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Screening for genomic rearrangements in *BRCA1* and *BRCA2* genes in Czech high-risk breast/ovarian cancer patients: high proportion of population specific alterations in *BRCA1* gene

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Abstract

Large genomic rearrangements (LGR) represent substantial proportion of pathogenic mutations in the *BRCA1* gene, whereas the frequency of rearrangements in the *BRCA2* gene is low in many populations.

We screened for LGRs in *BRCA1* and *BRCA2* genes by multiplex ligation-dependent probe amplification (MLPA) in 521 unrelated patients negative for *BRCA1/2* point mutations selected from 655 Czech high-risk breast and/or ovarian cancer patients. Besides long range PCR, a chromosome 17 -specific oligonucleotide-based array comparative genomic hybridization (aCGH) was used for accurate location of deletions.

We identified 14 patients carrying 8 different LGRs in *BRCA1* that accounted for 12.3% of all pathogenic *BRCA1* mutations. No LGRs were detected in the *BRCA2* gene. In a subgroup of 239 patients from high-risk families, we found 12 LGRs (5.0%), whereas two LGRs were revealed in a subgroup of 282 non-familial cancer cases (0.7%). Five LGRs (deletion of exons 1-17, 5-10, 13-19, 18-22 and 21-24) were novel; two LGRs (deletion of exons 5-14 and 21-22) belong to the already described Czech-specific mutations; one LGR (deletion of exon 1-2) was reported from several countries. The deletions of exons 1-17 and 5-14, identified each in four families, represented Czech founder mutations.

The present study indicates that screening for LGRs in *BRCA1* should include patients from breast or ovarian cancer families as well as high-risk patients with non-familial cancer, in particular cases with early-onset breast or ovarian cancer. On the contrary, our analyses do not support the need to screen for LGRs in the *BRCA2* gene. Implementation of chromosome-specific aCGH could markedly facilitate the design of primers for amplification and sequence analysis of junction fragments, especially in deletions overlapping gene boundaries.

Key words

BRCA1 gene; *BRCA2* gene; large genomic rearrangements (LGR); hereditary breast and ovarian cancer syndrome; multiplex ligation-dependent probe amplification (MLPA); chromosome-specific array comparative genomic hybridization (aCGH)

Introduction

BRCA1 (MIM# 113705) and *BRCA2* (MIM# 600185) are major predisposition genes in hereditary breast and ovarian cancer. Frequency of germline *BRCA1/2* mutations was estimated at 2-4.7% in unselected breast cancer (BC) patients from different populations [1-3]. Pathogenic alterations are predominantly represented by small deletions, insertions and point mutations leading to premature termination of translation and production of truncated proteins. Deletions and duplications of large genomic regions or alterations that combine both deletion and insertion events have been increasingly reported during period following the introduction of multiple ligation-dependent probe amplification (MLPA) testing [4]. The majority of large genomic rearrangements (LGR) at the *BRCA1* locus are unique and their frequency is not expected to vary markedly among various geographic regions [5]. They represented 8-10% of all *BRCA1* mutations in several countries [5-10]. The elevated incidence of LGRs in certain ethnic groups is caused by founder effect. In Dutch population, deletions of exon 13 or 22 represented 36% (12/33) of all *BRCA1* mutations found in 170 families [11]. In another group of 805 Dutch patients, these two deletions were detected in 23.1% (28/121) of *BRCA1* positive cases and all LGRs accounted for 27.3% of *BRCA1* mutations [4]. High proportion of LGRs was also found in Italy where LGRs accounted for 19% of *BRCA1* mutations [12].

Most of LGRs are the result of unequal homologous recombination between *Alu* repeats accumulated at the *BRCA1* locus [13]. Numerous recombination sites in homologous regions located in the *BRCA1* gene and the *BRCA1* pseudogene (*ψBRCA1*) were described by Puget and colleagues [10,14] and afterwards by other authors [5].

Contrary to *BRCA1*, the germline *BRCA2* rearrangements have not been identified in several populations [15-17] and in other geographic areas occurred rarely [7, 9, 18, 19]. LGRs in the *BRCA2* gene were most frequent in families with multiple BC cases including one or more male BC cases [20, 21].

