BRCA1 Promoter Region Hypermethylation in Ovarian Carcinoma: A Population-based Study

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Abstract

There is a clear association between germ-line BRCA1 mutations and inherited ovarian cancer; however, the association between BRCA1 mutations and sporadic ovarian cancer remains ambiguous. The frequency of BRCA1 promoter hypermethylation as an epigenetic means of BRCA1 inactivation was determined for a large, population-based cohort of ovarian cancer patients. BRCA1 promoter hypermethylation was determined by methylation-specific restriction digestion of tumor DNA, followed by Southern blot analysis and confirmed by methylation-specific PCR. BRCA1 promoter hypermethylation was observed in 12 of 98 ovarian tumors. BRCA1 methylation status of the primary tumor was conserved in six recurrent tumors after interim chemotherapy. None of the 12 tumors with BRCA1 promoter hypermethylation demonstrated BRCA1 protein expression by immunohistochemistry. BRCA1 methylation was only seen in ovarian cancer patients without a family history suggestive of a breast/ovarian cancer syndrome. Therefore, the 12 BRCA1 methylated tumors represented 15% (12 of 81) of the sporadic cancers analyzed in this study. Although the clinical significance of BRCA1 promoter hypermethylation is yet to be determined, promoter hypermethylation may be an alternative to mutation in causing the inactivation of the BRCA1 tumor suppressor gene in sporadic ovarian cancer.

Introduction

Links between BRCA1 mutations and inherited OC3 are well established; however, somatic mutations in BRCA1 do not seem to play a significant role in the etiology of sporadic OC (1–5). Despite the low frequency of BRCA1 mutations in sporadic OCs, LOH at 17q21, the BRCA1 locus, is seen in 40–70% of invasive OCs (6–9). In addition, BRCA1 mRNA levels are reduced or absent in a majority of sporadic breast cancers, and BRCA1 protein expression is absent or low in both breast and OCs (10, 11). These data support the hypothesis that BRCA1 plays an important role in sporadic ovarian tumorogenesis. Tumor suppressor gene function can be lost through epigenetic inactivation, resulting in altered gene expression or functional inactiva-
tion. The hypermethylation of normally unmethylated, promoter region CpG islands has been associated with the transcriptional inactivation of several tumor suppressor genes including hMut1, RB1, VHL, p15, and p16 (12–16). We hypothesized that BRCA1 methylation is regulated by promoter-region CpG island methylation in sporadic OC. Recently, the methylation pattern of the BRCA1 promoter has been examined and was found to be methylated in a subset of OCs and cell lines (17, 18). To accurately evaluate the hypermethylation of the BRCA1 promoter region, we analyzed a large, population-based cohort of OCs (n = 98) by Southern Blot analysis and confirmed our results by MSP. This study is the largest study to date and demonstrates methylation of the BRCA1 promoter in 15% of sporadic OCs. To gain an understanding of the functional significance that BRCA1 promoter methylation may play in ovarian carcinogenesis, we also evaluated alternative mechanisms of BRCA1 inactivation in samples with a hypermethylated BRCA1 promoter region, and we examined the relationship between the patient’s clinicopathological characteristics and BRCA1 methylation status.

Materials and Methods

Patients. The methylation status of the BRCA1 promoter-region CpG island was analyzed in a population-based cohort of OC patients. Ninety-eight OC and 12 normal ovarian specimens were obtained from the tissue bank maintained by the Gynecological Oncology Research Laboratory at Cedars-Sinai Medical Center. All tissue specimens were collected and immediately snap frozen in the operating room from patients undergoing surgery for OC. Women without a family history of OC undergoing oophorectomy for non-malignant indications contributed the normal ovarian specimens, which served as controls. All studies using human tissues were approved by the Cedars-Sinai Medical Center Institutional Review Board.

Southern Blot Analysis of CpG Methylation. Ten µg of genomic DNA isolated from snap frozen tissue was digested with AvaII alone or in combination with the methylation-sensitive restriction enzymes HpaII or CfoI, separated by gel electrophoresis, and transferred overnight to Hybond N+ membranes (Amersham, Buckinghamshire, United Kingdom) as described (17). To assess BRCA1 promoter region CpG island methylation, the membrane was prehybridized and hybridized using a random-labeled, 217-bp PCR-generated probe that spanned BRCA1 exon 1a (17).

