Large *BRCA1* Gene Deletions Are Found in 3% of German High-risk Breast Cancer Families

Carolin Hartmann\(^1\)\(^\dagger\), Anika L. John\(^2\)\(^\dagger\), Rüdiger Klaes\(^1\), Wera Hofmann\(^3\), Rainer Bielen\(^1\), Rolf Koehe\(^1\), Bart Janssen\(^1\), Claus R. Barthram\(^1\), Norbert Arnold\(^2\), and Johannes Zschocke\(^1\)*

\(^1\)Institute of Human Genetics, Ruprecht-Karls University Heidelberg, Germany; \(^2\)Department of Gynaecology and Obstetrics, Christian-Albrechts-University, Kiel, Germany; \(^3\)Department of Tumorgenetics, Max Delbrück Center for Molecular Medicine, Berlin, Germany

*Correspondence to: Johannes Zschocke, Institute of Human Genetics, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany; E-mail: johannes.zschocke@med.uni-heidelberg.de

\(^\dagger\)These authors contributed equally to the study.

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We have tested for large *BRCA1* gene rearrangements in German high-risk breast and ovarian cancer families previously screened negative for point mutations by dHPLC and sequencing. Using the novel MLPA method, two deletions of exons 1A, 1B and 2 and exon 17, respectively, were detected in four out of 75 families investigated in Southern Germany. An identical exon 17 deletion with the same breakpoints and a deletion of exons 1A, 1B and 2 were found by fluorescent multiplex PCR in two out of 30 families investigated in Northern Germany. Combining both populations, genomic rearrangements were found in 6% of the mutation-negative families and 3% of all high-risk families and account for 8% of all *BRCA1* mutations. Our data indicate that the exon 17 deletion may be a founder mutation in the German population. The prevalence of *BRCA1* gene deletions or duplications in our patients is similar to previous reports from Germany and France. Genomic quantification by MLPA is a useful method for molecular diagnostics in high-risk breast cancer families.

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INTRODUCTION

Point mutations and small insertions or deletions in the *BRCA1* and *BRCA2* genes (MIM# 113705 and 600185) are found in up to 50% of high-risk breast cancer families in Germany (Meindl, 2002; Meyer et al., 2003) and elsewhere. In contrast, large genomic rearrangements leading to functional loss of one of the two BRCA genes have rarely been recognised in most populations. This may be partly due to technical difficulties. Recently a novel method for genomic quantification, multiplex ligation-dependent probe amplification (MLPA) (Schouten et al., 2002), has been used to screen breast cancer families in The Netherlands and Italy. Large deletions and insertions in the *BRCA1* gene were found in up to 10% of high-risk families, accounting for up to one third of all *BRCA1* gene mutations (Hogervorst et al., 2003; Montagna et al., 2003). We have now used the MLPA method to test for genomic rearrangements in 75 Southern German high-risk breast and ovarian cancer families previously screened negative for point mutations by dHPLC and sequencing (Meyer et al., 2003). We also provide a follow-up to a

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previously published study on genomic rearrangements detected with a semi-quantitative fluorescent multiplex PCR method (Hofmann et al., 2003) in high-risk breast cancer families in Northern Germany.

SUBJECTS AND METHODS

Patients and Families

Following the guidelines of the German Consortium for Hereditary Breast and Ovarian Cancer (Meindl, 2002), families were classified into 6 categories: Families with 2 or more cases of breast cancer including at least two cases with onset under the age of 50 years (group A1), with at least one male breast cancer (group A2), with one or more cases of breast cancer and at least one ovarian cancer case (group B), with two or more cases of breast cancer including one case diagnosed before the age of 50 years (group C), with two or more cases of breast cancer diagnosed over 50 years (group D), and one sporadic breast cancer case that was diagnosed with an age of less than 35 years (group E). The comprehensive analysis of the BRCA1 and BRCA2 mutation spectra in Germany showed the highest mutation frequencies in patients of group B (53 %), A1 (37 %), and A2 (25 %), whereas the mutation frequencies were clearly lower in groups E (13 %), C (10%), and D (10 %), respectively (Meindl, 2002). Therefore, families pertaining to groups A1, A2, and B, respectively, were referred to as “high risk families”.

