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Deleterious *RAD51C* germline mutations rarely predispose to breast and ovarian cancer in Pakistan

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Abstract *RAD51C* plays a key role in homologous recombination-mediated DNA repair and maintenance of genomic stability. Biallelic *RAD51C* mutations cause Fanconi anemia, and monoallelic mutations predispose women to breast and ovarian cancer. Genetic variability of *RAD51C* and its impact in Asian populations have been poorly studied. Here, we report the results of comprehensive mutational screening of the *RAD51C* gene in 348 *BRCA1/2*-negative breast and/or ovarian cancer patients from Pakistan. Mutation analysis of the complete *RAD51C*-coding region was performed using denaturing high-performance liquid chromatography analysis, followed by DNA sequencing of variant fragments. Three novel protein-truncating mutations, c.204T>A, c.225T>G, and c.701C>G, were identified. c.204T>A was found in one out of 22 (4.5 %) early-onset (≤45 years of age) ovarian cancer patients and c.225T>G in one out of 119 (0.8 %) patients from breast cancer only families. c.701C>G was found in a 60-year-old control with no family history of breast/ovarian cancer. Furthermore, three novel in silico-predicted potentially functional mutations, a missense mutation, c.873T>G, a variant in 5′UTR, c.1-34T>G, and a recurrent intronic variant, c.965+21A>G, were identified. The missense mutation was observed in a patient with bilateral breast cancer from a breast and ovarian cancer family (HBOC), the 5′UTR variant was noted in an early-onset breast cancer patient, and the intronic variant in one early-onset breast cancer patient and one ovarian cancer patient from a HBOC family. Five of the six mutations described were not detected in 400 healthy controls. These findings suggest that *RAD51C* plays a marginal role in breast and ovarian cancer predisposition in Pakistan. Reliable estimation of the clinical implications of carrying a deleterious *RAD51C* mutation will require identification of additional mutation-positive patients/families.

Keywords *RAD51C* · Germline mutations · Familial breast and/or ovarian cancer · Pakistan

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Introduction

Approximately 30 % of familial breast cancer is explained by monoallelic mutations in the high and moderate breast cancer penetrance genes *BRCA1*, *BRCA2*, *ATM*, *CHEK2*, *BRIP1*, and *PALB2*. These genes play a key role in maintenance of genomic stability and have been functionally linked with homologous recombination (HR)-mediated DNA repair. Biallelic mutations in three of these genes, *BRCA2* [1], *PALB2* [2], and *BRIP1* [3] also cause Fanconi anemia (FA), establishing a strong link between breast cancer susceptibility and the FA pathway. This link was further strengthened by the identification of a fourth gene, *RAD51C* that is also involved in HR in these hereditary diseases. Biallelic *RAD51C* mutations were identified in two patients from a consanguineous Pakistani family with features of FA [4]. Subsequently, monoallelic mutations were found in families with breast and ovarian cancer [5].

In the first study on the role of *RAD51C* in breast and ovarian cancer predisposition, six monoallelic deleterious *RAD51C* mutations were identified by a combination of genetic and functional assays exclusively in 480 (1.3 %) German breast and ovarian cancer (HBOC) families, but not among any of the 620 families with breast cancer only (HBC) [5]. Since then, many studies have been conducted in Caucasian populations to validate these findings, which, however, yielded controversial results. While no obvious deleterious mutations were detected in various studies undertaken in *BRCA1/2*-negative breast and/or ovarian cancer families from Belgium, the Netherlands, Canada [6, 7], USA [8, 9], Australia [10], and Israel [6, 11], unequivocal deleterious mutations were identified in *BRCA1/2*-negative HBOC families from Spain [12, 13], UK [14], and Australia [15] at frequencies ranging from 0.6 to 1.3 % in studies comprising more than 100 cases and higher frequencies of 2.4 and 2.9 % in smaller studies [16, 17]. In the Finnish study, two deleterious *RAD51C* mutations were identified in eight cases with a personal or family history of ovarian cancer, which conferred moderate-to-high risk of ovarian cancer [18]. The observed mutation frequencies were 25 % (2/8) in ovarian cancer families (HOC) and 1 % in unselected ovarian cancer patients (4/409). These results substantiated the impact of *RAD51C* germline mutations on increased susceptibility and predisposition to breast and ovarian cancer, but also suggest that the *RAD51C* mutation frequency may be lower than initially stated [5].