MSP. The methylation status of all specimens was confirmed by MSP of sodium bisulfite-converted DNA. DNA was deaminated with the CpGenome DNA Modification kit following the manufacturer’s recommendations (Inter- gene, Purchase, NY). Bisulfite-modified DNA was amplified with PCR primers that distinguish methylated (M) and unmethylated (U) DNA. Primer sequences for U and M DNA were as follows: U forward, ggt taatt aag tgt agg gat g; U reverse, t cca aac act cac acc aca cca tca; M forward, ggt taatt aag tgt cgg gag c; and M reverse, tca aag aac tca cgg cgg cga atc g. These primers amplify a 182-bp product corresponding to nucleotides 3194–3375 (accession no. L78833), which immediately precede and include the initial portion of exon 1a. All amplifications used a hot start and AmpliTaq DNA Polymerase (Perkin-Elmer, Foster City, CA) with the following conditions: for M primers, 40 cycles of 94°C for 15 s, 65°C for 30 s, and 72°C for 30 s; for U primers, 35 cycles of 94°C for 15 s, 61°C for 30 s, and 72°C for 30 s. PCR products were analyzed on 2% NuSieve 3:1 Agarose gels (FMC Corp., Rockland, ME) in 1X TBE (89 mM Tris borate and 2 mM EDTA, pH 8.3).

Analysis of Allelic Loss. LOH was assessed by PCR amplification of tumor and normal DNA with four pairs of 3’-end labeled dinucleotide repeat markers, D17s855, D17s1323, D17s1327, and THRA-1 (Research Genetics, Inc., Huntsville, AL). These primers have been shown to amplify intragenic sequences and sequences closely associated with BRCA1 (19). DNA was

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3 The abbreviations used are: OC, ovarian carcinoma; LOH, loss of heterozygosity; MSP, methylation-specific PCR.
isolated from snap frozen tumor tissue, and normal control DNA was isolated from whole blood or archival specimens of nonmalignant tissue if blood was not available as described (20). One primer was end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (Life Technologies, Inc., Grand Island, NY), and PCR amplification and gel electrophoresis was performed as described (21). LOH was determined by comparing the intensity of the allelic bands in tumor and normal DNA. Samples were classified as heterozygous with loss, heterozygous without loss, or homozygous. Loss was defined as ≥40% reduction in band intensity of one band.

**Immunohistochemistry.** Paraffin-embedded tissues were used to examine BRCA1 protein expression in normal and malignant ovarian tissues using BRCA1 mouse monoclonal antibody Ab-1 (Oncogene Research Products, Cambridge, MA) and the conditions described by Wilson et al. (11). After deparaffinization and rehydration, slides were incubated at 95°C for 20 min in antigen retrieval solution (Dako Corp., Carpenteria, CA) and then cooled 10 min at room temperature. Endogenous peroxidase activity was inactivated by incubating slides in 3% H2O2 in methanol for 15 min. Nonspecific antibody binding was blocked with 2% BSA in PBS for 30 min at room temperature prior to incubation with murine anti-BRCA1 (1:150) overnight at 4°C in a humidified chamber. Control slides used to assess nonspecific, background staining were incubated in 2% BSA without primary antibody under identical conditions. All slides were incubated with LSAB + reagents (Dako Corp.) following the manufacturer’s instructions for the colorimetric detection of BRCA1 staining and evaluated by two independent reviewers (R. L. B. and I. C.). BRCA1-stained slides were not counterstained; therefore, corresponding serial sections were stained with H&E to reveal tissue architecture.

p53 expression was determined by immunohistochemical staining of paraffin-embedded tissues as described (22). Sections were incubated with antihuman p53 monoclonal antibody AB-6 (Clone DO-1; Oncogene Research Products, Cambridge, MA) at a 1:100 dilution and control sections with normal mouse IgG, also diluted 1:100. Antibody binding was detected with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine as color reagent following the manufacturer’s recommendations. Both the intensity and percentage of positivity of staining were evaluated by two observers. Staining intensity was graded as: −, negative; +, faint; ++, weak; +++, strong; and +++++, intense. The percentage of tumor cells exhibiting p53 staining was evaluated and determined to be <10%, 10–50%, or >50% positivity.

