Gross rearrangements in *BRCA1* but not *BRCA2* play a notable role in predisposition to breast and ovarian cancer in high-risk families of German origin

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Abstract   A total of 226 index cases from high-risk hereditary breast and ovarian cancer families of German origin who had tested negative for small nucleotide alterations in *BRCA1* and *BRCA2* were analyzed for gross genomic rearrangements at the two gene loci by the multiplex ligation–dependent probe amplification technique. Six large genomic alterations were identified in *BRCA1*, while no gross rearrangements were found in *BRCA2*. The six *BRCA1* mutations included two novel mutations including a deletion of exon 5, and a deletion comprising exons 5–7, as well as three distinct gross alterations previously reported, including a deletion of exons 1A, 1B, and 2, two duplications of exon 13, and a deletion of exon 17. To understand the mechanisms underlying the genomic rearrangements within the *BRCA1* gene and to provide a simple PCR-based assay for further diagnostic applications, we have defined the molecular breakpoints of the deletion/insertion mutations. In all cases, our data point to a mechanism by which illegitimate crossing over between stretches of direct repeat sequences as small as 9 base pairs (bp) and up to 188 bp may have occurred. Overall, we provide evidence that gross rearrangements within the *BRCA1* gene locus may be as frequent as 3% in primarily mutation-negative tested high-risk familial breast and ovarian cancer of German ancestry, while large alterations involving the *BRCA2* locus do not appear to play a significant role in disease etiology. These findings have important implications for genetic counseling and testing of high-risk breast and ovarian cancer families.   © 2006 Elsevier Inc. All rights reserved.

1. Introduction

Heterozygous germline mutations in the *BRCA1* and *BRCA2* genes confer a significantly increased lifetime risk for the development of breast and ovarian cancer [1–3]. For accurate risk assessment in members of families with a high incidence of early breast and ovarian cancer, it is therefore essential to identify the family-specific, disease-associated alteration. In the great majority of cases, the known mutation spectrum in the *BRCA1* and *BRCA2* genes includes small nucleotide alterations leading to premature termination of translation [4]. As a result, rearrangements encompassing genomic regions of the BRCA1 and BRCA2 loci are to be expected as part of the mutation spectrum of the two disease genes. Due to the widespread use of polymerase chain reaction (PCR)-based mutation identification technologies such as denaturating high-performance liquid chromatography or direct sequencing, such large genomic alterations may be greatly underrepresented in current mutation databases.

In 1997, Petrij-Bosch et al. [5] performed the first comprehensive *BRCA1* mutational analysis, which had an emphasis on large chromosomal rearrangements. Their data suggested that approximately one third of the identified *BRCA1* mutations in the Dutch breast and ovarian cancer population are attributable to gross deletions/insertions. This unexpectedly high proportion of gross rearrangements was shown to result from historical founder situations in the Dutch population. Hogervorst et al. [6] subsequently confirmed these findings by using a novel multiplex ligation-dependent probe amplification (MLPA) method. A similarly high rate of gross genomic alterations (40%)

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was also found in northern Italian breast/ovarian cancer families, although this number was calculated on a series of only 15 mutations and thus may not reflect the true proportion of *BRCA1* rearrangements in Italian hereditary breast cancer cases [7]. In a cohort of 71 American families, the identified rearrangements represented 8% based on all *BRCA1* mutations identified [8]. In a French study, five distinct rearrangements were detected in a cohort of 52 mutation-negative families, which were selected because of their high probability of *BRCA1*- or *BRCA2*-linked predisposition [9]. For the German population, reports indicate a lower incidence of gross alterations in *BRCA1*, ranging from 1 in 59 [10] to 6 in 105 [11] mutation-negative high-risk families. In contrast to *BRCA1*, reports of large rearrangements in the *BRCA2* gene are sparse [12–14].

