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Significant Contribution of Germline *BRCA2* Rearrangements in Male Breast Cancer Families

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Abstract

Although screening for large deletions or duplications of the *BRCA1* gene is becoming a routine component of the molecular diagnosis of familial breast cancer, little is known about the occurrence of such rearrangements in the *BRCA2* gene. Because of the high frequency of *BRCA2* mutations in breast cancer families with at least one case of male breast cancer, we selected a cohort of 39 such families, tested negative for mutations in the coding regions of *BRCA1* and *BRCA2*, and developed an assay for *BRCA2* rearrangements, based on quantitative multiplex PCR of short fluorescent fragments (QMPSF). We found three rearrangements: (1) a deletion of exons 12 and 13; (2) a duplication of exons 1 and 2; and (3) a complete deletion of *BRCA2*. We determined the boundaries of the deletion of exons 12 and 13, showing that it resulted from an unequal recombination between *Alu* sequences. We mapped the complete *BRCA2* deletion, which extends over at least 298 kb and showed that it does not affect *APRIN/AS3*, previously characterized as a tumor suppressor gene, but it comprises several loci corresponding to proven or putative transcripts of unknown functional significance. These data suggest that screening for *BRCA2* rearrangements should be done, especially in male breast cancer families tested negative for *BRCA1* and *BRCA2* mutations.

Introduction

Several studies have described large genomic deletions or duplications of the *BRCA1* gene in breast cancer or breast-ovarian cancer families (1–4), and sensitive assays designed to detect such rearrangements have been recently added to the routine molecular diagnosis of breast or breast-ovarian cancer predisposition (5, 6). Genomic rearrangements have been estimated to represent between 10 and 15% of the *BRCA1* defects (2, 7), but founder effects resulting in higher frequencies of germline rearrangements have been described in some populations (1). However, very little is known concerning the occurrence of germline rearrangements in the *BRCA2* gene. Besides the report of an *Alu* element insertion into exon 22 (8), only two examples of germline *BRCA2* rearrangements have been published: a deletion that comprises a portion of exon 3 and most of intron 3, resulting into an inframe exon 3 skipping (9) and a deletion/insertion mutation removing exons 12 and 13 (10). The former rearrangement was discovered at the cDNA level, whereas the latter was found at the genomic level, by Southern blot analysis. Therefore, we set out to develop for *BRCA2* a sensitive method, capable of analyzing rapidly all of the exons for copy numbers. To this end, we adapted to *BRCA2* the previously described quantitative multiplex PCR of short fluorescent fragments (QMPSF) method (5, 11). Breast cancer is rare in men, but *BRCA2* defects are frequent in families with one or more cases of male breast cancer. Therefore, we applied the new QMPSF assay to the detection of *BRCA2* rearrangements in families with multiple breast cancers, including one or more male breast cancers, and without detectable mutations within *BRCA1* and *BRCA2* coding sequences.

Materials and Methods

Patients. The inclusion criteria were as follows: at least one male breast cancer (any age) and at least one first- or second-degree relative with male breast cancer (any age) or female breast cancer (age of diagnosis below 60 years). In some cases, third-degree relatives were also considered if the intermediate was a male or an affected female. The index case was an affected male (any age) or female (diagnosed at age below 60 years) or, in a few cases, an obligate carrier. The diagnoses of the index case and of male breast cancer patients were verified by examining the histologic and/or the clinical records. All of the index cases had been found *BRCA1* and *BRCA2* negative on screening all of the exons for point mutations and microdeletions or -insertions by either denaturing high-performance liquid chromatography (dHPLC), denaturing gradient gel electrophoresis (DGGE), or by using different combinations of established methods [dHPLC, DGGE, fluorescence assisted mismatch analysis (FAMA), or the protein truncation test (PTT)].

