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Genomic rearrangements of the BRCA1 gene in Chilean breast cancer families: an MLPA analysis

Alejandro Sanchez · Paola Faundez · Pilar Carvallo

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Abstract Point mutations and small deletions and insertions in *BRCA1* and *BRCA2* genes are responsible of about 20% of hereditary breast cancer cases in Chilean population. Studies in other populations have identified the amplification and/or deletion of one or more exons in these genes as the cause of the disease. In this study the authors determined the presence of these types of alterations in *BRCA1* and *BRCA2*, in 74 Chilean families with breast/ovarian cancer that were negative for germline mutations in these genes. Since these alterations are not detectable using the conventional PCR-based methods, the authors use MLPA (multiplex ligation-dependent probe amplification) to detect amplifications and/or deletions in *BRCA1* and *BRCA2* genes. The authors identified two different alterations in *BRCA1*: exon 10 duplication in one family and amplification of exons 3, 5, and 6 in two families. Duplication of exon 10 contains intronic adjacent sequences suggesting gene duplication. The second rearrangement consist of a 4 times amplification of a fragment containing exons 3, 5, and 6 joined together with no introns, suggesting the presence of a processed pseudogene. No alterations were detected in *BRCA2*. In order to validate the MLPA results and characterize the genomic alterations the authors performed qPCR, long range PCR, and sequencing.

Keywords Hereditary breast cancer · *BRCA1* · *BRCA2* · Genomic rearrangements · MLPA

A. Sanchez · P. Faundez · P. Carvallo (✉)
Department of Cell and Molecular Biology,
Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile
e-mail: pcarvallo@bio.puc.cl

Introduction

Mutations in tumor suppressor genes *BRCA1* (OMIM# 113705) and *BRCA2* (OMIM#600185) confer high risk for breast and/or ovarian cancer [1]. The frequency of hereditary breast cancer families carrying nonsense mutations, small deletions or insertions in these genes, vary from 15 to 70% depending on the origin of the studied population [2–4]. In Chile, two different studies have defined that no more than 20% of hereditary breast cancer families carry this type of mutations in *BRCA1* or *BRCA2* [5, 6]. Large rearrangement mutations involving amplifications or deletions of one or several exons of *BRCA1* or *BRCA2* genes have been also reported as the pathogenic cause of breast cancer [7–9]. As of now, large deletions and insertions represent a 10.7% of all reported mutations in the *BRCA1* gene and a 2.7% of all reported mutations in the *BRCA2* gene, being large insertions less frequent than large deletions [10]. The frequency of large genomic rearrangements (LGRs) affecting these genes varies from one population to another [11]. For example, two different LGRs account for 24% of BRCA1 gene alterations in Dutch families [12], while in other populations LGRs are infrequent or absent such as French/Canadian or Iranian [13, 14]. The existence of several intragenic *Alu* repeats [15] and a *BRCA1* pseudogene 30 kb upstream the *BRCA1* gene locus [16], make this gene prone to LGRs through homologous recombination [17, 18]. The widely used mutation screening techniques based on polymerase chain reaction are incapable to detect LGRs because of a preferential amplification of normal allele and thus a misinterpretation of results. Therefore, the use of appropriated methodologies such as southern blot, semiquantitative-multiplex PCR, real-time PCR, restriction analysis, long range PCR, and sequencing have been used to detect and fully determine the

pathogenic nature of this type of mutations [19]. Recently, multiple ligation-dependent probe amplification (MLPA) [20] has been used to detect LGRs with the advantage of the simultaneous analysis of all *BRCA1* or *BRCA2* exons in several samples. This approach, although not validated for clinical diagnostic, is a powerful high throughput tool and allows establishing a priority in the subsequent characterization of the rearrangements. The MLPA results must be confirmed through other techniques in order to carry out a successful genetic counselling.

In this study the authors used the MLPA technique to detect large deletions or insertions in the *BRCA1* and *BRCA2* genes in 78 patients from 74 breast cancer families that were negative for mutations in these genes according to previous studies. It was also used a set of complementary techniques to confirm and characterize the genetic alterations detected.