**BRCA1 Mutation Analysis.** Genomic DNA from patients with methylated BRCA1 promoter regions was screened for the founder mutations commonly occurring in the Ashkenazi Jewish population: BRCA1 185delAG (exon 2), BRCA1 5382 insC (exon 20), and BRCA2 6174delT (exon 11; Ref. 23). A combination of single-strand conformational polymorphism analysis for BRCA1 exon 20, heteroduplex analysis of BRCA1 exon 2, and protein truncation testing of BRCA2 exon 11 was used to identify mutated DNA (22). The respective exons were amplified by standard PCR techniques for use in these analyses. Aberrant bands were sequenced using a standard protocol. All samples were tested for all three mutations.

**Statistics.** Comparisons of demographic variables between patients with and without a methylated BRCA1 promoter region were done by Fisher’s exact test.
Results and Discussion

The BRCA1 promoter region has a complex organization. It has two alternative first exons, 1a and 1b, each with its own promoter, $\alpha$ and $\beta$, respectively (Fig. 1; Ref. 24). The $\alpha$ promoter is bidirectional and shared with the NBR2 gene, which lies adjacent to BRCA1 in a head-to-head orientation (24, 25). Additionally, there is a partial duplication of exons 1a, 1b, 2, and intervening sequences forming the BRCA1 pseudogene. The BRCA1 pseudogene is located in a head-to-head orientation with NBR1, a candidate gene for the OC tumor marker CA125 (26). Methylation of the 2000-bp CpG island that encompasses both the BRCA1 $\alpha$ and $\beta$ promoter regions was analyzed in 98 epithelial OCs (27). The only selection criterion used in choosing cases was that there was enough tissue available to complete all planned analyses. We detected BRCA1 promoter methylation in 12% (12 of 98) OC by Southern Blot analysis. The majority of these cases (11 of 12) showed a heterogeneous methylation pattern. Two or more completely or partially uncleaved AvaII fragments were generated by further cleavage with the methylation-sensitive enzymes, HpaII and CfoI (Fig. 2A). Size mapping indicates that the CpG sites at the 5’ end of the AvaII fragment are always methylated, whereas the methylation of CpGs at the 3’ end lying within exon 1b had varying degrees of methylation (Fig. 1). All CpG sites were methylated in one case of 12.

Methylation of the BRCA1 promoter appears to be tumor specific because it was not detected in 25 normal ovarian specimens.

To rule out the possibility of false-positive results attributable to incomplete DNA digestion or the detection of partially digested fragments from the BRCA1 pseudogene, all tumors were also analyzed for hypermethylation by MSP (28). Results obtained by MSP were identical to the Southern Blot data and confirmed the BRCA1 promoter methylation status of all specimens (Fig. 2B). The probe we used for Southern Blot analysis is also able to detect methylation of the BRCA1 pseudogene (17). Methylation of the duplicate BRCA1 pseudogene promoter was detected in 10% (10 of 98) of OC, including one specimen with a methylated BRCA1 promoter.

It has been reported that CpG methylation patterns are replicated with DNA during S phase (29). One can envision that altered transcriptional regulation via aberrant promoter methylation plays a significant role in the carcinogenic process, and it will be interesting to determine whether BRCA1 promoter methylation is an early event in ovarian carcinogenesis. Our data support the maintenance of BRCA1 promoter methylation in recurrent cancer. Six of the 98 tumors examined had matched recurrent tumor tissue available for analysis. Two of the primary tumors were methylated, and four were not. Both the methylated and unmethylated phenotypes were maintained in 6 of

Fig. 3. BRCA1 immunohistochemistry. Positive BRCA1 staining is shown in normal ovarian epithelium and stroma (B, ×200). Positive BRCA1 staining in an ovarian adenocarcinoma with an unmethylated BRCA1 promoter (D, ×400) is shown. Lack of BRCA1 staining in an ovarian adenocarcinoma with a hypermethylated BRCA1 promoter (F, ×400) is also shown. H&E counter staining masks BRCA1 positivity. Therefore, BRCA1-stained sections were not counterstained, and separate H&E-stained serial sections are shown (A, C, and E).
6 tumors after tumor recurrence after interim chemotherapy. The consistent reproduction of BRCA1 methylation in recurrent OCs suggests it may be an important factor in causing and maintaining ovarian neoplastic transformation in these tumors.

