

Mutations of the *BRCA1* and *BRCA2* genes in patients with bilateral breast cancer

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Summary Mutations of the *BRCA1* or *BRCA2* genes have been shown to strongly predispose towards the development of contralateral breast cancer in patients from large multi-case families. In order to test the hypothesis that *BRCA1* and *BRCA2* mutations are more frequent in patients with bilateral breast cancer, we have investigated a hospital-based series of 75 consecutive patients with bilateral breast cancer and a comparison group of 75 patients with unilateral breast cancer, pairwise matched by age and family history, for mutations in the *BRCA1* and *BRCA2* genes. Five frameshift deletions (517delGT in *BRCA1*; 4772delA, 5946delCT, 6174delT and 8138del5 in *BRCA2*) were identified in patients with bilateral disease. No further mutations, apart from polymorphisms and 3 rare unclassified variants, were found after scanning the whole *BRCA1* and *BRCA2* coding sequence. Three pathogenic *BRCA1* mutations (Cys61Gly, 3814del5, 5382insC) were identified in the group of patients with unilateral breast cancer. The frequencies of common *BRCA1* and *BRCA2* missense variants were not different between the 2 groups. In summary, we did not find a significantly increased prevalence of *BRCA1* and *BRCA2* mutations in a hospital-based cohort of German patients with bilateral breast cancer. We conclude that bilaterality of breast cancer on its own is not strongly associated with *BRCA1* and *BRCA2* mutations when adjusted for age and family history. The high frequency of bilateral disease in multi-case breast cancer families may be due to a familial aggregation of additional susceptibility factors modifying the penetrance of *BRCA1* and *BRCA2* mutations. © 2001 Cancer Research Campaign <http://www.bjcancer.com>

Keywords: contralateral breast cancer; *BRCA1*; *BRCA2*

About 1 in 9 women in European and North American populations may develop breast cancer throughout their life-time, with rates having increased during the last decades (Philips et al, 1999). Each year there are approximately 46 000 new cases in Germany (Beckmann et al, 1997). Several risk factors have been defined including age, family history, smoking, hormonal factors and radiation exposure (Goss and Sierra, 1998; Olsen et al, 1999; Philips et al, 1999; Martin and Weber, 2000). Familial predisposition accounts for some 10% of breast cancer, and about half of these patients are thought to have a mutation in one of two large genes that function in DNA damage response, *BRCA1* and *BRCA2* (Kote-Jarai and Eeles, 1999; Martin and Weber, 2000).

Bilaterality of cancer, including breast carcinoma, is thought to be an indicator of a genetic predisposition (Anderson, 1971; Knudson, 1971), and studies of blood relatives and of twins affected by breast cancer have corroborated this view (Bernstein et al, 1992; Peto and Mack, 2000). Contralateral breast cancer accounts for about 2–10% of patients (Hislop et al, 1984; Adami et al, 1985; Healey et al, 1993; Mose et al, 1995) and is observed at an annual incidence of approximately 0.7% per year (Peto and Mack, 2000). The importance of *BRCA1* and *BRCA2* for the bilateral occurrence of breast cancer has been demonstrated in large multi-case families where heterozygosity for a *BRCA1* or *BRCA2* gene mutation strongly predisposes the affected patients towards the development of a contralateral breast cancer (Ford et al, 1994,

1998; Easton et al, 1995; Robson et al, 1998; Verhoog et al, 1998). Young age at onset appears to be a significant risk factor for contralateral breast cancer in the general population (Hislop et al, 1984; Adami et al, 1985) and also within *BRCA1* families, where the risk declines for patients with a postmenopausal primary tumour (Verhoog et al, 2000). Initial population-based studies of selected *BRCA1* and *BRCA2* mutations have provided first evidence for an apparently low proportion of mutation carriers among patients with bilateral breast cancer when their age at first onset was higher than 40 years (Eccles et al, 1998; Gershoni-Baruch et al, 1999). As an attempt to determine more closely the relative contribution of *BRCA1* and *BRCA2* mutations to bilateral breast cancer in relation to age and family history, we have performed a hospital-based study of the whole coding region of the *BRCA1* and *BRCA2* genes in 75 German patients with bilateral breast cancer, and compared the results with the prevalence of *BRCA1* and *BRCA2* mutations in a pairwise-matched cohort of patients with unilateral breast cancer.

PATIENTS AND METHODS

Patients

During the years 1996–2000, more than 1000 unselected patients with breast cancer received radiotherapy in the Department of Radiation Oncology of the Medical School Hannover. The patients were counselled about the possibility of a genetic predisposition of breast cancer, and anamneses were taken using questionnaires with regard to the family history and previous radiation exposure

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Received 15 February 2001

Revised 30 May 2001

Accepted 11 June 2001

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of the patients. After written informed consent had been obtained, blood samples were collected from 1000 participating patients. 75 patients had developed bilateral breast cancer and took part in this study. This proportion of 7.5% of cases with bilateral disease was the same as has been previously reported in another series of over 500 German breast cancer patients (Mose et al, 1995), indicating that our cohort is representative for a hospital-based cancer population in Germany. As a comparison group for mutation frequency determination, we selected 75 patients with unilateral breast cancer from the total cohort who were pairwise matched with respect to age and family history at the time of treatment.

