Letters to the Editor

J Med Genet 1999;**36**:262–263

BRCA1 expression is not affected by the intronic 12 bp duplication

EDITOR—About 3-10% of breast cancer cases have been estimated to result from a hereditary susceptibility to the disease.[1] A significant proportion of these are the result of mutations in the BRCA1 gene.[2] While several hundred BRCA1 gene mutations have been identified, the disease causing effect of some BRCA1 variants is a matter of current debate. In a recent issue of this journal, a 12 bp duplication in intron 20 of the BRCA1 gene has been proposed to be a regulatory mutation.[3] This intronic variant had initially been described in a North American patient and was tentatively classified as a splicing mutation.[2 4] It has also been detected in a population based sample of young breast cancer patients and was suggested to affect mRNA processing.[5] In another study of Polish patients, a splicing effect was excluded but mRNA reduction has been suggested as a possible consequence of the variant.[6] Robledo *et al*[3] provided experimental evidence to suggest that the intronic 12 bp duplication could act as a regulatory mutation resulting in the allele specific reduction of BRCA1 expression,[3] although the underlying mechanism for such an effect remained unclear. Here we have investigated the expression of BRCA1 mRNA transcribed from the allele carrying the 12 bp duplication and provide evidence against a regulatory effect of this variant.

We have screened genomic PCR products from lymphocytes of breast cancer patients for mutations in exon 20 and flanking intronic sequences of the BRCA1 gene. In a series of 700 unselected breast cancer patients who had given their written informed consent to be tested, two German patients (0.3%) were identified as carriers of the 12 bp duplication in intron 20, starting at position +48 (fig 1). The presence of this variant was uncovered by the length difference of the respective PCR products on agarose gel electrophoresis and was confirmed by direct sequencing. One of the two patients was 69 years old at diagnosis and did not report any family history of breast cancer. The other patient was diagnosed by the age of 60 years and reported one grandmother with breast cancer. Her mother had eventually died from ovarian cancer by the age of 78 years but no material was available for confirmation. In order to investigate whether the 12 bp duplication could be a functionally significant BRCA1 mutation in our two patients, additional peripheral blood samples were obtained from these two index cases and total RNA was extracted from their lymphocytes. After reverse transcription with random hexamer primers, selected regions of the BRCA1 cDNA were amplified using primers located in different exons. When we amplified a cDNA region spanning BRCA1 exons 19-21, only product of wild type length was observed and no evidence for any aberrant splicing could be obtained in either case, which is in agreement with the results of others.[3 6] We next investigated whether allele specific expression might be changed as a consequence of the intronic duplication. Because one of the two index patients (the one reporting a possible family history of breast cancer) was heterozygous for a neutral polymorphism, Ser1613Gly in exon 16 of the BRCA1 gene,[7] we investigated whether both alleles were equally present in the cDNA from this patient. We thus amplified a cDNA region spanning exons 15 to 17 using the two PCR primers 5'-ACTACCCATCTCAAGAGGAGC-3' and 5'-CTGGCAAACTTGTACACGAGC-3' under standard PCR conditions. The RT-PCR products were subsequently digested with the restriction enzyme *Scr*FI, because one new recognition site for this enzyme is created by the presence of the Gly1613 allele. In two different cDNA preparations from the index patient whose DNA was heterozygous for the allele carrying the Gly1613 substitution and the 12 bp duplication, a clearly heterozygous pattern was also observed in the RT-PCR product (fig 2). Similar heterozygous patterns were obtained when using the enzymes *Ava*II or *Nla*IV to distinguish between both alleles (data not shown). These findings indicate that both BRCA1 alleles are equally expressed in the lymphocyte mRNA from this carrier of the 12 bp duplication. Any allele specific alteration in the BRCA1 mRNA levels in this sample would have to have been very subtle to remain undetected by our RT-PCR based assay, and therefore would be unlikely to result in a loss of BRCA1 function.

