Frequency of BRCA1 Dysfunction in Ovarian Cancer

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Background: Ovarian cancer is one of the most common hereditary cancers in women. Mutations in the BRCA1 gene increase a woman’s risk of ovarian cancer. Testing for BRCA1 mutations is cumbersome and impractical for large populations. Therefore, we developed an efficient strategy to detect various types of BRCA1 dysfunction and also determined the relative frequency of BRCA1 dysfunction in ovarian cancer. Methods: Tumors from 221 patients with epithelial ovarian cancer were screened for loss of heterozygosity (LOH) at the BRCA1 locus. BRCA1 complementary DNA (cDNA) and genomic DNA from all cancers with BRCA1 LOH (106 tumors) or noninformative status (15 tumors) were polymerase chain reaction (PCR) amplified and analyzed for protein truncation in a coupled transcription/translation test. When truncated BRCA1 protein was detected, the BRCA1 gene from both the tumor and a paired blood sample was sequenced. When BRCA1 expression in tumor cDNA was not detected with a protein truncation test, a methylation-specific PCR was used to determine whether the promoter region of BRCA1 was methylated and thus inactivated. All statistical tests were two-sided. Results: Fifty-one (23.1%) of 221 tumors had BRCA1 dysfunction, including 18 with germline mutations, 15 with somatic mutations, and 18 with monoallelic or biallelic hypermethylated promoters. The consideration of only tumors with LOH or that were noninformative, the efficiency for detecting BRCA1 dysfunction improved to 45 (37.2%) of 121 tumors. Therefore, LOH/noninformative was a strong predictor of mutation status (Fisher’s exact test, P < .001). However, this subset of tumors did not include those with BRCA1 missense mutations (estimated at six [2.7%] of 221 not detected by our method) or biallelic promoter methylation (estimated at six [2.7%] of 221). Conclusions: BRCA1 dysfunction in ovarian cancer is common and occurs via multiple mechanisms. The use of LOH, rather than a family history of ovarian cancer, as a first step in a screening strategy, followed by protein truncation testing, appears to increase the chance of identifying tumors with BRCA1 dysfunction. [J Natl Cancer Inst 2002;94:61–7]

Ovarian cancer is one of the most common forms of hereditary cancer in adult females (1). It is the leading cause of death from gynecologic cancer in the United States (2,3). Mutations in the BRCA1 gene, located at 17q21, play an important role in the development of hereditary and sporadic ovarian cancers and increase a woman’s risk of ovarian cancer. A growing body of evidence suggests that the BRCA1 protein is involved in the DNA repair process (4). Because abnormal DNA repair mechanisms contribute to carcinogenesis and may modify the tumor’s response to chemotherapy, there is a great deal of interest in identifying cancers carrying mutations in their BRCA1 genes (5).

Testing for BRCA1 mutations in a large cohort is both cumbersome and impractical because of the large size of this gene, which is encoded by nearly 5500 bases located in an approxi-...
cancer was compared with LOH as the first step in determining on whom further testing should be performed. The purpose of this study was to develop an efficient screening strategy to detect various types of BRCA1 dysfunction that would have a high yield without compromising the efficiency of the process and to determine the frequency of BRCA1 dysfunction in ovarian cancer.

**MATERIALS AND METHODS**

This study was carried out in accordance with the standards of our Institutional Committee for the Protection of Human Subjects. All samples were obtained after patients signed a written informed consent. Initially, 14 BRCA1 mutations were detected by SSCP in a cohort of 81 patients with ovarian, peritoneal, or fallopian tube cancer (13). Recently, an additional nine mutations, not detected by SSCP, were found with a novel protein truncation test in an expanded cohort of 94 patients with ovarian cancer (6). Tumors from all of these patients were subjected to an LOH analysis that used three intragenic BRCA1 microsatellite repeat units. For this study, an additional 127 patients were added to bring the total number of ovarian cancers screened with LOH to 221. Patient selection was solely on the basis of the availability of snap-frozen tissue for protein truncation testing.

All 121 tumors with BRCA1 LOH or with a noninformative status were further screened by protein truncation testing. Sixty-six of 100 tumors that were diploid at the BRCA1 loci also underwent protein truncation testing.

