Bar code screening on combed DNA for large rearrangements of the BRCA1 and BRCA2 genes in French breast cancer families


Identification of the BRCA1 and BRCA2 genes was a major advance in the understanding of the familial forms of breast cancer, as alterations of these genes result in a high predisposition to breast cancer.\(^1\)\(^2\) To date, analysis of BRCA1 and BRCA2 coding sequences by mutation screening methods based on PCR sequencing protocols has allowed the identification of at least 900 different point or small disease causing germline alterations (Breast Cancer Information Core, BIC database). Furthermore, several large BRCA1 rearrangements have been reported, detected by Southern blotting, lymphocyte transcript analysis, or long range PCR.\(^3\)\(^4\) The majority of characterised rearrangements result from unequal recombination events between Alu sequences. We have estimated that the contribution of large rearrangements to the spectrum of BRCA1 mutations is close to 10% in French breast-ovarian cancer families.\(^5\)\(^6\) Only two rearrangements of the BRCA2 gene have been detected to date: a 5 kb deletion skipping exon 3 and a 6.2 kb deletion removing exons 12-13.\(^7\)\(^8\) However, few groups have systematically looked for BRCA2 rearrangements in defined series of breast cancer cases.\(^9\)\(^10\)

In order to detect such rearrangements, we have developed a bar code on combed DNA for the BRCA2 gene, which leads to a panoramic view of this gene and its flanking regions, as previously described for BRCA1.\(^10\)\(^11\) We have concurrently used BRCA1 and BRCA2 bar codes to analyse a series of 26 highly selected French patients with a family history of breast cancer only, who were previously found to be negative for point or small mutations in both BRCA1 and BRCA2 genes.

**METHODS**

From January 1991 to December 1999, women with breast cancer (index cases), ascertained in the Institut Curie cancer genetic clinic were referred to our laboratory for BRCA1/2 analysis. Familial criteria for genetic testing were: at least three cases of breast cancer at any age in the same lineage. Each of the index cases was an affected family member. The probability of being a carrier of a breast cancer predisposing allele (whatever the gene involved) was obtained by using the MLINK program of the LINKAGE package with the parameters of the Claus segregation model modified by Easton, as previously described.\(^10\)\(^11\) After informing index cases of the aims and limits of genetic testing, blood samples were collected with their written consent for DNA extraction and lymphoblastoid cell line establishment required for DNA combing and RNA studies. DNA was tested for point mutations in both BRCA1 and BRCA2 genes using a combination of DGGE-DHPLC and FAMA-PTT-DHPLC techniques, respectively.\(^12\)\(^13\)

DNA was extracted from lymphoblastoid cell lines using agarose blocks, as previously described.\(^14\)\(^15\) Combing relies on homogeneous stretching of DNA molecules at a constant rate of 2 kb/µm.\(^16\)\(^17\) It was performed using the Molecular Combing Apparatus\(^26\) (Institut Pasteur, Paris). Hybridisation and probe detection were performed as previously described.\(^18\)\(^19\) Full signals, corresponding to the BRCA1 or BRCA2 genes, were observed under an epiluminescence Leica DMRB microscope and captured with IPLab Spectrum-SU2 software (Vysis, Downers Grove, IL) using a NU 200 CCD camera (Photometrics, Tucson, AZ). The first view of full signal images is considered to constitute qualitative analysis. Full signals were captured during screening of the slide. To facilitate viewing and analyses, they were then aligned. Measurement of probes on each full signal was performed with Cartographix software using the 2 kb/µm factor, as previously described, and was considered to constitute quantitative analysis.\(^20\)\(^21\)

**RESULTS**

Twenty-six unrelated French cases with a personal and family history of breast cancer (three to eight cases per family), and with at least a 90% predisposition probability (90-98%) were selected for BRCA1 and BRCA2 bar code analyses as (1) they were previously negatively tested for BRCA1/2 point or small mutations and (2) a lymphoblastoid cell line was available (table 1).

