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## Original Article

# Molecular analysis of the BRCA2 gene in 16 breast/ovarian cancer Spanish families

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The recent isolated gene BRCA2 is responsible for about 45% of familial breast cancer and the majority of male breast cancer families. In order to evaluate the role of inherited BRCA2 mutations in Spanish families, the complete coding sequence of the gene was screened by SSCP/sequencing in 16 high-risk breast/ovarian cancer families. Four mutations were found that cause a premature termination codon. Two of them have been reported elsewhere and one is a novel mutation. In addition we have found seven polymorphisms, two of which have not been previously described. One of the mutations, 936delAAAC was found in two of our high-risk families. Because this mutation is considered as recurrent, we have tried to estimate its frequency in our breast cancer population. A total of 127 moderate- high-risk families were screened for this mutation and it was also found in another high-risk family. All the families carrying the 936delAAAC mutation harboured part of a common haplotype shared by other reported carriers, suggesting a possible founder effect for this mutation.

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Epidemiological studies have provided evidence for at least two genes conferring inherited susceptibility to breast and ovarian cancer: BRCA1 (17q21) (1) and BRCA2 (13q12) (2).

Germline mutations in these two genes, are thought to account for a great proportion of familial breast/ovarian and breast cancer (3, 4). The most obvious epidemiological difference between BRCA2 and BRCA1 is the higher risk of breast cancer among men that is associated with BRCA2 (5–7).

More than 100 BRCA2 mutations have been described to the date (8). Although this gene is characterized by the occurrence of multiple private mutations (5), few of them have been considered as recurrent as they have been identified in several families. These mutations are usually country specific and are often regarded as ancient mutations (9).

In this study we analysed the BRCA2 gene in 16 high-risk Spanish families, in order to evaluate the proportion of breast/ovarian cancer attributable to BRCA2 mutations and the possible existence of recurrent mutations in our population.

## Subjects and methods

### Subjects

In this study, 16 families with three or more cases of females affected with breast and/or ovarian cancer (at least one of them diagnosed before the age of 50) or at least one case of male breast cancer diagnosed at any age, were evaluated for germline mutations in the BRCA2 gene. The families did not belong to an ethnic subgroup, they were all ascertained in Spain and were representative of the general population. Partial results of this study have been previously published (10). For the screening of the 936delAAAC mutation, 127 moderate- to high-risk breast/ovarian cancer families were selected. Moderate risk families contained at least two cases of females affected with breast cancer (at least one of them diagnosed before the age of 50).

Although the families were not excluded as being linked to BRCA1, they were previously screened for the six more common mutations in this gene (17). These six mutations account for aproximately 30% of all mutation carrier families described (11).

Table 1. BRCA2 mutations detected in breast/ovarian cancer Spanish families

| Family | Female breast cancer | Average age | Male breast cancer | Cancers at other sites[b] | BRCA2 mutation | Exon | Codon | Effect |
|---|---|---|---|---|---|---|---|---|
| 21 | 3(2B) | 44.5 | — | Pan, Liv, Lung, Int. | delAAAC | 11 | 936 | ter956 |
| 59 | 4 | 38 | — | Prostate | delAAAC | 11 | 936 | ter956 |
| 87[c] | 1 | 44 | — | Ov | delAAAC | 11 | 936 | ter956 |
| 22 | 4(2B) | 44.7 | 3 | Leu, Ut, Osteo, Sto | del5 | 23 | 3009 | ter3015 |
| 24 | 1[a] | 49 | 1 | Ov(3), NHL, Sto | delAA | 14 | 2370 | ter2390 |

[a] Five paternal relatives with breast cancer at unknown ages and four with cancers at other sites.
[b] Pan = Pancreatic cancer, Liv = liver cancer, Int = Intestine cancer, Leu = Leukemia, Ut = Uterus cancer, Osteo = Osteosarcoma, NHL = No Hodgkins lymphoma, Sto = Stomach cancer, Ov = Ovarian cancer.
[c] Family detected in the 936delAAAC screening.

## Methods

The 26 BRCA2 coding exons were amplified under standard PCR conditions containing: 100 ng of genomic DNA, 10 × PCR buffer (Boehringer, Manheim), mix 200 mM of dATP, dTTP, dGTP and 10 mM of dCTP, 1 µl of 25 pmol each primer, 1.75 of Taq polimerase (Boehringer, Manheim) and 0.5 µCi 32P-dCTP (INC). In some cases 2% DMSO and 0.1% triton was added to improve amplification. Amplification conditions were as follows: 35 cycles of denaturing at 94° for 45 s, annealing for 30 or 45 s and in some cases extension to 72° for 30 s.

