Inactivation of BRCA1 and BRCA2 in Ovarian Cancer

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Background: Although BRCA1 and BRCA2 play important roles in hereditary ovarian cancers, the extent of their role in sporadic ovarian cancers and their mechanisms of inactivation are not yet well understood. Our goal was to characterize BRCA2 mutations and mRNA expression in a group of ovarian tumors previously evaluated for BRCA1 mutations and mRNA expression. Methods: The tumors of 92 unrelated women with “ovarian” cancer (i.e., ovarian, peritoneal, or fallopian tube cancer) were screened for BRCA2 null mutations using a protein truncation test. Methylation-specific polymerase chain reaction (PCR) was used to examine the BRCA2 promoter for hypermethylation in tumors that did not express BRCA2 mRNA. All statistical tests were two-sided. Results: Nine tumors had a germline (n = 5) or somatic (n = 4) BRCA2 mutation; each was associated with loss of heterozygosity. All of the somatic (1445delC, E880X, 4286del8, and 5783delT) and one of the germline (5984ins4) mutations were unique to this study. One tumor had somatic mutations in both BRCA1 and BRCA2. Two tumors are, to our knowledge, the first cases of germline BRCA2-associated peritoneal cancer. Twelve additional tumors lacked detectable BRCA2 mRNA, but the BRCA2 promoter was hypermethylated in only one of them, suggesting that other mechanisms effect transcriptional silencing of BRCA2. Tumors lacking BRCA1 mRNA were more likely to lack BRCA2 mRNA than tumors expressing BRCA1 mRNA (P<.001). Overall, 82% (95% confidence interval [CI] = 74% to 90%) of the tumors contained alterations in BRCA1, BRCA2, or both genes. Of 41 informative tumors with some alteration in BRCA2, 36 also had an alteration in BRCA1. The frequency, but not the mechanism, of BRCA1 or BRCA2 dysfunction in ovarian cancer was independent of family history. Conclusions: Multiple mechanisms cause nearly universal dysfunction of BRCA1 and/or BRCA2 in hereditary and sporadic ovarian carcinoma. Ovarian cancers with BRCA2 dysfunction often have simultaneous BRCA1 dysfunction. [J Natl Cancer Inst 2002;94:1396–1406]

The BRCA1 and BRCA2 genes play central roles in hereditary breast and ovarian cancer (1–3). However, they have generally been considered to have more limited roles in sporadic ovarian and breast cancer (4–10). Our group has challenged this concept for BRCA1 by reporting at least a 23% prevalence of BRCA1 dysfunction caused by multiple mechanisms in a consecutive series of ovarian cancers (11). Our ability to detect BRCA1 dysfunction at this level was due to our novel use of a protein truncation assay to screen cryopreserved ovarian cancers directly. Studies of BRCA2 mutations in sizable ovarian cancer cohorts (7–10,12) have been done less frequently than similar studies of BRCA1 mutations, at least in part because the BRCA2 gene is nearly twice as large as the BRCA1 gene. Moreover, only five studies to date have analyzed BRCA1 and BRCA2 mutations in the same ovarian cancer patients, and no population-based studies of both BRCA1 and BRCA2 dysfunction have been carried out using the same ovarian tumor specimens (10,12–16).

Knowledge of BRCA1 and BRCA2 gene status in the same cancer specimens may provide clinically useful information, because several lines of evidence suggest that the functions of the corresponding proteins may be interrelated. First, both proteins are expressed mainly in differentiating cells, with their mRNAs peaking simultaneously in the cell cycle between G1 and S. Thus, BRCA1 and BRCA2 may play roles in regulation of the cell cycle during proliferation and differentiation (17–21). Second, mouse embryos deficient in either BRCA1 or BRCA2 fail to develop beyond day 8.5 of embryogenesis in the absence of simultaneous p53 or p21 deficiency (22–29). Third, both the BRCA1 and BRCA2 gene products participate in DNA repair via the RAD51 complex. RAD51, a homologue of the bacterial RecA protein that is involved in double-stranded DNA repair and recombination, colocalizes with the BRCA1 and BRCA2 proteins in the cell nucleus (25,30–36). Nuclear BRCA1 and RAD51 are part of the RNA polymerase II complex (37). The nuclear expression pattern of RAD51 matches that of BRCA1 and BRCA2 (21), and knockout mice lacking the function of any of these three genes have a similar phenotype (38). Finally, DNA-damaging agents affect the expression of both BRCA1 and BRCA2, and the levels of expression of both genes are often associated with susceptibility to DNA damage (28,29,39).

