RESEARCH ARTICLE

MLPA Screening in the *BRCA1* Gene From 1,506 German Hereditary Breast Cancer Cases: Novel Deletions, Frequent Involvement of Exon 17, and Occurrence in Single Early-Onset Cases

Stefanie Engert,[1] Barbara Wappenschmidt,[2] Beate Betz,[3] Karin Kast,[4] Michael Kutsche,[5] Heide Hellebrand,[1] Timm O. Goecke,[6] Marion Kiechle,[1] Dieter Niederacher,[3] Rita K. Schmutzler,[2] and Alfons Meindl[1]*

[1]Department of Obstetrics and Gynecology, Division of Tumor Genetics, Technical University, Munich, Germany; [2]Department of Obstetrics and Gynecology, Division of Molecular Gynecooncology, Technical University, Cologne, Germany; [3]Department of Obstetrics and Gynecology, Heinrich Heine University, Düsseldorf, Germany; [4]Department of Obstetrics and Gynecology, Carl Gustav Carus University, Dresden, Germany; [5]Laboratory for Molecular Medicine, Hamburg, Germany; [6]Institute for Human Genetics, Heinrich Heine University, Düsseldorf, Germany

Communicated by Peter Oefner

We present a comprehensive analysis of 1,506 German families for large genomic rearrangements (LGRs) in the *BRCA1* gene and of 450 families in the *BRCA2* gene by the multiplex ligation-dependent probe amplification (MLPA) technique. A total of 32 pathogenic rearrangements in the *BRCA1* gene were found, accounting for 1.6% of all mutations, but for 9.6% of all *BRCA1* mutations identified in a total of 1,996 families, including 490 with small pathogenic *BRCA1/2* mutations. Considering only high risk groups for hereditary breast/ovarian cancer, the prevalence of rearrangements is 2.1%. Interestingly, deletions involving exon 17 of the *BRCA1* gene seem to be most frequent in Germany. Apart from recurrent aberrations like del ex17, dupl ex13, and del ex22, accounting for more than 50% of all *BRCA1* LGRs, we could fully characterize 11 novel deletions. Moreover, one novel deletion involving exons 1–7 and one deletion affecting the entire *BRCA1* gene were identified. All rearrangements were detected in families with: 1) at least two breast cancer cases prior to the age of 51 years; 2) breast and ovarian cancer cases; 3) ovarian cancer only families with at least two ovarian cancer cases; or 4) a single breast cancer case prior to the age of 36 years, while no mutations were detected in breast cancer only families with no or only one breast cancer case prior to the age of 51 years. Analysis for gross rearrangements in 412 high-risk individuals, revealed no event in the *BRCA2* gene and only two known *CHEK2* mutations. However, in an additional 38 high-risk families with cooccurrence of female breast/ovarian and male breast cancer, one rearrangement in the *BRCA2* gene was found. In summary, we advise restricting *BRCA1* MLPA screening to those subgroups that revealed LGRs and recommend *BRCA2* MLPA screening only for families presenting with cooccurrence of female and male breast cancer. Hum Mutat 0,1–11, 2008. **© 2008 Wiley-Liss, Inc.**

KEY WORDS: hereditary breast and ovarian cancer; *BRCA1* mutation spectra and prevalence; large genomic rearrangements; multiplex ligation dependent probe amplification

INTRODUCTION

Screening of more than 3,000 German breast/and ovarian cancer families by PCR-based techniques revealed mutation frequencies between 10 to 60% in the two known *BRCA* genes [Meindl and German Consortium of Hereditary Breast and Ovarian Cancer (GCHBOC), 2002]. Furthermore, mutations in other predisposing genes like *CHEK2* [Dufault et al., 2004] and *ATM* [Dörk et al., 2001] (GCHBOC, unpublished data) were detected with very low prevalences. As a significant part of families without small mutations in *BRCA1*, *BRCA2*, *CHEK2*, or *ATM* have pointed to a strong genetic history, there had been an urgent need to determine the precise prevalence of large genomic rearrangements (LGRs) within the two known *BRCA* genes. During the last years, multiplex ligation-dependent probe

The Supplementary Material referred to in this article can be accessed at http://www.interscience.wiley.com/jpages/1059-7794/suppmat.

Received 24 July 2007; accepted revised manuscript 10 December 2007.

*Correspondence to: Alfons Meindl, Department of Obstetrics and Gynecology at the Technical University, Division for Gynecological Tumor Genetics, Ismaninger Str. 22, 81675 Munich.
E-mail: alfons.meindl@lrz.tum.de

Stefanie Engert, Barbara Wappenschmidt, and Beate Betz contributed equally to this work.

DOI 10.1002/humu.20723
Published online in Wiley InterScience (www.interscience.wiley.com).

amplification (MLPA) has been established as a powerful and reliable method to screen for such gross aberrations.

High prevalences of large rearrangements in the *BRCA1* gene (MIM# 113705) have been demonstrated in several populations [Walsh et al., 2006; Mayozer, 2005; Petrij-Bosch et al., 1997; Montagna et al., 2003; Gad et al., 2002]. In contrast, inconsistent results have been obtained so far for the German population. First studies indicated a low frequency [Hofmann et al., 2002, 2003], while two subsequent studies [Hartmann et al., 2004; Preisler-Adams et al., 2006] suggested a more prominent role of *BRCA1* rearrangements. However, due to the low number of families included in these investigations, no conclusive recommendations for genetic counseling or testing could be drawn. Furthermore, no reliable data about the spectrum and precise prevalences of specific gross rearrangements have been reported. No LGRs within the *BRCA2* gene (MIM# 600185) have been described so far for the German population [Preisler-Adams et al., 2006], but have been reported for other populations [Tournier et al., 2004; Gutierrez-Enriquez et al., 2007; Mayozer, 2005]. In order to substantiate the preliminary data for our population, we screened another 450 high-risk individuals.

Most studies on gross aberrations in the two known *BRCA* genes have been performed only in so-called high-risk groups. To overcome this restriction, in the screening approach for the *BRCA* genes, we also included single early-onset cases and familial cases with a more modest risk for *BRCA* mutations. This should lead to the definition of more reliable inclusion criteria for MLPA screening.

SUBJECTS AND METHODS

Patients and Families

In order to obtain reliable data on the frequency and characteristics of *BRCA1* or *BRCA2* deletions, we screened 1,506 families that tested negatively for *BRCA1/2* mutations after applying PCR-based techniques, for rearrangements in the *BRCA1* gene. A total of 395 selected high-risk families for *BRCA2* deletions from the high-risk groups A, C, and E (see below) with or without male breast cancer. In addition, 55 early-onset cases were screened (group G, see below).