Patients, materials, and methods

Patients and controls

A total of 78 breast cancer patients from 74 families that were negative for *BRCA1* and *BRCA2* mutations [5 and unpublished results], were analyzed. These patients were primarily selected for hereditary breast cancer following these criteria: three cases of breast cancer in first degree relatives; or two cases of breast cancer in first degree relatives, one diagnosed before age 40; or one breast cancer and one ovarian cancer in first degree relatives. Genomic DNA was obtained from peripheral blood as previously described [21]. All patients signed an informed consent, and this protocol was approved by the Ethics Committee of the Faculty of Medicine, Pontificia Universidad Católica de Chile. The DNA from five individuals, not affected by cancer and not related to the families in study, was used as control for MLPA analyses.

Multiplex ligation dependent probe assay (MLPA)

Large genomic rearrangements in *BRCA1* and *BRCA2* were analyzed on genomic DNA using the SALSA MLPA commercial kits *BRCA1* P002-B1 lot.308 and *BRCA2* P045-B1 lot.108 (MRC-Holland, Amsterdam, The Netherlands). MLPA reaction was performed according to the manufacturer's recommendations, in 78 patient samples and 5 controls. PCR products were analyzed on an ABIPRISM 3100 capillary sequencer and Peak scanner v1.0 software. The data obtained was analyzed by using the Coffalyser software v.8 (MRC-Holland, Amsterdam, The Netherlands). This software normalizes all data, and calculates an average of the five peak areas corresponding to the five controls, for each probe result. A ratio for each probe, between samples and averaged controls, is represented in a chart, in which a normal copy number is considered between ratio values of 0.7–1.3. Ratios lower than 0.7 and higher than 1.3 are indicative of a deletion or amplification, respectively. Each result obtained by MLPA analysis was confirmed by two-independent experiments.

Quantitative real time PCR

To confirm the increased copy number of exons, primers localized at both ends of the respective exon were designed (Table 1) to generate an amplicon of approximately 80 bp suitable for qPCR analysis. For each exon analysis, one control DNA and DNA from all patients presenting the exon copy number variation, were serially diluted (100, 50, 25, and 12.5 ng/μl) and 1 μl, and subjected to PCR using the SYBR® GreenER™ qPCR SuperMix Universal (Cat. no. 11762-100, Invitrogen) in an Applied Biosystem 7500 thermal cycler. Calibration curves were constructed for each analyzed exon. Quantification of the altered exons was assessed in control DNA and in DNA from patients by comparing the crossing threshold (Ct) values determined by the Applied Biosystem 7500 System SDS software v.1.4.0. *BRCA1* exon 22 was used as reference of wild type allele dosage.

Long Range PCR

To determine if the extra copies of exons were present in the intragenic region of *BRCA1* it was used the Expand Long Range PCR System kit (Roche) to amplify twelve fragments ranging from 2 to 13 Kb, encompassing the whole gene. These PCR products were used later as a template for a nested PCR, using primers that specifically amplify the extra copies of exons.

Results

MLPA analysis

Two different LGRs were detected in *BRCA1* gene in different families. Two examples of these LGRs are shown in Fig. 1a. Figure 1a left, shows the presence of extra copies of exons 3, 5, and 6 in one of the two families. This family (70) has two breast cancer affected women, mother and daughter diagnosed at 40 and 48 years, respectively, presenting the same LGR (Table 2). In the second family (71), two siblings, mother and daughter, diagnosed at 73 and 42 years, were studied. Only the mother presented this rearrangement. Figure 1a right shows the MLPA results for one family (59) having an amplification of exon 10. In this

