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Large Genomic Deletions and Duplications in the *BRCA1* Gene Identified by a Novel Quantitative Method

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Abstract

We applied a novel method to detect single or multiple exon deletions and amplifications in the *BRCA1* gene. The test, called multiplex ligation-dependent probe amplification (MLPA), uses probes designed to hybridize adjacently to the target sequence. After ligation, the joined probes are amplified and quantified. Our two diagnostic laboratories have tested in the recent years 805 families by conventional PCR-based techniques, and found 116 BRCA1 and 28 BRCA2 mutation-positive families. Using MLPA, we have tested the remaining 661 noninformative breast cancer families and identified five distinct *BRCA1* germ-line mutations in five families: a deletion of exon 8, a deletion of exons 20–22, a duplication of exon 13 and exons 21–23, respectively, and a triplication, encompassing exons 17–19. Genomic deletions of *BRCA1* constitute a substantial fraction of mutations in Dutch breast cancer families. If MLPA had been included in our initial *BRCA1* testing, 33 families with a deletion or duplication would have been identified, representing 27% of the total 121 *BRCA1* mutation-positive families. The MLPA test for *BRCA1* ensures a sensitive and comprehensive high-throughput screening test for genomic rearrangement and can easily be implemented in the molecular analysis of *BRCA1*.

Introduction

Germ-line mutations in the *BRCA1* gene cause a hereditary predisposition to breast and ovarian cancer. Methods used to screen for mutations focus on genomic DNA and are usually PCR-based, enabling the detection of sequence alterations such as point mutations, and small deletions and insertions. By this approach, thousands of *BRCA1* families have been identified worldwide (the Breast Cancer Information Core on the Internet).[2] An increasing number of large genomic alterations have been described recently as laboratories put more effort into the detection of such alterations. At present, >18 different large genomic rearrangements have been characterized; they include both deletions and duplications of one or more exons in *BRCA1* (1–6). Although for some recurrent mutations a simple PCR test has been developed (4, 5), the gold standard to search for aberrant copy numbers of one or more exons is Southern blotting. However, this technique is time consuming and laborious and, therefore, screening is usually restricted to selected families (1–4, 7, 8). Ideally, all of the individuals eligible for *BRCA1* mutation screening should be screened for the presence of large genomic deletions and duplications. Therefore, we applied a new method, called MLPA,[3] which enables us to determine the relative copy number of all of the *BRCA1* exons simultaneously with high sensitivity in a high-throughput format (9). We have shown recently that this method is very successful in the identification of large genomic deletions in the *hMLH1* and *hMSH2* genes in hereditary nonpolyposis colorectal carcinoma families (10). The principle of this technique is shown in Fig. 1. In brief, only adjacently hybridized and subsequently ligated probes can be amplified by PCR. Because up to 40–50 target sequences can be analyzed simultaneously, the complete *BRCA1* gene can be screened in one single reaction. Fragment analysis is carried out on an automated, preferably capillary, sequencer identifying each of the fragments based on the specific PCR fragment length. The measured peak area is used to calculate the relative quantity of each probe. In this report we show the results of the MLPA-*BRCA1* analyses of >660 breast and/or ovarian cancer families, reveal alterations not reported before, and conclude that the method is reliable and very suitable to be included in the routine molecular analysis of predisposed families.

Materials and Methods

Family Ascertainment. Self-referred or physician-referred breast and/or ovarian cancer-prone families investigated at the family cancer clinics of the Netherlands Cancer Institute and the VU University Medical Center, which were opting for mutation screening, were the subject of this study. Between 1995 and 2001, individuals from 805 families have been tested in our laboratories of which 79% were breast cancer only families, 18% were breast and ovarian cancer families, and 3% were ovarian cancer only families. In general, these families had an *a priori* chance of ≥10% of harboring a deleterious *BRCA1* or *BRCA2* mutation (11).

