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BRCA1 genomic deletions are major founder mutations in Dutch breast cancer patients

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To date, more than 300 distinct small deletions, insertions and point mutations, mostly leading to premature termination of translation1, have been reported in the breast/ovarian-cancer susceptibility gene BRCA1. The elevated frequencies of some mutations in certain ethnic subpopulations2–4 are caused by founder effects5,6, rather than by mutation hotspots. Here we report that the currently available mutation spectrum of BRCA1 has been biased by PCR-based mutation-screening methods, such as SSCP, the protein truncation test (PTT) and direct sequencing, using genomic DNA as template. Three large genomic deletions that are not detected by these approaches comprise 36% of all BRCA1 mutations found in Dutch breast-cancer families to date. A 510-bp Alu-mediated deletion comprising exon 22 was found in 8 of 170 breast-cancer families recruited for research purposes and in 6 of 49 probands referred to the Amsterdam Family Cancer Clinic for genetic counselling. In addition, a 3,835-bp Alu-mediated deletion encompassing exon 13 was detected in 6 of the 170 research families, while an deletion of approximately 14 kb was detected in a single family. Haplotype analyses indicated that each recurrent deletion had a single common ancestor.

We selected four families (RUL5, RUL105, EUR9 and EUR21) for further study because they each showed strong evidence of linkage to BRCA1, although two index cases of each family had remained negative after the PTT of exon 11 (refs 4,7,8). All 22 coding exons and their immediate intron sequences were sequenced in one index case carrying the disease haplotype from each family. Other than a number of previously published exonic polymorphisms1, no conclusive mutations were found. A G→A mutation at position IVS22 + 5 was found to co-segregate with the disease haplotype in family EUR21. This G is conserved in 84% of splice-acceptor sites8, suggesting a splice-site mutation.

RT-PCR analysis of exons 20–24 revealed an additional smaller band in patient RUL105.6 and, surprisingly, also in the proband of family RUL105 (Fig. 1a), in which the IVS22 + 5G→A mutation had not been detected. In both cases, the sequence of these products revealed a deletion of exon 22. This deletion causes a frameshift and premature translation termination, which removes the last 60 amino acids of the protein. We conclude that in EUR21, but not in RUL105, the deletion of exon 22 from the mRNA is caused by the IVS22 + 5G→A mutation. The ratio between full-length and exon 22-deleted RT-PCR products was variable between duplicate experiments when lymphocyte RNA was used as starting material [Fig. 1b]. Given the very low expression levels of BRCA1 in these cells, we attribute this to coincidental preferential amplification. No alterations were detected in the RT-PCR products spanning exons 2–10 and 12–24 from patient EUR9.5 (data not shown). No RNA was available from any breast-cancer case in family RUL5, precluding RT-PCR analysis.

Two additional RT-PCR variants were found in eighteen samples derived from breast- or ovarian-cancer patients who requested genetic counselling at the Leiden Family Cancer Clinic (Fig. 1c). Patient B13 was found to contain a variant lacking exons 13–16 (801 bp), causing an in-frame deletion that removes residues 1396–1662 of the protein. The variant in patient B20 lacked exon 13 (172 bp), causing a frameshift and a stop codon at position 1398. Sequence analysis of the intron-exon borders flanking the deletions in these two patients did not reveal a change in the splice sites.

We performed Southern-blot analysis of genomic DNA derived from these patients. DNA samples were digested with either HindIII or BglII, and blotted with probes derived from

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In our total set of 170 research families, we had already found 21 sequence that is implied to result in nucleotide changes. These two 26-bp sequences that were strong homology with the ALU core, and one (Fig. 2d) shows a 14-kb part. Because of the absence of these distant large genomic deletions (Fig. 2d), one of these cases, it suggests that the heterozygous regions. From the combined data, we conclude the hemizygous regions.

...were found in samples from patient EUR21 (data not shown). Thus, it is highly unlikely that they represent polymorphic rearrangements. No changes in restriction patterns (data not shown) thus, it is highly unlikely that they represent polymorphic rearrangements. No changes in restriction patterns...
FIG. 2. Southern blot analysis. a-c. Geno-
BRCA1 and 9 BRCA2 mutations by PTT and PCR fragment-length analysis.2,4,5 Because strong founder effects have been shown for most recurrent Dutch BRCA1 mutations,6 we examined the remaining 137 breast-cancer research families for the occurrence of the 510- and 3,835-bp deletions. These were found in six and three additional families, respectively (Table 1). In all of these families, the deletion co-segregated with the disease. The rest were investigated for the presence of an Sp1 restriction site in intron 22, created by the IVS22 + 5G→A mutation as observed in EUR21; none were observed (data not shown). Together with previous mutation screening results, the two deletions thus comprise 12/33 (36%) of all mutations in families which a BRCA1 mutation has been detected. Furthermore, among the ten breast-cancer families predicted by linkage analysis8 to carry mutations in BRCA1, we have now detected a mutation in nine, three of which (33%) carry one of the large deletions described here. A further indication of the frequency of the 510-bp deletion came from another set of samples derived from the Family Cancer Clinic in Amsterdam. A deletion of exon 22 had been detected independently at the cDNA level in 6 of 49 probands analysed, without evidence for the IVS22 + 5G→A change. In a subsequent double-blind study examining the genomic DNA of these samples, our PCR-based assay identified the 510-bp deletion in all six cases (data not shown).