In this report, we investigated patients from 226 German breast and ovarian cancer families by the MLPA method to assess the frequency of genomic rearrangements in the *BRCA1* and *BRCA2* genes in a large cohort of German origin. Before this study, the patients' two breast cancer genes were pre-analyzed for small nucleotide alterations in their coding regions and immediately flanking intronic sequences, but no disease-associated alteration could be detected. Our data show that gross genomic alterations in the *BRCA1* gene occur in up to 3% (6/226) of such preselected high-risk breast and ovarian cancer families. The contribution of this type of mutation at the *BRCA2* locus appears to be of little or no significance. We therefore conclude that in addition to conventional DNA diagnostic testing by sequencing, familial breast cancer patients could greatly profit from searching for large genomic rearrangements at the *BRCA1* gene locus by MPLA analysis.

2. Materials and methods

2.1. Patient recruitment

Two-hundred twenty-six breast and/or ovarian cancer patients were recruited through a clinico-genetic counseling setting at the Familial Breast and Ovarian Cancer Centers at Würzburg and Münster, Germany. The Centers are part of the German Consortium for Hereditary Breast and Ovarian Cancer founded in 1996 by the Deutsche Krebshilfe in Bonn. Before this study, pathogenic point mutations and small nucleotide alterations in *BRCA1* and *BRCA2* were excluded in our patient cohort by PCR-based mutation detection techniques. According to established guidelines [15], the high-risk breast and ovarian cancer families were classified into five risk groups on the basis of their family history and tumor spectrum as follows: families with two or more cases of breast cancer, including at least two cases with onset under the age of 50 years (group A, $n = 111$); families with one or more cases of breast cancer and at least one case of ovarian cancer (group B, $n = 39$); families with at least one case of male breast cancer (group C, $n = 20$); families with three or more cases of breast cancer, of which

one case was diagnosed before the age of 50 years (group D, $n = 53$); and families with one case of breast cancer diagnosed under the age of 35 years (group E, $n = 3$).

2.2. DNA analysis

Genomic DNA from index cases was extracted from peripheral blood lymphocytes using standard procedures. Mutation analysis of *BRCA1* and *BRCA2* was done by single-stranded conformational analysis, denaturing high-performance liquid chromatography (dHPLC), protein truncation test, and direct sequence analysis essentially as described previously [4]. MLPA probes were designed by MRC-Holland (Amsterdam, The Netherlands) for genomic quantification of each of the 24 exons of *BRCA1* (Salsa P002 BRCA1) and 23 of the 27 exons of *BRCA2* (Salsa P045 BRCA2/CHEK2). It should be noted that *BRCA2* exons 5, 6, 23, and 27 were not amplified by the MPLA kit Salsa P045. Reactions were performed according to the manufacturer's instructions [6]. PCR products were quantified on an ABI310 or ABI3700 capillary sequencer (Applied Biosystems, Darmstadt, Germany). Genescan-ROX 500 was added to all probes to facilitate peak identification.

2.3. Quantitative and statistical analysis

For data analysis, the surface and area of each peak was calculated by Genescan software (Applied Biosystems, Foster City, CA) and imported into an Excel spreadsheet template. Relative peak ratios were calculated for each PCR amplicon by three methods relating the peak area to the following: (1) the neighboring reference peaks, (2) the flanking BRCA amplicon peaks, and (3) the overall peak areas. The values were subsequently normalized with corresponding values obtained from control individuals.

