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The c.156_157insAlu *BRCA2* rearrangement accounts for more than one-fourth of deleterious *BRCA* mutations in northern/central Portugal

Ana Peixoto · Catarina Santos · Patrícia Rocha · Manuela Pinheiro ·
Sofia Príncipe · Deolinda Pereira · Helena Rodrigues · Fernando Castro ·
Joaquim Abreu · Leonor Gusmão · António Amorim · Manuel R. Teixeira

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Abstract We evaluated the contribution of an Alu insertion in *BRCA2* exon 3 (c.156_157insAlu) to inherited predisposition to breast/ovarian cancer in 208 families originated mostly from northern/central Portugal. We identified the c.156_157insAlu *BRCA2* mutation in 14 families and showed that it accounts for more than one-fourth of deleterious *BRCA1/BRCA2* mutations in breast/ovarian cancer families originated from this part of the country. This mutation originates *BRCA2* exon 3 skipping and we demonstrated its pathogenic effect by showing that the *BRCA2* full length transcript is derived only from the wild type allele in carriers, that it is absent in 262 chromosomes from healthy blood donors, and that it

co-segregates with the disease. Polymorphic microsatellite markers were used for haplotype analysis in three informative families. In two of the three families one haplotype was shared for all but two markers, whereas in the third family all markers telomeric to *BRCA2* differed from that observed in the other two. Although the c.156_157insAlu *BRCA2* mutation has so far only been identified in Portuguese breast/ovarian cancer families, screening of this rearrangement in other populations will allow evaluation of whether or not it is a population-specific founder mutation and a more accurate estimation of its distribution and age.

Keywords c.156_157insAlu *BRCA2* mutation · Breast cancer · Hereditary predisposition

A. Peixoto · C. Santos · P. Rocha · M. Pinheiro · S. Príncipe ·
M. R. Teixeira (✉)
Department of Genetics, Portuguese Oncology Institute, Rua Dr.
António Bernardino de Almeida, 4200-072 Porto, Portugal
e-mail: mteixeir@ipoporto.min-saude.pt

D. Pereira · H. Rodrigues
Department of Medical Oncology, Portuguese Oncology
Institute, Porto, Portugal

F. Castro · J. Abreu
Department of Surgical Oncology, Portuguese Oncology
Institute, Porto, Portugal

L. Gusmão · A. Amorim
Institute of Molecular Pathology and Immunology, University
of Porto (IPATIMUP), Porto, Portugal

A. Amorim
Faculty of Sciences, University of Porto, Porto, Portugal

M. R. Teixeira
Institute of Biomedical Sciences (ICBAS), University of Porto,
Porto, Portugal

Introduction

Molecular analyses of the *BRCA1* and *BRCA2* genes in families suspected to have hereditary predisposition to breast/ovarian cancer have shown that most populations exhibit a wide spectrum of mutations throughout both genes. However, several founder mutations have been identified in individuals of different ancestries, including those of Ashkenazi Jewish (185delAG and 53832insC) [1], French Canadian (C4446T and 2953del3+C) [2, 3], Norwegian (1675delA and 1135insA) [4], Dutch (2804delAA and Alu-mediated deletions encompassing exons 13 and 22) [5, 6], Polish (5382insC, C61G, and 4153delA) [7], Greek (5382insC and G1738R) [8, 9], Icelandic (999del5) [10], Spanish (330A>G, 6857_6858del, and 9254_9258del) [11, 12], and Swedish (3171ins5) [13] origins. We have previously reported several novel mutations in a first characterization of the mutational spectrum of the entire coding sequences and exon–intron boundaries of the

BRCA1 and *BRCA2* genes and large *BRCA1* rearrangements in Portuguese breast/ovarian cancer families [14].

