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Advances in Brief

A 1-kb Alu-mediated Germ-Line Deletion Removing *BRCA1* Exon 17[1]

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Abstract

Although more than 100 different *BRCA1* germ-line mutations have already been identified in breast and/or ovarian cancer families, we report for the first time a deleterious genomic rearrangement in *BRCA1*. A 1-kb deletion comprising exon 17 was found in a large breast and ovarian cancer family, leading to a frameshift in the mutant mRNA due to the absence of exon 17. This deletion is probably the result of a recombination between two closely related Alu sequences. It was not detected by conventional PCR-based methods involving the genomic screening of the 22 coding exons or reverse transcription-PCR because the transcript without exon 17 is unstable in lymphoblastoid cell lines. Therefore, rearrangements in the *BRCA1* gene should be sought in breast/ovarian cancer families in which no mutations have been found by PCR-based methods in the coding region or in the splice sites.

Introduction

The first predisposing gene to breast and ovarian cancer to be cloned, *BRCA1*, has a coding sequence of 5592 nucleotides split into 22 coding exons distributed over roughly 100 kb of genomic DNA (1). Since its discovery in October 1994, more than 100 different germ-line mutations have been identified in breast and ovarian cancer families (2). These mutations can be classified into four categories: (*a*) nonsense mutations; (*b*) small insertions or deletions changing the reading frame; (*c*) splice-site mutations leading to deletion of exon(s) or insertion of intronic sequences; and (*d*) missense mutations, which mostly occur in the RING finger coding domain. Mutations presumed to result in the production of a truncated protein represent over 80% of all mutations.

In a previous study of 20 breast/ovarian cancer families, most of which showed positive evidence of linkage to 17q12–21, the sequencing of each exon of *BRCA1* and single-strand conformational polymorphism analysis of *BRCA1* cDNA allowed the identification of a mutation in 16 families (3). Among the four families for which no mutation could be found, only one, family 1816, was clearly linked to *BRCA1*, with a multipoint lod score of 3.62 using two markers flanking *BRCA1*. We now report the identification of the deleterious *BRCA1* mutation in this family, a 1-kb deletion that probably results from the recombination between two closely related Alu sequences, one in intron 16 and the other in intron 17. This deletion thus comprises exon 17, and the absence of exon 17 in the *BRCA1* mRNA produces a frameshift. This mutation is the first report of a major rearrangement in the *BRCA1* gene leading to predisposition to breast and ovarian cancer.

Materials and Methods

Family 1816 is followed at the Department of Preventive Medicine of the Creighton University School of Medicine in Omaha and has already been described (3, 4). Control individuals consisted of spouses within this family. DNA and RNA were extracted from EBV-immortalized lymphoblastoid cell lines using a standard procedure for DNA and Trizol reagent for RNA (Life Technologies, Inc.), according to the instructions provided by the manufacturer. RNA extracted from a spontaneously immortalized cell line (HI0106) established from a prophylactic oophorectomy sample of a carrier from family 1816 was kindly provided by A. K. Godwin (Fox Chase Cancer Center, Philadelphia, PA). PCR was performed in a GenAmp PCR system 9600 (Perkin-Elmer Corp.) using the Expand Long Template PCR system (Boehringer Mannheim) for long-range PCR, using the manufacturer's conditions, or with Red Hot Taq (Advanced Biotechnology, United Kingdom) for fragments of up to 2 kb. Amplification of a ~7-kb genomic fragment was performed in 25-μl volume reactions using forward primer 16BF (5′-AACCTCTGCATTGAAAGTTC-3′) and reverse primer 18R (5′-TCAGTGTCCGTTCACACACA-3′; Fig. 2). Ten μl of the reaction were digested with 5 units of *AluI* (Boehringer Mannheim), and the digested DNA was then loaded on a 1% low-melting point agarose (Life Technologies, Inc.) gel. The 480-, 420-, and 320-bp fragments were cut, purified using the Wizard PCR Preps DNA purification kit (Promega), and cloned in pBluescript vector (Stratagene). Positive clones were sequenced with primers M13F and M13R using the Sequenase PCR sequencing kit (United States Biochemical). Primers 1816F (5′-GCCGTGTCTGGCCCAGTAT-3′) and 1816R (5′-TTACAGGTGCCTGCCACC-3′) were designed (see Fig. 2) and used to amplify the wild-type sequence containing the deleted fragment. The 1.5-kb band generated was then used as a template for PCR with a combination of one of two primers located in exon 17 (17F, 5′-TTGCCAGAAAACACCACATC-3′; 17R, 5′-AGTGATGTGGTGTTTTCTGGC-3′) and either 1816F or 1816R. We obtained a PCR product of ~800 bp with 1816F and 17R and a PCR product of ~740 bp with 17F and 1816R, whereas no PCR product was obtained with either 1816R and 17R or 1816F and 17F. The 800- and 740-bp bands were cloned in a TA cloning vector (Invitrogen) and sequenced using an Applied Biosystems PRISM 377 semiautomated sequencing system with primers M13F and M13R, which allowed the whole sequence of the wild-type 1816R/1816F fragment to be known (European Molecular Biology Laboratory accession number, Y08757). For mutation screening, a second reverse primer was designed (1816R2, 5′-ATCCATGCTATGCTCAACAAA-3′) because 1816R is located in an Alu sequence and gives rise to unspecificity when used with 1816F on genomic DNA (Fig. 2).

