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ORIGINAL ARTICLE

Breast cancer risk associated with *BRCA1/2* variants in the Pakistani population

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Abstract

Background Majority of the *BRCA1* and *BRCA2* mutations are associated with the risk of sporadic and familial breast cancer. Since these genes are significant in DNA repair mechanisms, we focused homology-directed DNA repair (HDDR) and BRCA complex.

Methods We selected *BRCA1* variant (rs80356932, 4491C/T) and *BRCA2* variant (rs80359182, 319T/C) from the interaction region of BRCA complex and studied in 100 breast cancer patients and 100 controls using tetra-ARMS-PCR.

Results Here we show that *BRCA1* and *BRCA2* variants are significantly associated with high breast cancer risk (*BRCA1* rs80356932; Genotype T/T OR 8.66, 95% CI 3.16–23.71, $p < 0.0001$; Allele-T, OR 2.48, 95% CI 1.62–3.81, $p < 0.0001$ and *BRCA2* rs80359182; Genotype C/C OR 4.32, 95% CI 1.95–9.53, $p = 0.0001$; Allele-C, OR 2.19, 95% CI 1.43–3.34, $p = 0.0002$). Additionally, bioinformatics analysis showed that *BRCA2*-tryptophan > arginine substitutions result in altered interaction of BRCA1/PALB2/BRCA2/protein complex and impaired HDDR pathway. We also observed that breast cancer risk was significantly increased in over-weighted and obese women.

Conclusions Our results indicate that high risk of breast cancer is significantly associated with *BRCA1* and *BRCA2* variants, and mutations may alter the protein interactions of BRCA complex that results in tumor genesis.

Keywords BRCA complex · Breast cancer · Genetic variants · Homologue-directed DNA Repair

Introduction

Breast cancer is the second most frequent cause of cancer-related death among female population worldwide. About 1.2 million females are being diagnosed with breast cancer annually [1]. In comparison to other Asian countries, highest incidence rate of breast malignancy has been reported in Pakistan [2–4]. Generally, it is suggested that 30% of familial cancer cases occur due to mutations in certain genes. Majority of breast cancer genes code for DNA damage response (DDR) proteins that repairs double-strand breaks (DSBs) by homology-directed DNA repair (HDDR) [5, 6]. *BRCA1* (*Breast Cancer Susceptibility gene 1*) and *BRCA2* (*Breast Cancer Susceptibility gene 2*) are involved in the development of a significant proportion of Hereditary Breast Cancer (HBC) [7, 8]. Similar to *BRCA1* and *BRCA2, PALB2* (*Partner and Localizer of BRCA2*) mutations have also been involved in breast cancer risk, and suggests that these three proteins might be functionally linked [9, 10].

BRCA1 is a tumor suppressor gene that plays significant role in DNA repair and cell cycle regulation [11–13]. In HDDR pathway, it acts as a scaffolding protein to form BRCA complex in association with other protein mediators [13, 14]. *BRCA2* co-localizes with *BRCA1* and plays significant role in cell proliferation and HDDR pathway [15–18]. Although, there is no structural association between BRCA1 and BRCA2, PALB2 directly binds with BRCA1 and functionally associate it with BRCA2 [17–21]. In the BRCA complex, the coiled-coil domain of BRCA1 (1022–1186 a.

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a.) and N-terminus of BRCA2 (21–39 a. a.) are involved in structural interaction with PALB2 and act as bridging protein partner. In turn, the structural domains of PALB2 interact with both of the candidate proteins including the evolutionary conserved coiled-coil domain in the N-terminus to interact with BRCA1 and WD40 domain in the C-terminus to associate with BRCA2, respectively [22–26]. BRCA1 and BRCA2 potentially rely on constitutively occurring cooperative inter-linkage of BRCA1/PALB2/BRCA2 complex to become functionally active [18, 21, 27, 28]. Precisely, BRCA1 acts as a gatekeeper of genome integrity which control PALB2 as molecular switch of HDDR; whereas, BRCA2 regulates the formation of repair complex and act as caretaker of genome stability [6, 18]. The HDDR is one of the effective repair mechanisms of double-strand breaks (DSBs). The repair of DSBs controls the therapeutic response to radiation and chemotherapeutic agents [21]. Thus, BRCA1 and BRCA2 along with PALB2 ensures vital error-free repair of DSBs by HDDR [29]. Therefore, any genetic alteration or structural change in any of the partner of this complex may halt the formation of BRCA1/BRCA2/PALB2 assembly and compromise the homology-directed repair leading to cancer development [6, 18, 30, 31]. Similarly, Kang et al. [32] investigated 19 *BRCA1* genetic variants including 4491C/T nonsense mutation and reported rs80356932 variant to cause premature termination of translational process, and hence result in truncated BRCA1 protein as it changes glutamine residue (Glu; Q) in reference sequence to stop-gained codon (Amber;*). Furthermore, in 2006, *BRCA2* genetic variant (319T/C) was reported which result in T/C missense mutation at codon 324 and alter the protein sequence from tryptophan (Try; W) to arginine (Arg; R) [33]. Accordingly, we aimed to investigate association of *BRCA1* and *BRCA2* genetic variants with breast cancer risk in the population of Punjab, Pakistan. Furthermore, we analyzed the functional effect of genetic variants on protein structure and interactions of BRCA1 and BRCA2 with a third counterpart of BRCA complex (BRCA1/BRCA2/PALB2 complex), the PALB2 using bioinformatics.