The primers were designed to amplify fragments of 200–350 nucleotides. The sequence of the primers was gently yielded by Mike Stratton (Institute of Cancer Research, London). Given their big size, exons 10, 11, 14, 18, 25 and 27 were fragmented in overlapping segments. A total of 65 primer pairs were used to amplify BRCA2 genomic DNA. Reaction products were loaded in a SSCP electrophoresis gel under two different conditions (6% polyacrylamide, room temperature, 10 W and 10% polyacrilamyde, 4°C, 50 W), to increase sensibility, and this was done by manual radio-labeled analysis. Suspected BRCA2 mutants were identified as having a mobility shift and these samples were directly sequenced to verify the mutation. The DNA isolated from mutant allele carriers was amplified by PCR and the product was purified using Gene Clean (Progenetics) according to the manufacturer's specifications. The purified DNA was subjected to cycle sequencing using an automated fluorescence-based cycle sequencer (Abi Prism 310 Perkin-Elmer) and dye terminator system.

## Haplotype analysis

Members from the three families harbouring the 936delAAAC were genotyped using 10 microsatellite markers located within or near BRCA2 (12). From centromere to telomere these are D13S1444, D13S1700, D13S290, D13S267, D13S260, D13S1699, D13S1698, D13S171, D13S1695, D13S310. The radio-labeled PCR products were loaded on to a sequencing gel (8% polyacrylamide, 8 M urea) and run for 2–4 h. The bands were detected by autoradiography in an X-ray film after 30–60 min exposure.

## Results

BRCA2 mutations were found in four of our families (25%), all of them being small deletions that caused premature termination codons (Table 1). Two mutations, 936delAAAC and 3009del5, had been previously described (8), and one, 2370delAA, is a new mutation. Several polymorphisms were also detected at seven sites (Table 2), some of them had been reported elsewhere (8) and some are apparently new variants.

936delAAAC was found in two families (21 and 59). Family 21 contained three cases of women affected with breast cancer at ages 37, 39 and 48 years (two of them developed bilateral breast cancer). Family 59 contained four cases of women affected with breast cancer at ages 35, 35, 39 and 45 years. In both cases, cancers at other sites appeared in other members of the families. This mutation leads to a premature termination codon at position 956 and has been previously reported in seven other familial cases: three of them were ascertained in Great Britain, one in France, two in Canada and one in the United States, but all of them have European ancestry (9). Because this mutation is considered as recurrent, we tried to estimate its frequency in our breast cancer population. 127 moderate- high-risk families were screened for the mutation and it was found in another risk family which contained a woman who developed breast and ovarian cancer at the ages of 44 and 49 years, respectively. Although no more cases of breast or ovarian cancer were found in this family, some members of the family developed cancers at other sites. A collaborative study of

Table 2. Additional sequence variants

| Sequence variant | Exon | Codon | Amino-acid change | Frequency[b] |
|---|---|---|---|---|
| CAG-CAA | 2 | No | | 3/26 |
| AAT-CAT | 10 | 289 | Asn-His | 4/112 |
| TCA-TCG | 10 | 470 | Ser-Ser | 1/24 |
| AAA-GAA | 11[a] | 1286 | Lys-Glu | 19/80 |
| AGC-AGT | 11[a] | 1528 | Ser-Ser | 3/116 |
| ACG-ATG | 11 | 1915 | Thr-Met | 12/200 |
| TCA-TCG | 14 | 2414 | Ser-Ser | 3/24 |

[a] Polymorphisms not previously described.
[b] Frequency is given as number of variant alleles seen/chromosomes typed.

founder mutations in BRCA2 has been recently initiated by the Breast Cancer Linkage Consortium. This study is based on the haplotype analysis of individuals who carry any of the BRCA2 mutations that have been considered as recurrent. Our 936delAAAC carriers were analyzed for 10 microsatellite markers located within or near BRCA2. Carrier members from the three families shared part of the common haplotype described by Neuhausen et al. in the 11th Breast Cancer Linkage Consortium Meeting (Table 3).

3009del5 was found in family 22, which contained three cases of male breast cancer at ages 74, 81 and 87 years and four cases of women affected with breast cancer at ages 45, 50, 65 and 73 years (three of them developed bilateral breast cancer) (Fig. 1). This mutation leads to a premature termination codon at position 3015 and has been previously reported at least twice (8).