These observations have encouraged us to investigate whether the protein truncation testing strategy that we previously used to study BRCA1 (11,40) might be useful for determining mechanisms and frequency of BRCA2 inactivation in the same cohort of ovarian cancers (40). We have chosen to further develop this approach because 1) the size of BRCA2, at 10 254 nucleotides, makes large-scale sequencing impractical; 2) our BRCA1 analysis (40) suggested that protein truncation testing is far more sensitive and efficient than the single-strand conformational polymorphism (SSCP) test, which has been a cornerstone of BRCA2 mutation screening by other groups (9,10,13,15); 3) nearly three fourths of the 700 different BRCA2 mutations recorded in the Breast Cancer Information Core database (http://www.ncbi.nlm.nih.gov/Intramural_research/Lab_transfer/Bic) result in the truncated (and therefore nonfunctional) proteins that are readily detected by this methodology; and 4) our novel strategy of using both cDNA and DNA templates for protein truncation analysis will facilitate simultaneous identification of candidate ovarian cancers with BRCA2 gene silencing.
Materials and Methods

Tumor Samples

All samples were procured at University of Iowa Hospitals and Clinics between 1990 and 1999 from women undergoing primary surgery for ovarian cancer. The samples were collected in accordance with institutional policies dictated by the University of Iowa Committee for the Protection of Human Subjects, and written informed consent was obtained from all subjects. We used tumor samples from 92 patients whose tumors had previously been analyzed for BRCA1 mutations by protein truncation testing (40). (The 94 patients analyzed in that previous work contained two sets of sisters, and to be able to report frequencies in unrelated individuals in the present analysis, we removed one sister from each of these sets. All data for BRCA1 have been similarly modified for this paper.)

Our study included fallopian tube and primary peritoneal carcinomas as well as ovarian cancers. The frequency distribution of these three cancers in our study cohort—4.3%, 8.7%, and 87%, respectively—closely approximates the frequency distribution among the 788 ovarian cancers in our divisional tumor registry—3.4%, 8.8%, and 87.8%, respectively (Buller R: unpublished results). There are several justifications for including fallopian tube and primary peritoneal carcinomas with ovarian cancers. First, for any given histologic sample from a metastatic site, a gynecologic pathologist cannot differentiate between these three cancers at the level of the light microscope. Second, women with any of these three cancers are often allowed to enter the same “ovarian” cancer clinical trials. Some molecular differences clearly exist among the three cancers; e.g., primary peritoneal carcinomas have recently been characterized as polyclonal (41), in contrast to ovarian cancers, which are usually considered monoclonal (42). However, there are also likely to be molecular differences among different histologic types of ovarian cancer (43,44). It is also apparent from studies of BRCA1-related cancers that primary peritoneal, fallopian tube, and ovarian carcinomas can all be found in hereditary disease cohorts (14). Finally, these three epithelial cancers all appear to derive from the same coelomic epithelial precursor cells (45).

Protein Truncation Test

The open reading frame of BRCA2 (GenBank U43746 and Z74739) was divided into nine overlapping fragments for analysis, as shown in Fig. 1. The overlap increases the sensitivity of detecting truncating mutations that result in a nearly full-length protein that otherwise might be difficult to distinguish from a complete product. The protein sequence translated from the 5’ end of the overlapping adjacent polymerase chain reaction (PCR) fragment will truncate very early and may give no visible product at all in this protein truncation test, even though it yields a full-length PCR product. If an overlap strategy were not used, two apparently normal, full-length protein products would be observed. Genomic DNA was used to generate fragments encompassing exon 11. Genomic DNA could not be used for exons 2–10 or 12–27 because of the size of the intervening introns. In these regions, truncating mutations were detected by using PCR fragments amplified from appropriately designed in-frame primers for PCR amplification of cDNA. The protein truncation test cDNA primer sets also overlap with exon 11 to avoid failing to detect unusual splice variants, such as the complete absence of exon 11 from some BRCA1 mRNAs (46,47).

RNA was isolated and used for cDNA synthesis via reverse transcription PCR (RT–PCR), as previously described, starting from frozen tumor tissue stored at −140°C (48). cDNA quality and quantity were routinely estimated by amplifying a portion of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) housekeeping gene sequence with primers that straddled an intron. Failure to amplify a BRCA2 PCR product in several cDNA template reactions identified candidate tumors in which mRNA was not expressed because of epigenetic phenomena, such as promoter silencing. We confirmed that the absence of expression, when it was observed, was real and was not the result of poor-quality cDNA by amplifying portions of BRCA1 (fragment α), p53 (fragment 2), and the androgen receptor (exon 1 with CAG repeat included) (11,48–51).

Primers containing both a eukaryotic translation initiation site and a T7 promoter were used to generate PCR products for analysis by the protein truncation test. PCR primers and annealing parameters were as follows: initial denaturation at 94°C for 4 minutes, followed by 35 cycles (94°C for 40 seconds, annealing temperature for 40 seconds, 72°C for 3 minutes), completed with a terminal elongation step at 72°C for 5 minutes. The BRCA2 PCR products were translated in the presence of 35S-labeled methionine in the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI), essentially as described in the manufacturer’s protocol. After addition of the recommended sodium dodecyl sulfate (SDS) buffer, the samples were heated to 85°C for 2 minutes. An aliquot of the sample was...
then subjected to electrophoresis on an SDS–polyacrylamide (12%) gel until the dye front completely crossed the gel (60 W for 15 minutes). Gels were then fixed, dried, and exposed to Scientific Imaging Film (Kodak, Rochester, NY) overnight. Occasionally, nonspecific band patterns would appear on the protein truncation test gels. These patterns were determined to represent nonspecific PCR bands rather than alternatively spliced products, because they did not interfere with DNA- or cDNA-based sequencing of the appropriate fragments.

