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PRECLINICAL STUDY

Contribution of *BRCA1* large genomic rearrangements to early-onset and familial breast/ovarian cancer in Pakistan

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Abstract
Background Germline mutations in *BRCA1* and *BRCA2* (*BRCA1/2*) account for the majority of hereditary breast and/or ovarian cancers. Pakistan has one of the highest rates of breast cancer incidence in Asia, where *BRCA1/2* small-range mutations account for 17% of early-onset and familial breast/ovarian cancer patients. We report the first study from Pakistan evaluating the prevalence of *BRCA1/2* large genomic rearrangements (LGRs) in breast and/or ovarian cancer patients who do not harbor small-range *BRCA1/2* mutations.
Materials and methods Both *BRCA1/2* genes were comprehensively screened for LGRs using multiplex ligation-dependent probe amplification in 120 *BRCA1/2* small-range mutations negative early-onset or familial breast/ovarian cancer patients from Pakistan (Group 1). The breakpoints were characterized by long-range PCR- and DNA-sequencing analyses. An additional cohort of 445 *BRCA1/2* negative high-risk patients (Group 2) was analyzed for the presence of LGRs identified in Group 1.
Results Three different *BRCA1* LGRs were identified in Group 1 (4/120; 3.3%), two of these were novel. Exon 1–2 deletion was observed in two unrelated patients: an early-onset breast cancer patient and another bilateral breast cancer patient from a hereditary breast cancer (HBC) family. Novel exon 20–21 deletion was detected in a 29-year-old breast cancer patient from a HBC family. Another novel exon 21–24 deletion was identified in a breast-ovarian cancer patient from a hereditary breast and ovarian cancer family. The breakpoints of all deletions were characterized. Screening of the 445 patients in Group 2 for the three LGRs revealed ten additional patients harboring exon 1–2 deletion or exon 21–24 deletion (10/445; 2.2%). No *BRCA2* LGRs were identified.
Conclusions LGRs in *BRCA1* are found with a considerable frequency in Pakistani breast/ovarian cancer cases. Our findings suggest that *BRCA1* exons 1–2 deletion and exons 21–24 deletion should be included in the recurrent *BRCA1/2* mutations panel for genetic testing of high-risk Pakistani breast/ovarian cancer patients.

Keywords Large genomic rearrangement · *BRCA1* · *BRCA2* · Germline mutations · Breast and/or ovarian cancer · Pakistan

Abbreviations

DHPLC	Denaturing high-performance liquid chromatography
EOBC	Early-onset breast cancer
ER	Estrogen receptor
HBC	Hereditary breast cancer
HBOC	Hereditary breast and ovarian cancer
HER-2	Human epidermal growth factor receptor 2

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HGVS	Human Genome Variation Society
LGRs	Large genomic rearrangements
MLPA	Multiplex ligation-dependent probe amplification
PR	Progesterone receptor
RFLP	Restriction fragment length polymorphism
RPA	Ratio relative peak area
TNBC	Triple-negative breast cancer

Introduction

Approximately 5–10% of all breast and ovarian cancers are the result of an inherited predisposition due to germline alterations (including small-range mutations and large genomic rearrangements (LGRs)) in the two major high-penetrance breast cancer susceptibility genes, *BRCA1* and *BRCA2* (*BRCA1/2*) [1, 2]. Women with pathogenic *BRCA1/2* mutations have increased lifetime risk of developing breast and ovarian cancer as well as various other malignancies [3]. Identification of individuals harboring *BRCA1/2* mutations is clinically relevant and has significant impact on surveillance and management [4].

The presence of alterations in these genes is variable in different populations and may therefore account for a varying fraction of breast and ovarian cancer cases. In Pakistan, currently with one of the highest rates of breast cancer in Asia, *BRCA1/2* small-range mutations account for 17% of hereditary breast/ovarian cancer and early-onset breast and ovarian cancer cases [5]. The mutation frequency reported in Pakistani population may be underestimated as only PCR-based mutation screening methods have previously been applied that would have missed LGRs. Detection of LGRs in *BRCA1/2* is important because these alterations have been shown to significantly contribute to hereditary breast and/or ovarian cancer in some populations. At present, more than 100 different LGRs in *BRCA1/2* have been described among breast/ovarian cancer cases negative for *BRCA1/2* small-range mutations worldwide [6].