Methods

Study population

In the present case–control study, samples of breast cancer patients were collected from Jinnah Hospital, Fauji Foundation Hospital, and INMOL Hospital Lahore, Pakistan. The samples were collected during the period of September 2014 to April 2015. A total of 200 samples were recruited including 100 patients and 100 controls. The controls were taken from cancer-free females without positive family history of breast cancer. Informed consent was taken from each participant of the study. The demographic, clinical and pathological characteristics of patients and controls are given in Table 1. An ethical approval for the study was obtained from the institutional review board of University of the Punjab.

Sample collection

A 2–3 ml of blood sample was drawn from median cubital vein of participants under aseptic conditions and stored in EDTA vials at − 20 °C.

SNPs selection and genotyping

We selected two SNPs (*BRCA1* 4491C/T; rs80356932; and *BRCA2* 319T/C; rs80359182) that have been reported to increase breast cancer risk in other populations. The risk alleles were identified by using reference of NCBI SNP database.

Genomic DNA was isolated from 500 µl of venous blood by using Kit (Genomic DNA Extraction Blood DNA kit, FAVORGEN BIOTECH CORP, Taiwan) following the manufacturer's instructions. The primers sequences used and optimized PCR conditions are given in Table S1. For genotyping, we used already established protocol of Tetra primer ARMS-PCR [34] with minor modifications (Table S2). The PCR products were run on 1% agarose gel for 20–25 min to detect the bands specified to variants under UV illuminator.

Statistical analysis

We used Chi-square test to analyze the association demographic factors and variants with breast cancer risk using SPSS (version 22). Genetics statistics tools from Helmholtz Center Munich; Institute of Human Genetics (https://ihg.helmholtz-muenchen.de/ihg/) was used to calculate genotype and allele frequencies.

Bioinformatics tools for cross-validation of genetic data

The retrieved protein sequences of *BRCA1, BRCA2* and *PALB2* in FASTA format were converted into 3D structure file in PDB format using Protein Homology/AnalogY Recognition Engine (PHYRE2; version 2.0) for structural analysis of proteins and amino acid sequences. To further clarify the functional role of the W31R variant of *BRCA2*, ESE finder and FAS-ESS online software were used to predict any effect on sequences of Exonic Splicing Regulators (ESRs). We used an online molecular docking tool PatchDock to check the interaction and association of these protein structures. Visualization and modeling software "PyMol" was used to analyze the protein interactions and binding sites in a complex after docking.

Table 1 Demographic, clinical, and pathological characteristics of patients and controls

Variables	Characteristics	Patients	Controls	Chi-square	*p* value	Odds ratio (95% CI)	*p* value
Age	< 40	20	20	0.3	0.94	–	
	40–49	59	58				
	50–59	16	18				
	60–69	5	4				
Family history	Positive	23	0	–			
	Negative	77	100				
Marital status	Married	90	83	2.09	0.14	–	
	Single	10	17				
Occupation	Housewife	83	–				
	Working	12					
	Student	5					
Menstrual status	Premenopausal	32	–				
	Postmenopausal	68					
BMI (body mass index)	Obese	51	32	14.60	0.002*	4.09 (1.91–8.75)	0.0003*
	Over-weight	31	26			3.06 (1.36–6.87)	0.006*
	Normal weight	14	35			–	–
	Underweight	4	7			1.71 (0.41–7.00)	0.45
Localization	Bilateral	11	–				
	Unilateral right	39					
	Unilateral left	50					
Cancer stage	I	5	–				
	II	28					
	III	39					
	IV	28					
Histological tumor grade	I	9	–				
	II	37					
	III	52					
	IV	2					
Metastasis	Negative	58	–				
	Positive	42					
Treatment	Chemotherapy	52	–				
	Radiotherapy	19					
	Surgery	29					

