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## Molecular diagnosis of the Portuguese founder mutation *BRCA2* c.156\_157insAlu

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We wish to reply to the Letter to the Editor written by Machado and Vaz [1] concerning our recently published article on the c.156\_157insAlu *BRCA2* founder mutation [2], first discovered by Teugels et al. [3] in a Portuguese patient living in Belgium. That letter has several misstatements about our and others' work that must not remain unchallenged.

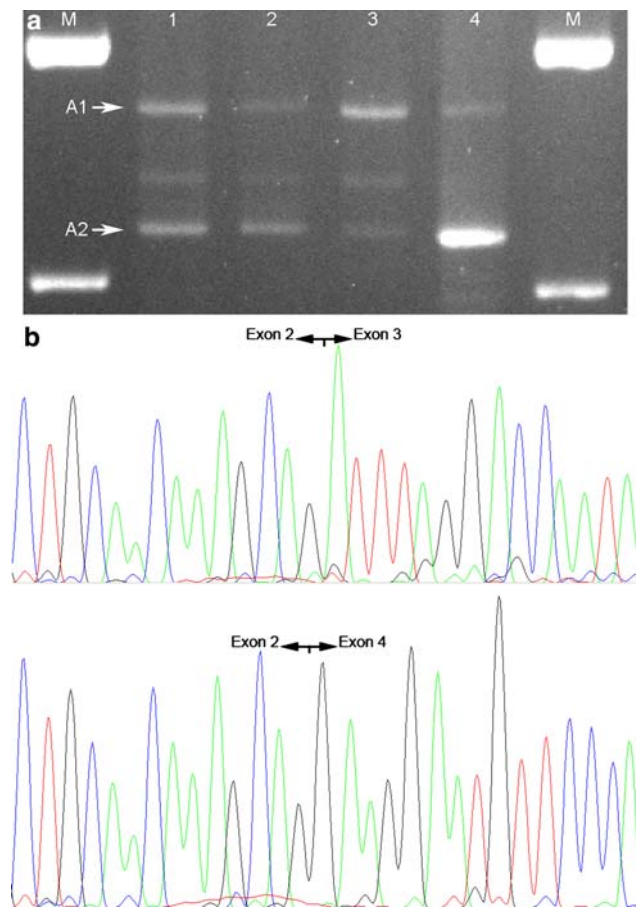
Machado and Vaz suggest in their letter [1] that we diagnose the c.156\_157insAlu *BRCA2* mutation by subjective evaluation of band intensities after RT-PCR. We think that it is clear in our paper [2], under the subheading "Screening for the c.156\_157insAlu *BRCA2* mutation", that we screen for this mutation by using two independent PCRs, one for exon 3 amplification and another specific for the Alu rearrangement. Using this strategy, in positive cases we expect two bands in the first PCR (one band if negative) and one band in the second PCR (none if negative). The second PCR helps to control the first PCR for eventual problems with preferential amplification of the shorter fragment (the normal), whereas the first PCR controls for eventual absence of amplification in the second PCR. This strategy of two independent PCRs (which differs from the nested PCR approach used by Machado et al. [4]), followed by sequencing of the genomic fragments in positive cases, allows the unambiguous detection of the c.156\_157insAlu *BRCA2* mutation. In the Results section of our article [2], the molecular diagnosis of families with the c.156\_157insAlu *BRCA2* mutation is also described under the separate subheading "Detection of the

c.156\_157insAlu *BRCA2* mutation", whereas any mention to RT-PCR data appears under the subheading "Analysis of RNA transcripts". Therefore, the reason why we performed RT-PCR with primers located in exons 1 and 6 was not to confirm the mutation, but to deliberately be able to compare the physiologic alternative splice lacking exon 3 in controls with the effect of the exon 3 Alu insertion in carriers. Taking advantage of a known polymorphism in *BRCA2* exon 2 (previously reported as c.-25G>A [2, 5, 6], but described as c.-26G>A according to the recommendations of the Human Genome Variation Society [7], we clearly demonstrated in Fig. 1 of our paper [2] the preferential production of the transcript without exon 3 by the allele with the Alu insertion and its (presumably residual) production by both alleles in non-carriers.