Clinical data of the patients with bilateral breast cancer were collected and compared with data of the whole series of 1000 breast cancer patients. In 29 patients, bilateral breast cancer occurred simultaneously. In the other 46 patients, contralateral breast cancer developed with a median time interval of 7.2 years (range 1 to 40 years). A time interval of at least 3 years was observed in 37 patients. The median age at diagnosis of the first breast cancer was 54 years, compared with 57 years in the total cohort. 22 patients (29.3%) were younger than 50 years and 9 patients (12%) younger than 40 years at first diagnosis. Self-reported family history revealed at least one first or second degree relative with breast cancer in 17 (22.7%) patients and ovarian cancer in 1 (1.3%) patient, which was not different from the figures obtained for the total group of unselected breast cancer patients ($n = 1000$).

Breast conservative surgery of the first and second tumour was performed in 48 (64%) and 65 (86.7%) patients, respectively. Postoperative radiotherapy was applied unilaterally in 18 patients (24%) and bilaterally in 57 patients (76%) with a median total dose of 50 Gy (single dose: 1.8 Gy). Of the latter group 18 patients received radiotherapy simultaneously to both sides without overlap of treatment fields. The median follow up for the first and second tumour was 6.3 and 2.5 years, respectively. 10 patients developed local (one patient bilaterally), 1 patient regional (axillary node) and 1 patient locoregional recurrence after a median time interval of 24 months. It is noteworthy that 3 of 6 (50%) identified carriers of a pathogenic *BRCA1* or 2 mutation showed local recurrence, whereas this was the case in only 9 of 69 noncarriers (13%). The actuarial locoregional relapse-free survival – as evaluated by Kaplan–Meier calculations – was significantly lower (log-rank test, $P = 0.0072$) for carriers (mean: 55 months, 95% CI: 36–74 months) compared to non-carriers (mean: 408 months, 95% CI: 354–462 months). Local relapse after initial radiotherapy occurred in 5 patients (5 of 126 irradiated breasts). 3 of these 5 patients (60%) were carriers of a pathogenic *BRCA1* or *BRCA2* mutation. Distant metastases developed in 14 patients (18.7%) with 10 patients having died of their disease at time of analysis.

Methods

EDTA blood samples from peripheral venous blood were collected from the patients of both groups, and genomic DNA was extracted from white blood cells according to a standard salting out procedure. PCR conditions were established to separately amplify all coding exons and exon-flanking intron sequences of both, *BRCA1* and *BRCA2* genes to investigate the samples obtained from patients with bilateral breast cancer (Appendix, Table 1). We designed a set of 26 primer pairs for the *BRCA1* gene and 29 primer pairs for the *BRCA2* gene based on the published genomic

sequence (GenBank Acc. No. L78833 for the *BRCA1* gene, Z74739 for the *BRCA2* gene). Due to their large size, the coding sequences of exon 11 of the *BRCA1* gene (3.4 kb) and exon 11 of the *BRCA2* gene (4.9 kb) were amplified in overlapping fragments. On the other hand, exons 5 and 6 and exons 23 and 24 of the *BRCA2* gene could be jointly amplified together with their intervening intron sequences.

Standard PCR amplifications were performed in 20 μ l reaction volumes containing 100–200 ng of genomic DNA, 0.2 mM of each dNTP and 1 Unit Taq DNA polymerase in 1 \times reaction buffer supplied by the manufacturer (USB/Amersham). Final primer concentrations were 0.5 μ M. As the standard procedure, 37 PCR cycles were performed with 1 min denaturation at 94°C, 1 min annealing, and 1 min elongation at 72°C. PCR products over 1 kbp were amplified at an elongation time of 100–150 s, and in 2 cases (primer pairs BS13/14 and BS15/16) the reaction mix was supplemented with 0.1 M betaine and 2% formamide. Some exons could be amplified and analysed in a duplex PCR format (Appendix, Table 2).

Restriction fragments of 150 to 400 bp (Appendix, Table 3) were subjected to single strand conformation polymorphism (SSCP) analysis. In brief, the products were separated on a 40 cm 5% non-denaturing polyacrylamide gel at 40 watts for 5 h in a cold room, and were subsequently visualized by conventional silver staining. Aberrantly migrating samples were sequenced on both strands using an ABI-dRhodamine Terminator Cycle Sequence Kit, and sequencing data were collected and evaluated on an ABI 310 Genetic Analyzer (Perkin Elmer). Identified variants were then screened by restriction enzyme-based assays (Appendix, Table 4a) in both groups of patients. Some tests required a mismatch primer to create recognition sites for the respective restriction enzymes (Appendix, Table 4b). In addition, the common restriction mutations 5382insC and Cys61Gly in the *BRCA1* gene and 6174delT in the *BRCA2* gene were analysed by restriction enzyme analysis in all patients, and a subset of patients in the comparison group who had a positive family history of breast cancer were scanned for mutations in the large exons of *BRCA1* (exon 11) and *BRCA2* (exons 10 and 11).

Statistical evaluation was performed using two-sided chi-square tests. The allele frequencies of truncating mutations were compared between both groups and results were considered to be significant for $P < 0.05$. In addition, the frequencies of missense substitutions that were found more than once, were compared between both cohorts and results and were considered to be significant if $P < 0.004$, following Bonferroni's correction for multiple testing with a number of 12 independent, i.e. unlinked, common substitutions.