To examine the functional significance of BRCA1 methylation, we correlated our results with BRCA1 protein expression by immunohistochemical analysis of paraffin-embedded tumor tissue sections. Positive BRCA1 staining was observed as a punctate nuclear pattern, as reported previously (11). BRCA1 protein staining was absent or observed as extranuclear weak staining in all methylated specimen (Fig. 3 and Table 1). As positive controls, nuclear staining was seen in the normal stroma of all tumors. Positive BRCA1 immunostaining was observed in 78% (7 of 9) or not detected in 22% (2 of 9) of tumors without hypermethylation of the BRCA1 promoter and in normal ovarian epithelium and stroma (Fig. 3 and Table 1). The absence of detectable BRCA1 staining in the 12 methylated tumors suggests that both BRCA1 alleles were inactive or that BRCA1 protein expression was inhibited or down-regulated by posttranscriptional inactivation.

Biallelic inactivation can occur by several mechanisms: biallelic methylation, or methylation in combination with mutation or allelic loss. The 12 tumors with BRCA1 promoter methylation were examined for LOH at the BRCA1 locus on chromosome 17q21. Of the 12 tumors demonstrating hypermethylation of the BRCA1 promoter region, matching normal DNA was available for 10 cases. All tumors were informative for at least one of the four microsatellite markers used for LOH analysis. Our data indicate that 50% (5 of 10) of the methylated tumors have LOH at the BRCA1 locus, a level consistent with the 40–70% LOH at 17q21 reported previously in OCs (Fig. 4; Refs. 6–9). Therefore, in 5 of the 12 hypermethylated tumors, the second hit knocking out BRCA1 is likely chromosomal loss at 17q21. This is in contrast to a recent report by Estreller et al. (30), showing a tight association between chromosome 17q21 LOH and BRCA1 promoter methylation. In their cohort of 31 OCs, 4 tumors (13%) were hypermethylated, and all 4 hypermethylated tumors showed 17q21 LOH. This difference can either be attributed to the smaller number of methylated tumors analyzed or to the particular LOH markers used for the analysis. Both studies, however, had an intragenic BRCA1-linked marker (D17s855) plus one other marker (THRA1) in common.

The 12 tumors with a hypermethylated BRCA1 promoter were also analyzed for the two BRCA1 founder mutations common in the Ashkenazi Jewish population, BRCA1 185delAG (exon 2) and BRCA1 5382 insC (exon 20), and found to be free of mutation (Table 1). These samples were also analyzed and found to be absent for the BRCA2 6174delT (exon 11) Jewish founder mutation. The lack of a Jewish founder mutation does not, however, rule out the possibility that other BRCA1 mutations are present. The absence of BRCA1 protein staining in 2 of 9 unmethylated OCs suggests that mechanisms other than promoter hypermethylation may also inhibit BRCA1 protein expression.

A relationship between the BRCA and p53 genes has long been suspected, based upon the higher incidence of p53 mutations in tumors with BRCA mutations than in sporadic carcinomas (31–33). In view of the critical role of p53 in cell cycle regulation, it has been postulated that BRCA1 mutant cells with wild-type p53 are less susceptible to carcinogenesis because they retain p53-mediated cell cycle arrest, whereas cells with both mutant BRCA1 and mutant p53 have lost critical cell cycle regulatory checkpoints and are more likely to proliferate (34, 35). The frequency of p53 somatic mutation in OCs associated with germ-line mutation of BRCA1 or BRCA2 is much higher (80%) than in OCs not associated with BRCA1 mutation (36). To determine whether the same association exists in BRCA1 hypermethylated OCs, we examined the p53 mutation status by immunohistochemistry in the 12 hypermethylated tumors. p53 overexpression was observed in 50% (6 of 12) of OCs with a methylated BRCA1 promoter (Table 1). This frequency of p53 mutation is consistent with that reported previously by us and other investigators in OCs and suggests that p53 mutation is not directly associated with the epigenetic inactivation of BRCA1 (33).