Rearrangements at the *BRCA1* locus represented 10.4% (10/96) of all *BRCA1* mutations identified in high-risk families in Moravia - the eastern part of the Czech Republic [22]. Further, one rearrangement in the gene was identified by RNA-based analysis performed in our previous study [23]. These results indicated that screening for LGRs in the *BRCA1* gene should be included in genetic testing in high-risk BC or ovarian cancer (OC) families. Nevertheless, only high-risk families were screened for LGRs at the *BRCA1* locus and LGRs

within the *BRCA2* gene have not been analyzed at all in the Czech population. In this study, MLPA analysis was applied to determine the frequency and spectrum of LGRs in the *BRCA1/2* genes in a group of patients from BC and/or OC families and a group of high-risk patients not selected on the basis of their family history of cancer. Oligonucleotide array-based comparative genomic hybridization (aCGH) was used for precise location of LGRs. The purpose of this study was to define reliable inclusion criteria for LGR testing.

Materials and methods

Patients and samples

Selection for genetic testing was performed between 2002 and 2009 at the Institute of Biology and Medical Genetics, at the Department of Oncology and at the Department of Obstetrics and Gynecology of the First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague. Selection criteria for *BRCA1/2* testing followed the national guidelines approved by the Czech Society for Oncology and the Czech Society for Medical Genetics published in 2003 and updated in 2009 [24, 25].

A series of 655 patients was first analyzed for the presence of point mutations and small deletions or insertions in *BRCA1/2* genes as described previously [26, 27] and those tested negative for mutations in coding gene regions were subsequently screened for LGRs at *BRCA1/2* loci. This series represented 521 cases including (i) 239 patients from high-risk families (referred herein as familial/hereditary cancer cases) and (ii) 282 patients selected regardless of the absence of reported family history of cancer (referred herein as sporadic/non-familial cancer cases). The first group met the following criteria in first- or second-degree relatives: two cases of either BC diagnosed before the age of 50 or OC diagnosed at any age; and three or more cases of BC or OC diagnosed at any age. A total of 161 families had a history positive for BC only (HBC families), 69 families had both BC and OC (HBOC families) and 9 families had OC only (HOC families). The second group included patients diagnosed with BC before the age of 36 (138 cases) or OC before the age of 40 (64 cases), patients with bilateral BC before the age of 51 (27 cases), patients with both primary breast and ovarian cancer (18 cases) or medullary breast carcinoma (19 cases) and cases of male BC (16 cases). The study was approved by the Ethical Committee of the First Faculty of

Medicine and the General University Hospital and all participants gave their written informed consent prior to genetic testing.

Genomic DNA was isolated from EDTA blood samples using the Wizard Genomic DNA Purification System (Promega, Madison, WI) according to the supplier's instructions.

Mutation probabilities calculation

To compare detected mutation frequencies in analyzed groups with expected mutation frequencies, we retrospectively calculated mutation probabilities in *BRCA1/2* genes using BRCAPRO BayesMendel model [28] with CancerGene software package (CaGene version 5.0; University of Texas Southwestern Medical Center) available at <http://www4.utsouthwestern.edu/breasthealth/cagene/>.

MLPA analysis

The SALSA MLPA kits for *BRCA1* (the primary screening kit P002B and the confirmatory kit P087) and *BRCA2* (kit P045-B1; MRC-Holland, Amsterdam, The Netherlands) were used for relative quantification of each of the *BRCA1/2* exons according to the manufacturer's instructions. Amplified products were separated on an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, USA). The collected data were analyzed using Gene Mapper v4.0 Software (Applied Biosystems) and evaluated by both visual comparison of chromatograms and quantitative comparison of peak areas exported to Excel spreadsheet. Samples differing in a relative peak area by more than 30% were reanalyzed with the P087 kit. Samples with single exon deletion were sequenced to exclude the presence of sequence variants affecting probe hybridization.

Array-based comparative genomic hybridization

The chromosome 17 –specific aCGH (Roche NimbleGen, Madison, USA) was used to locate deletion breakpoints in regions flanking the *BRCA1* gene. The arrays format was 385K; median oligonucleotide probe (50 to 75mer) spacing was 160 bp.

