Mutational Analysis of the BRCA1-Interacting Genes ZNF350/ZBRK1 and BRIP1/BACH1 Among BRCA1 and BRCA2-Negative Probands From Breast-Ovarian Cancer Families and Among Early-Onset Breast Cancer Cases and Reference Individuals

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Two potential breast cancer susceptibility genes, encoding the BRCA1-interacting proteins ZNF350 (or ZBRK1) and BRIP1 (or BACH1), have been identified in yeast two-hybrid screens. We sequenced these genes in probands from 21 families with potentially inherited breast/ovarian cancer, all of which were negative for BRCA1/BRCA2 mutations. Families had at least one case of male breast cancer, two cases of ovarian cancer, or three or more cases of breast and ovarian cancer. In addition, 58 early-onset (before age 35) breast cancer cases and 30 reference individuals were analyzed. Of 17 variants detected in ZBRK1, a missense mutation Val524Ile was identified in the proband of one high-risk family, but no other family members were available for testing. Of 25 variants identified in BRIP1, in addition to four common silent or missense mutations, we identified Gln540Leu, a non-conservative amino acid change, in a single familial proband with inflammatory breast cancer, but this mutation was not present in her three relatives with breast cancer. Haplotype analysis suggests that all ZBRK1 SNPs fall within a single block with two SNPs capturing 92% of the haplotype diversity, while the BRIP1 SNPs fall in two blocks, with five SNPs capturing 89% of the haplotype diversity. Based on sequencing of ZBRK1 and BRIP1 in 21 BRCA1/2-negative probands from inherited breast/ovarian cancer families, it appears unlikely that mutations in these genes account for a significant fraction of inherited breast cancer. Further analysis in unselected cases will be required to know whether the identified variants play a role in genetic predisposition to breast cancer in the general population. Hum Mutat 22:121–128, 2003. Published 2003 Wiley-Liss, Inc.

KEY WORDS: cancer; breast cancer; ovarian cancer; DHPLC; haplotype; linkage disequilibrium; ZNF350; ZBRK1; BRIP1; BACH1; BRCA1; BRCA2; SNP

DATABASES: BRIP1 – OMIM: 605882; GenBank: AF360549, NM_032043.1 (mRNA), NT_010783.13 (Chr 17 genomic contig) ZNF350 – OMIM: 605422; GenBank: AF295096, NM_021632.1 (mRNA), AC011460.3 (genomic) http://lpg.nci.nih.gov/LPG/struewing/pubs (Struewing Lab – additional data available)

INTRODUCTION
Among clearly inherited breast and ovarian cancer families (those with five or more early-onset cases of breast cancer, ovarian cancer, or male breast cancer), approximately two-thirds have germ line mutations in the BRCA1 (MIM# 113705) or BRCA2 (MIM# 600185)
genes that are detectable using PCR-based techniques [Szabo and King, 1997]. While some additional families may have abnormalities in BRCA1 or BRCA2, such as large genomic rearrangements or regulatory mutations, a sizeable fraction appear to be unexplained by mutations in these two genes. Linkage analyses of large collections of families without BRCA1/2 mutations are ongoing, but it does not appear that mutations in a single gene will account for a large proportion of the remaining families.

The BRCA1 and BRCA2 genes encode large, multifunctional proteins that do not share significant homology to other well-characterized proteins or to each other [Venkitaraman, 2002]. Biochemical studies have identified a large number of interacting proteins and upstream and downstream targets, many relating to DNA damage repair or transcriptional regulation. Two novel proteins that interact with BRCA1 were recently identified in yeast two-hybrid assays [Zheng et al., 2000; Cantor et al., 2001]. We studied whether mutations in the genes ZNF350 (originally termed ZBRK1; MIM# 605422) and BRIP1 (originally termed BACH1; MIM# 605882) that encode these BRCA1-interacting proteins might account for some proportion of BRCA1/2-negative probands from potentially inherited breast/ovarian cancer families. In addition, we screened for variants in an anonymized series of early-onset breast cancer cases and cancer-free reference individuals to identify variants that could be studied as lower-penetrance susceptibility alleles in other studies. We also studied linkage disequilibrium and haplotypes derived from the single nucleotide polymorphism (SNP) discovery and genotyping results emanating from this work.

