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To date, more than 300 distinct small deletions, insertions and point mutations, mostly leading to premature termination of translation, have been reported in the breast/ovarian-cancer susceptibility gene \textit{BRCA1}. The elevated frequencies of some mutations in certain ethnic subpopulations\textsuperscript{3-4} are caused by founder effects\textsuperscript{5,6}, rather than by mutation hotspots. Here we report that the currently available mutation spectrum of \textit{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 \textit{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 common ancestral.

We selected four families (RUL5, RUL105, EUR9 and EUR21) for further study because they each showed strong evidence of linkage to \textit{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 polymorphisms\textsuperscript{1}, 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 sites\textsuperscript{9}, 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 \textit{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 \textit{HindIII} or \textit{BglII}, and blotted with probes derived from

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{RT-PCR analysis of \textit{BRCA1} mRNA isolated from fresh lymphocytes using primers spanning exons 12–24. a, C1–C6: unrelated control individuals. Del74 denotes a fragment containing a 74-bp deletion corresponding to exon 22. b, Three independent RT-PCR experiments from the same DNA prepared from EUR21. c, Results from breast-cancer probands requesting genetic counselling. The Del172 fragment amplified in B20 represents a deletion of exons 13–16. In both B13 and B20, the presence of the transcript generated from the wild-type allele was confirmed using primers which map within the region deleted in the other allele (data not shown). M, molecular-weight marker.}
\end{figure}
In our total set of 170 research families, we had already found 21
sequences that were implicated to stimulate recognition
patterns. The 21 sequences that bear strong homology with the AHI
core promoter region were determined to be closely linked to other
sequences found in the promoter regions of other genes. The three
sequences perfectly match the observed restriction patterns (Fig. 2A).
The sizes of both deletions were also determined to be very
concordant with those of the AHI promoter region. This deletion
results in a 12 bp downstream of the promoter.

To further characterize these sequences, we used

PCR variants were

expected, as we were unable to detect the corresponding RT.

The finding that patient EU173 carries the exon 1-16 deletion was
inconsistent with the AHI promoter region. The expected
promoter region is 125 bp downstream of the exon 1-16 deletion
(Fig. 2B). The sizes of both deletions were also determined to be very
concordant with those of the AHI promoter region. This deletion
results in a 12 bp downstream of the promoter.

From the combined data, we conclude the heterozygous

exon II (p11) or exons 14-24 (p1424), respectively.

In the same DNA region as EU173, the

promoter region is 125 bp downstream of the exon 1-16 deletion
(Fig. 2B). The sizes of both deletions were also determined to be very
concordant with those of the AHI promoter region. This deletion
results in a 12 bp downstream of the promoter.

The findings suggest that these sequences

are likely to be restriction fragment-length polymorphisms. Patient
EU173 revealed aberrant patterns. With both restriction

patterns revealed from samples from patient EU173 (data not shown),

these results are highly unlikely that they represent

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FIG. 2. Southern-blot analysis. a-c: Geno-
BRCA1 and 9 BRCA2 mutations by PTT and PCR fragment-length analysis\textsuperscript{2,12}. Because strong founder effects have been shown for most recurrent Dutch BRCA1 mutations\textsuperscript{4}, 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 analysis\textsuperscript{8} 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 the 6 of 49 probes 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 with 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\textsuperscript{10}. A 1-kb Alu-mediated deletion affecting exon 17 of BRCA1 was recently detected in a single large French breast-cancer family\textsuperscript{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\textsuperscript{23}. 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 \textit{BRCA1} in sporadic breast cancer. Because strong founder effects have been observed for many \textit{BRCA1} mutations detected worldwide,\textsuperscript{16} 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 \textit{BRCA1} mutations predicted by linkage studies,\textsuperscript{16,17} and the actual prevalence established by mutation analysis of breast-cancer families\textsuperscript{3,8} and the actual prevalence established by mutation analysis of breast-cancer families\textsuperscript{3,8} having received a diagnosis before 50 years of age. Of the 170 thus 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.\textsuperscript{23} 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 probability of being carriers of a \textit{BRCA1} mutation\textsuperscript{23}.