Fig. 1 shows a schematic of our proposed screening strategy. LOH analysis is the first step narrowing the number of patients on which the protein truncation test must be performed. The protein truncation test then identifies important candidate truncating mutations and candidate tumors that failed to express BRCA1 protein for methylation-specific PCR (MS-PCR) analysis. This strategy will not detect missense mutations.

**Loss of Heterozygosity**

BRCA1 intragenic polymorphic loci (D17S855, D17S1322, and D17S1323) were amplified by PCR with radiolabeled primers by use of blood and snap-frozen tumor samples. PCR fragments were analyzed on 8% polyacrylamide sequencing gels that were fixed, dried, and exposed to Scientific Imaging Film™ (Kodak, Rochester, NY) for 30 minutes to 3 hours to show distinct bands for analysis. Mutations identified by direct sequencing from protein truncation test candidates provided additional LOH information when the polymorphic markers were not informative. To be informative, two alleles had to be visualized.
in the DNA PCR product from peripheral blood. LOH was recorded if the difference in intensity between two bands in the tumor DNA PCR product was visually greater than or equal to 2:1. The concepts of LOH and noninformative alleles are demonstrated in Fig. 2.

Protein Truncation Test Analysis

The open reading frame of BRCA1 was divided into five overlapping fragments as described previously (6). Both DNA and cDNA templates (generated from random hexamers) were used. DNA templates were used to generate fragments that encompass only exon 11. DNA cannot be used for exons 2–10 or 12–24 inclusive because of the size of the intervening introns. (In these regions, truncating mutations were detected by appropriately designed in-frame primers for PCR amplification of cDNA.) The technique for RNA isolation and cDNA synthesis for reverse transcription–PCR starting from snap-frozen tumor samples stored at −140 °C was described previously (14). Failure to amplify a product in the cDNA reactions also provided candidate tumors where epigenetic phenomenon, including promoter silencing, may occur (i.e., if the products were identified for genomic DNA [exon 11] but no products from cDNA [exons 2–11 and 11–24], despite appropriate amplification of a housekeeping gene sequence, such as glyceral 3-phosphate dehydrogenase [G3PD]).

Primers containing both a eukaryotic translation initiation site and a T7 promoter were used to generate PCR products that were amenable to protein truncation testing (6). The individual PCRs were performed with complete translation of the product in the TNT® Quick Coupled Transcription/Translation System (Promega Corp., Madison, WI) essentially as in the manufacturer’s protocol. After the addition of sodium dodecyl sulfate (SDS) buffer, the samples were heated to 85 °C for 5 minutes. An aliquot of the sample was then subjected to SDS–polyacrylamide gel electrophoresis until the leading dye completely crossed the gel (45 W for 15 minutes). Gels were then fixed, dried, and exposed to Scientific Imaging Film overnight.

Sequencing

Direct PCR-based sequencing was performed on any sample in which a shift in band pattern was observed on the protein truncation test. DNA samples were purified with the Wizard PCR DNA Purification System (Promega Corp.) or the QIAquick™ PCR Purification Kit (Qiagen Inc., Valencia, CA). The PCR sequencing reaction was completed by using the DNA Cyclic Sequencing System (Promega Corp.). Sequencing of the candidate mutations (with the use of Licor IR2; LI-COR, Lincoln, NE) based on protein truncation testing was carried out by selecting an appropriate region to sequence on a 41-cm, 7% Long Ranger™ polyacrylamide gel (FMC Bioproducts, Rockland, ME) (6). Specific M13-tagged primers were chosen on the basis of the size of the truncated protein product. Electrophoresis was performed at 50 °C, 31.5 W, and 35 mA for approximately 6 hours. Gels were evaluated by use of LI-COR ImagIR 4.2 data-collection software and image manipulator software (LI-COR). Mutations were confirmed with bidirectional sequencing of products from a second independent PCR. A germline mutation was determined by the presence of the same mutation in peripheral blood DNA that was identified in tumor DNA. In contrast, a somatic mutation was defined by a normal BRCA1 sequence from blood DNA in the region where a mutation was sequenced from tumor.