**Sequencing**

Direct PCR-based BRCA2 sequencing was performed on any sample in which the shifted band pattern was observed in the protein truncation test (11,49). The appropriate gene region to amplify was selected on the basis of the size of the truncated product (40). Primers for the appropriate regions were 5’ tagged with an M13 sequence (forward: 5’-CAGACGTGTAAAACGACGCTACTATAGGAACAGACCACCATGG; reverse: 5’-GGATAACAATTTCACACAGG). Individual DNA products were purified by using the Wizard PCR DNA Purification System™ (Promega). Cycle-based sequencing PCR products were completed by using the Sequitherm Excel DNA Sequencing Kit-LC (Epicentre Technologies, Madison, WI) with the inclusion of dye-labeled M13 forward (700 nm) and M13 reverse channel (800 nm) primers complementary to the M13 sequence. Following the addition of LI-COR IR 2 STOP Solution (LI-COR, Lincoln, NE), the PCR sequencing mixtures were heated to 95 °C for 3 minutes, and 1–1.5 μL of each sample was loaded on a 41-cm 6.5% KB-Plus Gel Matrix (LI-COR) polyacrylamide gel. The samples were subjected to electrophoresis at 50 °C, 31.5 W, and 35 mA for approximately 6 hours on the LI-COR IR2 DNA 4200 Sequencer (LI-COR). Electrophoresis patterns were evaluated using LI-COR Base ImagIR 4.2 data collection software and image analysis software (LI-COR).

All mutations were confirmed with bidirectional sequencing of products from a second independent PCR, as previously described (40). Finally, the germline status of candidate tumor mutations was determined by sequencing the same DNA region from a matched peripheral blood DNA sample.

**Loss of Heterozygosity Analysis**

Polymorphic loci (D13S1701, D13S1700, and BR2D13S) that closely flank BRCA2 were amplified by PCR with radiolabeled primers by using paired peripheral blood and tumor DNA samples, as described previously (50). Products were separated on 8% polyacrylamide sequencing gels. Gels were fixed, dried, and exposed to Scientific Imaging Film. The markers were informative when only two alleles could be visualized in the PCR product from peripheral blood. Loss of heterozygosity (LOH) was recorded if, on visual inspection, the two bands in the tumor PCR product differed in intensity by at least twofold (11). When the polymorphic markers were not informative, mutations identified with the protein truncation test or via direct sequencing provided additional LOH information.

**Methylation-Specific PCR**

When cDNAs from a tumor did not generate templates for protein truncation testing, we performed methylation-specific PCR (MS-PCR) on NaHSO3-converted DNA. NaHSO3 conversion of unmethylated cytosine to uracil was done as previously described (52–54). In brief, 54 μL (0.5–5 μg) of converted DNA was incubated with 6 μL of 3.0 N NaOH at 37 °C for 25 minutes. The alkalinized mixture was then treated with 431 μL of 3.6 M NaHSO3/1 mM hydroquinone overlayed with mineral oil at 55 °C for 14 hours. The bisulfite reaction was recovered and desalted with Promega Wizard Prep (Promega), as described by the manufacturer, except for the final elution, in which double-

### Table 1. Primers used in BRCA2 protein truncation test and methylation-specific polymerase chain reaction (PCR)

<table>
<thead>
<tr>
<th>Fragment (template)</th>
<th>Name</th>
<th>Base pairs</th>
<th>PCR annealing temperature, °C</th>
<th>Primer location*</th>
<th>Primer direction</th>
<th>Primer sequence (5’–3’) without T7 modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exons 2–10 (cDNA)</td>
<td>A</td>
<td>1613</td>
<td>64</td>
<td>238</td>
<td>F</td>
<td>GGTATCCAAAAGAGAGGCCAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1831</td>
<td>R</td>
<td>TCCAGTCCACTTTCAGAGGC</td>
</tr>
<tr>
<td>Exons 10–11 (cDNA)</td>
<td>B</td>
<td>1073</td>
<td>64</td>
<td>1381</td>
<td>F</td>
<td>AAGGAAAGTTGACCGCTTTTTT</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>2434</td>
<td>R</td>
<td>GTGACTGGTGACGATGCTG</td>
</tr>
<tr>
<td>Exon 11 internal</td>
<td>C</td>
<td>1486</td>
<td>55</td>
<td>3798</td>
<td>F</td>
<td>AAAGAAACTGTGACAGCTTC</td>
</tr>
<tr>
<td>Fragment 1 (genomic DNA)</td>
<td>D</td>
<td>1524</td>
<td>55</td>
<td>3397</td>
<td>F</td>
<td>CACAGGCGCAAGAAACTTC</td>
</tr>
<tr>
<td>Exxon 11 internal</td>
<td>E</td>
<td>1423</td>
<td>53</td>
<td>4901</td>
<td>F</td>
<td>GCCCTATGTTGAGCTAACC</td>
</tr>
<tr>
<td>Fragment 3 (genomic DNA)</td>
<td>F</td>
<td>1353</td>
<td>57</td>
<td>5659</td>
<td>F</td>
<td>GAGGAACCTGTAGACTGCTC</td>
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<tr>
<td>Fragment 4 (genomic DNA)</td>
<td>G</td>
<td>1461</td>
<td>62</td>
<td>6992</td>
<td>R</td>
<td>TCCACTTGGGAGCATGAG</td>
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<tr>
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<td>1649</td>
<td>62</td>
<td>5910</td>
<td>R</td>
<td>TTATCATCAGGCTATGCT</td>
</tr>
<tr>
<td>Exons 22–27 (cDNA)</td>
<td>I</td>
<td>1641</td>
<td>62</td>
<td>8992</td>
<td>F</td>
<td>ACCCTTGACAAGATGGTC</td>
</tr>
<tr>
<td>BRCA2 promoter</td>
<td></td>
<td></td>
<td></td>
<td>10613</td>
<td>R</td>
<td>CCGTACACACACACGGCTAGG</td>
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<tr>
<td>Methylation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmethylated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>

