LETTER TO JMG

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

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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.[3–14] 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.[15] 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.[16 17] However, few groups have systematically looked for *BRCA2* rearrangements in defined series of breast cancer cases.[17–19]

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*.[20 21] 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.

Key points

- In order to estimate the number of large rearrangements in the *BRCA1* and *BRCA2* genes, a series of 26 highly selected French patients with a family history of breast cancer only was analysed using *BRCA1* and *BRCA2* bar codes on combed DNA.
- One *BRCA1* deletion was detected, whereas no *BRCA2* rearrangement was identified.
- This study confirms the low frequency of *BRCA2* rearrangements in breast cancer families.

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.[22 23] 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.[23–25]

DNA was extracted from lymphoblastoid cell lines using agarose blocks, as previously described.[20 26] Combing relies on homogeneous stretching of DNA molecules at a constant rate of 2 kb/µm.[26 27] It was performed using the Molecular Combing Apparatus™ (Institut Pasteur, Paris). Hybridisation and probe detection were performed as previously described.[20 26] Full signals, corresponding to the *BRCA1* or *BRCA2* genes, were observed under an epifluorescence 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 26]

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 26] 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.[5 20] 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

Table 1 The 26 French breast cancer families analysed by *BRCA1* and *BRCA2* bar codes

IC family	Predisposition probability (%)	Age at diagnosis (y) of breast tumour for index cases	No of breast tumours in the family
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
1421	**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
833	97	27	3
2259	98	38	7

*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).

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[23] and negatively tested for *BRCA1* and *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 *BRCA1* gene was detected by either qualitative or quantitative analysis.

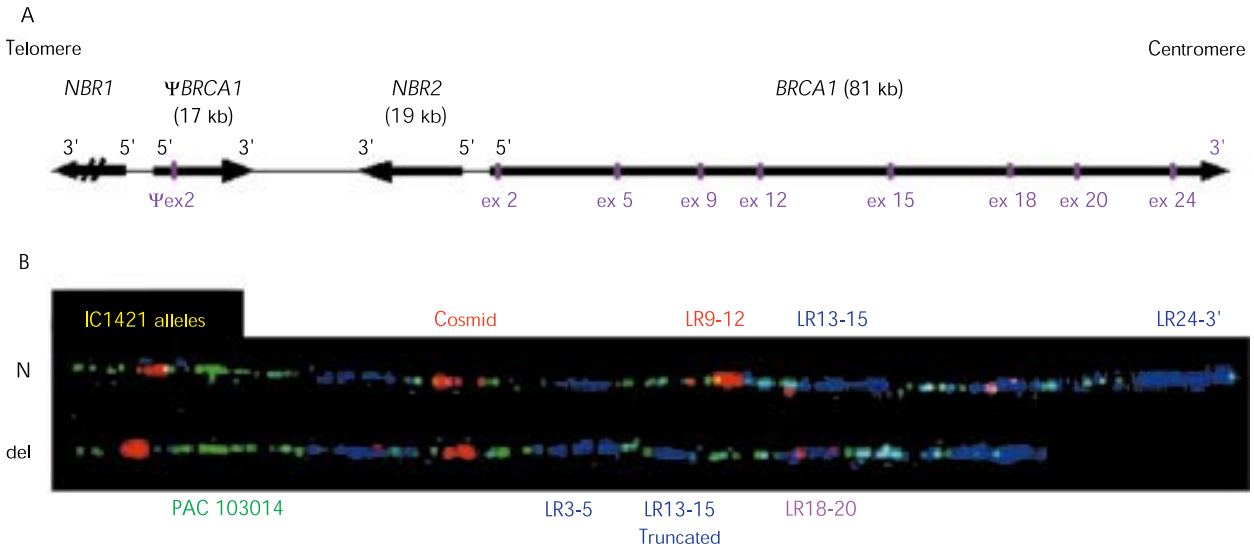

Figure 1 *BRCA1* bar code. (A) The *BRCA1* region in 17q21. The *BRCA1* gene, composed of 24 exons (22 coding exons), is spread over 81 kb and has a common promoter with the *NBR2* gene (Next to *BRCA1* gene 2).[32 36] The *BRCA1* pseudogene (Ψ*BRCA1*) lies next to *NBR2*, and corresponds to a partial duplication of *BRCA1*, from the promoter region to intron 2.[7 37 38] The *NBR1* gene is located 5′ to Ψ*BRCA1*.[37 38] The figure is not drawn to scale and is adapted from Puget *et al*[7], Barker *et al*,[37] and Brown *et al*.[38] (B) Analysis of DNA from patient IC1421 with the *BRCA1* bar code. (N) This full signal corresponds to the normal allele. The PAC 103O14 insert covers the region between the first exons of *NBR1* and the 3′ *BRCA1* UTR (exon 24), which is approximately 120 kb long.[32 36 38] The cosmid ICRFc105D06121 clone used in our experiment is rearranged, with an insert which is approximately 6 kb long and covers the region between *NBR2* intron 1 and *BRCA1* intron 2.[32 36 38] The other probes are long range (LR) PCR products, covering the region between the exons quoted, except LR24-3′, which covers *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 *BRCA1* gene in this patient.

