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ORIGINAL ARTICLE

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The *BRCA2* genetic variant IVS7 + 2T → G is a mutation

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Abstract Biochemical and genetic characterizations that support the conclusion that the variant *BRCA2* IVS7 + 2T → G represents a deleterious mutation are presented. RNA analysis from a breast cancer patient with *BRCA2* IVS7 + 2T → G showed that the productive message was produced from only one chromosome. A haplotype analysis confirmed that the intronic variant resides on the chromosome that does not produce the normal mRNA. Additionally, an RNA splicing product that deletes exon 7 was produced by the chromosome that carries *BRCA2* IVS7 + 2T → G. The deletion of exon 7 from the RNA alters the open reading frame by removing residues 249–287 and incorporating 18 abnormal amino acids before terminating with an opal stop codon. The experimental approach presented produces strong evidence of the presence of a deleterious mutation, because the contribution by both chromosomes to each RNA species analyzed was tracked using a coding region polymorphism as a marker. Furthermore, a single nucleotide polymorphism (SNP) haplotype analysis that confirms the location of the intronic variant and an associated family history that shows a high incidence of cancer supported these biochemical data.

Key words *BRCA2* · RNA · Splice · Mutation · Intron · Cancer

Introduction

The tumor suppressor breast cancer susceptibility gene, *BRCA2*, is located in a 70-kilobase region of chromosome 13 and produces an 11-kb mRNA encoded by 27 exons (Tavtigian et al. 1996). Mutations in *BRCA2* have been reported to increase the risk of a second contralateral breast cancer to 52% (Breast Cancer Linkage Consortium 1999) and the risk of ovarian cancer to 27% (Ford et al. 1998) by age 70, and are associated with increased risks for prostate (Breast Cancer Linkage Consortium 1999; Easton et al. 1997; Ford et al. 1994) and pancreatic (Breast Cancer Linkage Consortium 1999; Goggins et al. 1996; Phelan et al. 1996) cancer. Mutations in *BRCA2* have also been linked to male breast cancer (Easton et al. 1997; Couch et al. 1996).

Unlike many genes that contain mutation "hot-spots," genetic variants are dispersed throughout the *BRCA2* gene. Approximately 83 of 684 genetic variants recorded in *BRCA2* occur in introns (Breast Cancer Information Core; BIC 1999). Of these intronic variants, 26 were characterized as uncertain, 25 as splice mutants, and 32 as polymorphisms. Biochemical analysis is the most reliable method for establishing a causal association between the variant and disease predisposition, because the primary structure of the RNA molecules is determined. Other analyses may be confounded by undetected variants or genomic deletions that are in linkage disequilibrium with the intronic variant. Furthermore, the effects of a mutated splice consensus site could be negated by the activation of a nearby cryptic site.

Accurate RNA splicing requires conserved sequence motifs at the intron-exon junctions and at the branch site (Shapiro and Senapathy 1987). Genetic variants can disrupt splicing by altering consensus sites or by activating "cryptic" sites that are normally silent. Additionally, the splicing machinery normally produces more than one mature transcript, even in the absence of genetic variants. Many normal alternative RNA minor species can also be generated; commonly, incomplete removal of introns or the deletion of whole exons occurs. The presence of these alternative splice products complicates RNA analysis, because polymerase chair reaction (PCR) amplification from cDNA can generate numerous fragments whose distribution can vary depending upon sample processing.

The association of several variants with disease has been characterized by genetic linkage, population frequency analysis, or functional or biochemical approaches. Combi-

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nations of these approaches can provide supporting results that strengthen conclusions regarding the clinical significance of the variants. Here, results from biochemical analysis, family history, and haplotype analysis were combined to provide strong evidence for classifying this intronic variant as deleterious.

Subjects and methods

Subjects

Full sequence analysis of *BRCA1* and *BRCA2* coding regions and splice consensus sites (BRACAnalysis; Myriad Genetic Laboratories, Salt Lake City, UT, USA) of the proband, patient C, revealed only the uncertain genetic variant *BRCA2* IVS7 + 2T → G and single nucleotide polymorphisms, including *BRCA2* 5′ untranslated region (UTR)203G → A. The uncertain clinical significance of the intronic variant was reported to the patient through the patient's physician and genetic counselor. Following consultation, the patient elected to participate in this research protocol.

A control sample included in all analyses in this study is also heterozygous for the polymorphism *BRCA2* 5′UTR203G → A, among other single nucleotide polymorphisms (SNPs).