CI confidence interval

*Significant value

Results

In the present study, the mean age of patients and disease onset was 46.5 ± 11.6 and 44.3 ± 11.4 years, respectively. We analyzed the association of different demographic factors such as age, marital status and BMI with breast cancer risk. We found that breast cancer is significantly more likely in obese women as compared to controls (Chi-square test, $p = 0.002$, Table 1). Additionally, we did logistic regression analysis and observed that BMI significantly contribute to the risk of breast cancer (Table 1).

Association analysis of *BRCA1* and *BRCA2* variants in breast cancer cases and controls

ARMS-PCR was used for genotyping of selected SNPs, rs80356932 of *BRCA1* and rs80359182 of *BRCA2*. The variants showed deviation from Hardy–Weinberg equilibrium (HWE) presenting significant association with breast cancer in homozygous form ($p < 0.0001$; Table 2). The minor allele frequency of *BRCA1* SNP rs80356932 (T) was significantly higher (0.44) in breast cancer cases as compared to controls (0.24) and was associated

Table 2 Exact test for Hardy–Weinberg equilibrium ($n = 200$)

Subjects	*BRCA1* rs80356932				*BRCA2* rs80359182			
	C/C	C/T	T/T	*p* value	T/T	T/C	C/C	*p* value
Patients	50	12	38	< 0.0001*	51	11	38	< 0.0001*
Controls	57	38	5	0.79	58	32	10	0.12

*Significant value

with increased risk of breast cancer (OR 2.48, 95% CI 1.62–3.81, $p < 0.0001$). Additionally, the genotype T/T was associated with increased risk of breast cancer (OR 8.66, 95% CI 3.16–23.71, $p < 0.0001$) under co-dominant model when each genotype is analyzed independently keeping homozygous normal as reference; whereas, genotype C/T was associated with decreased in risk (OR 0.36, 95% CI 0.17–0.76, $p < 0.006$; Table 3). Similarly, *BRCA2* SNP rs80359182 showed high frequency of minor allele in patients (0.44) than in controls (0.26). The minor allele (C) was associated with increased risk of breast cancer in patients (OR 2.19, 95% CI 1.43–3.34, $p = 0.0002$). The genotype C/C was associated with increased breast cancer risk (OR 4.32, 95% CI 1.95–9.53, $p = 0.0001$) and the genotype T/C was protective against breast cancer (OR 0.39, 95% CI 0.17–0.85, $p = 0.01$; Table 3) as compared to reference T/T genotype.

We found that *BRCA1* SNP with recessive model (genotype T/T) was significantly associated with high risk of breast cancer (OR 11.64, CI 4.34–31.20, $p < 0.0001$) keeping genotype C/T and C/C as reference. However, the genotype C/T with over-dominant is protective against breast cancer (OR 0.22, 95% CI 0.10–0.45, $p < 0.0001$; Table 4) keeping C/C and T/T as reference. Similarly, in case of *BRCA2* SNP, high risk of breast cancer was associated with variant in recessive model (genotype C/C; OR 5.51, 95% CI 2.55–11.89, $p < 0.0001$). Under over-dominant model, the genotype T/C was protective against breast cancer (OR 0.26, 95% CI 0.12–0.55, $p = 0.0005$; Table 4).

Prediction of the functional effect of variants on protein interactions by bioinformatics analysis

Evolutionary conservation of genetic variants

To determine the evolutionary conservation and functional effect of genetic variants on protein function, sequence conservation analysis was carried out using Align-GVGD scores. It determines scores for GV (Grantham variation) and GD (Grantham deviation) by combining physio-chemical characteristics of amino acids and alignments of multiple protein sequences. These scores are used to evaluate the Align-GVGD grades. The C0 grade indicates neutral nature of amino acid while C65 shows pathogenic significance. We found that Q1458* (4491C/T) of *BRCA1* and W31R (319 T/C) of *BRCA2* are highly pathogenic based on the highest score grade C65. The tryptophan (Try; W) amino acid was found evolutionarily conserved from humans through sea urchin (Table 5).