Standard QMPSF Conditions. Exons of the *BRCA2* gene were analyzed by three QMPSF assays consisting of 8, 9, and 9 *BRCA2* amplicons, respectively, and a control from the *MLH1* gene. Amplicon sizes ranged between 172 bp and 300 bp. Reactions were done in 25 μL with 0.2 mmol/L of each deoxynucleoside triphosphate, 1.2 mmol/L $MgCl_2$, 1.5 units of Thermoprime Plus DNA Polymerase (ABgene, Epson, United Kingdom), 7.5% DMSO, 75 to 100 ng of DNA, and between 0.2 and 2.4 μmol/L of each PCR primer, one of them carrying a 6-FAM label. After an initial step of denaturation at 94°C for 4 minutes, 24 cycles were done consisting of denaturation at 94°C for 10 seconds, annealing at 48°C for 15 seconds, and extension at 72°C for 20 seconds, followed by a final extension step at 72°C for 5 minutes. DNA fragments generated by QMPSF were separated on an ABI Prism 377 DNA sequencer, and the resulting fluorescence profiles were analyzed with the GeneScan 3.1 Software. Primer sequences are available on request.

Special QMPSF Designs. The QMPSF assay for mapping the deletion of exons 12 and 13 comprised 7 amplicons as follows: an amplicon in exon 14 of *MLH1* as a control, the standard amplicons for exons 11 (3′end), 12, 13, and 14, and two additional amplicons in introns 11 and 13, respectively. To map

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the boundaries of the complete *BRCA2* deletion, amplicons were chosen in several loci in the *BRCA2* region that correspond to shown or putative genes. Three of them were located in the *13CDNA73* gene as follows: one in exon 21, one in exon 44, and the other one in exon 61. One amplicon was located in exon 7 of the *CG018* gene. Two amplicons were located in the *PFAAP5* gene–one in exon 2 and the other one in exon 6. One amplicon was located 6 kb from the 5′end of the *APRIN/AS3* gene, and the last one was located in exon 2 of the same gene. This QMPSF assay also contains the standard amplicon for *BRCA2* exon 17 and a control amplicon located in the *UFD1L* gene (22q11).

Other Methods. Long-range PCR was carried out with the Expand Long Template PCR System (Roche, Meylan, France) as described previously (5). The duplication of exons 1 and 2 of *BRCA2* in one family was confirmed with the multiplex ligation-dependent probe amplification (MLPA) kit for *BRCA2* from Microbiology Research Center-Holland (Amsterdam, The Netherlands).

Results and Discussion

BRCA2 mutations are frequent in families with multiple breast cancer cases including one or more male breast cancer cases. It has been estimated by linkage analysis that *BRCA2* defects are present in 76% of the male breast cancer families of the Breast Cancer Linkage Consortium, which are characterized by at least 4 breast cancers (age of diagnosis <60 years for females, any age for males; ref. 12). Large deletions or duplications of *BRCA2* are therefore likely in male breast cancer families tested negative after screening all exons and exon-intron junctions of *BRCA2* and of *BRCA1*. Of the 39 French families that fulfilled our criteria (Table 1), 5 had two male breast cancer cases and 11, including 2 families with two male breast cancer cases, had four or more breast cancer cases. Moreover, other characteristic features of the *BRCA2* phenotype (13) were found in several families of this cohort such as prostate cancer, pancreatic cancer, and melanomas (Table 1).

The QMPSF assay for *BRCA2* that we have developed consists of three multiplex PCRs and was validated with DNA from patients with known microdeletions or microinsertions in different *BRCA2* exons, which showed the expected size-shift of peaks because of the mutant amplicons and, in addition, a reduction to one half of the peaks of the corresponding wild-type allele (data not shown). We evaluated the sensitivity of this assay by using DNA from a chromosome 13 trisomy and found a ~50% increase of the signals corresponding to all of the *BRCA2* amplicons (data not shown). We then screened the index cases of the 39 families listed in Table 1 and found three rearrangements: a deletion of exons 12 and 13, a duplication of exons 1 and 2, and a complete deletion of *BRCA2* (families F1028, F1020, and F2832, respectively; shown in *bold* in Table 1). All three families were among those with the highest probability of *BRCA2*-related cancer predisposition. In family F1028, a total of 11 cancers had been found, including 2 male breast cancer cases, 8 breast cancer cases, and one prostate cancer case. In family F1020, two brothers had breast cancer. Family F2832 showed a pancreatic cancer, a male breast cancer, and a female breast cancer in the same lineage in subsequent generations.

