ELECTRONIC LETTER

Contribution of *BRCA2* germline mutations to hereditary breast/ovarian cancer in Germany

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J Med Genet 2002;39:e12 (<http://www.jmedgenet.com/cgi/content/full/39/3/e12>)

Breast cancer is the most common malignancy affecting women world wide.¹ Approximately 1 in 10 women will develop breast cancer during their life time² and 5-10% of all breast cancers, in particular those with an early age of onset, are the result of a genetic predisposition owing to the inheritance of a dominant susceptibility gene(s).

In the context of high risk families, one important gene is the *BRCA2* gene located on chromosome 13q12-13. *BRCA2* was localised to chromosome 13q by linkage analysis in 1994³ and cloned in 1995.^{4,5} To date, more than 250 *BRCA2* germline mutations have been identified in breast/ovarian cancer families.⁶ The majority of these mutations are nonsense mutations or frameshift mutations that generate premature termination codons.⁷ Recent studies on highly selected families with at least four cases of breast cancer suggest that *BRCA2* accounts for the majority of breast cancer families where males as well as females are affected, for about one third of the families with female breast cancer alone, and only for a few families containing multiple cases of breast and ovarian cancer.⁸ *BRCA2* encodes a nuclear protein of 3418 amino acid residues that is postulated to play a role in the regulation of gene expression⁹ and in DNA double strand break repair and homologous recombination.¹⁰

Estimates of the cumulative breast cancer risks by the age of 70 in *BRCA2* mutation carriers vary from 37-85%^{8,11} and the cumulative ovarian cancer risks from 16-27%.^{8,12} There is evidence for an increased risk of several other cancers including prostate cancer, pancreatic cancer, gall bladder and bile duct cancer, stomach cancer, and malignant melanoma.¹³ Analyses of *BRCA2* mutation data have provided evidence that the risks of breast cancer and ovarian cancer are related to the position of the mutation. Truncating mutations in families with the highest risk of ovarian cancer relative to breast cancer are clustered in the Ovarian Cancer Cluster Region (OCCR) in exon 11.^{14,15}

In Germany, 18% of the breast/ovarian cancer families are the result of *BRCA1* germline mutations.¹⁶⁻¹⁹ To determine the contribution of *BRCA2* to hereditary breast/ovarian cancer, we have performed a systematic study of the *BRCA2* gene in a series of 68 *BRCA1* negative breast/ovarian cancer families by using a combination of single strand conformational polymorphism analysis (SSCP) and the protein truncation test (PTT) followed by DNA sequencing analysis.

METHODS

Families

Patients were identified through an ongoing research family study of hereditary breast/ovarian cancer recruiting families from all over Germany. Families containing three or more members affected with breast/ovarian cancer with at least two breast cancer cases diagnosed under the age of 60 years and ovarian cancer diagnosed at any age were ascertained through gynaecologists or in response to press publicity in Germany. Each pedigree was extended through an index case and available related family members. All participants gave informed

consent and blood samples. Breast and ovarian cancer cases were verified by pathology reports, by reviewing the medical records, and by family studies. Malignancies other than breast and ovarian cancers were identified by detailed pedigree analysis.

Sixty-eight hereditary German breast/ovarian cancer families negative for a *BRCA1* germline mutation were included in this study. Sixty-one of these families have been described previously.^{17,18,20} Seven additional breast cancer families that fit the ascertainment criteria were also screened for *BRCA1* mutations and found not to harbour any changes in this gene (U Hamann, unpublished data). *BRCA1* mutation detection was performed using single strand conformational polymorphism analysis (SSCP) for all small coding exons, and the protein truncation test (PTT) for exon 11, followed by DNA sequencing analysis of all unusual conformers or prematurely truncated proteins. The classification of the 68 cancer families according to the occurrence of breast cancer, ovarian cancer, and other malignancies is shown in table 1.

Genomic DNA was isolated from blood samples according to standard procedures.²¹ One affected person per family was chosen for screening. Where possible the proband was the youngest affected person in the family. The average age of the 65 probands diagnosed with breast cancer was 43.7 years (range 26-76 years). Three probands were diagnosed with ovarian cancer at the ages of 49, 52, and 54 years.

