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BRCA2 germline mutations in Swedish breast cancer families

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Mutations in the breast cancer susceptibility gene (*BRCA2*) are believed to be responsible for a significant fraction of hereditary breast cancer. To determine the *BRCA2* mutation spectrum in a subset of Swedish breast cancer families, 162 families were screened for germline mutations in this gene. A combination of RT-PCR, PTT and direct DNA sequencing was used. Two mutations and one previously reported polymorphic variant resulting in a truncated protein were identified. Our data suggest that only a small proportion of Swedish breast cancer families is attributable to *BRCA2* germline mutations. This result, in combination with the low frequency of *BRCA1* germline mutations identified in our previous study, suggests additional high penetrant as well as low penetrant breast cancer susceptibility genes are involved in familial breast cancer.

Keywords: hereditary breast cancer; breast cancer susceptibility gene; *BRCA2*; cancer family; gene mutation; familial breast cancer; mutations screening

Introduction

Following the isolation of breast cancer susceptibility genes, *BRCA1* at 17q21[1] and *BRCA2* at 13q12–q13,[2,3] great interest has been devoted to what extent mutations in these two genes are inherited. It was estimated at an early stage that 45–50% of breast cancer families had *BRCA1* mutations[4] and about 35% *BRCA2* mutations.[5] Most hereditary breast/ovarian cancer is explained by *BRCA1* mutations, whilst male breast cancer families are over-represented among those with *BRCA2* mutations. A recent review cites the estimated frequency of *BRCA1* and *BRCA2* mutations in breast cancer families in various countries.[6] The proportion of hereditary breast and ovarian cancer attributable to *BRCA1* and *BRCA2* mutations varies among populations, and some have common founder mutations, whereas others have not. *BRCA1* mutations seem to be most common in Russia (79% of breast–ovarian families),[7] whilst a *BRCA2* mutation is exceptionally common in Iceland. In the Ashkenazim, two founder mutations, *BRCA1* 185del AG and *BRCA2* 6174delT, each appear in the general population at approximately 1% frequency; a third mutation, *BRCA1* 5382insC, occurs at a population frequency of 0.11%. The Icelandic founder mutation, 999del5 in exon 9 of *BRCA2*, is a frameshift mutation leading to an early termination in codon 273 and a highly truncated

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protein. A mutation screen revealed this mutation in 16/21 Icelandic breast ovarian cancer families with the shared *BRCA2* haplotype. It was found in 7.7% of female breast cancer patients and 40% of males with breast cancer in Iceland.[8,9]

The *BRCA2* has a large coding sequence, composed of 26 exons that span 10 254 bp and encode a protein of 3418 amino acids.[3] More than 90% of disease-causing *BRCA2* mutations result in a premature termination of translation or an absence of transcript (from Human Gene Mutation Database). The mutations are distributed throughout the gene without any clear evidence of clustering.

Since 1990 genetic counselling in cancer families has been offered to women in the Cancer Family Clinic at the Karolinska Hospital. All women with an increased risk of breast cancer based on family history have been offered screening. All families with increased risk (two or more close relatives with breast cancer regardless of age) have been invited to join our studies which aim to find the hypothetical genetic background for the increased risk. A blood sample has been obtained from at least one affected woman in each family willing to participate. Many studies have published frequencies of mutations in breast cancer families, usually with three or more affected women, and usually age of onset has been taken into consideration in the inclusion criteria. Since our criteria for increased risk has been based on earlier epidemiological studies, the majority of the families has few breast cancer cases and age of onset has not been used to exclude any family from the study. After cloning *BRCA1*, linkage data suggested that primary families with four or more cases of breast and/or ovarian cancer would have mutations. We included all available families (127) with three or more cases in this first study and found mutations in nine families, eight in breast–ovarian families.[10]

We have also an unpublished study on the families with only two cases of breast and/or ovarian cancer, which shows similar results, with mutations found only in families with breast and ovarian cancer, regardless of number of affected cases (unpublished data). Our results are similar to recent data based on clinical material.[11] When the *BRCA2* was cloned we wanted to establish the frequency of *BRCA2* mutations in the same cohort of patients. On this occasion we included all available patients, except those with *BRCA1* mutations (9 in the first study and 6 in the following), but including those new patients who had been recruited after the *BRCA1* studies.