Recently, Teugels et al. [15] identified an Alu insertion in *BRCA2* exon 3 (c.156_157insAlu) in a Portuguese breast cancer patient living in Belgium. This mutation was not detectable using the conventional mutation screening techniques and originates skipping of *BRCA2* exon 3 [15]. A regional founder effect has recently been described for this rearrangement in hereditary breast/ovarian cancer families mostly originated from central/southern Portugal [16]. In an attempt to gain insight into the ancestral origin and population spread of the c.156_157insAlu *BRCA2* mutation, we estimated its frequency in breast/ovarian cancer families originated mostly from northern/central Portugal and analyzed the chromosomal background upon which this mutation occurs using 10 microsatellite markers surrounding the *BRCA2* gene. Furthermore, because some doubts have been raised about the pathogenic effect of *BRCA2* exon 3 skipping [17], we have performed segregation studies and mRNA expression analysis of c.156_157insAlu carriers and disease-free controls.

Materials and methods

Patients

This study comprised the 78 Portuguese breast/ovarian cancer families in whom no clearly deleterious *BRCA1/BRCA2* mutations were found in our previous study [14] and an additional 130 *BRCA1/BRCA2* negative probands that had been selected using the following the criteria: (1) families with one or more breast cancers (one of them diagnosed before the age of 50 years) and one or more ovarian cancers (BC + OC); (2) families with two or more female breast cancers, one of them diagnosed before the age of 50 years (≥ 2 BC); (3) families with at least one case of breast cancer or ovarian cancer in association with at least one case of male breast cancer (MBC); (4) patients without family history of breast/ovarian cancer, but showing early-onset breast cancer (1BC, diagnosed before the age of 35 years), breast (diagnosed before the age of 60 years) and ovarian cancer (1BC + OC), bilateral breast carcinomas (1BIC, one of them diagnosed before the age of 50 years), or male breast cancer (1MBC, at any age). BRCAPRO mutation probabilities were estimated for all probands [18].

DNA isolation and *BRCA1/BRCA2* mutation screening

Genomic DNA was extracted from peripheral leucocytes according to standard procedures and subsequently

amplified using primers described by van der Hout et al. [19]. Mutation screening of the entire coding regions of *BRCA1* and *BRCA2* was performed by Denaturing Gradient Gel Electrophoresis (DGGE), using the IngenyphorU system (Ingeny; Goes). Sequence analysis of genomic fragments with altered DGGE mobility pattern was carried out on an Applied Biosystems' 310 automated DNA sequencer (Foster City), using the dye terminator method. Multiplex Ligation-dependent Probe Amplification (MLPA) (MRC-Holland, Amsterdam) was used to detect large exonic *BRCA1* and *BRCA2* rearrangements (only 23 probands were analyzed for the latter gene) in cases that were negative for pathogenic *BRCA1/BRCA2* point mutations.

Screening for the c.156_157insAlu *BRCA2* mutation

The c.156_157insAlu *BRCA2* mutation was screened in 208 probands and in 262 control chromosomes (healthy blood donors). In the first PCR we used primers for *BRCA2* exon 3 amplification (forward primer: GTCAGTGGT TAAACTAAGGTGGG and reverse primer: GAAGCCA GCTGATTATAAGATGGTT). The cycling conditions were 94°C for 1 min, 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 4 min, and a final extension of 72°C for 10 min. In the second PCR we used primers specific for the c.156_157insAlu *BRCA2* mutation (forward primer: GACACCATCCCGGCTGAAA and reverse primer: CCC CAGTCTACCATATTGCAT). The cycling conditions were 97°C for 15 min, 6 cycles of 97°C for 1 min, 68°C for 1 min, and 72°C for 1 min, 6 cycles of 97°C for 1 min, 66°C for 30 s, and 72°C for 1 min, 6 cycles of 97°C for 1 min, 64°C for 30 s, and 72°C for 1 min, 22 cycles of 97°C for 1 min, 60°C for 30 s, and 72°C for 1 min, and a final extension of 72°C for 10 min. Sequence analysis of genomic fragments with the insertion was carried out on an Applied Biosystems' 310 automated DNA sequencer (FosterCity), using the dye terminator method.