MLPA Reaction and Fragment Analysis. The *BRCA1*-MLPA test was developed and manufactured by MRC-Holland in close collaboration with one of the authors (G. P.). The preparation and sequences of the probes has been described elsewhere (9).[4] The *BRCA1*-MLPA test itself is commercially available at MRC-Holland. In short, 50–500 ng target DNA/5 μl of 10 mM Tris (pH 8)-0.1 mM EDTA was denatured for 5 min at 98°C after which 3 μl of the probe mix was added. The mixture was heated at 95°C for 1 min and incubated at 60°C overnight (16 h). Ligation was performed with the temperature-stable Ligase-65 enzyme (MRC-Holland) for 15 min at 54°C. Next, the ligase was inactivated by incubation for 5 min at 98°C. Ten μl of this ligation mix was premixed with 30 μl of PCR buffer and put in a PCR machine at 60°C. Subsequently, a 10-μl mix was added containing deoxynucleoside triphosphate, Taq polymerase, and one unlabeled and one carboxyfluorescein-labeled PCR primer, which are complementary to the universal primer sequences. PCR was carried out for 33 cycles (30 s at 95°C, 30 s at 60°C, and 60 s at 72°C). The fragments were analyzed on an ABI model 310 or 3700 capillary sequencer (Applied Biosystems) using Genescan-ROX 500 size standards (Ap-

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[2] Internet address: http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

[3] The abbreviations used are: MLPA, multiplex ligation-dependent probe amplification; nt, nucleotide; RT-PCR, reverse transcription-PCR.

[4] Internet address: http://www.mrc-holland.com.

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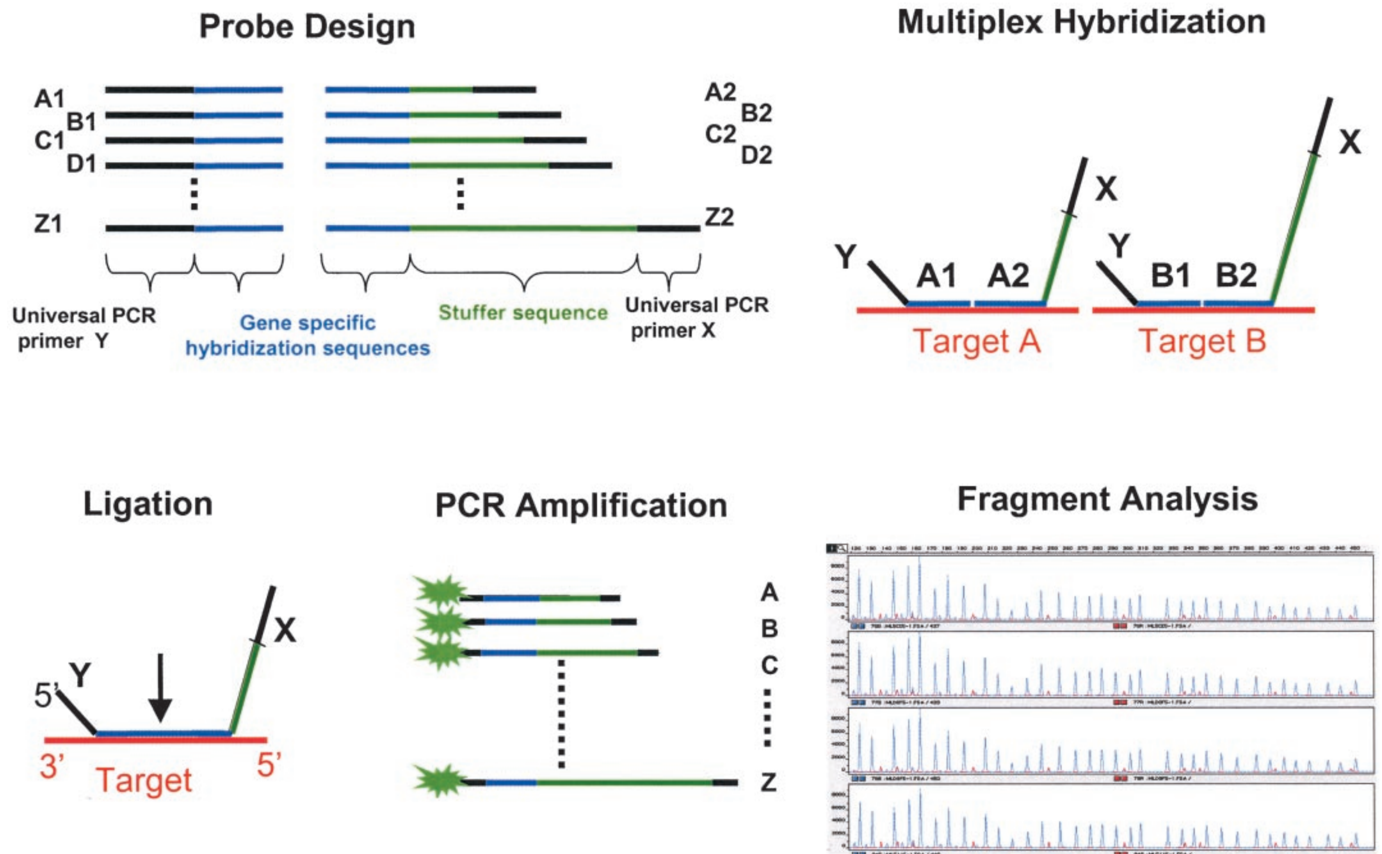