Results

Family 1816 contains 14 cases of breast cancer (mean age at diagnosis, 40.1 years) and 11 cases of ovarian cancer (mean age at diagnosis, 50.4 years; Ref. 4) and has a multipoint lod score of 3.62 with *D17S579* and *THRA1*. In 1991, this family alone reached a lod score of nearly 3 with the first marker reported to be linked to *BRCA1* and thus greatly strengthened the assignment of a breast cancer susceptibility locus to chromosome band 17q12–23 (5). Each exon of

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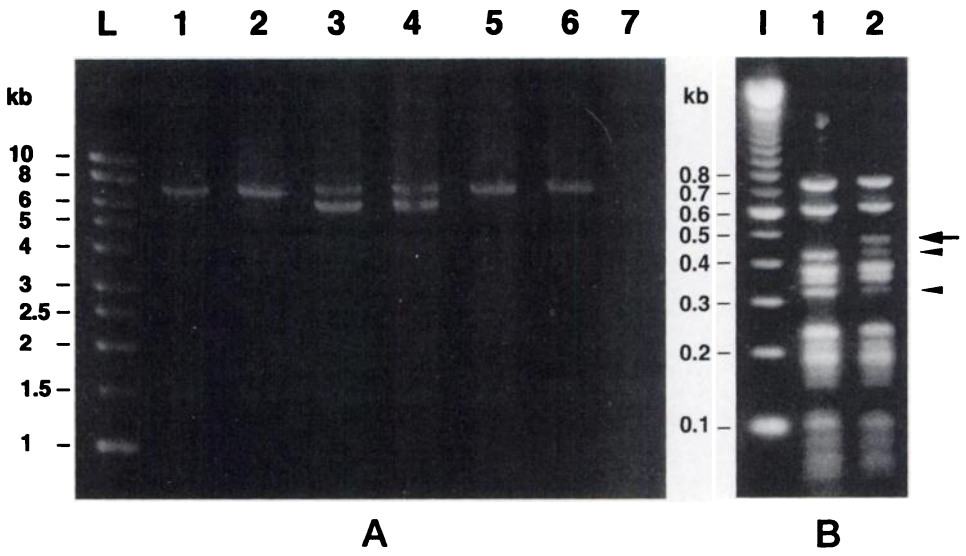

Fig. 1. Detection of a 1-kb *BRCA1* gene deletion in family 1816. *A*, a PCR reaction was performed with primers 16BF and 18R using genomic DNA from two control individuals (*Lanes 1* and *2*), two family 1816 disease haplotype carriers with a different wild-type chromosome (*Lanes 3* and *4*), or two family 1816 noncarriers (*Lanes 5* and *6*). *Lane L* contains a 1-kb ladder (Pharmacia). *Lane 7* contains the negative control. The PCR produces a 7-kb fragment with all DNAs. An extra band of 6 kb of equal intensity is present with both carriers. *B*, the PCR products generated with 16BF/18R and a control DNA (*Lane 1*) or a carrier (*Lane 2*) were digested by *AluI*. The *arrow* shows the ~0.48-kb extra band, whereas the *arrowheads* indicate the ~0.42-kb and the ~0.32-kb fragments that show a reduction of intensity. *Lane I* contains a 100-bp ladder (Life Technologies, Inc.).