RNA isolation and transcript analysis

Frozen cell pellets from nucleated cells of patients carrying the c.155_156insAlu *BRCA2* variant and from control individuals (without known *BRCA1* or *BRCA2* mutations) were used to extract total RNA using 1 ml of Tripure isolation reagent (Roche Diagnostics, Indianapolis, USA), according to the manufacturer's instructions. After quantification using a NanoDrop ND-1000 spectrophotometer, One-Step RT-PCR was performed in all cases using the Superscript One-Step RT-PCR System as suggested by the manufacturer (Invitrogen, Carlsbad, USA). We used primers spanning from exon 1 to exon 6 of *BRCA2* (forward primer B2Ex1F: GCTTACTCCGCCAAAAAAGA and reverse

primer B2Ex6R: GGTGTCTGACGACCCTTCACA). The cycling conditions were 50°C for 30 min, 95°C for 15 min, 40 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min. RT-PCR products were separated on a 2% agarose gel (SeaKem LE Agarose, Rockland, USA) and visualized with ethidium bromide in an image analyser ImageMaster VDS (Amersham Biosciences, Little Chalfont, UK). Individual bands were excised from the gel, purified using GFX™ PCR DNA and Gel Band Purification Kit (GE HealthCare) and sequenced as indicated above.

Microsatellite typing and haplotype analysis

A total of 14 probands and 14 family members were genotyped for polymorphic microsatellite markers flanking *BRCA2* and for the c.-25G>A polymorphism. The order of the markers, the consensus repeat, and the distances relative to each other and *BRCA2* are shown in Table 1. The physical distances of the genetic markers were derived from the Human Genome Browser assembly hg18 from the UCSC Genome Bioinformatics (<http://www.genome.ucsc.edu/>). The consensus pattern was obtained with the software Tandem Repeats Finder (<http://www.tandem.bu.edu/>). The primer sequences for the amplification of the markers were derived from the Human Genome database (<http://www.gdb.org>). All 10 markers were assayed by PCR using fluorescently end-labeled primers. PCR products were run on an ABI PRISM 310 Genetic Analyser (Applied Biosystems) together with a fluorescence labeled DNA fragment size standard. Genotyping of the c.-25G>A polymorphism was performed by sequencing. Haplotypes were reconstructed manually in the three informative families. The geographic origin of the c.156_157insAlu *BRCA2* positive families was inferred from the birthplace of the oldest carrier or of the oldest affected family member most likely to be a carrier.

Results

Detection of the c.156_157insAlu *BRCA2* mutation

We identified eight probands with the c.156_157insAlu mutation in our initial series of 78 patients in whom no deleterious *BRCA1/BRCA2* mutation had been found by DGGE and MLPA. This finding, together with the 22 pathogenic mutations previously reported [14], raised the deleterious mutation detection rate to 30% (30/100) of all cases studied and to 37% (29/79) of those probands with a family history of breast/ovarian cancer. The c.156_157insAlu *BRCA2* mutation accounts for 27% of the 30 probands with *BRCA1/BRCA2* pathogenic mutations and

Table 1 Microsatellite marker genotypes used to reconstruct the haplotypes in probands with the c.156_157insAlu *BRCA2* mutation