Index patients from 1,506 unrelated families (Table 1) were recruited through a clinicogenetic counseling setting at the Familial Breast and Ovarian Cancer Centers in Cologne, Düsseldorf, Dresden, and Munich as well as from a Laboratory for Molecular Medicine in Hamburg. They had been categorized in different risk groups: Group A: three or more affected females with breast cancer, two of them diagnosed before the age of 51 years (n = 489); Group B: three or more affected females independent of age (n = 247); Group C: two affected females with breast cancer, both of them diagnosed before the age of 51 years (n = 176); Group D: two affected females with breast cancer, one of them diagnosed before the age of 51 years (n = 170); Group E: at least one case of breast and of ovarian cancer each (n = 290); Group F: two or more cases of ovarian cancer (n = 31); and Group G: single case of breast cancer diagnosed before the age of 36 (n = 103).

All affected index patients had been screened for small nucleotide alterations in *BRCA1* and *BRCA2* by the PCR-based mutation detection techniques DHPLC and/or direct DNA sequencing [Meindl and GCHBOC, 2002] and tested negatively for pathogenic mutations out of 1,996 families.

All the tested patients gave informed consent and most of them shared genetic counseling.

MLPA Analysis

Analysis was performed by the MLPA technique [Schouten et al., 2002; Hogervorst et al., 2003]. Genomic DNA was isolated from leukocytes of affected females by applying standard techniques. Reagents for MLPA analyses were purchased from MRC-Holland B.V. (Amsterdam, the Netherlands; www.mrc-holland.com) and the MLPA procedure was performed as recommended by the manufacturer. The SALSA MLPA P002 kit (MRC-Holland, Amsterdam, The Netherlands) was used for genomic quantification of each of the 24 exons of the *BRCA1* gene, in case of positive results with the P002 kit, samples were reanalyzed with the SALSA MLPA P087 kit (MRC-Holland) for confirmation. The SALSA MLPA P045 kit (MRC-Holland) was used for genomic quantification of each of the 25 exons of the *BRCA2* gene (missing exons 5 and 23) and the exon 9–10del and 1100delC mutations in the *CHEK2* gene, respectively. MLPA data were analyzed using either the MRC Coffalyser (MRC-Holland) or the National Genetics Reference Laboratories (NGRL)-Manchester (www.ngrl.org.uk/Manchester) analysis sheet as recommended by the company or research institution.

Comparative Genomic Hybridization (CGH) by High-Definition 60-Oligomer Microarrays

The Agilent High-Definition 60-oligomer microarrays with a density of 244 K were hybridized by using Agilent reagents (Agilent Technologies, Santa Clara, CA) according to the Agilent CGH Protocol Version 5, June 2007. A total of 500 nanograms of total genomic DNA and the same amount of human female DNA (Promega, Mannheim, Germany) was used as a control. The arrays were scanned by applying an Agilent Microarray Scanner with the Agilent Scanner Control software v7.0 with a resolution of 5 μm. Data were extracted by the Feature Extraction software v9.5 (Agilent Technologies, Santa Clara, CA) and analyzed using CGH Analysis software v3.4 (Agilent Technologies). Statistically significant regions of aberration were determined by the ADM-2 algorithm at a threshold of 6.0.

Primer Pairs for Genomic Long-Range and Nested PCR

Primers used for long-range PCR, sequencing of junction fragments, and characterization of cDNAs are listed in Supplementary Table S1 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat) and Table 2.

Identification of the Genomic Breakpoints and Designation of Mutations

Positive MLPA results indicating deletion or duplication of single or multiple exons were validated by the identification and characterization of the genomic breakpoint containing region, and/or by RNA/cDNA analysis. In case of three families with suspected deletions in either exons 1–2 or exons 1–7, segregation analysis was also performed. In order to further characterize new rearrangements in the *BRCA1* gene as indicated by the MLPA kit and confirmed by RNA analysis, long-range PCR formats were designed to identify genomic breakpoints. Designation of the genomic breakpoints is based on the numbering of the genomic clones L78833.1 or AC060780 for *BRCA1* and AY436640 for *BRCA2*. Designations for RNA aberrations are based on U14680.1 for *BRCA1*, NM_000059 for *BRCA2*, and NM_007194 for *CHEK2*.

Sequence Analysis

Purified RT-PCR products were sequenced with the BigDye Terminator chemistry (DNA Cycle sequencing kit version 1.1;

Applied Biosystems, Darmstadt, Germany) and analyzed on an ABI 3100 automated sequencer. Data were depicted with the software package Sequencing Analysis version 3.7 and Sequencing navigator (all from Applied Biosystems).

RESULTS

Description and Prevalence of Rearrangements in the BRCA1 Gene

MLPA analysis of the *BRCA1* gene revealed deletions or duplications in 32 out of 1,506 affected females (Supplementary Table S2). In routine *BRCA1/2* mutation analysis of 1,996 families performing PCR-based techniques, we identified 327 mutations in the *BRCA1* gene and 163 mutations in the *BRCA2* gene. The prevalence of gross aberrations in the *BRCA1* gene for all families tested, excluding 455 carriers with small mutations was 2.1%; including them it was 1.6%. While the frequency varied in the groups C (1.0%), A (1.9%), E (2.5%), G (2.6%), and F (3.8%) from 1.0 to 3.8%, no deletions were found in groups B and D. Restricted to the familial high risk groups (A, C, E, and F), the mutation prevalence was 2.1% (Table 1).

The 32 identified *BRCA1* deletions account for 9.6% of all *BRCA1* mutations detected in a total of 1,996 families and for 6.6% of all *BRCA1/2* mutations. We identified at least 18 different gross rearrangements in the *BRCA1* gene, including 13 novel ones (Table 2). One (Del5) has been found once before also in the German population [Preisler-Adams et al., 2006], the remaining four (Del 13, Del 17, Del 22, and Dupl 13) are also present or common in other populations. One of the two identified deletions involving exons 1–2 revealed to be novel and could, like two other larger novel deletions (del ex1–7 and del ex1–24), be confirmed by array-CGH, using the Agilent High-Definition 60-oligomer microarrays.

Validation of Known Gross Aberrations in the BRCA1 Gene

In one family (Family CO499) with a deletion of exon 13, sequencing of the junction fragment confirmed the heterozygous presence of a 3.8-kb deletion comprising exon 13 (g.44513–48347) as described for Dutch families [Petrij-Bosch et al., 1997]. In four families we identified the duplication of exon 13 (g.44369–50449dup) that has been found in different populations including the German one [Puget et al., 1999; BRCA1 Exon 13 Duplication Screening Group, 2000; Hofmann et al., 2002]. Sequencing of the PCR-amplified genomic junction fragments revealed in all four cases identity with the known duplication of exon 13.