We used three intragenic and two flanking markers to reconstruct the disease haplotype for each of the research families carrying either the 510- or the 3,835-bp deletion (Table 1). Strong conservation of allele lengths was observed at the intragenic loci of the haplotypes carrying the same deletion, suggesting their descent from a common ancestor. The haplotype carrying the IVS22 + 5G→A mutation in EUR21 does indeed differ from the haplotype carrying the 510-bp deletion.

Our results are the first to show the importance of large genomic deletions in BRCA1 among breast-cancer families from a defined Caucasian subpopulation. It has been proposed that the unusually high concentration of Alu elements in the BRCA1 intronic regions might render the gene particularly prone to intragenic recombination/deletion events.10 A 1-kb Alu-mediated deletion affecting exon 17 of BRCA1 was recently detected in a single large French breast-cancer family.12 This lends further support to our hypothesis, and fuels the discussion of whether or not BRCA1 is inactivated by somatic mutations in sporadic breast cancer.13 Rather than small deletions and insertions, none of which have
been reported to occur somatically in breast tumours.\textsuperscript{14,15} Altered large genomic deletions might be a more common mechanism that inactivates BRCA1 in sporadic breast cancer.

Because strong founder effects have been observed for many BRCA1 mutations detected worldwide,\textsuperscript{6,9} large deletions may constitute a substantial proportion of the mutation spectrum in certain ethnic subpopulations, as evidenced here for the Dutch. This could explain the discrepancy between the proportion of BRCA1 mutations predicted by linkage studies,\textsuperscript{16,17} and the actual prevalence estimated by mutation analysis of breast-cancer families derived from a variety of ethnic backgrounds.\textsuperscript{6,18-22} Although such deletions are detectable by RT-PCR analysis, the latter has not yet been widely used for mutation detection, and might be prone to false-negative results (for instance, as seen in the case of EUR9.5), either because the low abundance of BRCA1 mRNA in lymphocytes causes coincidental preferential amplification of the wild-type transcript (Fig. 1b) or because transcripts carrying exonic deletions are more unstable than the full-length transcript.\textsuperscript{2,3} Our data clearly show that a comprehensive BRCA1 mutation test should include the examination of its genomic structure.

**Methods**

Family ascertainment. Research families were ascertained either by the Netherlands Foundation for the Detection of Hereditary Tumors or by the Rotterdam Family Cancer Clinic. They all contain at least three first-degree relatives with either breast or ovarian cancer, at least one of them having received a diagnosis before 50 years of age. Of the 170 thus recruited, there were 48 families with at least four cases of breast cancer diagnosed before age 60, in addition to any number of ovarian cancers. Patients entered the study by application or physician referral to a family cancer clinic or clinical genetics department in either Amsterdam or Leiden. In general, these patients have an a priori chance of 10% or higher of being carriers of a BRCA1 mutation.\textsuperscript{11}

DNA and RNA isolation, reverse transcription and nested PCR. Isolation of genomic DNA and total RNA from fresh blood samples and preparation of first-strand cDNA by reverse transcription have been described.\textsuperscript{12} For the RT-PCR analysis of exons 12-24, we used the following primers for the first PCR: forward (F), 5'-TCACAGTG CAGTGAATGACAG-3'; reverse (R), 5'-GTGACAGACCGAAGCTGAGCTG-3'. Nested primers were then used in a subsequent PCR to amplify exons 12-24 (F, 5'-GAAGAAGGAGAA GGGCTTG-3'; R, 5'-GCCACCTTGTGTAACCTGACATT-3'). For nested PCR of exons 20-24, the same reverse primer was used in combination with the 5'-5ACCAACAGGCTCCAAAGG-3' PCR conditions as were described previously.\textsuperscript{11}

<table>
<thead>
<tr>
<th>Family</th>
<th>Proband's diagnosis*</th>
<th>Allele frequency*</th>
<th>Linked allele length* at polymorphic marker (bp)</th>
<th>D17S855</th>
<th>D17S1322</th>
<th>D17S1323</th>
<th>D17S1327</th>
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<tbody>
<tr>
<td>B20</td>
<td>Br43</td>
<td>0.08</td>
<td>0.21</td>
<td>0.51</td>
<td>0.50</td>
<td>0.68</td>
<td>0.60</td>
</tr>
<tr>
<td>EUR9</td>
<td>Br28/Br46</td>
<td>0.15</td>
<td>0.32</td>
<td>0.55</td>
<td>0.50</td>
<td>0.68</td>
<td>0.60</td>
</tr>
<tr>
<td>EUR18</td>
<td>OV54</td>
<td>0.12</td>
<td>0.35</td>
<td>0.51</td>
<td>0.50</td>
<td>0.68</td>
<td>0.60</td>
</tr>
<tr>
<td>EUR19</td>
<td>Ov36/Br43/Br49</td>
<td>0.15</td>
<td>0.32</td>
<td>0.51</td>
<td>0.50</td>
<td>0.68</td>
<td>0.60</td>
</tr>
</tbody>
</table>