Markers	Position (Mb)	Consensus pattern	1	2	3	4	5	6	7	8	9	10	11	12	13	14
<i>cen</i>																
D13S1700	0.56	AGAA	257/265	249/257	261/317	309/321	257/309	317/317	257/317	317/325	265/317	313/317	261/317	309/317	253/313	313/317
D13S260	0.45	TG	160/164	160/164	160/160	160/160	162/168	160/168	158/160	168/168	160/160	158/160	160/160	160/160	160/160	160/168
D13S1699	0.28	TTTC	153/153	153/153	153/153	153/153	153/153	153/157	150/153	153/153	150/153	153/153	150/153	153/153	153/157	153/153
D13S1698	0.19	GT	156/156	156/160	156/160	156/156	156/160	156/160	156/160	156/174	156/156	156/156	156/166	156/166	156/176	156/160
c.-25G>A	Intragenic		G/G	G/G	A/G	A/G	A/G	G/G	G/G	G/G	G/G	A/G	G/G	G/G	G/G	G/G
<i>BRCA2</i>																
D13S1701	0.17	TTCC	299/299	295/299	299/299	291/299	295/299	291/299	295/299	295/299	291/299	299/299	295/299	299/307	299/307	291/299
D13S171	0.28	TG	230/230	230/240	230/240	230/230	226/230	226/230	230/230	230/230	230/240	230/230	230/230	226/226	230/240	230/230
D13S1695	0.55	AC	242/250	242/250	242/250	242/246	240/242	242/250	236/246	242/250	242/250	242/250	242/258	242/250	250/252	242/254
D13S1694	0.8	TG	228/234	228/234	228/228	234/236	228/232	228/234	234/234	228/232	234/234	228/234	228/234	234/234	228/234	234/238
D13S310	0.81	GT	144/144	144/144	144/144	148/148	144/144	144/144	148/166	144/148	144/150	144/148	144/148	144/144	148/150	144/148
D13S267	1.29	TG	156/158	144/156	156/158	144/150	156/158	156/158	144/150	144/150	144/144	146/158	150/158	144/158	146/158	144/156
<i>tel</i>																

53% of the 15 deleterious *BRCA2* mutations identified in that series of 100 probands from breast/ovarian cancer families. Furthermore, screening of the c.156_157insAlu *BRCA2* mutation in 130 additional probands in whom no germline *BRCA1/BRCA2* mutations have been detected by DGGE and MLPA (data not shown) revealed six additional families with this mutation. Of the 14 probands with the c.156_157insAlu *BRCA2* mutation, eight belonged to ≥ 2 BC families, three to MBC families, two to BC + OC families, and one was a 1BC + OC patient. The mutation was also detected in six affected family members but neither in eight healthy relatives nor in the 262 chromosomes of the control population.

Analysis of RNA transcripts

RNA splicing was evaluated by amplifying the *BRCA2* coding region from exon 1 through exon 6. When the products from these reactions were visualized on agarose gels, besides a normal transcript two shorter bands were common to both the patients and controls: a transcript representing an in frame skip of exon 3 (*BRCA2*- Δ ex3) and a transcript with deletion of exon 3 plus 109 bp of exon 4 (Fig. 1). However, the band representing the *BRCA2*- Δ ex3 transcript had much higher intensity in the patients with the c.156_157insAlu *BRCA2* mutation. When these fragments were excised from the gel, amplified, and sequenced, and because the PCR were designed to generate fragments that contained the c.-25G>A polymorphism, we could demonstrate that the controls heterozygous for the c.-25G>A polymorphism showed heterozygosity in the full length and the *BRCA2*- Δ ex3 transcripts, whereas the patients heterozygous for that polymorphism presented mainly one allele in the full length transcript and the other allele in the *BRCA2- Δ ex3 transcript (Fig. 1). These findings indicate that the normal mRNA for *BRCA2* is produced only from one chromosome in the patient.*

Haplotype analysis

Haplotypes were phased for most of the markers for only three out of the 14 families. The results of the haplotype analyses for the three informative families are shown in Fig. 2. In one informative family, the haplotype associated with the mutation could be inferred accurately as five carriers were genotyped. In the other two families the haplotype was inferred in most instances as two carriers per family were genotyped. One haplotype was found to segregate within two families (11 and 13). The shared haplotype covered eight markers, but varied for the D13S1701 and D13S1700 alleles. In family 12 the disease associated alleles from the *BRCA2* telomeric region were different from those associated with families 11 and 13. The genotypes found for the remaining probands are shown in Table 1 and the geographic origins of