**MATERIALS AND METHODS**

### BRCA1/BRCA2-Negative Probands

Within the NCI’s Family Cancer Registry, after excluding the 31 families with identified BRCA1 or BRCA2 mutations, there were 21 probands with breast or ovarian cancer who had no detectable deleterious mutations upon full sequencing of the BRCA1 and BRCA2 genes. The BRCA1/2-negative families had slightly fewer ovarian cancers (average 1.0 per family) compared to mutation positive families (2.8 per family), but similar numbers of breast cancer cases (3.1 vs. 3.3) [Struwing et al., 1995]. Each BRCA1/2-negative family in the present study had at least one case of male breast cancer, two cases of ovarian cancer, or three or more cases of breast and ovarian cancer. All families were of European ancestry.

### Early-Onset Breast Cancer Cases and Cancer-Free Reference Individuals

Among all 61 subjects diagnosed with breast cancer before age 35 from a national cohort of radiologic technologists [Moham et al., 2002], limited BRCA1 mutation testing was performed on samples rendered anonymous [Struwing et al., 1996] and the 38 negative cases were included in this analysis, having obtained a waiver of the requirement for full IRB review. The samples are linked only to disease status. The study was performed blinded as to case or reference status.

### Sequence Analysis

The intron–exon boundaries of ZBRK1 and BRIP1 were determined by aligning GenBank mRNA records (AF295096 and AF360549, respectively) with genomic sequence records (NT-010757 and NT-011091, respectively). ZBRK1 spans approximately 10 kb and is composed of four exons, while BRIP1 spans approximately 180 kb and is composed of 20 exons. PCR amplimers were designed to amplify the entire mRNA encoding portions and flanking intronic sequence from genomic DNA, using nine primer pairs ranging in size from 208 bp to 428 bp for ZBRK1 and 23 primer pairs ranging in size from 250 bp to 349 bp for BRIP1. The 21 BRCA1/2-negative probands were analyzed by sequencing all PCR products with both the forward and reverse primers used for amplification. The sequencing reaction included two microliters of PCR product as template in an eight-microliter reaction using 20 pmol of primer and Big Dye Terminator Ready Reaction Mix (Applied Biosystems, Foster City, CA) at 0.8 x concentration. The reactions were cleaned up using Microcon 96-well spin plates, dried down, resuspended in 10 microliters of deionized formamide and analyzed on an ABI 3100 sequencer (Applied Biosystems). Two reviewers inspected all sequence tracings, with the forward and reverse reads aligned using Vector NTI (Informax, Inc., North Bethesda, MD).

Analysis of the early-onset breast cancer cases and CEPH reference individuals included both denaturing high performance liquid chromatography (DHPLC) and sequencing. DHPLC analysis was performed on a WAVE System (Transgenicom, Inc., Omaha, NE) using the same PCR primers as for sequencing (gradients and DHPLC conditions are available at http://lpg.nci.nih.gov/LPG/dataRepository). Analysis of these 88 subjects was performed blinded as to case or reference status.

### Further Accession Numbers and Supplementary Data

Genomic records used were AC011460.3 and NM_010783.13 and mRNA records used were NM_021632.1 and NM_032043.1 for ZBRK1 and BRIP1, respectively. All variants were deposited into dbSNP with accession numbers ss6905442 to ss6905458 for ZBRK1 and ss6905459 to ss6905483 for BRIP1. Primer sequences, PCR and DHPLC thermal cycling conditions, and the genetic data for each subject are available at http://lpg.nci.nih.gov/LPG/struwing/pubs).

### Statistical Analysis

No formal statistical comparisons of variant allele frequencies between the early-onset breast cancer cases and reference individuals were made because the base populations for the two groups are likely to be dissimilar. Nucleotide diversity was calculated separately for the amino acid coding nucleotides (n = 1599 for ZBRK1 and n = 3750 for BRIP1) and non-coding nucleotides (n = 1084 for ZBRK1 and n = 5115 for BRIP1) that were analyzed [Hartl and Clark, 1997]. We used the publicly
available computer algorithms PHASE and SNPHAP to predict haplotypes, as implemented in the HapScope suite of programs [Johnson et al., 2001; Stephens et al., 2001; Zhang et al., 2002]. Haplotypes were estimated after excluding variants with allele frequencies below 1% (and before block boundaries were estimated), and the minimum set of variants required to distinguish all haplotypes with a frequency above 2% was calculated using a greedy partition algorithm as implemented in HapScope [Zhang et al., 2002]. The normalized measure of linkage disequilibrium, $D'$, and exact $P$ values for allelic association, were calculated for all pair-wise combinations of markers using Arlequin Version 2.001 software (http://anthro.unige.ch/arlequin).

