Original articles

BRCA1 mutations in a selected series of breast/ovarian cancer patients

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Abstract

Germline mutations in the BRCA1 gene have been associated with familial breast/ovarian cancer in large families showing high penetrance of the disease. Little is known, however, about the contribution of BRCA1 mutations to breast/ovarian cancer in small families with few affected members or in isolated early onset cases. Therefore we examined the BRCA1 gene in 63 breast/ovarian cancer patients who either came from small families with as few as one affected first degree relative, or in patients who had no family history but had developed breast cancer under 40 years of age. Using the protein truncation test, we were able to identify three unique BRCA1 germline mutations (4.8%). Two of the probands had only one affected first degree and several second degree relatives and the third had three affected first degree relatives including two sisters who, when tested, were also found to carry the mutation. There was no family history of ovarian cancer in any of the three families.

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Key words: breast cancer; BRCA1; mutations.

There are many factors associated with a woman's risk of developing breast cancer but by far the most consistent is a positive family history of the disease.¹ Hereditary breast cancer is characterised by a relatively early onset, an excess of bilateral disease, and in some families an over-representation of ovarian cancer and to a lesser extent other malignancies, such as colorectal and prostate cancer.² Segregation analysis provided evidence that familial breast cancer could be attributed to one or more highly penetrant autosomal dominant susceptibility genes.³

In 1990, a breast cancer susceptibility locus (BRCA1) was mapped to chromosome 17q21.⁴ The Breast Cancer Linkage Consortium confirmed these findings using 214 families having high numbers of breast and ovarian cancer patients,⁵ and further refined the locus. The BRCA1 gene was recently isolated⁶ and is believed to confer an 85% risk of developing

breast cancer by the age of 70 years in women identified as harbouring germline mutations in this gene and a 63% risk of developing ovarian cancer by the same age.⁷ The BRCA1 gene codes for a 7.5 kb transcript which is spread over 100 kb of genomic DNA. The coding sequence consists of 24 exons of which 22 are coding. The first 10 and the last 13 are relatively small, whereas exon 11 accounts for about 60% of the entire coding region of the gene.⁶

Mutation analysis of the BRCA1 gene shows a wide spectrum of mutations occurring throughout the gene with little evidence of mutational hot spots. There remains some debate as to whether a phenotypic gradient exists in the BRCA1 gene as mutations reported at the 3' end appear to predispose to breast cancer, whereas those towards the 5' end appear to be associated with an increased likelihood of ovarian cancer.⁸ Somatic BRCA1 mutations have not been found in breast tumours,⁹ but have been found in a small percentage of sporadic ovarian tumours.¹⁰ To date most BRCA1 mutations have been identified in large families where there is a high level of penetrance and consequently there is good evidence that a breast cancer susceptibility gene is involved. However, germline BRCA1 mutations should also account for some of the breast cancer cases found in people lacking an obvious family history. Small family size, paternal transmission, a high proportion of males, and incomplete or inaccurate family histories can obscure the presence of a breast cancer susceptibility gene in a given kindred.

We attempted to determine the role of BRCA1 in a group of 63 breast/ovarian cancer patients using selection criteria that maximised the probability of detecting germline mutations given that there was no strong family history. The entire BRCA1 coding region was examined using the protein truncation test and the mutations thus identified were sequenced. The results indicate that three of the 63 patients (4.8%) had previously unidentified BRCA1 mutations which we believe are causative.

