Genomic rearrangements account for more than one-third of the BRCA1 mutations in northern Italian breast/ovarian cancer families

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The recent identification of major genomic rearrangements in breast and breast/ovarian cancer families has widened the mutational spectrum of the BRCA1 gene, thus increasing the number of informative patients who can benefit from molecular screening. Numerous types of alterations have been identified in different populations with variable frequencies, probably due to both ethnic diversity and the technical approach employed. In fact, although several methods have been successfully used to detect large genomic deletions and insertions, most are laborious, time-consuming, and of variable sensitivity. In order to estimate the contribution of BRCA1 genomic rearrangements to breast/ovarian cancer predisposition in Italian families, we applied, for the first time as a diagnostic tool, the recently described multiplex ligation-dependent probe amplification (MLPA) methodology. Among the 37 hereditary breast/ovarian cancer (HBOC) families selected, all had a high prior probability of BRCA1 mutation, and 15 were previously shown to carry a mutation in either the BRCA2 (five families) or BRCA1 gene (10 families, including one genomic rearrangement). The application of BRCA1-MLPA to the remaining 22 uninformative families allowed the identification of five additional genomic rearrangements. Moreover, we observed that loss of constitutive heterozygosity of polymorphic markers in linkage disequilibrium is predictive of such BRCA1 alterations. By means of this approach, we demonstrate that BRCA1 genomic deletions account for more than one-third (6/15) of the pathogenic BRCA1 mutations in our series. We therefore propose to systematically include MLPA in the BRCA1 mutational analysis of breast/ovarian cancer families.

INTRODUCTION

BRCA1 and BRCA2 are the two most important genes associated with a high predisposition to breast/ovarian cancer. Studies based on linkage analysis have indicated that BRCA1 is the major gene responsible for those hereditary breast/ovarian cancers that occur in families with at least one ovarian cancer patient (i.e. breast/ovarian cancer families) (1). Nonetheless, molecular typing of BRCA1 point mutations in such families has consistently failed to identify disease-causing mutations at the expected rate. A partial explanation of this discrepancy lies in the complexity of the BRCA1 mutational spectrum. Indeed, besides point mutations and small deletions and insertions, which are by far the most frequent mode of BRCA1 inactivation, regulatory mutations as well as major genomic rearrangements have also been described (Breast cancer Information Core database; www.nhgri.nih.gov/ Intramural_research/Lab_transfer/Bic/). The latter have probably been underestimated since they escape the currently employed PCR-based genomic screenings and require specific technical approaches.

Large genomic deletions and duplications involving one or more exons of the BRCA1 gene have been reported in recent years (2–16). Most of them are caused by recombination between Alu repeats that are particularly numerous in the BRCA1 locus (17). Moreover, a duplicated region at the 5’ end of the BRCA1 gene and containing the BRCA1 pseudogene has recently been shown to represent an additional source of illegitimate recombination (16). The proportion of genomic rearrangements over all BRCA1 gene mutations in breast and/or ovarian cancer families seems to be population-dependent varying from 15% in a series of American families (4) to 36%.

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Human Molecular Genetics, Vol. 12, No. 9 © Oxford University Press 2003; all rights reserved
in the Dutch patients (2). However, in most cases, these studies were probably limited by the complexity and sensitivity of the technical approaches employed. For instance, in order to achieve a good accuracy, the commonly used Southern blot analysis should be carried out using several well-designed probes, possibly prepared from genomic DNA, in combination with a number of restriction enzyme digestions. Long-range PCR approaches are prone to miss deletions that include the primer sequences, and molecular dynamic DNA combing is very sensitive for large and intermediate size rearrangements but has a lower limit of approximately 2 kb. Therefore, the current figures on the involvement of such alterations in breast/ovarian cancer predisposition might still be imperfect.

