Identification of a 14 kb deletion involving the promoter region of **BRCA1** in a breast cancer family

Jeff Swensen¹, Michael Hoffman¹, Mark H. Skolnick¹,² and Susan L. Neuhausen¹,*  

¹Division of Genetic Epidemiology, Department of Medical Informatics, University of Utah School of Medicine, 391 Chipeta Way, Suite D-2, Salt Lake City, UT 84108, USA and ²Myriad Genetics Inc., Salt Lake City, Utah, USA

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**BRCA1** is a breast and ovarian cancer susceptibility gene. An inferred germline regulatory mutation was previously reported in the **BRCA1**-linked kindred K2035, based on the absence of transcripts from the **BRCA1** allele associated with the cancer susceptibility haplotype. In this study, the promoter region of **BRCA1** was examined in individuals from K2035 for evidence of a mutation which could halt transcription. Evaluation of a polymorphism located within intron 2 of **BRCA1** gave results consistent with the presence of a large deletion in K2035 mutation carriers. Southern blot analysis identified unique restriction fragments which occurred in K2035 mutation carriers. Transcription was found to start from one of two alternatively spliced first exons (exon 1a or 1b). Both splice variants were present in the tissue types examined in the study (11). Consequentially, for a mutation to halt transcription from **BRCA1**, the promoters associated with both exon 1a and exon 1b needed to be affected. To rule out a deletion, genomic sequence at the 5′ end of **BRCA1** was surveyed for polymorphisms which could demonstrate the heterozygosity of **CSH** carriers. PCR primers were positioned around the poly(A) tract of an Alu repeat in intron 2. The PCR product was amplified from the DNA of several K2035 **CSH** carriers and electrophoresed. All individuals appeared heterozygous; however, one had an allele (allele 2) which was a different size than the allele of the others (allele 1). Thus, the haplotype was seemingly not conserved.

DNA preparations from a sibling and the parents of the individual with allele 2 were amplified with the primer set (Fig. 1). The non-**CSH** carrying father and sibling were heterozygous (alleles 1, 2) while the **CSH** carrying mother appeared homozygous (allele 1). No amplification was seen from the mother’s chromosome in the affected daughter. Although this could have resulted from a localized alteration in the sequence, it was consistent with the locus being hemizygous in **CSH** carriers due to a deletion. A 60 kb **P1** clone (P1 1141) was known to extend from exon 11 through the promoter region of **BRCA1** (12). Digestion of **P1**

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**RESULTS AND DISCUSSION**

A mutation affecting the promoter region of **BRCA1** was considered a likely reason for the absence of transcription from the **BRCA1** allele associated with the K2035 CSH. The structure of the promoter region had been previously reported (11). Transcription was found to start from one of two alternatively spliced first exons (exon 1a or 1b). Both splice variants were present in the tissue types examined in the study (11). Consequently, for a mutation to halt transcription from **BRCA1**, the promoters associated with both exon 1a and exon 1b needed to be affected.

**INTRODUCTION**

Germline mutations in **BRCA1** cause a hereditary predisposition to breast and ovarian carcinomas. Mutations are distributed throughout the gene and most frequently lead to truncation of the protein. It has been estimated that mutations which affect expression, splicing or stability of the transcript may account for 15–20% of the total (1). In a previous study, mutation carrier status of a **BRCA1**-linked kindred, K2035, had been assigned based upon a shared haplotype segregating with breast and ovarian cancers. It was determined that genomic DNA of a cancer susceptibility haplotype (**CSH**) carrier from K2035 was heterozygous for a series of polymorphisms located in the distal 3.5 kb of the **BRCA1** transcript, while cDNA from the same individual appeared homozygous at the loci (2). Based on these results, the presence of a regulatory mutation which halted transcription from the shared allele was inferred. A similar loss of transcription from **BRCA1** has been reported in three additional families (3,4), suggesting such mutations occur at some frequency.