Table 1 Primers for MLPA, qPCR, Long Range PCR, and sequencing

Primer name	Sequence 5′ > 3′	Location	Used for
B1.3intF	TCTGGAGTTGATCAAGGAACC	Exon 3	qPCR
B1.3intR	TTGCAAAATATGTGGTCACAC	Exon 3	qPCR
B1.5intF	AGATTTTGCATGCTGAAACTT	Exon 5	qPCR
B1.5intR	ACCTTTTGGTTATATCATTCTT	Exon 5	qPCR
B1.6intF	GAGCCTACAAGAAAGTACGA	Exon 6	qPCR
B1.6intR	CACTCCAAACCTGTGTCAAG	Exon 6	qPCR
B1.10F	TGGTCAGCTTTCTGTAATCG	Intron 10	qPCR, Restriction analysis, Long range PCR
B1.10R	GTATCTACCCACTCTCTTCTTCTTCAG	Intron 10	qPCR, Restriction analysis, Long range PCR
B1.11AR	CTTCCAGCCCATCTGTTATGTTG	Exon 11	Restriction analysis
B1.9F	CCACAGTAGATGCTCAGTAAATA	Intron 9	Restriction analysis
B1.10probeF	TCAAGGAACCAGGGATGAAATC	Exon 10	Long range PCR
B1.10probeR	TGATTTGTAACAATTCTTGATCTC	Exon 10	Long range PCR
B1.1F	TAGCCCCTTGGTTTCCGT	5′UTR	Long range PCR
B1.3R	TTGGATTTTTCGTTCTCACTT	Intron 3	Long range PCR
B1.3F	TCCTGACACAGCAGACATTTA	Intron 2	Long range PCR
B1.7BR	AGGACTGCTTCTAGCCTGG	Exon 7	Long range PCR
B1.7AF	CACAACAAAGAGCATACATAGGG	Intron 6	Long range PCR
B1.12R	TGTCAGCAAACCTAAGAATGT	Intron 12	Long range PCR
B1.12F	GTCCTGCCAATGAGAAGAAA	Intron 11	Long range PCR
B1.13R	ATGTTGGAGCTAGGTCCTTAC	Intron 13	Long range PCR
B1.13F	AATGGAAAGCTTCTCAAAGTA	Intron 12	Long range PCR
B1.14R	GTGTATAAATGCCTGTATGCA	Intron 14	Long range PCR
B1.14F	CTAACCTGAATTATCACTATCA	Intron 13	Long range PCR
B1.19R	CATTGTTAAGGAAAGTGGTGC	Intron 19	Long range PCR
B1.19F	CTGTCATTCTTCCTGTGCTC	Intron 18	Long range PCR
B1.20R	ATGGGTGGGGTGAGATTTTTGTC	Intron 20	Long range PCR
B1.20F	CCTGATGGGTTGTGTTTGGTTTC	Intron 19	Long range PCR
B1.21F	AAGCTCTTCCTTTTTGAAAGTC	Intron 20	Long range PCR
B1.21R	GTAGAGAAATAGAATAGCCTCT	Intron 21	Long range PCR
B1.22R	GAGAAGACTTCTGAGGCTAC	Intron 22	Long range PCR
B1.22F	TCCCATTGAGAGGTCTTGCT	Intron 21	Long range PCR
B1.23R	ACTGTGCTACTCAAGCACCA	Intron 23	Long range PCR
B1.23F	CAGAGCAAGACCCTGTCTC	Intron 22	Long range PCR
B1.24R	GTAGCCAGGACAGTAGAAGGA	Intron 24	Long range PCR

family it was analyzed two breast cancer affected sisters, diagnosed at 50 and 51 years, only one presenting this rearrangement (Table 2), indicating that this extra copy does not segregate with the disease. The amplification ratio for exons 3, 5, and 6 is over 4.0 in both families suggesting that several copies of these exons may be present in the genome. For exon 10 the amplification ratio was 1.6, indicating that this is probably a duplication.

Quantitative PCR analysis

In order to corroborate the amplifications detected by MLPA the authors performed quantitative PCR using primers located at both ends of exons 3 and 5 (coding sequence) and two intronic primers flanking exon 10 (Table 2). In the case of exon 6 qPCR did not revealed an extra copy, probably because of a failure of forward primer for this exon. The presence of an extra copy of exon 6 was demonstrated in an additional experiment shown in Fig. 2a. As shown in Fig. 1b extra copies of exons 3, 5, and 10 were confirmed by the relative amplification values obtained by qPCR. Amplification values are higher for exon 3 and 5 than for exon 10, as it was defined by MLPA ratios. Concordant to MLPA results, qPCR relative amplification values show that both siblings (M138, M139) from family 70, have extra copies of exons 3 and 5, but in families 71