Another Dutch founder mutation, deletion of exon 22 [Petrij-Bosch et al., 1997], was found in three families with multiple cases of breast cancer (risk groups A and E) and one early-onset case (group G). Sequence analysis of cDNA and of genomic junction

TABLE 1. **Prevalence of *BRCA1* Rearrangments in Different Risk Groups**

	LGR+	B1/2–	%	All[a]	%
A	**13**	**489**	**2.7**	**668**	**1.9**
B	**0**	**247**	**–**	**281**	**–**
C	**2**	**176**	**1.1**	**210**	**1**
D	**0**	**170**	**–**	**192**	**–**
E	**12**	**290**	**4.1**	**478**	**2.5**
F	**2**	**31**	**6.5**	**53**	**3.8**
High risk (A,C,E,F)	**29**	**986**	**2.9**	**1409**	**2.1**
G	**3**	**103**	**2.9**	**114**	**2.6**
Total	**32**	**1506**	**2.1**	**1996**	**1.6**

[a]**Including small *BRCA1/2* mutations.**

TABLE 2. **Novel Characterized Rearrangments in the *BRCA1* Gene***

BRCA1 exons involved	Notes	Genomic clone	Effect on transcript	Detection method	Confirmation	cDNA primer forward	cDNA primer reverse
Del1-24	min 259138–max 344632		Loss	MLPA	Agilent	–	–
Del1-7	min 200333–max 284794		Loss	MLPA	Agilent	–	–
Del1-2M	g.33267_70035del36769	AC060780	–	MLPA	Segregation	5′-ACCCTCTGCTCTGGGTAAAG-3′	5′-TTGGAAGGCTAGGATTGACA-3′
				LR-PCR	Junction fragment	5′-TGTCTCCACAAAGTGTGACC-3′	5′-TTGGAAGGCTAGGATTGACA-3′
Del8D	g.25529_31240del5712	L78833	Stop	MLPA	RNA	5′-CAGCTTGACACAGGTTTGGAG-3′	5′-CCTTGAGGGGTGATTTGTAAC-3′
				LR-PCR	Junction fragment		
Del8H	g.26181_29711del3531	L78833	Stop	MLPA	RNA	5′-CAGCTTGACACAGGTTTGGAG-3′	5′-CCTTGAGGGGTGATTTGTAAC-3′
				LR-PCR	Junction fragment		
Del5-14	g.21716_53298del31583	L78833	Stop	MLPA	RNA	5′-TCGCGTTGAAGAAGTACAAA-3′	5′-GATTCCAGATTCCAGGTAAGG-3′
				LR-PCR	Junction fragment		
Del16M	g.56074_58704del2631	L78833	Stop	MLPA	RNA	5′-GTGAATACCCTATAAGCCAG-3′	5′-ACCACATCTCCTCTGACTTC-3′
				LR-PCR	Junction fragment		
Del17	g.58530_61209del2680	L78833	Stop	MLPA	RNA	5′-ACTACCCATCTCAAGAGGAG-3′	5′-ACCACATCTCCTCTGACTTC-3′
				LR-PCR	Junction fragment		
Del18-19	g.63335_70579del7245	L78833	Stop	MLPA		–	–
	g.99286_99312ins27	AC060780		LR-PCR	Junction fragment		
Del20	g.65740_73887del8148	L78833	Stop	MLPA	RNA	5′-CTAGGAATTGCGGGAGGA-3′	5′-AAGGCCACTTTGTAAGCTCAT-3′
				LR-PCR	Junction fragment		
Del23-24	g.80280_88398del8119	L78833	Stop	MLPA		–	–
				LR-PCR	Junction fragment		
Del24	Monoallelic expression		Loss	MLPA	RNA	5′-TGGCTGAACTAGAAGCTGTG-3′	5′-ACCACATCTCCTCTGACTTC-3′
	g.82974_84478del1505	L78833		LR-PCR	Junction fragment		

*GenBank reference sequence: U14680.1.
LR, long-range.

fragments confirmed identity with this founder mutation (g.79505–80014del) and the skipping of exon 22 (p.Asp1778GlyfsX26).

One mutation with loss of exon 5, recently described in a German family with multiple cases of breast cancer [Preisler-Adams et al., 2006], could be demonstrated for the second time in our population in a single early-onset breast cancer case diagnosed at the age of 30 years (ML447). Likewise, sequencing of the genomic junction fragment revealed an identical deletion of 224 basepairs and cDNA analysis demonstrated the heterozygous loss of exon 5, causing a heterozygous in-frame deletion of 26 amino acids (p.Phe46_Arg71del26).

Deletions Involving Exon 17 Are Most Common in the German Population

To validate deletions involving exon 17, we designed primer pairs according to the two distinct mutations that have been identified in German families previously [Hartmann et al., 2004; Preisler-Adams et al., 2006]. The generation of a 4.5-kb large junction fragment with a primer pair spanning intron 15 to intron 18 (Fig. 1a) pointed to a known deletion described by Preisler-Adams et al. [2006]. Sequence analysis of the junction fragments confirmed a 3,117-bp deletion for six families (Supplementary Table S2). However, in contrast to Preisler-Adams et al. [2006], we could not generate specific junction fragments with intronic primer pairs located close to the breakpoint. Therefore, this recurrent exon 17 deletion should either be confirmed by specific primer pairs located in intron 16 and 18, respectively (Fig. 1a; Supplementary Table S1), or by RT-PCR analysis (Fig. 1b; Table 2). Loss of exon 17 is causing a frameshift, resulting in a truncated protein (p.Val1665SerfsX7).

In order to determine whether this del ex17 is a founder mutation, we performed haplotype analysis with five polymorphic markers located within or adjacent to the *BRCA1* gene. As seen in Table 3, the six unrelated females share an identical haplotype. The informativeness of the markers is demonstrated by the combination of six different wild-type haplotypes with the common disease haplotype. Furthermore, DHPLC and sequencing data of these families revealed that the disease haplotype is linked to a common cluster of polymorphisms, such as 2201C>T, 2430T>C, 2731C>T, 3232A>G, 3667A>G, 4227T>C, and 4956A>G [Durocher et al., 1996].

In a seventh family, a novel rearrangement involving exon 17 was found. Long-range PCR analysis with the primers described above generated a slightly larger junction fragment. Sequencing of this fragment confirmed a smaller deletion encompassing 2,680 bp instead of 3,117 bp (Supplementary Tables S1 and S2).

Further Cloning of Breakpoints in Novel Deletions

Including the novel ex17 deletion, 11 novel *BRCA1* deletions could be fully characterized on the genomic and in most cases on cDNA level as well. Furthermore, two putative novel rearrangements involving exons 1–7 or the entire *BRCA1* gene were found in two families and made to be likely by segregation analysis and confirmation by a CGH microarray technique (Table 2).