*To whom correspondence should be addressed. Tel: +1 801 581 6144; Fax: +1 801 585 7272; Email: susan@morgan.med.utah.edu
Figure 1. Amplification of the intron 2 polymorphism in the branch of K2035 which demonstrates the hemizygosity of CSH carriers. The affected daughter (#14) received allele 2 (A2) from her father (#13), but did not receive allele 1 (A1) from her apparently homozygous, CSH carrying mother (#12). The heterozygous sibling (#15) does not carry the CSH.

1141 with EcoRI, HindIII, BamHI and EcoRV restriction endonucleases yielded fragments used to construct a restriction map (Fig. 2), that was later supplemented with the recently published BRCA1 genomic sequence (ref. 13; GenBank number: L78833). Since it had previously been demonstrated that CSH carriers were heterozygous in exon 11 (2), the search for a deletion breakpoint within BRCA1 was limited to the region between exon 2 and exon 11.

Southern blots were prepared from genomic DNA of one CSH carrier and one non-CSH carrier which had been digested with HindIII, EcoRI, BamHI, EcoRV, BglII and PvuII. PCR products obtained from the promoter-distal ends of EcoRI restriction fragments were used as hybridization probes. Hybridization with probe A from intron 2 (Fig. 2) revealed unique bands in the CSH carrier on Southern blots prepared from BamHI, EcoRV, BglII and PvuII digests (three of the blots are presented in Fig. 3). This result was consistent with the presence of a deletion breakpoint.

Probe B (Fig. 2) was used to locate the upper deletion breakpoint which was presumed to lie across the BRCA1 promoter. This probe hybridized to a unique EcoRV band in the CSH carrier which corresponded to the one identified with probe A. Altered HindIII and EcoRI bands were also identified (Fig. 3).

Probe C (Fig. 2), which was located closer to the upper breakpoint, revealed unique bands of the expected sizes with all six enzymes in the CSH carrier (Fig. 3). These results were consistent with the presence of a 14 kb deletion which spanned the BRCA1 promoter region (Fig. 2).

Probes B and C hybridized to multiple sets of normal bands in most of the blots. This likely occurred as a result of the ∼30 kb duplication which was identified in the region (14). These extra bands produced a more complex hybridization pattern, but did not interfere with the identification of unique fragments.
Figure 3. Southern blots prepared from the genomic DNA of K2035 cancer susceptibility haplotype carriers (C) and noncarriers (N). The enzyme used to digest the DNA is indicated for each blot (blots prepared with EcoRI, BglII, and PvuII are not shown). The Southern blots have been sequentially hybridized with probes A, B and C. Unique restriction fragments which result from the deletion are labeled with arrows.

Figure 4. Alignment of the Alu elements involved in the unequal crossover in K2035 mutation carriers. Recombination occurred in the region between the arrows.

Oligonucleotides were designed to amplify a PCR product across the deletion. PCR primers, which normally annealed to regions separated by ~15 kb, specifically amplified a 1.3 kb product in the CSH carriers which could be reamplified with nested primers into a 650 bp product. Sequencing revealed that the PCR product contained the expected sequence on each of its ends. Within the product, the right arm of an Alu repeat in intron 2 was found to be joined to an Alu fragment from the upstream region in a way which preserved the consensus sequence of an Alu element (Fig. 4). This result was consistent with the deletion having occurred as a result of unequal crossover between the Alus in a region of 23 bp of perfect sequence identity.

The PCR product spanning the deletion was amplified in 55 individuals and their spouses from K2035. An additional 50 random individuals were examined to confirm the specificity of the PCR product. Amplification occurred only in the 23 CSH carriers. These results were consistent with a previous haplotype analysis with six markers across a 625 kb region spanning the BRCA1 gene. Eight of the ten breast cancer cases in the family were tested, and all carried the mutation. The ninth case was deceased and inferred to be a mutation carrier as several of her children were positive and her spouse was negative. It was not possible to determine the mutation status of the tenth case.

