tumor with respect to that in nontumor specimens. Interestingly, of the 20 cases showing reduced *BRCA1* expression and the 24 cases showing elevated *BRCA2* expression, 14 of the 24 cases (58.2%) showing aberrant *BRCA* expression demonstrated opposing mRNA expression patterns of the *BRCA1* and *BRCA2* genes within the same case. This suggests that both *BRCA1* and *BRCA2* are involved, but may play different roles in the development of tumorigenesis in sporadic ovarian cancer.

Eight of the 12 cases found to have allelic loss at *BRCA1* locus, demonstrated significantly decreased *BRCA1* mRNA expression. Although allelic loss on *BRCA1* can explain aberrant *BRCA1* mRNA expression in a portion of our cases, *BRCA2* gene amplification results did not yield a significant correlation to explain the observed elevated mRNA expression levels.

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,<sup>4</sup> 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 *hMih1* (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

<sup>4</sup> Internet address: <http://www.cbrc.jp/research/db/TFSEARCH.html>.

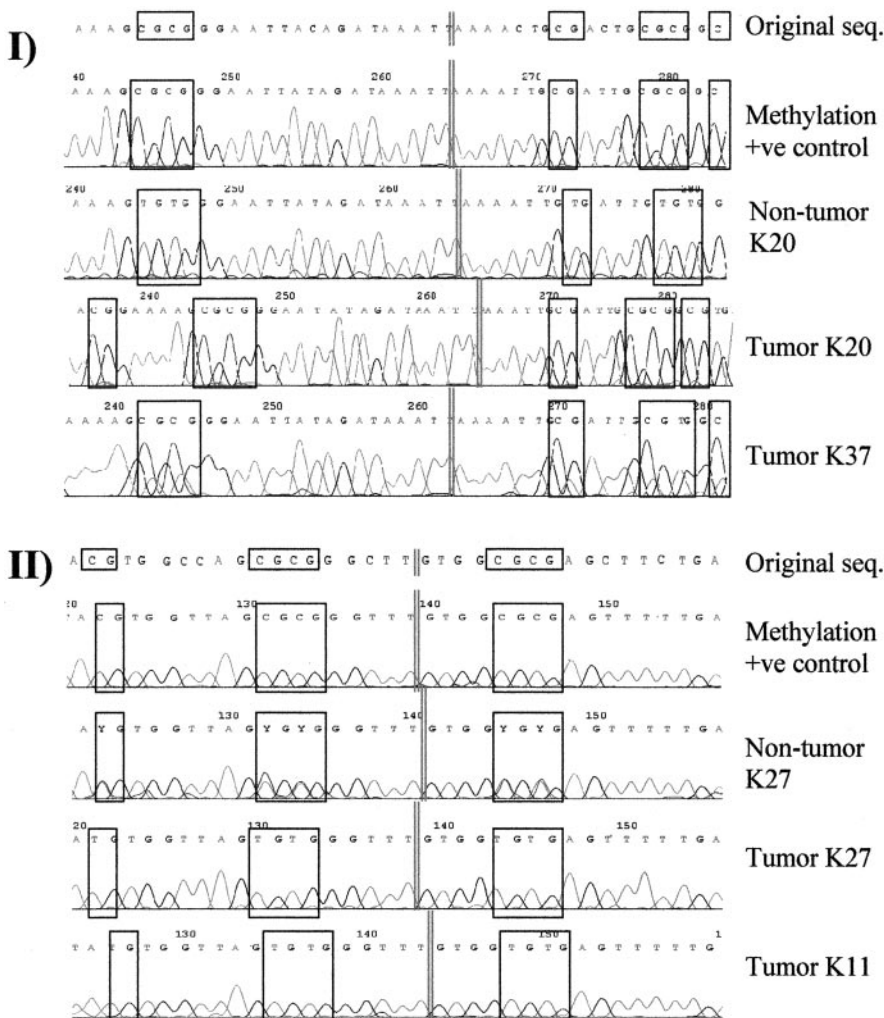


Fig. 3. I, *BRCA1* promoter methylation bisulfite sequencing results: all of the CpG island are in boxes. The double vertical lines represent the transcriptional initiation site. The upper panel shows the original sequence of the part of the *BRCA1* promoter. Methylation +ve control was from the *SssI* methylase *in vitro* methylated DNA. Unmethylated CpG islands were found in the nontumor DNA, whereas there were methylated CpG island in tumor cases K20 and K37. A mixture of methylated and unmethylated CpG islands was indicated in tumor case K37. This illustrates the heterogeneous methylation pattern in DNA. II, *BRCA2* promoter methylation bisulfite sequencing result: CpG islands in tumor cases K27 and K11, in contrast, had an absence of methylation, whereas in the nontumor, there was a mixture of methylated and unmethylated CpG islands.

*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- $\kappa$ B to be bound to the *BRCA2* promoter, thus causing transcription up-regulation (23). Nuclear factor- $\kappa$ B 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

1. Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., and Micklem, G. Identification of the breast cancer susceptibility gene *BRCA2*. *Nature*, 378: 789-792, 1995.
2. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., and Skolnick, M. A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science*, 266: 66-71, 1994.
3. Khoo, U. S., Ozcelik, H., Cheung, A. N., Chow, L. W., Ngan, H. Y., Done, S. J., Liang, A. C., Chan, V. W., Au, G. K., Ng, W. F., Poon, C. S., Leung, Y. F., Loong,