Mutation screening

The entire coding region of the *BRCA2* gene (Genbank accession number U43746) was examined in 32 families. Thirty-six families with a low a priori probability of being linked to *BRCA2* were partially screened. In these families, 70% of the coding region was analysed, including exons 9, 10, 11, 23, and 27, in which recurrent mutations have been identified,²² and exon 14 because of the presence of a disease associated mutation in one of the families included in this study. Mutation analysis of the small exons was done by SSCP analysis. PCR, primer pairs, and cycling conditions have been described previously.^{5,23}

SSCP analysis

Amplified samples were diluted 1:10 in formamide buffer (98% formamide, 10 mmol/l EDTA, pH 8, 0.025% bromophenol blue, 0.025% xylene cyanol), heated to 95°C for five minutes and chilled on ice for five minutes. Three to five µl of this mixture were loaded on vertical non-denaturing 6% polyacrylamide gels and run at 6 W at constant power for 7-16 hours in 0.6 × TBE at 4°C. In addition, samples were run on

Abbreviations: HBC, hereditary breast cancer; HBOC, hereditary breast/ovarian cancer; SSCP, single strand conformational polymorphism; PTT, protein truncation test; OCCR, ovarian cancer cluster region

Table 1 Classification of the 68 German cancer families according to the number of cancers

No of breast cancers/family	No of families	No of cancers																		Other sites
		Female breast	Male breast	Ov*	Brain	Cervix	Colon	Gyn	Leuk	Liver	Lung	Skin	Panc	Pro	Sarc	Sto	Ut	Vulva	Testis	
1-2	4	7	0	9	0	1	3	0	0	3	0	0	0	0	0	1	0	0	0	0
3	25	74	1	3	1	2	3	2	4	4	4	3	1	1	0	9	2	1	0	7
4	17	68	0	4	0	0	3	0	1	3	0	2	1	1	1	1	2	1	2	4
5	13	63	2	8	2	0	4	1	1	2	1	1	0	1	1	2	5	0	1	6
6+	9	62	1	6	0	1	3	0	1	2	2	1	0	1	0	3	0	1	0	5

Ov, ovarian cancer; Gyn, gynaecological cancer; Leuk, leukaemia; Panc, pancreatic cancer; Pro, prostate cancer; Sarc, sarcoma; Ut, uterine cancer; Sto, stomach cancer.

*Including one borderline tumour and three ovarian or uterine cancers.

†One parotid, one Hodgkin's lymphoma, two eye, one larynx, two bone, one anus, one small intestine, one nose, one oesophagus, one kidney, one fallopian, one rectum, one urinary organ, two bladder, and five unknown sites.

non-denaturing 6% polyacrylamide gels containing 5% glycerol at room temperature. Variant bands were detected by autoradiography.

PTT analysis

BRCA2 exons 10 and 11 were screened with the protein truncation test (PTT) on genomic DNA. Primers used for this analysis have been described elsewhere.^{24, 25} For exon 11, overlapping PCR products were amplified from genomic DNA in 1.2-1.8 kb pieces. PCR amplification of the segments was as follows: 50 ng genomic DNA was used as template in a 25 µl reaction volume using standard PCR conditions except that the primer concentration was 500 nmol/l. Samples were amplified using the following cycling conditions: 90°C for one minute, 52°C for 30 seconds, 72°C for two minutes, 35 times. Coupled transcription and translation was done using 1-3 µl of PCR product in a total volume of 7.5 µl which included 3 µl of TNT T7 reticulocyte lysate (Promega, Madison) and 0.5 µl of ³⁵S-cysteine (ICN, Irvine). Labelled protein products were denatured and size fractionated on 12% SDS-PAGE, dried, and autoradiographed.

DNA sequence analysis

Each sample showing a variant band on SSCP and PTT gels was sequenced on a CEQ 2000 automated DNA sequencer (Beckman) according to the manufacturer's instructions. Both strands of genomic DNA were sequenced to confirm a mutation.