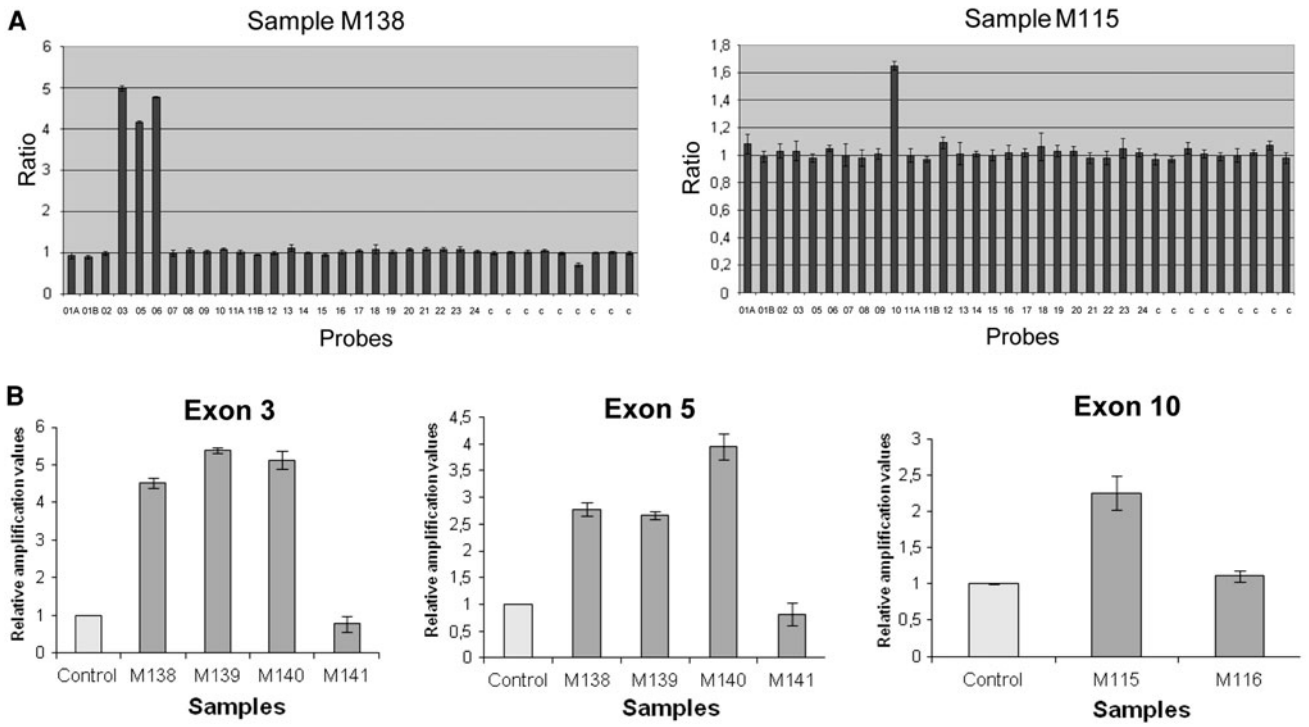

Fig. 1 MLPA analysis and qPCR results for *BRCA1*. **a** MLPA charts obtained after the analysis with Coffalyser v.8 software. The ratio of the averaged fluorescence of control DNA and sample DNA probes (*Y axis*) for each one (*X axis*) is represented. *Left chart* shows the amplification of exons 3–6 in DNA from sample M138 (ratio values of 5.01, 4.13, and 4.73 for exons 3, 5, and 6, respectively). *Right chart* shows the results for patient M115 from family 59 where an amplification of exon 10 is observed (ratio value of 1.63). **b** Amplification of exons 3, 5, and 10 were confirmed by qPCR taking as reference control DNA. The determination was assessed using the $2^{\Delta\Delta ct}$ method

Table 2 Description of cancer cases, MLPA, and qPCR results for each family

Family	Type and number of cancer cases	Sample	MLPA result	qPCR product
70	Breast (3), lung (1), lymphoma (1)	M138	Exons 3–6 amplification	Amplified
		M139	Exons 3–6 amplification	Amplified
71	Breast (3), brain (1), testicle (1), prostate (1), uterus (1)	M140	Exons 3–6 amplification	Amplified
		M141	No rearrangement	No amplified
59	Breast (2), ovary (2), lung (2), lymphoma (1), pancreas (1)	M115	Exon 10 amplification	Amplified
		M116	No rearrangement	No amplified

(exons 3 and 5) and 59 (exon 10), only one relative (M140, M115) show a higher value reflecting the presence of extra copies. From this observation the authors confirmed that in these two families the rearrangement does not segregate with the disease, strongly suggesting that the extra copies of the mentioned exons are not affecting the expression or function of the *BRCA1* gene.