- F., Ip, P., Chan, G. S., Andrulis, I. L., Lu, J., and Ho, F. C. Somatic mutations in the *BRCA1* gene in Chinese sporadic breast and ovarian cancer. *Oncogene*, *18*: 4643–4646, 1999.
4. Khoo, U. S., Ngan, H. Y., Cheung, A. N., Chan, K. Y., Lu, J., Chan, V. W., Lau, S., Andrulis, I. L., and Ozcelik, H. Mutational analysis of *BRCA1* and *BRCA2* genes in Chinese ovarian cancer identifies 6 novel germline mutations. *Hum. Mutat.*, *16*: 88–89, 2000.
  5. Hosking, L., Trowsdale, J., Nicolai, H., Solomon, E., Foulkes, W., Stamp, G., Signer, E., and Jeffreys, A. A somatic *BRCA1* mutation in an ovarian tumour. *Nat. Genet.*, *9*: 343–344, 1995.
  6. Beckmann, M. W., Picard, F., An, H. X., van Roeyen, C. R., Dominik, S. I., Mosny, D. S., Schnurch, H. G., Bender, H. G., and Niederacher, D. Clinical impact of detection of loss of heterozygosity of *BRCA1* and *BRCA2* markers in sporadic breast cancer. *Br. J. Cancer*, *73*: 1220–1226, 1996.
  7. Smith, S. A., Easton, D. F., Evans, D. G., and Ponder, B. A. Allele losses in the region 17q12–21 in familial breast and ovarian cancer involve the wild-type chromosome. *Nat. Genet.*, *2*: 128–131, 1992.
  8. Thompson, M. E., Jensen, R. A., Obermiller, P. S., Page, D. L., and Holt, J. T. Decreased expression of *BRCA1* accelerates growth and is often present during sporadic breast cancer progression. *Nat. Genet.*, *9*: 444–450, 1995.
  9. Ozcelik, H., To, M. D., Couture, J., Bull, S. B., and Andrulis, I. L. Preferential allelic expression can lead to reduced expression of *BRCA1* in sporadic breast cancers. *Int. J. Cancer*, *77*: 1–6, 1998.
  10. Russell, P. A., Pharoah, P. D., De Foy, K., Ramus, S. J., Symmonds, I., Wilson, A., Scott, I., Ponder, B. A., and Gayther, S. A. Frequent loss of *BRCA1* mRNA and protein expression in sporadic ovarian cancers. *Int. J. Cancer*, *87*: 317–321, 2000.
  11. Bieche, I., Nogues, C., and Lidereau, R. Overexpression of *BRCA2* gene in sporadic breast tumours. *Oncogene*, *18*: 5232–5238, 1999.
  12. Ohtani-Fujita, N., Dryja, T. P., Rapaport, J. M., Fujita, T., Matsumura, S., Ozasa, K., Watanabe, Y., Hayashi, K., Maeda, K., Kinoshita, S., Matsumura, T., Ohnishi, Y., Hotta, Y., Takahashi, R., Kato, M. V., Ishizaki, K., Sasaki, M. S., Horsthemke, B., Minoda, K., and Sakai, T. Hypermethylation in the retinoblastoma gene is associated with unilateral, sporadic retinoblastoma. *Cancer Genet. Cytogenet.*, *98*: 43–49, 1997.
  13. Dong, S. M., Kim, H. S., Rha, S. H., and Sidransky, D. Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. *Clin. Cancer Res.*, *7*: 1982–1986, 2001.