Inclusion/exclusion of exon by genetic variants

We found that the *BRCA1* mutation results in protein truncation (Q1458*) as protein synthesis was stopped due to

Table 3 Allele and genotype frequencies in patients and controls

Allele/genotype	Patients	Controls	Odds ratio (95% CI)	Chi-square	*p* value
BRCA1 rs80356932					
C	0.56	0.76	2.48 (1.62–3.81)	17.83	< 0.0001*
T	0.44	0.24			
C/C	0.5	0.57	Reference		
C/T	0.12	0.38	0.36(0.17–0.76)	7.37	0.006*
T/T	0.38	0.05	8.66 (3.16–23.71)	21.94	< 0.0001*
BRCA2 rs80359182					
T	0.56	0.74	2.19 (1.43–3.34)	13.51	0.0002*
C	0.44	0.26			
T/T	0.51	0.58	Reference		
T/C	0.11	0.32	0.39 (0.17–0.85)	5.74	0.01*
C/C	0.38	0.1	4.32 (1.95–9.58)	14.23	0.0001*

CI confidence interval

*Significant value

Table 4 Genetic contrast model

Model	Genotype	Patients	Controls	Odds ratio (95% CI)	*p* value	AIC
BRCA1 rs80356932						
Dominant	C/C	50	57	Reference	0.32	280.3
	C/T–T/T	50	43	1.32 (0.75–2.31)		
Recessive	C/C–C/T	62	95	Reference	< 0.0001*	245.6[a]
	T/T	38	5	11.64 (4.34–31.20)		
Over-dominant	C/C–T/T	88	62	Reference	< 0.0001*	262.5
	C/T	12	38	0.22 (0.10–0.45)		
BRCA2 rs80359182						
Dominant	T/T	51	58	Reference	0.32	280.3
	T/C–C/C	49	42	1.32 (0.75–2.31)		
Recessive	T/T–T/C	62	90	Reference	< 0.0001*	258.7[a]
	C/C	38	10	5.51 (2.55–11.89)		
Over-dominant	T/T–C/C	89	68	Reference	< 0.0005*	267.7
	T/C	11	32	0.26 (0.12–0.55)		

CI confidence interval, *AIC* Akaike information criterion

*Significant value

[a]Best associated model

Table 5 Analysis of evolutionary conservation for genetic variants

Genetic variant	Align-GVGD		Grade	Effect on protein	Splicing effect	Pathogenicity
	GV	GD				
Q1458*	1.04	1.55	C65	Truncation	Disruption	Pathogenic
W31R	1.022	1.35	C65	Disruption	Skipping of exon 3	Pathogenic

GV Grantham variation, *GD* Grantham deviation

this mutation. Splicing and regulation for alternative was disrupted by this mutation. In case of *BRCA2* mutation, full-length protein was observed with substitution of arginine instead of normal tryptophan (W31R). We found that W31R (319 T>C) located in exon 3 have a putative binding site for SRp40 splice protein (–TTAATCG–) as shown in Table 5. It suggests that 319 T>C (W31R) causes alternative splicing by exon skipping, but does not alter any exonic silencer sequence site (ESS site) shown by FAS-ESS (fluorescence-activated screen for exonic splicing silencers).

Prediction of 3D protein structure and molecular docking of proteins

The 3D structure of interacting domains of BRCA1 (coiled-coil domain 21–39 amino acids) and PALB2 (coiled-coil domain 1022–1863 amino acids) were not available in any protein databases. The respective sequences were then subjected to de novo modeling process using TASSER-I online software.

Docking in pair BRCA2–PALB2 proteins was done by using 'Cluspro', a web server for computational docking of proteins. The interaction analysis was done by comparing mutant and normal 3D structure after docking for difference in bonding type, bond length, and nature of amino acid (Table 6). We found that normal BRCA2 protein interacts with PALB2 protein through arginine residue as shown in Fig. 1a, b; whereas, when there is mutation from T to C allele, it results in the substitution of arginine residue instead of tryptophan (Fig. 1c, d). This substitution results in conformational changes, differences in bonding, bond lengths, protein structure, folding and altered interaction (Table 6) with PALB2 that may lead to tumor genesis.