the *BRCA1* gene, as well as the splice junctions, was sequenced using the genomic DNA of one patient bearing the disease haplotype, and no significant change could be detected (3). The analysis by single-strand conformational polymorphism of the cDNA prepared from a lymphoblastoid cell line from this individual revealed no mutation either (3). A comparison of the genomic sequence and the cDNA sequence at a polymorphic site in exon 11 showed a slight reduction in the level of the transcript produced by the mutant allele, but this reduction was not important enough to be taken into account (3). However, subsequent analysis of a cDNA synthesized with RNA extracted from a spontaneously immortalized ovarian cell line from another patient from family 1816 revealed the presence of a transcript, at a reduced level, without exon 17 (data not shown). This transcript without exon 17 was then shown not to be stable in the lymphoblastoid cell lines of three patients bearing the disease haplotype. Genomic DNA contamination was found in the RNA sample used in the first place for the loss of transcript assay, which explains why this absence of the mutant transcript was not detected before because the primers used (in exon 11) give rise to the same product from genomic DNA or cDNA (3).

Because the mutation was not located at the splice junctions, it was likely to affect the introns surrounding exon 17. We thus performed a long-range PCR to amplify the *BRCA1* gene from exon 16 to exon 18 (Figs. 1*A* and 2). The PCR produced a ~7-kb fragment when the genomic DNA of two controls (Fig. 1*A*, *Lanes 1* and *2*) and of two individuals from family 1816 not carrying the disease haplotype (Fig. 1*A*, *Lanes 5* and *6*) were used as a template. When the genomic DNA of two carriers with a different wild-type chromosome were used, an extra band of ~6 kb of equal intensity was present (Fig. 1*A*, *Lanes 3* and *4*). The PCR product was then digested by *AluI*; in carriers, it produced an extra band of ~480 bp, whereas at least two bands (one of ~420 bp and one of ~320 bp) showed a reduction of intensity (Fig. 1*B*, compare *Lane 2* to *Lane 1*). The ~320- and ~480-bp bands were then cloned and sequenced. The ~320-bp fragment consisted of 50 bp of intron 16, the entire exon 17 (88 bp), and 190 bp of intron 17. The ~480-bp band, which contained the breakpoint of the deletion, consisted of an unknown sequence (only a very small portion of introns 16 and 17 was known at this time). Two primers were then designed, 1816F and 1816R, to amplify the deleted sequence (Fig. 2). This