Among high-risk breast/ovarian cancer patients, the prevalence and spectrum of LGRs in *BRCA1/2* vary significantly with the geographic distribution, ethnicity, and selection criteria of study population. LGRs in *BRCA1/2* have been identified in studies (considering those with more than 100 high-risk cases) with frequencies varying from 0 to 5% in Europe [7–16], 3.3 to 4.4% in Australia/New Zealand [17, 18], and 4.8 to 11.7% in US [19–21]. However, increased frequency of LGRs in *BRCA1* was reported in those populations where founder effects or recurrent mutations had been identified [8, 10, 16, 22–25]. Contrary to *BRCA1,* LGRs in *BRCA2* were absent in several populations [8, 12, 14–16, 26, 27] or reported rarely [7, 10, 17, 19–21, 28–30].

The LGRs in *BRCA1/2* have been reported with frequencies varying from 0.4 to 3.0% in Asia [26–30]. Studies from Asia assessing the contribution of *BRCA1/2* LGRs to breast/ovarian cancer cases have been small in size [31–34] or analyzed only *BRCA1* [31, 34–36]. We report the results of a large study from Pakistan which evaluated the prevalence of LGRs in the *BRCA1/2* genes in 565 high-risk breast/ovarian cancer patients who did not harbor small-range *BRCA1/2* mutations. Initially, 120 patients (Group 1) were comprehensively screened by multiplex ligation-dependent probe amplification (MLPA) for all exons of *BRCA1* and *BRCA2*. An additional cohort of 445 high-risk cases (Group 2) was analyzed for those LGRs identified in Group 1.

Materials and methods

Study population

Breast and/or ovarian cancer families were identified at the Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH & RC) in Lahore, Pakistan, from June 2001 to August 2015. Index patients diagnosed with invasive breast cancer or epithelial ovarian cancer from 565 unrelated breast and/or ovarian cancer families were included in the current study based on following criteria: (A1) families with one female breast cancer diagnosed $\leq$30 years of age, (A2) families with two first- or second-degree (through a male) female relatives diagnosed with breast cancer; at least one diagnosed $\leq$50 years of age, (A3) families with at least three cases of breast cancer; at least one diagnosed $\leq$50 years of age, (A4) families with one male breast cancer case diagnosed at any age, (B) families with at least one female breast cancer and one ovarian cancer at any age, (C1) families with one ovarian cancer diagnosed $\leq$45 years of age, and (C2) families with at least two ovarian cancers; at least one diagnosed $\leq$45 years of age. Bilateral breast cancer or breast and ovarian cancer in the same patient were counted as two independent diagnoses. A detailed description of the 565 index cases is shown in Table 1. All study participants signed informed written consent. The study was approved by the institutional review board of the SKMCH & RC.

Genomic DNA was extracted as described elsewhere [37]. One hundred and twenty cases underwent comprehensive screening for *BRCA1/2* using protein-truncation test, single-strand conformational polymorphism analysis and denaturing high-performance liquid chromatography (DHPLC) analysis followed by DNA sequencing of variant fragments and confirmed to be negative for *BRCA1/2* small-range mutations as described previously were assigned to Group 1 [5]. Small-range mutations affecting

Table 1 Phenotypic characteristic of 565 index patients from Pakistan

Characteristic	Group 1 ($n = 120$)	Group 2 ($n = 445$)	Total ($N = 565$)
Gender, No (%)			
Male	11 (9.2)	22 (4.9)	33 (5.8)
Female	109 (90.8)	423 (95.1)	532 (94.2)
Female breast cancer, No (%)	108 (90.0)	389 (87.4)	497 (88.0)
Age at diagnosis, years			
Mean	30.4	35.8	34.6
Range	21–70	18–78	18–78
Male breast cancer, No (%)	11 (9.2)	22 (4.9)	33 (5.8)
Age at diagnosis (years)			
Mean	48.0	52.5	51.0
Range	30–73	27–69	27–73
Ovarian cancer, No (%)	5 (4.2)	43 (9.7)	48 (8.5)
Age at diagnosis (years)			
Mean	31.8	38.8	38.1
Range	29–34	22–66	22–66
Ethnicity, No (%)			
Punjabi	92 (76.7)	295 (66.3)	387 (68.5)
Pathan	19 (15.8)	64 (14.4)	83 (14.7)
Others	9 (7.5)	86 (19.3)	95 (16.8)
LGRs in *BRCA1*, No (%)	4 (3.3)	10 (2.2)	14 (2.5)
Exon 1–2 deletion	2 (1.7)	7 (1.6)	9 (1.6)
Exon 20–21 deletion	1 (0.8)	0	1 (0.2)
Exon 21–24 deletion	1 (0.8)	3 (0.7)	4 (0.7)
LGRs in *BRCA2*, No (%)	0	0	0
Pathology of breast cancer, No (%)			
TNBC	51 (48.1)	89 (23.2)	140 (28.6)
Non-TNBC	55 (51.9)	294 (76.8)	349 (71.4)
Unknown	13	28	41
Frequency of LGRs based on pathology[a], No (%)			
TNBC	4 (8.2)	5 (5.6)	9 (6.4)
Non-TNBC	0	3 (1.0)	3 (0.9)