RESULTS

All coding exons and flanking intronic regions of the *BRCA1* and *BRCA2* genes were amplified by PCR from genomic DNA samples of 75 patients with bilateral breast cancer and were investigated by single-strand conformation polymorphism (SSCP) and sequencing analysis. Detected sequence alterations were subsequently confirmed by restriction enzyme analyses and screened in the comparison cohort of 75 pairwise-matched patients with unilateral breast cancer. In addition, a screening of the 3 most common pathogenic mutations as well as a mutation scanning of the largest exons of the *BRCA1* and *BRCA2* genes was also performed in the comparison cohort (see Patients and Methods

Table 1 Mutations of the *BRCA1* and *BRCA2* genes in patients with bilateral and unilateral breast cancer

Gene	Exon	Mutation	Predicted effect	Patient no. (status)	
				Bilateral	Unilateral
<i>BRCA1</i>	5	T300G	Cys→Gly at 61		1 het
	7	517delTG	Truncation	1 het	
	11	3814del5	Truncation		1 het
	20	5382insC	Truncation		1 het
<i>BRCA2</i>	11	4772delA	Truncation	2 het(*)	
	11	5946delCT	Truncation	1 het	
	11	6174delT	Truncation	1 het	
	17	8138del5	Truncation	1 het	

Identified mutations are designated according to their nucleotide position within the *BRCA1* or *BRCA2* cDNA sequences. het: heterozygote. (*): two 4772delA heterozygous patients were mother and daughter, respectively.

section). A total of 8 different pathogenic *BRCA1* and *BRCA2* gene mutations were identified, with 5 heterozygous carriers among the patients with bilateral breast cancer and 3 heterozygous carriers among the patients with unilateral breast cancer (Table 1). All 5 mutations of the patients with bilateral breast cancer were small frameshift deletions of 1–5 basepairs leading to a premature translation termination codon. 4 truncating mutations (5946delCT, 6174delT, 8138del5, and the new 4772delA mutation) were found in the *BRCA2* gene, and one frameshift deletion (517delTG) was uncovered in the *BRCA1* gene (Figure 1, Table 1). The mutations

in the group of patients with unilateral breast cancer were all in *BRCA1* and include the Cys61Gly substitution of the RING finger motif, the 5382insC insertion, and a 5-basepair deletion, 3814del5 (Figure 1, Table 1). There was no concordant occurrence of *BRCA1* or *BRCA2* mutations in paired samples of our matched cohorts. The 3814del5 mutation in the *BRCA1* gene and the 4772delA mutation in the *BRCA2* gene may be confined to the single families described here since they had not been reported previously to the Breast cancer Information Core (BIC) database (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/BIC/).

The clinical characteristics of the mutation carriers are summarized in Table 2. In the study cohort of patients with bilateral breast cancer, one carrier of the 6174delT mutation in the *BRCA2* gene had developed a synchronous bilateral breast cancer by the age of 29 years, and she died from metastatic cancer 6 months after diagnosis. This patient also had juvenile-onset diabetes as an additional complication, eventually accelerating the progression of the disease. Another patient, and her mother, who also had bilateral breast cancer, were heterozygotes for the 4772delA mutation in the *BRCA2* gene. The younger patient had her first cancer by the age of 54 years and a contralateral breast tumour by the age of 59, while her mother developed synchronous bilateral breast cancer by the age of 73 years as well as a histologically distinct recurrence in her first breast; she died from her cancer by the age of 79 years (Table 2). The other 3 *BRCA1* or *BRCA2* mutation carriers had developed metachronous contralateral cancer after time intervals of 2 y, 4.5 y and 12.5 y, respectively (in comparison with a median

