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


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 adjacent 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). 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.

Materials and Methods

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).

MLPA was carried out for 33 cycles (30 s at 95°C, 30 s at 60°C, and 60 s at 72°C). Ligation was performed with the temperature-stable Ligase-65 enzyme (MRC-Holland) for 15 min at 54°C. Next, the ligase was ligated 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 preincubated with 30 μl of PCR buffer and put in a PCR machine at 60°C. Subsequently, a 10-μl mix was added containing deoxyxynucleoside triphosphate, Taq polymerase, and one unlabelled 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-
applied 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 automated sequencer, 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'-GTGACCGCTTCAAATGATCTGTTCC-3' and reverse primer (nt 29530) 5'-CCTATTGCTACTCTCGATCTTTCCC-3'. The deletion of exons 20–22 could be detected using forward primer (nt 67941) 5'-AGATGAAACAGTGGTGGTGGCATGA-3' and reverse primer (nt 59849) 5'-GATGAAAATCTCTAGGAAGG-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 BamHI or EcoRV, 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 quant-
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.

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.

To test the sensitivity and specificity of the BRCA1-MLPA, we...
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 (4535delETTinsG). The Genescan electropherograms 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. 4a). The BamHI 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. 4b). In case 1673, the EcoRI digestion generated an extra band of ~8 kb (Fig. 4a). 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. 4b). 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
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.

References


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

Frans B. L. Hogervorst, Petra M. Nederlof, Johan J. P. Gille, et al.