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The variants *BRCA1* IVS6-1G>A and *BRCA2* IVS15+1G>A lead to aberrant splicing of the transcripts

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Abstract The majority of *BRCA1* and *BRCA2* deleterious mutations and variants of unknown significance have been identified in genomic DNA and their effects at the mRNA level have not been reported. Our aim was to ascertain the pathological effect of the *BRCA1* IVS6-1G>A (c. 302-1G>A) and the *BRCA2* IVS15+1G>A (c. 7617+1G>A) variants detected in Spanish breast/ovarian cancer families. Sequencing of cDNA from the *BRCA1* IVS6-1G>A allele revealed an inappropriate splicing of exon 7. The analysis of

the *BRCA2* IVS15+1G>A allele showed the skipping of exon 15. Both alterations predicted the appearance of premature stop codons. Our findings highlight the importance of studying mutations at DNA and RNA levels in order to clarify the effect of the suspected mutation and to provide adequate counseling for breast/ovarian cancer families.

Keywords Hereditary breast cancer · *BRCA1* · *BRCA2* · Splicing mutation

An RNA analysis published online recently, showed that the *BRCA2* IVS15+2T>G caused skipping of exon 15 in RNA isolated from skin fibroblasts [Vreeswijk MPG, Kraan JN, van der Klift HM et al (2008) Intronic variants in *BRCA1* and *BRCA2* that affect RNA splicing can be reliably selected by splice-site prediction programs. Hum Mutat Published Online. doi:10.1002/humu.20811].

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Introduction

A family history of breast cancer (BC) is one of the main risk factors for developing the disease. It is currently estimated that about 5–10% of BC are due to an inherited predisposition. Although, the number of predisposing genes is unknown, epidemiological studies provide evidence of at least two high penetrance susceptibility genes: *BRCA1* (17q21) (MIM# 113705) [1] and *BRCA2* (13q12–13) (MIM# 600185) [2].

Extensive analyses of the *BRCA1* and *BRCA2* genes have resulted in the identification of a large number of different disease-causing germ-line mutations (listed online at the Breast Cancer Information Core) [3]. Most reported disease-associated alleles of *BRCA1* and *BRCA2* have been attributed to frame-shift, nonsense or splice site alterations that lead to truncation of *BRCA1* or *BRCA2* proteins.

A large number of missense mutations, translationally silent mutations, and intron variants (especially those affecting splice sites positions) routinely encountered in clinical and research laboratories cannot be readily distinguished as either disease-associated mutations or benign polymorphisms, and they are classified as variants of uncertain significance (BIC database). This fact poses a

real challenge for risk assessment and genetic counseling of patients from cancer-prone families.

Mutations that affect intron consensus sequences at the splice and branch sites, and those that create ectopic splice sites account for 15% of point mutations associated with human genetic diseases [4]. A number of studies have demonstrated the effect of some of these splice site variants in the *BRCA1* and *BRCA2* genes at mRNA level [5–14].

Nevertheless, the majority of *BRCA1* and *BRCA2* variants have been identified in genomic DNA and their effects at the mRNA level have not been reported. Our aim was to ascertain the pathological effect of two *BRCA1* and *BRCA2* variants detected in the genetic analysis of two Spanish breast/ovarian cancer families.

Patients and methods

As part of genetic studies carried out on breast/ovarian Spanish families in the Hospital Vall d'Hebron (Barcelona), germ-line *BRCA1* and *BRCA2* mutations were screened in a proband of each family. Individuals underwent genetic counselling and signed an informed consent prior to testing.

DNA was extracted from whole blood using the Puregene Genomic DNA purification kit (Gentra System, Minneapolis, MN, USA). DNA was amplified by PCR using primers specific for the coding sequence and exon-intron boundaries. Analysis of the entire coding and flanking sequences was carried out by sequencing using BigDye Terminator V3.1 Cycle Sequencing Kit on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Both forward and reverse strands were sequenced.

Two sequence variants were analyzed in our study: the *BRCA1* IVS6-1G>A (c. 302-1G>A according HGVS), and the *BRCA2* IVS15+1G>A (c. 7617+1G>A according HGVS). These variants were named according to the GenBank entries NM_007294 (*BRCA1*) and NM_000059 (*BRCA2*). Their pathological significance was unknown although their location suggested that they could be deleterious.

Patients

The *BRCA1* IVS6-1G>A mutation was present in the proband and in her healthy brother. The proband was diagnosed as having bilateral BC at 40 and 52 years of age, and her mother died of BC at 61 years. No other neoplasias were reported in her family.

The *BRCA2* IVS15+1G>A mutation was detected in the proband, who was diagnosed with BC at 48 years, and in one sister, who also presented with OC and BC both diagnosed at 61 years.

Reverse transcription and amplification of the *BRCA1/BRCA2* coding regions

Patient peripheral blood samples were collected in EDTA tubes. Fresh peripheral blood was treated with erythrocytes lysis solution (RBC lysis solution, Qiagen Sciences, Maryland, USA). Total RNA was extracted from peripheral-blood leukocytes of carrier patients and controls using TRIzol reagent (Invitrogen, Paisley, United Kingdom) according to the manufacturer's protocol. Isolated RNA was further processed on columns from RNeasy Kit and RNase-Free DNase set to cleanup RNA and remove any DNA traces, according to the protocols provided by the manufacturer (Qiagen Sciences, Maryland, USA). The integrity of RNA purified was checked in an 1.5% agarose gel stained with ethidium bromide.