The rearrangement in family F1028 is illustrated in Fig. 1. On superimposition of the fluorescence profiles of control DNA and of DNA from the index case of this family, we found a reduction of the

Table 1 *Characteristics of the 39 families studied*

Family *	Male breast cancers † *n* (age at diagnosis ‡)	Female breast cancers † *n* (age at diagnosis ‡)	Other cancers	Tested *BRCA1* and *BRCA2* negative with
ACT853	1 (65)	2 (50 §, 81/81)		DHPLC
ACT1006	1 (39)	2 (44 §, 62/62)		DHPLC
ACT1369	1 (67)	3 (46 §, 36)	ovary (38)	DHPLC
EXT292	1 (52, melanoma 58)	2 (59 §)	melanoma (42)	DHPLC
ACT773/IC423	1 (71, testis 31)	2 (42 §)		DHPLC
EXT/Ang53	1 (64)	1 (49 §)		DHPLC
F 1214	1 (76)	2 (47 §)		DHPLC
F 1028	**2 (57, 63)**	**8 (56/63 §,¶, 36)**	**prostate (52)**	**DHPLC**
F 1020	**2 (51 §, 48)**	**0**		**DHPLC**
F 2045	1 (62)	1 (48 §)	pancreas (45)	DHPLC
F2816	1 (53 §)	3 (45)		DHPLC
F2833	1 (77 §)	1 (36)		DHPLC
F2686	1 (49 §)	1 (48)		DHPLC
F2814	1 (69)	3(36 §)	prostate (58); ovary (55); kidney (69); lung (65)	DHPLC
F2832	**1 (72 §)**	**1 (45)**	**pancreas (63)**	**DHPLC**
F1932	1 (48)	3 (47 §, 45)		DHPLC
F1565/ACT660	1 (63)	3 (59 §,‖, 40/44 **)		DHPLC
IC154	1 (32 §)	1 (42)		DGGE, FAMA
IC344	1 (33 §)	1 (60)		DGGE, FAMA
IC469	1 (51)	2 (45 §)		DGGE, FAMA
IC503	1 (73 §)	4 (66/77, 31)	ovary (38), uterus (61)	DHPLC
IC738	1 (32 §)	1 (39)		DGGE, FAMA
IC894	1 (63)	5 (38 §, 52/53, ov61)	head-and-neck, bone (48, 64)	DGGE, DHPLC
IC1110	1 (60)	2 (45 §, 42)		DHPLC, PTT
IC1828	1 (74 §)	1 (33/37)		DHPLC, PTT
IC2232	1 (70)	3 (45 §)		DHPLC, PTT
IC2494	1 (62 §)	2 (47)	pancreas (25), kidney (42), corneal epithelioma	DGGE, DHPLC
IC2684	1 (68, prostate 78)	1 (47 §)		DHPLC
IC2841	1 (56)	2 (35/35 §, 45/45)		DHPLC
IC2967	1 (60)	2 (66 § obligate carrier, 52)		DHPLC
IC3839	1 (70)	2 (51 §, 49)		DHPLC
IC3591/CAL9705034	2 (55 §, 65 ††)	1 (52)		DGGE, DHPLC
IC3850/R4465	1 (53 §,‡‡)	2 (39)		DHPLC
IC/Lim2118	1 (60)	1 (45 §)		DHPLC, PTT
IC/CRG2723	2 (45 §, 35)	1 (51)		DHPLC, PTT
GE0068	2 (61 §, 73)	2 (38)		DHPLC
GE0194	1 (58)	4 (34 §)		DHPLC
CFB/Ang189	1 (60)	1 (36)	pancreas (62), ovary (72), colon polyps/obligate carrier §	DHPLC
L98011	1 (42)	1 (34 §)		Heteroduplex analysis

* Families in which germline *BRCA2* rearrangements were found are marked with bold characters.

† The patient tested is marked by a section symbol (§).

‡ Age at diagnosis of patient tested followed by the earliest diagnosed breast cancer case, if different, and by all bilateral or controlateral breast cancer cases; ¶ also esophagus (62); ‖ also non-Hodgkin lymphoma (72); ** also head-and-neck cancer; †† also cancer of the bladder (68); ‡‡ also cancers of thyroid (26) and colon (54).