2.4. Breakpoint analysis of novel rearrangements

To characterize the rearrangements identified by MPLA, long-range PCR formats were designed to successively narrow the respective breakpoint regions. The genomic locus of *BRCA1* was established by aligning the mRNA sequence (NM_007296) to the finished human reference genome (built October 2004). Oligonucleotide primer pairs tightly flanking the respective breakpoint regions were as follows: BC1_Pseudo2F: 5'-acc taa aat tcc ttc tgc tgg ac-3'/ Chr17_IB_R7: 5'-caa aga acg act aac ctg gca-3' (*BRCA1* deletion exon 1A, 1B, 2); (BC_del5F5) 5'-gct tgt aat tca cct gcc a-3'/ (BC_del5R2) 5'-tga cag agc agg acc ctg tc-3' (*BRCA1* deletion exon 5); BC1_del5-7F3: 5'-acc tag tca ccc cct tca cc-3'/ BC1_del5-7R4: 5'-cca gcc cca gaa tga tta ctt-3' (*BRCA1* deletion exon 5-7); dup13F: 5'-gat tat ttc ccc cca ggc ta-3'/ dup13R: 5'-aga tca tta gca agg acc tgt g-3' (*BRCA1* duplication exon 13) [16]; (BC_del17F2) 5'-aca tgg gtg aat tta cca gc-3'/(BC_del17R2) 5'-aca aga atc tcg ctc ttg tc-3' (*BRCA1* deletion exon 17). PCR amplifications were performed in a 25-μL reaction

containing 50 ng genomic DNA, 10 pmol/μL each of forward and reverse primer (Sigma-Aldrich Chemie, Munich, Germany), 2.5 μL reaction buffer (0.5 mol/L KCl, 0.2 M Tris, 10 mmol/L MgCl$_2$), 1.25 mmol/L dNTPs, and 1 unit Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany).
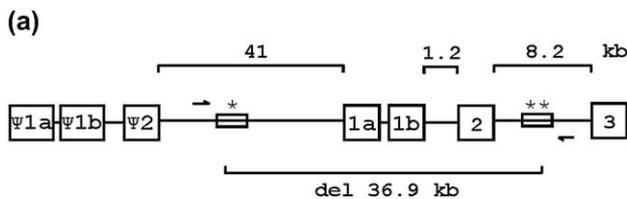
2.5. Sequence analysis

Purified PCR products were subjected to fluorescence-labeled cycle sequencing with BigDye Terminator chemistry (DNA Cycle Sequencing kit version 3.1; Applied Biosystems, Darmstadt, Germany) and analyzed on an ABI 3700 automated sequencer. Data were evaluated with the software package Sequencing Analysis version 3.6.1 and Sequencing Navigator.

3. Results

The 226 unrelated breast cancer cases that tested negative for *BRCA1* and *BRCA2* point mutations and small insertions/deletions were analyzed by the MLPA method for gross genomic rearrangements at the two breast cancer gene loci. Gene electropherograms showed reproducible reductions/increases of specific fluorescence signals in six cases at the *BRCA1* locus compared to normal control samples (data not shown). There was no indication of gross rearrangement at *BRCA2*. For the six *BRCA1* alterations identified, the molecular breakpoints were determined (Fig. 1; Table 1).

In family pC2, we found a deletion of *BRCA1* exons 1A, 1B, and 2 in three affected siblings (Fig. 1, a and d; Table 1). PCR with oligonucleotide primers located in intron 2 of the *BRCA1* pseudogene (Ψ*BRCA1*) and intron 2 of the *BRCA1* gene specifically amplified a 1.1-kb product in the three patients but not in controls (Fig. 1d). Sequence alignment of the PCR product to the human genome sequence assembly (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html) revealed a fusion sequence resulting from a 36.9-kb deletion that extends from intron 2 of Ψ*BRCA1* to intron 2 of the functional *BRCA1* gene and,
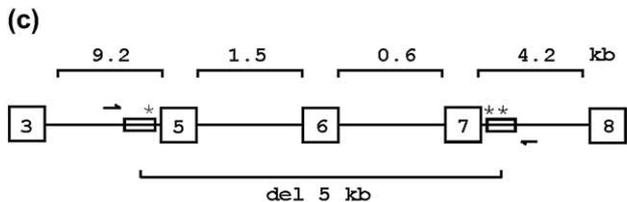

Fig. 1. Breakpoint identification in patients with gross rearrangement in the *BRCA1* gene. Schematic representation of the genomic alterations leading to del ex1a, 1b, 2 (a), del ex5 (b), and del ex 5-7 (c). Partial sequences of the upstream and downstream breakpoint regions are given. Identical sequences are indicated by vertical bars. The genomic rearrangements identified in this study were confirmed by mutation-specific PCR assays (d). Oligonucleotide primers were used with relative location as indicated in a–c (half arrows).