PCR amplification analysis of the fragment containing a fusion of exons 3–6

A PCR amplification of exons 3 to 6 was performed using exonic primers at the 5′ end of exon 3 (B1.3intF) and at the 3′ end of exon 6 (B1.6intR) (Table 2), in the three patients presenting the extra copies of these exons (M138, M139, M140). As shown in Fig. 2a, a PCR product close to 220 bp was obtained, which is consistent with the sequence of 221 nucleotides corresponding to the joining of complete exonic sequences 3, 5, and 6. The genomic fragment containing exons 3–6, with the respective introns, is too large (10 kb) to be amplified in these conditions, though no PCR product was obtained. It is important to note that a PCR amplification reaction using primers located in intron 2 and intron 6 did not lead into any product, indicating that the extra copy of exons 3–6 is not flanked by their respective introns. To compare the DNA sequence of the

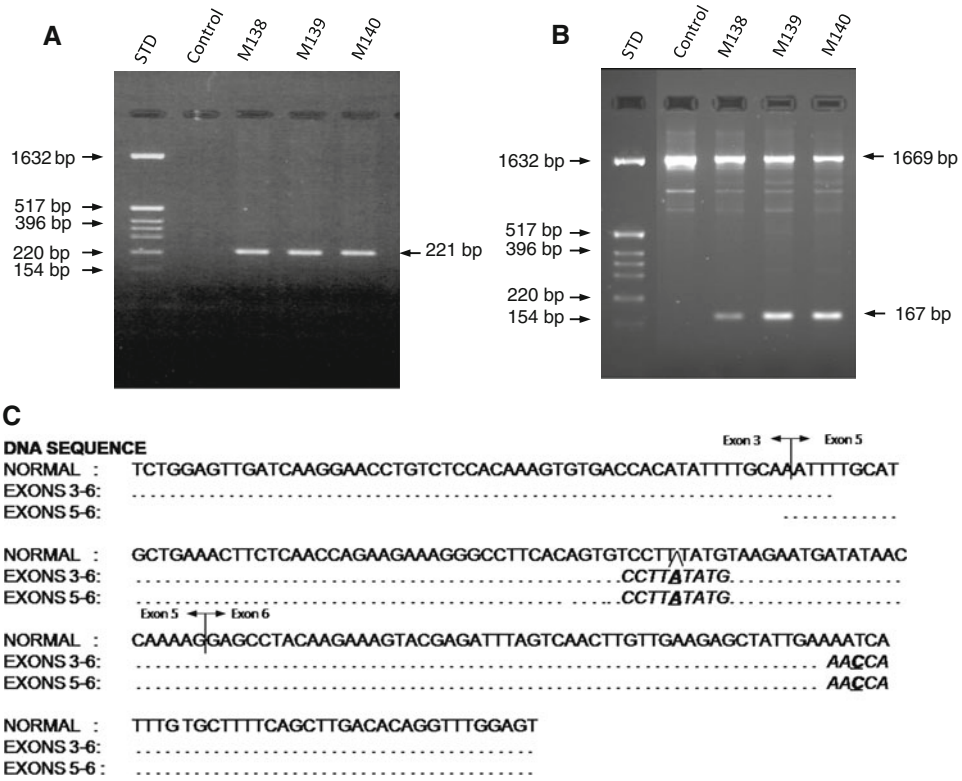

Fig. 2 DNA structure of the extra copy involving exons 3,5, and 6. **a** DNA from patients showing amplification of exons 3–6 and one control, were amplified by PCR with primers B1.3intF and B1.6intR **b** or primers B1.5intF and B1.6intR **c** DNA sequence of amplified fragments containing exons 3–6 or 5–6. *Dots* indicate no change, exon boundaries are indicated, and the two nucleotide changes are in *bold* and *underlined*