Sample and reference DNA (from blood samples of 100 random blood donors) were fragmented by sonication and further processed according to the supplier's protocol (Roche NimbleGen). Briefly, 1 µg of sample DNA was labeled with Cy3- and the same amount of reference DNA with Cy5-dye using a NimbleGen Dual-Color DNA Labeling Kit. Differentially labeled DNA samples were purified, combined (6 µg of each labeled DNA) and

hybridized to arrays for 18 hours at 42°C. The slides were washed, dried and fluorescence was recorded at 3µm resolution using microarray fluorescent scanner Innoscan 700 (INNOPSYS, France). Scanned images were analyzed using NimbleScan software. Fluorescence signals were normalized with qspline algorithm [29] to compensate for inherent differences in signal between the two dyes. Copy number analysis was performed using the segMNT algorithm [30], and statistically significant regions of aberration were visualized using SignalMap software (Roche NimbleGen).

DNA breakpoint analysis

Large deletions detected by MLPA and located by aCGH were validated by identification and characterization of the genomic breakpoints. Long-range PCR (LR-PCR) implementing Expand Long Template PCR System (Roche Applied Science) was performed according to the manufacturer's instructions with primers flanking the breakpoints (Table 1) to amplify the junction fragment carrying the deletion. The junction fragments of previously reported rearrangements were obtained with published primers [16, 22, 23]. Amplified PCR fragments were separated on agarose gels and visualized by ethidium bromide staining. PCR products, corresponding to the deleted allele, were purified with ExoSAP-IT (USB Corp., Cleveland, USA) according to the manufacturer's instructions and sequenced using the BigDye v3.1 terminator cycle sequencing kit (Applied Biosystems) on an ABI 3130 genetic analyzer.

Nomenclature of mutations

Mutations were described according to the recommended nomenclature system described by the Human Genome Variation Society (HGVS) [31]. Designation of the genomic rearrangements in the *BRCA1* gene is based on the reference sequence L78833.1; deletions of exons 1-17 and exons 1-2 on the reference sequence NC_000017.9 complement (38300000..38760000) [32]. Whole genomic locale is from March 2006 assembly of the UCSC Genome Browser on Human, NCBI Build 36.1 (available at <http://genome.ucsc.edu>).

Haplotype analysis

DNA samples carrying deletion of exons 1-17 or 5-14 in the *BRCA1* gene were genotyped for two *BRCA1* intragenic microsatellite markers (D17S855, D17S1322), and two markers (D17S1320, D17S1325) flanking *BRCA1* gene on the centromeric and telomeric side,

respectively. Each marker was amplified by PCR using FAM-labeled forward primers as described previously [33] and analyzed on an ABI 3130 genetic analyzer.

Results

Prevalence of genomic rearrangements in BRCA1/2 genes

LGRs were examined in 521 unrelated patients negative for *BRCA1* and *BRCA2* point mutations. A series of 239 high-risk patients was selected from 161 HBC families and 78 HBOC or HOC families. A second series of 282 participants represented non-familial cancer cases. MLPA analysis revealed 8 different LGRs in *BRCA1* gene in 14 (2.7%, 14/521) probands (Table 2). The frequency of LGRs in the whole group of *BRCA1/2* tested patients (including 134 patients with small mutations) was 2.1% (14/655) (Table 3). Deletions were detected in the entire *BRCA1* locus and varied in size from less than 4 kb to more than 80 kb. The five rearrangements detected in eight families were novel (Fig. 1). One of the identified mutations (deletion of exons 1-2) occurred repeatedly in several countries [5, 7, 14, 34, 35]; two other mutations (deletion of exons 5-14 and 21-22) have already been detected in the Czech population [22, 23]. No LGRs were identified in the *BRCA2* gene in 521 analyzed samples of high-risk patients.

LGRs were significantly more frequent in the group of patients from high-risk families (12/239, 5.0%) compared to the group of non-familial cancer cases (2/282, 0.7%, $P = 0.002$). In high-risk families, the frequency of LGRs was higher among patients from HBOC and HOC families (6/78, 7.7%) than that from HBC families (6/161, 3.7%, $P = 0.2$). Similar to LGRs, the highest frequency of *BRCA1* point mutations was also found in a group of HBOC and HOC families (Table 3). Among non-familial cancer cases two LGRs were found, a deletion of exons 5-14 in a woman diagnosed with medullary BC at the age of 39, and a deletion of exons 21-22 in a woman with OC at the age of 36.