Thus, our observations are in agreement with the previously reported lack of aberrant splicing resulting from the intronic 12 bp duplication, but they differ from the results reported by Robledo *et al*[3] with respect to the relative expression of this variant allele. The reasons for these differences are unknown at present. It is possible, for example, that the presence of an undetected BRCA1 mutation could have resulted in the transcript instability

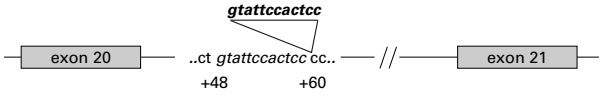

Figure 1 Sequence and location of the 12 bp duplication within intron 20 of the BRCA1 gene. The duplicated sequence between positions +48 and +60 of intron 20 is shown in italics. One out of 13 possible 12 bp insertion events resulting in the duplication of this sequence is illustrated. Exons and introns are not drawn to scale.

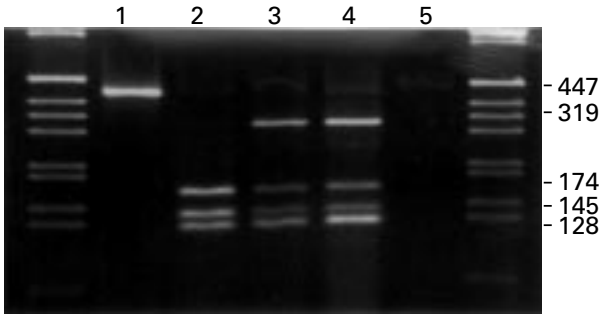

Figure 2 Allele specific analysis of RT-PCR products spanning exons 15-17 of the BRCA1 cDNA. Lane 1: undigested RT-PCR product (447 bp). Lanes 2-5: RT-PCR products digested with ScrFI; lane 2: patient 1, homozygous for the G1613 allele; lanes 3 and 4: two samples from patient 2, heterozygous for the S/G1613 polymorphism; lane 5: control without reverse transcriptase. Outer lanes: size marker (kb ladder from Gibco BRL). ScrFI digestion of the product carrying the Gly1613 codon yields fragments of 174, 145, and 128 bp, whereas ScrFI digestion of the product carrying the Ser1613 codon yields fragments of 319 bp and 128 bp, respectively.

reported in the previous study.[3] One caveat of both studies is that expression patterns in lymphocytes may not necessarily reflect expression patterns in other tissues such as breast epithelium. In addition, previous investigations have shown that BRCA1 mRNA expression in normal mammary epithelial cells in culture is highly sensitive to growth conditions and cell cycle status.[8] Given these complexities of BRCA1 regulation, it is hard finally to rule out the possibility that the intronic 12 bp duplication could have a regulatory effect in any particular cell population under certain physiological circumstances. Although the intronic 12 bp duplication was seen only twice in our patient cohort, its relatively high incidence in some previously reported series indicates that a closer examination of this allele is important with respect to genetic diagnosis and counselling of breast cancer families. Because our present study, within its above mentioned limitations, failed to confirm the proposal that the presence of the intronic 12 bp insertion alone would be sufficient to have a real effect on BRCA1 expression in breast cancer, we suggest that this variant should not be regarded as a disease causing mutation unless definite proof can be obtained to show any impairment of BRCA1 level or function in carriers of the duplication.

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J Med Genet 1999;**36**:263–264

First putative sequence alterations in the minimal CFTR promoter region

EDITOR—In the February 1998 issue of the Journal, Verlingue *et al*[1] reported an absence of mutations in the promoter region of the CFTR (cystic fibrosis transmembrane conductance regulator) gene. They analysed a region that spans over 3.9 kb of sequences upstream of the first CFTR exon, including the CFTR promoter, down to 1.3 kb within the first intron. These sequences, shown previously to contain potential regulatory elements,[2–4] had been selected on the basis of conservation throughout evolution (phylogenetic footprints) from rodents to primates.[2 3] Verlingue *et al*[1] analysed a cohort of 205 subjects including patients with classical cystic fibrosis (CF), disseminated bronchiectasis, or congenital bilateral absence of the vas deferens (CBAVD), carrying either one or no mutation after scanning all 27 CFTR exons by DGGE (denaturing gradient gel electrophoresis). They further screened 5.2 kb of targeted sequences spanning the CFTR promoter region, but were unable to detect any putative disease related mutation in their sample.