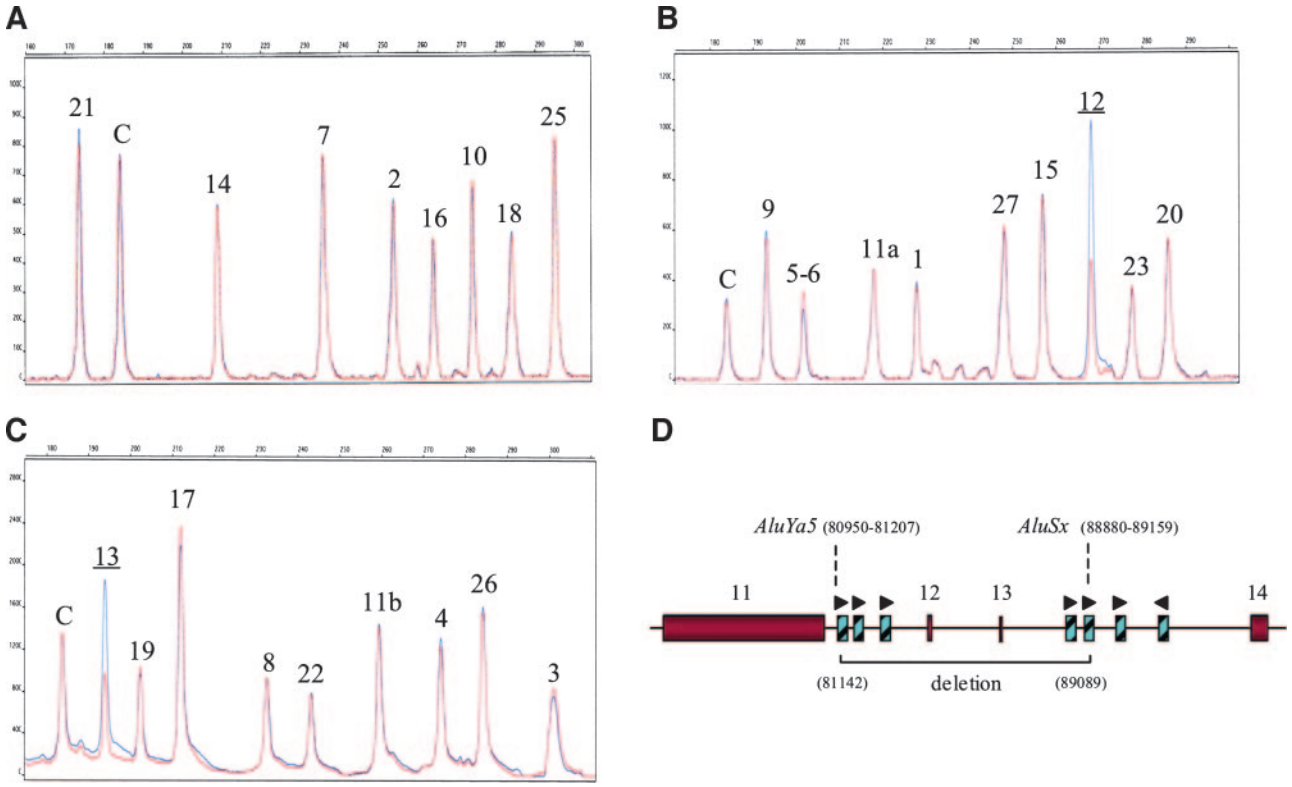

Fig. 1. Detection and characterization of a *BRCA2* deletion involving exons 12 and 13. *A–C* correspond to QMPSF 1, 2, and 3 of *BRCA2*, respectively. The horizontal scale is in bp, the vertical one is in arbitrary units of fluorescence. Peak numbers refer to *BRCA2* exons and correspond to amplicons with sizes ranging from 172 to 300 bp. The fluorescence profile obtained from a patient from family F1028 (*red*) is superimposed with the one obtained from a normal subject (*blue*) by adjusting to the same level the height of the control peaks (amplicon C, which targets exon 14 of *MLH1*). The heterozygous deletion of exons 12 and 13 is detected by a 2-fold reduction of the intensity of the corresponding peaks. *D* is a schematic representation of the region deleted. *BRCA2* exons are shown as *red boxes*, *Alu* repeat elements are shown as *green striped boxes*, with orientations shown by *arrowheads*. Sequence coordinates are from GenBank (accession no. Z74739). The deletion spans 7947 bp, as determined by sequencing a junction PCR fragment obtained by long-range PCR with the forward primer 5′-TTTGGGAAAAGAACAGGCTT-3′ in exon 11 and the reverse primer 5′-CCTCTGCTAGGTTAAGACCTGTA-3′ in intron 13. Note that on this schematic, the 11-bp sequence (TGCAGTGAGC) of perfect homology in which the recombination has occurred has been attributed to the *AluYa5* element of intron 11, and, therefore, the left boundary of the deletion is arbitrarily considered to be at position 81142.

peak intensities of amplicons corresponding to exons 12 and 13, shown in Fig. 1, *B* and *C*, respectively. To define the boundaries of this deletion, we designed a specific QMPSF assay for this region (not shown), which included additional amplicons in introns. This specific assay allowed us to reduce the target size for long-range PCR and to amplify from the mutant allele a fragment containing the deletion boundaries. Sequencing of the junction showed that the deletion had occurred by unequal recombination between an *Alu*Ya5 sequence in intron 11 and an *Alu*Sx sequence in intron 13 and that its size was 7947 bp (Fig. 1*D*). This deletion therefore differs from the one described by Wang *et al.* (10), which was shorter (6.2 kb) and could not be explained by a simple unequal recombination event between *Alu* repeats.