We first paid special attention to the Icelandic founder mutation 999del5, to see whether that was also common in Sweden. Iceland was settled in the 9th century by people mainly of Scandinavian and Celtic origin. It is possible that this mutation is common in Scandinavian areas. It has been observed in two Finnish breast cancer families.[12] We examined a cohort of 222 patients from 222 families with hereditary breast or breast–ovarian cancer as described (3). All patients were normal and thus none of these Swedish families showed the Icelandic founder mutation. It suggests that this founder mutation may not be of Swedish origin. Subsequently, we wanted to outline the *BRCA2* germline mutation spectrum in Sweden. We selected PTT (protein truncation test),[13] a rapid and easy screening method which was expected to detect all truncating mutations, to evaluate the importance of *BRCA2* in our samples.

Patients and Methods

Patients

The patients were recruited through a study of familial breast cancer in Stockholm in 1989[14] and through the cancer family clinic at Karolinska Hospital in Stockholm. The inclusion criteria were: at least two close relatives (first or second degree) with breast and/or ovarian cancer, regardless of age, and one affected relative willing to participate. The study was approved by the local ethical committee (no 89:144).

We examined 222 breast cancer patients from 222 families for the Icelandic 999del5 mutation. These included 129 families with three or more close relatives with breast or breast–ovarian cancer and 93 families with only two close breast cancer relatives.

The second part of the study looked at 162 patients who are part of the above cohort of 222 families. The type of family is shown in Table 1. These families were screened for mutations by being divided into three groups. The first group included 74 families from whom DNA and RNA were available. The entire coding sequence was examined for *BRCA2* mutation in these families. The second group consisted of 79 families where only DNA was available. The screening was only applied to exon 10 and 11 (60% of the *BRCA2* coding sequence). The third group was composed of only 9 families where only RNA was available. We examined all exons except exons 10 and 11. Of these 162 families, 149 were screened for *BRCA1* mutations and no mutation was found,[11] and unpublished data].

Methods

DNA and RNA preparation DNA was isolated from blood by standard phenol/chloroform extraction. Total RNA was extracted from Epstein-Barr virus-transformed lymphocytes by acid guanidine thiocyanate-phenol-chloroform extraction.[15]

Table 1 Characteristics of 162 Swedish breast cancer families

Type of families	No. of families screened				No. of mutations	Polymorphism
	Ex. 2–27 (group 1)	Ex. 10+11 (group 2)	Ex. 2–9+12–27 (group 3)	Total		
>3br	16	24	0	40	1	1
3 br	25	40	1	66	0	0
br+ov(≥3)	11	11	1	23	1	0
2 br	22	4	7	33	0	0
Total	74	79	9	162	2	1

br, breast cancer case; ov, ovarian cancer case; Ex, exon.

PCR and RT-PCR For PTT analysis RT-PCR and PCR were performed for the 26 coding exons (Table 2). Uncoding exon 1 and the 3'-untranslated region contained in exon 27 were not examined. PCR reactions were carried out in a volume of 50 μl containing 50 ng genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 200 μM each of dATP, dGTP, dCTP and dTTP, each primer at 0.2 μM, and 2 units Taq (Perkin Elmen, Branchburg, New Jersey, USA) or DynaZyme (Finnzymes Oy, Espoo, Finland) polymerase. Amplification was run for 35 cycles of 40 s at 94°C, 50 s at 53–57°C, 1 min 30 s at 72°C, and a final extension step for 10 min at 72°C. For RT-PCR 2–5 μg total RNA was reverse transcribed with random hexamers using the superscript cDNA preamplification kit (Life Technologies, Gaithersburg, USA) to generate cDNA. A 2 μl aliquot of cDNA was used for subsequent PCR. PCR was performed under the same conditions as the genomic DNA PCR, except cycles were increased to 45–50 because of the low expression of *BRCA2* in lymphocytes.

PTT Analysis *BRCA2* has two large exons, exon 10 (1116 bp) and 11 (4929 bp), which represent 60% of the coding region. For these exons genomic DNA can be used as a PCR template. Exon 10 was amplified in one segment and exon 11 in four segments. Exons 2–9 and 12–27 were amplified in four segments because of their small sizes, and RT-PCR was needed to produce transcription templates for PTT. All the sequences of primers are given in Table 2. Each forward primer carried a T7 promoter and an eukaryotic translation initiation sequence. PTT reaction was carried out according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA).