Statistical analysis

The association between *BRCA2* mutation frequency and family profile was assessed using Fisher's exact test and the exact Cochran-Armitage trend test. Two sided p values of 0.05 or less

were considered significant. All results were calculated using StatXact 4 for Windows (Cytel Inc, Cambridge, USA).

RESULTS

BRCA2 germline mutations

Sixty-eight probands from 68 breast/ovarian cancer families were studied for germline mutations of the *BRCA2* gene. Eight different *BRCA2* germline mutations (12%) and one polymorphism were identified by using a combination of SSCP, PTT, and DNA sequencing analysis. Probands from families harbouring *BRCA2* germline mutations (n=7) were significantly younger than probands from *BRCA1/2* negative families (n=58) with a median age at diagnosis of breast cancer of 36 years and 44.5 years, respectively (Wilcoxon rank sum test, p=0.03). There was no difference in the age of breast cancer diagnosis of probands of *BRCA1* families (n=13), median age of 37 years,¹⁷⁻¹⁹ from probands of *BRCA2* families (Wilcoxon rank sum test, p=0.98).

Three mutations were detected in fully screened families and five mutations and one polymorphism were detected in partially screened families. The germline mutations included four frameshift mutations in exons 11, 14, and 27, three nonsense mutations in exon 11, and one splice mutation in exon 23 in four HBC and four HBOC families (table 2). One polymorphism,²⁶ an I3412V missense mutation in exon 27, was identified in one HBC family. The *BRCA2* germline mutations were deemed causative as they were predicted to result in premature termination codons. Among these, four mutations (50%), including one that we previously reported,²⁷ have not been described in other populations. These novel mutations were not found in 50 controls.

Family 67 carries a 7296delTC mutation in exon 14.²⁷ In this family, 13 *BRCA2* mutation carriers were identified (10 direct

Table 2 *BRCA2* germline mutations and cancer phenotypes of German breast/ovarian cancer families

Family	Exon	Nucleotide	Codon	Base change	Amino acid change	Designation	Type*	Reported in BIC†	No of cancers		
									Breast (bilateral)	Breast ≤50 y	Ovarian
98	11	3036	936	delACAA	Stop 958	3036del4	FS	24×	6‡	1	3§
86	11	3279	1017	delC	Stop 1042	3279delC	FS	–	3	1	1
83	11	4780	1518	G→T	Glu→Stop	E1518X	NS	–	1	0	3¶
33	11	5910	1894	C→G	Tyr→Stop	Y1894X	NS	14×	8 (1)	4	–
53	11	6265	2013	A→T	Lys→Stop	K2013X	NS	–	3 (2)	2	–
67	14	7296	2356	delTC	Stop 2358	7296delTC	FS**	–	3 (1)	2	–
76	23	9345	3039	G→A	Pro→Pro	9345G>A	SP	2×	4 (1)	4	1
97	27	9894	3222	delT	Stop 3248	9894delT	FS	1×	4 (1)	1	–

*FS, frameshift mutation; NS, nonsense mutation; SP, splice site mutation.

†BIC, Breast Cancer Information Core database.

‡More than one patient's age of diagnosis is unknown.

§Including two ovarian or uterine cancers.

¶Including a borderline tumour.

**Described by Hamann *et al.*²⁷

Figure 1 Pedigree and *BRCA2* mutation carriers of the breast cancer family 67. Symbols with filled left upper quadrant: unilateral breast cancer; symbols with filled left and right upper quadrants: bilateral breast cancer; symbols with filled right lower quadrant: other cancer. Br: breast cancer; Sto: stomach cancer. The numbers following these abbreviations indicate age at diagnosis. The index patient is indicated by an arrow. M: mutation carrier, N: non-carrier; NT: not tested.

and three obligatory) (fig 1). The two obligatory female carriers (II.1 and II.3) and one male carrier (II.12) both had children who harboured the same mutation as the three living affected patients. Among these were three females, of whom two (II.1 and II.9) were diagnosed with breast cancer at 41 and 55 years of age and one (II.5) who developed bilateral breast cancer at the ages of 40 and 58 years. One mutation carrier (II.11) developed stomach cancer at 65 years. A second gene carrier (II.1) was diagnosed with skin cancer at 48 years of age. Pedigree and mutation carriers of family 67 are shown in fig 1.