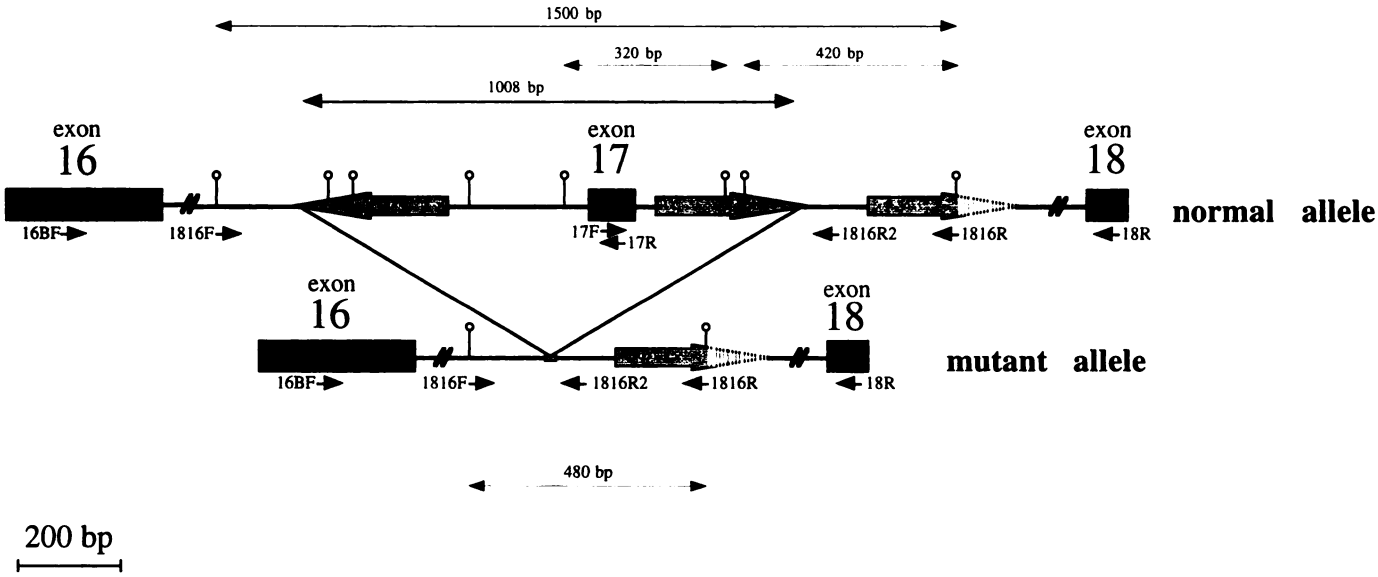

Fig. 2. Representation of the 1-kb deletion in the *BRCA1* gene in family 1816. Schema of the normal and the mutant alleles between exons 16 and 18, showing the location of the 1008-bp deletion. *Gray arrows*, Alu sequences and their orientation (one was sequenced partially and is thus represented with a *hatched arrow*); ↑, *AluI* sites; *small black arrows*, primers used. The fragments referred to in "Results" are indicated with a *double arrow*.

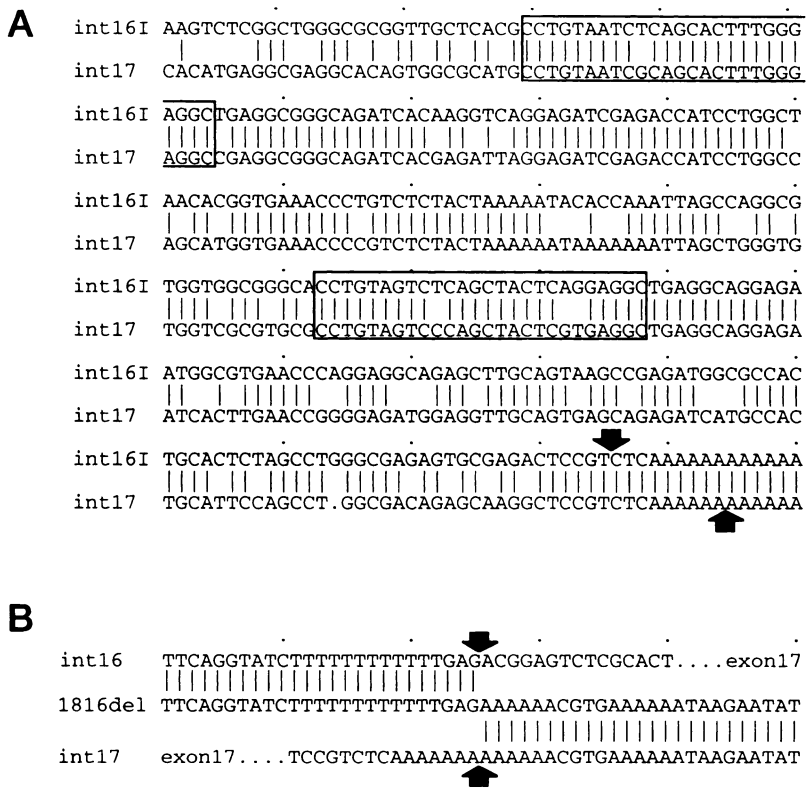

Fig. 3. *A*, homology between the two Alu sequences involved in the homologous recombination. The Alu sequence located in intron 16 is shown in the inverse strand and orientation (*int16I*) to allow comparison with the one in intron 17 (*int17*). The 26-bp core sequence reported to stimulate recombination (see text) is *boxed* in the *left* and *right arms*. *Black arrows*, the position of the crossover. *B*, nucleotide sequences across the breakpoint junctions of the 1-kb deletion in family 1816. The deleted allele (*1816del*) sequence is shown with the corresponding normal intron 16 (*int16*) and intron 17 (*int17*) sequences *above* and *below*, respectively. *Black arrows*, the position of the crossover.

amplification led to the production of a 1.5-kb band in control DNA, whereas the PCR gave rise to two bands of 1.5 and 0.48 kb in carrier DNA (data not shown). A comparison of the sequences of the PCR products obtained with 1816F and 1816R when amplifying the normal or the mutant allele confirmed that the deletion involved a 1008-bp fragment encompassing exon 17 (Fig. 2 and 3*B*). Because both breakpoints occurred within Alu sequences, the deletion is probably the consequence of a recombination between two Alu repeat elements, one in intron 16 and the other in intron 17.