A heterozygous loss of *BRCA1* exon 8 was identified in an index case (Family DU 570) with bilateral breast cancer (34 years and 38 years) by MLPA analysis. A single 6,900-bp PCR fragment was amplified in controls using in the first PCR the ex7-forward and ex9-reverse DHPLC primers and in the second nested PCR a primer pair including the exon 7 and exon 9 MLPA probe sequences. In patients' DNA an additional 1,200-bp fragment was identified indicating a deletion of 5,700 bp. The putative breakpoint was localized to a region of about 250 bp by restriction fragment analysis of the truncated fragment using informative restriction sites. From DNA sequencing with primers flanking this 250-bp region, the breakpoint of the *BRCA1* exon 8 deletion g.25529_31240del5712 could be identified. RNA/cDNA analysis confirmed the skipping of exon 8, leading to a frameshift and protein truncation (p.Glu148AspfsX50).

Another novel deletion involving exon 8 was found in a family presenting with three ovarian cancer cases (Table 2). A 784-bp patient-specific junction fragment was obtained; sequencing

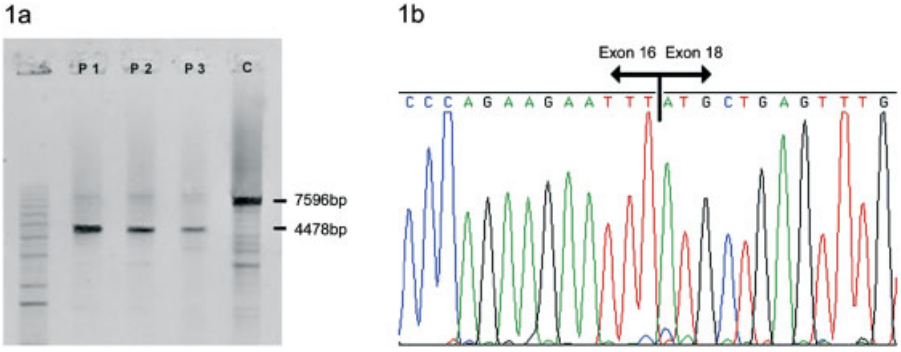

FIGURE 1. **a: A 4,478-bp junction fragment obtained from three patients with the recurrent del ex 17 (lanes P1–P3). The wild-type fragment of 7,596 bp is seen in the control (lane C). b: Confirmation of del ex 17 on RNA level by cDNA sequencing. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

TABLE 3. **Common Haplotype for Del Exon 17**

Fam. ns.:	105		571		730		1196		71		448	
D17S1326	88*	105	88	88	88	105	88	103	88	107	88	105
D17S1323	157	151	157	157	157	151	157	151	157	151	157	151
D17S1322	120	117	120	123	120	120	120	117	120	117	120	117
D17S855	140	150	140	142	140	148	140	150	140	152	140	152
D17S1328	214	199	214	226	214	197	214	197	214	199	214	197

*Sizes of the according microsatellite markers.

revealed a deletion of 3.5 kb containing exon 8, which was confirmed by RT-PCR analysis (Table 2).

A deletion comprising exons 13–15 that was previously described for an American breast and ovarian cancer family [Gad et al., 2001] was found in Family CO1085. It causes a frameshift and a severely truncated protein (p.Gln1396ArgfsX41). Analysis of the genotypes in this family showed the clustering of common polymorphisms on one allele [Durocher et al., 1996]. In agreement with the MLPA data, this haplotype lacks the polymorphisms located in exons 13–15. After confirming the deletion of exons 13–15 at the RNA level, we determined the breakpoint for this rearrangement. A family specific junction fragment could be amplified with a forward primer located in intron 12 and a reverse primer located in intron 15 (Supplementary Table S1). Sequence analysis of the junction fragment demonstrated a deletion of 11.6 kb, identical in length with the one found in the American family. However, careful analysis of the junction fragment revealed a breakpoint 22 bp proximal from the one described in the American family.

A novel deletion involving exon 16 of the gene was found in two families. It was first confirmed in Family CO767 by cDNA analysis, where two fragments were obtained—the wild type and the aberrant fragment. Sequencing of the shorter aberrant fragment revealed the lack of exon 16, resulting in a frameshift mutation and a truncated protein (p.E1559DfsX15). To characterize this rearrangement on the genomic level, primers suitable for long-range PCR were designed. With PCR primers located in intron 15 and 16 (Supplementary Table S1), a 1.2-kb PCR product specifically amplified the patient's DNA, but not that in controls. Alignment of the sequenced junction fragment to the *BRCA1* reference sequence revealed a fusion sequence resulting from a 2,631-bp deletion. The identical junction fragment was obtained in the second family (Family DU353) and sequence analysis confirmed the same deletion breakpoint (g.56074_56704del2631).

A novel deletion comprising exons 4–14 occurred in Family DU396. Primer pairs were designed to obtain a patient-specific junction fragment with a length of about 600 bp (Supplementary Table S1) and cloning of the breakpoint revealed a genomic deletion of 31,583 nucleotides (Fig. 2a; Table 2). RNA/cDNA analysis demonstrated heterozygous loss of exons 5–14 (*BRCA1* exon 4 is noncoding) and the aberrant cDNA sequence confirmed a deletion of 4,350 nucleotides (Fig. 2b), resulting in an in frame deletion of 1,450 amino acids (p.F46_R1495del1450).

Recently, a rearrangement involving exon 18 and exon 19 has been published [Montagna et al., 2003]. Deletion of these two exons causes a frameshift and a shortened protein (p.Asp1692AlafsX1). MLPA analysis in Family CO1051 also indicated heterozygous loss of these two exons, but characterization of the breakpoints revealed a novel recombinational complex event (Table 2; Fig. 3a). Interestingly, the recombination of Alu sequences caused the deletion of 7,218 bp, and the insertion of a 27-bp stretch derived from intron 1 of the *NBR1* gene (Fig. 3b).