Owing to the small number of families with disease associated *BRCA2* germline mutations, it was impossible to identify any genotype-phenotype correlation. However, all three HBOC families harboured mutations in the OCCR region in exon 11, whereas in the four HBC families one mutation was located in the OCCR region and three were located outside this region.

Frequency of *BRCA2* and *BRCA1* germline mutations according to family profiles

Of the deleterious *BRCA2* mutations, four were identified in 51 HBC families (8%) and four were found in 17 HBOC families (24%) (table 3). No *BRCA2* mutations were found in four HBC families with male breast cancer. HBC families with bilateral breast cancer patients showed a higher *BRCA2* mutation frequency (4/13, 31%) than those with unilateral breast cancer patients (0/34, 0%) (Fisher's exact test, $p=0.01$). Three unrelated breast cancer patients with bilateral disease were diagnosed at the ages of 30 and 32 years, 34 and 57 years, and 40 and 58 years, respectively. One family with two women with bilateral disease developed breast cancers aged 50 and 51 years, and 50 years and an unknown age. There was no trend in number of families with a *BRCA2* mutation corresponding to the average age of diagnosis of breast cancer (Exact Cochran-Armitage trend test, $p=0.71$) and ovarian cancer

Table 3 Frequency of *BRCA2* and *BRCA1* mutations according to family characteristics

	<i>BRCA2</i> mutation screening		<i>BRCA1</i> mutation screening*	
	No of families	No of families with mutations (%)	No of families	No of families with mutations (%)
All families†	68	8 (12)	74	13‡ (18)
Breast/ovarian cancer families	17	4 (24)	23	6 (26)
Average age of diagnosis of breast cancer (y)				
<40	4	1 (25)	8	4 (50)
40–49	5	2 (40)	6	1 (17)
≥50	8	1 (13)	9	1 (11)
Average age of diagnosis of ovarian cancer (y)				
<40	1	0 (0)	1	0 (0)
40–49	5	0 (0)	8	3 (38)
≥50	8	2 (25)	11	3 (27)
Unknown	3	2	3	
Breast cancer families	51	4 (8)	51	7 (14)
Female and male breast cancer	4	0 (0)	3	0 (0)
Female breast cancer	47		48	
No of breast cancer patients				
3	21	2 (10)	20	2 (10)
4	13	1 (8)	15	3 (20)
5+	13	1 (8)	13	2 (15)
Laterality				
Unilateral	34	0 (0)	33	3 (9)
Bilateral	13	4 (31)	15	4 (27)
Average age of diagnosis (y)				
<40	2	0 (0)	6	4 (66)
40–49	18	2 (11)	17	3 (18)
≥50	27	2 (7)	25	0 (0)

*Breast/ovarian cancer families and *BRCA1* mutations have been described previously.^{17–19}

†The average age of diagnosis of female breast cancer was 48.3 years in the *BRCA2* positive families, 39.7 years in the *BRCA1* positive families, and 50.6 years in the mutation negative families. The average age of diagnosis of ovarian cancer was 55.5 years in the *BRCA2* group, 52.9 years in the *BRCA1* group, and 50 years in the mutation negative group.

‡Including one previously identified *BRCA1* mutation (U Hamann, unpublished data).

(Exact Cochran-Armitage trend test, $p=0.56$) in HBOC families and the number of breast cancer patients and the average age of diagnosis of breast cancer in HBC families (Exact Cochran-Armitage trend test, $p=1$).

The analysis of 74 breast/ovarian cancer families previously screened for *BRCA1* germline mutations^{17–19} showed a significant trend in the number of families with a *BRCA1* mutation and a decreasing average age of diagnosis of breast cancer in HBC families (Exact Cochran-Armitage trend test, $p<0.001$). No such trend was observed in HBOC families (Exact Cochran-Armitage trend test, $p=0.1$). There was no association between the number of families with a *BRCA1* mutation and the average age of ovarian cancer diagnosis (Exact Cochran-Armitage trend test, $p=1$) nor between the number of breast cancer patients in HBC families (Exact Cochran-Armitage trend test, $p=0.63$). There was no significant difference in the number of *BRCA1* positive HBC families with bilateral breast cancer patients (4/15, 27%) compared to families with unilateral breast cancer patients (3/33, 9%) (Fisher's exact test, $p=0.18$). *BRCA2* and *BRCA1* mutation frequencies according to family profiles are shown in table 3.