**RESULTS**

A total of 12 nucleotide variants were identified in ZBRK1 and 11 variants were identified in BRIP1 among the 21 BRCA1/2-negative probands (Tables 1 and 2 and Fig. 1). The ZBRK1 mutations Q393Q (g.1362A>G) and V524I and a variant in the 3' UTR (c.1845C>T) were identified in one proband each, but no other affected family members were available to determine whether these variants segregated with cancer in the family. Neither the silent nor the missense mutations were observed in the 58 early-onset breast cancer cases or 30 reference individuals, but the 3' UTR variant was present in 7% and 8% of these subjects, respectively. The other variants observed in the BRCA1/2-negative probands had allele frequencies of 10% or greater, were observed in the 88 other individuals analyzed, and are unlikely to be related to the cancer clustering in these families (Table 1). A missense mutation Q540L and an intronic variant IVS14+26delT were identified in BRIP1 in one proband each; neither was observed in the early-onset breast cancer cases nor in reference individuals. Another intronic variant, IVS5–31G>C, was identified in two probands but in many of the other 88 individuals. The other six variants identified in the high-risk family probands were common, and had allele frequencies of 25% or greater in the reference individuals (Table 2).

The glutamine to leucine change at residue 540 (Q540L) in BRIP1 is a non-conservative change that does not fall within any of the helicase domains of this protein. Samples from additional family members were available for analysis, but neither the proband's daughter nor her two nieces who had breast cancer carried this mutation. No other family members were available for analysis of the BRIP1 IVS14+26delT variant, nor for the one family in which the proband had the IVS5–31G>C. In the second family with the intron 5 variant, all three relatives with breast or ovarian cancer also carried this mutation. One other female over age 50 without cancer from this family was available for analysis, and she also carried the IVS5–31G>C.

Analysis of the early-onset breast cancer cases and the reference individuals revealed five additional ZBRK1 variants and 14 additional BRIP1 variants that were not observed in the BRCA1/2-negative probands (Tables 1 and 2). Of seven additional BRIP1 missense mutations identified, four were identified in the 30 reference individuals and not in the early-onset breast cancer cases. Four breast cancer cases carried both the T385T (g.1338A>C) and S472P variants in ZBRK1, while none of the reference individuals did, but the frequencies of most variants were similar between the