A novel deletion involving exon 20 was identified in Family DU406, which presented with multiple cases of breast cancer. Sequence analysis of aberrant migrating PCR fragments amplified from patient's genomic DNA identified a 8,148-bp deletion

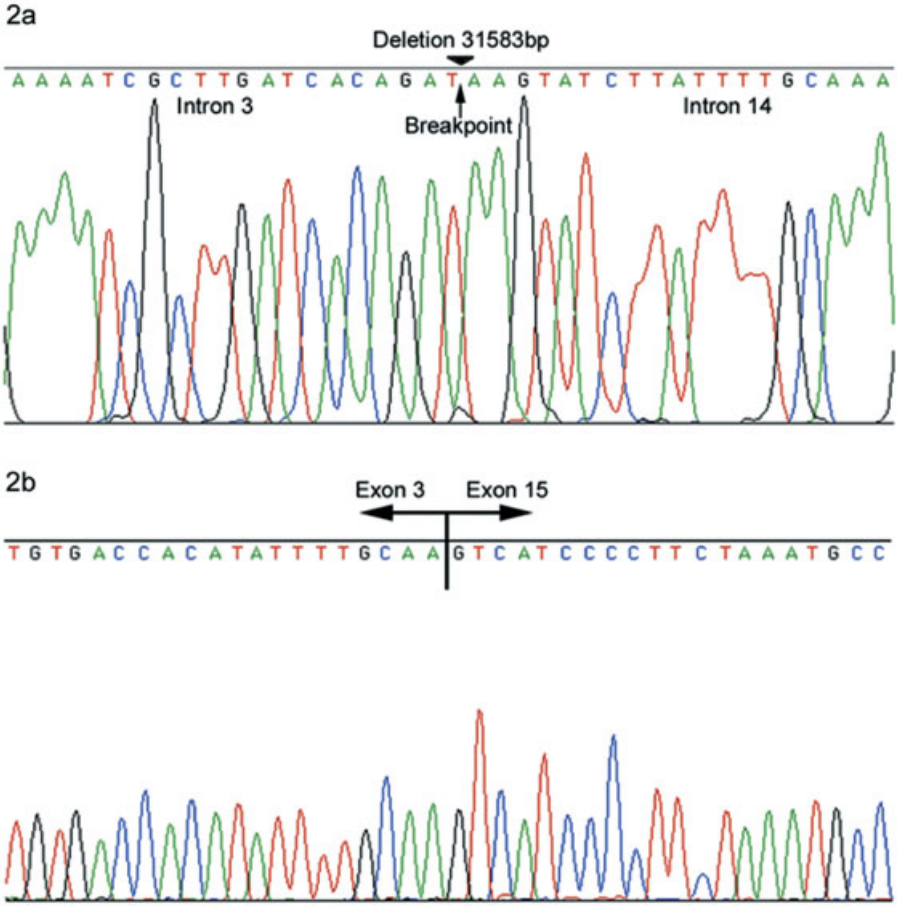

FIGURE 2. **a: Sequencing of the genomic breakpoint in a patient demonstrating a novel deletion of exons 5–14. b: Confirmation of del ex 5–14 by cDNA analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

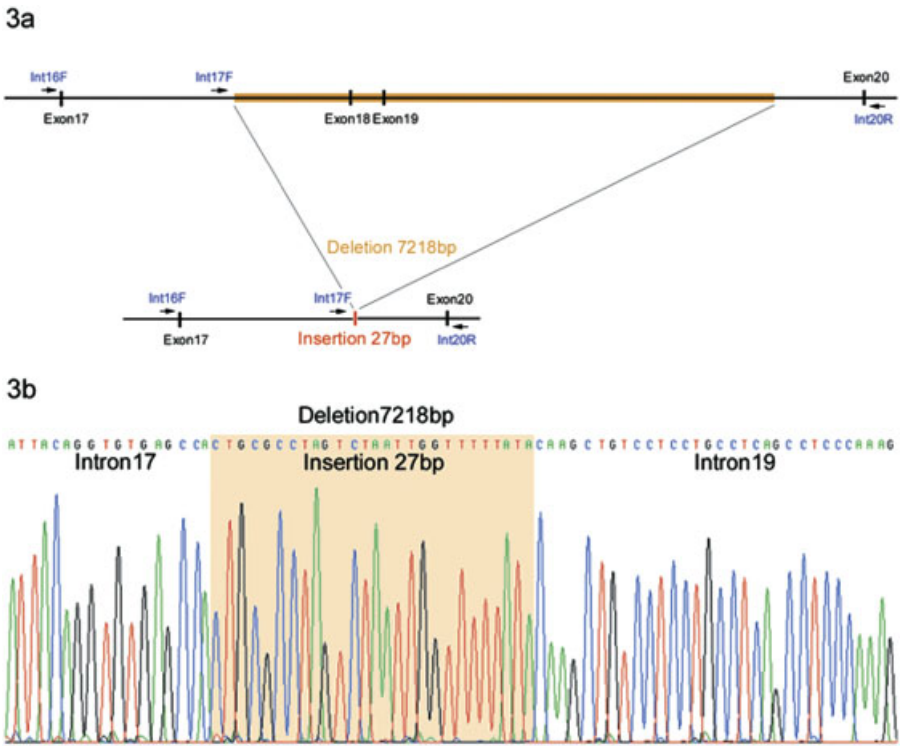

FIGURE 3. **a: Complex recombinatorial event in a patient with a novel del ex 18–19. Alu-Alu HR causes a deletion a deletion of 7,218 bp and an insertion of 27 bp of a *NBR1* motif. b: Sequencing of the complex genomic breakpoint. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

(g.65740–73887). The expected in-frame skipping of 28 amino acids (p.His1732_Lys1759del28) was confirmed by cDNA sequencing. With a nearly complete loss of the intron 19 sequence, this novel mutation is about twice as large as compared to the exon 20 deletions identified so far in one Canadian [Carson et al., 1999] and one Italian family [Montagna et al., 2003]. Segregation analysis in the family was not possible as all the other affected females were deceased, indicating a severe phenotype for this alteration. Pathogenicity of the in-frame loss of 28 amino acids is indicated by the observation that the substitution of one of these amino acids (G1738R) is very likely pathogenic [Easton et al., 2007].

One deletion involving exon 24 and encompassing 4.4 kb has been reported so far [Armaou et al., 2007]. In the MLPA analysis of Family DD1091, which presented with two cases of ovarian cancer, we also found a reduced peak for exon 24. As we hypothesized that this deletion might cause loss of *BRCA1* expression due to nonsense mediated decay [Perrin-Vidoz et al., 2002], we first performed RNA analysis. While sequencing of the genomic DNA revealed a silent substitution in exon 13 (c.4427T>C), analysis of the RT-PCR product spanning this position demonstrated expression from only one allele (Fig. 4a). To determine the extension of the deletion, we again performed long-range PCR with several primers located in intron 23 and in the 3′UTR of the *BRCA1* gene. A junction fragment specific for this family was obtained by using a primer located in intron 23 and 3′UTR (Fig. 4b). Sequential sequencing of this fragment revealed a 1,506-bp deletion ranging from nucleotide 82974 to nucleotide 84479 (g82.974_84.478del1505) (Fig. 4c), making it different from the one found in the Greek family.

Also once reported has been a deletion involving exons 23–24. It was found recently in a Spanish family [De la Hoya et al., 2006]. A patient-specific junction fragment with a length of 6.5 kb (Supplementary Table S1) was found in a family with two affected females diagnosed before the age of 50 years for breast cancer. Sequencing of this fragment revealed a deletion of 8.1 kb (Table 2), excluding the identical event as found in the Spanish family. Analogous for the patient harboring the deletion of exon 24, we would predict monoallelic expression of the *BRCA1* gene.