In total, 14 large deletions or complex rearrangements were detected at the *BRCA1* locus in 521 independent families, which accounted for 12.3% (14/114) out of all identified *BRCA1* mutations (Table 3). To compare the number of actually detected mutations in *BRCA1* gene with expected mutation frequencies in our study groups, we implemented BRCAPRO computational model to retrospectively estimate the likelihood of finding germline *BRCA1* mutations. Compared to BRCAPRO, we obtained slightly higher frequencies of *BRCA1*

mutations in all analyzed patients and in high-risk families and non-familial cancer cases subgroups (Table 4). Small discrepancies in mutation rates may be influenced by the fact that the BRCAPro can underpredict the numbers of mutations, especially in low carrier probability categories [36-38]

Characterization of LGRs and determination of breakpoints

Specification of families with identified mutations and characterization of LGRs are described in Table 2. A deletion of exons 1-2 was found in a woman from HBOC family No 779. Sequencing of the junction fragment amplified from a patient DNA sample revealed a deletion of 36,934 bp (g.197575_234508del36934) with identical breakpoints as described previously in families from the USA [14], Italy [34] and Germany [7]. A deletion of exons 5-14 was identified in two high-risk HBOC families, one HBC family encompassing four BC cases (including two bilateral BC cases) and one sporadic BC patient with medullary BC. LR-PCR and sequence analysis of the amplified junction fragment confirmed in all four cases a deletion of 31,583 nucleotides (g.21716_53298del31583) resulting in an in frame deletion of 1,450 amino acids, which was detected in four patients from Moravia, Czech Republic [22] and in one from Germany [7]. In frame deletion involving exons 21-22 and simultaneous insertion of a 236-bp intronic fragment into the site of a deleted sequence (g.77128_80906del3779ins236) was detected once in our laboratory [23] and two times in Moravia [22].

A large deletion affecting exons 1-17 was identified in two HBC families and in two high-risk HBOC families. Chromosome 17 -specific aCGH was used to map accurately borders of the deletion extending the 5' boundary of the *BRCA1* gene, which enabled precise design of PCR primers and sequencing of breakpoints (Fig. 2a). Junction fragments of 1,311 bp were obtained from patients' genomic DNA with primers spanning the region from the end of the *NBR2* gene to intron 17 of *BRCA1*. Sequence analysis of amplified fragments revealed a deletion of 80,496 bp (g.208928_289423del80496) in all four families with breakpoints occurring in a 37-bp region of identical sequence within two *AluY* repeats located more than 20 kb upstream of the *BRCA1* translation start site and in intron 17 of *BRCA1*. The deletion spanned more than 90% of the *BRCA1* coding sequence together with coding sequence of the

NBR2 gene. The deletion of exons 1-17 was also described in Sweden [18], but the alteration had different breakpoints and included 284,479 bp.

A complex recombination event characterized by the deletion of exons 5-10 and the insertion of a short nucleotide stretch derived from the sequence in intron 3 of the *BRCA1* gene (Fig. 3) was identified in a HBOC family No 1190. With PCR primers located in introns 3 and 10, a fragment of 816 bp was amplified from patient's DNA but not from control DNA. Sequencing of the PCR product ascertained a deletion of 15,552 bp with concurrent insertion of a 35-bp fragment (g.17696_33247del15552ins35). Deletion breakpoints were found in the *AluSx* and *AluJb* sequences located in introns 3 and 10, respectively. Inserted fragment corresponded to the reverse complement of the sequence derived from *AluY* repeat located in intron 3 of *BRCA1* (g.18725-18759). Breakpoints were located in a sequence comprising 10 identical nucleotides within *AluSx* (g.17686-17695) and *AluY* (g.18760-18769) repeats in intron 3 and a stretch of 29 identical nucleotides located in the *AluY* (g. 18696-18724) and *AluJb* (g.33248-33276) repeats in introns 3 and 10, respectively. The complex rearrangement led to the loss of exons 5-10 that caused a shift in the reading frame and truncation of the *BRCA1* protein (p.Lys45AsnfsX3).

A deletion comprising exons 13-19 was found in a woman with bilateral BC from HBC family No 571. The specific junction fragment of 716 bp was obtained from patient's DNA using primers located in introns 12 and 19 (Fig. 4). Sequence analysis identified a deletion of 25,201 bp (g.41850_67050del25201). Despite the deletion of exons 13-19 maintains the *BRCA1* reading frame, elimination of the gene region coding for 336 amino acids (p.Gln1396_Glu1731del) is probably of pathogenic nature. The deleted region comprises the substantial part of the functional *BRCT* (*BRCA1* C-terminal) domain (amino acid residues 1,646–1,859) [39] affected by several reported missense and nonsense mutations associated with BC [40].