TNBC triple negative breast cacner

[a] Two LGRs in *BRCA1* were found in index patients diagnosed with ovarian cancer

one or a few nucleotides included frameshift deletions or insertions, nonsense mutations, or splice junction alterations. 445 cases additionally enrolled were subsequently analyzed for *BRCA1/2* using DHPLC- and DNA-sequencing analyses and found negative for small-range mutations were assigned to Group 2. Ninety-eight patients from Group 1 and 311 cases from Group 2 have previously been included in a Pakistani breast cancer study [38].

After screening for LGRs in the complete *BRCA1/2* coding regions in Group 1 ($n = 120$), Group 2 ($n = 445$) was screened for the three identified LGRs.

MLPA analyses

For Group 1 cases, comprehensive MLPA analyses was performed for *BRCA1* (using probe mix P002 for primary screening and probe mix P087 for confirmatory screening) and *BRCA2* (using probe mix P045), as described by manufacturer (MRC Holland, Amsterdam, The Netherlands). Separation and relative quantification of the amplified product was obtained using the Beckman CEQ 8000XL DNA analysis system (Beckman Coulter, Fullerton, USA). A positive control with a known LGR in *BRCA1* (exon 1–2 deletion, exon 1–7 deletion, exon 3–16 deletion, exon 9–12 deletion, exon 13–15 deletion, exon 13 duplication, exon 14 deletion, exon 14–20 deletion, exon 17–19 deletion, exon 20 deletion, exon 21–24 deletion, exon 22 deletion, exon 23–24 deletion and exon 24 deletion) or *BRCA2* (exon 1–2 deletion) was included in the MLPA analyses. For quality control of each experiment, visual peak pattern evaluation was performed as described by manufacturer (MRC Holland, Amsterdam, The

Netharlands). The proportion of each peak relative to the height of all peaks was calculated for every single patient sample and then compared to proportions for the corresponding peak averaged for a set of healthy control samples using a commercially available SeqPilot software (JSI medical systems GmbH, Ettenheim, Germany). The ratio relative peak area (RPA) defined as the RPA of the patient result file divided by the RPA of the control result file was calculated. If this value was around 100%, the RPA of the patient was about the RPA of the control indicative of no copy number change. Samples revealing RPA ratios of $\leq$50% were considered as deletions and RPA ratios of $\geq$150% as duplications. Each positive result was independently confirmed in a second experiment. DNA-sequencing analyses was performed in samples showing single exon deletion or duplication to exclude the presence of polymorphisms at the probe ligation sites.

Identification of genomic breakpoints and confirmation

DNA samples with a positive MLPA result indicating exon 1–2 deletion, exon 20–21 deletion, and exon 21–24 deletion in the *BRCA1* gene were subjected to further analysis.

For characterization of exon 1–2 deletion, long-range PCR was performed using long template enzyme mix (Fermentas, Vilnius, Lithuania) with the primer pair as previously reported [39].

For characterization of exon 20–21 deletion and exon 21–24 deletion, a combined approach of long-range PCR and restriction fragment length polymorphism (RFLP) was used to narrow down the breakpoint regions with restriction endonucleases PstI or PstI and XmnI, respectively (New England Biolabs, Ipswich MA, USA). The primer pairs (sequences are available upon request) that eventually allowed for characterization of exon 20–21 deletion and exon 21–24 deletion were *P1* and *P2* (located in intron 19 and 21) and *P3* and *P4* (located in intron 20 and $\sim$25 kb downstream of the *BRCA1* stop codon), respectively (Fig. 1a). Mutation-specific PCR products of 580 and 536 bp containing breakpoints of exon 20–21 deletion and exon 21–24 deletion were obtained, respectively (Fig. 1b, c). PCR products containing the breakpoint regions were purified using QIAquick® PCR purification kit (Qiagen, Hilden, Germany) and bidirectionally sequenced with BigDye Terminator v3.1 Cycle Sequencing kit on the ABI 3130 genetic analyzer (Applied Biosystem, Foster City, CA, USA).