*Primer location for protein truncation test primers is based on GenBank cDNA sequence accession No. U43746; primer location for methylation-specific PCR primers is based on GenBank genomic sequence accession Z74739. All forward primers for protein truncation test are 5’ tagged with T7 promoter and initiation codon: GCTAATACGACTATAGGG.
distilled H₂O was incubated on the column at room temperature for 5 minutes. The eluate (50–54 μL) was then incubated with 6 μL of 3.0 N NaOH at 37 °C for 15–30 minutes before the addition of 26 μL of 10.0 M ammonium acetate and 300 μL of 95% ethanol. After a 20-minute incubation at –20 °C, the mixture was centrifuged at 18,620 g (4 °C) for 30 minutes. The pelleted DNA was then lyophilized and resuspended in 100 μL of double-distilled H₂O.

MS-PCR was performed on the converted DNA using the primers listed in Table 1. These BRCA2 primers cover a portion of the promoter CpG island (55). The primer that matches methylated DNA is situated 135 bp upstream of the transcription start site, and the primer that matches unmethylated DNA is situated 211 bp upstream of the transcription start site. The methylated product was 250 bp, and the unmethylated product was 337 bp. CpGenome–Universal Methylated DNA (Intergen Co., Gaithersburg, MD) was used as methylated control DNA after NaHSO₃ conversion. DNA samples from non-neoplastic ovarian epithelium and human placental tissue after NaHSO₃ conversion were used as unmethylated controls. An additional control sample consisted of all reagents except the DNA template. Thermocycling parameters were as follows: initial denaturation at 94 °C for 5 minutes, 35 cycles (94 °C for 30 seconds, annealing temperature [62 °C for methylated specific primers; 56 °C for primers specific for the unmethylated sequence] for 30 seconds, 72 °C for 30 seconds); and a terminal elongation at 72 °C for 4 minutes.

Statistical Analyses

Statistical analyses, including chi-square and Fisher’s exact tests, were performed using SPSS for Windows, version 11.0 (Statistical Package for Social Sciences, Chicago, IL). All statistical tests were two sided, and P values less than .05 were considered statistically significant.

RESULTS

Demographic and pathologic characteristics of the 92 women whose tumors were used in this study are summarized in Table 2. At the time of registration, only 1% of the individuals in this study, and indeed of all women diagnosed with ovarian cancer at the Holden Comprehensive Cancer Center of the University of Iowa, stated a Jewish religious preference, indicating a low expected prevalence of the Ashkenazi Jewish founder mutations of BRCA1 and BRCA2. To our knowledge, none of the individuals in this study were related. A total of 34 women (37%) reported a positive family history of breast or ovarian cancer, which we defined arbitrarily as one additional family member with ovarian cancer or two additional family members with breast cancer in a three-generation maternal/paternal pedigree (40). Mean age at diagnosis for this cohort was 59.2 years (range = 30.8–84.6 years), which matches the SEER registry age incidence data and the ovarian cancer age distribution seen at the Holden Comprehensive Cancer Center of the University of Iowa. As Table 2 shows, the majority of tumors in our study were serous, high-grade, and of advanced International Federation of Gynecology and Obstetrics (FIGO) stage, as is typical for ovarian cancer. However, all histologic types of epithelial ovarian cancers were represented. Seven of the 92 individuals (7.6%) had synchronous or metachronous cancers.

Protein truncation testing showed that nine of the 92 tumors (9.8%) contained a truncating mutation in BRCA2 (Table 3).

The mutations, which were all different, included five germline and four somatic mutations. Fig. 2 shows a representative protein truncation test result from two individuals whose tumors contained somatic BRCA2 mutations. With just one exception, both somatic and germline BRCA2 mutations were associated with BRCA2 LOH. The lone exception, tumor 89, had functional LOH secondary to promoter methylation (see below) of the wild-type BRCA2 allele.

Only one of the women with a BRCA2 mutation in her tumor was under the age of 50 years at the time of diagnosis. This woman, from family 357, carried a germline BRCA2 mutation and was 48 when diagnosed with stage IIIC serous ovarian cancer. None of the tumors from individuals with metachronous or synchronous breast cancer was found to contain a germline BRCA2 mutation; one tumor from an individual with metachronous breast cancer contained a somatic mutation. Of the nine BRCA2 mutations we identified, only four (all germline mutations) were previously recorded in the Breast Cancer Information Core database; the other five mutations are new.

