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LETTER TO THE EDITOR

BRCA1 c.4987-3C>G is a pathogenic mutation

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To the Editor,

In our previously published article by Brandão et al. [1], we reported that the unclassified variant (UV) *BRCA1* c.4987-3C>G gives rise to *BRCA1*Δ17. However, since we could not exclude residual expression of the full-length transcript from the variant allele, we have classified the variant as likely pathogenic. We now have evidence that the variant is indeed pathogenic.

Allele-specific PCRs are useful to determine the relative contribution of each allele to the synthesis of full-length or alternative transcripts. This can be achieved with, for instance, Sanger sequencing of the RT-PCR products and the use of a heterozygous variant to establish allelic expression ratios [1]. Unfortunately, in this family neither the proband nor the relatives tested for the BRCA1 c.4987-3C>G variant were heterozygous for a polymorphism in the region of the spliced exon. Since the UV is not exonic, it could not be used to determine allelic expression. Consequently, in our previously published article, using RT-PCRs on RNA isolated from primary cultured lymphocytes, we failed to determine whether the UV allele was still giving rise to some full-length BRCA1 transcript. To establish the pathogenicity of this variant, we performed an additional study using an ex vivo assay based on a splicing reporter minigene. We selected the exon-trapping vector pSPL3b [2] (a kind gift from Dr. R Sedlmeier, Ingenium Pharmaceuticals GmbH), previously used for similar studies [3–5]. Initially, *BRCA1* exon 17 was amplified including the surrounding intronic regions from the proband's DNA and one additional control, using primers that contained restriction sites XhoI and EcoRV in their 5′ ends. PCR products and vector were digested with the two enzymes and subsequently used in a ligation reaction, after purification with QIAquick PCR Purification Kit (QIAGEN). The structure of the minigenes is shown in Fig. 1a. After transformation of competent *E. coli* DH5α (Invitrogen), independent colonies were selected with minigenes containing the variant allele from the patient, WT alleles from the patient and from the controls. All constructs were verified by sequence analysis and confirmed the absence of changes in the constructs. These minigenes and the empty vector were transfected into HeLa cells. Transfection was performed in duplicate with FuGENE HD transfection reagent (Promega) according to the manufacturer's protocol.

After RNA extraction from the transfected HeLa cells and reverse transcription, the cDNA was amplified using primers in each of the flanking exons of the vector pSPL3b, which we named A and B for clarity (Fig. 1a). The results obtained are shown in Fig. 1b. The WT allele of the patient and of a healthy control show only one band in the agarose gel, with a larger size than that observed for the empty vector. Sequencing confirmed that the RT-PCR products contain exon 17 (Fig. 2). The variant allele of the patient shows a prominent lower band of the same size as the empty vector indicating exon 17 skipping, which was confirmed by sequencing. In addition, a faint unexpected upper band can be observed for the variant allele. Sequencing of the two bands separately, by excision of the bands from the gel, revealed this to be a transcript

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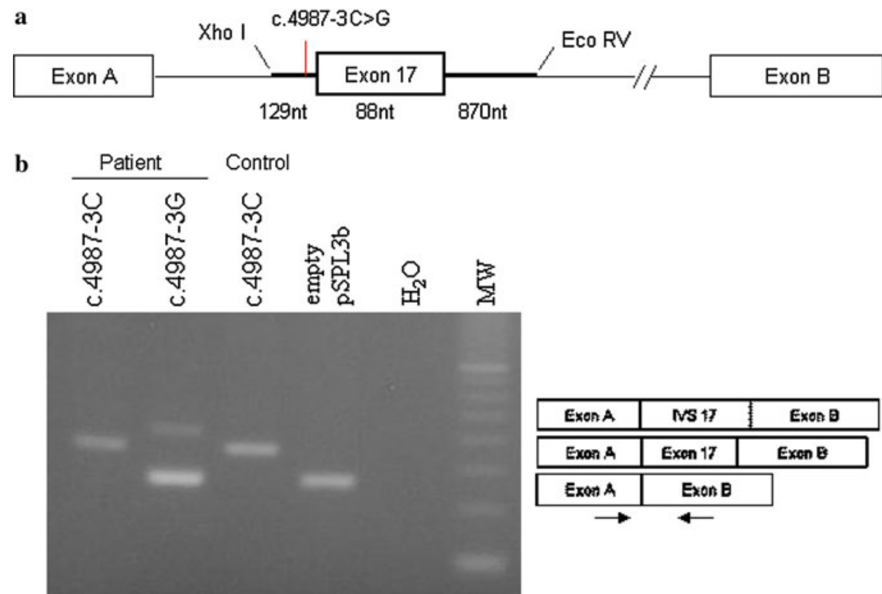

Fig. 1 Ex vivo assay using pSPL3b vector. **a** Structure of the pSPL3b vector containing exon 17 and flanking intronic regions with the mutant or wild-type allele (c.4987-3C>G). The size of the exon, intronic regions and the restriction sites included in the primers are indicated. **b** Transcript analysis by electrophoresis of the RT-PCR products obtained after transfection of HeLa cells using primers hybridizing to the exons of the vector. *H_2O* negative PCR control, *MW* molecular weight ladder XIV (Roche). *Boxes* next to the PCR bands indicate the exon composition and the position of the primers used is shown with *arrows*