Materials and methods

PATIENT SELECTION

For this study we selected 63 women who had as minimum requirements (1) breast cancer

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Table 1 Disease characteristics of the 63 breast cancer families

Number of families with only breast cancer
24 families with only 1 breast cancer patient
21 families with only 2 breast cancer patients
6 families with only 3 breast cancer patients
3 families with only 4 breast cancer patients
7 bilateral breast cancer patients
Number of families with breast and ovarian cancer
1 family with 1 ovarian cancer patient (1 breast cancer)
2 families with 2 ovarian cancer patients (1 breast cancer)
63 total number of patients examined

under the age of 40 years (24 patients), (2) breast cancer under 50 with at least one first degree relative having breast cancer under 50 (37 families), (3) breast cancer under 50 with a first degree relative having ovarian cancer at any age (one family), and (4) ovarian cancer at any age with a first degree relative having ovarian cancer at any age (one family). Although not applied in these criteria, many of the index patients had affected second degree relatives. All patients signed a declaration of informed consent before entering this study. The family characteristics of the 63 index patients are presented in table 1 indicating the number of sporadic early onset and familial cases.

CELL LINES

EBV transformed B cell lines GM 13705, GM 13708, and GM 13713 were purchased from the Coriell Institute (Camden, New Jersey) and were grown in RPMI/10% fetal calf serum.

TEMPLATE PREPARATION

Genomic DNA was prepared from lymphocytes obtained from 10 ml EDTA blood using the protocol described by Miller *et al.*¹¹ Total RNA was prepared from PBLs isolated by ficoll-hypac centrifugation (within 24 hours after donation) of 10 ml heparin treated blood by the acid guanidinium isothiocyanate-phenol-chloroform extraction method.¹² cDNA was made using 10 µg of total RNA, 500 ng random hexamer, and 400 units of M-MLV reverse transcriptase in a 50 µl volume according to the manufacturer's protocol (BRL Life Technologies).

PCR AND SEQUENCING

Five overlapping fragments of the BRCA1 coding sequence were PCR amplified from either genomic DNA or cDNA, as shown in fig 1, using the primers given in table 2. Segments II, III, and IV were PCR amplified from 200 ng of genomic DNA template in a 25 µl reaction volume using standard conditions except that the primer concentration was 200 nmol/l. The

samples were cycled at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for two minutes, 35 times. Segments I and V were amplified from cDNA using the following procedure: 0.5 µl (5 - 10 ng) of cDNA was used in a nested PCR procedure in which the external primers (P1, P5 for segment I and P10, P13 for segment V) were included for 20 cycles of amplification in a 10 µl reaction volume. A 1 µl aliquot of this reaction was removed and used as template for an additional 30 cycles of amplification in a 25 µl reaction in the presence of the internal primers. The temperature and time parameters were as above. Oligonucleotide primers were purchased from Microsynth (Balgach, Switzerland) and were based on the BRCA1 cDNA sequence (Genbank accession number U14680) except for P13 in the 3'UTR which was taken from Friedman *et al.*¹³ Dideoxy sequencing of the PCR products was performed using Thermosequenase (Amersham, Buckinghamshire, UK) and ³²P labelled primers according to the manufacturer's protocol.

PROTEIN TRUNCATION TEST (PTT)

Transcription and translation of segments I-V was performed using 1 µl of PCR product in a 5 µl reaction using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI). In each reaction 0.2 µl of EasyTag ³⁵S Methionine (NEN, Boston, Mass) were included for labelling purposes. Labelled protein products were size fractionated on 12% SDS-PAGE gels, fixed, dried, and exposed to Biomax film (Kodak, Rochester, New York).

Results and discussion

We screened the entire coding region of BRCA1 from 63 breast/ovarian cancer patients using either genomic DNA or cDNA as template. Recently, missense mutations in exons 11 and 13 of BRCA1 have been shown to result in the absence of mutant BRCA1 message,¹⁴ presumably because of nonsense mediated decay of RNA transcripts that leads to a reduction of mutant BRCA1 mRNA making it difficult to detect under normal conditions. We found, however, that when nested PCR is used, we could distinguish identical mutations in exon 11 using either cDNA or genomic DNA as template. To verify that we could identify mutations using cDNA as template we amplified segment IV of BRCA1 by nested PCR (using P14 and P15 as external primers) from two cell lines known to have truncating mutations (GM13705 and GM13713) and a negative control (GM13708). Since segment IV is also present as continuous sequence in genomic DNA within exon 11, it was necessary to show that

Figure 1 The coding sequence of BRCA1 is divided into five overlapping segments that can be amplified using the primer shown.