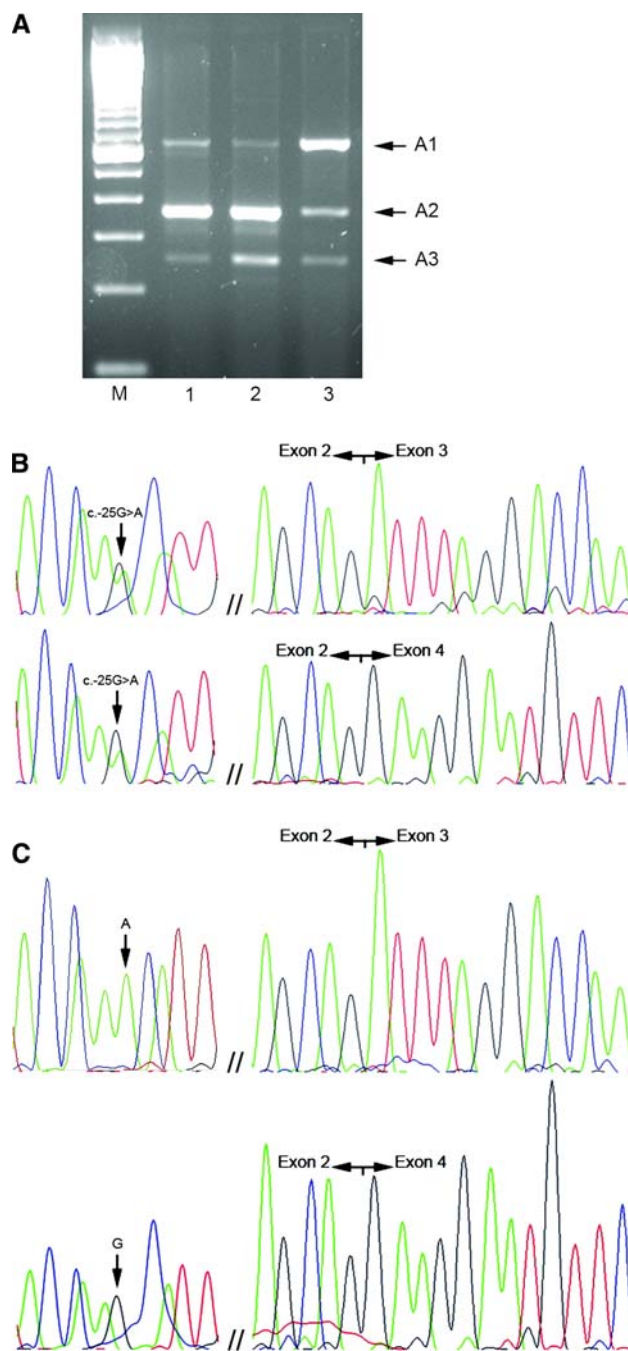
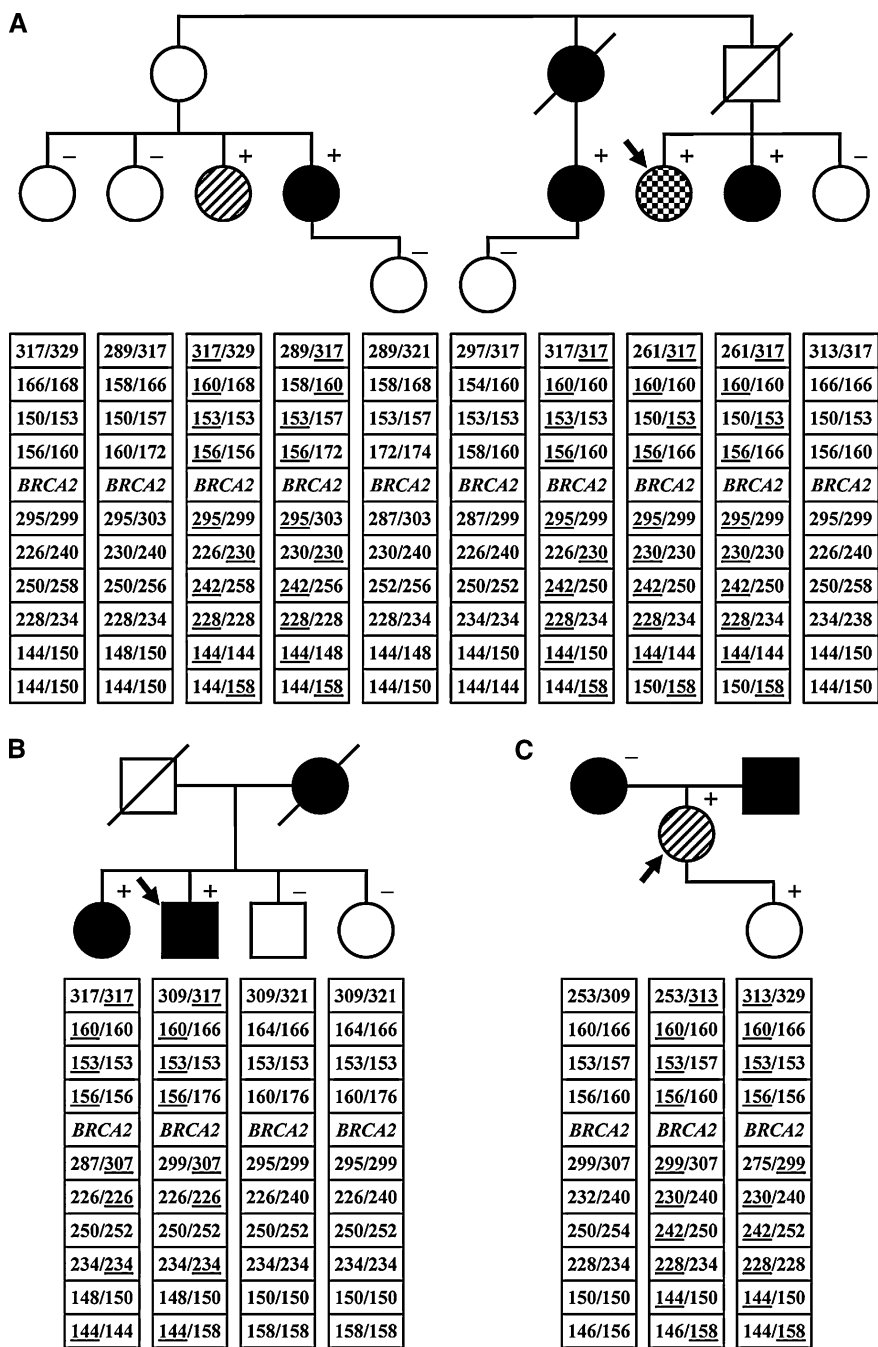