In Asia, only one study conducted in an East Asian population from China has previously addressed the contribution of *RAD51C* germline mutations toward increased predisposition to breast and ovarian cancer. In this study, no unequivocal deleterious mutations were identified in 273 patients from breast and/or ovarian cancer families [19]. Of the eight identified variants, two were in silico predicted to be potentially functional. Given the paucity of data on genetic variability of *RAD51C* in Asian populations and the fact that germline mutations in the high-penetrance genes *BRCA1*, *BRCA2*, and *TP53* and the moderate-penetrance gene *CHEK2* account for only 20 % of early-onset or familial breast/ovarian cancer in Pakistan [20–22], we assessed the prevalence of *RAD51C* germline mutations in 348 high-risk *BRCA1/2*-negative early-onset and familial breast and/or ovarian cancer patients from Pakistan. An additional group of 400 healthy female Pakistani controls was screened for the identified *RAD51C* mutations.

Materials and methods

Study subjects

The study included index patients from 348 breast and/or ovarian cancer families who were diagnosed with invasive breast cancer or epithelial ovarian cancer at the Shaukat Khanum Memorial Cancer Hospital and Research Center (SKMCH & RC) in Lahore, Pakistan, from June 2001 to June 2011. The index cases were categorized into seven categories based on family history of cancer: (1) families with one female breast cancer diagnosed ≤30 years of age; (2) families with two first- or second-degree (through a male) female relatives diagnosed with breast cancer; at least one diagnosed ≤50 years of age; (3) families with at least three cases of breast cancer; at least one diagnosed ≤50 years of age; (4) families with one male breast cancer case diagnosed at any age; (5) families with at least one female breast cancer and one ovarian cancer at any age; (6) families with one ovarian cancer diagnosed ≤45 years of age; and (7) families with at least two ovarian cancers; at least one diagnosed ≤45 years of age. Cases fulfilling the criteria (2) and (3) were considered as HBC families, those with criterion (5) as HBOC families, and those with criterion (7) as HOC families. All index cases were tested negative for deleterious mutations in the *BRCA1* and *BRCA2* genes [20, 22]. Bilateral breast cancer and breast and ovarian cancer in the same patient were counted as two independent cases. A detailed description of the 348 index cases is provided in Table 1.

The control population comprised 400 healthy Pakistani women. These were either attendants of the patients registered at the hospital or visiting the cancer center for medical reasons other than cancer. The study was approved by the Institutional Review Board of the SKMCH & RC. All study participants signed informed written consent.

Mutation screening

Genomic DNA was extracted as previously described [20]. The complete coding sequence and exon–intron junctions of the *RAD51C* gene (Genbank accession number NM_058216.1) were screened in the 348 index patients by denaturing high-performance liquid chromatography (DHPLC) analysis. DHPLC analysis was performed with the WAVE system (Transgenomics, Omaha, NE, USA). PCR primer pairs were according to Meindl and colleagues [5]. Melting temperatures for the amplicons were predicted by the Transgenomic Navigator software (Version 1.7.0). The setup of PCR reactions, cycling conditions, and DHPLC running conditions are available on request.

The presence of the *RAD51C* mutations identified in the 348 *BRCA1/2*-negative index cases was subsequently evaluated in 400 controls.

DNA sequence analysis

Each sample revealing variants identified by DHPLC analysis was sequenced using an automated 3730XL DNA

Table 1 Description of index cases screened for *RAD51C* mutations

Cancer type of index case	Phenotype of families[a]	Number of families	BC index cases		Mean age of index cases in years (age range)
			Unilateral	Bilateral	
Females	Breast cancer				
BC	Early-onset BC (1 case ≤30 years)	175	175	–	27.2 (19–30)
BC	HBC (2 cases, ≥1 diagnosed ≤50 years)	60	41	19	39.7 (21–59)
BC	HBC (≥3 cases, ≥1 diagnosed ≤50 years)	59	51	8	43.8 (24–73)
	Breast and ovarian cancer				
BC	HBOC (≥2 cases)	9	7	2	48.6 (25–67)
BC + OC[b]	HBOC (≥2 cases)	6	4	2	38.8 (29–59)
OC	HBOC (≥2 cases)	5	–	–	40.4 (33–60)
	Ovarian cancer				
OC	Early-onset OC (1 case ≤ 45 years)	22	–	–	32.9 (22–45)
OC	HOC (2 cases, ≥1 diagnosed ≤45 years)	1	–	–	31
Males	Breast cancer				
BC	Male BC (1 case)	11	11	–	48 (30–73)
All cases[c]		348	289	31	