Sequence traces were aligned to the February 2009 assembly of the Human Genome Browser (available at: http://genome.ucsc.edu/). Mutations were described according to the recommended nomenclature system described by the Human Genome Variation Society (HGVS) [40]. Designation of the exon 1–2 deletion and exon 20–21 deletion in *BRCA1* was based on the reference sequence NG_005905.2. Designation of the *BRCA1* exon 21–24 deletion was based on the chromosome 17 reference sequence from February 2009 assembly of the Genome Reference Consortium Human genome build 37 (GRCh37/hg19) (available at: https://genome.ucsc.edu). The sequences at breakpoints were also analyzed by RepeatMasker program (http://www.repeatmasker.org). A second blood sample was taken from patients identified with LGR. LGRs were confirmed by PCR-based assays as described above.

The *BRCA1* LGRs identified in Group 1 were subsequently analyzed in the 445 cases in Group 2 by deletion specific PCR-based assays using primer pairs flanking the deletion breakpoints. A positive control with a known *BRCA1* exon 1–2 deletion, exon 20–21 deletion, or exon 21–24 deletion was included in each experiment. PCR products containing the breakpoint regions were bidirectionally sequenced to confirm the deletions.

Results

Characteristics of the study participants

Characteristics of the 565 unrelated index patients from Pakistani breast and/or ovarian cancer families are shown in Table 1. Of the index cases, 530 patients had a diagnosis of invasive breast cancer—497 females and 33 males. Forty-eight patients had a diagnosis of epithelial ovarian cancer. Majority of the index patients belonged to the Punjabi (68.5%) or Pathan (14.7%) ethnic group. Of the index patients who presented with invasive breast cancer, 140 (28.6%) had triple-negative breast cancer (TNBC), i.e., lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) expression, while 349 (71.4%) were diagnosed with non-TNBC phenotype. In Group 1, the median age of disease onset was 30.4 years (range 21–70) for female breast cancer ($n = 108$), 48.0 years (range 30–73) for male breast cancer ($n = 11$), and 31.8 years (range 29–34) for ovarian cancer ($n = 5$). Seventeen of 120 women (14.2%) were diagnosed with bilateral breast cancer. In Group 2, the median age of disease onset was 35.8 years (range 18–78) for female breast cancer ($n = 389$), 52.5 years (range 27–69) for male breast cancer ($n = 22$), and 38.8 years (range 22–66) for ovarian cancer ($n = 43$). Twenty-nine of 445 (6.5%) women were diagnosed with bilateral breast cancer. All index patients were previously tested and found to be negative for germline small-range mutations in the *BRCA1/2* genes [5, 38].