Fig. 1. Principle of MLPA. For each specific target, a set of two probes was designed that hybridize immediately adjacent to each other on the same target strand. Both probes consist of a short (22–43 nt) target-specific sequence and a universal forward or reverse PCR primer-binding site. In addition, one of the probes contains a so-called stuffer sequence. For each probe, the stuffer part has a specific length (19–364 bp) and sequence. The long probes are M13-derived. The short probes are synthetic. After an overnight hybridization to the target DNA, the two parts of each hybridized probe are joined by a ligation reaction. Next, a PCR is carried out with a single fluorescent-labeled primer pair, which ensures that the relative yield of the PCR products is proportional to the amount of target. The fragment analysis is preferably carried out on an automated capillary sequencer. The multiple fragments can be distinguished based on different length. The peak area value of each product is used to calculate the relative quantity.

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plied Biosystems). Fragment analysis was performed using Genescan and Genotyper software.

Quantitative and Statistical Analysis. To automate the interpretation of the fragment analysis, the relative quantity of the amplified probes in each sample was determined using an Excel template. For this purpose, the relative peak areas for each probe were calculated as fractions of the total sum of peak areas in a certain sample. Subsequently, the fraction of each peak was divided by the average peak fractions of the corresponding probe in control samples. Finally, the values were normalized using the values obtained for the autosomal control probes, which served as a reference for the copy number of 2.0. Between 2 and 8% of the samples showed a variation >20% in the control genes. They were removed from the quantification calculations and retested. The great variation was because of either the high quantity of the input DNA or the DNA extraction method, in particular, because of the presence of residues of agents (like phenol). In general, reduction of the amount of input DNA yielded less variation.

Confirmation of Rearrangements by PCR. Primers for the exon 13 duplication were described by Puget *et al.* (5). For the newly discovered deletion of exons 8 and 20–22, primers were designed located in the flanking introns. The primer combination for the exon 8 deletion is: forward primer (nt 27164; GenBank accession no. L78833) 5′-GTTGGGCTTTTAAAT-ACTCGTTCC-3′ and reverse primer (nt 29530) 5′-CCTATTGCTACTCTC-CATCTTTCC-3′. The deletion of exons 20–22 could be detected using forward primer (nt 67911) 5′AGATAGGCCAATGTTGGTCG- 3′ and reverse primer (nt 81183) 5′-ACTGTGCTACTCAAGCACCA-3′. The recombination site was sequenced using forward primer (nt 68664) 5′-GCTGGGAATGGT-GGCATGTG-3′. The primer combination for the elucidation of the exons 21–23 duplication is forward primer (nt 81291) 5′-GGGTGGGAGTAA-GATATGAAT-3′ and reverse primer (nt 75461) 5′-TGGAGCCAAATGCT-GACATGA-3′. The primer combination used for the triplication of exons 17–19 is: forward (nt 66183) 5′-AAGAAACAGTCCTTTCTTTGGG)-3′ and reverse (nt 58949) 5′-GATGAAAAATCCTAGGAAGG-3′.