The BRCA1 bar code used has been previously described.\(^20\)\(^21\) In this series of breast cancer cases, microscopic analysis of IC1421 DNA showed BRCA1 signals without LR9-12 and with half of the LR13-15 probes (fig 1). This deletion resembled the previously reported 23.8 kb deletion of exons 8-13 of family IC568.\(^22\)\(^23\) The use of primers specifically designed for genetic testing of IC568 family members detected an identical 923 bp PCR product in patient IC1421, suggesting that these two deletions resulted from a common mutation event in these two unrelated families. This was confirmed by the characterisation of a common haplotype associated with the carrier status in the two families, namely tel - D17S1327, 131 bp - D17S1323, deleted - D17S1322, 121 bp - D17S855, 141 bp - D17S1185, 216 bp - cen (data not shown). In order to examine
the frequency of this large deletion among breast cancer only families, we screened for the presence of the specific 923 bp PCR product a series of 245 women with breast cancer, ascertained at the Institut Curie cancer genetic clinic according to previously reported family criteria\textsuperscript{23} and negatively tested for \textit{BRCA1} and \textit{BRCA2} point mutations. No 923 bp fragment was observed, suggesting that this large deletion is not frequent in the study population (data not shown). No other rearrangement of the \textit{BRCA1} gene was detected by either qualitative or quantitative analysis.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
IC family & Predisposition probability (%) & Age at diagnosis (y) of breast tumour for index cases & No of breast tumours in the family \\
\hline
25 & 90 & 33 & 3  \\
343 & 90 & 46 & 5  \\
1008 & 90 & 44 & 5  \\
1077 & 90 & 33 & 8  \\
1110 & 90 & 45 & 3*  \\
1185 & 90 & 43 & 4  \\
1205 & 90 & 47 & 4  \\
1427 & 90 & 36 & 4  \\
1422 & 90 & 30 & 3  \\
1679 & 90 & 48 & 5  \\
1782 & 90 & 44, 67 & 5  \\
2054 & 90 & 47 & 3  \\
2232 & 90 & 44 & 4*  \\
2240 & 91 & 56 & 4  \\
648 & 92 & 37, 38 & 3  \\
59 & 93 & 56 & 6  \\
392 & 93 & 61, 65 & 4*  \\
995 & 93 & 35 & 3  \\
1189 & 93 & 32 & 3  \\
1843 & 93 & 29 & 4  \\
2229 & 93 & 37 & 4  \\
2208 & 94 & 46 & 6  \\
1743 & 95 & 46 & 7  \\
179 & 97 & 36 & 6  \\
383 & 97 & 27 & 3  \\
2259 & 98 & 38 & 7  \\
\hline
\end{tabular}
\caption{The 26 French breast cancer families analysed by \textit{BRCA1} and \textit{BRCA2} bar codes}

*Three of the 26 families comprised a male breast cancer. Predisposition probabilities of index cases range from 90 to 98\% (average 92\%). The number of breast tumours in the family varies from 3 to 8 (average 4.5).

Figure 1 \textit{BRCA1} bar code. (A) The \textit{BRCA1} region in 17q21. The \textit{BRCA1} gene, composed of 24 exons (22 coding exons), is spread over 81 kb and has a common promoter with the \textit{NBR2} gene [Next to \textit{BRCA1} gene 2].\textsuperscript{22-26} The \textit{BRCA1} pseudogene (Ψ\textit{BRCA1}) lies next to \textit{NBR2}, and corresponds to a partial duplication of \textit{BRCA1}, from the promoter region to intron 2.\textsuperscript{27-29} The \textit{NBR1} gene is located 5′ to Ψ\textit{BRCA1}.