  14. Nakajima, T., Akiyama, Y., Shiraishi, J., Arai, T., Yanagisawa, Y., Ara, M., Fukuda, Y., Sawabe, M., Saitoh, K., Kamiyama, R., Hirokawa, K., and Yuasa, Y. Age-related hypermethylation of the hMLH1 promoter in gastric cancers. *Int. J. Cancer*, *94*: 208–211, 2001.
  15. Catteau, A., Harris, W. H., Xu, C. F., and Solomon, E. Methylation of the *BRCA1* promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. *Oncogene*, *18*: 1957–1965, 1999.
  16. Rice, J. C., Ozcelik, H., Maxeiner, P., Andrulis, I., and Futscher, B. W. Methylation of the *BRCA1* promoter is associated with decreased *BRCA1* mRNA levels in clinical breast cancer specimens. *Carcinogenesis (Lond.)*, *21*: 1761–1765, 2000.
  17. Collins, N., Wooster, R., and Stratton, M. R. Absence of methylation of CpG dinucleotides within the promoter of the breast cancer susceptibility gene *BRCA2* in normal tissues and in breast and ovarian cancers. *Br. J. Cancer*, *76*: 1150–1156, 1997.
  18. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.*, *22*: 2990–2997, 1994.
  19. Khoo, U. S., Chan, K. Y. K., Cheung, A. N. Y., Xue, W. C., Shen, D. H., Fung, K. Y., Ngan, H. Y. S., Choy, K. W., Pang, C. P., Poon, C. S. P., Poon, A. Y. A., and Ozcelik, H. Recurrent *BRCA1* and *BRCA2* germline mutations in ovarian cancer: a Founder mutation of *BRCA1* identified in the Chinese population. *Hum. Mutat.*, *19*: 307–308, 2002.
  20. Mancini, D. N., Rodenhiser, D. I., Ainsworth, P. J., O'Malley, F. P., Singh, S. M., Xing, W., and Archer, T. K. CpG methylation within the 5' regulatory region of the *BRCA1* gene is tumor specific and includes a putative CREB binding site. *Oncogene*, *16*: 1161–1169, 1998.
  21. Rainier, S., Johnson, L. A., Dobry, C. J., Ping, A. J., Grundy, P. E., and Feinberg, A. P. Relaxation of imprinted genes in human cancer. *Nature (Lond.)*, *362*: 747–749, 1993.
  22. Davis, P. L., Miron, A., Andersen, L. M., Iglehart, J. D., and Marks, J. R. Isolation and initial characterization of the *BRCA2* promoter. *Oncogene*, *18*: 6000–6012, 1999.
  23. Wu, K., Jiang, S. W., Thangaraju, M., Wu, G., and Couch, F. J. Induction of the *BRCA2* promoter by nuclear factor-kappa B. *J. Biol. Chem.*, *275*: 35548–35556, 2000.
  24. Sharan, C., Hamilton, N. M., Parl, A. K., Singh, P. K., and Chaudhuri, G. Identification and characterization of a transcriptional silencer upstream of the human *BRCA2* gene. *Biochem. Biophys. Res. Commun.*, *265*: 285–290, 1999.