Sequence Analysis. Purified PCR products, encompassing the recombination sites, were subjected to cycle sequence reactions either by BigDye Terminators or by BigDye Primers (DNA Sequencing kit; Applied Biosystems). The sequenced fragments were precipitated and analyzed using an automated sequencer (ABI 310, 377, or 3700).

Southern Blotting. Genomic DNA was digested with *Bam*H1 or *Eco*R1, and run on agarose gels (0.8%) for 16 h in Tris-borate EDTA buffer. DNA was denatured and transferred to nylon membranes (Hybond N+; Amersham). Two different probes were generated using RT-PCR, and primers in exons 16 and 20 or 16 and 24. Purified fragments were radioactive labeled using the Random labeling kit (Roche). The hybridizations were carried out overnight at 65°C in Quick-hyb (Stratagene). After washing, the filters were exposed to a phosphorimager screen (Fuji) for quantification or to Kodak X-Omat film.

Results and Discussion

For each of the 24 exons of *BRCA1* we designed probes, including two probes for the large exon 11 to establish the exon-specific quantification. Polymorphic sequences were avoided, because they could hamper hybridization and quantification. Furthermore, the ligation sites of the exon 1a, 1b, and 2 probes were located at sequences differing from the pseudo-*BRCA1* exons (12). Probes located more upstream of the *BRCA1* gene were not included in the test, because the rearrangements reported thus far always included one or more exons of *BRCA1* gene. An additional seven control probes served as quan-