Direct DNA sequencing PTT or RT-PCR variants were subjected to cycling sequencing according to the manufacturer's instructions (Amersham Life Science) (Figures 1 and 2). For PTT variants the same PCR products were used for sequencing. For the RT-PCR variant the aberrant band

Table 2 Primers used for *BRCA2* PCR-PTT analysis

Fragments	Sense (5′-3′)/positions of primers	Antisense (5′-3′)/positions of primers	Size (bp)
2–9	GATCCAAAGAGAGGCCAACA 239–258	TCTTTCTTTTGTTCTCTGTG 1537–1518	1335
10	CGCTTCTGTTTTATACTTTAACAGG (1022–23)–1022	GGAATCGTCATCTATAAAAC (2137+58)–(2137+39)	1196
11A	GTTTATTGCATTCTTCTGTG 2138–2157	TGACTTCCTGATTCTTCTAA 3599–3580	1461
11B	AATGGGCAGGACTCTTAGG 3203–3221	TTTCATCACGTTCGGGTTGT 4771–4752	1605
11C	CTACTAAAACGGAGCAAAAT 4460–4479	TCGTAACAACCTGCCATAAT 5912–5893	1452
11D	ATGCATACCCACAAACTGTA 5621–5640	CCAAGTCTACTGAATAAACAC (7069+68)–(7960+48)	1516
12–17	TGGGAAAAGAACAGGCTTCA 6767–6786	GCTGTGTCATCCCTTTCCAT 8273–8254	1506
17–23	GAATACAGTTGGCTGATGGT 7961–7980	AATCCTATTAGGTCCACCTC 9458–9439	1497
23–27	GGAAGTTGCGTATTGTAAGC 9137–9156	CTGGAAAGGTTAAGCGTCAA 10531–10512	1394

To all the sense primers were added a T7 promoter sequence and translation start codon at their 5′ end. The T7 promoter sequence is: 5′-GCTAATACGACTCACTATAGGAACAGACCACCATGG-3′. The primers for exon 10 and the antisense primer of fragment 11D are located in their corresponding introns.

was removed from a 1% low-melting agarose gel and purified using Wizard Minicolumns (Promega) before sequencing.

Results and Discussion

The *BRCA2* was screened for germline mutations in breast cancer patients representing 162 families with increased risk for breast cancer (Table 1). The entire coding sequence of *BRCA2* gene was examined in 74 breast and/or ovarian cancer families and 60% of coding sequence (exon 10 and 11) was screened in 79 families. The other 9 families where only RNA was available were screened for all exons except exon 10 and 11 (Table 1). Of these families, 149 has previously been screened for *BRCA1* mutations and no mutation was found.

We identified two germline mutations and one polymorphic variant (Table 3). The first mutation was found in a woman who had breast cancer at 72 years of age and was a member of a breast–ovarian cancer family (4064) (Figures 1, 2a, 3a). It was a frameshift mutation in exon 11, 6819delTG, resulting in a loss of two bases in codon 2196, and a translation termination just four codons downstream. A second mutation was identified in a woman with breast cancer at 42 years of age from family 4081 (Figures 1, 2b, 3b). It was a microdeletion of five bases in exon 10, 2024del5, also leading to a frameshift and an immediate stop. Both these mutations have previously been reported.[16] The polymorphic alteration identified in family 1416 is a base

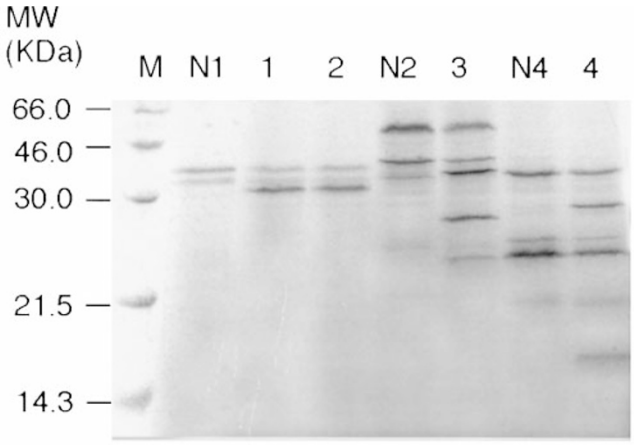

Figure 1 *PTT analysis of two germline mutations and the polymorphism Lys3326ter. M, molecular weight marker; N1, normal control – fragment 10; 1 and 2; two patients with the mutation 2024del5 from family 4081; N2, normal control – fragment 11D; 3, one patient with mutation 6819delTG; N4, normal control – fragment 23–27; 4, a patient with the polymorphism Lys3326ter.*