In HBC family No 897 affected with six cases of BC, a deletion of exons 18-22 was revealed. Chromosome 17 -specific aCGH was used to map the deletion breakpoints (Fig. 2b). LR-PCR with primers located in introns 17 and 22 amplified a family specific fragment of 3,738 bp. Sequencing of the junction fragment showed that recombination took place at highly homologous *AluY* and *AluSx* sequences in introns 17 and 22 and demonstrated a deletion of 16,316 bp (g.63647_79962del16316). Out of frame deletion of exons 18-22 predicted truncation of the *BRCA1* protein (p.Asp1692GlyfsX26).

A deletion of exons 21-24 was revealed in a woman from HBC family No 609 with invasive BC diagnosed at the age of 38. Chromosome 17 -specific aCGH analysis localize the deletion breakpoints in intron 20 and in a region of about 18 kb downstream of the *BRCA1* stop codon (Fig 2c). Primers were designed to amplify a specific junction fragment of 259 bp from patient's DNA sample. Sequence analysis demonstrated the recombination of non-homologous sequences leading to the deletion of 25,639 nucleotides and the insertion of a trinucleotide TAG (g.75489-101127del25639insTAG). Due to homozygosity in the coding region of the gene, study of allelic expression was not possible, however, monoallelic expression of the *BRCA1* gene was observed in a patient with the deletion involving exon 24 [7]. In addition, the deleted gene region coding for terminal 104 amino acid residues includes an essential part of the C-terminal BRCT domain of the BRCA1 protein. The similar deletion of exons 21-24 was also described in the USA in a family with Irish ancestry [41], but in this 19,245-bp long deletion, *Alu* repeats were involved in recombination.

Analysis of BRCA1 Haplotypes

To evaluate whether the repeatedly occurring deletions in the *BRCA1* gene involving exons 1-17 and 5-14 (both found in four families) represent founder mutations, haplotype analysis was performed in families carrying these alterations. One specific ~756-kb haplotype defined by D17S855, D17S1322, D17S1320 and D17S1325 markers was common for each carrier with a given type of alteration, which indicates the same origin for each mutation (Table 5).

Discussion

The frequency of LGRs in our study (12.3% of all mutations found in *BRCA1* gene) was lower than that observed in the Netherlands (27.3%) [4] and Italy (19%) [12], but it was in agreement with data reported from Denmark (12.5%) [9], France (12%) [8], Germany (9.6%) [7] and Spain (8.2%) [6]. Frequency of LGRs described in Moravia (10.4% of all detected *BRCA1* mutations) [22] was similar to our results. However, only two mutations (deletions of exons 5-14 and 21-22) were identical in both parts of the country. The Czech-specific mutations, deletions of exons 1-17 and 5-14 in the *BRCA1* gene, comprised more than 50% (8/14) of identified LGRs in our study. While the occurrence of exon 1-17 deletion was restricted to the Prague area so far, the deletion of exons 5-14 was also detected four times in Moravia and likely represents the most frequent LGR in the Czech Republic. The complex rearrangement involving deletion of exons 21-22 was identified two times in Moravia and once in our laboratory and may represent further population specific *BRCA1* mutation. Nevertheless, haplotype analysis was not carried out in affected families. The deletion of exons 1-2 was the only identified mutation spread throughout Europe [5]. In the presented study, five novel LGRs were found. Frequent occurrence of unique mutations is in agreement with the results obtained from other countries [5].

The whole group of 521 high-risk patients was tested for LGRs in the *BRCA2* gene, however, no alterations were found. Very low frequency of LGRs was reported from Germany, where a gross rearrangement in the *BRCA2* gene was found only in one family with male BC out of 450 analyzed families [7]. In Denmark, a group of 642 patients was screened for LGRs and one deletion in *BRCA2* was also described in a family with male BC [9]. Higher frequency of rearrangements in the *BRCA2* gene was found in Spain (2/20) [20] and France (3/39) [21] in families with female and male BC. In our study, only a small group of 5 families with female and male BC was analyzed with negative results for rearrangements in *BRCA2*. On the other hand, our results are not in agreement with analyses performed in France [42], Spain [20] and the USA [41] where genomic rearrangements of the *BRCA2* gene have been found in families with no male BC cases.