Only breast cancers and one ovarian cancer are observed in K2035. There are no prostate or colon cancers, which are often present in BRCA1 families, although there are three obligate male mutation carriers in their 60s. The breast cancer cases in this kindred descend from two sisters who were both unaffected until their deaths in their 80s. There is an additional obligate female carrier in her 60s who has not had any cancer. Lifetime cumulative penetrance was estimated at 85%, and by age 50 was 75%. This is higher than the 59% cumulative overall reported for BRCA1 at age 50 (15), although it is not significant due to the large confidence intervals associated with the small sample size.

Alu repeats are abundant in the human genome and provide numerous potential target sites for recombination events such as unequal crossover (5–10). The importance of Alu-mediated recombination as a source of germline mutations in cancer susceptibility genes has recently been demonstrated by the identification of 3.5 kb (8) and 22 kb (9) Alu-mediated deletions in the hereditary nonpolyposis colorectal cancer (HNPCC) gene, hMLH1, as well as a 1 kb Alu-mediated deletion in BRCA1 (10). Our results are consistent with a 14 kb Alu-mediated deletion which eliminates transcription from the shared BRCA1 allele in K2035 CSH carriers by removing both known transcription start sites as well as exon 2. Deletions of this type may also be responsible for other reported germline regulatory mutations in BRCA1 (3,4).

An estimate of the number of BRCA1 mutations which are deletions may be calculated from the data of the Breast Cancer...
Linkage Consortium. Thirty families, with a LOD score greater than 1.0 for linkage to BRCA1 were previously screened for mutations. Mutations were identified in 21 (70%) of the families (D. Easton, personal communication). Of the nine families where mutations were not found, loss of transcription from the CSH-associated allele was detected in two (including K2035 reported here). The Alu-mediated deletion of exon 17 (10) was later identified in a third family. cDNA analysis in the remaining six families was either not informative or not performed, so a percentage of them may have had large deletions as well. These results suggest that 10–30% of germline mutations in BRCA1 may be large deletions, of which half may be deletions which affect the promoter region and eliminate transcription. The potential presence of such deletions clearly needs to be considered when screening individuals for BRCA1 germline mutations.

MATERIALS AND METHODS

Family

Members of K2035 contacted us and asked to participate in our genetic study. All participants signed informed consent documents, and this research project was approved by the University of Utah School of Medicine Institutional Review Board. Individuals completed a questionnaire and gave a sample of blood. The family was extended to all possible mutation carriers and their relatives. It is an extended family of 55 individuals, including spouses, with seven cases of unilateral breast cancer (age of onset 27–53 years), two cases of bilateral breast cancer (age of onset 33/34 and 46/55 years) and one case of bilateral breast and ovarian cancer (diagnosed at age 45 and 62 years with breast cancer, and age 59 years with ovarian cancer). It had been linked previously to BRCA1 with a LOD score of 2.25 at D17S1327 (2). CSH carrier status had been assigned based on a shared, six-marker haplotype segregating with the disease.

DNA extraction

Nuclear pellets were prepared from 16 ml of ACD blood, then extracted with phenol and chloroform. DNA was precipitated with ethanol and resuspended in Tris–EDTA.

Restriction mapping

DNA from P1 1141 was digested with HindIII and EcoRI, and Southern blots were prepared. BRCA1 exons 2–11 were radiolabeled by PCR, then hybridized singly and in combination to the blot. Some EcoRI fragments were also used as probes. A restriction map was created based on the locations of the exons, hybridization of EcoRI fragments, double digests and bands shared with other clones in the region. Recently published BRCA1 genomic sequence (ref. 13; GenBank number: L78833) was used to confirm and complete portions of the map. Additional mapping was later performed with EcoRV and BamHI.