genomic alleles and the extra copy of exons 3–6, the authors PCR amplified exons 5 and 6 in a sample of the three patients presenting this rearrangement. As shown in Fig. 2b, the authors obtained two different products, one corresponding to the exon–intron structure (1669 bp), and another close to 160 bp, corresponding to exons 5 and 6 joined (167 bp) (Fig. 2b). The sequence analysis of fragments 3–6 (Fig. 2a) and 5–6 (Fig. 2b), are shown in Fig. 2c. Two nucleotide changes were found in the fragment containing the joined 3–6 exons: an adenine insertion, c.306_307insA, leading to a stop at codon 65 (p.L63Yfs X65), and c.385 T > C (p.I89T), both previously described as a pathogenic mutation [5] and allelic variant [22], respectively. These variants are present in the three patients showing this insertion, suggesting a common ancestor or origin. On the other side, these two variants are not present in the corresponding exons of the *BRCA1* alleles of the three patients, confirming that these are extra copies. In addition an intronic variant c.332-161A > G was found in intron 5 of the gene allele (fragment of 1669 bp, Fig. 2b), in a homozygous form in patient M139 (mother), and in a heterozygous form in the daughter M138. The third patient does not have this polymorphism. The absence of the c.306_307insA in exon 5 belonging to the *BRCA1* gene is an additional proof for the presence of an exon five extra copies in these three patients.

Long range PCR analysis of the whole BRCA1 gene region

To address the question of the localization of the BRCA1 extra copies of exons 3–6 and exon 10, long range PCR of *BRCA1* to search for these exons inside the sequence was performed. For this purpose it was divided the whole sequence in 12 fragments (Fig. 3a), and used them as templates for PCR reactions with primers able to amplify the described extra copies. None of the *BRCA1* fragments revealed the presence of an extra copy of exons 3–6 or exon 10. Since fragments C and D contain the wild type copy of exon 10, the authors followed a different strategy to seek for an extra copy of exon 10 within introns 9 or 10. As shown in Fig. 3a (above exon 10), four different PCR reactions were done to analyze the structure of the gene region containing exon 10 and adjacent introns. Primers B1.10probeF and B1.10probeR, were designed in opposite direction and complementary to the sequence where the MLPA probe anneal. The strategy of this PCR reaction was to search for a probable presence of exon 10 within one of the adjacent introns. The authors did not obtain any PCR