Five individuals who carry *BRCA2* IVS7 + 2T → G, designated patients A–E, were identified at Myriad Genetic Laboratories. The proband, patient C, was diagnosed with ovarian cancer in her sixties; her sister and mother were diagnosed with breast cancer in their forties and sixties, respectively. Patient A was diagnosed with breast cancer in her forties; her mother, an aunt, and a grandmother were diagnosed with breast cancer in their forties, fifties, and sixties, respectively. Patient A also has two sisters (patients D and E) who have not been diagnosed with cancer, but are among the five patients at Myriad Genetics who carry the *BRCA2* IVS7 + 2T → G variant; another, unaffected, sister tested negative for the intronic variant. Patient B was diagnosed with breast cancer in her forties and has three sisters who have been diagnosed with breast cancer (sister 1 and sister 2 [bilaterally]), pancreatic cancer (sister 3), and ovarian cancer (sister 2) at unreported ages. She also has a brother with prostate cancer (age at diagnosis unreported) and two aunts with breast cancer.

Nucleic acid techniques

The RNA isolation from peripheral blood leukocytes and cDNA synthesis used previously described techniques (Scholl et al. 1999). Primer selection permitted the specific amplification or discrimination of products derived from DNA or RNA. In this way, primers targeted to residual DNA in these samples permitted confirmation of the genotypes that were identified by the clinical test.

The primers used in this analysis conform to the following designations and gene-specific sequences (all numeric base pair designations conform to GenBank submission U43746) (Tavtigian et al. 1996): 1AF 5′-GCTTCTGAAAC TAGGCGGCAGA (bases 11-32), 1BF 5′-GAGAAGCGT GAGGGGACAGATT (bases 106-127), 3AR 5′-CAGAG TCAGCCCTTGCTCTTTGA (bases 492-470), 3BR 5′-GCCAGCTGATTATAAGATGGTTTCCTT (bases 452-426), 5AF 5′-GTCTTAGTGAAAGTCCTGTTGTTCTA CA (bases 641-668), 5BR 5′-GTCCTGTTGTTCTACAAT GTACACATGT (bases 652-680), 10BR 5′-TGGCATTG ACTTTCCAATGTGGTCTT (bases 1092-1067), 6/8R 5′-GATGCTTCTTCATTTCTGA/CTTCACAAACT (bases 878-860 and 744-734). Primer 10AR was previously described (Tavtigian et al. 1996). The slash in primer 6/8R sequence indicates the juxtaposition of sequence for exons 6 and 8. M13 tails with the sequences 5′-GTTTTCCCAGTC ACGACG for forward (designated by an F) or 5′-AGGA AACAGCTATGACCAT for reverse (designated by an R) were incorporated on all primers to facilitate nucleotide sequencing. Sequencing employed dye-primer chemistry detected on automated instruments (ABI Prism Model 377; Applied Biosystems, Foster City, CA, USA).

BRCA2 SNP haplotyping

The following common *BRCA2* SNP loci were used for haplotype analysis, in the manner described previously for *BRCA1* (Shattuck-Eidens et al. 1997): 5′UTR203G → A, H372N, K1132K, V1269V, T1915M, S2414S, and IVS16-14C → T.

Results

Reactions with DNA-specific primers verified that the patient sample contained the uncertain variant *BRCA2* IVS7 + 2T → G and the polymorphism *BRCA2* 5′UTR203G → A, while the control carried only the polymorphism (data not shown).

Amplification with primer pair 1AF and 10AR, using patient and control cDNA as targets, produced bands of a size that corresponded to the wild-type transcript. These bands were excised from a 1% low-melt agarose gel and used as targets in subsequent PCR reactions. There, each band was reamplified and sequenced with the primer pairs 1BF-3AR and 5AF-10BR. These experiments are depicted schematically in Fig. 1 (amplicon 1). Sequencing results from both the patient and control showed correct splicing for exons 5 through 10 (Fig. 2A, only junctions 6–9 shown). However, analysis of the polymorphism in the patient sample, using primers 1BF and 3AR, showed that only the chromosome carrying G at position 203 of the 5′UTR contributed to the normal mRNA, while both chromosomes contributed to this product in the control (Fig. 2B). Because the patient is heterozygous at this polymorphic base, this result indicates that the other chromosome, which carries adenine at position 203, does not contribute to the production of normal *BRCA2* protein, because its transcript is missing.