In a previous study using Southern blot analysis with probes prepared from BRCA1 cDNA, we identified a genomic rearrangement in two out of 70 Italian breast and/or ovarian cancer families that were not informative in the BRCA1 and BRCA2 mutational analyses (7). Exploiting the recently described MLPA methodology (18) we analyzed 22 breast/ovarian cancer families selected for a high gene carrier probability but with no BRCA1 point mutations identified, and showed that genomic rearrangements may account for a large proportion of all BRCA1 mutations in Italy.

RESULTS

Strategies for identification of carriers of BRCA1 genomic rearrangements

Due to the strong linkage disequilibrium that characterizes the BRCA1 locus (19–21), most of the single-nucleotide polymorphisms (SNP) located in the BRCA1 gene result in only two most common haplotypes; accordingly, the vast majority of the individuals are either homozygous or heterozygous for most of these SNPs. We took advantage of this information to pinpoint cases that might have harbored major genomic deletions. In particular, using DHPLC to screen the 23 BRCA1 exons and exon–intron boundaries for point mutations, we routinely genotype 11 common polymorphisms located across the entire gene sequence (introns 1, 8, 18 and exons 11, 13, 16). Therefore, heterozygous (i.e. informative) individuals can easily be screened for loss of constitutive heterozygosity.

Using this type of analysis in 22 HBOC families that were selected for a high probability of BRCA1 mutation but did not disclose any point mutation when analyzed by single-strand conformation polymorphism/heteroduplex analysis (SSCP/HA), protein truncation test (PTT) or denaturing high-performance liquid chromatography (DHPLC), we identified two cases that showed a putative hemizygosity for common polymorphisms located in intron 1 (patient B319), and introns 8, 18 and exons 11, 13, 16 (patient B212). In order to investigate the presence of major genomic rearrangements, the DNA of the two patients was analyzed by MLPA. This recently described methodology (18) allows the quantitative analysis of all BRCA1 exons in a single reaction that involves annealing and subsequent amplification of 40 different exon-specific probes. Deletions or insertions of one or more exons are scored as a change in the peak height of the corresponding amplification product. In agreement with the SNP data, when applied to patients B319 and B212, MLPA resulted in a decrease of the peaks corresponding to exons 1a-2 and 9–19, respectively, (Fig. 1, first two panels), thus confirming the possibility that deletions of these exons might have occurred in these two cases.

The same methodology was also applied to the remaining 20 patients and led to the identification of three additional cases with putative genomic rearrangements. In particular, patient B306 displayed a MLPA profile identical to patient B319 (Fig. 1), suggesting the occurrence of a similar, if not identical, rearrangement involving exons 1a, 1b and 2. A decrease in peak height was also found in patients B333 and B242, indicating the putative loss of exons 20 and 18–19, respectively (Fig. 1).

Characterization of the aberrant BRCA1 transcripts

BRCA1 transcripts of all five patients with suspected genomic deletions were subsequently analyzed by means of RT–PCR to evaluate the effect of the presumed rearrangements. Since the identical MLPA profile of patients B319 and B306 indicated the absence of exon 1a, 1b and 2, we reasoned that the promoter region might also have been involved in the deletions.
that occurred in the two patients. As mentioned above, patient B319 was heterozygous for the common polymorphic sites in exon 11; therefore, we used one of these polymorphisms (2201C/T) to evaluate the presence of the mutant transcript. As shown in Figure 2, the DHPLC profile obtained from the RT–PCR of the polymorphic region, lacked the heteroduplex peak present in the genomic DNA as well as in a heterozygous control. Transcript analysis was also undertaken for sample B306; in this case, however, the patient was homozygous for the common polymorphisms, thus precluding a simple investigation of the mutant allele expression. Any attempt to amplify a possible chimeric transcript derived form the fusion of the two alleles in patient B212 more than one aberrant PCR product with the loss of one of the two forward oligonucleotides was located either in the exons 1 and 2 with BRCA1 exon 3 appeared unsuccessful, thus suggesting the likely absence of any chimeric mutant allele.