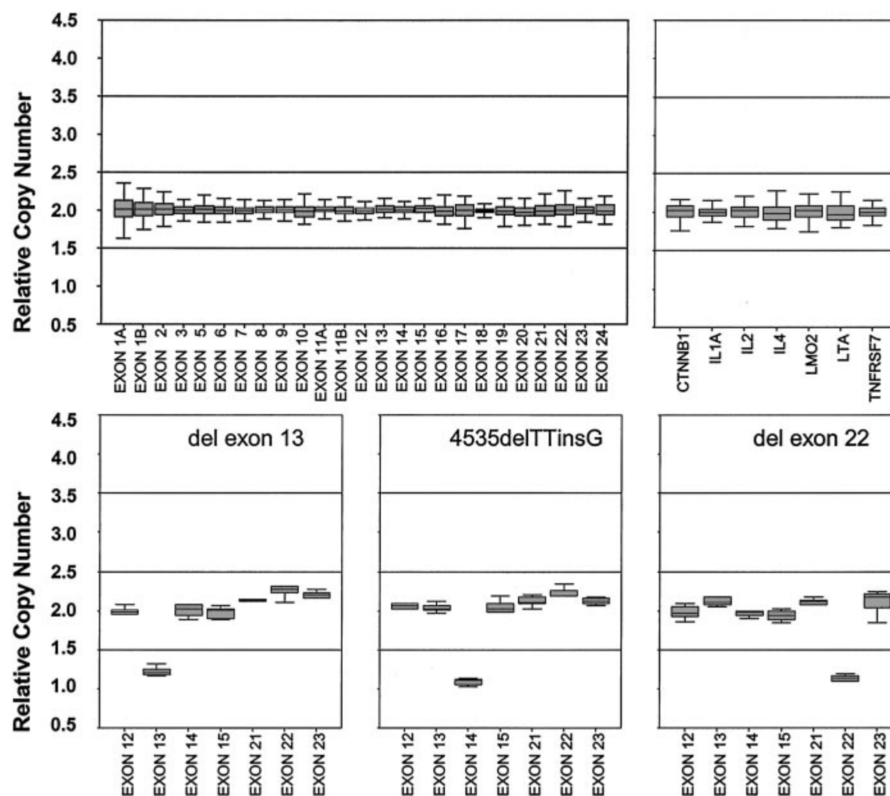

Fig. 2. Quantitative MLPA analysis of controls and *BRCA1* mutation carriers. The quantitative results are represented as box plots with median relative copy numbers; 50% of the samples are within the *box*, 95% within the *error bars*. The first and second panels show the quantitative analysis of the peak areas for the *BRCA1* and non-*BRCA1* probes in 90 control samples. The SD is 3–11% depending on the exon. The *bottom panels* show the quantitative analysis of known *BRCA1* mutation carriers, which were used to validate the method. Data were obtained from independent experiments ($n = 6$) and show SDs from 3 to 6%, depending on exon and sample; *bars,* ±SD.

tification reference, allowing the accurate detection of a copy number change of *BRCA1* exons or the entire gene.

To automate the interpretation of the fragment analysis we normalized the relative quantity of each of the amplified probes in each sample, using the relative copy number of two for the control probes. The MLPA analysis of 90 control samples yielded highly reproducible results, both for *BRCA1* and non-*BRCA1* probes (Fig. 2, *top panels*). The limited variation allows an accurate discrimination between carriers of deletions and duplications or noncarriers.

To test the sensitivity and specificity of the *BRCA1*-MLPA, we

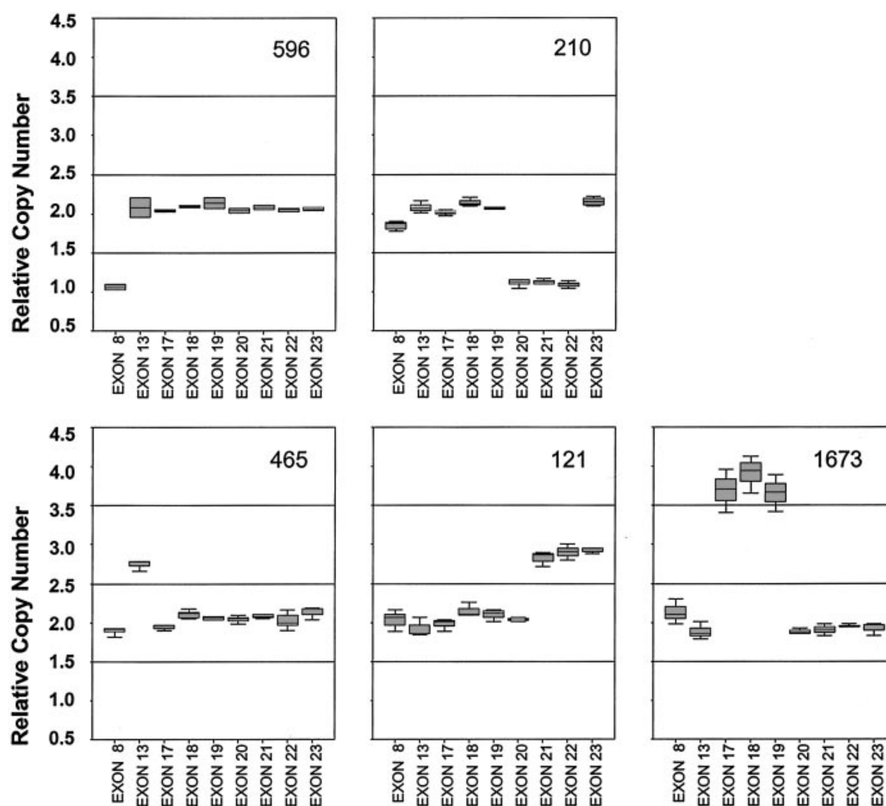

Fig. 3. MLPA identifies 5 different mutations in 5 families. Out of 661 families analyzed by MLPA, 5 families were shown to harbor aberrant gene copy numbers of one or more *BRCA1* exons. In the box plot-panels only part of the complete *BRCA1* quantitative MLPA analysis is shown. Results were obtained from three to six independent experiments; *bars,* ±SD.