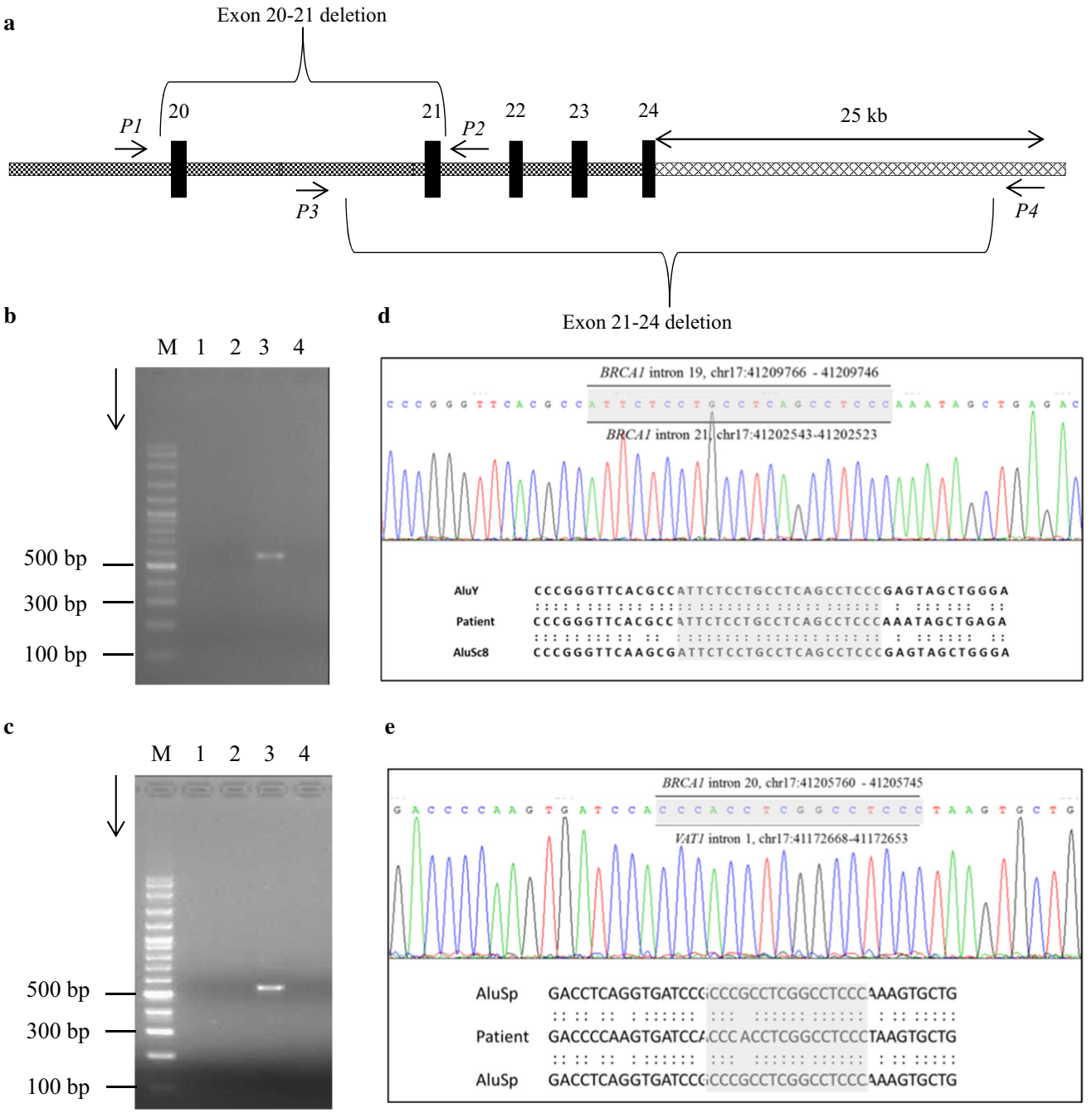

Fig. 1 Diagram of the *BRCA1* exon 20–21 deletion and exon 21–24 deletion assays. **a** Genomic structure of breakpoint region with numbered exon. *Solid blocks*, *BRCA1* exon 20–24; *dotted bars*, intron 19–23; *crossed-pattern bar*, 25 kb genomic region downstream from *BRCA1* stop codon; *Brackets* above and below the diagram, positions of the respective breakpoints. Primer positions for the specific rearrangement assay (*P1, P2* and *P3, P4*) are indicated along with respective orientation. Photographs of ethidium bromide-stained gels of exon 20–21 deletion specific PCR (**b**), and exon 21–24 deletion specific PCR (**c**). *M*, DNA 100 bp marker; *Lanes 1, 2*, predicted wild-type allele products of 7.8 kb (**b**) and 33.6 kb (**c**) that were not detectable in assays; *Lane 3*, mutant alleles with a deletion resulting in 580 bp (**b**) and 536 bp (**c**) fragments; *Lane 4*, no template control. DNA sequence analysis of the fragments with breakpoints for exon 20–21 deletion (**d**) and exon 21–24 deletion (**e**). *Boxes shaded in gray*, the 21-bp core identical sequences for both the *Alu*Y and *Alu*Sc present in intron 19 and 21, respectively (**d**), and 16-bp core identical sequences for the *Alu*Sp present in intron 20 of *BRCA1* and intron 1 of *VAT1* (**e**), where the recombination events occur

LGRs identified in Group 1

All index patients of Group 1 ($n = 120$) were comprehensively screened for *BRCA1/2* LGRs using MLPA analyses. Genomic breakpoints were identified by the joint approach of long-range PCR-, RFLP-, and DNA-sequencing analyses. Three LGRs were identified in four patients (4/120, 3.3%) (Table 2). All of them were deletions in *BRCA1*. The frequency of *BRCA1* LGRs by family phenotype was 1.4% (1/72) for early-onset breast cancer (EOBC), 6.7% (2/30) for hereditary breast cancer (HBC), and 14.3% (1/7) for hereditary breast and ovarian cancer (HBOC) families (Table 2). All deletion carriers were diagnosed with invasive ductal carcinoma, grade 3 tumor, and displayed TNBC features. No LGRs were detected in the *BRCA2* gene.

BRCA1 exon 1–2 deletion

A recurrent and previously reported LGR, *BRCA1* exon 1–2 deletion [39] was identified in two patients of Punjabi ethnicity: one with EOBC at 25 years of age (II:3) (Supplementary Fig. 1, FP 89) and another patient with bilateral breast cancer at age 28 and 33 (III:5) and also reported a family history of HBC (Supplementary Fig. 1, FP 147). Characterization of the genomic breakpoints of this LGR revealed a 36,934 bp deletion, which is similar to the deletion previously described [39].

BRCA1 exon 20–21 deletion

A novel LGR, exon 20–21 deletion was identified in a 29-year-old breast cancer patient (III:1) of Punjabi background. The mother (II:4) and maternal uncle (II:2) of the index patient were diagnosed with breast cancer or intestinal cancer at age 47 and 61, respectively (Supplementary Fig. 1, FP 187).