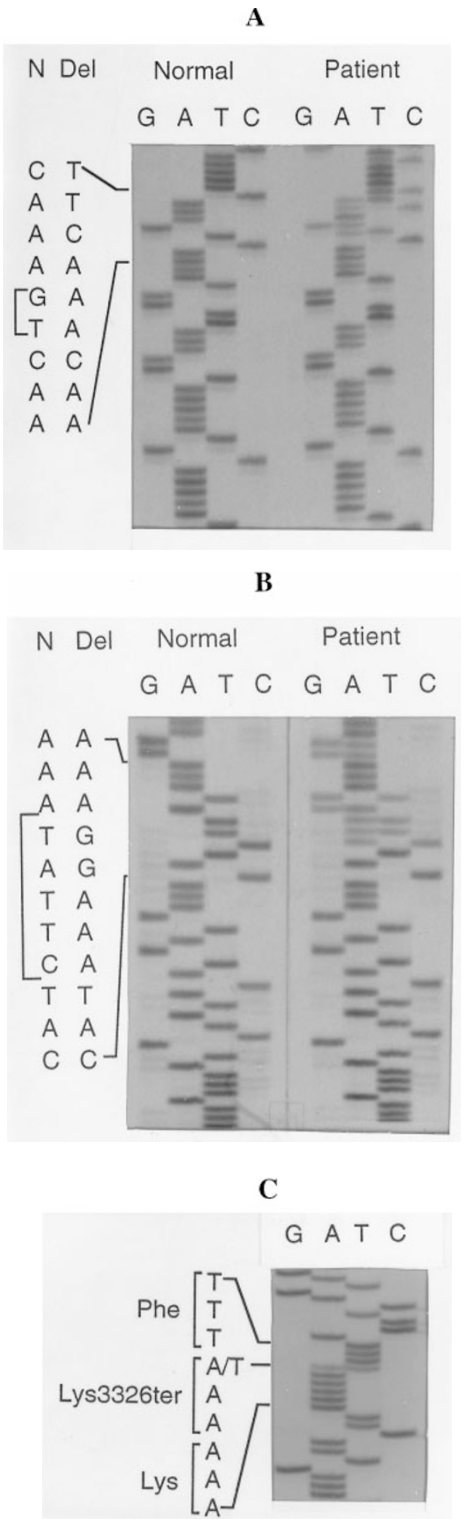

Figure 2 *The cycling sequencing analysis of two mutations and one polymorphism*
A One normal control and patient with 2024del5 mutation. **B** One normal control and patient with 6819delTG. **C** Polymorphism Lys3326ter, A T. N, normal DNA sequence, Del, deleted sequence.

Table 3 *BRCA2* germline mutations and polymorphism identified in 162 Swedish breast cancer families

Family no.	*Type of family*	*Exon*	*Codon*	*Nucleotide change*	*Effect on protein*
4081	7 br	10	598	2024del5	Truncation
4064	1 br+2ov	11	2196	6819delTG	Truncation
1416	4 br	27	3326	AAA(Lys)-TAA(Ter)	Polymorphism

br = breast cancer; ov = ovarian cancer.

substitution in exon 27, leading to a change of AAA (Lys) to TAA (Ter) at codon 3326 (Figure 2c). This variant results in the deletion of the last 93 amino acids of the *BRCA2* protein. This alteration was reported recently to be a polymorphic stop codon.[17] In our family, two close relatives with breast cancer did not carry this variant. Neither did the patient carry the 6503delTT variant which was found sometimes on the same haplotype with ter3326 in breast cancer.[17]

Inherited mutations in *BRCA2* are believed to be responsible for a significant fraction of hereditary breast cancer. However, our study suggests quite a low frequency (< 5%). The observed prevalance of *BRCA2* mutations in our study may be underestimated, primarily since half (79/162) of the patients were only examined for 60% of the coding region.

Our mutational screening approach was not designed to identify uncommon missense mutations or possible mutations in the *BRCA2* gene regulatory region. However, PTT detects all types of mutations, except missense mutations, and almost all clearly disease-causing mutations identified so far in *BRCA2* result in a premature termination of translation or an absence of transcript. Of these mutations, approximately 70% are small deletions, 15% small insertions and 10% base substitutions leading directly to termination codon-sTEXTREF ID = "rf18" > 18 (from Human Gene Mutation Database). Occasional missense mutations and mutations resulting in splice errors have also been described,[12,19,20] but it is difficult at present to distinguish missense mutations from rare polymorphisms.