Methylation-Specific Polymerase Chain Reaction

MS-PCR was performed on NaHSO_3-converted DNA from tumors that failed to express BRCA1 messenger RNA (mRNA). The NaHSO_3 reaction has been described previously (15–17). From 0.5 to 5 μg of DNA was incubated first with 0.3 M NaOH at 37 °C. The alkalinized mixture was exposed to 3.6 M NaHSO_3 and 1 mM hydroquinone at 55 °C for 14 hours before recovering and desalting the products with Promega® Wizard Prep (Promega Corp.). The desalting was performed as recommended by the manufacturer, except for the last elution in which double-distilled H_2O was incubated on the column at room temperature for 5 minutes before the final centrifugation (18 620 g for 2 minutes at 4 °C). The eluate was then incubated with 0.3 M NaOH at 37 °C again before the addition of 3 M ammonium acetate and 95% ethanol. The mixture was next incubated at −20 °C for 20 minutes and then centrifuged at 18 620 g at 4 °C for 30 minutes. The supernatant was removed, and the DNA was lyophilized and then resuspended in 100 μL of double-distilled H_2O.

MS-PCR was then performed on the converted DNA with the primers and conditions described previously (18). The primers covered a portion of the CpG island in the BRCA1 promoter (GenBank accession number U37574) that flanks the transcription start site. The methylated product was 75 base pairs (bp), and the unmethylated product was 86 bp. CpGenome™ Universal Methylated DNA (Intergen Company, Gaithersburg, MD) was used as the methylated control after NaHSO_3 conversion. DNA from non-neoplastic ovarian epithelium and human placental tissue after NaHSO_3 conversion were used as unmethylated controls. An additional control included all reagents except DNA template.

Pedigree Analysis

Three-generation pedigrees are routinely obtained on patients seen at the Gynecologic Oncology Clinic of the Holden Comprehensive Cancer Center, Iowa City, by individuals with special genetics training. A positive family history of ovarian cancer was arbitrarily designated on the basis of a three-generation maternal/paternal pedigree, with at least one family member with ovarian carcinoma or two family members with breast carcinoma (13).

Statistical Analyses

Statistical analyses, including Fisher’s exact test, were performed by use of SPSS for Windows version 10.0 (Statistical
RESULTS

LOH analyses were carried out on tumors from 221 patients with ovarian (n = 187), peritoneal (n = 22), or fallopian tube (n = 12) cancer. One hundred six (48.0%) tumors demonstrated LOH, 100 (45.2%) retained both BRCA1 alleles, and 15 (6.8%) were noninformative.

All tumors (n = 121) determined to have LOH or that were noninformative at the BRCA1 locus were analyzed by the protein truncation test. Thirty-three tumors (27.3%) had a truncated BRCA1 protein. All 33 abnormalities identified by the protein truncation test were confirmed by direct sequencing to be secondary to BRCA1 null mutations (18 germline and 15 somatic mutations; Table 1). A single tumor originally classified as of unknown germline status may be a somatic mutation as shown by a 68-year-old woman with no female relatives in a three-generation pedigree who developed breast or ovarian cancer. However, because no germline cDNA was available, her true germline status is not known. Among 66 of 100 tumors diploid at the BRCA1 locus studied by the protein truncation test, no null mutations were found. Therefore, LOH/noninformative was shown to be a strong predictor of mutation status (Fisher’s exact test, P < .001).

The site of disease and histology were examined in relation to the presence of BRCA1 LOH and BRCA1 mutation in Table 2. Clear cell carcinomas were the least likely to demonstrate both LOH and BRCA1 mutation. Poorly differentiated ovarian adenocarcinomas were most likely to contain BRCA1 mutations. The frequency of BRCA1 LOH (47.1%, 50.0%, and 58.3%) and BRCA1 mutation (15.5%, 9.1%, and 16.7%) was approximately the same for ovarian, peritoneal, and fallopian tube cancers, respectively.