BC breast cancer, *HBC* hereditary breast cancer, *OC* ovarian cancer, *HBOC* hereditary breast and ovarian cancer, *HOC* hereditary ovarian cancer

[a] See "Study subjects" in the Method section

[b] Bilateral breast cancer and breast and ovarian cancer in the same patient ($n = 6$) were counted as two independent cases

[c] Including 309 female BCs, 34 OCs, and eleven male BCs

sequencer (Applied Biosystems, California, USA) according to the manufacturers' instructions. Amplicons were bi-directionally sequenced to confirm the occurrence of a mutation.

In silico analyses

The *RAD51C* missense variants were analyzed for their potential effect on protein function using the default settings of web tools SIFT (http://sift.jcvi.org), Panther (http://www.pantherdb.org/tools/csnpScoreForm.jsp), PhD-SNP (http://snps.uib.es/phd-snp/phd-snp.html), SNAP (http://www.rostlab.org/services/snap/submit), and PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/). Furthermore, all intronic variants were analyzed for their potential effect on splicing using the splice prediction algorithms SpliceSiteFinder-like (http://www.umd.be/searchSpliceSite.html), MaxEntScan (http://genes.mit.edu/burgelab/maxent/), NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html), GeneSplicer (http://ccb.jhu.edu/software/genesplicer/), and HumanSplice Finder (http://www.umd.be/HSF/) via the Alamut software interface (Interactive Biosoftware) in default settings.

Results

Total of 348 *BRCA1/2*-negative Pakistani breast and ovarian cancer patients were screened for germline mutations in the *RAD51C* gene using DHPLC followed by DNA sequencing analysis of variant bands. Of these patients, 175 were early-onset breast cancer cases (≤30 years of age), 60 belonged to families with two breast cancer cases, 59 to families with three or more breast cancer cases, 20 to families with both breast and ovarian cancer, 22 were early-onset ovarian cancer cases (≤45 years), one belonged to a HOC family, and eleven to families with male breast cancer (Table 1). The median age of disease presentation was 30 years (range 19–73) for female breast cancer ($n = 309$), 45 years (range 30–73) for male breast cancer ($n = 11$), and 33.5 years (range 22–60) for ovarian cancer ($n = 34$). Thirty-one of 309 (10 %) women were diagnosed with bilateral breast cancer.

In total, we detected 17 different *RAD51C* variants. These comprised nine novel heterozygous variants including three protein-truncating mutations, one nonsynonymous and five noncoding variants, as well as eight variants that were previously identified (Table 2).

One truncating mutation, a nonsense substitution of T to A at nucleotide position 204 in exon 2, c.204T>A (C68*), was identified in a 45-year-old ovarian cancer patient (III:10) of Punjabi ethnicity, who presented with a grade 3 endometrioid carcinoma (Fig. 1a) but no family history of breast/ovarian cancer. The mutation was also identified in an unaffected 51-year-old sister of the index patient (III:8) and in one of her sons (IV:2), who was diagnosed with leukemia at age 20. In this family, four other cancers were reported: two leukemias diagnosed at ages <20 (III:2) and 25 (V:2), one uterine tumor at unknown age (V:1), and one

Table 2 *RAD51C* germline alterations in familial breast/ovarian cancer patients and controls from Pakistan