Homologous recombination between *Alu* sequences is associated with most reported rearrangements at the *BRCA1* locus [5]. High percentage of LGRs identified in our study group of patients was also caused by recombination of *Alu* repeats (Table 2). Deletions of

exons 5-14, 13-19 and 21-24 were not mediated by *Alu/Alu* recombination and the deletion of exons 1-2 resulted from recombination of a sequence in intron 2 of the *BRCA1* gene with the homologous sequence from the ψ *BRCA1* gene [14].

Sequencing of the junction fragment amplified by LR-PCR that confirms MLPA results and characterizes deletion breakpoints is a necessary step in the LGR analysis. However, location of the breakpoints and PCR amplification may fail when the deletion interferes with large introns or regions beyond the gene boundaries, where the location of the breakpoints may be difficult. In tested carriers, we initially failed to amplify specific junction fragments in two novel LGRs with borders located in regions adjacent to the *BRCA1* gene (deletions comprising exons 1-17 and 21-24). Chromosome-specific aCGH with a high density of oligonucleotide probes is a method that enables accurate mapping of the deleted region. In this study, we implemented commercially available chromosome 17 -specific oligonucleotide arrays (Roche NimbleGen) that have comparable density of probes as custom made arrays described recently [18]. The technique was validated by analysis of a sample with already identified breakpoints (deletion of exons 18-22) and consequently two novel *BRCA1* rearrangements with unknown boundaries were analyzed. In all cases, the range of the deletion was accurately mapped, which enabled convenient PCR design and amplification of a short junction fragment (less than 1,500 bp) suitable for sequence analysis (Fig. 2). The sample No 609 with deleted exons 21-24 (deletion of 25.639 kb) was tested by both chromosome-specific (median probe spacing ~160 bp) and whole-genome aCGH (median probe spacing ~7000 bp) (Roche NimbleGen). The deletion was identified by chromosome-specific aCGH, while the resolution power of the whole-genome aCGH with lower density of oligonucleotide probes was not satisfactory (data not shown).

In conclusion, analysis performed in 521 high-risk families and patients with non-familial cancer demonstrated that LGRs at the *BRCA1* locus account for 12.3% of all disease-causing mutations found in the *BRCA1* gene. The two Czech-specific founder mutations, the deletions of exons 1-17 and 5-14, were dominant gene alterations. Our results indicate that screening for LGRs in the *BRCA1* gene performed in high-risk families can also be recommended to patients with non-familial cancer; in particular cases with early-onset BC or OC. Screening for LGRs in *BRCA2* may be restricted to high-risk families with male breast cancer, in which large rearrangements were identified in several studies. Characterization of deletion breakpoints belongs to essential steps in analysis of LGRs that could be substantially

improved by chromosome-specific aCGH suitable for accurate detection of deletion boundaries.

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Tables

Table 1 Primers used for amplification of junction fragments

Exon (length of deletion)	Primers (forward and reverse)	Position of primers ^{a/b}	Amplified junction fragment	Detection method
1A-2 (36.934 kb)	¹ F: ACCTAAAATTCCTTCTGCTGGAC R: CAAAGAACGACTAACCTGGCA	^a 197249-235352	1.2 kb	MLPA, LR-PCR
1-17 (80.496 kb)	F: TATCCCAGTGACTTTCCAGC R: TGAGAGGGATGGGAGAATAC	^a 208205-290011	1.3 kb	MLPA, aCGH, LR-PCR
5-10 (15.552 kb)	F: AGGCAGTCATGCATTAGTTTG R: CCGTCTCAGAAAATTCACAAG	^b 17563-33895	0.8 kb	MLPA, LR-PCR
5-14 (31.583 kb)	² F: CCTTACCTACCTACATTCAC R: CTTTATGTAGGATTCAGAGTA	^b 19244-54463	3.6 kb	MLPA, LR-PCR
13-19 (25.201 kb)	F: ACCAGTTTATGCTCTTCCAG R: GCCTATAAGATGCATTCCCT	^b 41617-67533	0.7 kb	MLPA, LR-PCR
18-22 (16.316 kb)	F: GCTCGTGTACAAGTTTGCCAGA R: ATGGAAGCCATTGTCTCTGTG	^b 61040-81093	3.7 kb	MLPA, aCGH, LR-PCR
21-22 (3.779 kb)	F: AGCATGATTTTGAAGTCAGAGGAG R: GTCCAGGCATCTGGCTGCACAACCACAA	^b 71596-81074	5.9 kb	cDNA analysis, LR-PCR
21-24 (25.639 kb)	F: TTATCTCATAACTAGGCATCC R: TTCCTGAAAGTCCTCAGAGC	^b 75369-101263	0.3 kb	MLPA, aCGH, LR-PCR