As for the other three cases with positive MLPA results (patients B333, B242 and B212), cDNA analysis was performed using primers located in the exons flanking the putative deletions. As shown in Figure 3A, all three patients displayed an abnormal band pattern fitting with the loss of one or more exons. In particular, the analysis of BRCA1 transcript in patient B333 resulted in a single aberrant band which was not present in the normal control cDNA. As expected, sequencing of this mutant transcript revealed the skipping of the entire exon 20 corresponding to 28 amino acids. The junction of exon 19 to exon 21 of BRCA1 preserves the correct reading frame thus maintaining the potential for coding all the remaining part of the protein. As no premature termination codons are created, this transcript is not subject to the nonsense-mediated mRNA decay (NMD) associated with the majority of BRCA1 and BRCA2 truncated mutations (22,23)

and the two alleles are equally expressed. Differently, in patients B212 and B242 more than one aberrant PCR product was co-amplified together with the wild-type transcript (Fig. 3A); sequence analysis of the gel purified bands revealed transcripts missing those exons that showed hemizygosity in the MLPA analysis as well as transcripts that lacked exons otherwise present in the genomic DNA (Table 1). These unexpected additional products are likely derived from alternative aberrant splicing events. Similar to the common observation made with certain splice site mutations, these data confirm the concept that acceptor and donor splicing sequences require some degree of specificity for their recognition. In both cases, the aberrant transcripts, lacking those exons deleted in genomic DNA, contained premature termination codons and consequently were expected to be expressed at low level. While this was apparent in patient B242, the preferential amplification of the faster migrating band in patient B212 was probably caused by the different size of the wild-type (4991 bp) versus the main mutant (344 bp) transcript. To precisely assess the relative expression level of the two alleles in patient B212, we designed a PCR experiment using three oligonucleotides for the amplification of specific PCR products of similar size. In particular, while the reverse primer recognized both transcripts, the two forward oligonucleotides were located either in the deleted region, allowing amplification of the wild-type only, or adjacent to it for selective amplification of the mutant transcript due to the large size of the intervening wild-type sequences. As expected, this experiment finally demonstrated the preferential decay of the mutant transcript lacking exons 9–19 (Fig. 3B).

Identification of the genomic break-points

The final and conclusive characterization of a genomic rearrangement usually relies on the molecular definition of the break-point sequences that disclose the mechanism underlying the mutation. For the three patients with intragenic rearrangements (B333, B242, B212) we designed a set of long-
range PCR experiments using primers located in the exons surrounding the deletions. In this way, we successfully amplified abnormal migrating fragments ranging in size from 6 to 9 kb in all three patients (Fig. 4). Subsequent narrowing of the breakpoint-containing regions was achieved by comparison between restriction enzyme profiles of the amplification products and the corresponding undelated regions (data not shown). Finally, 2–3 kb PCR products containing the breakpoints were sequenced following their cloning in plasmid vectors; this step could not be avoided since the low Taq polymerase processivity on homonucleotide tails of Alu repeats caused unreadable sequences. In all three cases, the sequences of the breakpoint limits revealed the presence of Alu repeats belonging to different sub-families (Table 1); overall, the degree of identity between the recombining sequences was very high, ranging from 75 to 84%.

Differently, patients B306 and B319, in which deletions occurred in the first part of the gene (exons 1a–2), were analyzed using primers recently described for the screening of the rearrangements due to improper pairing of the BRCA1 gene with its S' duplicated region containing exons 1a, 1b and 2 of the psiBRCA1 gene (16). Using these primers, a faint band, expected for deletions occurring in that region, was obtained only in patient B306 (data not shown), thus mapping the breakpoint of the rearrangement to a region containing four blocks of strong homology. In order to investigate whether this rearrangement was identical to one of the two previously described (16), we used the specific primers pintron 2.2f and intron 2r2, located in the psiBRCA1 (forward primer) and BRCA1 gene (reverse primer), respectively. A clearly positive result was obtained in patient B306; however, an identical size band, albeit of lower intensity, was also apparent in patient B319 as well as in the normal controls (data not shown).