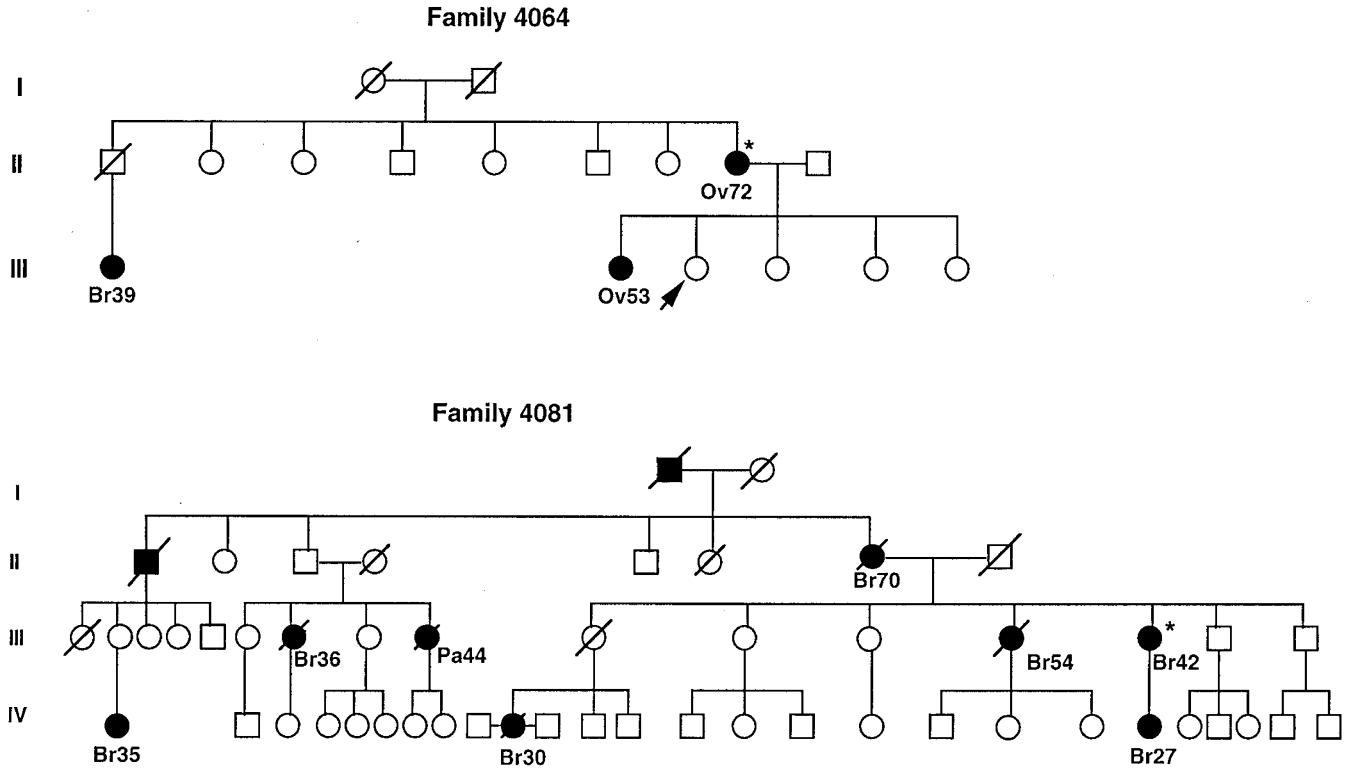

Figure 3 *Pedigrees of breast cancer families with mutations 6819delTG (family 4064) or 2024del5 (family 4081). Br, breast cancer, Ov, ovarian cancer, Pa, pancreatic cancer, the numbers following Br, Ov and Pa are the patient's age of onset. The mutations were identified from the patients marked*.*

In this study, exon 10 and 11 were screened using DNA, while the other exons used RNA-based screening. *BRCA2* is expressed in lymphocytes to quite a low extent. It is also possible that some mutant transcript is absent or lower in abundance than its wild type counterpart and escapes from detection by PTT. This possibility, in combination with undetected missense mutations, may also contribute to an underestimated frequency.

We found only two mutations in the 162 families recruited because of an increased risk based on family history. Although we may have missed mutations, used wider inclusion criteria, and some families might be explained by non-genetic factors, we still believe that the figure is representative for this cohort of patients. A low incidence of *BRCA2* mutations has also been reported in other populations.[6,21–23]

Epidemiological studies suggest an increased risk of breast cancer in our cohort of breast cancer families. We believe that many of these families have an increased risk of breast cancer based on a genetic factor. However, less than 10% (primarily in breast–ovarian cancer families) are attributable to mutations in *BRCA1* and *BRCA2*. This implies that another gene or genes may be involved in the rest of familial breast cancers.

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