Next, we compared the utility of BRCA1 LOH data with family history of breast/ovarian cancer as predictors of the likelihood of finding a BRCA1 null mutation. Three-generation pedigrees were obtained for 205 (92.8%) of 221 patients in the entire cohort and 32 (97.0%) of 33 patients with mutations. Direct comparison of LOH and a family history of ovarian cancer yielded differences in sensitivity, specificity, positive predictive value, and negative predictive value that are reported in Table 3. Independent of mutation type (somatic or germline), the best sensitivity (100%) for BRCA1 mutation detection and negative predictive value for the absence of BRCA1 tumor mutation (100%) was obtained from the BRCA1 LOH data by itself. The optimal specificity and positive predictive values were obtained from the combination of BRCA1 LOH and family history, regardless of mutation type, with the predictable exception that BRCA1 LOH alone yielded the highest positive predictive value (14.4%) for somatic BRCA1 tumor mutations. Among patients with available family data, positive family histories were found for 11 (64.7%) of 17 individuals with germline mutations but for only two (13.3%) of 15 individuals whose tumors contained somatic mutations (P < .001). Fifty-three (25.9%) of 205 patients with known family data exhibited positive family histories of breast/ovarian cancer. Thirteen (40.6%) of 32 patients with null mutations and available family data had a positive family history of ovarian cancer. Although this difference was statistically significant (P = .01), it was neither as significant nor as useful as the relationship exhibited between LOH/noninformative status and mutation status. All BRCA1 mutations, whether somatic or germline, were in LOH/noninformative tumors. Thus, a screening strategy based on tumor BRCA1 LOH/noninformative status is clearly a better first step than ascertaining a family history of breast/ovarian cancer.

We were unable to generate BRCA1 cDNA PCR products from 16 of the 187 ovarian cancers tested, including 12 (9.9%) of 121 tumors that were noninformative for BRCA1 or had BRCA1 LOH and four (6.1%) of 66 tumors that were diploid for BRCA1. In each case, G3PD mRNA was detected as a control. Fig. 3 shows a representative agarose gel, where G3PD expression is readily detectable for both tumors 525 and 616, but BRCA1 mRNA was only detected in tumor 525. Consequently, we used MS-PCR to determine whether this expression pattern was the result of BRCA1 hypermethylation. Fig. 4 demonstrates promoter methylation of tumors 316 and 89 but not of tumor 525. The finding of biallelic BRCA1 promoter hypermethylation in four of 66 cancers without BRCA1 LOH indicates that BRCA1 dysfunction is caused by epigenetic factors that may occur independently of LOH.

Table 4 summarizes our findings and estimates the frequency with which each type of BRCA1 alteration can be detected with the proposed strategy of Fig. 1. Overall, BRCA1 gene changes appeared to approach 26%. Approximately two thirds of BRCA1 dysfunction is due to mutation, and one third is due to promoter hypermethylation. Considering only the BRCA1 abnormalities that lead to obvious dysfunction, the extrapolated frequency of BRCA1 dysfunction is 51 (23.1%) of 221 (18 germline mutations, 15 somatic mutations, and 18 tumors with monoallelic or biallelic hypermethylated promoters; missense mutations, not detected with this method, are not included in this total). Focus-
ing only on the cancers with BRCA1 LOH/noninformative as proposed by our screening strategy (Fig. 1), the probability of identifying BRCA1 dysfunction increases from 23.1% in the entire cohort to 37.2% (45 of 121) patients in the targeted subset for screening. Limitations of this strategy include failure to detect BRCA1 missense mutations (estimated at six [2.7%] of 221) and cancers with biallelic promoter methylation (estimated at six [2.7%] of 221).

**Discussion**

BRCA1 dysfunction in ovarian cancer is common and occurs via multiple mechanisms. The identification of germline and somatic mutations of BRCA1 in ovarian cancers is important for several reasons. Because germline mutations of this gene are inherited in an autosomal-dominant manner, the finding of a germline mutation in a proband raises a daughter’s risk of ovarian cancer by up to 25 times (19). The identification of both germline and somatic mutations may allow novel therapeutic interventions, including replacement gene therapy (20,21), which may be an improvement over current chemotherapeutic regimens that affect host cells and cancer cells almost equally.