In family F1020 (Fig. 2), an ~50% increase of the intensity of the peaks corresponding to exons 1 and 2 was observed (Fig. 2, *A* and *B*), and this difference in the QMPSF profile was not seen in any of the >100 individuals tested in this study. Moreover, this profile was identical, concerning exons 1 and 2, to that of our duplication control obtained with DNA from a chromosome 13 trisomy (data not shown). However, we were unable to amplify the duplicated segment by PCR and to characterize it at the nucleotide level. We therefore confirmed our QMPSF result using a commercially available multiplex ligation-dependent probe amplification (MLPA) assay for *BRCA2*, which is based on single-stranded probes that hybridize at adjacent positions to exonic targets and can then be joined covalently by ligase and amplified by PCR (6). The relevant portion of the MLPA profile is shown in Fig. 2*C* and confirms the duplication of exons 1 and 2. Our failure to obtain duplication-specific PCR fragments, despite the numerous combinations of primers used in long-range PCR experiments, may indicate that this rearrangement is more complex than a tandem duplication of exons 1 and 2.

A complete *BRCA2* deletion was found in family F2832. Fig. 3 shows our analysis of the boundaries of this large deletion, particularly with regard to the known genes or open reading frames surrounding *BRCA2*. The 13q12.3 region (schematically shown in Fig. 3*B*) contains (in the centromer to telomer direction) the following: the large gene *13CDNA73* encoding the hypothetical protein CG003; the *BRCA2* gene; the *PRO0297* gene, inferred from a human fetal liver cDNA (data not shown); the hypothetical gene *CG018*, with opposite transcriptional orientation; a locus with unknown significance (*LOC88523*; data not shown), also in opposite orientation; the *PFAAP5* gene, predicted to encode phosphonoformate immunoassociated protein 5, with unknown function; and the *APRIN* gene, also known as *AS3* gene (henceforth named *APRIN/AS3*), which is transcribed from the same strand as *BRCA2*, and whose 5′ end lies 187 kb downstream of the 3′ end of *BRCA2*. It is especially noteworthy that the AS3 protein (androgen-induced prostate proliferative shutoff associated protein) is a mediator of the proliferative arrest of prostate epithelial cells (14) and that the gene coding for this protein had previously been found affected by large somatic deletions of the *BRCA2* region, described initially in a pancreatic carcinoma (ref. 15 and the references therein). It was therefore of great interest to define the boundaries of the germline deletion found in family F2832, and particularly the telomeric one. To this end, we designed additional