Characterization of Families With del ex1-2, del ex1-7, and del ex1-24

In two families we found a deletion involving exons 1–2 (Families CO231 and MT375). Rearrangements affecting these exons have been described already in four other German families [Hartmann et al., 2004; Preisler-Adams et al., 2006]. Like Hartmann et al. [2004], we initially failed to clone the breakpoints from the genomic DNA of the patients. Although we used different primer pair combinations based on confirmed rearrangements found in other families [Preisler-Adams et al, 2006; Montagna et al., 2003; Puget et al., 2002; Swensen et al., 1997], no junction fragments could be obtained. Therefore, we also performed MLPA analysis in two additional affected females from Family CO231 and one additional affected female in Family MT375 and saw segregation of the putative deletion in exons 1–2 in both.

In order to confirm the indicated deletions, we applied an alternative technique. The DNAs of the index patients from both families were hybridized against array-CGH microarrays containing 60mer oligonucleotides (Agilent) distributed across the genome, including the *BRCA1* locus. Measurement of the hybridization signals confirmed loss of exons 1–2. Interestingly, by using alternative primer pairs (see Supplementary Table S1), we now could obtain junction fragments for both families. Sequencing of the junction fragments revealed, first, identity of the deletion from Family CO231 with the one described in Montagna et al. [2003]

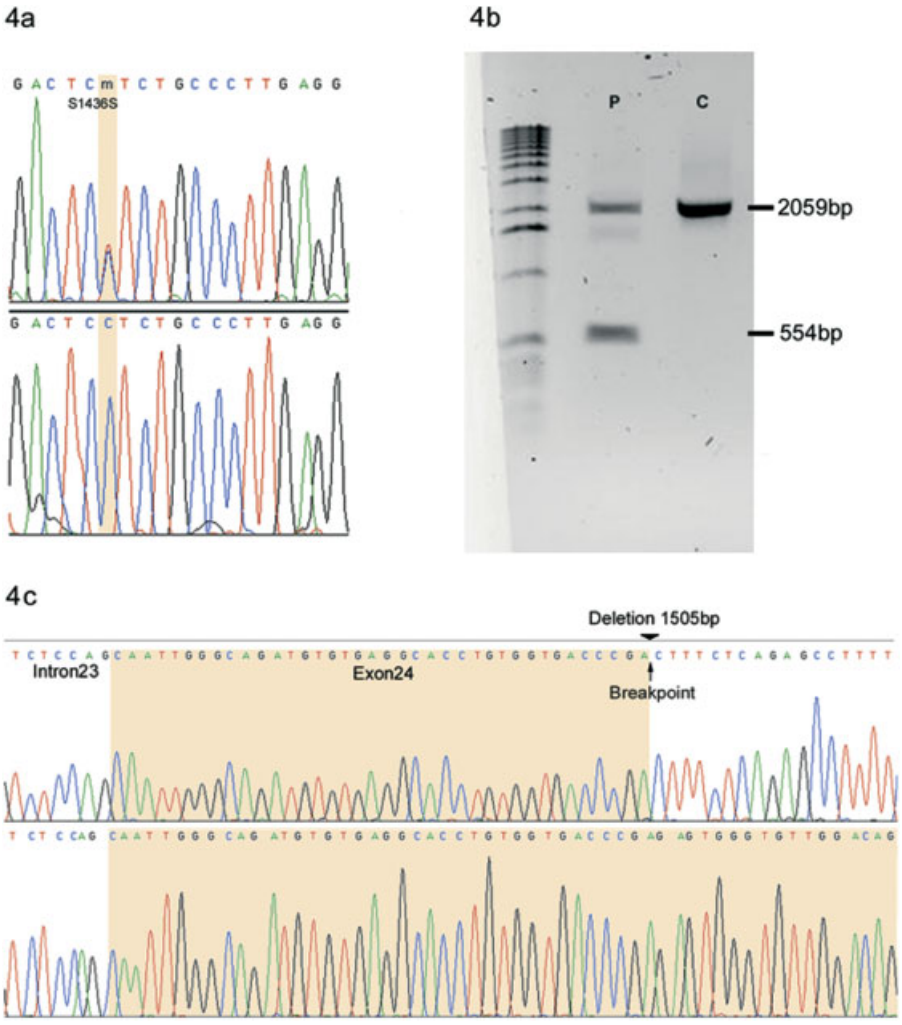

FIGURE 4. **a: Monoallelic expression of the *BRCA1* gene in a patient with a novel exon 24 deletion. Top: Partial genomic sequencing of exon 13 shows heterozygosity at position 4427 (T and C). Bottom: Partial cDNA sequencing of exon 13 shows only a C residue at position 4427. b: Junction fragment of 554 bp (lane P). The wild type fragment of 2,059 bp can be seen in the patient and homozygous in the control (lane C). c: Genomic sequencing reveals a 1,505-bp deletion starting in exon 24 toward the 3′ UTR of *BRCA1*. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

and, second, a novel rearrangement for Family MT375 (see Table 2).

While, due to homozygosity within the entire coding region, allelic expression studies were not possible in Family CO231, we were able to perform such studies for the second del ex1-2 identified in Family MT375. As PCR-based mutation screening revealed extensive heterozygosity (Fig. 5a), cDNA analysis was eligible. Surprisingly, cDNA analysis with primer pairs spanning exons 3–11 revealed biallelic expression, while cDNA analysis with primers located in exon 1 and exon 11 demonstrated monoallelic expression (Fig. 5b and c). Furthermore, the patient is missing part of the cluster polymorphism [Durocher et al., 1996] located in intron 1. This strongly indicates that exons 3–24 of the wild-type *BRCA1* allele was recombined to an aberrant 5′UTR-exon1-exon2 motif of a *BRCA1*-like gene. Additional experiments excluded the *NBR1* gene (data not shown).

A more extending novel deletion was found in Family MT455. MLPA analysis strongly suggested the heterozygous absence of exons 1–7. The deletion was also present in the predicted carrier male, the father of the patient, who is suffering from colon cancer and had two sisters deceased from breast cancer. In addition, we could find a deletion encompassing the entire *BRCA1* gene. Both LGRs could also be confirmed by applying the CGH 60-oligomer microarray technique. Based on these results, the minimum size of del ex1-7 is about 200 kb and the maximum size is about 285 kb, while the minimum size for del ex1-24 is about 259 kb and the maximum size is 345 kb. Both deletions encompass the entire *NBR1* region, which is located distally to the *BRCA1* gene (Table 2).