**TABLE 1. Results of Mutational Analysis of ZNF350/ZBRK1**

<table>
<thead>
<tr>
<th>Number</th>
<th>Protein variant (aa codon)</th>
<th>DNA variant</th>
<th>Segment</th>
<th>Genomic location</th>
<th>BRCA1/2 Negative</th>
<th>Breast Ca cases dx &lt;35</th>
<th>Reference group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(D35)</td>
<td>288T&gt;C</td>
<td>Exon 2</td>
<td>29248</td>
<td>7/42 (0.17)</td>
<td>26/116 (0.22)</td>
<td>16/60 (0.27)</td>
</tr>
<tr>
<td>2</td>
<td>L66Fp1</td>
<td>380T&gt;C</td>
<td>Exon 3</td>
<td>29671</td>
<td>7/42 (0.17)</td>
<td>14/116 (0.12)</td>
<td>11/60 (0.18)</td>
</tr>
<tr>
<td>3</td>
<td>IVS3+18A&gt;G</td>
<td>IVS3+46A&gt;T</td>
<td>Intron 3</td>
<td>29730</td>
<td>6/42 (0.14)</td>
<td>25/116 (0.22)</td>
<td>16/60 (0.27)</td>
</tr>
<tr>
<td>4</td>
<td>IVS3+6C&gt;G</td>
<td>IVS3+6C&gt;G</td>
<td>Intron 3</td>
<td>29758</td>
<td>7/42 (0.17)</td>
<td>14/116 (0.12)</td>
<td>11/60 (0.18)</td>
</tr>
<tr>
<td>5</td>
<td>IVS3+6C&gt;G</td>
<td>IVS3+6C&gt;G</td>
<td>Intron 3</td>
<td>29774</td>
<td>6/42 (0.14)</td>
<td>27/116 (0.23)</td>
<td>16/60 (0.27)</td>
</tr>
<tr>
<td>6</td>
<td>(C236)</td>
<td>891C&gt;T</td>
<td>Exon 4</td>
<td>32545</td>
<td>6/42 (0.14)</td>
<td>14/116 (0.12)</td>
<td>10/60 (0.27)</td>
</tr>
<tr>
<td>7</td>
<td>(P373)</td>
<td>1302C&gt;A</td>
<td>Exon 4</td>
<td>32956</td>
<td>4/42 (0.10)</td>
<td>14/116 (0.12)</td>
<td>9/60 (0.15)</td>
</tr>
<tr>
<td>8</td>
<td>(T385)</td>
<td>1338A&gt;C</td>
<td>Exon 4</td>
<td>32992</td>
<td>0/42</td>
<td>4/116 (0.03)</td>
<td>0/60</td>
</tr>
<tr>
<td>9</td>
<td>(Q393)</td>
<td>1362A&gt;g</td>
<td>Exon 4</td>
<td>33016</td>
<td>1/42 (0.02)</td>
<td>0/116</td>
<td>0/60</td>
</tr>
<tr>
<td>10</td>
<td>S472Pp</td>
<td>1597T&gt;C</td>
<td>Exon 4</td>
<td>33251</td>
<td>0/42</td>
<td>4/116 (0.03)</td>
<td>0/60</td>
</tr>
<tr>
<td>11</td>
<td>(S476)</td>
<td>1611C&gt;T</td>
<td>Exon 4</td>
<td>33265</td>
<td>0/42</td>
<td>0/116</td>
<td>1/60 (0.02)</td>
</tr>
<tr>
<td>12</td>
<td>RS501S</td>
<td>1686A&gt;T</td>
<td>Exon 4</td>
<td>33340</td>
<td>4/42 (0.10)</td>
<td>14/116 (0.12)</td>
<td>9/60 (0.15)</td>
</tr>
<tr>
<td>13</td>
<td>V524Ip</td>
<td>1753G&gt;A</td>
<td>Exon 4</td>
<td>33407</td>
<td>1/42 (0.02)</td>
<td>0/114</td>
<td>0/60</td>
</tr>
<tr>
<td>14</td>
<td>1845C&gt;Tg</td>
<td>1845C&gt;T</td>
<td>Exon 4</td>
<td>33509</td>
<td>1/42 (0.02)</td>
<td>8/116 (0.07)</td>
<td>5/60 (0.08)</td>
</tr>
<tr>
<td>15</td>
<td>1947T&gt;A</td>
<td>1947T&gt;A</td>
<td>Exon 4</td>
<td>33601</td>
<td>0/42</td>
<td>0/116</td>
<td>1/60 (0.02)</td>
</tr>
<tr>
<td>16</td>
<td>1955T&gt;A</td>
<td>1955T&gt;A</td>
<td>Exon 4</td>
<td>33609</td>
<td>0/42</td>
<td>0/116</td>
<td>1/60 (0.02)</td>
</tr>
<tr>
<td>17</td>
<td>2067C&gt;T</td>
<td>2067C&gt;T</td>
<td>Exon 4</td>
<td>33721</td>
<td>4/42 (0.10)</td>
<td>14/116 (0.12)</td>
<td>9/60 (0.15)</td>
</tr>
</tbody>
</table>

*aFrequency of the second allele listed.
*bSNP number for cross-referencing to Figure 2.
*cNumbering based on RefSeq NM_002632.1 (AF295096), with most common allele listed first.
*dNucleotide position within AC011460.3.
*eBased on complete sequencing.
*fBreast cancer cases diagnosed before age 35 from radiologic technologist cohort; reference group consists of 30 CEPH parents.
*gIn dbSNP as of Nov. 1, 2002.
*hOne subject was untyped for this SNP.
early-onset breast cancer cases and reference individuals (no formal statistical comparisons were made).