Machado and Vaz [1] state that RT-PCR must be used to confirm the c.156\_157insAlu *BRCA2* mutation in each patient and that a reliable confirmation of this rearrangement must be done using the primers in exons 2 and 10 first described by Nordling et al. [8]. Both these claims are unfounded for the following reasons. First, it is not necessary to confirm one given mutation at the RNA level in every new patient once its pathogenic nature is well established, as it is now the case for this *BRCA2* rearrangement. Second, Machado and Vaz claim [1] that these primers amplify specifically the transcript produced from the allele with the Alu insertion, and not the ubiquitous alternative splice transcript, allegedly because only the former transcript includes at least the first part of *BRCA2* exon 10 [1]. They present no data to support this assumption nor do they exist in the literature. In fact, Zou et al. [9] have shown that the full length transcript and the ubiquitous alternative splice differ only by the lack of exon 3 in the latter, so exon 10 does not distinguish them. Additionally, the forward primer described by Nordling

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**Fig. 1** RT-PCR analyses from exon 1 to exon 10 of the *BRCA2* gene in three normal controls and in one c.156\_157insAlu *BRCA2* mutation carrier. **a** Lanes M, 1 Kb DNA standard; Lanes 1, 2 and 3, controls without the c.156\_157insAlu *BRCA2* mutation; Lane 4, c.156\_157insAlu *BRCA2* mutation carrier; A1 represents the full length transcript and A2 the *BRCA2*- $\Delta$ ex3 transcript. **b** Sequencing of the RT-PCR products (A1 band above and A2 band below) from a control individual showing that both the wild type and the ubiquitous transcript lacking exon 3 can be detected in negative controls

et al. [8] (5'-GCTTATTTACCAAGCATTGGA) has two features that might interfere with the amplification process: first, it contains a mismatch in the first base (A>G); second, it is positioned on the SNP c.-26G>A in exon 2, which has an allelic frequency of 28% [5]. Indeed, Bonnet et al. [10] have recently used a monoallelic RT-PCR protocol based on the SNP c.-26G>A to demonstrate the complete skipping of exon 3 induced by the presence of the c.316+5G>C *BRCA2* mutation. In addition to these methodological flaws, it is likely that Machado et al. [4] were not able to amplify the transcript lacking exon 3 in controls because they try to amplify a large transcript produced at very low level in non carriers, whereas it can more easily be detected in carriers as that transcript is produced at high levels from the mutated allele. To address this possibility, we have analyzed freshly

collected blood samples from five negative controls (confirmed not to carry the c.156\_157insAlu *BRCA2* mutation). Total RNA was isolated using 1 ml of Tripure isolation reagent (Roche Diagnostics, Indianapolis, USA) and One-Step RT-PCR was performed (QIAGEN GmbH, Germany), as suggested by the manufacturers. Using a 5'UTR *BRCA2* primer that does not include the SNP c.-26G>A (5'-GCTTACTCCGGCCAAAAAAGA) and a reverse primer located in exon 10 (5'-TGGTCACATGAAGAAATATGCAATAG) that allows the detection of its first 469 bp, we were able to amplify both the wild type and the ubiquitous transcript lacking exon 3 in all five negative controls (Fig. 1). These data contradict the assumption made by Machado and Vaz [1, 4] that the alternative splice transcript in negative controls lacks part of exon 10, as we here show using optimized methods for RNA extraction and analysis. We therefore conclude that RT-PCR studies aiming to detect exon 3 skipping should not be performed for molecular diagnosis of the c.156\_157insAlu *BRCA2* mutation; instead, this Portuguese founder mutation can reliably be detected by genomic PCR followed by sequencing as we demonstrated in our article [2].

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