After quantification with a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA), one microgram of total RNA was reverse transcribed using Transcriptor High Fidelity cDNA Synthesis Kit with anchored-oligo (dT)₁₈ (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers' protocols.

Primers (5'–3') used to amplify the *BRCA1* coding region from exon 3 through exon 8 were: TCAAGGAACCTGTCTCCACA (forward), and GGTTGTATCCGCTGCTTTTGT (reverse). Primers used for exon 14 through exon 16 *BRCA2* coding region were: ACAGGCAGACCAACCAAAGT (forward) and TCCACCATCAGCCAAGTGT (reverse).

For IVS6-1G>A PCR was performed to amplify the cDNA sample from one carrier and three normal controls. For the study of IVS15+1G>A a number of samples were analyzed in parallel: two positive samples and one without the mutation (all of them belonging to the same family), and one normal sample as control.

Results

After cDNA amplification of each variant the PCR products were visualized by agarose gel electrophoresis.

For *BRCA1* fragment (exons 3–8) a product of the expected size (approximately 427 bp) was obtained from all samples. Sequencing of cDNA from the carrier of the *BRCA1* IVS6-1G>A allele revealed that this allele causes inappropriate splicing of exon 7 (Fig. 1), resulting in a 10-base frameshift deletion at the beginning of exon 7 (c. 302_311del10). This alteration in the cDNA creates 14 new residues (101[^]114) and a predicted stop codon at residue 115 (p.Tyr101SerfsX15). The 10 bp differences between the two alleles made difficult to distinguish two different wild-type and mutant bands in agarose gels.

For the *BRCA2* cDNA amplified sequence (exons 14–16), the expected 561 bp DNA fragment was obtained

classified as variants of uncertain significance (BIC database). We evaluated at the mRNA level the molecular nature and pathological effect of one *BRCA1* and *BRCA2* variants of uncertain significance, located within consensus splicing sequences. We demonstrated by cDNA sequencing the existence of aberrant splicing in both *BRCA1* IVS6-1G>A and *BRCA2* IVS15+1G>A mutations.

The *BRCA1* c. 302-1G>A (HGVS) variant has been reported once to the BIC database (IVS6-1G>A). In the same nucleotide another variant (IVS6-1G>T) was reported twice. Our analysis of IVS6-1G>A cDNA products demonstrates that this intronic substitution activates another cryptic acceptor splice site, which results in a 10 bp frameshift deletion at the beginning of exon 7. This frameshift deletion results in the addition of 14 new residues and a premature stop codon.

Other different variants located in the last intronic bases in the same splicing consensus sequence have also been reported to the BIC database: IVS6-2delA (11 times), IVS6-2A>C (one time), IVS6-2A>T (one time) and IVS6-3C>G (three times). The splice site variant IVS6-2delA has been described previously and extensively tested [15, 16]. The IVS6-2delA change clearly appeared to affect the production of mRNA from the variant allele in carrier individuals [15]. Although sequencing of the cDNA product failed to detect any deficiency in the splicing of exon 6 to exon 7, it was not possible to demonstrate if the transcript represented one or two *BRCA1* alleles, and the lack of expression in the MCF-7 cells carrying the construct containing the IVS6-2delA mutation demonstrated the disruption of the normal splicing of *BRCA1* [15]. The authors also found that the mutation directly affected mRNA stability. Furthermore, by combining these data with an assessment of the histopathological features of the breast carcinomas in carriers and mutation penetrance estimates, the authors concluded that this *BRCA1* variant conveyed an increased risk of breast cancer [15]. More recently, Chen et al. conducted a RT-PCR of the IVS6-2delA mutation and the sequencing analysis revealed the same result that we obtained studying the IVS+1G>A variant: a 10-base frameshift deletion at the beginning of exon 7. Therefore, these previous data and our present work make very likely that the other observed variants that involve exon 7 splicing consensus sequences such as IVS6-1G>T, IVS6-2A>C, IVS6-2A>T and IVS6-3C>G (BIC database) lead to the same splicing product.

The *BRCA2* c. 7617+1G>A (HGVS) mutation has been reported four times in the BIC database (IVS15+1G>A), and a similar variant (IVS15+1G>T) has been reported once. The *BRCA2* IVS15+1G>A mutation affects the first position of intron 16, and can be considered pathogenic. We have demonstrated that it causes skipping of exon 15, with the inclusion of 7 new amino acids in the *BRCA2*

protein and the appearance of a premature stop codon. Other variants reported in BIC that affect exon 15 splicing consensus sequences, such as IVS15+2T>G and IVS15+4T>C, could probably lead to the same splicing product but as far as we know no mRNA analysis has been reported (see article note).

In conclusion, our study has evaluated the *BRCA1* c. 302-1G>A variant and the *BRCA2* c. 7617+1G> variant located within intron-exon boundaries. We have demonstrated that both mutations cause frameshift in the translational reading frame, contributing to the pathogenesis of breast and/or ovarian cancer.

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