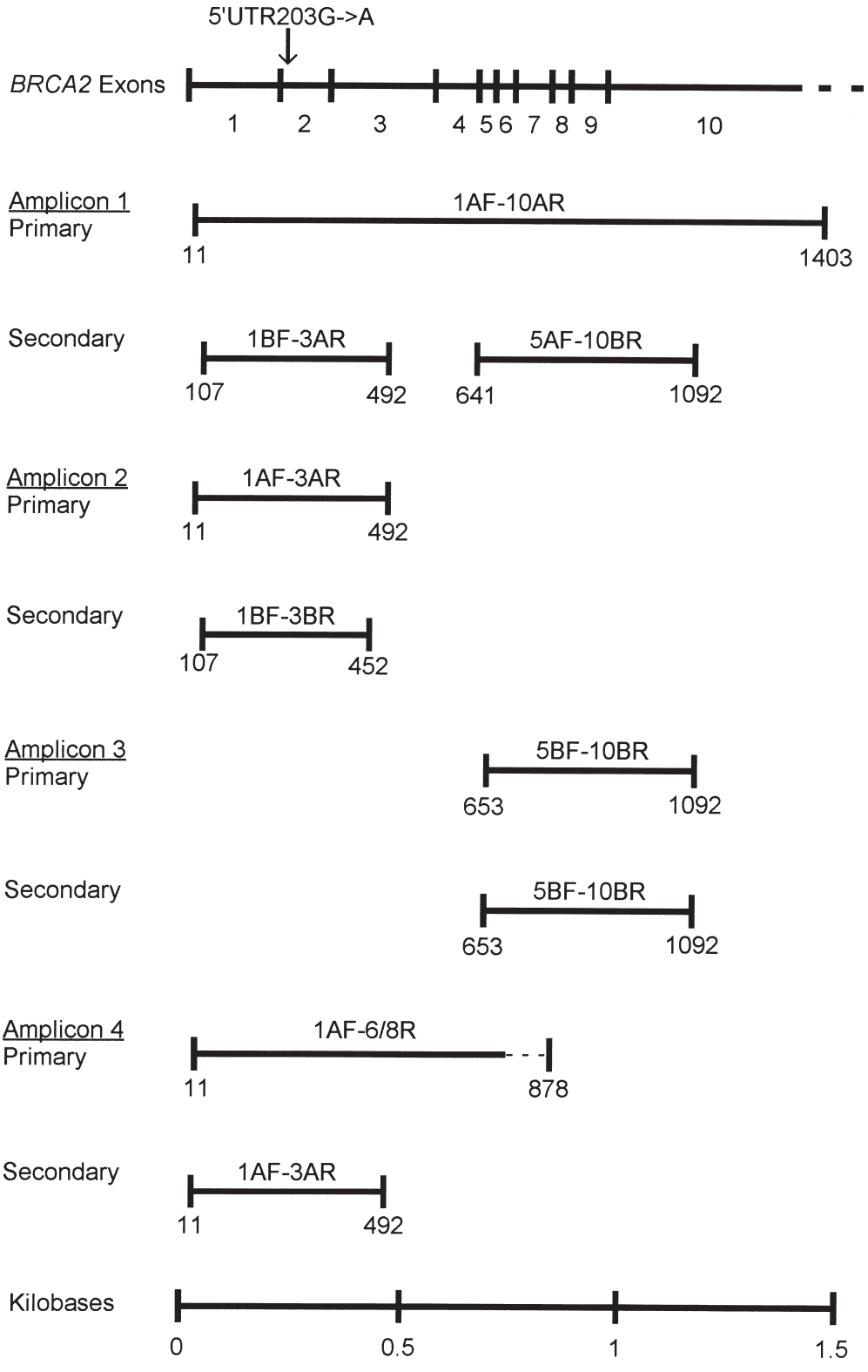

Fig. 1. Schematic of fragments amplified and sequenced to analyze *BRCA2* IVS7 + 2T → G mRNA 5′ of exon 10. The figure schematically depicts the exons of *BRCA2*. The position of the 5′UTR203G → A polymorphism is indicated (*arrow*). The primer pairs used to amplify fragments are mapped below the RNA with boundaries in base position corresponding to GenBank submission U43746 (Tavtigian et al. 1996). Note that, in amplicon 4, the primer 6/8R spans exon 6 and 8 (deletion of exon 7) and so is represented by a *dotted line*. *Primary* is used to designate polymerase chain reactions (PCRs) that were targeted to cDNA, while *secondary* reactions used amplified products, usually gel-purified, as targets