Hybridization probes and sequencing

Restriction fragments were cut from agarose gels and purified with a GeneClean kit (BIO 101). A vector [pBluescript II (Stratagene), digested to have a compatible end and treated with calf intestinal phosphatase, was ligated onto one or both ends of the fragments. The random priming technique (RPT) was then employed as previously described (16) to create PCR products using one specific primer in combination with an array of single, randomly selected oligonucleotides. After incubation, the ligation reactions were diluted 1:100 for use as PCR templates. T3 and T7 oligonucleotides were employed as specific primers for the RPT. Products were sequenced with a Cyclist kit (Stratagene) to determine if repetitive sequences were present which would interfere with their use as hybridization probes. A probe’s location on the restriction map was confirmed by its pattern of hybridization to the Southern blots.

Probe A was obtained from the promoter-distal end of a 2.3 kb EcoRI fragment located upstream of exon 3. Probe B was created on the promoter-distal end of a 6.8 kb HindIII fragment located upstream of the first exon. Since probe B detected an altered band on the Southern blot of the EcoRV digest, but not the Southern blot of the BamHI digest, the breakpoint was localized to a 1.3 kb BamHI/EcoRV fragment. Probe C was obtained from this fragment. The probes ranged in size from 350 to 600 bp in length, including the vector sequence.

Southern blot analysis

DNA preparations from one CSH carrier and one noncarrier (5 µg for each) were digested individually with BamHI, EcoRI, HindIII, EcoRV, PvuII and BglII. The digests were electrophoresed and transferred to Hybond N+ nylon membrane (Amersham). Blots were crosslinked by exposure to UV light and were prehybridized for 2 h at 65°C in a solution consisting of 10% PEG 8000, 7% SDS, 5x SSPE, and 200 µg/ml of sheared salmon sperm DNA which had been boiled prior to addition.

Probes for hybridization consisted of RPT PCR products which were reamplified in a 30 µl volume for 45 cycles. Reaction mixtures contained 200 µM dGTP, dTTP and dATP; 5 µM dCTP; 15 µCi [α-32P]dCTP; 0.5 µM each primer; 1 U of AmpliTaq DNA Polymerase (Perkin Elmer); GeneAmp buffer (Perkin Elmer) and template.

The radiolabeled PCR products were washed through NucTrap Purification Columns (Stratagene) and boiled for 5 min prior to the addition of ~2× 106 CPM to the hybridization mixtures. The blots were incubated overnight at 65°C, and were washed in 1× SSPE/0.1% SDS at 55–65°C for ~15 min. Additional washes were performed in 0.1x SSPE/0.1% SDS if required.

PCR primers

The primer sequences for the ~130 bp intron 2 polymorphism were: 5'-AACTCCAGCGCAGAAGCTAA-3' (forward), and 5'-TTGTAAGAGACAGGCCAGTTTCA-3' (reverse). The product was amplified and electrophoresed using standard techniques for genotyping.

Primers which amplified the PCR product across the deletion junction came from sequence contained within probe C on the upstream end. Primers on the intron 2 side were placed below an Alu cluster near the distal end of the 7.1 kb EcoRI fragment containing exon 2. The primer sequences for the 1.3 kb external product were: 5'-CCACTGGAGCACTAAAGCATAAA-3' (forward), and 5'-GA-TATTGTAGGGAAGAGATCATCAG-3' (reverse). The nested primers used to amplify the 650 bp product were: 5'-GGGA-
PCR reactions were performed with a GeneAmp™ PCR System 9600 thermal cycler (Perkin Elmer), using standard reagents. The external product was amplified for 30 cycles of: 94°C for 15 s, 55°C for 15 s, and 72°C for 1.5 min. These reactions were diluted 1:100 and reamplified with nested primers using a similar protocol. To ensure that the reactions worked, primers which amplified a ∼300 bp product from another locus were included in each mix.

Statistical analysis

For penetrance estimates, a non-parametric Kaplan–Meier analysis was used to estimate the probability of women developing cancer as a function of age. Unaffected women were classified as censored at their current age or age at death, if deceased.

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REFERENCES