Table 2 Primers used for PCR and sequencing

Primer	Sequence 5'-3'	Position in BRCA1
P1	TCTGCTCTGGGTAAAGTTCA	85-104
P2	T7-GATTTATCTGCTCTTCGCGT	123-142
P3	GTTCTCATGCTGTAATGAGC	961-980*
P4	T7-GCTTGTGAATTTCTGAGAC	792-811
P5	TCATCACTTGACCATTCTGCTCC	1736-1758*
P6	T7-TTTGGGAAAACCTATCGGAA	1500-1519
P7	CGTTGCCTCTGAAGTGAATGATAG	2979-3003*
P8	T7-CGCCAGTCATTTGCTCCGTTTTC	2715-2737
P9	TAAGTTTGAATCCATGCTTTGC	4193-4214*
P10	TGTCTAAGAACACAGAGGAG	3874-3893
P11	T7-GCAAAGGCATCTCAGGAACATCA	3948-3970
P12	TCAGTAGTGGCTGTGGGGGATC	5690-5711*
P13	TAGCCAGGACAGTAGAAGGA	3'UTR
P14	CCACAGTCGGGAAACAAGCA	2633-2652
P15	TGATGGAAGGGTAGCTGTTAGAAGG	4392-4416*
P16	CTTACTGGCCAGTGATCCTC	1409-1428
P17	ATTC AAGCAGAACTAGGTAG	3321-3340

T7- refers to the T7 polymerase promoter and Kozak consensus 5'-GGATCCTAATACGACTCACTATAGGAGACCACCATG-3'. The numbering of BRCA1 is that used in Genbank accession number U14680.

*= the reverse complement of the given BRCA1 sequence.

Figure 2 PTT of segment IV amplified from genomic DNA (lanes 1, 3, 5) or cDNA (lanes 2, 4, 6) derived from the negative control cell line GM13708 (lanes 1 and 2) and the mutant cell lines GM13705 (lanes 3 and 4) and GM13713 (lanes 5 and 6).

the template being amplified was cDNA and not contaminating genomic DNA. Wild type segment IV encodes a 500 residue polypeptide, whereas GM13705 and GM13713 cell lines are heterozygous for mutations that result in truncated products of 396 and 384 residues, respectively. The wild type and truncated proteins encoded by genomic DNA from these cell lines are seen in lanes 3 and 5 of fig 2. The same truncated proteins are also present in lanes 4 and 6 (although at slightly reduced levels), showing that BRCA1 mutations can be detected by PTT when cDNA is used as template.

Genomic DNA and cDNA from our series of patients were used to amplify segments I-V of BRCA1, and the PCR products were analysed by PTT. PTT should detect those BRCA1 mutations (approximately 90% of the total) which result in premature translational termination. In our series we detected three truncated products generated by segment II (lanes

Figure 4 Sequences of the three BRCA1 mutations. (A) Segment II of patient 10 (even lanes) and a control (odd lanes) sequenced with oligonucleotide P16. Position C 1648 is indicated by the arrow. (B) Segment II of patient 46 sequenced with P5. C 1611 is indicated. (C) Segment IV of patient 20 sequenced with P17. The additional A residue at position 3449 is indicated by an arrow.

Figure 3 Three unique mutations in BRCA1 detected by PTT. Segment II (lanes 1, 2, 3) from a control (lane 1) and two patients (lanes 2 and 3) and segment IV (lanes 4-7) from a control (lane 4), patient 20 (lane 5), and two of her sisters (lanes 6 and 7).

2 and 3 of fig 3), and one patient had a truncated segment IV product (lane 5). This patient has two sisters with breast cancer who, when tested, were found to harbour the same mutation (lanes 6 and 7).