### Methods

\textbf{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.\textsuperscript{23} 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 probability of being carriers of a \textit{BRCA1} mutation.\textsuperscript{23}

### 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{24} For the RT-PCR analysis of exons 12–24, we used the following primers for the first PCR: forward (F), 5'-TCACAGTCAGTGAATGGGACG-3'; reverse (R), 5'-TCTCAGAACACGATGCTTGG-3'. Nested primers were then used in a subsequent PCR to amplify exons 12–24 (F, 5'-GAAGAAAA-GAGGACCGGCTTGG-3'; R, 5'-GGCCACTTTGTAAGCTTACATC-3'). For nested PCR of exons 20–24, the same reverse primer was used in combination with the F: 5'-AACGACAGGTCACAGGACG-3'. PCR conditions were as described previously.\textsuperscript{24}

### 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 AmpliTag 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 Optiprimer buffer with (Strategene), supplemented with 0.1 mM dNTPs for exon 13. For exon 13, the primers were (F) 5'-CAACGTTCGCTCGCCACT-3' and (R) 5'-ACTGCTGTACACACTAGACTATTAA-3', while an internal control fragment from exon 11 was co-amplified simultaneously (primers F, 5'-GTACGTAACGACATATTGCCGG-3'; R, 5'-TCTGAGAAACACTGTGAGATG-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'-TCCATTGGAGGCTTGGTCTG-3' and (R) 5'-ACTGCTGCTACTGCAAGACCTACCGA-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.

### Genomic DNA isolation and dideoxy sequencing

Genomic DNA (5 mg) was digested with 

<table>
<thead>
<tr>
<th>Family</th>
<th>Proband's diagnosis\textsuperscript{*}</th>
<th>Linked allele length\textsuperscript{d} at polymorphic marker (bp)</th>
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<tr>
<td></td>
<td></td>
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<td>Br28/Br46</td>
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<tr>
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<td>EUR92</td>
<td>Ivs22C</td>
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</table>

\textsuperscript{a}Br43 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 to determine phasing, both alleles are listed. Of most commonly shared allele only, as measured among 168 unrelated Dutch control individuals.\textsuperscript{15}

### Table 1 - Disease-linked haplotypes of families with a recurrent deletion mutation

Because strong founder effects have been observed for many \textit{BRCA1} mutations detected worldwide,\textsuperscript{16} 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 \textit{BRCA1} mutations predicted by linkage studies,\textsuperscript{16,17} and the actual prevalence established 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 \textit{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{25} Our data clearly show that a comprehensive \textit{BRCA1} mutation test should include the examination of its genomic structure.
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\(^2\)\(^5\).

Polymorphic marker analysis. Five polymorphic microsatellite markers located on chromosome 17q, spanning an approximately 3-cM interval including \(\text{BRCA1}\), were used in a radioactive PCR under conditions described elsewhere\(^4\). \(\text{D17S1322}\) has been mapped to intron 12 of \(\text{BRCA1}\), \(\text{D17S1322}\) to intron 19, and \(\text{D17S855}\) to intron 20 (ref. 10). THRA1 is approximately 2 cM proximal of \(\text{BRCA1}\), and \(\text{D17S1327}\) 100 kb distal\(^5\) to \(\text{BRCA1}\). CEPH sample 1347 was used as a reference to estimate the size of alleles in base pairs.

Acknowledgements

We thank the families for their cooperation and I. van Leeuwen for ascertainment and taking blood samples. G. Brink is acknowledged for his expert technical assistance. A plasmid clone containing the full-length \(\text{BRCA1}\) cDNA was the gift of B. Weber and F. Couch. This work was supported by the Dutch Cancer Society (grant RUL95-1035) and the Nijmegen Medical Foundation.

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