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used samples from known *BRCA1* mutation carriers of a deletion of exon 13 or 22, both of which are found frequently in the Dutch population, or a mutation located at the ligation site of the exon 14 probes (4535delTTinsG). The Genescan electrophorerograms of the exon 13 deletion showed a clear reduction of the fluorescence signal of this exon compared with a normal control sample (data not shown). Similar results were obtained for the exon 22 deletion and the exon 14 ligation site mutation. After quantification, the reduction of the relative copy numbers for exons 13, 14, or 22 (Fig. 2, *bottom panels*) was estimated to be 40–50%, which results in a relative copy number of one for the exons involved.

Among 805 families tested between 1995 and 2001 in our two diagnostic laboratories (Netherlands Cancer Institute and VU University Medical Center) a mutation with a predicted pathogenic effect was identified in 144 families, using denaturing gradient gel electrophoresis, denaturing high performance liquid chromatography, protein truncation test, or mutation-specific tests. A *BRCA1* mutation was detected in 116 families, 28 families of which with a deletion of exon 13 or 22. A *BRCA2* mutation was found in 28 families. Using MLPA, we have screened the remaining 661 families (843 individuals) and detected 5 families with aberrant exon copy numbers (Fig. 3). Two novel deletions were identified: a deletion of exon 8 in case 596 and a deletion of the exons 20–22 in case 210. In addition, two duplications were found: a duplication of exon 13 (case 465) and a novel duplication of exons 21–23 (case 121). Finally, in case 1673 MLPA suggests a relative copy number of four for exons 17–19, being the first example of exon triplication in the *BRCA1* gene. Repeated experiments showed similar results as can be seen in the box-plot representations.

Regarding the cancer history of the identified families, there are no additional characteristic features compared with already identified *BRCA1* families. Except for case 1673, the families can straightforwardly be classified as high-risk families because of multiple cases of breast cancer, and within some families ovarian cancer or bilateral breast cancer (13). Case 1673 is part of a very small family in which no other cancers has been diagnosed thus far. She has been tested because she had breast cancer at the age of 31.

PCR and/or Southern blotting were carried out to verify the gene rearrangements detected by MLPA. PCR analysis showed that the duplication of exon 13 in case 465 was identical to the duplication described by Puget *et al.* (Ref. 5; data not shown). For the novel deletions, we used various combinations of PCR primers to obtain a junction fragment for sequence analysis. We found that the exon 8 deletion in case 596 starts at position IVS7–1129 and comprises 1458 bp including exon 8. The deletion of exons 20, 21, and 22 starts at position IVS19–2840 and comprises 11,395 nt. Southern blotting was used to investigate the amplifications of exons 21–23 and 17–19 (Fig. 4*a*). The *Bam*H1 digestion of case 121 resulted in an extra fragment of ~7.5 kb. Additional analysis by PCR and sequencing showed that the duplication of exons 21–23 consisted of a tandem duplicated fragment of 7654 nt starting at position IVS23 + 343 (Fig. 4*b*). In case 1673, the *Eco*R1 digestion generated an extra band of ~8 kb (Fig. 4*a*). Quantitative analysis of this band confirmed the presence of two additional copies of the exons 17–19. Combined with the fact that case 1673 is heterozygous for several *BRCA1* polymorphisms, we conclude that one allele contains three copies of exons 17–19 in tandem repeat. Sequence analysis showed that the triplication of exons 17–19 starts at IVS19 + 1681 and comprises an amplicon of 8352 nt (Fig. 4*b*). Because >40% of the *BRCA1* genomic sequence consists of *Alu* repeats it is not surprising that all five of the genomic breakpoints are located in *Alu* elements and, thus, are likely caused by *Alu* recombination (14).