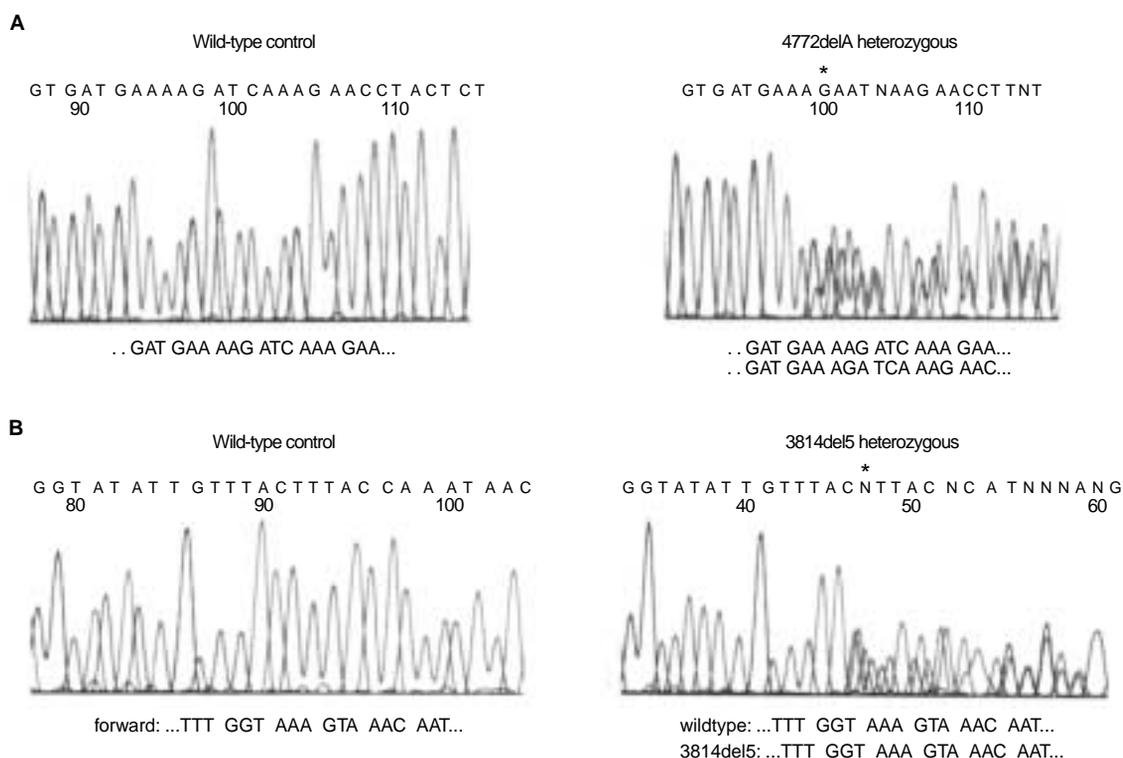


Figure 1 Identification of new truncating mutations in the *BRCA1* and *BRCA2* genes. **(A)** Direct sequencing of *BRCA2* mutation 4772delA in a patient with bilateral breast cancer. Left: wildtype control, right: heterozygous mutation carrier. An asterisk marks the start of the frameshift. **(B)** Direct sequencing of *BRCA1* mutation 3814del5 in a patient with unilateral breast cancer. The antisense strand is shown. Left: wildtype control, right: heterozygous mutation carrier. An asterisk marks the start of the frameshift

interval of 7.2 y in the total cohort). In 3 of the 6 carriers with bilateral cancer, one of both tumours was multifocal. The family history of cancer in 1st and 2nd degree relatives was negative for 3 of the 5 unrelated patients who had bilateral cancer and a *BRCA1* or *BRCA2* truncating mutation.

Apart from these clearly pathogenic mutations, a further 11 amino acid variants and polymorphisms were found in the coding region of the *BRCA1* gene and 12 were detected in the *BRCA2* gene (Table 3). Common alterations with more than one occurrence were observed also in the comparison group, and their allele frequencies were not significantly different between the patients with bilateral breast cancer and the patients with unilateral breast cancer. There was no significant deviation in the genotype frequencies from those expected for Hardy–Weinberg equilibrium. 7 polymorphisms of the *BRCA1* gene (2201C/T, 2430T/C, Pro871Leu, Glu1038Gly, Lys1183Arg, 4426C/T, Ser1613Gly) and 3 polymorphisms of the *BRCA2* gene (Asn289His, 1593A/G and Asn991Asp) were in absolute linkage disequilibrium, in accordance with previous findings in other populations (Durocher et al, 1996; Dunning et al, 1996; Southey et al, 1999; Wagner et al, 1999). A slightly higher prevalence of the Gln356Arg substitution was observed among patients with bilateral cancer (9/75 vs. 6/75, including one patient with unilateral breast cancer carrying the Cys61Gly mutation in cis with the Arg356 allele), a non-significant trend which may deserve further attention in larger cohorts taking into account that the Arg356 substitution is located within the ZBRK1-binding region and has recently been described to impair the *BRCA1*-mediated induction of GADD45 expression (Zheng et al, 2000). In addition, 2 unclassified missense variants of *BRCA2* (Pro1819Ser, Lys2950Asn) were found only in the cohort with bilateral disease, which can not be adequately addressed for their functional impact at the present time. Altogether, despite these subtle tendencies, the number and frequencies of missense substitutions did not differ significantly between the 2 cohorts of unilateral and bilateral breast cancer patients.

DISCUSSION

Contralateral breast cancer risk appears to be strongly increased in multi-case families with a genetic predisposition through *BRCA1* and *BRCA2* gene mutations (Ford et al, 1994, 1998; Easton et al, 1995; Robson et al, 1998; Verhoog et al, 1998). These may act in concert with environmental factors such as radiation (Goss and Serra, 1998; Roberts et al, 1999), as there is accumulating evidence to implicate the *BRCA1* and *BRCA2* gene products in the cellular responses to radiation-induced damage (for review see Kote-Jarai and Eeles, 1999; Haber, 2000; Welch et al, 2000). It thus seems on solid biochemical grounds to assume that *BRCA1* and *BRCA2* mutations predispose towards the development of bilateral breast cancer via their impairment of DNA repair. On the other hand, the first population-based studies of a limited number of *BRCA1* and *BRCA2* gene mutations have revealed mutations only in a small minority of patients with bilateral disease, and most of these also had an early onset at first primary (Eccles et al, 1998; Gershoni-Baruch et al, 1999).

To gain more insight into the proportion of bilateral breast cancer that can be explained by *BRCA1* and *BRCA2* mutations in a hospital setting, we analysed the whole *BRCA1* and *BRCA2* coding region in 75 consecutive patients with bilateral breast cancer not selected for age or family history. Somewhat unexpectedly, only 5 unrelated carriers of a truncating mutation were identified. Family history would not have been a good indicator for *BRCA1* or *BRCA2* testing

in these cases. Our finding that 3 unrelated patients and one mother–daughter pair with bilateral breast cancer had frameshift deletions in their *BRCA2* genes, does not support previous suggestions that *BRCA2* mutation carriers may be less prone to develop a contralateral cancer than *BRCA1* mutation carriers (Verhoog et al, 1999, 2000). However, in view that 4 of the 5 *BRCA2* deletion carriers developed their contralateral breast cancer after a first diagnosis at an age >45 years, it is possible that the age at first primary could be less influential in case of *BRCA2* mutations than it is for *BRCA1* gene mutations (Verhoog et al, 2000). The higher number of *BRCA2* carriers among patients with bilateral cancer compared with *BRCA1* carriers among the patients with unilateral breast cancer may deserve further investigation but could also reflect a maldistribution by chance due to the small numbers involved.