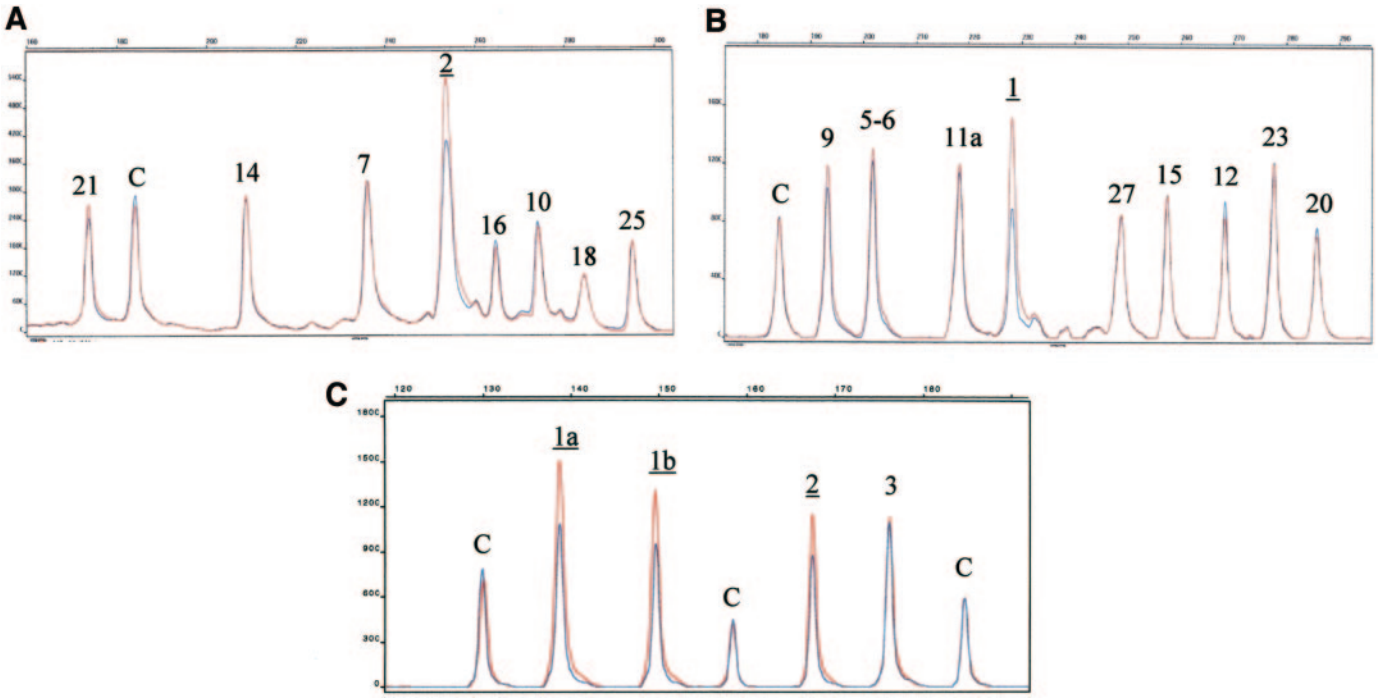

Fig. 2. Detection of a duplication involving exons 1 and 2 of *BRCA2*. *A* and *B* correspond to *BRCA2* QMPSF 1 and 2, respectively. The *red profiles* are from a patient from family F1020, and the *blue profiles* are from a control subject. The duplication of exon 1 and exon 2 was revealed by a 50% increase in the corresponding peak height. *C* represents the relevant portion of the fluorescent profiles obtained from the same DNA samples with the *BRCA2* MLPA kit from Microbiology Research Center-Holland, which targets twice the exon 1 region (peaks *1a* and *1b*).