Fig. 1 RT-PCR analyses from exon 1 to exon 6 of the *BRCA2* gene in c.156_157insAlu *BRCA2* mutation carriers and controls. (a) Lane 1, 100 bp DNA standard. Lanes 2 and 3, patients with the c.156_157insAlu *BRCA2* mutation. Lane 3, wild type control sample. A1 represents the full length transcript, A2 the *BRCA2*- Δ ex3 transcript and A3 the transcript with deletion of exon 3 plus 109 bp of exon 4 (data not shown). (b) Sequencing of the RT-PCR product (A1 band top and A2 band bottom) from a control individual with the c.-25G>A germline polymorphism (noted by an arrow), showing heterozygosity in the full length and the *BRCA2*- Δ ex3 transcripts. (c) Sequencing of the RT-PCR product (A1 band top and A2 band bottom) from a c.156_157insAlu *BRCA2* mutation carrier with the c.-25G>A germline polymorphism (noted by an arrow), showing mainly one allele in the full length transcript and the other allele in the *BRCA2*- Δ ex3 transcript

Fig. 2 Pedigrees of the three informative breast/ovarian cancer families and haplotype results (families 11, 12 and 13 in **a**, **b** and **c**, respectively). The order of the microsatellite markers is the same as in Table 1 and the alleles that segregate with the mutation are underlined. Unaffected individuals are indicated with open symbols, patients affected with breast cancer with black symbols. Bilateral breast disease is represented by striped circles and breast/ovarian cancer patients by dotted circles. Plus and minus signals represent family members with and without the c.156_157insAlu *BRCA2* mutation, respectively



the c.156_157insAlu *BRCA2* positive families are shown in Fig. 3.

