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

U Hamann, X Liu, S Lange, H U Ulmer, A Benner, R J Scott

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 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. 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criteria were also screened for BRCA1 mutations and found not to harbour any changes in this gene (U Hamann, unpublished data).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. 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 43.5 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, 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.

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...
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. 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 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* 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, 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 non-sense mutations in exon 11, and one splice mutation in exon 23 in four HBC and four HBOC families (table 2). One polymorphism, 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, 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. In this family, 13 *BRCA2* mutation carriers were identified (10 direct

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

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

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

<table>
<thead>
<tr>
<th>Family</th>
<th>Exon</th>
<th>Nucleotide Codon</th>
<th>Base change</th>
<th>Amino acid change</th>
<th>Designation</th>
<th>Type*</th>
<th>Reported in BIC†</th>
<th>Breast (bilateral)</th>
<th>Breast ≤50y</th>
<th>Ovarian</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>11</td>
<td>3036</td>
<td>936</td>
<td>delACAA</td>
<td>Stop 958</td>
<td>3036delA</td>
<td>FS</td>
<td>24×</td>
<td>6×</td>
<td>1</td>
</tr>
<tr>
<td>86</td>
<td>11</td>
<td>3279</td>
<td>1017</td>
<td>delC</td>
<td>Stop 1042</td>
<td>3279delC</td>
<td>FS</td>
<td>–</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>83</td>
<td>11</td>
<td>4780</td>
<td>1518</td>
<td>G→T</td>
<td>Glu→Stop</td>
<td>E1518X</td>
<td>NS</td>
<td>–</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>33</td>
<td>11</td>
<td>5910</td>
<td>1894</td>
<td>C→G</td>
<td>Tyr→Stop</td>
<td>Y1894X</td>
<td>NS</td>
<td>14×</td>
<td>8 (1)</td>
<td>4</td>
</tr>
<tr>
<td>53</td>
<td>11</td>
<td>6265</td>
<td>2013</td>
<td>A→T</td>
<td>Lys→Stop</td>
<td>K2013X</td>
<td>NS</td>
<td>–</td>
<td>3 (2)</td>
<td>2</td>
</tr>
<tr>
<td>67</td>
<td>14</td>
<td>7296</td>
<td>2356</td>
<td>delTC</td>
<td>Stop 2358</td>
<td>7296delTC</td>
<td>FS*</td>
<td>–</td>
<td>3 (1)</td>
<td>2</td>
</tr>
<tr>
<td>76</td>
<td>23</td>
<td>9345</td>
<td>3039</td>
<td>G→A</td>
<td>Pro→Pro</td>
<td>9345G&gt;A</td>
<td>SP</td>
<td>2×</td>
<td>4 (1)</td>
<td>4</td>
</tr>
<tr>
<td>97</td>
<td>27</td>
<td>9894</td>
<td>3222</td>
<td>delT</td>
<td>Stop 3248</td>
<td>9894delT</td>
<td>FS</td>
<td>1×</td>
<td>4 (1)</td>
<td>1</td>
</tr>
</tbody>
</table>

* 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

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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.

**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.

**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

<table>
<thead>
<tr>
<th></th>
<th>BRCA2 mutation screening</th>
<th>BRCA1 mutation screening*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of families</td>
<td>No of families with mutations (%)</td>
</tr>
<tr>
<td>All families†</td>
<td>68</td>
<td>8 [12]</td>
</tr>
<tr>
<td>Breast/ovarian cancer families</td>
<td>17</td>
<td>4 [24]</td>
</tr>
<tr>
<td>Average age of diagnosis of breast cancer (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>4</td>
<td>1 [25]</td>
</tr>
<tr>
<td>40–49</td>
<td>5</td>
<td>2 [40]</td>
</tr>
<tr>
<td>Average age of diagnosis of ovarian cancer (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>1</td>
<td>0 [0]</td>
</tr>
<tr>
<td>40–49</td>
<td>5</td>
<td>0 [0]</td>
</tr>
<tr>
<td>≥50</td>
<td>8</td>
<td>2 [25]</td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Breast cancer families</td>
<td>51</td>
<td>4 [8]</td>
</tr>
<tr>
<td>Female and male breast cancer</td>
<td>4</td>
<td>0 [0]</td>
</tr>
<tr>
<td>Female breast cancer</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>No of breast cancer patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>2 [10]</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>1 [8]</td>
</tr>
<tr>
<td>Laterality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilateral</td>
<td>34</td>
<td>0 [0]</td>
</tr>
<tr>
<td>Bilateral</td>
<td>13</td>
<td>4 [31]</td>
</tr>
<tr>
<td>Average age of diagnosis (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>2</td>
<td>0 [0]</td>
</tr>
<tr>
<td>≥50</td>
<td>27</td>
<td>2 [7]</td>
</tr>
</tbody>
</table>

*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).
The analysis of 74 breast/ovarian cancer families previously screened for BRCA1 germline mutations showed a significant trend in the number of families with a BRCA1 mutation and a decreasing average age of diagnosis of breast cancer in HBOC 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 and the average age of ovarian cancer diagnosis (Exact Cochran-Armitage trend test, p=0.56) in HBOC families.

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. Similar mutation frequencies have been obtained in Austrian (8%), Finnish (8%), and Dutch (12%) families. A higher mutation frequency was found in US families (27%).

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.

Because of the strong association between BRCA1 mutations and ovarian cancer, a family history of ovarian cancer may be involved in the pathogenesis of male breast cancer in these families. These data suggest that other genes besides BRCA1 and BRCA2 genes should be performed in HBOC families. Furthermore, given that the number of BRCA2 mutations identified in our HBOC families, it is important that these families are also screened for both BRCA1 and BRCA2 mutations.

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. In the Dutch study, it was shown that this mutation, which affects the last nucleotide of exon 23, results in aberrant splicing. Recently, several other silent mutations in the fibrillin-1, coagulation factor VIII, and the ataxia-telangiectasia genes have been shown to induce exon skipping. 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 polymorphism changing Ile to Val was identified in family 134.

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.

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 BRD 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. 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.

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. In the Dutch study, it was shown that this mutation, which affects the last nucleotide of exon 23, results in aberrant splicing. Recently, several other silent mutations in the fibrillin-1, coagulation factor VIII, and the ataxia-telangiectasia genes have been shown to induce exon skipping. 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 polymorphism 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 calculated for by the PTT as it is incapable of identifying missense changes. Because only the coding region, and not 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 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, in 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.

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.

Because of the strong association between BRCA1 mutations and ovarian cancer, a family history of ovarian cancer 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. 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
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% to 19% and those of BRCA2 in only 12% of the families. In Finland, the corresponding percentages were 10% and 11% to 12% and in southern Sweden 23% and 10% to 11%, 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.

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