Discussion

In the present study, we found that demographic factors such as obesity may increase the risk of breast cancer (Table 1). Our results indicate that breast cancer was more likely in obese women as compared to controls. Besides demographic factors, genetics play significant role in cancer development. For this purpose, we investigated the association of *BRCA1* and *BRCA2* variants (rs80356932 and rs80359182) with breast cancer risk in female population of Pakistan. We found that high risk of breast cancer was significantly associated with these variants. The *BRCA1* variant (T) and *BRCA2* variant (C) increases the risk of breast cancer more

Table 6 Difference in bond lengths of *BRCA2* genetic variant after docking

BRCA2 with wild-type allele (bond length)	*BRCA2* with variant allele (bond length)	Type of bonding	Difference in bond length
TRP31:NE1-MET1022: O (10.51)	ARG31: NE-MET1022: O (11.19)	Ionic bonding	0.68
TRP31:NE1-LEU1026: CD1 (14.69)	ARG31:NE -LEU1026: CD1 (14.13)	Van der Waals bonding	0.56
TRP31:NE1-MET1067:CE (10.37)	TRP31:NE1-MET1067:CE (12.42)	Van der Waals bonding	2.05
TRP31:NE1-LEU1027:CD2 (12.90)	TRP31:NE1-LEU1027:CD2 (12.63)	Van der Waals bonding	0.27
TRP31:NE1-PRO1097: O (6.93)	TRP31:NE1-PRO1097: O (5.66)	Ionic bonding	1.27
TRP31:NE1-LYS1048:C (12.96)	TRP31:NE1-LYS1048:C (11.46)	Van der Waals bonding	1.5

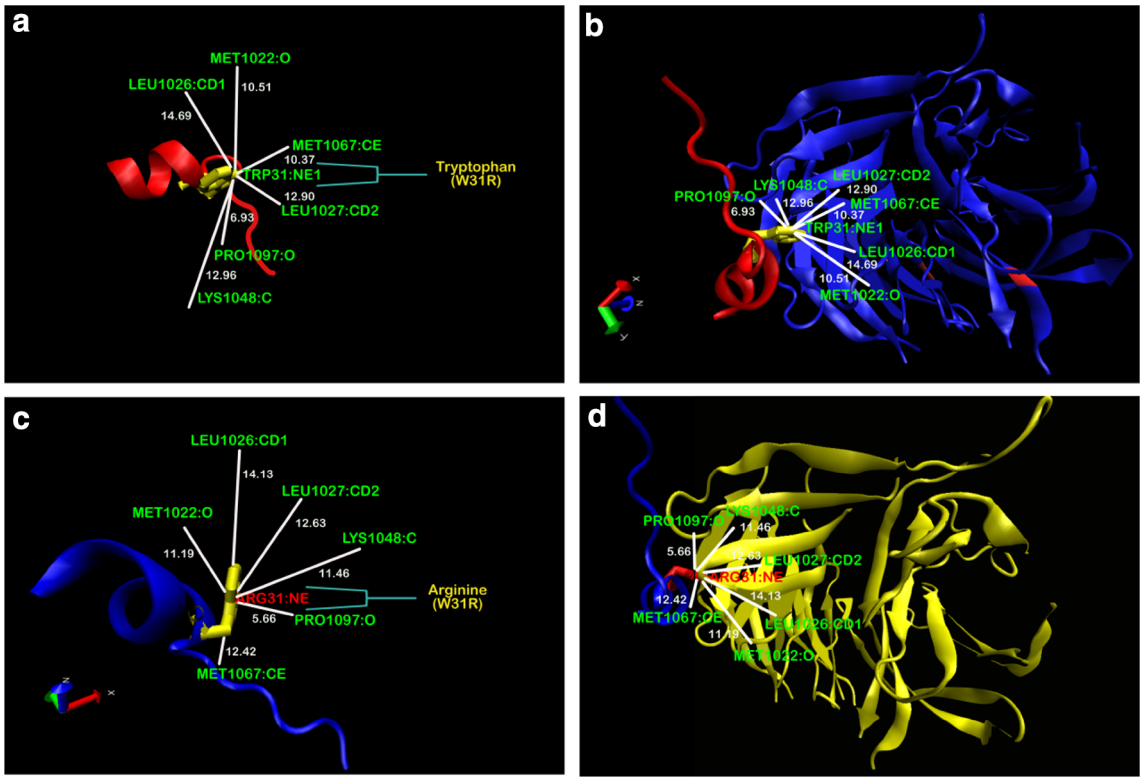

Fig. 1 Molecular docking of BRCA2 and PALB2 proteins. **a** Normal BRCA2 protein with normal allele for tryptophan (BRCA2, red; tryptophan, yellow). **b** Interaction of normal BRCA2 protein with PALB2 (BRCA2, red; tryptophan, yellow; and PALB2, blue). **c** Mutant BRCA2 protein with arginine substitution instead of tryptophan due to variant allele (BRCA2, blue; arginine, yellow), and **d** interaction of mutant BRCA2 with PALB2 proteins (BRCA2, blue; arginine, red; and PALB2, yellow). The white lines and white text represent bonds lengths between tryptophan (without variant allele) and arginine (with variant allele) residue of BRCA2 and various amino acids residues of PALB2 protein. The green text presents different amino acid residues. As a result of mutation from T to C, arginine is substituted instead of tryptophan (marked red in **c** and **d**) and brings conformational changes in the protein interactions and differences in bond length