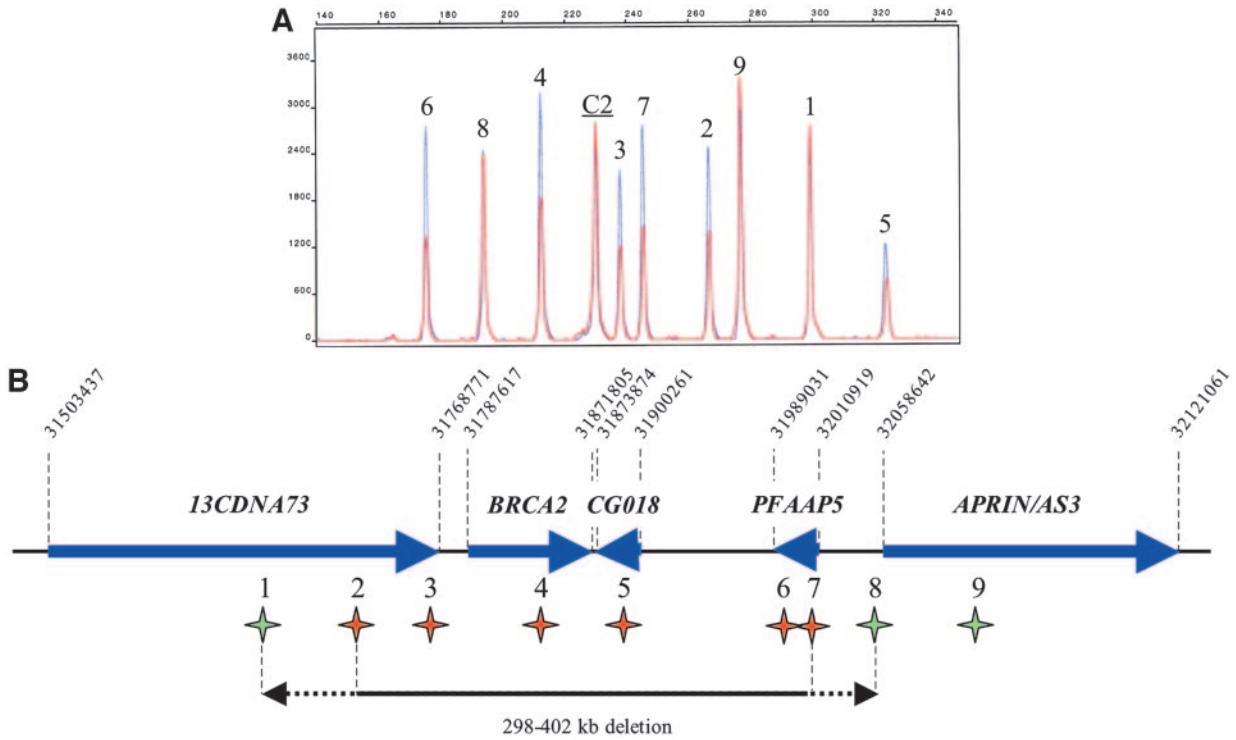

Fig. 3. Characterization of a complete *BRCA2* deletion in family F2832 (*red profile*). The deletion of all exons was detected (data not shown) with the three QMPSF assays already illustrated in Fig. 1. *A* represents the specially designed QMPSF assay that allowed us to define the intervals containing the deletion boundaries. Amplicons were chosen in proven or putative genes of the *BRCA2* region. *B* is a schematic representation of the deleted region. Coordinates are from the UCSC Browser database (www.genome.ucsc.edu/), May 2004 freeze. *Red stars* mark the position of deleted amplicons, whereas *green stars* mark the nondeleted ones. The deletion size ranges between 298 and 402 kb. This large heterozygous deletion comprises the 3′ end of *13CDNA73* [amplicons 2 (31709855-31710099) and 3 (31767461-31767676) for exons 44 and 61, respectively, are deleted, whereas amplicon 1 (31650317-31650595) corresponding to exon 21 is not deleted], the *BRCA2* gene [amplicon 4 (31834781-31834971) for exon 17], the *CG018* locus [amplicon 5 (31874381-31874687) for exon 7], and the *PFAAP5* gene [amplicons 6 (31989871-31990024) and 7 (32008056-32008278) for exons 6 and 2, respectively]. It does not affect the tumor suppressor gene *APRIN/AS3*, because amplicons 8 (32052956-32053128) and 9 (32120807-32121061), located 6 kb from the 5′ end of *APRIN/AS3* and within *APRIN/AS3* exon 2, respectively, are not deleted.