Location	Coding (c.) DNA Sequence[a]	Effect	SNP Link[b]	Classification	Prevalence		Previously described
					Cases ($N = 348$) N (%)	Controls ($N = 400$) N (%)	
Exon 2	**c.204T>A (C68*)**	**Nonsense**	**–**	**M**	**1 (0.3)**	**0 (0.0)**	**No**
Exon 2	**c.225T>G (Y75*)**	**Nonsense**	**–**	**M**	**1 (0.3)**	**0 (0.0)**	**No**
Exon 2	c.195A>G (R65R)	Silent	rs45511291	P	1 (0.3)	0 (0.0)	Yes[d]
Exon 2	c.376G>A (A126T)	Missense	rs61758784	P	1 (0.3)	1 (0.25)	Yes[e]
Exon 4	**c.701C>G (S234*)**	**Nonsense**	**–**	**M**	**0 (0.0)**	**1 (0.2)**	**No**
Exon 5	c.790G>A (G264S)	Missense	–	P	1 (0.3)	0 (0.0)	Yes[f]
Exon 6	**c.873T>G (D291E)**	**Missense**	**–**	**M?[c]**	**1 (0.3)**	**0 (0.0)**	**No**
5′UTR	c.1 −26C>T	5′UTR	rs12946397	P	74 (21.3)	105 (26.3)	Yes[g]
5′UTR	**c.1 −34T>G**	**5′UTR**	**–**	**M?[c]**	**1 (0.3)**	**0 (0.0)**	**No**
Intron 2	c.404 +63_71dup9	Intronic	–	P	2 (0.6)	0 (0.0)	Yes[h]
Intron 2	**c.405 −111A>G**	**Intronic**	**–**	**P**	**1 (0.3)**	**0 (0.0)**	**No**
Intron 3	c.572 −17G>T	Intronic	–	P	1 (0.3)	1 (0.2)	Yes[i]
Intron 6	c.904 +34T>C	Intronic	rs28363318	P	109 (31.3)	125 (31.3)	Yes[j]
Intron 7	**c.965 +21A>G**	**Intronic**	**–**	**M?[c]**	**2 (0.6)**	**0 (0.0)**	**No**
Intron 8	**c.1026 +13T>C**	**Intronic**	**–**	**P**	**2 (0.6)**	**0 (0.0)**	**No**
Intron 8	**c.1027 −17T>C**	**Intronic**	**–**	**P**	**1 (0.3)**	**0 (0.0)**	**No**
3′UTR	c.*25C>G	3′UTR	rs28363336	P	64 (18.4)	49 (12.3)	Yes[k]

Novel germline mutations are marked in bold

P polymorphism, *M* mutation, *M?* potentially deleterious mutation

[a] Nomenclature follows Human Genome Variation Society (HGVS) (http://www.hgvs.org). Numbering starts at the first A of the first coding ATG (located in exon 1) of NCBI GenBank Accession NM_058216.1

[b] Link to NCBI SNP database (http://ncbi.nlm.nih.gov/projects/SNP/)

[c] Classification of the mutation is based on in silico analyses

[d] [7, 9, 15]

[e] [5, 6, 32]

[f] [5, 15, 18]

[g] [9, 18, 32]

[h] [9]

[i] [13, 15, 32]

[j] [9, 13, 32]

[k] [7]

abdominal cancer of unknown primary origin at age 23 (III:1).

The second truncating mutation, another nonsense substitution of T to G at nucleotide 225 also in exon 2, c.225T>G (Y75*), was identified in a patient from Kashmir, who was diagnosed with breast cancer at age 51 (III:1) and reported a family history of breast cancer only (Fig. 1b). The mutation was also found in a maternal aunt of the index patient diagnosed with breast cancer at age 49 (II:11) and not in her unaffected sister (III:3). Both mutation carriers presented with invasive ductal carcinoma (IDC), with no lymph node involvement, and were negative for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).

The third truncating mutation, another nonsense substitution of C to G at nucleotide 701 in exon 4, c.701C>G (S234*), was identified in a 60-year-old control with no family history of breast/ovarian cancer implying that she was an asymptomatic mutation carrier.

The remaining six novel variants were analyzed for their potential functional effect by in silico analyses (Table 3). The coding variant c.873T>G (p.D291E) is predicted to be likely pathogenic by three of the five prediction tools, and the two noncoding variants c.1-34T>G in the 5′UTR and c.965+21A>G in intron 7 were predicted to create a splice donor site by four of the five splice-site prediction algorithms integrated in the Alamut software implying that they are disease-causative.