The last mutation, 2370delAA, has not been previously described and it was found in family 24, which contained a case of male breast cancer at the age of 67, one women affected with breast cancer at the age of 49 and three cases of women affected with ovarian cancer, two of them developed the cancer within 30 years and in one case the age of onset was unknown.

## Discussion

Twenty-five percent of our high-risk families (4/16), were found to carry a mutation in the BRCA2 gene. This percentage is quite high, considering the small number of families analyzed and suggests an important role of inherited BRCA2 mutations in breast/ovarian cancer Spanish families. A larger study is required to establish the proportion of these families attributable to BRCA2 in Spanish population and to compare it with the different data obtained from other European countries (9).

### Genotype–phenotype correlation

Two of the four families with BRCA2 mutations carried the same alteration, 936delAAAC in exon 11, which had been previously reported in several families of different ascertainment, and is thus considered as recurrent (9). Only one of the 11 reported families in which these mutations have been observed, contained a case of male breast cancer. 936delAAAC begins one nucleotide 5′ of the suggested ovarian cancer-cluster region of BRCA2 and extends into this region (13); despite this, only one of our three carrier families contained a case of ovarian cancer.

The mutation in exon 14 has not been previously reported, and it was found in a family containing a case of male breast cancer and three cases of ovarian cancer (10).

In general, our results support the idea that there is no clear evidence for a genotype–phenotype correlation in BRCA2 mutation carriers. Only one of the three families with mutations in the BRCA2 OCCR, had persons affected with ovarian cancer and, on the other hand, family 24, harbouring a mutation in exon 14 (which is not in this region), contained three cases of ovarian cancer.

The mutation in exon 23 was found in a family containing three cases of male breast cancer. This mutation has been previously reported at least twice (8) but, as far as we know, our family is unique in including some cases of male breast cancer. Previous studies have suggested that, in general, mutations in families with male breast cancer begin in exon 2 and extend to exon 23 (2, 5–7, 14, 15). Our results support the idea of a lack of correlation between the location of a BRCA2 mutation and male breast cancer risk. It has been recently suggested the possible existence of elements that could modify the risk of male breast cancer in BRCA2 mutation carriers and make male breast cancer cluster in particular branches of BRCA2 mutation carrier families, this element

Table 3. Haplotype analysis of 936delAAAC mutation carriers

| Microsatellite marker | D13S1444 | D13S1700 | D13S290 | D13S267 | D13S260 | D13S1699[e] | D13S1698[e] | D13S171[e] | D13S1695[e] | D13S310 |
|---|---|---|---|---|---|---|---|---|---|---|
| Patient 1[a] | 11 | — | 12 | 13 | 9 | **10** | **10** | **10** | 7 | 7 |
| Patient 2[b] | 8/10 | — | 13 | 4/12 | 6/8 | 8/**10** | **10** | 3/**10** | 7 | 4/10 |
| Patient 3[c] | 5/10 | — | 13 | 3/2 | — | 9/**10** | 12 | — | 7 | 7 |
| Common haplotype[d] | | | | | 7 | **10** | **10** | **10** | 7 | |

[a] Proband from family M21. In this case the study was extended to the whole family, and all the mutation carriers shared the same haplotype.
[b] Proband from family M56. In this case there was no sample available from the rest of the family.
[c] Proband from family M86. In this case there was no sample available from the rest of the family.
[d] Data from the International study about recurrent mutations reported by Neuhausen et al. (6).
[e] Intragenic markers.

could be for example a cytogenetic abnormality (Savelyeva et al., personal communication). In the case of family 22, we could not detect any cytogenetic abnormality by conventional methods.

Recent studies have shown that the lifetime risks of breast cancer, associated with BRCA1 and BRCA2, are approximately equal, but that the age at onset is later among BRCA2 mutation carriers (16). In this case, we did not observe a later age at onset for our affected mutation carriers, compared to our own BRCA1 carrier data (17).

Not all the affected members from our five families could be analysed, but there was at least one 936delAAAC woman carrier in family 21, that had not already developed cancer at the age of 54 years, while her sister (37), and her mother (48) developed breast cancer and, in both cases, developed bilateral breast cancer. At least in this family, the mutation seems to be not completely penetrant.

A number of other malignancies such as pancreas, stomach and liver cancer, are present in the families studied. As we did not have DNA available from these members, we do not know their mutation carrier status. Our results support the idea that BRCA2 linked families have a higher incidence of cancer at other sites.