Sequence analysis revealed a deletion of 7223 bp (chr17:41,202,523–41,209,745) (Fig. 1a, b, d). Sequence analysis at the junction point showed that the exon 20–21 deletion fused the *Alu*Y sequence of intron 19 with the *Alu*Sc of intron 21 (Fig. 1d). The deletion involved an unequal homologous recombination between these two *Alu* sequences which shared 98% homology. The sites of crossover event were present within a 21-bp sequence.

BRCA1 exon 21–24 deletion

Another novel LGR, exon 21–24 deletion, was identified in a patient (IV:6) from Balochistan, who was diagnosed with breast and ovarian cancer at age 29 and 31, respectively (Supplementary Fig. 1, FP 193). The mutation co-segregated with the disease in two sisters of the index patient, one diagnosed with breast cancer (IV:8) at age 44 and the other diagnosed with ovarian cancer (IV:5) at age 30. Another sister (IV:1) was diagnosed with breast cancer at age 37. Three other breast cancers (II:3, III:6, III:7) and one uterine cancer (II:1) were also reported in this family.

Sequence analysis revealed a deletion of 33,092 bp (chr17:41,172,653–41,205,744) (Fig. 1a, c, e). Sequence analysis at the junction point showed that the exon 21–24 deletion fused the *Alu*Sp sequence of intron 20 with *Alu*Sp of *VAT* intron 1, present ~25 kb downstream of stop codon of *BRCA1* (Fig. 1e). The deletion involved an unequal homologous recombination between these two *Alu*

Table 2 Frequencies of LGRs in *BRCA1/2* according to family structure

Risk group	Phenotype of families	Group 1[a]			Group 2[b]	
		No. of families	Families with LGRs *N* (%) in		No. of families	Families with LGRs *N* (%) in *BRCA1*
			BRCA1	*BRCA2*		
	Female breast cancer families	102	3 (2.9)	0 (0)	365	7 (1.9)
A1	1 case ≤30 years (EOBC)	72	1 (1.4)	0 (0)	182	2 (1.1)
A2 + A3	≥2 cases, ≥1 diagnosed ≤50 years (HBC)	30	2 (6.7)	0 (0)	183	5 (2.7)
A4	Male breast cancer families					
	≥1 case of male breast cancer	11	0 (0)	0 (0)	22	0 (0)
B	Breast-ovarian cancer families (HBOC)					
	≥1 breast cancer and ≥1 ovarian cancer	7	1 (14.3)	0 (0)	32	2 (6.2)
	Ovarian cancer families	0	0 (0)	0 (0)	26	1 (3.8)
C1	1 case diagnosed ≤45 years	0	0 (0)	0 (0)	23	0 (0)
C2	≥2 cases, ≥1 diagnosed ≤45 years (HOC)	0	0 (0)	0 (0)	3	1 (33.3)
	All families	120	4 (3.3)	0 (0)	445	10 (2.2)

EOBC early-onset breast cancer, *HBC* hereditary breast cancer, *HBOC* hereditary breast and ovarian cancer, *HOC* hereditary ovarian cancer

[a] Comprehensive MLPA analysis

[b] PCR-based assay for LGRs identified in group 1

sequences which shared 100% homology. The sites of crossover event were present within a 16-bp sequence.

LGRs identified in Group 2

Screening for patients in Group 2 for the presence of the three *BRCA1* LGRs identified in Group 1 revealed ten deletions. Exon 1–2 deletion was detected in seven patients, all of Punjabi ethnic origin (Supplementary Fig. 1, FP 229, FP 498, FP 379, FP 406, FP 549, FP 314, and FP 291). Exon 21–24 deletion was detected in three patients of Punjabi background (Supplementary Fig. 1, FP 261, FP 719, and FP 191). The frequency of *BRCA1* LGRs by family phenotype was 1.1% (2/182) for EOBC, 2.7% (5/183) for HBC, 6.2% (2/32) for HBOC, and 33.3% (1/3) for hereditary ovarian cancer (HOC) families (Table 2).

Several other cancers were also reported among families harboring LGRs from Group 1 and Group 2 (Table 3).

Discussion

In the current study, we investigated the frequency of *BRCA1/2* LGRs in 565 breast and/or ovarian cancer cases, who tested negative for *BRCA1/2* small-range mutations. Initally, index patients from Group 1 ($n = 120$) were comprehensively screened and characterized for LGRs in *BRCA1/2*. The LGRs identified in Group 1 were next screened in Group 2 ($n = 445$). Three different LGRs in *BRCA1* were identified and characterized in Pakistani high-risk cases, with an overall frequency of 3.3% (4/120) in Group 1 and 2.2% (10/445) in Group 2. No LGRs were detected in the *BRCA2* gene.