DISCUSSION

This report describes the analysis of the *BRCA2* gene in 68 German breast/ovarian families. Families included contained at least three cases of breast or ovarian cancer and two cases diagnosed before the age of 60 years. All families have previously been screened for *BRCA1* germline mutations and have been found to be negative.

A disease causing *BRCA2* germline mutation was identified in 12% of the 68 German breast/ovarian cancer families. This mutation frequency is in agreement with the frequency previously reported in 110 families from northern Germany.²⁸ Similar mutation frequencies have been obtained in Austrian (8%),²⁶ Finnish (8%),²⁹ Scandinavian (11%),³⁰ and Dutch (12%)³¹ families. A higher mutation frequency was found in US families (27%).³² However, this may be because of different selection criteria as only families with at least four breast or ovarian cancer cases were included in the study.

Many germline mutations have been identified within the *BRCA2* coding region. Most of these mutations are nonsense or frameshift mutations resulting in truncated proteins. Using the combined approach of SSCP, PTT, and DNA sequencing analysis, we identified eight different disease associated *BRCA2* germline mutations and one polymorphism. Four of these mutations have not been reported in other populations. Three mutations resulted in stop codons, four mutations caused a frameshift, and one mutation was silent.

Three of the novel *BRCA2* mutations described in this study are located in the short BRC repeat sequences in exon 11. Using in vitro studies, it has been shown that these repeats interact with the double strand DNA repair protein RAD51.³³ Inactivating mutations in the RAD51 binding sites may be responsible for breast and ovarian cancer predisposition in these families. Another possibility is that these mutations are non-functional because they do not translocate the protein to the nucleus. Recently it has been shown that two nuclear localisation signals reside in the last 159 amino acid residues of *BRCA2* and that loss of these signals results in mislocation of the protein to the cytoplasm.³⁴

The splice site mutation in the last nucleotide of exon 23 was identified in HBOC family 76. This mutation has previously been reported in one Dutch family with nine breast cancer cases and one ovarian cancer case and one US breast/ovarian cancer family patient.⁶ In the Dutch study, it was shown that this mutation, which affects the last nucleotide of exon 23, results in aberrant splicing.³¹ Recently, several other silent mutations in the fibrillin, coagulation factor VIII, and the ataxia-telangiectasia genes have been shown to induce exon skipping.^{35–37} These results imply that silent

mutations at the very end of exons associated with hereditary breast/ovarian cancer might be of functional relevance. Therefore, further tests analysing the effect of these mutations on RNA stability and splicing need to be developed. Finally, a previously reported polymorphism³⁶ changing Ile to Val was identified in family 134.

Since only nine changes were identified, we cannot exclude the possibility that some mutations have been missed by the strategy adopted and the methods of mutation detection used in the present study. Whereas SSCP may have missed some single base pair substitutions, PTT would have missed missense mutations and some truncating mutations. The low frequency of missense mutations identified in this study can be accounted for by the PTT as it is incapable of identifying missense changes. Because only the coding region, and in half the families only 70% of the coding region, was analysed, regulatory mutations outside these regions, which affect transcription as well as mutations in the unanalysed section of the coding region would not have been detected. Large genomic deletions and rearrangements, similar to those that have recently been reported by Nordling *et al*³⁸ may have been missed. Finally, given that some of the breast cancer patients have developed disease at the same age as those without any family history, there remains the possibility that those probands are phenocopies rather than gene carriers.