than twofold in patients in comparison to normal alleles (Table 3). The clinical significance of *BRCA1* (rs80356932) has already been described as pathogenic and associated with increased breast cancer risk [32]. Consistent with an earlier investigation of Japanese population [35], our study also reveals highly significant association of *BRCA2* variant rs80359182 with increased risk of breast cancer. The genotype analysis revealed that the genotype T/T of *BRCA1* variant increased the risk up to 9 times in patients and the genotype C/C of *BRCA2* variant increased the risk up to 4 times in breast cancer patients as shown in Table 3. However, in heterozygous form (C/T of BRCA1 and T/C of BRCA2) when there is only one copy of variant allele, these variants do not confer any risk of breast cancer (OR 0.36 and

0.39, respectively) as shown in Table 3. These results suggest that normal alleles of BRCA1/2 have dominant effect on variant alleles and one copy is enough to protect against disease. But, when there are two copies of BRCA1/2 variants (homozygous form), breast cancer risk is increased up to ninefold.

Furthermore, the recessive genotype model was significantly associated with BRCA1/2 variants. As evident from above results also, the T variant (*BRCA1*) with genotype T/T under recessive model ($p < 0.0001$ and lowest AIC value 245.6) was associated with up to 12-fold increased breast cancer risk.

Similarly, in case of C variant (*BRCA2*) with genotype C/C under recessive model too ($p < 0.0001$ and lowest AIC value 258.7) was associated with up to fivefold increased risk of breast cancer. The over-dominant genotype model for both SNPs also showed significant *p* values and appeared to have protective effect against breast cancer where single copy of normal allele is enough to have dominant effect on one copy of variant allele to protect against breast cancer (OR 0.22 and 0.26, respectively) as shown in Table 4.

Furthermore, multifunctional proteins perform various functions by concurrent interaction with unique set of protein partners. Only based on the statistical association, the direct association of non-synonymous missense genetic variants of uncertain clinical significance with disease risk is not sufficient. Therefore, in addition to statistical association, it is important to analyze the functional consequences.

The functional analyses showed that the genetic variant of *BRCA1* rs80356932 confers nonsense mutation in mRNA transcript resulting in premature termination of protein by replacing glutamine (Glu;Q) to *Amber* stop codon (Glu1479>*). The clinical significance of rs80356932 has already been described as pathogenic and associated with increased breast cancer risk [32]. The *BRCA2* variant rs80359182 has uncertain clinical significance [36]. This variant results in missense mutation changing the amino acid codon 319T>C from tryptophan (Trp; W) to arginine (Arg; R) resulting in altered amino acid sequence W31>R which subsequently leads to altered protein structure and function [33].

The evolutionary conservation analysis confirmed that tryptophan is highly conserved in multiple protein sequences of *BRCA2* protein in different organisms. Moreover, W31R (319T/C) located in exon 3 have a putative binding site for SRp40 splice protein (–TTAATCG–) that causes alternative splicing of exon and skips exon 3 but does not alter any exonic silencer sequence site (ESS site). Bioinformatics analysis-based protein docking confirmed the drastic effect of W31R (319T/C) on *BRCA2* protein structure. Replacement of tryptophan (Trp; W) with arginine (Arg; R) caused drastic conformational change in *BRCA2* protein and alters interaction ability with *PLAB2* (Fig. 1d).

However, large-scale population-based studies are required for replication and cross-validation of our research findings to eliminate the possible off-target effects. These studies should include the effects of multiple variants and pathological characteristics. Genetic testing for *BRCA1* and *BRCA2* in routine clinical practice could be helpful in diagnosis and treatment of breast cancer.

Conclusion

In conclusion, our findings elucidate strong association of *BRCA1* and *BRCA2* genetic variants with increased risk of breast cancer in Pakistan. These variants also affect the functional binding of proteins and inhibit several essential mechanisms of DNA repair.

Compliance with ethical standards

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

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