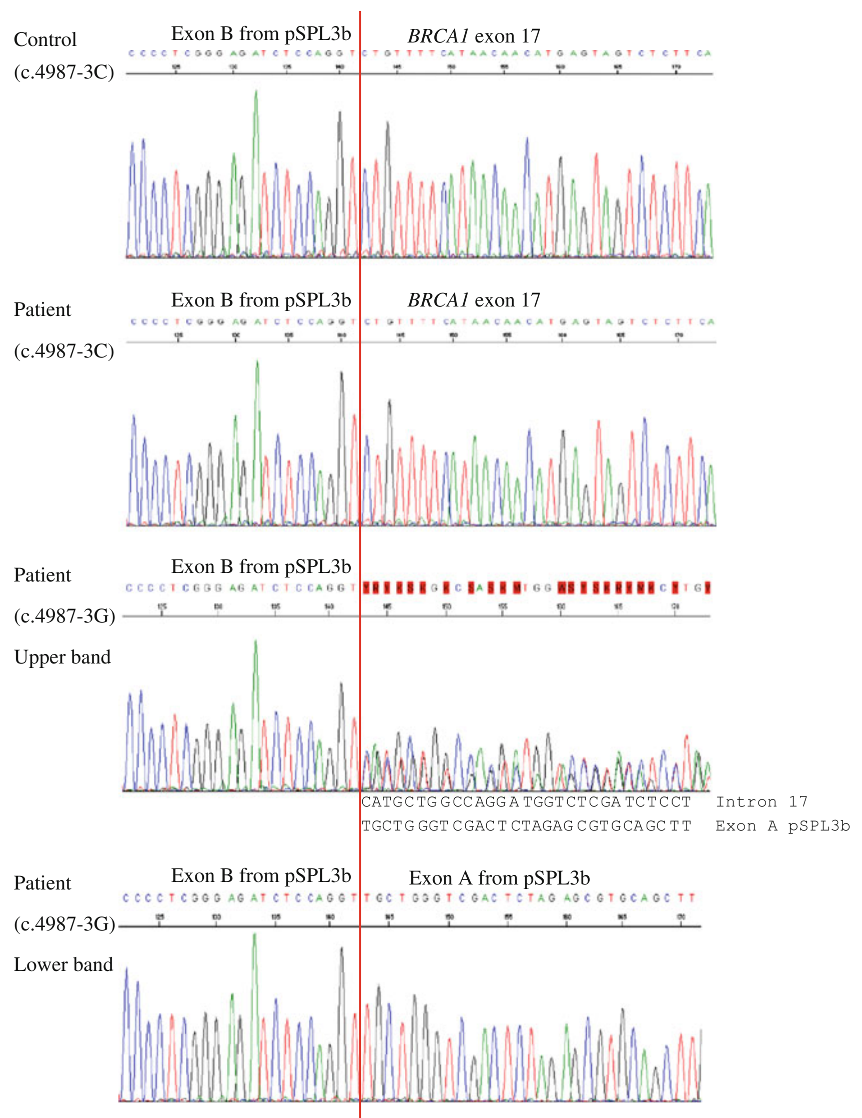

Fig. 2 Reverse sequence of amplification products observed in Fig. 1b, using primers in the exons A and B of the pSPL3b vector. The two fragments of the sample containing the c.4987-3G variant were excised from the gel and sequenced separately. As expected, both the samples with WT sequence c.4987-3C contain exon 17. Vector containing the c.4987-3G variant gave rise to a transcript where the exons of the vector are adjacent, revealing exon 17 skipping (lower band, Fig. 1b), and another transcript with inclusion of part of intron 17 (upper band, Fig. 1b). The latter contained a heteroduplex of the two described transcripts, which formed due to the high similarity between the two fragments

containing part of intron 17. In Fig. 2, the sequence of this transcript is shown but it contains a background sequence of the transcript lacking exon 17 (the lower band on the gel). This is due to heteroduplex formation between the two fragments, which occurs due to the high similarity between them as both contain exons A and B. The intron 17 retention starts at the beginning of intron 17, position c.5074+1, and the donor splice site is at position c.5074+153. This is possible since the original donor splice site of exon 17 is also predicted to be a strong acceptor splice site (AG|GTATAC, 76% score) by the Splice Site Finder-like algorithm and Human Splice Finder (recently added to the Alamut software), as observed using Alamut (Interactive Biosoftware), which integrates several splice site prediction algorithms. The c.5074+153 is predicted to be a weak donor splice site by two algorithms (5 and 11% by MaxEntScan and GeneSplicer, respectively) but strongly predicted by the Human Splice Finder algorithm (79%). This intronic region is not observed in the WT alleles used in the ex vivo assay, neither in the previous results from the IL2/PHA stimulated lymphocytes. We conclude that the intron retention is an artefact in the in vitro system caused by skipping of exon 17 and activation of cryptic splice sites.

Summarising, using an ex vivo assay to complement the previous RT-PCR analysis on RNA from IL2/PHA stimulated lymphocyte cultures [1], we were able to show that the variant allele from the patient results only in exon 17 skipping since a transcript containing exon 17 was not detected. The deletion of BRCA1 exon 17 is a frameshift event that leads to a truncated protein: p.Val1665SerdelfsX9. In combination with the results from the previously reported RT-PCR analysis, we are now confident that the *BRCA1* c.4987-3C>G variant is pathogenic and can be genetically counselled as such.

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Conflict of interest The authors declare that they have no conflict of interest.

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