Consistent with other reports,^{14,15} the current study supports the notion of there being a specific region within the *BRCA2* gene which is associated with an increased predisposition to develop ovarian cancer. Three out of four HBOC families harboured mutations within the OCCR region, whereas three out of four HBC families had changes outside this region. Three of the 35 mutation carriers tested had neoplasms other than breast or ovarian cancer, one was diagnosed with skin cancer, another with stomach cancer, and one with cervical cancer. All these cancers have been shown to be part of the phenotypic spectrum of disease associated with germline mutations of *BRCA2*.¹³

In our study, the number of HBC families with mutations in *BRCA1* increased with a decreasing average age of diagnosis of breast cancer, while the number of families with a *BRCA2* mutation remained unchanged. These results are similar to those previously reported.⁴⁰

Because of the strong association between *BRCA1* mutations and ovarian cancer, a family history of ovarian cancer has been taken as an indicator to look for a mutation in this gene.³⁹ However, a large number of *BRCA2* mutations (18/59) were detected in probands from ovarian cancer families.⁴⁰ In this study, four of the eight deleterious *BRCA2* mutations occurred in HBOC families. This finding supports the notion that mutation analysis of both the *BRCA1* and *BRCA2* genes should be performed in HBOC families. Furthermore, given the number of *BRCA2* mutations identified in our HBC families, it is important that these families are also screened for both *BRCA1* and *BRCA2* mutations.

The male breast cancer risk in *BRCA2* mutation carriers has been reported to be 6% by 70 years of age, which is greater than male breast cancer risk associated with *BRCA1* mutation carriers.⁴¹ Three of our families without detectable germline mutations in the *BRCA1* and *BRCA2* genes contained individual cases of affected males. These families have been included in a linkage study and two have yielded negative lod scores at both loci on chromosomes 17q12 and 13q12–13.²⁰ These data suggest that other genes besides *BRCA1* and *BRCA2* may be involved in the pathogenesis of male breast cancer in these families.

Breast Cancer Linkage Consortium data on 237 breast/ovarian cancer families showed that 52% were linked to *BRCA1* and 32% to *BRCA2*.⁸ These were special families selected by virtue of there being at least four breast cancers in women diagnosed under the age of 60 years or in males diagnosed at any age, irrespective of any ovarian cancer in the

- The purpose of this study was to establish the contribution of *BRCA2* to hereditary breast/ovarian cancer in 68 German breast/ovarian cancer families negative for *BRCA1* germline mutations.
- Eight different deleterious *BRCA2* germline mutations (12%) were identified, including four frameshift mutations in exons 11, 14, and 27, three nonsense mutations in exon 11, and one splice site mutation in exon 23. Four mutations (50%) are novel and have not been described in other populations. *BRCA2* mutations were identified in four breast cancer (HBC) and four breast/ovarian cancer (HBOC) families.
- Three out of four HBOC families had mutations in the Ovarian Cancer Cluster Region (OCCR) in exon 11, whereas three out of four HBC families had non-OCCR mutations. In HBC families, *BRCA2* mutation frequency was higher in families with bilateral breast cancer (4/13, 31%) than in families with unilateral breast cancer (0/34, 0%) ($p=0.01$).
- Our results show that *BRCA2* is implicated in a small fraction of HBC/HBOC families, supporting the notion that additional susceptibility gene(s) may be important in Germany.

family. These families were chosen specifically for the identification of genes associated with breast cancer. Population based family studies, including this one, identified smaller proportions of families attributable to *BRCA1* and *BRCA2*. In Germany, *BRCA1* mutations were found in only 18%¹⁷⁻¹⁹ and those of *BRCA2* in only 12% of the families. In Finland, the corresponding percentages were 10% and 11%²⁹ and in southern Sweden 23% and 10%,³⁰ suggesting that the three populations are not significantly different from one another. The substantial proportion of families without any identifiable mutation support the notion that other genes may be important determinants of familial risk.

In conclusion, our data suggest that genetic testing for predisposing *BRCA2* germline mutations is justified for any family in which *BRCA1* has been excluded where there are at least three early onset breast/ovarian cancers. We propose that the most efficient strategy for mutation detection in breast/ovarian cancer families is first to test the *BRCA1* gene and then, if this is negative, test for mutations in the *BRCA2* gene.

ACKNOWLEDGEMENTS

We are grateful to all family members for their participation in this study. This study was supported in part by the Swiss Cancer League AKT 463 and the Hunter Area Pathology Service establishment grant. We thank Antje Seidel-Renkert, Michaela Schleicher, and Michael Gilbert for expert technical assistance.

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