Screening for Gross Rearrangements in the BRCA2 Gene

First, 412 index patients belonging either to the familial high-risk groups A, C, or E, or to the early onset group G were screened for rearrangements in the *BRCA2* gene or the *CHEK2* gene, respectively. For all these patients, the MLPA kit P045 indicated no *BRCA2* deletion. In contrast, analysis of additional 38 families from the high-risk groups A, C, or E presenting at least one male breast cancer led to the identification of one deletion in *BRCA2*. Characterization of the identified del ex21 found in Family DU223 revealed identity to the one described recently [Walsh et al., 2006].

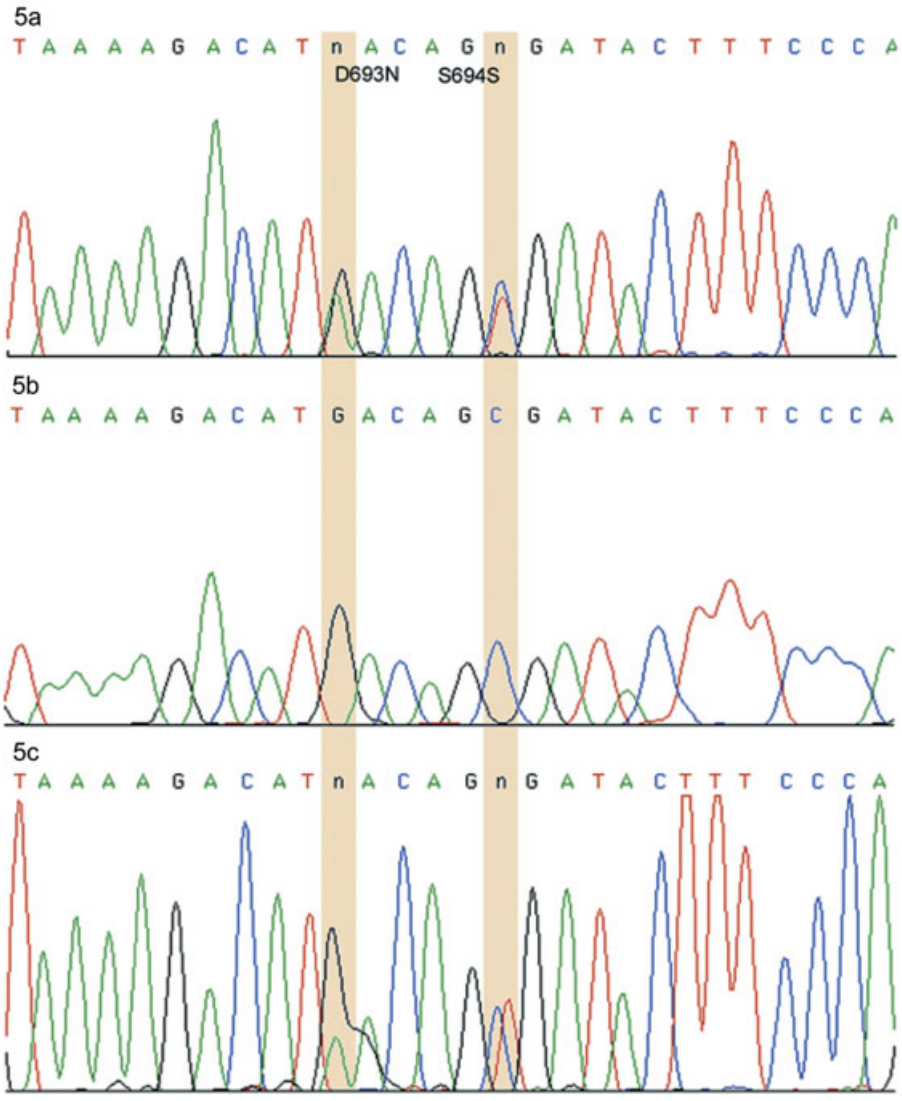

FIGURE 5. **Expression of a hybrid *BRCA1* mRNA in a patient with del ex 1–2. a: Partial genomic sequence of exon 11 reveals heterozygosity at position 2196 (D693N) and 2201 (S694S). b: Monoallelic expression by using exonic primer pairs from exons 1 and 11. c: Biallelic expression by using exonic primers from exons 3 and 11. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

Two aberrations within the *CHEK2* gene could be observed in the breast/ovarian cancer families screened for BRCA2 LGRs using the SALSA MLPA P045 kit (MRC-Holland). A mutation in exon 10 of the *CHEK2* gene was found in one family (Family ML671). Sequencing of exon 10 of the *CHEK2* gene in this family revealed a deletion of C at nucleotide position of 1100. This causes a premature stop codon at amino acid position 381 (T367Mfs14X). In this family, four women were affected by breast cancer, two of them diagnosed before the age of 50 years. As the other three females of the family were deceased, no segregation analysis was possible. A peak reduction was found for exon 9 in the index patient of another family. Deletions involving exons 9–10 have been described recently for the Slavish population [Cybulski et al., 2007] and the usage of the published primer pairs generated the expected junction fragment. The deletion causes an in-frame loss of 88 amino acids (L303Fdel304_391) and is present in a female diagnosed with having breast cancer at the age of 33 years, while the obligate carrier mother had complete surgery in the womb area at the age of 38 years and still has no disease at the age of 57 years.

DISCUSSION

Including the 194 German families formerly screened for gross aberrations in the *BRCA1* gene by other groups [Hofmann et al., 2002, 2003; Hartmann et al., 2004; Preisler-Adams et al., 2006], data from 1,700 families are now available to calculate reliable mutation prevalences for high-risk individuals in the German population. In total, 45 intragenic deletions, duplications, or losses of promoter region could be demonstrated in 1,700 families found to be negative for *BRCA* mutations after screening with PCR-based techniques. The overall frequency of *BRCA1* rearrangements (2.1%) in German high-risk families as identified in this study is comparable with the results reported for North Americans [Hendrickson et al., 2005], Australians [Woodward et al., 2005], or Eastern Asians [Lim et al., 2007], but is significantly lower than that reported for the Dutch [Hogervorst et al., 2003], Northern Italian [Montagna et al., 2003], French [Gad et al., 2002], or Czech population [Vasickova et al., 2007]. Similar low prevalences have been also reported for the Finnish [Laurila et al. 2005] and Danish population [Thomassen et al. 2006]. Whether this points

to a geographical tendency or simply reflects absence of frequent founder mutations, like in the Dutch [Petrij-Bosch et al., 1997] or Mexican population [Weitzel et al., 2007], is still questionable.