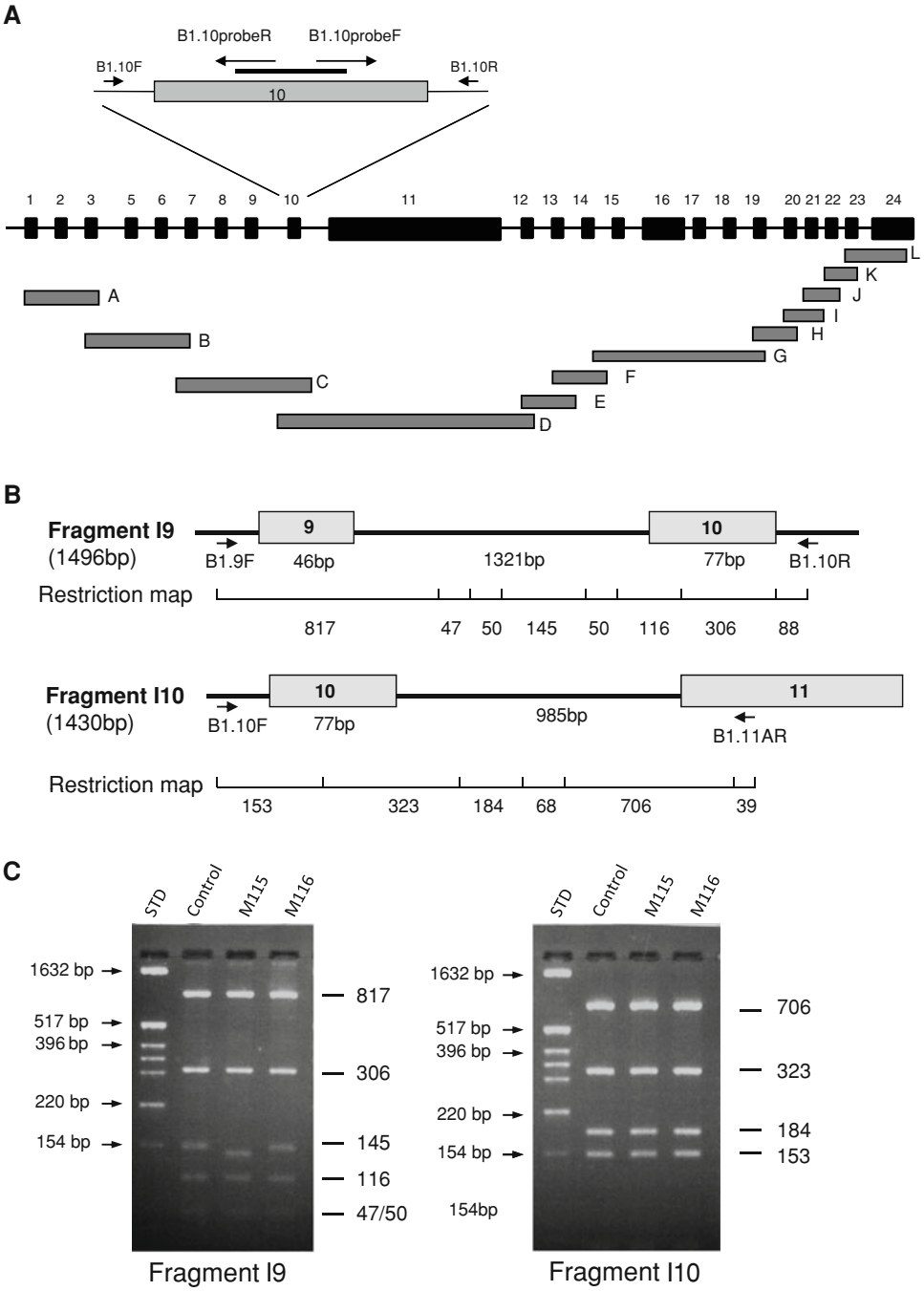

Fig. 3 Long range PCR and restriction analysis of *BRCA1*. **a** The structure of *BRCA1* and the fragments amplified by long range PCR to cover all genomic region is shown. Above, an amplified view of exon 10 with the location of the MLPA probe and the primers B1.10probeF, B1.10probeR used to verify the presence of an extra copy of exon 10 in the adjacent intronic regions of exon 10. **b** Restriction map of fragment I9 containing exon 9-intron 9-exon 10 and fragment I10 containing exon 10-Intron 10 with BstNI. **c** Electrophoresis of digestion fragments I9 and I10 from one control, samples M115 and M116. Restriction digestion products were resolved in a NuSieve: agarose gel (2:1), and the restriction fragment pattern was analyzed

product indicative of the presence of exon 10 duplicated in the adjacent introns.

Two additional PCR fragments were analyzed seeking for the extra copy of exon 10, as shown in Fig. 3b. These PCR fragments were restriction digested with BstNI to confirm the DNA map of this region. As shown in Fig. 3c the electrophoresis pattern of fragments I9 and I10 shows no differences between controls and samples. Only sample M115, shows a band smaller than the expected 145 bp product. The smaller size is because of a less number of T in a tract of 18 T present in this fragment, which was demonstrated by sequencing (not shown). In conclusion the extra copy of exon 10 is not present in the region involving exon9-intron9-exon10-intron10-exon 11.

Discussion

The discovery of the first *BRCA1* rearrangement gave a new sight in the mutational screening of *BRCA1* and *BRCA2* genes [17]. As of now, studies in various populations have found a differential contribution of LGRs to

hereditary breast cancer. This is the first MLPA study in Chilean hereditary breast cancer patients detecting LGRs in *BRCA1*. No LGRs involving *BRCA2* were detected in this study. Among different populations LGRs in *BRCA1* are 4 times more frequent than in *BRCA2* [11], and in families with male breast cancer cases the incidence of *BRCA2* LGRs is higher [23]. In the group of families, only two present male breast cancer cases and none of these families have shown mutation or LGRs in *BRCA2*.