Discussion

Germ-line rearrangements have been shown to account for 15% of all mutations in the *RB1* gene in a series of 119 patients (6), 10% of all mutations in the *NF1* gene in a series of 200 patients (7), 22% of all mutations in the *VHL* gene in a series of 116 patients (8), and for at least 7% of all mutations in the *ATM* gene in a series of 44 patients (9). It thus seemed sensible to speculate that such rearrangements might be part of the mutation spectrum of *BRCA1*, especially because in at least eight families with a lod score > 1 with markers close or within *BRCA1*, no mutation could be found, although an extensive mutation search has been performed in the coding region and in splice sites (3, 10–12). The different strategies currently used to look for mutations in the *BRCA1* gene all share a preliminary step: they necessitate PCR reactions, whether the template is genomic DNA, cDNA, or both. That means that in the case of a rearrangement, only the wild-type allele would be amplified, and as a result, such mutations would not be detected.

We report here for the first time a germ-line rearrangement in the *BRCA1* gene leading to predisposition to breast and ovarian cancer in a large family with 14 cases of breast cancer and 11 cases of ovarian cancer (lod score, 3.62 with markers flanking *BRCA1*). This is a deletion of a 1-kb fragment containing exon 17, which leads to the apparition of a premature termination codon in the transcript (ter1672) and results in a drastic reduction of the mutant *BRCA1* mRNA level in lymphoblastoid cell lines. An altered reading frame has frequently been shown to result in a reduction in mRNA abundance in many genetic diseases (13). In four families in the literature, only mRNA transcribed from the wild-type *BRCA1* allele was found to be present in lymphoblastoid cell lines, and the undetected mutations were suggested to lie in the regulatory regions (1, 3, 10) rather than supposed to have a destabilizing effect on mRNA. On the other hand, two families with a mutant *BRCA1* transcript without exon 3 or exon 20, for which no genomic changes have been identified at the splice junctions, have been reported in the Breast Cancer Information Core database on the Internet (2). In the light of our results, rearrangements should be sought in all these families. We have investigated the genomic structure of the *BRCA1* gene to perform Southern analysis on our breast and ovarian cancer families with undetectable *BRCA1* or *BRCA2* mutation when screened by PCR-based methods. The Southern method seems to be the most appropriate because RNA is not always available for mutation screening, and even if it is, the mutant transcript might not be stable in lymphoblastoid cell lines.

In family 1816, the comparison between the normal and mutant alleles revealed that the breakpoint junction occurred between two repetitive elements of the Alu family that are oriented in opposite direction (Fig. 2). Most parts of both Alu sequences are removed in the mutant allele. Sequence analysis showed a 81.3% identity in the 300-bp region of the parental sequences involved in the rearrangement (Fig. 3*A*). This suggests that the deletion may be due to a homologous recombination involving the two Alu sequences on a single strand of DNA, as was reported in the low-density lipoprotein receptor gene (14). Recently, a 3.5-kb deletion in MLH1 was found to be the first example of an Alu-mediated recombination causing a prevalent, dominantly inherited predisposition to cancer (15). A 26-bp core sequence of the Alu elements was found to be systematically present at or close to the site(s) of recombination in rearrangements involving Alu repeats, suggesting that this sequence is a recombinational hot spot (16).

However, none of the two breakpoints disrupted this core sequence in family 1816 (Fig. 3*A*), suggesting the existence of other recombinational sequence(s) within Alu elements. Three Alu sequences were found in the 1.4-kb sequenced portion of introns 16 and 17; this finding confirms the very high frequency of Alu sequences reported recently in the *BRCA1* gene (density of 40.5%, which is ~1 Alu/0.65 kb; Ref. 17). As a consequence, Alu-mediated recombination in this gene might be a common mechanism in the generation of mutations predisposing to breast and ovarian cancer.

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