The proportion of 6.7% *BRCA1* and *BRCA2* deletion carriers among the patients with bilateral breast cancer in our study lies well within the range of 5–10% of breast cancer cases that are generally assumed to be caused by a strong familial predisposition (Kote-Jarai and Eeles, 1999; Martin and Weber, 2000). 3 carriers (4%) were found also in the comparison group indicating that the prevalence of ‘classical’ deleterious *BRCA1* and *BRCA2* mutations is not significantly different in patients with bilateral and unilateral disease from the same department matched by age and family history. We thus investigated whether more common missense substitutions of *BRCA1* or *BRCA2* might play a role as predisposing factors towards contralateral cancer. Missense substitutions in other tumour suppressor genes have been described as low-penetrance mutations that may predispose to certain malignancies or modify disease progression (Larson et al, 1998; Otterson et al, 1999; Varley et al, 1999; Lumin et al, 2000; Rebbeck et al, 2000), and a few missense variants of the *BRCA1* and *BRCA2* proteins, including the Gln356Arg, Arg841Trp and Leu871Pro substitutions of *BRCA1* and the Asn372His substitution of *BRCA2*, have previously been implicated in the susceptibility to breast or ovarian cancer (Barker et al, 1996; Durocher et al, 1996; Dunning et al, 1996; Healey et al, 2000). However, none of the common *BRCA1* and *BRCA2* variants occurred at a significantly increased prevalence in our patients with bilateral cancer, nor did we find a deviation of the genotype distributions from Hardy–Weinberg equilibrium. Hence, there was no evidence to support a strong predisposition by any of the common missense substitutions. Our study had 80% power to detect relative risks of 2.5 or greater for polymorphisms with an allele frequency of about 0.3, therefore more subtle effects or moderate risks for less frequent variants cannot be finally excluded.

Altogether, these results suggest that patients with bilateral breast cancer, if not selected for age and family history, are not significantly more likely to be carriers of a *BRCA1* or *BRCA2* alteration than patients with unilateral breast cancer. In fact, the proportion of detected *BRCA1* and *BRCA2* mutations among patients with bilateral disease appeared much lower than would have been calculated from high-penetrance breast cancer families (Ford et al, 1994, 1998; Easton et al, 1995). If, for example, a *BRCA1* or *BRCA2* mutation confers a risk of about 50% to develop a contralateral breast cancer by the age of 54, we might have expected that about 1 in 4 patients with bilateral breast cancer in a similarly aged population-based cohort were carriers of a *BRCA1* or *BRCA2* mutation, a proportion to be detected with 80% power in our study. Instead, our present data are more consistent with an odds ratio of 1.7 (95% CI 0.4–5.0) in favour of *BRCA1* or *BRCA2*

Table 2 Clinical characteristics of breast cancer patients carrying *BRCA1* or *BRCA2* mutations

	Mutation	Age at diagnosis	Family history (1° and 2° relatives)	Histology	Treatment	Course of disease	Follow up
Bilateral breast cancer							
1	BRCA 1 517delTG	46 ys and 48 ys	none	BC 1: multifocal invasive-ductal,EIC recurr. BC 1: invasive-ductal BC 2: invasive-ductal	BCT: BC 1 and 2 CTX:BC 2 HT: BC 1 RT: BC 1 and 2	BC 1: local failure after 25 mo.	42 mo.
2a	BRCA2 4772delA	54 ys and 59 ys	BC: 1 (bilat., 2b)	BC 1: invasive-ductal BC 2: invasive-ductal	BCT: BC 1 and 2 RT: BC 1 and 2 HT: BC 1 and 2	NED	17 mo.
2b	BRCA2 4772delA	73 ys (synchr.)	BC: 1 (bilat., 2a)	BC 1: invasive-lobular recurr. BC1: invasive-ductal BC 2: invasive-tubular	mastectomy: BC 1 BCT: BC 2 RT: BC 1, BC 2 and recurr. BC 1 HT: BC 1, BC 2, and recurr. BC 1	BC 1: local failure after 64 mo. distant failure after 70 mo.	76 mo., deceased
3	BRCA 2 5946delCT	63 ys and 65 ys	none	BC 1: microinvasive-ductal BC 2: invasive-ductal	BCT: BC 1 and 2 RT: BC 1 and 2 HT: BC 1 and 2	NED	9 mo.
4	BRCA 2 6174delT	29 ys (synchr.)	none	BC 1: multifocal invasive-ductal BC 2: invasive-ductal	BCT: BC 1 and 2 CTX RT: BC 1 and 2	distant failure after 4 mo.	6 mo., deceased
5	BRCA 2 8138del5	46 ys and 59 ys	BC: 2	BC 1: invasive-lobular recurr.: invasive-ductal BC 2: multifocal tubular	BCT: BC 1 and 2 RT: BC 1 and 2 HT: BC 1 and 2	BC 1: local failures after 43 mo., 57 mo. and 133 mo.	10 mo.
Unilateral breast cancer							
6	BRCA 1 Cys61 Gly	49 ys	OC: 1	medullary	BCT, CTX, RT	NED	45 mo.
7	BRCA 1 3814del5	54 ys	BC: 3	multifocal invasive-ductal	mastectomy, CTX, HT, RT (after local failure)	local failure after 9 mo.	24 mo., deceased
8	BRCA 1 5382insC	42 ys	BC: 1 (bilat.)	invasive-ductal	BCT, CTX, RT	NED	49 mo.

Abbreviations: BCT = breast conservative therapy; RT = radiotherapy; CTX = chemotherapy; HT = hormonal therapy; BC = breast cancer; OC = ovarian cancer; NED = no evidence of disease, EIC = extensive intraductal component. Patients 2a and 2b are daughter and mother, respectively.