Application of novel molecular therapeutic strategies requires an efficient screening system to detect and characterize BRCA1. Our group (6) has demonstrated previously that the protein truncation test is a more efficient screening technique. However, even with the protein truncation test, much work and expense are required to identify the cancers containing BRCA1 mutations. Several investigators (1,12) have demonstrated the importance of both a family history of ovarian cancer and BRCA1 LOH to the risk of ovarian cancer. Using a large unselected cohort, we have shown that BRCA1 LOH is a better

![Fig. 3. Agarose gel demonstrating lack of BRCA1 messenger RNA (mRNA) expression by polymerase chain reaction (PCR) amplification from complementary DNA (cDNA) of BRCA1 exons 11–24. Lane 1 shows a 100-base-pair (bp) DNA ladder. cDNA from tumor 525 (stage II, endometrioid ovarian carcinoma with BRCA1 mRNA expression) is used in lanes 2 and 3, and cDNA from tumor 616 (stage III, serous ovarian carcinoma lacking BRCA1 mRNA expression) is used in lanes 4 and 5. Lanes 2 and 4 show cDNA-based PCR amplification of exons 11–24 of BRCA1 (1798 bp); lanes 3 and 5 show cDNA-based PCR amplification of glyceral 3-phosphate dehydrogenase.](https://academic.oup.com/jnci/article-abstract/94/1/61/2519694)

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**Table 2. Disease site, histologic type, BRCA1 loss of heterozygosity (LOH), and rate of BRCA1 mutations**

| Site and histologic type* | No. of tumors | % of tumors with BRCA1 LOH | \( P_{†,‡} \) | % of tumors with BRCA1 null mutations | \( P_{‡,§} \) |
|--------------------------|--------------|---------------------------|-------------|--------------------------------------|-------------|
| Ovary                    | 187          | 47.1                      | .021        | 15.5                                 | .60         |
| Serous                   | 111          | 53.2                      |             | 15.3                                 |             |
| Mucinous                 | 25           | 24.0                      |             | 12.0                                 |             |
| Endometrioid             | 34           | 55.9                      |             | 17.6                                 |             |
| Clear cell               | 9            | 11.1                      |             | 0.0                                  |             |
| Adenocarcinoma (NOS)     | 7            | 42.9                      |             | 42.9                                 |             |
| Transitional cell        | 1            | 0.0                       |             | 0.0                                  |             |
| Peritoneum               | 22           | 50.0                      | .33         | 9.1                                  | .67         |
| Serous                   | 20           | 55.0                      |             | 10.0                                 |             |
| Adenocarcinoma (NOS)     | 2            | 0.0                       |             | 0.0                                  |             |
| Fallopian tube           | 12           | 58.3                      | .36         | 16.7                                 | .73         |
| Serous                   | 9            | 66.7                      |             | 22.0                                 |             |
| Clear cell               | 1            | 0.0                       |             | 0.0                                  |             |
| Adenocarcinoma (NOS)     | 2            | 0.0                       |             | 0.0                                  |             |

* NOS = not otherwise specified.
†Univariate analysis of BRCA1 LOH frequency among histologic types within each histologic site.
‡All statistical tests were two-sided.
§Univariate analysis of BRCA1 null mutation frequency among histologic types within each histologic site.

**Table 3. BRCA1 loss of heterozygosity (LOH) versus family history as a first step in determining BRCA1 null mutations**

| Mutations                  | First-step screen | Sensitivity, % | Specificity, % | Positive predictive value, % | Negative predictive value, % |
|----------------------------|-------------------|----------------|----------------|-------------------------------|-----------------------------|
| All                        | BRCA1 LOH         | 100            | 57.8           | 31.1                          | 100                         |
| Family history             |                   | 40.6           | 76.9           | 24.5                          | 87.5                        |
| BRCA1 LOH and family history |                 | 40.6           | 89.1           | 40.6                          | 89.1                        |
| Germline                   | BRCA1 LOH         | 100            | 53.2           | 17.0                          | 100                         |
| Family history             |                   | 64.7           | 77.7           | 20.8                          | 96.1                        |
| BRCA1 LOH and family history |                 | 64.7           | 88.9           | 34.4                          | 96.6                        |
| Somatic                    | BRCA1 LOH         | 100            | 52.4           | 14.4                          | 100                         |
| Family history             |                   | 13.3           | 73.2           | 3.8                           | 91.4                        |
| BRCA1 LOH and family history |                 | 13.3           | 84.3           | 6.3                           | 92.5                        |
Table 4. Estimate of the extent of BRCA1 dysfunction in ovarian cancer (n = 221)

| Mechanism          | No. of cases | Frequency, % | Detected by screening strategy |
|--------------------|--------------|--------------|--------------------------------|
| Mutation           | 39           | 17.6         |                                |
| Germline null      | 18           | 8.1          | Yes                            |
| Somatic null†      | 15           | 6.8          | Yes                            |
| Missense†          | 6            | 2.7          | No                             |
| Promoter methylation| 18           | 8.1          |                                |
| Haploid or noninformative BRCA1† | 12 | 5.4 | Yes |
| Diploid BRCA1‡     | 6            | 2.7          | No                             |