Discussion

Our findings show that the specific detection of the c.156_157insAlu *BRCA2* mutation in Portuguese breast/ovarian cancer families allows a significant increase in the detection rate of *BRCA1/BRCA2* mutations from 24.7% to

37% of those with a family history of cancer. This mutation rate is slightly above what has been reported in the majority of populations using similar criteria for referral to genetic counseling and testing [12, 20–22], something that might reflect the fact that we are now able to detect a mutation that escaped conventional mutation screening techniques. If we had used more strict selection criteria like those of the other major report on *BRCA1/BRCA2* analysis of Portuguese breast/ovarian cancer families (BRCAPRO risk >25%, resulting in a mutation detection rate of 36%, one-

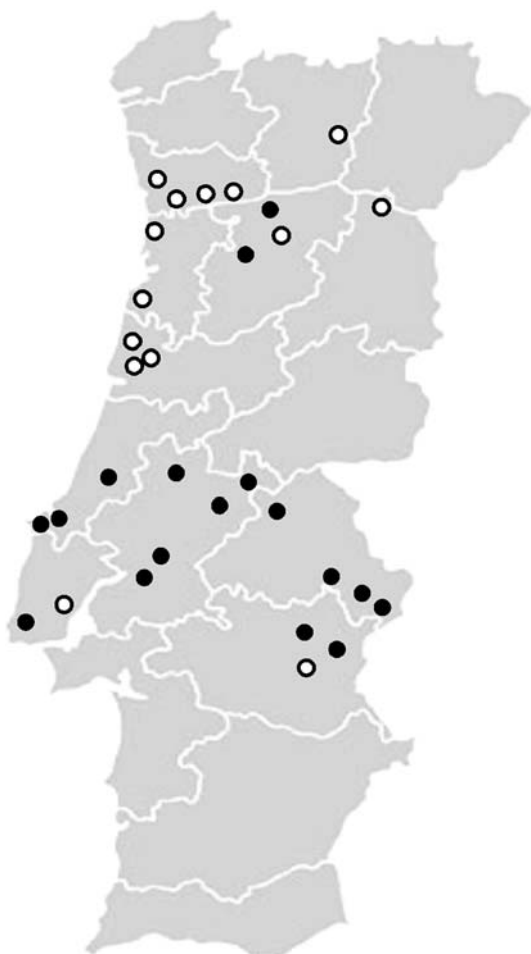


Fig. 3 Map of Portugal showing the known geographical origin of the families with the c.156_157insAlu *BRCA2* germline mutation. The open circles indicate the origin of the 14 families here reported and the black circles the families previously described by Machado et al. [16]

sixth being the c.156_157insAlu *BRCA2* mutation [16]), our mutation detection rate would have been 41%. Although this mutation rate can be seen as relatively high, it also indicates that other *BRCA1/BRCA2* mutations undetectable by current-day screening methodologies may remain to be uncovered in high-risk breast/ovarian cancer families. On the other hand, five out of the 30 pathogenic mutations would have been missed had we used the generally recommended higher than 10% mutation probability criteria for genetic testing (8 of 30 if >25%). Careful selection of families after genetic counseling is therefore recommended to increase the chance of identifying all families that can benefit from genetic diagnosis of inherited predisposition to breast/ovarian cancer.

Teugels et al. [15] demonstrated that the c.156_157insAlu mutation originates an in-frame deletion of exon 3 from the *BRCA2* mRNA encoding a transcriptional activation domain. On the other hand, Díez et al. [17] issued a word of