Table 3 Polymorphisms and rare amino acid substitutions of *BRCA1* and *BRCA2*

Gene	Exon	Codon	Amino acid substitution	Nucleotide	Nucleotide	Allele frequency no./total (rel. fraction)		
						Bilateral	Unilateral	
<i>BRCA1</i>	11	356	Gln/Arg	1186	CAG-CGG	9/150 (0.06)	6/150 (0.04)	
	11	694	none	2201	AGC-AGT	56/150 (0.37)	52/150 (0.35)	
	11	771	none	2430	TTG-CTG	56/150 (0.37)	52/150 (0.35)	
	11	871	Pro/Leu	2732	CCG-CTG	56/150 (0.37)	52/150 (0.35)	
	11	1038	Glu/Gly	3232	GAA-GGA	56/150 (0.37)	52/150 (0.35)	
	11	1040	Ser/Asn	3238	AGC-AAC	3/150 (0.02)	3/150 (0.02)	
	11	1183	Lys/Arg	3667	AAA-AGA	56/150 (0.37)	52/150 (0.35)	
	11	1347	Arg/Gly	4158	AGA-GGA	2/150 (0.01)	3/150 (0.02)	
	13	1435	none	4426	TCT-TTT	56/150 (0.37)	52/150 (0.35)	
	15	1512	Ser/Ile	4655	AGT-ATT	1/150 (0.01)	2/150 (0.01)	
	16	1613	Ser/Gly	4956	AGT-GGT	56/150 (0.37)	52/150 (0.35)	
	<i>BRCA2</i>	10	289	Asn/His	1093	AAT-CAT	6/150 (0.04)	9/150 (0.06)
		10	372	Asn/His	1342	AAT-CAT	43/150 (0.29)	42/150 (0.28)
		10	455	none	1593	TCA-TCG	6/150 (0.04)	9/150 (0.06)
11		991	Asn/Asp	3199	AAC-GAC	6/150 (0.04)	9/150 (0.06)	
11		1132	none	3624	AAA-AAG	45/150 (0.30)	32/150 (0.21)	
11		1172	Ser/Leu	3742	TCG-TTG	2/150 (0.01)	1/150 (0.01)	
11		1269	none	4035	GTT-GTC	28/150 (0.19)	26/150 (0.17)	
11		1819	Pro/Ser	5683	CCC-TCC	1/150 (0.01)	0/150	
11		1915	Thr/Met	5973	ACG-ATG	4/150 (0.03)	2/150 (0.01)	
14		2414	none	7470	TCA-TCG	24/150 (0.16)	23/150 (0.15)	
18		2728	Val/Ile	8410	GTT-ATT	1/150 (0.01)	1/150 (0.01)	
22		2950	Lys/Asn	9078	AAG-AAT	1/150 (0.01)	0/150	

Codon and nucleotide positions were designated according to the *BRCA1* and *BRCA2* cDNA sequences. Nucleotide substitutions are shown within their codon context. Allele frequencies are given as allelic counts out of the total chromosomes and as relative frequencies (in brackets). Haplotype or genotype frequencies were not significantly different between both groups for either of the substitutions (see text).

mutation carriers in bilateral breast cancer. Although the confidence intervals are wide and the results have to be confirmed with larger sample sizes, the identification of only 5 mutation carriers among 75 patients with bilateral breast cancer suggests that the carrier frequency in our hospital-based sample may be about two- or threefold lower than expected from multi-case family studies. An important difference between population-based association studies and family-based studies lies in the fact that the magnitude of relative risks in the latter is conditional on shared genetic and environmental influences. It is therefore possible that the high frequency of bilateral disease in multi-case breast cancer families may be due to a familial aggregation of other susceptibility factors modifying the penetrance of *BRCA1* and *BRCA2* mutations. Such a view would be supported by the recent observation that within *BRCA1* families the contralateral breast cancer risk is also associated with the age at onset of the primary tumour (Verhoog et al, 2000).

These apparent differences between family-based and population-based studies may have implications for therapeutic decision making. For instance, it has been proposed that the magnitude of life expectancy gains from cancer prevention strategies, including prophylactic contralateral mastectomy, strongly depends on the penetrance of the *BRCA* mutation (Schrage et al, 2000). Our results suggest that the penetrance with regard to contralateral disease is – at least in our hospital-based sample – not necessarily a feature determined only by the nature or location of the *BRCA* mutation itself, as the mutational types and frequencies were similar in our patients with unilateral and bilateral breast cancer. There may be additional, yet undetermined, factors that influence the contralateral breast cancer risk. Some of the known putative genetic modifiers include ATM mutations, rare H-ras alleles or androgen receptor gene variations (Phelan et al, 1996; Larson et al, 1998;

Rebbeck et al, 1999; Broeks et al, 2000). We have presently no evidence that bilaterality of breast cancer alone were a strong indicator of *BRCA1* and *BRCA2* mutations if adjusted for age and family history.

ACKNOWLEDGEMENTS

We wish to thank Andrea Korte, Hildegard Frye and PD Dr Manfred Stuhmann for their support with DNA extractions. Part of this work was funded by a grant from the Medical School Hannover to MB and TD.