*Includes a sample that is most likely somatic but no peripheral blood complementary DNA was available for analysis.
†Estimated on the basis that 85% of BRCA1 mutations detected by complete sequencing are null; the remainder are missense mutations. Thus, we expect 330.85 = 39 total mutations for the cohort based on the 33 null mutations actually detected. We have reported previously four missense mutations for a 94-tumor subset, demonstrating the consistency of this logic.
‡We actually detected four biallelic methylated BRCA1 promoters in the 66 cancers tested without BRCA1 loss of heterozygosity. This extrapolates to an expected six cases from the entire subset of 100 ovarian cancers that were diploid at the BRCA1 locus. These candidates for promoter methylation are detectable by the addition of a single polymerase chain reaction for exons 11–24 on all tumors.

The effective screening strategy (Fig. 1) not only eliminates the need for evaluating genomic DNA of all patients but also may identify types of mutations and gene dysfunction that are missed by genomic DNA-based screening. Unusual splicing mutations and promoter-silencing DNA methylation are two examples not detected by SSCP (6).

These important mechanisms of BRCA1 dysfunction demonstrate the limitations of screening a population with no predisposition to a specific disease. Several authors (1,8,9,22) have tried to use Ashkenazi or other ovarian cancer-founder mutations to screen the population in question. Garvin et al. (23) used only a protein truncation test to screen exon 11 in a series of patients with breast/ovarian cancer. In our population, this type of screening does not appear to be adequate because 50% of the mutations detected occurred in the 40% of the open reading frame not encoded by exon 11 and the paucity of Ashkenazi-founder mutations (three of 33) in our heterogeneous population with European roots.

The rate of truncating BRCA1 mutations in the entire cohort of patients with ovarian cancer (33 [14.9%] of 221) is higher than the rate from other nonselected cohorts (3%–8%) (24–27) but is similar to the rate in a consecutive series of genomically sequenced tumors (12.6%) reported by Berchuck et al. (12). The differences stem from the number of somatic mutations reported. Our number is consistent with the BRCA1 somatic mutation frequency reported by Berchuck et al. (12). Thus, the effectiveness of this protein truncation test screening strategy approaches that of genomic sequencing for the detection of both germline and somatic BRCA1 mutations.

Strong protein truncation test bands resulted from the exon 2–11 and exon 11–24 PCR fragments amplified from cDNA and detected on agarose gels before carrying out protein truncation test-coupled translation to protein products (6). However, in 16 (8.6%) of the 187 tumors screened, no BRCA1 transcription product was obtained. In each case, G3PD mRNA and other mRNAs were readily detectable. By extrapolation, we would predict that BRCA1 mRNA would be absent in 18 (8.1%) of the entire 221 tumor cohort, an observation that likely reflects BRCA1 promoter hypermethylation (18,28). To confirm this observation, we carried out MS-PCR to analyze the BRCA1 promoter for CpG island methylation near the transcription start site. Indeed, hypermethylation was found, which may have resulted in the absence of BRCA1 in all of the 16 cancers with absent BRCA1 mRNA. In contrast to the report by Esteller et al. (18), we have found that BRCA1 promoter hypermethylation may occur in BRCA1 diploid tumors. The extrapolated 8.1% (18 of 221) incidence of promoter hypermethylation in our entire cohort is slightly lower than that reported by Baldwin et al. (28) (13.3%) who also used MS-PCR.

All germline BRCA1 null mutations should be detected with protein truncation tests. The additional dysfunction caused by promoter biallelic methylation could be detected with a single cDNA PCR amplifying the fragment from exons 11 to 24. On the basis of this frequency of BRCA1 dysfunction in unselected cancers, we urge that the development of BRCA1-targeted therapeutics continue.
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Notes

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