caution when interpreting as pathogenic alterations causing *BRCA2* exon 3 skipping, as exon 3 splicing can also be detected in normal tissues. In order to better characterize the pathological and clinical significance of this mutation, we have looked for this mutation in disease-free controls, analyzed in more detail the mRNA in carriers and noncarriers, and reconstructed haplotypes in informative families. Three separate findings corroborate the conclusion that the c.156_157insAlu *BRCA2* mutation is indeed deleterious. First, *BRCA2* full length transcript is derived only from the wild type allele in patients carrying the c.156_157insAlu *BRCA2* mutation, whereas the *BRCA2*- Δ ex3 transcript was transcribed mostly from the mutated allele. This was demonstrated using a heterozygous polymorphism to evaluate the relative contribution of each allele to normal and *BRCA2*- Δ ex3 transcripts in patients and controls [23], the latter showing both alleles giving rise to a small proportion of the *BRCA2*- Δ ex3 transcript. Second, if the abnormal transcript with the in-frame deletion of exon 3 is fully functional, as it was suggested by Díez et al. [17], then this variant would have to be considered a polymorphism given its high frequency (8%) in families suspected to have hereditary predisposition to breast/ovarian cancer. However, we did not find the c.156_157insAlu in 262 chromosomes from healthy blood donors, allowing us to dismiss the hypothesis that it is a neutral polymorphism. Third, we could show co-segregation of this alteration and disease in six affected (but not in healthy) family members belonging to the informative families. These pieces of information allow us to confidently conclude that the c.156_157insAlu *BRCA2* mutation is associated with the hereditary predisposition to breast/ovarian cancer in these families.

Alu elements are believed to insert randomly in the host genome, so the fact that this particular Alu is inserted at exactly the same position in *BRCA2* exon 3 in all 14 families is by itself sufficient evidence that this represents a founder effect and not a recurrent mutation [24]. Additionally, in all c.156_157insAlu mutation carriers heterozygous for the c.-25G>A polymorphism there was a physical association between the mutation and the allele that did not contribute to wild type transcript. Nevertheless, analysis of the chromosomal background upon which a mutation occurs can be used to reconstruct its origin and age. We have performed haplotype reconstruction with ten microsatellite markers in three informative families. In two of the three families one haplotype was shared for all but two markers, whereas in the third family all markers telomeric to *BRCA2* differed from that observed in the other two. A recombination event around *BRCA2* could account for the latter haplotype difference, whereas the variance in allele size of one tetranucleotide repeat unit within the D13S1701 allele in two families may have been

caused by slippage during DNA replication of this repeat sequence. It is not possible to determine if this marker exhibits a higher mutation rate than other markers in the *BRCA2* region, as it was suggested by other studies [25], or if that mutation occurred early on during in the spreading of the population carrying the c.156_157insAlu *BRCA2* mutation. If we compare the D13S1701 genotypes for all 14 c.156_157insAlu positive probands, it is tempting to say that all mutation carriers with unphased genotypes harbored the same 299 allele, but in two out of three informative families we observed that this allele did not segregate with the mutation. The possibility exists that this variation in D13S1701 is more frequent than that observed by Machado et al. [16], so care should be taken when constructing haplotypes from independent families. If that is so, estimation based on the closest recombinant markers could greatly underestimate mutation age. Although Machado et al. [16] claim that the c.156_157insAlu *BRCA2* mutation has its origin 2,400–2,600 years ago in what is now central/southern Portugal, it is likely that it merely reflects their target population for genetic testing. In fact, we here show that the c.156_157insAlu *BRCA2* mutation accounts for 27% of the *BRCA1/BRCA2* deleterious mutations in northern/central Portugal (our main target population; Fig. 3), as compared to 16% in central/southern Portugal [16]. Although all known c.156_157insAlu *BRCA2* mutations have so far been identified in Portuguese breast/ovarian cancer families, we cannot rule out that this mutation may be present in other populations and so it might have been introduced in Iberian Peninsula from somewhere else.

In summary, our study provides significantly new evidence to substantiate the pathogenicity of the c.156_157insAlu *BRCA2* rearrangement and shows that this mutation is responsible for more than one-fourth of deleterious *BRCA1/BRCA2* mutations in northern/central Portugal. To further evaluate whether or not it constitutes a population-specific founder mutation, one needs to perform the screening of the c.156_157insAlu *BRCA2* rearrangement in other populations, which will allow for a more accurate estimation of its distribution and age.

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