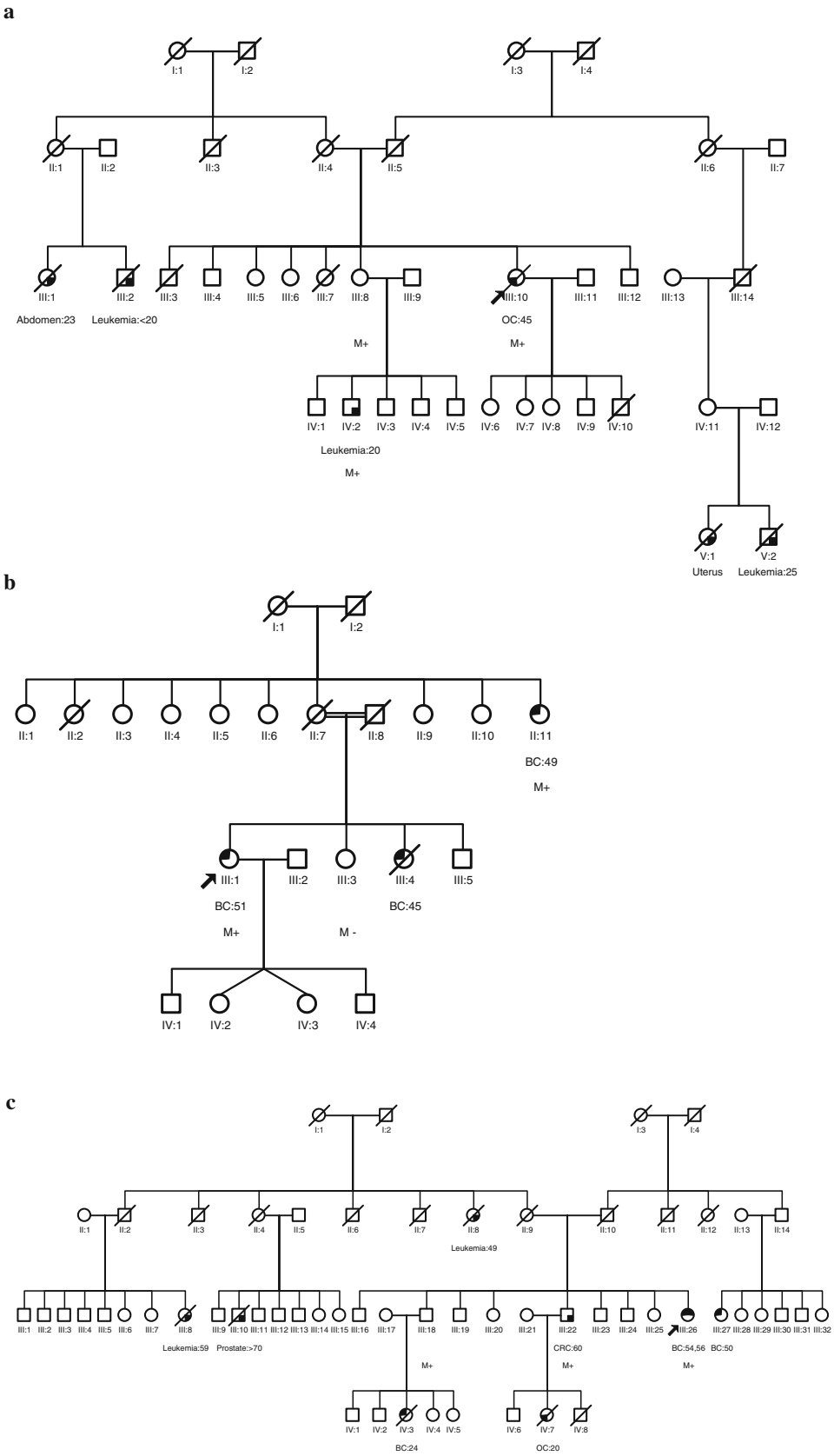

Fig. 1 Pedigrees of breast and ovarian cancer patients with *RAD51C* mutations. **a** Family 70 carrying the c.204T>A **(p.C68*)** mutation; **b** Family 245 carrying the c.225T>G **(p.Y75*)** mutation; **c** Family 517 carrying the c.873T>G (**p.D291E)** mutation. *Circles* are females, *squares* are males, and a *diagonal slash* indicates a deceased individual. *Symbols* with filled *left upper* quadrant: unilateral breast cancer. *Symbols* with *filled upper half*: bilateral breast cancer. *Symbols* with *filled left* lower quadrant: ovarian cancer. *Symbols* with *filled right lower* quadrant: cancer other than breast or ovarian cancer, the name of that cancer is mentioned. Identification numbers of individuals are below the *symbols*. The index patient is indicated by an *arrow*. *BC* breast cancer, *CRC* colorectal cancer, *OC* ovarian cancer. The numbers following these abbreviations indicate age at cancer diagnosis. M+ mutation positive, M− mutation negative