Table 1
Summary of gross insertions/deletions in the *BRCA*1 gene and associated malignancies in families

Risk group (no. of patients)	Family ID	Findings in *BRCA1*	Breast/ovarian cancers and other tumors
A (n = 111)	p250	del ex 5	4 unilateral breast cancer ≤ 50 yr, 1 unilateral breast cancer > 50 yr, no other malignancies reported
	p74	dup ex 13	4 unilateral breast cancer ≤ 50 yr, no other malignancies reported
	p4	del ex 17	3 unilateral breast cancer ≤ 50 yr, 1 unilateral breast cancer > 50 yr, no other malignancies reported
B (n = 39)	pC2	del ex1-2	1 bilateral breast cancer ≤ 50 yr, 6 unilateral breast cancer ≤ 50 yr, 2 unilateral breast cancer > 50 yr, 1 ovarian cancer, 1 colorectal cancer, 2 undefined gynecological tumors, 1 melanoma, 1 testicular cancer, 1 thyroid cancer
	pG6	del ex5-7	2 unilateral breast cancer ≤ 50 yr, 1 ovarian cancer, 1 stomach cancer
	pF2	dup ex 13	1 bilateral breast cancer ≤ 50 yr, 1 unilateral breast cancer ≤ 50 yr, 1 unilateral breast cancer 50 yr, 1 ovarian cancer, 1 stomach cancer

in addition, includes the entire *NBR2* gene locus [17]. The immediate breakpoint region comprises 188 bp of perfect nucleotide (nt) matches between Ψ*BRCA1* and sequences in intron 2 of *BRCA1*. Based on the sequences of BAC clone AC060780, the breakpoints occurred between nt 71053 and nt 71240 in Ψ*BRCA1*, and nt 34118 and nt 34305 in *BRCA1* intron 2. At the mRNA level, this deletion hypothetically generates a chimeric *BRCA1* transcript consisting of Ψ*BRCA1* exons 1A, 1B, and 2 fused to *BRCA1* exons 3–24, which lack the *BRCA1* 5'-UTR and the *BRCA1* translation initiation codon. Thus far, it is not known whether the chimeric gene is transcribed and possibly translated [17]. If so, an alternative in-frame methionine start codon may be used deleting the immediate N-terminal 47 amino acids of the BRCA1 protein (p.Met1_Cys47del).

In family p250, a 244-bp deletion was identified encompassing the entire coding region of *BRCA1* exon 5, including the consensus splice acceptor and donor sequences (Fig. 1, b and d; Table 1). The breakpoint region constitutes a stretch of 9 bp that flanks exon 5 as a direct repeat in the normal genomic sequence located at nt 21060 to nt 21068 in *BRCA1* intron 4 and nt 20816 to nt 20824 in *BRCA1* intron 5 (GenBank accession no. AC060780). As a result of the deletion, the mRNA of the mutant transcript lacks exon 5 and results in a fusion of *BRCA1* exon 3 to exon 6. This should lead to an in-frame deletion of 26 amino acids (p.Phe46_Arg71del).

In family pG6, a 5-kb deletion including *BRCA1* exons 5–7 was identified (Fig. 1, c and d; Table 1). The deletion breakpoints are located in an upstream AluSx (*BRCA1* intron 4 at nt 22035 to nt 22049, GenBank accession no. AC060780) and a downstream AluSc sequence (*BRCA1* intron 7 at nt 17011 to nt 17025, GenBank accession no. AC060780) and reveal a 15-bp region of complete sequence identity between the proximal and distal Alu repeat units (Fig. 1c). The removal of exons 5–7 causes a frameshift in protein translation and thus should result in a truncated protein lacking the immediate C-terminal 102 amino acids which are replaced by a short peptide sequence of 14 novel amino acids (p.Lys45_Lys147delinsAsnfsX162).