We therefore sequenced all PCR products and found out that while the lower intensity bands of patient B319 and control samples derived from the improper, nearly complete, annealing of the forward primer to the BRCA1 gene, the more intense PCR product of patient B306 was the result of a 37 kb deletion from intron 2 of the psiBRCA1 to the corresponding region of the BRCA1 gene including the NBR2 gene. Therefore, as in the family F32 described by Puget and colleagues, the breakpoint of patient B306 occurs in a 237 bp region that share 100% identity in the two recombining sequences (Table 1).

Rearranged bands were not obtained in patient B319 with either one of the above-mentioned primers. Therefore, in order to exclude a very large deletion extending beyond the psiBRCA1 gene, we took advantage of the heterozygous status of the patient to limit the deletion. As mentioned above, the linkage disequilibrium of the BRCA1 SNPs extends upstream of the gene through a segment that includes in the order: NBR2, psiBRCA1, NBR1 and the snRNA (RNU2) genes. We therefore typed two genetic markers located at the S' end of the psiBRCA1 gene (SNP E) (19) and upstream of the NBR1 gene, before the RNU array (D17S1327). In both cases, retention of heterozygosity was observed (data not shown), thus locating the S' breakpoint limit between the psiBRCA1 and BRCA1 genes. No informative results were obtained using several couples of primers for long-range PCR, most likely reflecting the complex structure of this genomic region; similar observations were recently made by colleagues attempting to characterize major genomic rearrangements in the very same region (16,24).

DISCUSSION

The screening for BRCA1 mutations in breast and/or ovarian cancer families has become more and more complex due to the occurrence of different mutation types, such as major genomic rearrangements, detectable only when specific technical approaches are employed. Here we demonstrate that the information gained from the search of point mutations might also be valuable for the identification of patients harboring major genomic deletions. This is achieved simply by examining the genotype of the numerous SNPs that are spread throughout the BRCA1 sequences and that show strong linkage disequilibrium, so that heterozygosity or homozygosity usually extends over most polymorphic sites. In fact, we show that loss of constitutive heterozygosity in specific exons of informative B212 and B319 patients reflected the presence of two genomic rearrangements. Obviously, this kind of analysis has two major limitations: (1) it only monitors the loss of polymorphic exons; and (2) it is not applicable to homozygous (i.e. uninformative) individuals that represent approximately 54% of the samples (19). Nonetheless, it is readily applicable to those patients already tested for point mutations who represent the vast majority of the cases analyzed to date, worldwide. Moreover, since the nearly complete linkage disequilibrium extends upstream of the first BRCA1 exon throughout a genomic region that also contains NBR2, psiBRCA1, NBR1 and RNU2 genes (19), ‘regulatory mutations’ due to rearrangements of the BRCA1 promoter/enhancer sequences of the BRCA1 gene might also be investigated by exploiting this same strategy.

When no clues are provided from the analysis of the BRCA1 sequence, methods such as long-range PCR, DNA combing, or the most commonly used Southern blot analysis can be successfully employed for the identification of major genomic rearrangements. In alternative, methods such as the recently described quantitative multiplex PCR of short fluorescent fragments (QMPSF), which involves four distinct quantitative
PCR reactions to analyze the whole BRCA1 gene, seem very promising (25). Most of these techniques, however, are technically demanding, time-consuming and, most importantly, their sensitivity is not always very high. Here we make use of MLPA methodology that was recently shown to properly detect two cases with already characterized genomic rearrangements of the BRCA1 gene (18). MLPA outperforms the above-mentioned methods in terms of both sensitivity and speediness, being even much more rapid and simple than BRCA1 point mutation analysis. Moreover, it directs the molecular characterization of the rearrangement as all the involved exons are specifically identified by a decrease/increase of the corresponding peaks; this clearly represents a major advantage in the laborious and time-consuming molecular characterization of the genomic rearrangement.