Table 3 In silico analysis of the novel *RAD51C* variants

Coding variant	In silico predictions					
	PolyPhen2	SIFT	PANTHER	PhD-SNP	SNAP	Consensus[a]
c.873T>G (D291E)	Benign	Tolerated	Deleterious	Disease	Disease	Deleterious
	Splice-site predictions					
Noncoding variant	SpliceSiteFinder-like	MaxEntScan	NNSPLICE	GeneSplicer	HumanSpliceSite Finder	Consensus[a, b]
c.1 −34T>G	D (0 → 71.2)[c]	D (0 → 3.5)[c]	NE	D (0 → 4.9)[c]	D (0 → 82.7)[c]	Deleterious
c.405 −111A>G	NE	D (0 → 4.8)	NE	NE	D (0 → 74.0)	Benign
c.965 +21A>G	D (0 → 78.0)[d]	D (0 → 4.2)[d]	D (0 → 0.7)[d]	NE	D (0 → 70.7)[e]	Deleterious
c.1026 +13T>C	NE	NE	NE	NE	NE	Benign
c.1027 −17T>C	NE	NE	NE	NE	NE	Benign

A acceptor, *D* donor, *NE* no effect

[a] The variant is considered as deleterious by three of the five protein function prediction algorithms

[b] >20 % change in score (i.e., a wild-type splice-site score decreases, and/or a cryptic splice-site score increases) is considered as significant

[c] Creates a cryptic splice donor site at c.1−35

[d] Creates a cryptic splice donor site at c.965+16

[e] Creates a cryptic splice donor site at c.965+20

The novel potentially functional missense mutation, c.873T>G (p.D291E), was identified in a patient of Hindko origin, who was diagnosed with bilateral metachronous breast cancer at ages 54 and 56 (III:26) and reported a family history of breast and ovarian cancer (Fig. 1c). The index case presented with a grade 2, lymph node-positive, IDC that was ER/PR positive. On the contralateral side, she presented with a grade 2, lymph node-positive, ER/PR-negative, and HER2-positive IDC. The mutation was also identified in two brothers of the index patient; one was diagnosed with colorectal cancer at age 60 (III:22), and the other was unaffected at age 72 (III:18). A daughter of III:18 had been diagnosed with breast cancer at age 24 and died at 26 (IV:3) implying that she may have inherited the mutation from her unaffected father. In this family, another breast cancer case and one ovarian cancer case were reported with diagnoses at 50 (III:27) and 20 (IV:7) years of age, respectively, and two leukemias at 49 (II:8) and 59 (III:8) years of age and one prostate cancer diagnosed above age 70 (III:10).

The c.1-34T>G variant was observed in an early-onset breast cancer diagnosed at age 25 who presented with an ER/PR/HER2-negative grade 3 IDC. The other variant (c.965+21A>G) was found in two patients: one with early-onset diagnosis who presented at age 29 with an ER/PR/HER2-positive grade 3 IDC, and the other diagnosed with serous ovarian cancer at age 34. The index patient's mother was diagnosed with breast cancer at age 38, and her mother's maternal aunt was affected with breast cancer at an unknown age.

The truncating mutations c.204T>A (p.C68*) and c.225T>G (p.Y75*) and the three in silico-predicted potentially functional variants were not identified in 400 controls further supporting their pathogenicity (Table 2).

The remaining eight variants have been detected in other recent *RAD51C* mutation studies and classified as polymorphisms (Table 2). Of these, c.790G>A, encoding a p.G264S amino acid residue change, has shown evidence of association with cancer [14].

Discussion

The significance of *RAD51C* gene as a potential moderate-to-high-penetrance breast and/or ovarian cancer susceptibility gene has generated significant interest and has been intensively studied in Caucasian populations. In the current study, we investigated the prevalence of *RAD51C* germline mutations in *BRCA1/2*-negative early-onset and familial breast and/or ovarian cancer patients from Pakistan. While only one previous study conducted in an East Asian population from China has assessed the *RAD51C* mutation frequency in familial breast and/or ovarian cancer patients [19], our study conducted in Pakistan provides additional information for a South Asian population.

We identified two novel protein-truncating *RAD51C* mutations, p.C68* and p.Y75*, and three novel in silico-predicted potentially functional mutations, p.D291E, c.1-34T>G, and c.965+21A>G, the latter of which was detected in two unrelated patients. These mutations were not detected in 400 healthy controls further supporting the notion that they may be associated with disease. Additionally, a third novel truncating mutation, p.S234*, was identified in a 60-year-old control with no family history of

breast/ovarian cancer. This nonsense mutation is likely to be pathogenic and, in view of the relatively advanced age of the woman, probably of low penetrance. In contrast to our study, no clearly pathogenic mutations were identified in the Chinese study, in which two out of eight identified variants were in silico predicted to be possibly functional [19]. These findings suggest that the prevalence and spectrum of *RAD51C* mutations may vary within Asian populations.