In this study the authors detected two different amplifications in *BRCA1* in approximately 3.8% of the 74 studied families, having no point mutation in *BRCA1* or *BRCA2*. The great majority of LGRs described up to now are deletions involving *BRCA1* or *BRCA2*, being amplifications very infrequent [11]. In addition, deletions are normally well described and validated by confirming the interruption of BRCA1 mRNA open reading frame, but amplifications or duplications have been validated in very few cases. Some of these are, the duplication of exon 13 [24] which has been widely described up to now, duplication of exons 3–8 [25] in a French group of patients, triplication of exons 17–19, and duplication of exons 21–23 [12] in a Dutch population, duplication of exon 20 in Italian patients [26], and duplication of exons 18 and 19 in USA [27]. Other duplications have not been exactly localized in the genome, though the significance of these LGRs is not certain yet. In this study the authors demonstrated through MLPA two different rearrangements validated by qPCR that resulted to be localized outside *BRCA1*.In addition, the LGRs do not segregate with breast cancer in two families (family 71 for exons 3–6 and family 59 for exon10; Table 2), revealing no association of the amplification with the pathology. Recently, in an Asian sporadic breast cancer patient, an amplification of exon 10 of *BRCA1* has been reported [28] which is similar to the one found in this study. Amplification of *BRCA1* exons 3–6 has not been described to date.

The amplification of exons 3, 5, and 6 of *BRCA1* consists in a fragment of 221 bp containing the exons 3, 5, and 6 joined without introns. This fragment is restricted to the 5′ coding region of exon 3 and the 3′ coding region of exon 6, harboring the deleterious mutation c.306-307insA in exon 5 and the allelic variant c.385C > T in exon 6. The presence of the 3 exons with no introns strongly suggests that this LGR is a processed pseudogene product of a reverse transcription of part of the *BRCA1* mRNA and the following insertion in the genome [29]. The existence of retropseudogenes has been reported for many human genes as β-tubulin [30], β-actin [31], and dihydropholate reductase [32]. In relation to exon 10 duplication, the sequence has been detected by PCR amplifying with primers localized at nucleotides −110 and +53 relative to exon 10. This finding better corresponds to gene duplication more than a retro-transposition. This genomic duplication is not inside *BRCA1*, though it could be close to the gene, far in the same chromosome o in a different chromosome, either way it does not affect the *BRCA1* open reading frame.

Chilean population resulted mainly by an admixture of Amerindian and Spaniards, though the interest in searching for founding mutations in breast cancer has been focused in the Spanish population. In relation to point mutations in *BRCA1* and *BRCA2* in Chilean breast cancer families, it was found that 40% of mutations detected are from Spanish origin [5], being recurrent mutations in the population. In relation to LGRs in *BRCA1* found in the Spanish population, the reports describe mainly deletions [33–36] and few duplications. Among duplications one study reported an extra copy of exon 20 in *BRCA1* [33], but no segregation or other analysis were performed in order to determine the pathologic relevance of this duplication. Interestingly, Agata et al. [26] have already described the same duplication, in Italian patients, confirming that both copies of exon 20 are together between exons 19 and 21. If this duplication is the same among the Italian and Spanish patients remain to be clarified. The other two duplications in *BRCA1*described in Spanish breast cancer families for exons 1–2 and 9–24 [36], were not confirmed as being deleterious for gene expression. In summary none of the deletions or duplications in *BRCA1* or *BRCA2* found in Spanish breast cancer patients was found in the Chilean study.

This study shows clearly that not all LGRs have a clear pathogenic effect, especially in terms of duplications or amplifications detected by MLPA and even validated by qPCR. It is extremely important to define co-segregation of the LGR with the pathology in the family, to determine the localization of the extra copies found through MLPA, to assess functional studies, as mRNA structure and sequencing, and to determine if the duplication/amplification found affect gene function. MLPA is a great technique to start seeking for rearrangements in genes involved in diverse pathologies, but it must be complemented with other approaches before genetic counselling.

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