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APPENDIX

Table A1 Primers used for the genomic PCR amplification of *BRCA1* and *BRCA2* gene segments

Exon	Forward primer	Reverse primer	Annealing (°C)	Size (bp)
<i>BRCA1</i>				
2	GTGTTAAAGTTCATTGGAACAG	GCATAGGAGATAATCATAGGAATCC	58	162
3	TGAGGCCTTATGTTGACTCAG	CTGGGTATGAAGGACAAAAAC	62	220
5	CTCTTAAGGGCAGTTGTGAG	TTCCTACTGTGGTTGCTCC	64	234
6	GGTTGATAATCACTTGCTGAG	GCAAACCTCCTGAGTTTTCATG	62	200
7	GCATACATAGGGTTTCTCTTGG	AGAAGAAGAAGAAAACAATGG	59	258
8	CTGGCCAATAATGCTTGACTG	CTATAAGATAAGGAATCCAGC	60	207
9	GTTGAATATCTGTTTTTCAAC	CCAGCTTCATAGACAAAGGTTT	62	172
10	TTGACAGTTCTGCATACATG	AGGTCCCAAATGGTCTTCAG	62	232
11/F	CACCTCCAAGGTGTATGAAG	CTCTAGGATTCCTGAGCATGG	58	506
11/1	CCAAAGCGAGCAAGAGAATCTC	ATGAGTTGTAGGTTTCTGCTGTG	63	1337
11/2	ACAATTCAAAAGCACCTAAAAAG*	AACCCCTAATCTAAGCATAGCATTC	63	1463
11/3	CACCACCTTTTCCCATCAAGTC*	TTATTTTCTTCCAAGCCCGTTCC	63	1123
11/R	CTACTAGGCATAGCACCGTTGC	CACCTCTATAATAGACTGGG	58	419
12	AAGACACAGCAAGTTGCAAGCG	GGGATACACTACTGAAATGC	64	224
13	TAATGGAAGCTTCTCAAAG	TCCTTACTCTTCAGAAGGAG	58	306
14	TCACTATCAGAACAAGCAG	GATGTCAGATACCACAGCATC	58	220
15	TGCCAGCAAGTATGATTTG	TTGTTCCAATACAGCAGATG	59	289
16	CTTAACAGAGACCAGAACTTTG	GTCATTAGGGAGATACATATGG	58	415
17	GTGTGCTAGAGGTAACATCATG	GCAGCAGATGCAAGGTATTC	58	177
18	TCTTAGGACAGCACTTCCCTG	CTGAGGTGTTAAAGGGAGGAG	64	190
19	AGCACGTTCTTCTGCTGTATG	TCTGGTTAGTTTGTAAACATC	58	120
20	TCTCTTATCCTGATGGGTTG	GGGAATCCAAATTCACACAGC	62	338
21	CATCAGGTGGTGAACAGAAG	GTAAGACAAGGGCTGGTGTCTG	64	243
22	GGTAGAGGGCCTGGGTTAAG	CAGGTGCCAGTCTTGCTCAC	64	208
23	TCCAGTAGTCTACTTTGAC	ACCCCATATAGCACAGGTAC	60	149
24	GATTGATTAGAGCCTAGTCCAGG	CAGCCTGAATAGAAAGATAGGGC	64	519
<i>BRCA2</i>				
2	TATTTACCAAGCATTGGAGG	TAGAAAACACTTTCTCGGTG	64	156
3	ACAAATTTGTCTGTCACTGG	GTAGTTCTCCCCAGTCTACC	64	338
4	TCATTTCCAGTATAGAGGAG	CTTCTACCAGGCTCTTAGCC	59	329
5/6	AAGATAAACTAGTTTTTGCCAG	GGGCAAAGGTATAACGCTATTGTC	56	328
7	ATATCCTTAATGATCAGGGC	GAGATGACAATTATCAACCTC	59	231
8	GTAATCAAATAGTAGATGTGC	TGTTAGCAATTTCAACAGTC	59	190
9	AAGTGAACCATGGATAAGGG	CACGGGAGGCAGAGGTTGCGG	61	322
10/F	TCTATGAGAAAGGTTGTGAG	CAAAGACGGTACAACCTCCTTG	59	418
10	TATACTTTAACAGGATTTGG*°	ACACAGAAGGAATCGTCATC	53	1116
11/F	CTTCCTGCCTCAGCCTCCCAAAG	GCTAGTTAAGGACAAAGTTGG	58	154
11/1	TTTTTATGTTTAGGTTTATT*°	TGCATTCCTCAGAAGTGGTC	57	1569
11/2	AAACCAAGCTACATATTGC*°	TAATTTCTACATAATCTGCAG	59	1751
11/3	TGGCTTAGAGAAGGAATAT*°	AAAATAGTGATTGGCAACACG	63	1769
12	AATGGTCTATAGACTTTTGAG	ATCCATACCTATAGAGGGAG	59	218
13	ACAGTAACATGGATATTCTC	ACGAGACTTTTCTCATACTG	59	187
14	TGAGGGTCTGCAACAAAGGC	CAGGACATTATTTAACAACGG	64	573
15	GTGCCTGGCCAGGGTTGTGC	CTCTGTCATAAAGCCATCAG	60	281
16	ATTGTGTGATACATGTTTAC	GTTTCGAGAGACAGTTAAGAG	59	322
17	TTCAGTATCATCCTATGTGG	TACTGCGTATATGATTACG	59	267
18	ACAGTGAATTCTAGAGTCAC	TTTAACTGAATCAATGACTG	56	460
19	AAGGCAGTTCTAGAAAGATG	AAGAGACCGAAACTCCATATC	54	339
20	CCACTGTGCCTGGCCTGATAC	GTCTCTAAGACTTTGTTCTC	64	283
21	CTTTGGGTGTTTTATGCTTG	CTTGAATAATCATCAAGCCTC	59	251
22	CCATCTAGTTACAATAGATG	ATCATTTTGTAGTAAGGTC	54	339
23	GATAATCACTTCTCCATTGC	TAAACTAACAAGCACTTATC	54	260
23/24	GATAATCACTTCTCCATTGC	AACTGGTAGCTCCAATAATC	64	531
25	GGCATATTAGAGTTTCCTTTC	CAAATGTGTGGTGTAGCTG	64	361
26	CATCGGCATGTTGACAATTGG	CATTTTACCATGTTTACTAGG	56	390
27	TAGGGGAGGGAGACTGTGTG	AACTGAAAGGTTAAGCGTC	64	779

/° A T7 promoter and a eukaryotic translation initiation sequence with an ATG start codon were added at the 5'-end for further use of this primer in a protein truncation test.