\*No. indicates that age of onset of breast cancer was 43 y; OV54 indicates that age of onset of ovarian cancer was 54 y. When marker was uninformative, both alleles are listed. Of most commonly shared allele only, as measured among 168 unrelated Dutch control individuals.\textsuperscript{11}

Genomic PCR of the deletions spanning exons 13 or 22. A PCR reaction of 30 ml typically contained 200 ng of genomic DNA, 10 pmol primers, 0.75 U AmpliTaq DNA polymerase (Perkin Elmer/Cetus) and 5 ml of either x10 RM buffer (500 mM KCl, 100 mM TRIS-HCl pH 8.4, 25 mM MgCl\textsubscript{2}, 2 mg/ml BSA, 2 mM dNTPs) for exon 13 or Optiprime buffer #6 (Stratagene), supplemented with 0.1 mM dNTPs for exon 13. For exon 13, the products were (F) 5'-CACTTGTTCTCCTGCCACT-3' and (R) 5'-ACACTGGAGACACAGATATAAT-3', while an internal control fragment from exon 11 was co-amplified simultaneously (primers F, 5'-GTACAGTGACACATATGCG-3'; R, 5'-TCTCAGAACAACCCCTAGAGTC-3'). This mixture was heated at 94 °C for 1 min, followed by 32 cycles of PCR (1 min at 94 °C, 1.5 min at 56 °C and 2 min at 73 °C) on a Perkin Elmer/Cetus DNA thermal cycler. For exon 22, the primers were (F) 5'-TCCCTAGGATGCTCTGTCCT-3' and (R) 5'-ACTGTGGCTGATCT CAAGACAGCC-3'. After heating at 94 °C for 5 min, the PCR consisted of 35 cycles (45 s at 94 °C, 1 min at 52 °C and 2.5 min at 72 °C). All PCRs were concluded by an incubation at 73 °C for 4 min. The PCR products (5 ml) were analysed on a 1.5% agarose gel.

Southern analysis. Genomic DNA (5 μg) was digested with BglII or HindIII according to the supplier's protocols (Pharmacia). Agarose gels (0.8%) were run at 30 V for 16 h in TAE buffer (40 mM Tris-HAc pH 8.3, 1 mM EDTA). Procedures for denaturing and transferring the separated DNA to nylon membranes (Hybond N+, Amersham) have been previously described.\textsuperscript{24} As probes we used PCR products obtained from a clone containing the complete BRCA1 cDNA, and purified with the QIAquick PCR Purification Kit (Qiagen). Probe 11 (p11) derives entirely from exon 11 and was obtained with the primers (F) 5'-GAAA AAA AAAAA GTA CAAG CAAATGCC-3' and (R) 5'-AGGCCATCTTGAAGTACGGAAC-3'. Probe 1424 (p1424) contains exons 14-24 and was obtained with the primers (F) 5'-TACCCTTATAAGCCGAGATCTGAGAC-3' and (R) 5'-GAGTTTGCAGCTATTCAAGCGAC-3'. Purified fragments were labelled using the Megaprime DNA labelling system (Amersham) according to the supplier's protocols. Hybridizations were carried out overnight at 65 °C in 125 mM Na\textsubscript{2}PO\textsubscript{4}, 20% SDS, 10% PEG-6000, 1 mM EDTA. Final washes were in 45 mM Na\textsubscript{2}CO\textsubscript{3} 4.5 mM Na citrate pH 7.0, 0.1% SDS, at 65 °C for 30 min. Filters were exposed overnight to a Phosphorimage screen (ImageQuant, Molecular Dynamics) and subsequently to Kodak X-Omat autoradiograms for 3 days at -70 °C.
Sequencing. Direct sequencing of PCR fragments was performed with M13-tailed and biotinylated primers used for PCR and subsequent solid-phase sequencing with fluoresceinated primers on a Pharmacia A.L.F. Sequencer. Primer sequences have been published previously.25

Polymorphic marker analysis. Five polymorphic microsatellite markers located on chromosome 17q, spanning an approximately 3-cM interval including BRCA1, were used in a radioactive PCR under conditions described elsewhere4. D17S1322 and D17S855 have been mapped to intron 12 of BRCA1, D17S1322 to intron 19, and D17S127 to intron 20 (ref. 10). THRA1 is approximately 2-cM proximal of BRCA1, and D17S127 100 kb distal5,16 to BRCA1. CEPH sample 1347 was used as a reference to estimate the size of alleles in base pairs.

6. Szabo, C.I. & King, M.-C. M.-C. Polymorphic marker analysis. Five polymorphic microsatellite markers including BRCAl, were used in a radioactive PCR under conditions described elsewhere4.

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