QMPSF assays containing amplicons distributed over the ~500-kb region shown in Fig. 3 (an example of these assays is shown in Fig. 3*A*). These analyses showed that the germline deletion extended over at least 298 kb and did not affect the *APRIN/AS3* gene (Fig. 3*B*). Because of the undefined function of genes surrounding *BRCA2* (except for *APRIN/AS3*), one cannot predict which phenotypes could have been expected for this family, because of the large deletion. It is, however, noteworthy that the cancer characteristics observed in this family (a pancreatic cancer, a male breast cancer, and a female breast cancer in the same lineage in subsequent generations) are typical of the *BRCA2* phenotype (13).

Finally, we screened for *BRCA2* rearrangements our collection of 91 breast-ovarian cancer families that had been tested *BRCA1* and *BRCA2* negative, except for five distinct rearrangements detected in *BRCA1* (5, 16). We found no *BRCA2* rearrangement in this large breast-ovarian cancer cohort, a result consistent with the notion that in breast-ovarian cancer families *BRCA1* defects are predominant, although *BRCA2* mutations are also found, particularly in the ovarian cancer cluster region (17).

This work shows that large constitutive rearrangements are not uncommon in the *BRCA2* gene. Two studies had previously addressed this question by Southern blot analysis, but they were not based on families selected for particularly high probability of BRCA2-related cancer predisposition. In a study of 82 Finnish breast and/or ovarian cancer families, no germline rearrangement was found in *BRCA1* or *BRCA2* (18). Moreover, no *BRCA2* rearrangement was found in 81 Dutch breast and/or ovarian cancer families (19). These negative results therefore indicate the absence, in these populations, of *BRCA2* rearrangements with a founder effect, as described for *BRCA1* (1), but leave open the possibility of a variety of *BRCA2* rearrangements such as those that we have described.

The ability of both QMPSF and MLPA to detect the duplication of exons 1 and 2 (Fig. 2) illustrates the high sensitivity of these methods and indicates that the vast majority of rearrangements should be detected, if one uses target DNAs of good and homogeneous quality. Nevertheless, particular types of rearrangements could be missed if they involve only portions of exons, because only short sequence stretches are targeted in each exon. The cost per sample of screening for *BRCA2* rearrangements is the same as that of screening for *BRCA1* large germline deletions or duplications, which is already being implemented routinely in many laboratories, but screening for *BRCA2* rearrangements is presently limited to selected families with high probability of a *BRCA2* defect, tested negative for *BRCA1* and *BRCA2* mutations.

Our cohort of *BRCA1*- and *BRCA2*-negative male breast cancer families was composed of families studied in several French laboratories and did not result from consecutive cases. Therefore, it would be highly troublesome to evaluate directly the contribution of the three rearrangements found to the total number of *BRCA2* defects. However, the criteria for inclusion of the families analyzed here are similar to those of 29 of the 33 families analyzed by Evans *et al.* (20). They found *BRCA2* point mutations or microdeletions/insertions in 41% of these families (12 of 29). Assuming a similar proportion in the families from which our cohort of 39 *BRCA1*- and *BRCA2*-negative families was derived, one can estimate that *BRCA2* point mutations or microdeletions/insertions accounted for not more than 30 defects. Although these estimates have been extrapolated and should be taken with caution, they suggest that the 3 *BRCA2* rearrangements found represented an additional 10% of *BRCA2* defects detected in our families. Moreover, considering that the *BRCA2* rearrangements described here have different predicted consequences at the RNA level and that no genotype-phenotype correlation is known between *BRCA2* mutations and male breast cancer, it is very likely that germline *BRCA2* rearrangements are also present in a fraction of families with large numbers of breast cancers, regardless of the presence of male breast cancer cases.

Consequently, it seems appropriate to add the search of large deletions/duplications of *BRCA2* to the molecular diagnosis of male breast cancer families and possibly also of breast cancer families with large numbers of cases, also considering the sensitivity and low cost of both assays (*i.e.,* QMPSF and MLPA) that are now available.

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Significant Contribution of Germline *BRCA2* Rearrangements in Male Breast Cancer Families

Isabelle Tournier, Brigitte Bressac-de Paillerets, Hagay Sobol, et al.

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