The figure is not drawn to scale and is adapted from Puget et al\textsuperscript{7}, Barker et al\textsuperscript{37} and Brown et al\textsuperscript{38} (B) Analysis of DNA from patient IC1421 with the \textit{BRCA1} bar code. (N) This full signal corresponds to the normal allele. The PAC 103014 insert covers the region between the first exons of \textit{NBR1} and the 3′ \textit{BRCA1} UTR (exon 24), which is approximately 120 kb long.\textsuperscript{32-36} The cosmid ICRFc105D06121 clone used in our experiment is rearranged, with an insert which is approximately 6 kb long and covers the region between \textit{NBR2} intron 1 and \textit{BRCA1} intron 2.\textsuperscript{32-36, 38} The other probes are long range (LR) PCR products, covering the region between the exons quoted, except LR24-3′, which covers \textit{BRCA1} exon 24 to 10 kb downstream. (del) This full signal lacks LR9-12 and half of the LR13-15 probes, which represents a first indication of a deletion of at least exons 9 to 13 in the \textit{BRCA1} gene in this patient.
The BRCA2 gene sequence is present in PACs 214K23 and 92M18 corresponding to exons 1-24 and exons 25-27 respectively (Genbank accession numbers Z74739 and Z73359). In the “Human BAC End” database (The Sanger Centre), BAC 486O17 was found to comprise both 5’ and 3’ ends of the BRCA2 gene, spreading over 84 kb. Introns sizes were precisely determined based on available data on sequences of both BRCA2 exons and PACs. Long range (LR) PCR primers were designed to obtain probes for the BRCA2 bar code (available on the Institut Curie web site), varying from 3.2 to 9 kb and spanning over several exons. This bar code is slightly different from the BRCA1 bar code, since all but two of the exons (exons 10 and 11, owing to their large sizes) are included in LR probes, and large introns are covered by the green BAC 486O17 (fig 2). A total of eight probes designed the bar code, splitting the BRCA2 gene into 13 fragments. The series of 26 breast cancer cases was analysed with the BRCA2 bar code. No rearrangement of the BRCA2 gene, on either qualitative or quantitative analysis, was detected.

**DISCUSSION**

The aim of the present study was to estimate the number of rearrangements in the **BRCA1** and BRCA2 genes in a series of highly selected French breast cancer families. A set of 26 cases defined by an at least 90% predisposition probability (that is, an average of 4.5 breast tumours per family), absence of a point mutation in BRCA1/2, and availability of a lymphoblastoid cell line, was analysed by **BRCA1** and **BRCA2** bar codes on combed DNA. The previously reported 23.8 kb **BRCA1** deletion of exons 8-13 was detected in one family, whereas no **BRCA2** rearrangement was identified in this set of families.

Several studies have examined the **BRCA1** gene for rearrangements over the past five years, but few have analysed the **BRCA2** gene. Only two **BRCA2** deletions have been reported to date, with one detected by KF-PCR as a case report and the second by Southern blotting. Three series looked for **BRCA2** rearrangements: a total of 187 families with breast only or breast-ovarian cancer history were analysed and only one deletion was detected (0.53%). We have developed another approach, a **BRCA2** bar code, in order to look for deletion, duplication, or inversion in the **BRCA2** gene. As no **BRCA2** rearrangement was observed in our family series, assuming the detection of a rearrangement at least 2 kb long, this study confirms the low frequency of **BRCA2** rearrangements in breast cancer families.

The frequency of large gene rearrangements in human diseases varies from 5 to 50%.[15,20] The contribution of rearrangements to the **BRCA1** mutation spectrum ranges from 10 to 15%.[16,5] In the Dutch population, an even higher value is observed, owing to founder effects, since two deletions account for 36% of the alterations.[4] The lower contribution of rearrangements to the **BRCA2** mutation spectrum as compared to **BRCA1** may be explained by two hypotheses: (1) the longer coding sequence (10 257 v 5992 bp) split into about 20 exons (26 v 22) and encompassing similar genomic sequence sizes (84 v 81 kb) and (2) the lower intrinsic density of Alu sequences (20 v 41.5%) which are the most frequently repetitive elements involved in the appearance of large gene rearrangements.[25,27]

Despite complete screening for point mutations and large rearrangements in both **BRCA1** and **BRCA2**, only one family among the 26 highly selected families of this series was explained by the identification of a **BRCA1** alteration. Therefore, 25 families including three with male breast cancer still remained unexplained. This is striking as the contribution of rearrangements to the **BRCA1** mutation spectrum as compared to **BRCA1** may be explained by two hypotheses: (1) the longer coding sequence (10 257 v 5992 bp) split into about 20 exons (26 v 22) and encompassing similar genomic sequence sizes (84 v 81 kb) and (2) the lower intrinsic density of Alu sequences (20 v 41.5%) which are the most frequently repetitive elements involved in the appearance of large gene rearrangements.[25,27]

The search for **BRCA1** rearrangements may now be systematically included in genetic testing. However, because of the lack of evidence for a significant contribution of **BRCA2**
rearrangements, it does not seem justified to include the search for them in routine genetic testing at the present time.

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