Table A2 Duplex PCR conditions

Gene/Exon	Annealing (°C)	Primer ratio
BRCA 1/9 + 20	60	3:1
BRCA 2/2 + 3	62	1:1
BRCA 2/4 + 12	57	1:1
BRCA 2/7 + 8	59	1:1
BRCA 2/13 + 17	57	1:1
BRCA 2/16 + 21	57	1:1

Table A3 Restriction fragments for SSCP analysis

Gene/Exon	Restriction enzyme	Size of fragments (bp)
1/11 F	<i>EcoRI</i>	187/319
1/11/1	<i>AlwI, HphI</i>	363/270/372/332
1/11/2	<i>DdeI</i>	238/207/225/306/383
1/11/3	<i>FokI, EcoNI</i>	358/371/186/208
1/11R	<i>EcoNI</i>	135/284
1/16	<i>MboI</i>	131/284
1/24	<i>MboI</i>	173/346
2/10F	<i>NlaIII</i>	171/289
2/10	<i>DpnII, BsaAI</i>	297/250/236/333
2/11/1	<i>HphI, SpeI</i>	297/316/290/306/360
2/11/2	<i>PstI, BclI, BsaI, ScrFI</i>	366/318/273/169/216/389
2/11/3	<i>HphI, BsaAI</i>	379/384/272/175/273/294
2/14	<i>DraI</i>	303/270
2/18	<i>DdeI</i>	290/170
2/23+24	<i>MspI</i>	208/323
2/26	<i>DpnII</i>	213/177
2/27	<i>DrdI/NlaIV/MboI</i>	218/169/257/133

Table A4a Restriction-enzyme based mutation screening assays

Gene/Exon	Primer pair	Size (bp)	Annealing	Substitution	Enzyme	Fragments
BRCA1/5	BR5F/BR5R	234	64	Cys61Gly	<i>Avall</i>	Cys 234, Gly 154/80
BRCA1/11	BS1/BS2	1337	63°C	Gln356Arg	<i>AlwNI</i>	Gln 396/941, Arg 1337
BRCA1/11	BS5/BS4	325	59°C	Glu1038Gly	<i>NlaIV</i>	Glu 325, Gly 172/153
BRCA1/11	BS5/BS4	325	59°C	Ser1040Asn	<i>AluI</i>	Ser 178/147, Asn 325
BRCA1/11	BS5B/R1347G	341	60°C	Arg1347Glu	<i>BsuF5/EcoNI</i>	Arg 189/152, Glu 189/134/18
BRCA1/15	BR15F2/BR15S-I	145	64°C	Ser 1511 ile	<i>AlwNI</i>	Ser 145, ile 125/20
BRCA1/16	BR16F/BR16R	415	58°C	Ser1613Gly	<i>Avall, ScrFI, NlaIV</i>	Ser 415, Gly 179/236
BRCA1/20	BS9/BS10	270	60°C	5382insC	<i>ScrFI</i>	wildtype 270, mutant 248/22
BRCA2/10	BS11/1342D	359	56°C	Asn372His	<i>NlaIII</i>	wildtype 214/22, mutant 236
BRCA2/10	BS11/1342D	359	56°C	Asn289His	<i>NlaIII, AflIII</i>	Asn 359, His 334/25
BRCA2/11	BS14/3624D	111	54°C	A3624G	<i>MwoI</i>	Asn 359, His 274/85
BRCA2/11	BS15/16	1751	59°C	Ser1172Leu	<i>Taq</i>	A 111, G 86/25
BRCA2/11	BS16B/4035D	361	56°C	T4035C	<i>TthIII</i>	Leu 1751, Ser 1630/121
BRCA2/11	BS19/18B	436	59°C	Thr1915Met	<i>NsiI</i>	T 361, C 336/25
BRCA2/11	6174delT/BS20A	375	61°C	6174delT	<i>PflMI</i>	Thr 436, Met 325/111
BRCA2/18	BR2-18F/V2728I-R	286	60°C	Val2728Ile	<i>HpaI</i>	mutant 375, wildtype 354/21
						Val 265/21, Ile 286

Table A4b Sequences of mismatch primers used in restriction-enzyme based screening assays

Gene/Exon	Gene alteration	Designation	Sequence (5'-3', mismatch)	Use in Table 4a
BRCA1/11	A4158G	R1347G-R	ATTTTCTTCCAAGCCCGTTCA T C	Reverse primer
BRCA1/15	G4654T	BR15-S/I	CTATTCTGAAGACTCCCA C AGC	Reverse primer
BRCA1/20	5382insC	BS10	CCAAAGCGAGCAAGAGAATC I C	Forward primer
BRCA2/10	C1342A	1342D	CTTCCACTCTCAAAGGGCTTCT C AT	Reverse primer
BRCA2/11	A3624G	3624D	GTCAGTTTGAATTTACTCAG C TTAG	Forward primer
BRCA2/11	T4035C	4035D	TCTTCAAGTTTTTGTGATGAC T CTG	Forward primer
BRCA2/11	6174delT	6174delT	GTGGGATTTTTAGCC C AGCAAG	Forward primer
BRCA2/18	G8410A	V2728I-R	GGGAGGATCTAACTGGGC T TAA	Reverse primer