Figure 1: Electropherograms in normal and abnormal samples. a: Control sample, b and c: patient samples. Each peak represents one BRCA1 exon, recognised by a specific fragment size (x-axis: fragment size, y-axis: fluorescence intensity). Note decreased peak heights of exons 1A, 1B and 2 in Figure 1b and of exon 17 in Figure 1c.
As previously described, index patients from 251 Southern German families were investigated in Heidelberg for BRCA1 and BRCA2 mutations by denaturing high-performance liquid chromatography (dHPLC) analysis and sequencing (Meyer et al., 2003). 140 families belonged to high-risk groups, and trait-causing mutations (48 BRCA1 mutations and 17 BRCA2 mutations) were detected in 65 (46 %) families. DNA samples from 75 BRCA1/2-negative index patients were assessed using the MLPA technique, as described below. Expanding on a previously published study on BRCA1 rearrangements in high-risk breast cancer families from Berlin (Hofmann et al., 2003), we also investigated 30 previously unreported BRCA1/2 mutation-negative families out of a total of 65 high-risk families at the Center in Kiel. Patients fulfilled identical selection criteria as the Heidelberg patients and were tested with a semi-quantitative multiplex PCR method as described (Hofmann et al., 2003).

**MLPA analysis**

Genomic DNA was isolated from leucocytes using standard methods. Reagents for MLPA analysis were obtained from MRC Holland (Amsterdam, The Netherlands). One hundred ng of DNA were diluted in 4 µl DHPLC-H2O and denatured at 98°C for 5 minutes before adding 3 µl of "SALSA" probe mix and MLPA buffer. To ensure hybridisation of the probes with their target sequences the reaction mixture was denatured for 1 min and incubated for 16 hours at 60°C. 32 µl of a ligase/buffer mixture were added at 54°C and the samples were incubated for another 15 minutes. Termination of the ligation reaction was performed by heating to 98°C for 5 minutes. PCR amplification (36 cycles) of ligation products was carried out using standard conditions according to manufacturer’s instructions. One µl of each PCR product was diluted in 0.5 µl of Genscan-Rox 500 size standards and 8.5 µl of deionised formamide for fragment analysis on an ABI 3100 capillary sequencer using Genescan software (Applied Biosystems).

For statistical analysis we transferred the raw data (see Fig. 1) of each individual peak area into an Excel data sheet (Microsoft, Redmond, USA). The relative peak area (RPA) for each probe (exon) was determined by calculating the ratio of each single peak area to the total area of all peaks (including non-BRCA1 targets) in the sample. In order to obtain a normal "expected value" we pooled data from five control samples and calculated the mean RPA for each peak (corresponding to each probe or locus). The resulting value was taken as 100 %, corresponding to two autosomal gene copies. Standard deviations were calculated to assess the variability of the results in the control samples and thus the quality of the run. It was decided that the standard deviation of single peaks in the control samples should not exceed 25 %, and the mean standard deviation of all controls for each peak should not exceed 15 %. Repeat analysis was considered to be necessary when these criteria were not met.

For the assessment of test samples we compared the RPA for each probe to the expected RPA, resulting in a percentage value (RPA ratio, norm 100 %; see Fig. 2). RPA ratios of individual exons were considered normal and reliable if they differed less than 25 % from the expected RPA in controls. Results were considered indicative of a...
deletion of this probe or exon when RPA ratios were below 75% (see Fig. 2b), and indicative of a duplication when they were above 125%. MLPA analysis was repeated in these samples; the results were considered reliably abnormal when they were consistent in both analyses.

Fluorescent multiplex PCR

The method used for the identification of genomic rearrangements in the Northern German cohort involved multiplex amplification of short exon fragments corresponding to the 22 protein-coding exons and the 5’ untranslated region of the BRCA1 gene and visualisation on an ALFexpress sequencer, as previously described (Hofmann et al., 2003).