Using MLPA we have identified five genomic rearrangements involving various BRCA1 exons in individuals belonging to families with a strong history of breast/ovarian cancer. The molecular characterization of the rearrangements has revealed the already known mechanism of Alu-mediated recombination in at least three cases; the BRCA1 intronic sequences have, in fact, an above-average density of Alu repeats (17). On the other hand, the rearrangement of patient B306, removing exons 1–2, supports a different mechanism that involves recombination between sequences located in intron 2 of the BRCA1 gene and the corresponding region of the BRCA1 gene (16). The duplicated genomic region localized upstream of the BRCA1 gene includes exons 1a, 1b and 2 and retains a high degree of homology with the original sequence. In particular, Puget and colleagues have recently mapped in the two sequences 14 blocks of strong homology all of which might prompt aberrant unequal recombination events. As in the French family F32 (16), the genomic deletion of patient B306 is mediated by sequences that display 100% identity over the 237 nucleotides at the breakpoint; only haplotype analysis could reveal whether these mutations derive from the same ancestor. Including patients B306 and B319, the apparently unrelated families with BRCA1 exons 1a–2 deletions described to date total five in all, thus suggesting that the 5' duplicated region might represent a new recombinational hot spot for such kind of mutations as recently suggested (16). Indeed, except for a few mutations showing founder effects (2,26), the rearrangements detected thus far in independent families are spread all over the BRCA1 locus.

We have carried out a detailed analysis of the mutant allele expression and showed that, with the exception of patient B333 who carries an in-frame deletion, the remaining four rearrangements display allelic imbalance in the expression of the mutant versus wild-type allele. Indeed, in addition to patients B319 and B306 in which absent expression of the mutant allele was observed, both the other two rearrangements (i.e. patient B212 and B242) caused NMD-mediated degradation of the truncating mutation bearing transcripts. This agrees with our previous finding that most of the BRCA1 truncating mutations are associated with NMD (23), later also confirmed in a larger number of BRCA1 families (22). Therefore, these data reinforce the concept that most of the BRCA1 carriers could be identified by methods such as allele-specific gene expression (AGE) analysis (23) that evaluate the relative abundance of mutant versus wild-type transcript.

In this study we have identified BRCA1 genomic rearrangements in five out of 22 breast/ovarian cancer families highly selected for the presence of a BRCA1 mutation (i.e. HBOC). Including the other 15 families, who matched the same selection criteria, but had point mutations identified by SSCP, PTT and DHPLC (27 and unpublished data), or genomic rearrangements detected by Southern blot (7), the rate of mutation detection in our series of HBOC families is close to 40% considering only BRCA1 gene, and rises to 54% when also including the BRCA2 mutations. Notably, major genomic rearrangements represent more than one-third (40%) of the mutations of the BRCA1 gene in these families. This high proportion of genomic rearrangements is unlikely to be biased.

<table>
<thead>
<tr>
<th>Patient</th>
<th>MLPA (exons)</th>
<th>Mutant transcripts (expression level)</th>
<th>Mutation designation</th>
<th>Breakpoint coordinatesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>B212</td>
<td>9–19</td>
<td>Deleted exon 9–19 (+++) ter184 (U14680)b Deleted exon 8–19 (+) (in frame)</td>
<td>g.29197_65577del36381 (L78833)</td>
<td>Alu-Sp 29188/97 Exon 9 start 31443 Exon 19 end 65400 Alu-Sp 65569/78</td>
</tr>
<tr>
<td>B319</td>
<td>1a–2</td>
<td>Absent</td>
<td>—</td>
<td>Alu-Sx 62090/115 Exon 18 start 64782 Exon 19 end 65400 Alu-Sg 66916/41</td>
</tr>
<tr>
<td>B242</td>
<td>18–19</td>
<td>Deleted exon 18–19 (+) ter1693 (U14680) Deleted exon 17–19 (+) (in frame)</td>
<td>g.62115_66940del4826 (L78833)</td>
<td>N.D.</td>
</tr>
<tr>
<td>B306</td>
<td>1a–2</td>
<td>Absent</td>
<td>g.34439_71373del36935 (AC060780)</td>
<td>Intron 2 ψBRCA1Alu-Sx 71374/610 Exon 1a 39879 Exon 2 38505 Intron 2 BRCA1 34439/675</td>
</tr>
<tr>
<td>B333</td>
<td>20</td>
<td>Deleted exon 20 (++++) (in frame)</td>
<td>g.68028_72355del4328 (L78833)</td>
<td>Alu-Sx 68021/28 Exon 20 start 71598 Exon 20 end 71681 Alu-Jb 72349/56</td>
</tr>
</tbody>
</table>