The frequency with which BRCA2 mutations were identified in fallopian tube cancer (one of four, or 25%) and primary peritoneal cancer (two of eight, or 25%) was not statistically significantly different from the frequency with which they were identified in “true” ovarian cancer (six of 80, or 7.5%, χ² = 3.62, P = .16). However, the small number of fallopian tube and primary peritoneal cancers limits the ability to draw meaningful conclusions about differences in frequencies. The primary peri-
Table 3. BRCA2 mutations identified by protein truncation test in 92 cancers

<table>
<thead>
<tr>
<th>Patient</th>
<th>Exon</th>
<th>Mutation*</th>
<th>Age at diagnosis, y</th>
<th>Site</th>
<th>Histology</th>
<th>FIGO stage†</th>
<th>Grade</th>
<th>No. of relatives with breast or ovarian cancer</th>
<th>LOH‡</th>
<th>Germline</th>
</tr>
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<tbody>
<tr>
<td>2-E</td>
<td>11</td>
<td>5579insA</td>
<td>59</td>
<td>Ovary</td>
<td>Serous</td>
<td>IIIC</td>
<td>2</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>81</td>
<td>3</td>
<td>295–7 T→A</td>
<td>51</td>
<td>Peritoneum</td>
<td>Serous</td>
<td>IIIC</td>
<td>1</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>354</td>
<td>27</td>
<td>K3326X</td>
<td>69</td>
<td>Peritoneum</td>
<td>Serous</td>
<td>IIIC</td>
<td>2</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>357</td>
<td>11</td>
<td>6630del5</td>
<td>48</td>
<td>Ovary</td>
<td>Serous</td>
<td>IIIC</td>
<td>2</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>84</td>
<td>11</td>
<td>5783delT</td>
<td>65</td>
<td>Ovary</td>
<td>Serous</td>
<td>IIIC</td>
<td>2</td>
<td>1</td>
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<tr>
<td>87§</td>
<td>10</td>
<td>1445delC</td>
<td>83</td>
<td>Ovary</td>
<td>Clear cell</td>
<td>IA</td>
<td>3</td>
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<tr>
<td>89∥</td>
<td>11</td>
<td>E880X</td>
<td>52</td>
<td>Ovary</td>
<td>Clear cell</td>
<td>IA</td>
<td>3</td>
<td>3</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>122</td>
<td>11</td>
<td>4286del8</td>
<td>66</td>
<td>Ovary</td>
<td>Serous</td>
<td>IIIC</td>
<td>3</td>
<td>0</td>
<td>Yes</td>
<td>No</td>
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</table>

*Mutations 1445delC, 4286del8, 5984ins4, 5783delT, and E880X were newly identified in this study; mutations 5579insA, 6630del5, and K3326X had previously been reported in breast cancer but not in ovarian, primary peritoneal, or fallopian tube cancer; and mutation 295–7 T→A was previously reported in a breast/ovarian cancer family. LOH = loss of heterozygosity.

†FIGO = International Federation of Gynecology and Obstetrics (56).
‡Among first-, second-, or third-degree relatives.
§Patient had metachronous breast cancer.
∥Tumor had concomitant promoter hypermethylation of both the wild-type BRCA1 and BRCA2 alleles, with BRCA1 and BRCA2 mutations expressed from unmethylated alleles. This situation is functionally equivalent to LOH at both loci.

The relationship of BRCA1 and BRCA2 mRNA expression and the histopathologic characteristics of the tumors deficient in either BRCA1 or BRCA2 expression are detailed in Table 4 and Fig. 3. BRCA2 mRNA was undetectable in 12 (13%) of the 92 ovarian tumors while BRCA1 mRNA was undetectable in eight (8.7%) of the same 92 tumors (11). Lacking BRCA1 mRNA expression were more likely to lack BRCA2 mRNA expression than tumors that expressed BRCA1 mRNA. Five tumors demonstrated the simultaneous absence of both BRCA1 and BRCA2 mRNA. Fig. 3, A, shows the simultaneous absence of BRCA1 and BRCA2 mRNA in tumor 373, from which mRNAs for G3PD, androgen receptor, and p53 were readily amplified. In contrast, all five mRNAs were readily amplified from tumor 89. Only seven of 84 tumors expressing BRCA1 mRNA failed to express BRCA2 mRNA (Fisher’s two-sided exact test, P < .001).

To determine whether the absence of BRCA2 mRNA was associated with promoter methylation, we carried out MS-PCR on all tumors lacking BRCA2 mRNA. A representative gel is shown in Fig. 3, B. Just one of the 12 tumors lacking BRCA2 mRNA demonstrated BRCA2 CpG island hypermethylation. In contrast, all eight of the tumors without detectable BRCA1 mRNA demonstrated BRCA1 promoter CpG island hypermethylation (11). Of note, tumor 89, which contained somatic mutations in both BRCA1 and BRCA2, also demonstrated promoter hypermethylation of the wild-type allele for each gene, without LOH at either locus; consequently, because of functional LOH, only the mutant BRCA1 and BRCA2 sequences were transcribed.