The sizes of the truncated products were used to estimate the positions of the mutations within each segment, and the heterozygous PCR products containing the mutations were sequenced. Patient 10 has a C→G transversion at base 1648 (fig 4A). The transversion results in a Ser 510 (TCA) Stop 510 (TGA) nonsense mutation which generates a segment II protein of 288 residues, 34 amino acids shorter than the wild type product. Patient 46 harbours a frameshift deletion of C, position 1611 (fig 4B). The frameshift introduces a stop codon 12 bases 3' to the deletion, resulting in a truncated segment II protein of 279 residues. Patient 19 has an additional A residue inserted in the stretch of six As between bases 3443 and 3448 (fig 4C). The reading frameshift introduces a stop codon 18 bases 3' to the insertion and results in a segment IV product truncated by 247 residues to a size of 253 residues.

The criteria used for choosing patients include those having one or more first degree relatives with breast/ovarian cancer. The pedigrees of the three families (fig 5) show that each of the BRCA1 carriers does have affected first degree relatives. In each case the mutation appears to be maternally transmitted, perhaps reflecting a bias introduced in the selection criteria (paternally transmitted mutations would not result in an affected parent). Furthermore, our results confirm the finding that only persons with a family history of breast/ovarian cancer appear to have mutations.

Our BRCA1 mutation detection frequency of 4.8% (three of 63) is 38 fold higher than the 0.125% frequency of BRCA1 carriers in the general population calculated by Ford *et al*¹⁵ using population based studies of cancer mortality in the relatives of breast and ovarian cancer patients. Ford *et al*¹⁵ also estimated that BRCA1 mutations are causative in 5.3% of all breast cancers diagnosed before the age of 40, yet only 1.7% of all breast cancers diagnosed before the age of 70.¹⁵ Recently, Fitzgerald *et*

Figure 5 Pedigrees of (A) patient 10, (B) patient 16, (C) patient 20. The index patients are indicated by arrows and the age of diagnosis (dark symbols) is given when known. The two affected sisters of patient 20 are indicated by asterisks.

*al*¹⁶ performed a population based BRCA1 screening of women with breast cancer under the age of 30 and found that five of 30 (17%) women harboured BRCA1 mutations, while Langston *et al*¹⁷ detected BRCA1 mutations in 7.5% of 80 women having breast cancer under the age of 35. We tested patients ranging in age from 27 to 74 years with an average age of 41.5 years and detected BRCA1 mutations at a frequency similar to that expected in all women under 40 years of age with breast cancer, regardless of family history. The selection criteria in this study were aimed at small families and sporadic early onset breast cancer patients, whereas previous studies relied on large well documented families that had high a priori probabilities of being linked to BRCA1. The results of this investigation indicate that it is worthwhile studying a selected series of women based on restrictive criteria, as the incidence of BRCA1 mutations appears to be similar to that found in other investigations based on large informative families.

Given that our frequency of mutation detection is in accordance with that estimated, it appears that the PTT is a valuable method for rapidly screening the BRCA1 gene. Nevertheless, it is important to note that we did not compare this method of detection to any other and thus do not know if mutations were missed. In a recent study by Dobbie *et al*¹⁸ using the PTT to detect mutations in the adenomatous polyposis coli (APC) gene, it was found that this method could only identify 66% of all chain terminating mutations even though genomic DNA was used as template. Notwithstanding, given the size and structure of the BRCA1 gene we conclude that PTT screening is a rapid and efficient way of detecting BRCA1 mutations in subjects lacking or

having an unconvincing family history of breast/ovarian cancer.