Characterisation of the detected deletions

In order to further characterise the deletions of BRCA1 we performed breakpoint analysis by long-range PCR (Expand Long Template PCR System, Roche Diagnostics, Germany) using manufacturer’s conditions. To define the genomic breakpoints, aberrant fragments were sequenced by a primer walking strategy. Because characterisation of the breakpoint junction of deletions involving exons 1A, 1B and 2 failed, we performed RT-PCR to determine the significance of these mutations. RNA preparations from peripheral blood leukocytes were carried out by using TRIzol reagent as recommended by the manufacturer (Life Technologies). Total RNA (1 µg) was reverse transcribed using an oligo(dT)$_{17}$-primer and 200 units of MMLV reverse transcriptase (SuperScript, Life Technologies) for 1 h at 42°C. RT-PCR products (primer sequences available on request) were subsequently analyzed by fluorescent dye-terminator sequencing. All nucleotide numbers refer to the wild type cDNA sequence of BRCA1 (accession # U14680, version # U14680.1) as reported in GenBank. The BRCA1 numbering for the traditional mutation nomenclature used in the BIC Database (http://research.nhgri.nih.gov/bic/) is based on the reference sequence as stated above where the A of the ATG translation initiation codon is at the position of 120 of BRCA1. The approved systematic nomenclature follows the rule that the A of the ATG translation initiation codon is +1 (den Dunnen and Antonarakis 2000) and numbers based on this nomenclature are provided in parenthesis.

Figure 3: Breakpoint analysis of exon 17 deletion (Heidelberg). Long-range PCR involving exons 16-18 results in a 7.5 kb amplicon (wt) whilst an additional 2.5 kb product was detected in the patients with deletion of exon 17 recognised by the MLPA analysis (arrow). Sequence analysis (right) showed a deletion of 5105 bp spanning from intron 16 to intron 17, denoted as IVS17+891del5105 (systematic nomenclature: c.4986+891del5105).
RESULTS

MLPA analysis consistently revealed large deletions in the **BRCA1** gene in four out of 75 independent Southern German families (Heidelberg). In two families, abnormal RPA ratios were detected for exons 1A (65 % and 51 %, respectively), 1B (70 % and 50 %) and 2 (56 % and 69 %), indicative of a large deletion of these exons. Figures 1b and 2b illustrate the results in one of these families. In two other families, abnormal RPA ratios were detected for exon 17 (56 % and 57 %, respectively; see also Figures 1c and 2c) indicative of a deletion of exon 17. In addition we confirmed a previously recognized exon 22 deletion and an exon 13 duplication in two control samples (data not shown). Deletions of exons 1A, 1B and 2, and exon 17, respectively, were also identified by fluorescent multiplex PCR in two out of 30 Northern German families (Kiel).

Breakpoint analysis of the exon 17 deletion by long-range PCR and sequencing in all three families showed loss of 5105 bp including the entire exon 17, formally described as IVS17+891del5105 (systematic nomenclature: c.49865105+891del5105, see Figures 3 and 4). This deletion is predicted to lead to a frameshift at the mRNA level and a non-functional protein. It is interesting to note that two families with this deletion originated from Southern Germany whilst the third family originated from Northern Germany.

Breakpoint analysis did not succeed for the deletions involving exons 1A, 1B, and 2 (Del. 1-2) despite extensive efforts by two different research teams and use of the same materials and methods as described elsewhere (Puget et al., 2002). We predicted that the Del. 1-2 would disrupt the promoter region of the **BRCA1** gene leading to a complete loss of mRNA transcripts of this allele. Therefore, we performed cDNA analysis from an index patient with the Del. 1-2 and with multiple common polymorphisms in the coding region of **BRCA1** as identified during mutational screening. Heterozygous signals were obtained from genomic DNA, whereas sequencing of RT-PCR products from the same patient displayed **BRCA1** expression from only one allele (Figure 5). These results clearly demonstrate the existence of the Del. 1-2 and its significance for the expression of **BRCA1**. Unfortunately, no heterozygous polymorphisms in the coding region were detected in the other two patients with this deletion. Presence of the mutation in the second Heidelberg family was proven by repeated MLPA analyses of independent samples from the same patient. The mutation in the Kiel patient was confirmed by MLPA.
DISCUSSION