We report the first three nucleotide alterations in the CFTR minimal promoter, defined as a 250 bp fragment upstream of the ATG translation start codon.[4] These sequence changes were identified by DGGE followed by direct DNA sequencing on an ABI 377 automated sequencer by using primers specially designed for optimal amplification of this GC rich region (forward: 5'-GGCTCGAGGCTGGGAGTC-3', reverse: 5'-TTCCATGGTCTCTCGGGCGCTGGGGT-3'). From a total of 450 patients referred to our laboratory for molecular analysis of the CFTR gene since 1989, we detected 118 different mutations responsible for CF or CBAVD (M Claustres, unpublished data). The high allelic heterogeneity of the populations studied might have raised the chances of discovering putative mutations in regulatory sequences.

Mutation −33G→A was identified in a male adult with CBAVD from southern France with an as yet undefined second mutation. The patient was homozygous for allele 7T at the splice acceptor site in intron 8 and heterozygous for M470V in exon 10.

Mutation −94G→T was characterised in a South African Black CF female. She had been diagnosed with CF on the basis of two positive sweat tests and severe chronic lung disease. The other allele remains unknown.

Mutation −102T→A was detected in two unrelated CF patients from southern France previously found to be compound heterozygotes for two CF mutations in the coding portion of CFTR. Careful familial segregation studies showed that −102T→A was associated in cis (on the same allele) with the mutation S549R (T→G). One female patient, born in early 1992 and diagnosed when she was 5 years old (sweat chloride value 118 mmol/l), had a genotype −102T→A+S549R (T→G)/ΔF508. The male patient, born in 1980 and diagnosed at the age of 9 (sweat test 74 and 96 mmol/l), had a genotype −102T→A+S549R (T→G)/S945L. Both patients had mild pulmonary disease and were classified as pancreatic sufficient by the clinicians. As the S549R mutation has been previously described as a "severe allele" associated with pancreatic insufficient status,[5] we wondered whether −102T→A could modulate the clinical phenotype in these patients. A small number of complex alleles containing more than one mutation have previously been observed in CF,[6 7] and at least two of them, R553Q/ΔF508[6] and 7T-R117H[8] have been shown to contribute to a milder phenotype by inducing changes in the conformation of mutant protein or in the splicing of mRNA mutant transcripts or both. We have undertaken a collaborative study of phenotype/genotype correlations in patients carrying S549R (T→G),

with or without −102T→A, and functional analyses in transient transfections in order to support our preliminary findings.

None of these sequence alterations was detected in a further 238 normal, 376 CF, and 158 CBAVD chromosomes from our sample, which suggests that they are either very rare polymorphisms or potential regulatory mutations. It is noteworthy that each of these variations is located in evolutionarily conserved sequences (S Vuillaumier, unpublished data). The −102T→A alteration was detected within a 28 bp sequence that shares 89% homology with a segment of the human α1(I) collagen promoter involved in the regulation of the gene.[9] A computer aided search for the presence of transcription factor binding sites, using the Patsearch program,[10] indicated that −33G→A is located in a PEA3-like motif, −94G→T in a GC box, and −102T→A in potential binding sites CCAAT-like or CarG-like among other possible cis acting elements. Interestingly, computer analysis of the antisense strand, determined that −102T→A creates a YY1 repressor site, which matches completely with the consensus sequence.[11]

There is a relative paucity of information regarding naturally occurring variation in the 5' upstream regions of human genes, with available data indicating low nucleotide diversity at these sites. So far, only four other sequence changes have been described in the 5' region of CFTR, three polymorphisms, −966T→G,[1] −895T→G, and −816C→T,[12] and a putative deleterious mutation −741T→G.[12] Moreover, in spite of several studies on the patterns of CFTR expression in men and rodents, the molecular mecanisms involved in CFTR gene regulation remain unknown. Further functional analyses will be required in order to determine whether the base pair substitutions reported in this study could lead to subtle variations in levels of CFTR expression.

This study was supported by AFLM (Association Française de Lutte contre la Mucoviscidose), by AFM (Association Française contre les Myopathies), and the ICF(M)A (International Cystic Fibrosis (Mucoviscidosis) Association). We thank the CF families and the clinicians who collaborated in this study, particularly Dr Lesbros.

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