The clinicopathological characteristics of the BRCA1 methylated samples were compared with the unmethylated BRCA1 OC cohort. Patient’s age, tumor stage, grade, and histological types were similar in both groups (Table 2). Because BRCA1 mutation status was not available for the whole study group, these two subcohorts were compared for a family history suggestive of a breast/ovarian cancer syndrome. A family history for a breast/ovarian cancer syndrome was

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**Table 1 Molecular characteristics of OCs with hypermethylated and unmethylated BRCA1 promoters**

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>BRCA1 methylation</th>
<th>BRCA1 protein staining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BRCA1/2 mutation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p53 staining</th>
</tr>
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<tbody>
<tr>
<td>T853</td>
<td>+</td>
<td>Not detected</td>
<td>None</td>
<td>++</td>
</tr>
<tr>
<td>T900</td>
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<td>Not detected</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>T924</td>
<td>+</td>
<td>Not detected</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>T925</td>
<td>+</td>
<td>Not detected</td>
<td>None</td>
<td>++</td>
</tr>
<tr>
<td>T953</td>
<td>+</td>
<td>Not detected</td>
<td>None</td>
<td>++</td>
</tr>
<tr>
<td>T955</td>
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<td>Not detected</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>T975</td>
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<td>None</td>
<td>++</td>
</tr>
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<td>Not detected</td>
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<td>++</td>
</tr>
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<td>Not detected</td>
<td>None</td>
<td>++</td>
</tr>
<tr>
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<td>Not detected</td>
<td>None</td>
<td>++</td>
</tr>
<tr>
<td>T1026</td>
<td>+</td>
<td>Not detected</td>
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<td>+</td>
</tr>
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<td>+</td>
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<td>Not detected</td>
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<td>–</td>
</tr>
<tr>
<td>T921</td>
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<td>Faint</td>
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<td>–</td>
</tr>
<tr>
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<td>Not detected</td>
<td>NA</td>
<td>–</td>
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<td>T856</td>
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<td>None</td>
<td>++</td>
</tr>
<tr>
<td>T958</td>
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<td>++</td>
</tr>
<tr>
<td>T859</td>
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<td>Not detected</td>
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</tr>
<tr>
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<td>++</td>
</tr>
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<td>T974</td>
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<td>++</td>
</tr>
<tr>
<td>T980</td>
<td>+</td>
<td>Not detected</td>
<td>NA</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> Tumors were analyzed for the three founder mutations common in Ashkenazi Jews.  
<sup>b</sup> IHC staining intensity was scored as: –, negative; +, faint; ++, strong; and ++++, intense.
defined as having at least one first-degree relative with OC or as having two or more first- or second-degree relatives with breast cancer and/or OC. Zero of 12 patients in the BRCA1 methylated group had a family history suggestive of a breast/ovarian cancer syndrome, whereas 17 of 86 (20%) of the unmethylated group qualified as having familial disease. This difference did not reach statistical significance (P = 0.12), although it suggests a trend. Hereditary OC is thought to represent ~10% of OCs. Twenty % (17 of 98) of our population were classified as “familial,” possibly because of the large number of Ashkenazi Jewish women in this cohort (29 of 98). The absence of Jewish founder mutations in the methylated cohort may be attributable to the presence of only one Jewish woman without a family history suggestive of a breast/ovarian cancer syndrome. When analyzed as a portion of sporadic OCs, the 12 methylated tumors represent 15% (12 of 81) of the sporadic population and leaves the role of BRCA1 methylation in familial OC tumors unclear.

We have shown that the BRCA1 gene promoter region is hypermethylated in a significant percent of sporadic OCs. These data support the hypothesis that BRCA1 promoter methylation plays a role in the functional inactivation of BRCA1. As additional follow-up clinical data are accumulated, the impact of BRCA1 methylation on OC survival will be revealed.

References

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