Table 5 summarizes the BRCA2 alterations that we characterized for this study in relation to the BRCA1 alterations that we previously characterized in the same 92 cancers (11). The ratio of germline defects in BRCA1 (14%) to germline defects in BRCA2 (5.4%) was approximately 3:1. Somatic defects in both genes were common as well, at 9.8% and 4.3% for BRCA1 and BRCA2, respectively. A total of 67 of the 92 tumors (73%) demonstrated some sort of BRCA1 alteration (germline mutation, somatic mutation, lack of mRNA, or LOH in the absence of associated gene mutation or silencing), and 44 (48%) demonstrated some sort of BRCA2 alteration. Thirty-six of the 67 BRCA2-informative tumors with BRCA1 dysfunction (54%)
also had BRCA2 dysfunction, and 36 of the 41 BRCA1- informative tumors with BRCA2 dysfunction (88%) also had BRCA1 dysfunction. Only 15 tumors (16%) appeared completely normal at both loci. Thirty tumors had BRCA1 dysfunction without associated BRCA2 dysfunction, and five tumors had a BRCA2 dysfunction without associated BRCA1 dysfunction. Analysis of two (2.2%) of the 92 tumors was incomplete because of noninformative BRCA status at one or the other locus.

To test the hypothesis that the BRCA1 and BRCA2 genes are simultaneously inactivated in ovarian cancers, we constructed a 2 × 2 contingency table for BRCA1 (dysfunction/no dysfunction) versus BRCA2 (dysfunction/no dysfunction). For this comparison, dysfunction meant any mutation, gene silencing, or LOH. Fisher’s two-sided exact test yielded a P value of .02 in support of the hypothesis. We then compared the frequency distributions for type of BRCA dysfunction between cancers stratified on the basis of a positive family history (Table 5). Although the mechanism of dysfunction varied with family history, there was an overall 82% (95% confidence interval [CI] = 74% to 90%) prevalence of BRCA1 or BRCA2 dysfunction independent of family history (80% prevalence among those with a positive family history and 83% among those with a negative family history).

**DISCUSSION**

In this study, we used a protein truncation test to screen the entire BRCA2 open reading frame of tumors from 92 unrelated women with ovarian cancer whose BRCA1 mutation status was already known. We identified five truncating germline BRCA2 mutations and four truncating somatic BRCA2 mutations. All of the somatic mutations, but only two of the germline mutations, were found in true ovarian cancers; the other three germline mutations were found in fallopian tube (one mutation) and primary peritoneal (two mutations) cancers. Germline BRCA2 mutation-associated fallopian tube and primary peritoneal cancers have not been previously recorded in the Breast Cancer Information Core database. Indeed, only isolated case reports of germline BRCA2 mutations associated with fallopian tube cancer have been recorded (58–60). To our knowledge, tumors 81 and 354 represent the first cases of primary peritoneal cancer attributed to germline BRCA2 mutations. Surprisingly, two somatic BRCA2 mutations were found in the only two clear-cell carcinomas in this series. Interestingly, two of six tumor BRCA2 mutations previously reported by Takahashi (9) were also in clear cell ovarian carcinomas. Further study of this subgroup of relatively uncommon and aggressive ovarian cancers should be undertaken to determine if the mutation of BRCA2 is a key molecular event in the etiology of this disease.

Only about 60 of the unique BRCA2 mutations recorded in the Breast Cancer Information Core database have been found in ovarian cancers. None of the nine BRCA2 mutations identified in this study (Table 3) is currently listed in the Breast Cancer Information Core database as occurring in ovarian cancer. Five of these mutations (all four somatic and one germline mutation) had not been previously reported in breast cancer either. Five mutations, including two of the five germline mutations, fall outside the cluster region (nucleotides 4235–6504), originally thought to be important for BRCA2-related hereditary ovarian cancer (62). Other authors (63,64) have suggested extending this region so that it includes nucleotides 3035–7069. Nevertheless, two of the germline mutations in our cohort fall outside even this extended region (Table 3). One of these mutations, the K3326X truncation, is the second most common mutation in BRCA2 reported in the Breast Cancer Information Core database. Despite the deletion of the terminal 126 amino acids of the BRCA2 protein, it has been suggested that the K3326X truncation may be a non-disease-related polymorphism (65). Malone et al. (66) found this germline mutation/polymorphism in peripheral blood DNA from two young breast cancer patients of 386 tested (0.5%). They also found it in peripheral blood DNA of three of 71 control subjects under age 45 years (4.2%), but each of these control subjects had at least one first-degree relative with breast cancer. The other noncluster region mutation, 295–7 T→A,
The protein truncation test we used to identify BRCA2 mutations has several advantages over SSCP screening and may even detect mutations overlooked by DNA-based sequencing, as previously reported for BRCA1 (40,68). For example, it detects nonsense mutations often missed by SSCP and can even detect mutations such as the BRCA1 exon 13 duplication, which evades identification by conventional DNA sequencing (40). The protein truncation test allowed for efficient analysis of the BRCA2 gene using only nine PCR amplifications and their coupled translation reactions, as opposed to the 78 reactions necessary for SSCP analysis of this gene (9). An additional benefit of this approach is that the use of a cDNA template for a portion of the reactions provides an opportunity to semiquantitatively measure mRNA levels. The use of a DNA template for exon 11 allowed us to rule out simultaneous loss of both alleles as an etiology for complete BRCA2 mRNA deficiency in some ovarian cancers.