In the present study conducted in Pakistan, no unequivocal deleterious *RAD51C* mutation was identified in HBOC families although the sample size of this subgroup of patients was relatively small ($n = 20$). In the studies describing *RAD51*C mutation frequency in Caucasian population, variable results have been reported. In the initial German study, the frequency of mutations in HBOC families was 1.3 % (6/480) [5], that is the same as reported in a Spanish study (4/300) [12]. Lower frequency of mutation was observed in other larger studies with more than 100 families conducted in Australia (2/335; 0.6 %) [15], UK (8/1102; 0.7 %) [14], and Spain (1/101; 1 %) [13], while no disease-causative mutations were detected in families from Belgium, Netherlands, Canada ($n = 351$) [6, 7], USA ($n = 92$) [9] and ($n = 192$) [8], Australia ($n = 70$) [10], and Israel ($n = 100$) [6] and ($n = 206$) [11]. These findings suggest that the prevalence of *RAD51C* mutations in HBOC families may be lower than that initially described [5].

In this study, we identified one deleterious *RAD51C* mutation in the small subset of 22 early-onset ovarian cancer patients (4.5 %). Deleterious *RAD51C* mutations were also identified in unselected Australian or Finnish ovarian cancer patients (1/267; 0.4 % and 4/409; 1 %, respectively) [15, 18] and in HOC families from the UK (1/30; 3.3 %) [14], Australia (1/21; 4.8 %) [15], and Finland (2/8; 25 %) [18] confirming the role of *RAD51C* as a potential susceptibility gene predisposing to ovarian cancer.

While evidence for *RAD51C* conferring susceptibility to ovarian cancer is convincing, albeit with low penetrance, its role in conferring susceptibility to breast cancer is less clear. Few studies have identified pathogenic *RAD51C* mutations in HBC families. A deleterious mutation, p.Gln143Arg, was previously identified in a Spanish HBC family [12], and several in silico-predicted potentially functional missense variants were described in Australian families [15]. In our study, we identified a deleterious mutation in one of the 119 HBC families from Pakistan (0.8 %) supporting the low potential contribution of *RAD51C* in predisposition to breast cancer.

The contributory role of *RAD51C* mutations to risk of early-onset breast cancer was investigated in a previous study on 34 early-onset breast cancer cases (diagnosed below age 35), and it showed no mutations to be associated [13]. In contrast, the present study showed two of the 175 early-onset breast cancer patients diagnosed below age 30 to harbor an in silico-predicted potentially functional mutation suggesting an association.

No pathogenic *RAD51C* mutation was observed in eleven male breast cancer patients from Pakistan. A similar observation was previously reported by Silvestri and colleagues who screened 97 *BRCA1/2*-negative male breast cancers from Italy [23]. This finding supports the notion that *RAD51C* may not confer susceptibility to breast cancer in males.

Two of the *RAD51C* mutations identified in this study, p.C68* and p.D291E, occurred in families with multiple cases of leukemia. In the p.C68*-positive family, the mutation was also found in a nephew of the index patient who was diagnosed with leukemia at age 20, implying that a monoallelic *RAD51C* mutation may predispose to leukemia. However, further evidence for this could not be provided because DNA samples from the two additional family members affected with leukemia were not available for analysis. Similarly, lack of DNA samples prevented further evaluation of the two leukemic patients from the p.D291E-positive HBOC family and three leukemic patients from a previously described Finnish HBOC family positive for the deleterious c.-13_14del27 *RAD51C* mutation [17].

In the present study, no recurrent pathogenic mutation in the *RAD51C* gene was detected. Worldwide, only four recurrent mutations have been described. Two founder mutations (c.93delG and c.837 +1G>A) were identified in Finnish breast and/or ovarian cancer families/unselected ovarian cancer patients [18]. Another mutation (c.774delT) was detected in one unselected ovarian cancer patient from Sweden and one HBOC family from Spain [13, 17]. The Spanish patient inherited the mutation from her unaffected Swedish mother, suggesting that c.774delT is a recurrent Swedish mutation. Recently, one gross deletion encompassing exons 5–9 was identified in one HBC and one HBOC family from Germany [24]. However, due to their low prevalence, estimates of the breast and ovarian cancer risk associated with the recurrent mutations are unknown.