Although the breast/ovarian cancer patients used in this study fulfilled our selection criteria, only a small percentage of them appeared to harbour mutations in BRCA1. Those persons who did carry BRCA1 mutations came from families where there is a clear clustering of breast cancer at young ages in both first and second degree relatives. In the remaining families where the majority of patients were isolated cases under 40 years of age, it appears, from this limited study, that BRCA1 does not account for early onset disease in the absence of a substantial family history. Sporadic early onset breast cancer may well be the result of other factors affecting proliferating breast tissue, such as exposure to ionising radiation.¹⁹

In conclusion, given that only relatively few persons in this study are carriers of BRCA1 mutations and that virtually all BRCA1 mutations described to date appear to be associated with a familial clustering of breast/ovarian cancer, we would recommend that only women with a positive family history of early disease be considered for BRCA1 screening. Sporadic early onset breast cancer patients (those under 40 years of age) represent a special problem in relation to BRCA1 screening (especially in light of the paucity of de novo BRCA1 mutations). From this limited study it appears that BRCA1 mutations do not play a major role in early breast cancer susceptibility in women without a family history. Testing for other breast cancer susceptibility genes, such as BRCA2, and the ataxia telangiectasia gene (ATM) in this instance may be worthwhile.

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- 1 Newman B, Austin MA, Lee M, King MC. Inheritance of human breast cancer: evidence for autosomal dominance transmission in high-risk families. *Proc Natl Acad Sci USA* 1988;**85**:3044-8.
- 2 Black DM. The genetics of breast cancer. *Eur J Cancer* 1994;**13**:1957-61.
- 3 Narod SA, Amos C. Estimating the power of linkage analysis in hereditary breast cancer. *Am J Hum Genet* 1990;**46**:266-72.
- 4 Hall JM, Lee MK, Newman B, *et al*. Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 1990;**250**:1684-9.
- 5 Easton DF, Bishop DT, Ford D, Crockford GP. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. *Am J Hum Genet* 1993;**52**:678-701.
- 6 Miki Y, Swensen J, Shattuck-Eidens D, *et al*. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;**266**:66-71.
- 7 Easton DF, Ford D, Bishop DT, Consortium BC. Breast and ovarian cancer incidence in BRCA1-mutation carriers. *Am J Hum Genet* 1995;**56**:265-71.
- 8 Gayther SA, Warren W, Mazoyer S, *et al*. Germline mutations of the BRCA1 gene in breast and ovarian families provide evidence for a genotype-phenotype correlation. *Nature Genet* 1995;**11**:428-33.
- 9 Futreal PA, Liu Q, Shattuck-Eidens D, *et al*. BRCA1 mutations in primary breast and ovarian carcinomas. *Science* 1994;**266**:120-2.
- 10 Merajver SD, Pham TM, Caduff RF, *et al*. Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. *Nature Genet* 1995;**9**:439-43.
- 11 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;**16**:1357-65.
- 12 Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162**:156-9.
- 13 Friedman LS, Ostermeyer EA, Szabo CI, *et al*. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nature Genet* 1994;**8**:399-404.

- 14 Friedman, LS, Szabo CI, Ostermeyer EA, *et al.* Novel inherited mutations and variable expressivity of BRCA1 alleles, including the founder mutations 185delAG in Ashkenazi Jewish families. *Am J Hum Genet* 1995; 57:1284-97.
- 15 Ford D, Easton DF, Peto J. Estimates of the gene frequency of BRCA1 and its contribution to breast and ovarian cancer incidence. *Am J Hum Genet* 1995;57:1457-62.
- 16 Fitzgerald MG, MacDonald DJ, Krainer M, *et al.* Germ-line mutations in Jewish and non-Jewish women with early-onset breast cancer. *N Engl J Med* 1996;334:143-9.
- 17 Langston AA, Malone KE, Thompson JD, Daling JR, Ostrander EA. BRCA1 mutations in a population-based sample of young women with breast cancer. *N Engl J Med* 1996;334:137-42.
- 18 Dobbie Z, Spycher M, Mary JL, *et al.* Correlation between the development of extracolonic manifestations in FAP patients and mutations beyond codon 1403 in the APC gene. *J Med Genet* 1996;33:274-80.
- 19 Bhatia S, Rodison LL, Oberlin O, *et al.* Breast cancer and other second neoplasms after childhood Hodgkin's disease. *N Engl J Med* 1996;334:747-51.