No other obvious bands that might represent additional splicing products were detected in the agarose gel. Because aberrant transcripts, especially those that contain premature nonsense codons in the 5′ region of the transcript, can have reduced stability (Culbertson 1999; Frischmeyer and Dietz 1999; Beelman and Parker 1995; Sachs 1993), abnormal RNA splicing products resulting from IVS7 + 2T → G could be of low abundance. Intronic variants in acceptor splice sites commonly lead to exon skipping, while those in donor sites may lead to the inclusion of all or portions of an intron (Pohlenz et al. 1998; Talerico and Berget 1990). In light of these results, several attempts were made to detect

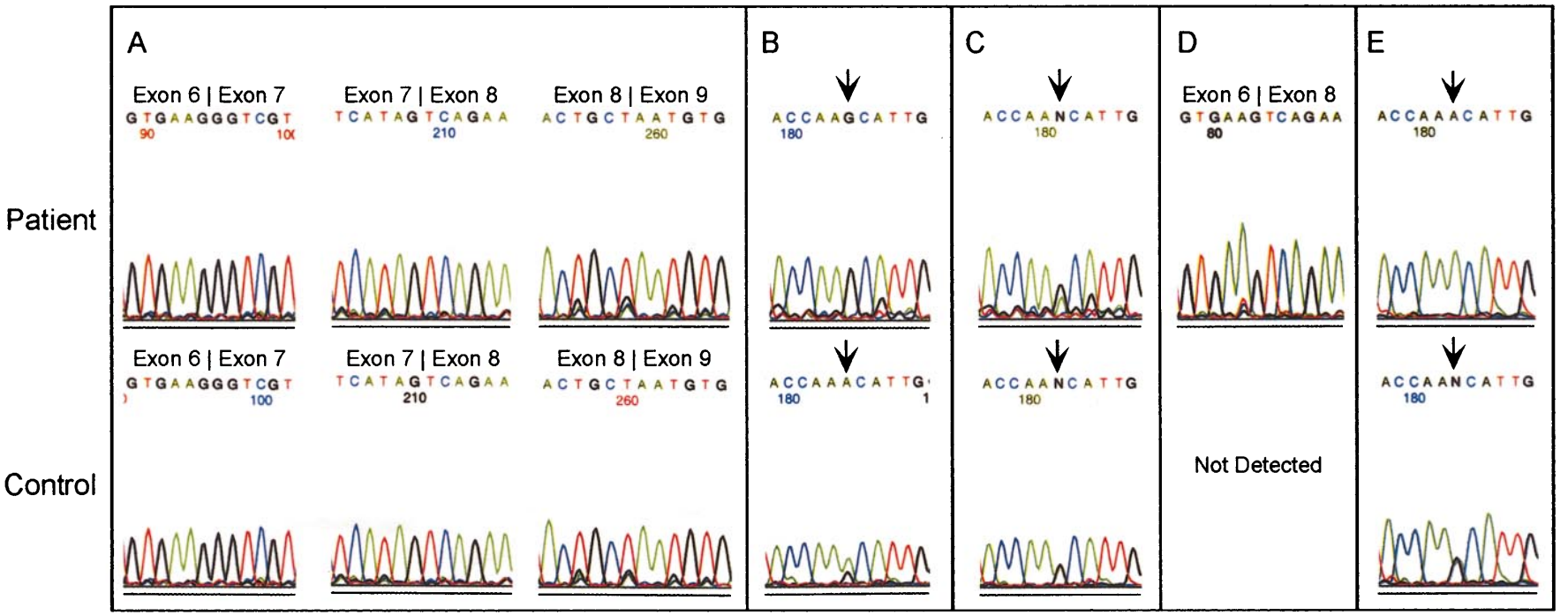

Fig. 2A–E. Sequencing results from *BRCA2* IVS7 + 2T → G amplifications. The sequencing results from amplicons depicted in *Fig. 1* are shown for the patient and control. **A** Sequencing products from amplicon 1 show correctly spliced mRNA for exons 6 through 9. **B** Sequencing results of amplicon 1 for the patient show contribution to the correctly spliced product by only the chromosome carrying G at *BRCA2* 5′UTR203, while both chromosomes contributed to this product in the control. In the control sample, the heterozygous base is clearly visible as reduced A and increased G signals, despite the "A" base designation by the software basecaller. **C** Results from amplicon 2 show the presence of transcripts from both chromosomes in the patient and control. **D** The results of amplicon 3 produced a product that deletes exon 7 that was visible only in the patient (see Fig. 3 and text). **E** The results of amplicon 4 show that the chromosome carrying A at 5′UTR203 produces the vast majority of the transcript that deletes exon 7 in the patient, while both chromosomes produce approximately equal amounts in the control. The transcript that deletes exon 7 appears to be a normal low-level alternative product, whose abundance in the patient is greatly increased by the mutation of the donor site (see text)