¹⁻² Primers according to ¹Preisler-Adams et al. [16]; ²Vasickova et al. [22]; ^a GenBank reference sequence NC_000017.9 complement (38300000..38760000); ^b GenBank reference sequence L78833.1

Table 2 Germline rearrangements in the *BRCA1* gene

Exon	Chromosomal breakpoints ^a , mut. Designation – gDNA ^b , mut. Designation – cDNA ^{c/d}	Predicted mutation effect	Recombination	Patient No.	BC & OC cases ^e (age at diagnosis)	Other tumors	Reference
1A-2	^a chr17: 38,525,493-38,562,426 ^b g.197575_234508del 36934 ^c c.1-32787_80+4067del 36934	No transcript?	<i>ΨBRCA1/BRCA1</i>	779	BC (<u>58*</u>); OC (<u>51*</u> , 47)	-	7, 14, 34
1-17	^a chr17:38,470,578-38,551,073 ^b g.208928_289423del 80496 ^c c.1-21434_5075-1084del 80496	Loss of ~90% of coding sequence	<i>AluY/AluY</i>	206 225 269 416	BC (<u>28</u> , 50, 50) BC (27); OC (<u>40</u>) BC (<u>35</u> , 36) BC (39, 62*); OC (<u>42</u> , 64*)	Uterus (3x) Colon, lung - Lymphoma	novel
5-10	^a chr17:38,501,028-38,516,581 ^b g.17696_33247del 15552ins35 ^d c.135-4505_670+361del 15552ins35	p.Lys45Asn fsX3	<i>AluSx/AluY/AluJb</i>	1190	BC (50, 50); OC (<u>46</u>)	-	novel
5-14	^a chr17:38,480,980- 38,512,561 ^b g.21716_53298del 31583 ^d c.135-485_4485-913del 31583	p.Lys45_Lys1495del	LINE/-	278 453 862 938	BC (<u>40/41</u> , 63, 69, 35/35) BC (<u>39</u>) BC (<u>44</u> , 48*); OC (60, 64*) BC (<u>32</u> , ?*, ?); OC (?*)	Pancreas, uterus - Prostate -	7, 22
13-19	^a chr17:38,467,226-38,492,424 ^b g.41850-67050del 25201 ^d c.4185+4062_5193+1650del 25201	p.Gln1396_Glu1731del	<i>-/AluSx</i>	571	BC (<u>56/56</u> , 48, 44)	-	novel
18-22	^a chr17:38,454,318-38,470,629 ^b g.63647_79962del 16316 ^d c.5075-1135_5046+347del 16316	p.Asp1692Gly fsX26	<i>AluY/AluSx</i>	897	BC (<u>35</u> , 64, ?, ?, ?, ?)	-	novel
21-22	^a chr17:38,453,374- 38,457,152 ^b g.77128-80906del 3779ins236 ^d c.5278-492_5407-128del 3779ins236	p.Ile1760_Thr1802del	<i>AluSx/AluJb</i>	102	OC (<u>36</u>)	-	22, 23
21-24	^a chr17:38,433,150- 38,458,787 ^b g.75489_101127del 25639 insTAG ^d c.5278-2135_5592+18067del 25639insTAG	No transcript? p.Ile1760 fs	<i>-/-</i>	609	BC (<u>38</u> , ?, ?)	-	novel

^a Genomic locale for chromosome 17 is from the UCSC genome browser, Mar 2006 assembly; ^b GenBank reference sequence L78833.1; ^c GenBank reference sequence covering the analyzed region: NC_000017.9 complement (38300000..38760000); ^d Mutation numbering: +1 corresponds to the A of the ATG translation initiation codon in the reference sequence U14680; ^e BC and/or OC cases in proband and its relatives. Age of onset in proband is indicated in bold underlined numerals; asterisks (*) mark the patient with both BC and OC; ages at diagnosis of bilateral BC cases are separated by a slash (/)

Table 3 Frequencies of genetic changes in relation to classification of patients and families