Association of *RAD51C* mutations with the development of two or more tumors has previously been suggested in an Australian study, in which two of three truncating *RAD51C* mutations were observed in women with breast and ovarian cancer [15]. Additionally, a Spanish study showed four of the seven affected women harboring a deleterious mutation developed bilateral tumors, either breast or ovarian, including one patient diagnosed with breast cancer at age 64 and bilateral ovarian cancer at age 74 [12]. These findings contrast with that of our study, in which both patients with a deleterious mutation presented with unilateral disease.

The proportion of missense mutations differs greatly between genes affecting either no or few alleles, as is the case for *BRCA1* and *BRCA2* (BIC database, http://research.nhgri.nih.gov/bic/) or affecting the majority of alleles, as is the case for *TP53* [25] and apparently also *RAD51C* [5, 12, 13, 19]. Interestingly, in the Pakistani study, only three out of 17 *RAD51C* variants were of missense type, whereas in the Chinese study, four out of eight were missense mutations suggesting that the nature of *RAD51C* mutations may vary between populations.

Many methods have been developed for the in silico prediction of the phenotypic effect of nonsynonymous SNPs (nsSNPs) that may affect gene function through their effect on structure and/or function of the encoded protein. The methods differ in the properties of the variants they take into account in the prediction, as well as in the nature and possible training of the classification method used for decision making. Due to these differences, the prediction performance of these methods for a given variant can differ. Recently, it was shown that a consensus prediction using multiple algorithms improved the prediction performance [26]. However, since none of the methods are 100 % predictive, they are considered as one tool to help interpret nsSNPs according to their predicted pathogenicity. The definite proof for the pathogenicity of the variants, however, has to be provided by functional in vitro assays, which are often complicated and time consuming as is the case for *RAD51C*. For that reason, most studies including the present one used multiple methods to classify *RAD51C* variants [5, 7–9, 12, 13, 15, 17, 19]. Two studies that in addition functionally characterized the variants yielded similar results [5, 12], supporting the notion for good performance of prediction methods.

In contrast to the mean age of breast cancer onset of 31 years (range 22–49) in Pakistani patients harboring *BRCA1* germline mutations [20], the mean age of breast cancer onset of the two Pakistani patients with the p.Y75* *RAD51C* truncating mutation was 50 years (range 49–51), which is similar to mean age of 53 years previously reported in Caucasians [5].

The present study included 175 female early-onset breast cancer patients with diagnosis $\leq$30 years of age. The mean age of the remaining familial breast cancer cases ranged from 39.7 to 43.8 years. It is of note that in Pakistan, women usually present with breast cancer below 40 years of age [27]. The Karachi Cancer Registry also reported the highest risk of breast cancer in women between 25 and 39 years of age [28]. Recently, similar findings were observed in two other studies showing that Pakistani women residing in the US more often presented with breast cancer before age 40 compared to Caucasian women [29, 30].

The breast tumors of the two p.Y75* *RAD51C* mutation carriers were of IDC histology, lymph node negative, and negative for ER, PR, and HER2 expression. This is in keeping with findings from various previous studies showing that *RAD51C*-linked breast tumors were predominantly IDC, lymph node negative, and HER2 negative [5, 12, 18], whereas results of ER and PR status were not consistent.

RAD51C mutations have been shown to confer a six-fold increased risk of ovarian cancer in Caucasians, which constitutes a greater than 9 % cumulative risk by age 80 [14]. Conversely, the risk of breast cancer and other cancer types including leukemia [17] and in present study, non-Hodgkin lymphoma, lower abdominal tumor [5], cancers of the bowel, rectum [15], and skin [18], as well as sporadic squamous cell carcinomas of the head and neck [31], are minimal or unknown due to low mutation prevalence in these tumor phenotypes. In Asian populations, data on the prevalence of *RAD51C* mutations are even rarer and data on risks and tumor phenotypes associated with *RAD51C* mutations are lacking.

In this study, we have identified two novel deleterious mutations in the Pakistani patients/breast/ovarian cancer families. The *RAD51C* mutation frequency was 0.8 % (1/119) in HBC families and 4.5 % (1/22) in early-onset ovarian cancer patients, while no mutations were detected in the 20 HBOC families and the 175 early-onset breast cancer cases. These findings suggest that *RAD51C* mutations may not have substantial contribution toward breast and ovarian cancer predisposition in Pakistan. These findings imply that in Pakistan, larger studies are required to assess the genotype–phenotype correlation in regard to tumor spectrum and penetrance associated with *RAD51C* mutations before a genetic test can be introduced in the clinical setting.

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

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