We found LGRs in risk groups where we found mutation prevalences for *BRCA1/2*, in the range from 20 to 60% (C, A, E, and F): the higher the prevalence of LGRs, the higher the overall mutation prevalence in the *BRCA1/2* genes. The only exception of this rule concerns risk groups B, D, and G. All these risk groups show mutation prevalences for *BRCA1/2* of about 10% (Meindl and GCHBOC, unpublished data). However, while the low-risk breast cancer cases exhibit no LGRs, we could find LGRs in early-onset cases. This observation might indicate that in the low-risk groups B (two or more cases of female breast cancer, only one diagnosed before 51 years of age) and D (exactly two cases of female breast cancer, with one diagnosed before 51 years of age) sporadic cases are accumulating, while early-onset cases exhibit a rather high proportion of novel mutations in the known *BRCA* genes.

As families with at least three postmenopausal cases of breast cancer (group B) or two cases with only one premenopausal case (group D) did not reveal gross aberrations, we suggest restricting screening for gross rearrangements to certain risk groups, i.e., families with three or more cases of breast cancer, including two premenopausal cases (group A), families with two premenopausal cases of breast cancer (group C), families with breast and ovarian cancer (group E), families with two or more cases of ovarian cancer (group F), and early onset cases diagnosed before the age of 36 years (group G).

Most of the gross rearrangements found in the German population are recurrent and have also been found in other populations. Likewise, duplication of exon 13, one frequent *BRCA1* rearrangement in our population (described seven times), has been identified throughout Europe [BRCA1 Exon 13 Duplication Screening Group, 2000]. The deletion of exon 22 (described five times), another common aberration, has been found frequently in the Dutch population [Petrij-Bosch et al., 1997]. However, the most frequent German specific aberrations affect exon 17. Here, we describe six times a deletion of 3.1 kb containing this exon that has only been found before in two Italian [Montagna et al., 1999], and one British, one French, and one German family [Walsh et al., 2006; Mayozer, 2005; Preisler-Adams et al., 2006]. Moreover, this deletion could be found in another German family (B. Schlegelberger, Institute of Experimental Pathology, University of Hannover, personal communication). Haplotype analysis revealed that it is a recurrent founder mutation in the German and presumably the Caucasian population (Table 3). In addition, a possible German specific founder, yet another deletion involving exon 17, has been described three times in other breast/ovarian cancer families [Hartmann et al., 2004]. And finally, we detected a novel deletion affecting exon 17, making gross aberrations involving exon 17 to the most common events in our population. Gross rearrangements occurring in exons 13, 17, and 22, respectively, account for more than 50% (24/43) of all deletions/duplications found so far within the German population and are also present in other populations. In contrast, together with the above mentioned 5.1 kb deletion in exon 17 [Hartmann et al., 2004], all novel rearrangements reported in this work might be country-specific.

Alu repeats are frequently associated with the generation of deletions or duplications and most gross aberrations in the *BRCA1* gene are caused by homologous recombinations of *Alu*-elements [Walsh et al., 2006; Mayozer, 2005]. Most of the German-specific deletions also result from such events. Only the deletions of exon 5 and exon 24 are not mediated by Alu-Alu recombinations. Homologous recombinations between Alu elements could have also mediated the loss of exons 1–2 [Swensen et al., 1997]; however, in most cases an Alu element is recombining with a stretch from the *BRCA1* pseudogene [Mayozer, 2005]. Although the breakpoints for some families with such a deletion could be identified [Mayozer, 2005], the genomic breakpoint could only be found in only 1 out of 4 German families [Preisler-Adams et al., 2006]. Interestingly, the identical deletion could be found and confirmed in a Czechian high-risk family with cooccurrence of breast and ovarian cancer [Vasickova et al., 2007].

Like others, we also initially failed to identify the precise breakpoints in our families. However, confirmation of the two exon 1–2 deletions by using High-Definition 60-oligomer microarrays (Agilent) encouraged us to design alternative primer pairs, which then allowed to generate junction fragments (Table 2). The 60-oligomer microarray technique could also confirm the novel deletions exons 1–7 and of the entire *BRCA1* gene. With this technique not only LGRs in the two *BRCA* genes can be validated, but also the genomic integrity in other hereditary diseases. Interestingly, for one of our families lacking exons 1–2, we could demonstrate the generation of an expressed hybrid RNA containing exons 3–24 from wild-type *BRCA1* and exons 1–2 from an yet unknown *BRCA1* pseudogene.

As indicated by the first study addressing gross aberrations in the *BRCA2* gene for German families [Preisler-Adams et al., 2006], such events are very rare in our population. Together with the 125 high-risk families studied by Preisler-Adams et al. [2006], 575 high-risk families from risk groups A, C, and E and 55 early-onset cases have now been screened for rearrangements in the *BRCA2* gene. Gross aberrations could only be detected in 1 out of 65 (38 in this study; 20 in Preisler-Adams et al. [2006]; and another seven were found to be negative at the Institut of Human Genetics, Heidelberg, Dr. C. Sutter, personal communication) families presenting with female breast/ovarian and male breast cancer. This is in slight agreement with studies from other populations, where *BRCA2* rearrangements were also found in families with female and male breast cancer [Tournier et al., 2004; Gutierrez-Enriquez et al., 2007]. Therefore, for our high-risk population, we would recommend MLPA screening within the *BRCA2* gene only for a subgroup of families presenting with at least one case of female breast/ovarian cancer and male breast cancer.

Furthermore, we identified only one frameshift mutation in exon 10 and one deletion of exons 9–10 in the *CHEK2* gene within the 450 high-risk individuals investigated in this study. This confirms our recent finding that *CHEK2* mutations, now generally considered as being of moderate penetrance [CHEK2 Breast Cancer Case-Control Consortium, 2004], are infrequent in our population [Dufault et al. 2004] as well.

In conclusion, screening of more than 1,500 families for gross aberrations in the *BRCA1* gene revealed a low but notable number of such events in high-risk families and early-onset cases. Presence of gross aberrations in the *BRCA2* gene could be demonstrated only for one family with female and male breast cancer and deleterious mutations in the *CHEK2* gene were confirmed to be very rare within our high-risk population. Therefore, while genetic testing for gross aberrations in the *BRCA1* gene can be recommended for different familial risk groups, deletion screening for the *BRCA2* gene is currently only indicated for a few families. Finally, our study suggests that the confirmation of MLPA results is not only obligatory to avoid artifacts, but also reveals novel interesting rearrangements like the complete deletion of *BRCA1*, a

complex event associated with the loss of exons 18–19, and the generation of a hybrid *BRCA1* RNA by the loss of exons 1–2, which points to an yet unknown regulatory motif for *BRCA1*.

ACKNOWLEDGMENTS

We thank the families who participated in the study and the German Consortium for Hereditary Breast and Ovarian Cancer. This Consortium was founded by the German Cancer Aid (Deutsche Krebshilfe), which also supported this work. Special thanks go to Dr. Andreas Polten from Agilent Technologies, who supported our experiments performed with the 60-oligomer CGH-microarray technique. Finally, we are obligated to Dr. Juliane Ramser for technical help.

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