We report the result of quantitative genomic analyses in the BRCA1 gene in German high-risk families for familial breast/ovarian cancer in whom previous analyses of the BRCA1 and BRCA2 genes by dHPLC and sequencing had failed to identify mutations. MLPA analysis of the BRCA1 gene in 75 Southern German high-risk breast cancer families led to the identification of Deletions of exons 1A, 1B and 2 in two independent families and a deletion of exon 17 in another two families. This number represents 5\% (4/75) of mutation-negative families after initial screening or 3\% (4/140) of all high-risk families. Large rearrangements represent 8\% (4/52) of all BRCA1 mutations in the study. Identical or similar mutations were identified in two out of 30 independent families investigated in Kiel. In this Northern German cohort, genomic rearrangements were found in 7\% (2/30) of mutation-negative families or 3\% (2/65) of all high-risk families, representing 9\% (2/22) of all BRCA1 mutations. Combining both cohorts, genomic rearrangements were found in 6\% of mutation-negative families and 3\% of all high-risk families; they account for 8\% of all BRCA1 mutations.

The prevalence of genomic rearrangements of the BRCA1 gene in our population is thus in line with previously reported data from France (9\%) (Casilli et al., 2002; Gad et al., 2002) but is lower than in the previously reported Dutch and Italian studies in which deletions and duplications represented one quarter to one third of BRCA1 gene mutations (Hogervorst et al., 2003; Montagna et al., 2003). Overall mutation detection rates were not calculated in these studies. The discrepancies in deletion/duplication detection rates are unlikely to be due to methodological differences since all three studies used the same commercially available MLPA kit with only small differences in the data analysis approach. The most likely explanation for the lower prevalence of large deletions in our German patients is a different genetic background of the investigated populations. It has been previously shown that one deletion comprising exon 22 is a common founder mutation in The Netherlands (Petrij-Bosch et al., 1997); excluding this deletion and the previously recognized deletion of exon 13, genomic rearrangements were detected in only five out of 661 mutation-negative families investigated in the Dutch study (Hogervorst et al., 2003). The

Figure 5: cDNA analysis from a patient with a deletion of exons 1A, 1B, and 2 of the BRCA1 gene. Sequencing of genomic DNA revealed multiple common polymorphisms at various positions (e.g. c.2430, c.3232, and c.4956) of the BRCA1 gene (upper panel), whereas cDNA analyses from the same patient showed expression of the BRCA1 gene from only one allele (lower panel).
absence of the common Dutch deletions in our patients is in line with evidence that the German population is different from the Dutch population and may be more closely related to Eastern European populations (Zschocke, 2003).

Comparing the intron sequences containing the breakpoints of the 5.1 kb deletion revealed a high similarity of two Alu sequences, an AluSp sequence in intron 16 and an AluY sequence in intron 17. Thus the deletion of \textit{BRCA1} exon 17 is the result of a recombination between these closely related sequences. Most of the reported large rearrangements detected in the \textit{BRCA1} gene were considered to be Alu-mediated. The here described deletion is the third one resulting in a deletion of exon 17 and all are Alu-mediated (Puget et al., 1997; Montagna et al., 1999). It should be noticed that three times the same region is concerned, but the recombination of different Alu sequences results in different deletion breakpoints. Although there are various possibilities of Alu-mediated recombination in this region (intron 17 contains 5 Alu sequences; intron 16 4 Alu sequences) the breakpoints found in the three independent families of our study were identical. This suggests that the described exon 17 deletion may be a founder mutation in the German population.

Our data confirm that large \textit{BRCA1} deletions or duplications account for a relatively small proportion of familial breast cancer cases in Germany. Nevertheless, considering costs and sensitivity of dHPLC/sequencing vs. MLPA for \textit{BRCA1} mutation analysis, it may be argued that MLPA could be employed as a first-line mutation detection method in familial breast cancer. MLPA analysis would have removed six families from complete point mutation screening in the two cohorts, with cost savings exceeding the calculated expenditure of first-line MLPA analysis in all patients. Our data confirm that MLPA is a rapid, reliable and cost-efficient method for the identification of large \textit{BRCA1} deletions or duplications. Data analysis is still elaborate but may be advanced by a standardised sequence of operations and statistical analysis as developed by us.

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\section*{REFERENCES}