Our extensive genetic analysis of these two genes in 218 chromosomes allowed us to examine diversity and linkage disequilibrium measures. The calculated amino acid encoding nucleotide diversity was approximately 10–4, respectively. The \( D' \) values were 1.2 times higher for the BRCA1 and BRCA2 genes than for the \( D' \) values of the other genes.

## DISCUSSION

Approximately one-third of families with inherited forms of breast and ovarian cancer do not segregate mutations in BRCA1 or BRCA2, two genes identified through linkage analysis in multiple-case families. Additional linkage studies in families that are negative for BRCA1/2 suggest that mutations in no single gene account for a large proportion of families. If mutations in several genes acting in concert account for small subsets of these BRCA1/2-negative families, identifying such genes by linkage analyses and positional cloning will be difficult. An alternative approach is to analyze candidate genes to search for mutations that alter the normal biological function of the gene or its product and that segregate with cancer in a family. Some breast cancer
families have been attributed to such mutations [Vahteristo et al., 2001; Chenevix-Trench et al., 2002].

The biological basis of the cancer predisposition in BRCA1 and BRCA2 mutation carriers has not been clearly elucidated. BRCA1 and BRCA2 are large, multi-functional proteins, involved in DNA damage sensing and repair, transcriptional regulation, and transcription-coupled repair, but whether these or other functions account for their carcinogenic effect are not known. They interact with many different proteins, including two that were identified through yeast two-hybrid analyses. The ZBRK1 gene is a novel gene identified using an N-terminal fragment of BRCA1 as bait [Zheng et al., 2000]. This gene encodes a protein with a KRAB domain and eight zinc fingers involved in repression of GADD45 and, potentially, other targets. No mutational analysis has been published, but there are four SNPs in dbSNP that map to the mRNA. The BRIP1 gene, originally termed BACH1, was identified using a C-terminal fragment of BRCA1 as bait [Cantor et al., 2001]. BRIP1 contains six DEAH-helicase homology regions and interacts with the BRCT domains of BRCA1. Mutations within the helicase domains of BRIP1 interfered with double-strand DNA break repair in a BRCA1-binding dependent manner, and two germline missense mutations in BRIP1 were identified among 65 breast cancer cases [Cantor et al., 2001]. One of these, P47A, identified in a patient with a strong family history of breast and ovarian cancer who was negative for BRCA1/2 mutations, affected a conserved residue in the helicase domain and produced an unstable protein. No other family members from the two missense-carrying cases were available for analysis, but this finding does raise the possibility that mutations in BRIP1 may account for some (presumably small) fraction of inherited breast cancer cases.

We screened the ZBRK1 and BRIP1 genes in a series of individuals at high risk of genetic forms of breast cancer, including 21 probands from families with multiple cases of breast and/or ovarian cancer who were negative for BRCA1/2 mutations, and 58 individuals who were diagnosed with breast cancer before age 35 [Struwing et al., 1995, 1996; Mohan et al., 2002]. We did not identify any mutations that were clearly related to breast cancer. For BRIP1 we did not observe the two previously reported mutations P47A and M299I, but we did identify
a non-conservative missense mutation, Q540L, in a BRCA1/2-negative proband with breast cancer. Analysis of seven additional relatives, however, suggested that this mutation was not the underlying cause of the breast cancer in this family, as none of the three other relatives with breast cancer carried this mutation. The conservative BRCA2 missense mutation V524I was identified in a BRCA1/2-negative proband and in none of the early onset breast cancer cases or reference individuals, but no other family members were available for analysis and it is unclear whether this is related to the cancer occurrence. There were only two other ZBRK1 or BRIP1 variants observed exclusively in the BRCA1/2-negative probands; one was a silent mutation and the other an intronic variant that appear unlikely to be causative mutations. If we assume none of the 21 probands carries a disease-related mutation, the upper 95% confidence limit on this proportion (0/21) would be 13% [Hanley and Lippman-Hand, 1983].