Like all mutation detection techniques, however, the protein truncation test has some limitations—most obviously its inability to detect missense mutations. Missense mutations make up approximately 28% of the unique BRCA2 mutations in the Breast Cancer Information Core database. However, until a functional assay is developed for the BRCA gene products, most missense mutations will remain of undetermined relevance, particularly when cosegregation with disease cannot be determined or could be due to chance alone (e.g., in small families or families with limited cases of disease). Eng et al. (69) have recently summarized the relative sensitivities of SSCP, confirmation-sensitive gel electrophoresis (CSGE), two-dimensional gene scanning (TDGS), and denaturing high-performance liquid chromatography (DHPLC) to detect 58 distinct BRCA1 mutations in a blinded set of 65 DNA samples previously sequenced by Myriad Genetic Laboratories (69). DHPLC was found to detect all of the mutations in this sample and would appear to offer the advantage of minimizing false-negatives for screening. However, although such a comparison is useful, the best comparison would use a consecutive set of truly unknown samples that were not preselected on the basis of sequencing data. Furthermore, even DHPLC, like full gene sequencing, will not detect cryptic splicing or BRCA dysfunction that is due to gross genomic rearrangements. Only protein truncation testing or complete cDNA-based gene sequencing can detect these defects, which are nearly certain to produce gene dysfunction. Both DHPLC and gene sequencing have associated cost and efficiency issues not seen with the protein truncation test. The protein truncation test also offers the unique ability to detect BRCA dysfunction that is due to gene silencing. Finally, because the purpose of our study was to identify multiple mechanisms contributing to BRCA2 gene dysfunction in ovarian cancer, our results should only be strengthened if additional BRCA2 mutations are detected in the same cohort.

The frequency of tumor BRCA1 or BRCA2 mutations (germline, 20%; somatic, 13%) in this cohort is second only to the 40.3% prevalence reported by Mosleh et al. (14) in a selected population of Ashkenazi Jewish women with ovarian cancer. By contrast, a population-based study of mutations detected by SSCP in both candidate tumor suppressor genes in epithelial ovarian cancer patients by Rubin et al. (10) found only a 10% prevalence of germline mutations (somatic mutations were not evaluated). Several explanations for these divergent results are possible. First, as originally constructed, our patient population
Table 5. Tumor alterations of BRCA1 and BRCA2 and relation to family history in 92 patients with ovarian cancer*

<table>
<thead>
<tr>
<th>Alteration</th>
<th>BRCA1, No. (%)</th>
<th>BRCA2, No. (%)</th>
<th>Either BRCA1 or BRCA2, No. (%)</th>
<th>Positive, No. (%) (n = 34)</th>
<th>Negative, No. (%) (n = 57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germline</td>
<td>13 (14)</td>
<td>5 (5.4)</td>
<td>18 (20)</td>
<td>13 (38)</td>
<td>4 (7.0)</td>
</tr>
<tr>
<td>Somatic</td>
<td>9 (9.8)</td>
<td>4 (4.3)</td>
<td>12 (13)§</td>
<td>3 (8.8)</td>
<td>9 (16)</td>
</tr>
<tr>
<td>Absent mRNA</td>
<td>8 (8.7)</td>
<td>12 (13)</td>
<td>13 (14)‡</td>
<td>3 (8.8)</td>
<td>10 (18)</td>
</tr>
<tr>
<td>Isolated LOH¶</td>
<td>37 (40)</td>
<td>23 (25)</td>
<td>32 (35)§</td>
<td>8 (24)</td>
<td>24 (42)</td>
</tr>
<tr>
<td>No alterations</td>
<td>20 (22)</td>
<td>47 (51)</td>
<td>15 (16)</td>
<td>6 (18)</td>
<td>9 (16)</td>
</tr>
<tr>
<td>Unknown**</td>
<td>5 (5.4)</td>
<td>1 (1.1)</td>
<td>2 (2.2)</td>
<td>1 (2.9)</td>
<td>1 (1.8)</td>
</tr>
</tbody>
</table>

*Data on BRCA1 are from (11). LOH = loss of heterozygosity.
†Family history is defined as positive if the patient had one relative with ovarian cancer or two relatives with breast cancer in a three-generation pedigree.
‡One individual who had a germline mutation in BRCA1 was adopted and did not know her family history, so she was excluded from the analysis of family history.
§One tumor (from patient 89) contained somatic mutations in both BRCA1 and BRCA2, without LOH but with associated gene silencing of the wild-type allele of each gene.
¶LOH without associated gene mutation or silencing.
#Five tumors lacked both BRCA1 and BRCA2 mRNA.
‡LOH without associated gene mutation or silencing.
Twelve of 14 tumors with isolated BRCA2 LOH also demonstrated simultaneous BRCA1 LOH. One tumor with BRCA2 LOH was not informative at the BRCA1 locus.
**No abnormalities detected but not informative for LOH at either the BRCA1 or BRCA2 locus.