Classification	Number of families/patients	Small mutations in <i>BRCA1</i> (%)	Small mutations in <i>BRCA2</i> (%)	LGRs in families in the study (%)	LGRs in families negatively tested for <i>BRCA1/2</i> mutation (%)
HBC families	206	26 (12.6)	19 (9.2)	6 (2.9)	6/161 (3.7)
HBOC+HOC families	138	48 (34.8)	12 (8.7)	6 (4.3)	6/78 (7.7)
High-risk families	344	74 (21.5)	31 (9.0)	12 (3.5)	12/239 (5.0)
Sporadic cases	311	26 (8.4)	3 (1.0)	2 (0.6)	2/282 (0.7)
Overall	655	100 (15.3)	34 (5.2)	14 (2.1)	14/521 (2.7)

HBC, hereditary breast cancer; HBOC, hereditary breast and ovarian cancer; HOC, hereditary ovarian cancer

Table 4 The mean predicted probabilities calculated using BRCAPRO and actual occurrence of *BRCA1* mutations

Classification	Number of families/patients	Mutations in <i>BRCA1</i> (%)	BRCAPRO Mean (\pm SD) Range
High-risk families	344	86 (25.0)	0.229 (\pm0.269) 0.001-0.975
Sporadic cases	311	28 (9.0)	0.086 (\pm0.122) 0.001-0.838
Overall	655	114 (17.4)	0.162 (\pm0.225) 0.001-0.975

Table 5 Haplotype association with deletion of exons 1-17 and 5-14

	Patient No	D17S1325	D17S1322	D17S855	D17S1320
Del ex 1-17	206	*186 194	115 124	147 139	167 171
	225	186 192	115 115	147 145	167 173
	269	186 194	115 115	147 141	167 173
	416	186 192	115 115	147 145	167 169
Del ex 5-14	278	192 188	115 121	145 139	173 160
	453	192 200	115 118	145 143	173 173
	862	192 194	115 115	145 147	173 169
	938	192 204	115 118	145 139	173 179

* Sizes of microsatellite markers (bp)

Legends for figures

Fig. 1 Five novel deletions in *BRCA1* gene identified by MLPA analysis. Control sample is indicated by wt, numbers denote exons of *BRCA1*, C – control DNA sequences. Arrows indicate exons interpreted as deleted.

Fig. 2 Breakpoint analysis of three novel genomic rearrangements. Left - copy number analysis at the *BRCA1* locus by aCGH. The y-axis indicates log₂ ratios of Cy5/Cy3 fluorescent intensity. Red lines represent the results of segMNT analysis. Deleted regions are shown by lower red lines. The schemes show the position and orientation of *BRCA1*, *NBR2* and *RND2* genes and position of deleted regions (in red). Genomic locale is from the UCSC genome browser, Mar 2006 assembly. Right - analysis of PCR products on agarose gels and breakpoint verification by sequencing. Carriers of the deletion are indicated by numbers; lane C - negative control; lane M - size standards. Breakpoints are indicated by dashed lines. (a) *Alu*-mediated 80.5-kb deletion comprising exons 1-17 and the *NBR2* gene; (b) *Alu*-mediated 16.3-kb deletion comprising exons 18-22; (c) a 25.6-kb deletion spanning exons 21-24 and the last three exons of *RND2* gene.

Fig. 3 Breakpoint analysis of a complex genomic rearrangement comprising deletion of exons 5-10 and insertion of 35 nucleotides using LR-PCR (left) and sequencing (right). The carrier of the deletion is indicated by a number; lane C - negative control; lane M - size standards. Breakpoints verified by sequencing are indicated by dashed lines. The upper part of the scheme shows the deleted sequence in red; exons are denoted as red bars; position (out of scale) and orientation of *Alu*-elements are indicated by arrowheads. *Alu*-elements involved in rearrangement are labeled. Sequence coordinates are according to the GenBank reference sequence L78833.1. The lower part of the scheme shows sequences upstream and downstream to the breakpoints. Identical sequences, where the recombination events occur, are indicated by vertical lines. The green box highlights an insert of 35-bp. The underlined sequence corresponds to the junction fragment.

Fig. 4 Breakpoint analysis of a 25.2-kb deletion of exons 13-19 by LR-PCR (left) and sequencing (right). The carrier of the deletion is indicated by a number; lane C - negative control; lane M - size standards. Breakpoints verified by sequencing are indicated by dashed line.