We identified many sequence variants in the two genes, including two silent mutations in ZBRK1 and two silent and one missense mutation in BRIP1 that had allele frequencies of at least 10% but are not in dbSNP (as of September, 2002). The observed nucleotide diversity was about twice as high for ZBRK1 as BRIP1, but in comparison with 15 other genes analyzed in 93 subjects of worldwide distribution, only four genes had higher amino-acid coding diversity than BRIP1, suggesting that neither gene is under strong functional constraint [Thorstenson et al., 2001]. Our linkage disequilibrium and haplotype analyses appear to be consistent with recent evidence suggesting that SNPs fall within blocks of extended disequilibrium [Rioux et al., 2001; Gabriel et al., 2002]. All ZBRK1 variants were in

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Schematic representation of calculated haplotypes as displayed by HapScope, with minor modifications. The exons and introns are drawn to scale (different for each gene) at the top of the diagram as horizontal lines, with nucleotide positions of the genomic record indicated by the ruler. Repetitive elements are shown as blackened areas in the genomic record bar. Symbols (diamonds = nucleotide substitutions, triangles = insertion/deletion) below the ruler represent the SNPs and their relative location indicated by lines connecting to the ruler. Small squares below a SNP symbol indicate a minimum set required to identify all haplotypes with a frequency greater than 2%. Numbers immediately below some SNP symbols correspond to the variant numbers from Tables 1 and 2, for cross-reference). Haplotypes are shown in the lower half of the figure, with each SNP used to calculate the haplotypes represented by a circle; darkened circles represent the common allele; open circles represent the variant allele. The number of occurrences and percent frequency are shown to the left of each haplotype. A: ZNF350/ZBRK1 showing the 12 SNPs with allele frequencies greater than 1% used to estimate haplotypes. D35D and P373P are indicated with small squares as the minimum SNPs required to identify the three haplotypes with a frequency greater than 2%. B: BRIP1/BACH1 showing the nine SNPs with allele frequencies greater than 1% used to estimate haplotypes, and the two estimated haplotype blocks. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
strong linkage disequilibrium, and analyzing the D35D and L66P variants would capture the common haplotype diversity across this gene, which appears to fall within a single block. While less diverse at the nucleotide level, BRIP1 showed considerably more haplotype diversity, with the variants in BRIP1 falling within two haplotype blocks.

Our SNP discovery effort in the early-onset breast cancer cases and reference individuals identified several common variants that may warrant further study in breast cancer or other cancer case-control studies. Within ZBRK1, the two non-conservative missense mutations L66P and R501S are common (allele frequencies of 10% or greater). Another non-conservative mutation, S472P, was present in 7% of early-onset breast cancer cases but none of the reference individuals. Two BRIP1 variants, the non-conservative missense mutation (P919S) that is very common (allele frequency >30%), and a newly described, common variant 64 nucleotides upstream from the transcription start site (c.–64G>A) that might affect gene regulation, may warrant further investigation as susceptibility alleles.

In summary, we performed mutational analysis of the genes ZBRK1 and BRIP1, encoding two recently identified BRCA1-interacting proteins, as possible explanations for inherited breast and ovarian cancer clustering in families that are negative for germline mutations in BRCA1 and BRCA2. We did not identify any mutations that appear to be causative in these families and it appears unlikely that mutations in these genes account for a large fraction of inherited forms of breast cancer. Additional analysis in early-onset breast cancer cases and reference individuals identified several newly described variants that make attractive candidates as susceptibility alleles for breast and other cancers.

REFERENCES


FIGURE 3. Graphical representation of measures of linkage disequilibrium between SNPs with allele frequencies greater than 1%. The relative location of SNPs are indicated by lines connecting to the linear representation of the genomic structure of the gene, which are drawn to a different scale for each gene; a 500-bp line above the gene line indicates the scale. For each pair-wise comparison, the upper half of the square indicates the magnitude of D', with dark shading representing values >0.9, light shading representing values between 0.8 and 0.9, and open rectangles representing values <0.8; the lower half represents the exact P-value for the 2×2 association between SNPs, with dark shading representing values <0.001, light shading representing values between 0.05 and 0.001, and open rectangles representing values >0.05. A: ZNF350/ZBRK1. B: BRIP1/BACH1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]