The exon 1–2 deletion is the most commonly reported LGR in *BRCA1*. This deletion is pathogenic as it predicts for disruption of the *BRCA1* promoter and abolishes the transcription from the mutant allele [41, 42]. In the present study, it was identified in nine Punjabi families from Group 1 and Group 2 including one HBOC, two EOBC, and six HBC families. This deletion comprised 36,934 bp including *BRCA1* exon 1–2 and the intergenic region between *ΨBRCA1* and *BRCA1*. It was previously identified in several other European and North-American studies [7, 8, 12, 16, 19, 21, 39, 41–51] and was associated with different breakpoints. Breakpoints similar to those detected in the Pakistani families were observed in one HBOC family from USA [39] and subsequently detected in families from Italy [42], Germany [8], The Netherlands [50], and Czech Republic [16]. Different [12, 21, 47, 51] or uncharacterized breakpoints were reported in several other studies from Europe and Turkey [7, 19, 41, 43–46, 48, 49].

BRCA1 exon 21–24 deletion is another recurrent LGR detected in our study. This deletion is disease-causative as it is predicts to remove the polyA tail and 3′-UTR regions of *BRCA1,* hence abolishing the transcription from the mutant allele [6]. It was detected in four Pakistani families including one HBC, one HBOC, one HOC family from Punjab, and one HBOC family from Balochistan. This novel large deletion co-segregated with the disease in the Balochi family FP 193 as it was identified in the index patient́s two sisters affected by breast and ovarian cancer at age 44 and 30, respectively, suggesting that this mutation predisposes to breast and ovarian cancer. Her niece, who was unaffected, tested negative for this mutation. The deletion comprised a 33,092 bp fragment, with breakpoints within highly similar duplicated sequences, i.e., *Alu* elements (*Alu*Sp). These findings support the notion that the *BRCA1* exon 21–24 deletion is a result of *Alu* repeat-mediated recombination event. The *BRCA1* sequence is composed of about 42% *Alu* sequences, thereby suggesting that majority of LGRs in this gene are due to *Alu* repeats [52]. *BRCA1* exon 21–24 deletion has previously been reported with the different breakpoints in one HBOC family from Ireland [21] and one HBC family from Czech Republic [16]. It has also been described with uncharacterized breakpoints [19, 20, 45, 46]. The results of these studies indicate that different hotspots are present in intron 20 and downstream of the *BRCA1* gene, which result in deletion fragments of different sizes.

A *BRCA1* exon 20–21 deletion was identified in one HBC family of Punjabi background, suggesting it is a rare alteration in Pakistan. This deletion is novel and has not been previously reported. It comprised a 7223 bp fragment with breakpoints within *Alu* elements, *AluY* and *AluSc*. This finding supports the notion that the *BRCA1* exon 20–21 deletion is because of *Alu* repeat-mediated recombination event.

In the current study, *BRCA1* LGRs were identified in 3.3% of Pakistani patients with EOBC or family history of breast/ovarian cancer. Lower *BRCA1* LGR frequencies ranging from 0.4 to 2.1% were observed in other Asian studies from China, Malaysia, Indonesia, Singapore, or Korea, considering those with more than 100 EOBC or familial patients [26–30]. Frequencies ranging from 0 to 10.7% have been reported by other investigators in studies conducted in Finland, Spain, Italy, Germany, Denmark, Czech Republic, the Netherlands, USA (including Hispanics), and Australia [7, 8, 10, 12, 15–17, 19, 21, 24, 53, 54]. These discrepant results may be explained by differences in inclusion criteria, sample size, the sensitivity of the LGR detection assay used, different ethnic or geographic origins of study participants, and founder effects.

The *BRCA1*-associated breast tumors are reported to have significant numbers of TNBC phenotype [55]. In the current study, the breast tumors linked with *BRCA1* LGRs in majority of Pakistani index patients exhibited TNBC