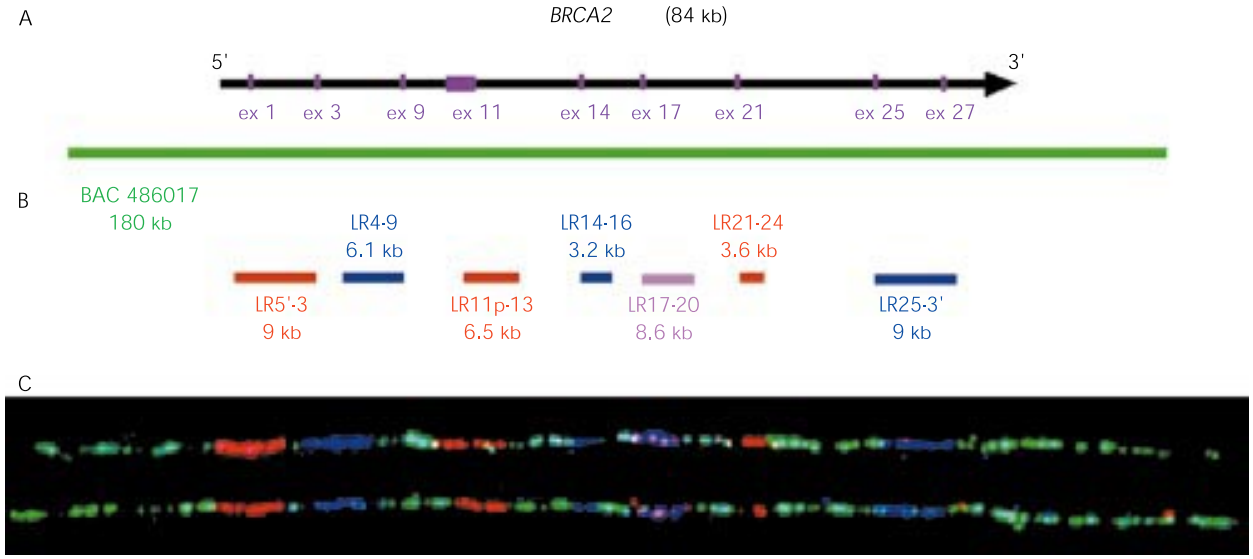

Figure 2 *BRCA2* bar code. (A) The *BRCA2* gene in 13q12-13 (not drawn to scale). The *BRCA2* gene is composed of 27 exons (26 coding exons) and is spread over 84 kb.[2 28] Introns range in size from 91 bp (intron 5) to 14.5 kb (intron 24). (B) Probes used for the design of the *BRCA2* bar code. They are presented in the colour of their revelation step (five layers of antibodies): BAC 486O17 insert (approximately 180 kb long, The Sanger Centre) covers the entire region, the other probes are long range (LR) PCR products, ranging in size from 3.2 to 9 kb, covering several exons and generated from the BAC. Owing to the 5 kb length of exon 11, probe LR11-13 covers only from the 3′ end of exon 11 to exon 13. (C) Control DNA: two full signals showing the *BRCA2* bar code (among 46 signals captured after screening of the slide). A total of eight probes designed the bar code, splitting the *BRCA2* gene into 13 fragments.

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. Intron sizes were precisely determined based on available data on sequences of both *BRCA2* exons[28] 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 spreading 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[5] 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 RT-PCR as a case report[16] and the second by Southern blotting.[17] Three series looked for *BRCA2* rearrangements[17–19]: a total of 187 families with breast only or breast-ovarian cancer history were analysed and only one deletion was detected (0.53%).[17] 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%.[29–31] The contribution of rearrangements to the *BRCA1* mutation spectrum ranges from 10 to 15%.[5 15] In the Dutch population, an even higher value is observed, owing to founder effects, since two deletions account for 36% of the alterations.[3] 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* 5592 bp) split into about 20 exons (26 *v* 22) and encompassing similar genomic sequence sizes (84 *v* 81 kb)[28 32] and (2) the lower intronic density of Alu sequences (20 *v* 41.5%) which are the most frequently repetitive elements involved in the appearance of large gene rearrangements.[29 32 33]

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 *BRCA1/2* mutations, estimated from linkage analyses, is 65% and 96% in breast only cancer families and in families with at least one male breast cancer, respectively.[34] The failure to detect mutations may be the result of mutations not detected by the methods used, especially mutations located in introns and far from splice sites. Index cases may also be sporadic cases occurring in families with a segregating predisposing mutation. Finally, such large families with a high predisposition probability may be the result of a single gene with a recessive transmission mode or more likely additional gene variations with multiplicative effects.[35] Considerable work remains to be done to explain breast cancer families with no *BRCA1/2* mutation.

The search for *BRCA1* rearrangements may now be systematically included in genetic testing.[15] 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. Additional studies are needed to define clearly their role in breast cancer genetic testing.

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Database information: Breast Cancer Information Core (BIC): http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/
The Institut Curie web site: www.curie.net/genetique
The Sanger Centre: www.sanger.ac.uk/HGP

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