In the two apparently unrelated families p74A and pF2, a heterozygous duplication of *BRCA1* exon 13 was found (Fig. 1d). By sequencing the duplication junction, we determined the exon 13 duplication to be identical to the 6-kb duplication, ins6kbEx13, reported earlier with breakpoints occurring in a 23-bp region of perfect identity within two AluSx sequences located at nt 44346 to nt 44368 in *BRCA1* intron 12 and nt 50427 to nt 50449 in *BRCA1* intron 13 (GenBank accession no. L78833) [16]. The duplication of exon 13 leads to a frameshift and consequently to a premature termination of translation after adding another six novel amino acid residues at the C terminus (p.Val1454GlufsX1460).

In family p4, a deletion of *BRCA1* exon 17 was identified. PCR amplification and sequencing of the resulting 623-bp fragment demonstrated that this deletion is identical to the mutational event reported previously by Montagna et al. [18]. A 3-kb deletion is mediated by a recombination between an AluSp in *BRCA1* intron 16 (nt 58736 to nt 58758, clone L78833) and an AluSq in intron 17 (nt 61853 to nt 61875, clone L78833). Removal of exon 17 results in an out-of-frame translation leading to a truncated protein that lacks the original C-terminal 29 amino acids, which are replaced by 9 novel amino acids (p.Met1663_Thr1691delinsMetfsX1701).

4. Discussion

The present study provides a comprehensive survey of large genomic alterations at the two known breast cancer gene loci, *BRCA1* and *BRCA2*, in high-risk breast and ovarian cancer families of German origin. We show that *BRCA1*, but not *BRCA2*, is affected by this type of mutation, with gross genomic rearrangements in *BRCA1* accounting for approximately 3% of the 226 mutation-negative familial breast and ovarian cancer cases. In earlier studies, the frequency of large genomic rearrangements in *BRCA1* in cases of German ancestry preanalyzed for pathogenic point mutations, small insertions, or deletions varied between 1.7% (1/59) [10] and 5.7% (6/105) [11]. Overall,

these findings are in good agreement with our data with the variability probably due to differences in patient sample size and/or the selection criteria for high-risk breast and ovarian cancer families.

Subsequent to this study, we further analyzed 57 independent high-risk families by dHPLC, direct sequencing, and MLPA of the *BRCA1* gene. In this cohort, we identified a total of 10 pathogenic *BRCA1* mutations, including two large genomic rearrangements resulting in a relatively high proportion of gross genomic alteration to the overall number of BRCA1 mutations (2/10 or 20%). This is in contrast to findings in the two previous German studies revealing a ratio of 8.3% (3/36) [10] and 8.1% (6/74) [11] and is likely explained by the small sample size of the families analyzed in our study. Comparable data available from other European populations such as the French [9,19–21] or Italians [7] are still too scarce to make further conclusions regarding population-specific differences in their frequency.

Of the six *BRCA1* alterations identified, only duplication ex13 was found to be recurrent and was detected twice in our sample of 226 index cases. This finding is consistent with earlier reports demonstrating a common, ancestral founder for the *BRCA1* dup ex13 alteration [16,22]. The 3.1-kb deletion of *BRCA1* exon 17, although identified only once in our patient cohort, was found previously in two independently ascertained Italian families [18]. Another exon 17 deletion encompassing 5.1 kb of genomic DNA was identified in three German high-risk breast and ovarian cancer families [11], while a 1-kb deletion of exon 17 was detected in a single family of French descent [23].