Table 3 Germline large genomic rearrangements identified in the *BRCA1* gene

Exon deletion	Chromosomal breakpoints[a], mutation designation–gDNA[b], mutation designation–cDNA[c]	Recombination	Family no.	Number of BC or OC (age at onset in years)[d]		Other cancer(s) (age at onset in years)	Ethnicity	Previously described
				BC	OC			
1–2	chr17:41,271,967-41,308,900[a]	*ΨBRCA1/BRCA1*	89[e]	1 (**25**)	–	–	Punjabi	Yes[g]
	g.61101_98034del36934[b]		229[f]	1 (**27**)	–	–	Punjabi	
	c.1-32787_80 + 4067del36934[c]		498[f]	2 (40, **41**)	–	–	Punjabi	
			379[f]	2 (29, **31**)	–	Liver (38)	Punjabi	
			406[f]	2 (**39**, 40)	–	Abdomen (65)	Punjabi	
			549[f]	2 (36, **71**)	–	Bone (> 60), unknown	Punjabi	
			147[e]	2 (**28/33**, < 50)	–	–	Punjabi	
			291[f]	3 (39, 42, 48)	1 (**48**)	Stomach, brain	Punjabi	
			314[f]	6 (**32**, 42, 48, 56, 59, ?)	–	Brain (63), uterus (54), stomach (70), abdomen	Punjabi	
20–21	chr17:41,202,523-41,209,745[a]	*Alu*Y/*Alu*Sc8	187[e]	2 (**29**, 47)	–	Intestine (61)	Punjabi	No
	g.160256_167478del7223[b]							
	c.5194-593_5332 + 557del7223[c]							
21–24	chr17:41,172,653-41,205,744[a]	*Alu*Sp/*Alu*Sp	191[f]	0	2 (**37**, 38)	Lung (53), abdomen (42, 50), leukemia (16)	Punjabi	No
	c.5278-2610_5592 + 25013del33092[c]		261[f]	2 (33, **34**)	–	–	Punjabi	
			719[f]	3 (> 40, **42/42**, ?)	1 (?)	–	Punjabi	
			193[e]	6 (**29***, 35, 37, 44, 55, ?)	2 (30; **31***)	Uterus	Balochi	

Age at diagnosis of bilateral BC case is separated by a slash (/)

BC breast cancer, *OC* ovarian cancer

[a] Genomic locale for chromosome 17 is from the UCSC genome browser, Feb 2009 assembly

[b] GenBank reference sequence NG_005905.2

[c] Mutation numbering: +1 corresponds to the A of the ATG translation initiation codon in the reference sequence U14680

[d] Age at diagnosis of proband and relatives. Age at onset in proband is indicated in *bold* numerals

[e] Families identified in Group 1

[f] Families identified in Group 2

[g] [39, 42, 50]

* The patient with both BC and OC

? The age of BC or OC is unknown

features (9/12; 75%). This observation is in agreement with previous findings that breast tumors associated with LGRs in *BRCA1* predominantly present with TNBC phenotype [29].

In the present study, no LGR in *BRCA2* was detected. Similar findings have been reported in Asian studies from Indonesia and Korea [26, 27] or European studies performed in Germany, Poland, and Czech Republic [8, 12, 14, 16]. LGRs in *BRCA2* were identified at low frequencies ranging from 0.2 to 2.0% in some Asian studies from China, Singapore, and Malaysia [28–30], European studies conducted in Spain, Denmark [7, 9, 10, 13], or studies from the USA [19, 21] and Australia [17, 18], suggesting a minimal contribution of *BRCA2* LGRs to breast and/or ovarian cancer. The low contribution or lack of LGRs in the *BRCA2* gene may be a reflection of the fact that it is composed of few *Alu* sequences.

No *BRCA1/2* LGRs were identified in 33 male breast cancer families included in our study. In agreement with our findings, no LGRs in *BRCA1/2* have previously been reported among male breast cancer patients from Italy [56], Finland [57], USA [58], and Turkey [59]. However, LGRs in *BRCA2* only have been identified in small subset of male breast cancer patients (7.7%; 3/39) from France [60]. Taken together, the results suggest a marginal contribution of *BRCA2* LGRs to hereditary male breast cancer.

In summary, this is the first comprehensive study conducted among Pakistani high-risk breast/ovarian cancer patients to assess the prevalence and spectrum of LGRs in the *BRCA1/2* genes. We have not observed any LGR in *BRCA2* indicating that such type of alterations may be rare or absent in our population. However, LGRs in *BRCA1* are found with a considerable frequency in Pakistani breast/ovarian cancer cases. Two recurrent alterations, *BRCA1* exon 1–2 deletion and exon 21–24 deletion, were identified in multiple high-risk breast/ovarian cancer patients. Potential founder effects of the recurrent mutations will be investigated in future haplotype analyses. Our findings suggest that these recurrent LGRs in *BRCA1* should be included in the panel of recurrent point mutations in *BRCA1/2* for further early-onset and familial-based genetic testing. Our data may improve genetic counseling and may help effective detection strategy in Pakistani early-onset and familial breast/ovarian cancer patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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