### Frequent polymorphisms

A total of seven sequence variants, that may be common polymorphisms were found. Until a functional test is available, general population frequency analysis of BRCA2 sequence variants in the coding region, is useful to support the putative role of missense mutations. Three of the sequence variants result in a change of amino acid, two of them have been previously described and are considered as frequent polymorphisms (BIC), the last one (1286 Lys-Glu) is a new variant and it was found in approximately 24% of our control population. Three variants are silent mutations and the one in exon 2 is not in the coding region.

With the exception of 289Asn-His (AAT-CAT) in exon 10 and the CAG/CAA change in exon 2, all the polymorphisms are due to C→T or A→G substitutions, which is in agreement with the excess of transition-type mutations in vertebrate genomes (18, 19).

### Haplotype analysis

The appearance of the same alteration, 936delAAAC, in two of 16 high-risk families (12.5%), suggested the idea of a higher recurrence for this mutation in the Spanish population. In order to estimate the frequency of this alteration, 127 moderate- high-risk breast/ovarian cancer families were screened, and the same mutation was found in another high-risk family.

The existence of the 936delAAAC mutation in 2.3% of our risk families (3/127), suggested the idea of a possible founder effect. Carrier members from the three families were tested for 10 microsatellite markers located within or near BRCA2. Preliminary studies by Neuhausen et al. concerning founder effects in BRCA2, have shown a common haplotype in several 936delAAAC mutation carriers, for five of the 10 microsatellite markers analyzed (Table 3) (11th Breast Cancer Linkage Consortium Meeting). In families 21 and 59, carrier members analyzed, may harbour the same haplotype reported by Neuhausen et al., except for the D13S260 marker. Family 86 could not be analyzed for the five markers, but we observed that at least for D13260 and D131698, allele sizes were different than the reported for the common haplotype (Table 3). D13S260 is an extragenic marker and D13S1698 is intragenic. The other three markers are intragenic (12); our results suggest that the common region could be restricted to

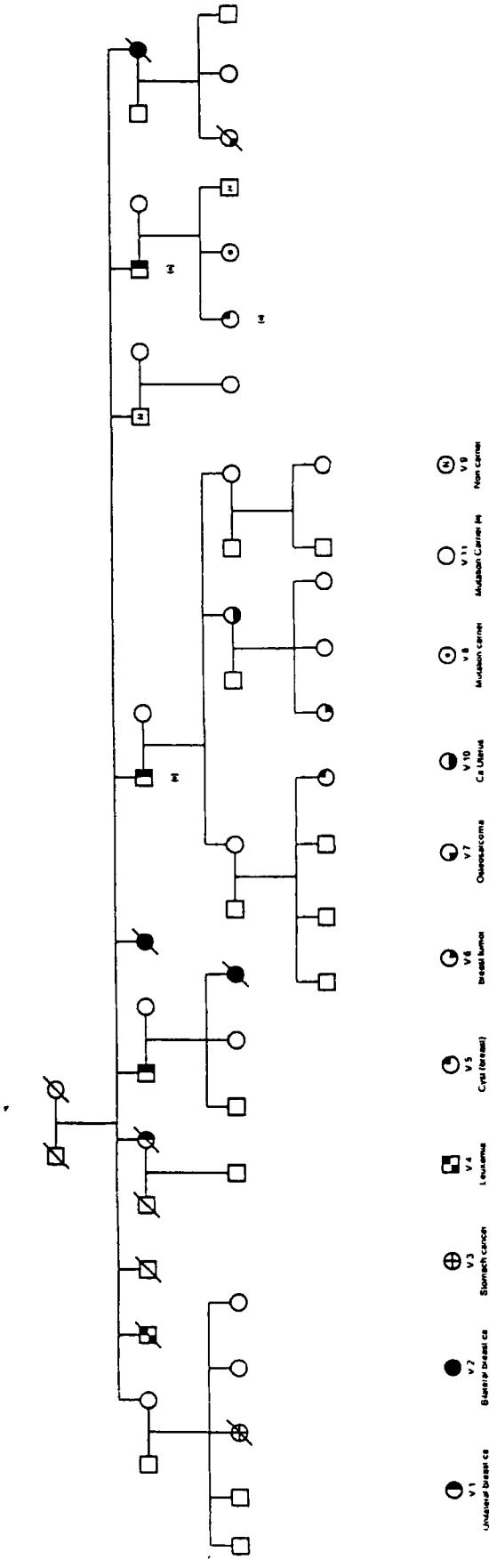

*Fig. 1.* Pedigree of family 22.

part of the intragenic region and may be smaller than that suggested in the preliminary studies. An other possibility could be the existence of two different origins for the same mutation. It is necessary to analyse more mutation carriers to confirm a founder effect and more microsatellite markers to refine the shared DNA segment.

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