To determine the underlying mechanisms causing the frequent rearrangements at the *BRCA1* locus but also to design simple PCR-based assays for their rapid analysis, we have characterized the molecular breakpoints in detail. In the majority of cases, the rearrangements result from well-established mechanisms mediated by *Alu* repeat elements. For example, the *BRCA1* exon 5–7 deletion results from a nonallelic homologous recombination between AluSx in intron 3 and AluSc in intron 7. Both *Alu* repeats share a homologous region of 15 bp at the site of crossover. *Alu*-mediated recombination is known as a common mutational mechanism in the *BRCA1* gene and can be attributed to the high proportion (41.5%) of *Alu* sequences within this gene locus [24]. In agreement with this, three of the five distinct *BRCA1* alterations identified in this study (deletion of exons 5–7, duplication of exon 13, and deletion of exon 17) are likely to be caused by *Alu*-mediated recombination.

Another important cause of unequal recombination is the presence of nonfunctional pseudogenes in the human genome with high sequence homology to at least parts of the functional counterpart. In the case of the *BRCA1* deletion encompassing exons 1A, 1B, and 2, the breakpoint junctions are located in highly homologous regions in intron 2 of both the Ψ*BRCA1* and the *BRCA1* gene loci. Interestingly, Puget et al. [17] identified two such deletions with differing breakpoints in intron 2 of *BRCA1* at nt 34439

(family F32) and nt 34339 (family F3514; GenBank accession no. AC060780). Analysis of our index patient from family pC2 revealed a third breakpoint in *BRCA1* intron 2 at nt 34118, equally resulting in the removal of exons 1A, 1B, and 2. This provides further evidence of a recombinational hot spot in the 5' region of the *BRCA1* locus.

Gross chromosomal deletions/insertions may also be mediated by tandemly arranged short sequence repeats. Such a mechanism likely underlies the 244-bp deletion encompassing exon 5, which was found in patient p250. Our data suggest a nonallelic, nonhomologous crossing within a short direct 9-bp repeat flanking the deletion region. Such short repeats are thought to cause a slipped mispairing during replication, causing deletions of varying sizes [25,26].

While three of the five rearrangements identified (deletion of exons 5–7, duplication of exon 13, and deletion of exon 17) cause a frameshift resulting in premature protein termination, the mutation removing exon 5 should not affect the reading frame but should be likely to result in an in-frame deletion of 26 amino acids affecting residues 46–71 of the BRCA1 protein. As this region of BRCA1 is part of a conserved RING finger domain (residues 23–64), the deletion may affect the dimerization of BRCA1 with BARD1 [27]. The importance of this motif for proper BRCA1 function has been further emphasized by the disease-associated *BRCA1* missense mutation Cys61Gly [28].

In contrast to *BRCA1*, gross alterations in *BRCA2* seem to be rare not only in our sample set but also in other studies analyzing different ethnic populations. Accordingly, our results (0/226) are similar to data from studies in the Netherlands (0/58) [29], Finland (0/82) [30], and Israel (1/47), where only one family of Turkish ancestry was shown to be carrier of an approximately 6.2-kb deletion in *BRCA2* [13]. In a French cohort, *BRCA2* rearrangements were found in 3 out of 39 cases analyzed [14]. In this study, selection criteria included families with at least one case of male breast cancer. This study group was therefore enriched for patients with a higher probability of carrying a *BRCA2* mutation. Although our study included 20 families with male breast cancer, we could not identify a *BRCA2* rearrangement. In general, the greatly reduced incidence of large genomic alterations affecting *BRCA2* compared to *BRCA1* is likely to result from differences in the density of *Alu* repeat sequences at the two loci, with *BRCA2* revealing a low frequency of *Alu* sequences of only 17%.

In conclusion, our study shows that gross genomic alterations in *BRCA1*, but not *BRCA2*, account for a substantial proportion of familial breast and ovarian cancer cases in Germany. As a result, our findings suggest that index cases from high-risk familial situations testing negative for small nucleotide alterations in *BRCA1* and *BRCA2* should be re-analyzed for possible genomic rearrangements in *BRCA1*. This will further strengthen risk prediction for members of high-risk breast and ovarian cancer families who are seeking genetic counseling.

Acknowledgments

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