MLPA testing of 661 families with a previously negative test result during regular screening yielded two deletions and three duplications, of which four were novel. Because these mutations occurred only once, it is unlikely that in addition to the exon 13 and exon 22 deletion founder mutations, another high frequency exon copy number mutation is present in the Dutch population. Furthermore, if MLPA had been used as the initial screening technique, 33 families with a deletion or duplication in *BRCA1* would have been identified, representing 27% of the total 121 pathogenic *BRCA1* mutations. In parallel to the Dutch situation, the fraction of mutations missed by direct sequencing or other mutation detection methods based on PCR amplification of small fragments could be high in any population. Regarding the mutation status of the 805 families tested thus far for the *BRCA1* (including MLPA) and *BRCA2* genes, 13.2% of the breast cancer only families were mutation positive, 15% of the ovarian cancer only families, and 54.6% of the breast and ovarian cancer families. Furthermore, the presence of genomic rearrangements was not confined to a particular family cancer history, as in the two major categories (breast only, and breast and ovarian cancer families) the genomic rearrangements made up to 25% of the mutations in the *BRCA1* mutation-positive families.

Our results show that MLPA is a rapid, reliable, and sensitive technique, which allows high-throughput screening. This is of importance, as many diagnostic laboratories have collected in the recent years hundreds or even thousands of individuals/families with a predisposition to breast/ovarian cancer. With the *BRCA1*-MLPA test, it is feasible to analyze all of these individuals and families for the

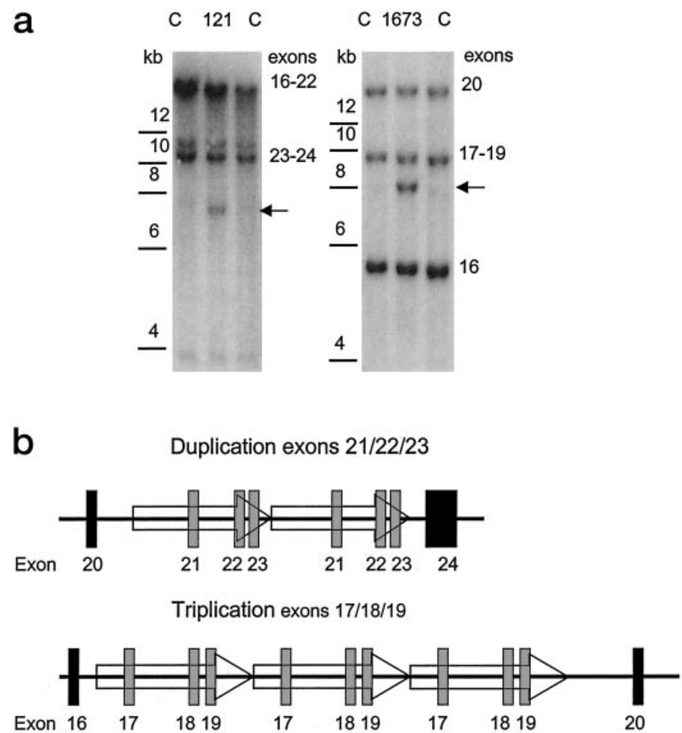

Fig. 4. Southern blot and PCR confirm the novel amplifications in *BRCA1*. *a,* genomic DNA from case 121, suspected carrier of an amplification of exons 21–23, and two controls were digested using *Bam*H1 and, after blotting, hybridized using a RT-PCR probe containing exons 16–24. The autoradiogram shows a novel fragment of 7 kb only present in case 121. DNA from case 1673, identified by MLPA as carrier of two additional copies of exons 17–19, was digested using *Eco*R1 and, after blotting, hybridized with a RT-PCR probe encompassing exons 16–20. An additional band of 8 kb is observed in the mutant sample only. This band is of similar intensity as the wild-type *Eco*R1 band of 9.5 kb containing exons 17–19. *b,* the genomic organization of the amplifications was subsequently assessed by PCR and sequence analysis using different primer combinations. The size of the amplified sequences as determined by sequencing is comparable with the size of the extra bands observed after Southern blot analysis, which indicates that the amplified sequences are amplified in tandem.

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presence of large genomic deletions and duplications. And, therefore, we consider the *BRCA1*-MLPA test a major improvement on the current diagnostic testing for hereditary breast and/or ovarian cancer.

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