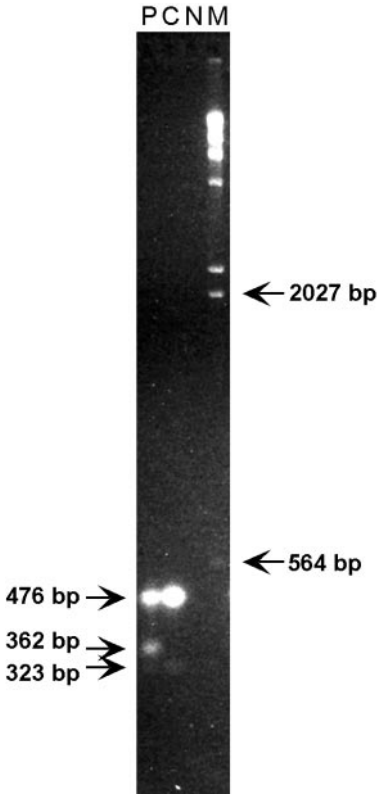

Fig. 3. Agarose gel showing PCR fragments that delete exon 7. The results of amplicon 3 are shown. The *arrows* indicate the positions and sizes of the products that were excised from the gel. The *upper band* (present in both patient and control) is produced by the correctly spliced transcript. The *lower band* (very faint in both patient and control) is produced by an alternative splicing event (see text). The *middle band*, detected only in the patient, is the result of the deletion of exon 7 from the mRNA. *P*, Patient; *C*, control; N, negative control; *M*, Marker (λ *Hind*III fragments)

RNA species that contained sequence from intron 7 by placing reverse primers in this region. No RNA products that included intron 7 were detected, despite tests with several primer pairs.

Two additional PCR reactions that generated short fragments centered around the polymorphism and exon 7 (Fig. 1, amplicons 2 and 3) were performed as a sensitive measure to detect RNA species from the chromosome carrying adenine at position 203. Amplicon 2 (Fig. 1) was performed to determine whether any transcripts produced by that chromosome were present in the patient sample. The bands were excised from the gel, amplified with primers 1BF and 3BR, and sequenced. These results show that some transcripts derived from the chromosome carrying A at 5′UTR203 (Fig. 2C) are present in the patient sample.

Amplicon 3 (Fig. 1) was tested to detect transcripts that delete exon 7. These reactions produced three bands in the patient and two in the control (Fig. 3). The bands were excised from the gel, reamplified using the same primers, and sequenced. The upper band (in Fig. 3) is the wild-type product, while the lowest band is an alternative product that deletes exon 7 and all of exon 6, except for the first two bases (data not shown). The middle band (in Fig. 3), which is visible in the patient, but not in the control, deletes exon 7 (Fig. 2D). These results suggest that an aberrant transcript that deletes exon 7 is produced in the patient sample that may be caused by the intronic variant. No products from amplicon 1 that might contain a deletion of exon 7 were detected on agarose gels. These fragments either did not resolve from the wild-type product on agarose gels or were too scarce to visualize. There is an apparent difference in the patient between the ratio of the wild-type and mutant products detected on agarose gels (Fig. 3) and the fluorescent signal intensities for the A and G bases (Fig. 2C). These results are not discrepant, because they are derived from the employment of different semi-quantitative techniques with different amplicons.