was skewed based on selection of individuals with a positive family history, a personal history of breast cancer, or early onset ovarian cancer (70). The larger, revised cohort that forms the basis for this study lost much of that bias, because we added only cases with a negative family history and removed several individuals with tumor BRCA1 mutations for whom no fresh tissue was available (40). However, our inclusion criteria were far less stringent than those of other investigators who studied only very high-risk women and reported a lower percentage of mutations (13,16,63). Indeed, Garvin et al. (63) studied a more skewed population selected on the basis of early onset breast or ovarian cancer and reported only a 10% prevalence of germline BRCA1 or BRCA2 mutations. By contrast to the population studied by Garvin et al., our study population contains a 61% prevalence of cancers that are assumed to be sporadic because of a negative family history. Finally, the protein truncation test, although insensitive to the detection of missense mutations, appears much more sensitive than SSCP for detecting null mutations (40). For example, our use of the protein truncation test as a screening strategy may explain why we identified a higher percentage of BRCA2 mutations than Gras et al. (15), who screened genomic DNA for BRCA2 mutations by SSCP for exons 2–9 and 12–27 and used a DNA-based protein truncation test for exons 10 and 11. Indeed, the only mutations Gras et al. found were detected by the protein truncation test (15). As we have done for BRCA1 (11), we are currently screening a larger, unscreened cohort to better understand the true prevalence of BRCA2 mutations in ovarian cancer. Further speculation about the overall frequency of BRCA1 and BRCA2 mutations in ovarian cancer permits an exploratory analysis of the frequency distribution of the various types of BRCA1 and BRCA2 inactivation mechanisms between familial and sporadic cases. As reported in Table 5, women with and without a family history had essentially the same overall frequency of BRCA1 and/or BRCA2 gene dysfunction (80% and 83%, respectively). However, the frequency of the various mechanisms of dysfunction was different in the two groups. For example, germline defects in BRCA1 or BRCA2 were seen in 38% of the women with a positive family history but in just 7% of the women without a family history. Conversely, alternative inactivating mechanisms (somatic mutation, gene silencing, or LOH) were seen in 76% of women without a family history and 50% of women with a family history.

The 82% prevalence of either a BRCA1 or BRCA2 defect associated with the 92 ovarian cancers in our study suggests an interrelationship between the two candidate tumor suppressor genes in ovarian cancer that is more than coincidental. The case for simultaneous inactivation of both BRCA1 and BRCA2 in ovarian cancer is based on the observation that 36 of 41, or 88%, of the informative tumors with any BRCA2 defect also demonstrated a BRCA1 defect (P = .02; Fisher’s exact test, twosided). Some may dispute the notion that LOH is a form of gene dysfunction; however, two studies of the activity of the BRCA1 locus in sporadic breast cancer showed an association between LOH and decreased mRNA and/or protein expression (71,72). A third study (73) did not show such an association. We have not actually measured BRCA1 or BRCA2 protein levels or precisely quantified mRNA levels in the tumors with LOH. However, five of the eight tumors lacking BRCA1 mRNA also lacked detectable BRCA2 mRNA. There have been two case reports of ovarian cancer patients with simultaneous germline mutations in both BRCA1 and BRCA2 (15,74), but our findings provide the first broad evidence that simultaneous dysfunction of both the BRCA1 and BRCA2 tumor suppressor genes may be common in ovarian cancer.

The absence of BRCA2 mRNA, seen in 12 of the 92 tumors in our study (13%), effectively results in BRCA2 null ovarian cancers. Although we (11) and others (54,75–80) have reported that BRCA1 methylation is a common mechanism to explain the absence of BRCA1 mRNA in ovarian cancer, no previous cases of BRCA2 promoter methylation have been observed (15,81). We identified one tumor with apparent biallelic BRCA2 promoter methylation (tumor 133, Table 4) and one tumor with wild-type BRCA1 promoter methylation associated with a so-
mative BRCA2 mutation in the other allele (tumor 89, Table 3). Therefore, additional mechanisms must be responsible for BRCA2 inactivation in the 11 other tumors that lacked BRCA2 mRNA expression. These potential mechanisms include BRCA2 promoter mutation, failure to identify the correct CpG island of the true BRCA2 promoter, and lack of function of a gene whose product is required for BRCA2 transcription. Because seven of the 11 informative tumors without BRCA2 mRNA expression failed to demonstrate LOH, we favor the third possibility, which would affect both BRCA2 loci simultaneously. Loss of such a trans-acting factor in the germline could explain the apparent hereditary ovarian cancers in family 11 [see Table 4; (82)], for which no truncating BRCA1 and BRCA2 mutations have been detected (Buller RE: unpublished data). Alternatively, biallelic methylation of the true promoter site or biallelic promoter mutation would have to be invoked to explain this data.

In summary, protein truncation analysis of BRCA2 represents a powerful exploratory tool for the study of BRCA2 expression in ovarian and related cancers. Using this tool, we have determined that BRCA2 dysfunction in the ovarian tumors in our study was usually accompanied by simultaneous BRCA1 dysfunction. Furthermore, our data indicate that some degree of BRCA1 and/or BRCA2 dysfunction may be of nearly universal importance for the process of ovarian carcinogenesis and that multiple genetic mechanisms are responsible for the dysfunction of these critical candidate tumor suppressor genes in ovarian tumors.

REFERENCES


NOTES

1 Editor’s note: SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

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