aFor the Alu repeats, coordinates refer to the region of identity between the recombining sequences.
bNumbers between brackets correspond to GenBank sequences.
N.D., not determined.
by an eventual underestimation of the BRCA1 point mutations. Indeed the point mutation frequency itself (40%) is high compared with those usually reported, and this is probably due to the highly selective operational criteria adopted as well as the extensive mutational screening that was carried out by both SSCP/HA plus PTT, and DHPLC analysis.

In conclusion, even though the number of families analyzed in this study is small, we believe that our estimation of the contribution of BRCA1 genomic rearrangements in the Italian breast/ovarian cancer families is realistic, and is mainly due to the high sensitivity of the methodology used. Given these figures and considering the quickness of MLPA analysis over the currently used point mutation detection methods, we suggest that this analysis not only is mandatory but should also be considered as an early step in the molecular characterization of individuals at high risk for breast/ovarian cancer.

MATERIALS AND METHODS

In the last 8 years, 230 patients with either breast (151 females; nine males) or ovarian (n = 48) cancer or both (n = 22), attending the Division of Medical Oncology of the University Hospital in Padua, were defined as probands belonging to apparently unrelated high-risk breast and breast/ovarian cancer families living mainly in the Veneto region of northeast Italy. Following genetic counselling sessions, these families were classified, according to published operational criteria (28), as affected by either the inherited or the familial tumor form. Of all pedigrees, 37 were classified as affected by hereditary breast/ovarian cancer (HBOC) since their family history included no less than three tumor cases (among them at least one ovarian cancer), at any age, in a minimum of two generations on the same parental branch, among first-degree relatives, with the exception of disease-transmitting males.

Blood samples were obtained from each affected proband following informed consent on the aims of the study, and EBV immortalized lymphoblastoid cell lines were established from peripheral blood lymphocytes. DNA was extracted by means of standard phenol–chloroform techniques, and RNA was isolated using RNAzol reagent (Tel-Test, Friendwood, TX, USA).

The MLPA procedure was performed according to the instructions provided by the manufacturer (MRC Holland, Amsterdam, the Netherlands) on a PTC 200 (MJ Research Inc., Waltham, MA, USA) thermalcycler. In particular, ~100 ng of denatured genomic DNA were used in the overnight annealing of the exon-specific probes and subsequent ligation reaction; PCR was carried out with FAM-labeled primers using 10 µl of ligation reaction (alternative protocol 2). Separation and relative quantification of the amplification products was obtained with the ABI Prism-310 Genetic Analyser and Genescan 3.1 software (Applied Biosystems, Foster City, CA, USA). Variations in peak height were evaluated by comparison of each sample with a normal control and also by cumulative comparison of four to six samples, always from the same experiment.

RT–PCR analysis of the BRCA1 transcripts was carried out using the Superscript™ RT RnaseH− reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with gene-specific primers for the cDNA synthesis. DHPLC analysis of the mutant and wild-type transcripts of patient B319 was carried out with the Wave DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA).

All sequence reactions were performed with the DNA sequencing kit (Applied Biosystems, Foster City, CA, USA) and subjected to capillary electrophoresis with the ABI Prism-310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). When necessary, cloning of PCR products for subsequent sequencing was carried out using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA).

Long-range PCR were performed with the Expand Long Template PCR System (Roche Indianapolis, IN, USA) according to the suggestions provided by the manufacturer.

Primers couples, employed to amplify the breakpoint region from cDNA, or genomic DNA, are available upon request.

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