A reverse primer that juxtaposes exons 6 and 8 (6/8R) was synthesized to specifically amplify products that were missing exon 7. This primer was used in conjunction with primer 1AF to amplify across the polymorphism at 5′UTR203 (Fig. 1, amplicon 4). While no fragment from primary amplification was visible on agarose gels, semi-nested reamplification of this reaction mixture, using primers 1AF and 3AR, produced visible products that were gel-purified and sequenced in the patient and the control (Fig. 2E). This result shows that the vast majority of the transcript that deletes exon 7 in the patient is derived from the chromosome carrying A at position 203 of the 5′ UTR, while the control produces this transcript equally from both chromosomes. It seems likely that RNA splicing that deletes exon 7 is more common in the patient than in controls, because the exon 7 deletion fragment from amplicon 3 was visible only in the patient. The transcript that deletes exon 7 appears to be a normal low-level alternative product, whose abundance is greatly increased by the mutation of the donor site in the patient. If IVS7 + 2T → G prevents normal splicing, more RNA could enter the pathway that results in the deletion of exon 7. In PCR, the abundance of deletion product from the chromosome with A at position 203 masks the small amount that is alternatively produced by the chromosome with G at position 203. Therefore, only the adenine peak may be detected in the sequence shown in Fig. 2E.

SNP haplotype analysis (Fig. 4) shows the common haplotypes for the carriers of *BRCA2* IVS7 + 2T → G whose entire *BRCA2* gene was sequenced, and the corresponding haplotype pairs. Within the patient database at Myriad Genetic Laboratories, two additional patients were positive for *BRCA2* IVS7 + 2T → G, but were not tested at the SNPs and are therefore not included. It is likely that this variant has occurred only once in evolution (Shattuck-Eidens et al. 1997; Neuhausen et al. 1996) and resides on haplotype 9, which is shared by all three informative patients. These results indicate that IVS7 + 2T → G occurs on haplotype 9. Of 306 occurrences of haplotype 9 among the patients at Myriad Genetic Laboratories who were tested at all of the relevant SNP loci, three carried IVS7 + 2T → G. Given that this cohort of patients was selected on the basis of likely genetic predisposition to breast and/or ovarian cancer and is highly enriched in *BRCA2* gene mutations, it

A

Haplotype	5′UTR203G->A	H372N	K1132K	V1269V	T1915M	S2414S	IVS16-14C->T	Haplotype designation
		■		■			■	4
	■	■	■			■		8
	■	■	■		■	■		9

B

Anon ID	5′UTR203G->A	H372N	K1132K	V1269V	T1915M	S2414S	IVS16-14C->T	Haplotype Pairs
A	2	2	2	0	2	2	0	9 + 9
B	2	2	2	0	1	2	0	9 + 8
C	1	2	1	1	1	1	1	9 + 4

Fig. 4A,B. Single nucleotide polymorphism (SNP) haplotype analysis of carriers of *BRCA2* IVS7 + 2T → G. **A** SNP haplotype designations. *A solid box* indicates the presence of a nonconsensus base. **B** Polymorphisms among *BRCA2* IVS7 + 2T → G carriers (*A*, *B*, *C*, Patients A, B, C). The number of nonconsensus alleles in each locus is depicted (0, 1, or 2). The combinations of haplotypes that account for these nonconsensus alleles are given. Patient *C* is the index case from which *BRCA2* IVS7 + 2T → G was analyzed biochemically. *Anon ID*, anonymous identification

is likely that IVS7 + 2T → G is found on fewer than 0.1% of haplotype 9 carriers in the general population.

Discussion

These results support the conclusion that *BRCA2* IVS7 + 2T → G is a deleterious mutation that increases predisposition to cancer. Most significantly, only the chromosome carrying G at position 203 of the 5′ UTR contributed to the normal mRNA. Also, an aberrant transcript that deletes exon 7 was produced by the chromosome carrying the mutation at greatly increased levels in the patient, but was very rare and produced by both chromosomes in the control. The deletion of exon 7 is a reasonable product of a lesion at this highly conserved base in the donor splice site. Also, the data from the haplotype analysis associates the intronic variant with the defective chromosome.

The family history of cancer (described in the "Subjects and methods" section) is extensive among patients who carry *BRCA2* IVS7 + 2T → G. The high incidence of cancer among those families that carry *BRCA1* IVS7 + 2T → G supports the conclusion that the variant increases predisposition to disease.

The deletion of exon 7 alters the *BRCA2* reading frame by removing residues 249–287 and incorporating 18 abnormal amino acids before terminating at an opal stop codon. Like the majority of deleterious mutations in *BRCA1* and *BRCA2*, this intronic variant is infrequently observed (Shen et al. 1998). In the more than 5800 patients who submitted to sequence analysis at Myriad Genetic Laboratories, only five occurrences of this variant have been identified. The high incidence of novel mutations warrants full sequence analysis, rather than screening techniques, to provide the best opportunity to identify mutations in these genes.

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