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A report of novel inactivating missense mutations of *BRCA1* detected in patients with acute myeloid leukemia

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Abstract

Introduction Exon 11 and exon 14 of *BRCA1*, a tumor suppressor gene, are known to be mutated in various cancers.

Methodology We screened 24 samples (13 from patients with acute myeloid leukemia and 11 from normal controls) using polymerase chain reaction to detect *BRCA1* exon 11 and exon 14 mutations, if any. Purified polymerase chain reaction-amplified products of four samples (cases 1–4) were subjected to bidirectional sequence analysis.

Results The sequence analysis revealed in total 20 mutations. One novel frameshift mutation, that is, c.2339insG (p.E781fs), observed in case 1 promoted the synthesis of truncated protein (protein 1). In case 2, five novel insertions mutations c.2376insC (p.K793fs), c.2380insT (p.A794fs), c.2443insT (p.I815fs), c.2533insG (p.I845fs), and c.2511insG (p.N838fs), two novel deletion mutations, c.3762delG (p.N1255fs) and c.3830delC (p.A1277fs), along with one reported mutation,c.3621delG (p.Lys1208fs), were observed which resulted in a truncated protein (protein 2). Besides these, one nonsense c.2383A > T (p.K795X), and four missense c.2429A > C (p.N810T), c.2430C > A (p.N810K), c.3644A > T (p.N1215I), and c.3740 T > A (p.V1247D) were also observed in case 2. In case 3, a single missense c.3577 T > C (p.F1193L) and three silent mutations c.3576 T > C (p.P1192P), c.3579C > T (p.F1193F), and c.4311 T > C (p.S1437S) were noted, having no impact on the encoded protein's function. However, a unique silent mutation c.4252 T > C (p.L1418L) observed in case 4 did not influence the protein sequence. Bioinformatics analysis revealed that both truncated proteins (protein 1 and 2) have higher pI and considerably lower molecular weight than the wild-type *BRCA1* protein. The molecular weight of protein 3 having one alteration (phenylalanine to leucine) varied slightly, and there was no difference in the characteristics of wild-type *BRCA1* and protein 4.

Conclusion This study reveals the mutational spectrum of *BRCA1* in patients with acute myeloid leukemia, representing unique missense and novel pathogenic mutations that can be further targeted for designing diagnostic and therapeutic strategies for acute myeloid leukemia.

Clinical practice points

Both the *BRCA1* and *TP53* genes are major participants in the DNA damage repair process. *TP53* is a well-studied gene and known to be mutated in leukemia. However, no study has been carried out to explore the mutational status of *BRCA1* in leukemia. Considering the connection between *BRCA1* and abnormal behavior of *TP53* in the DNA repair

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process in patients with leukemia, this study was initiated to document leukemia-associated *BRCA1* mutations, if any. Exon 11 and exon 14 of *BRCA1* comprise BRCT and DNA binding domains, respectively. During the present study, both exons of *BRCA1* (11 and 14) were amplified using genomic DNA of patients with leukemia ($n = 13$) and normal individuals ($n = 11$) using PCR. This study is the first to report leukemia-associated *BRCA1* mutations in the Pakistani population, which can be employed for early diagnosis, monitoring treatment success, and designing drugs specifically targeting leukemia.

Keywords *BRCA1*, Acute myeloid leukemia, Missense mutations, Non-sense mutation, Premature termination codon, Truncated *BRCA1* protein

Introduction

Hematological malignancies can develop at any stage of blood cell formation, affecting the synthesis and function of cells and being classified into three types of blood cancer, that is, leukemias, lymphomas, and myeloma [1]. These blood cancers arise either in the bone marrow or immune cells, dysregulating the formation of normal blood cells [2]. Leukemias are neoplastic proliferations of hematopoietic cells, comprising a heterogeneous group of tumors including acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML) [1]. Lymphomas are caused by malignant alterations of cells residing mainly in the lymphoid tissues and are usually subdivided into non-Hodgkin's lymphoma (NHL) and Hodgkin's disease (HD) [1, 3].

Children are majorly diagnosed with ALL and AML. Leukemia incidences have been majorly observed between the ages of 1–4 and 9–19 years. Incidence and mortality due to leukemia are higher in males than females. In 2020, 58% of incidences were reported for males [4]. By 2024 in the USA, 62,770 new cases of leukemia in both genders (36,450 males and 26,320 females) and 23,670 deaths in males (13,640) and females (10,030), have been estimated [5]. According to the latest data from the Leukemia & Lymphoma Society, from 2015 to 2019, leukemia was reported as the sixth most prevalent death-causing cancer in males and the seventh most deadly cancer in females in the USA [6]. According to the World Health Organization (WHO), in 2020, almost 80,491 childhood leukemia cases were reported worldwide, with a fatality rate of 32,765 cases per year [4]. For AML, the highest death rate, i.e., 15.19 deaths per 10,000 population, has been reported in Australia [7]. In Canada, the incidence of AML was estimated to be 2.79/100,000 persons per year [8]. AML is the most common cancer in Pakistan. According to available reports from 2015 to 2019, in Pakistan, a total of 5510 new cases of leukemia were reported, including 3489 males, 2021 females, 1626 children, 503 adolescents (15–19 years), and 3318 adults (20 years) [9].

Most leukemias and lymphomas are sporadic. Leukemia risk factors are categorized as environmental (ionizing radiation, chemotherapy drugs), familial, lifestyle (obesity, diet, and smoking), and genetics [10] such as telomere length [11]. Further, EBV or HHV-6 viral infection in young children, allergies involving Th1/Th2 lymphocyte-dependent process, and genetic disorders such as Down syndrome and Fanconi anemia enhanced the risk of acute leukemia [4]. Cytogenetic analysis unveils the genetic basis of AML, depicting aberration at the chromosomal level including deletions, translocations, inversion, insertions, polyploidy, trisomy, and monosomies [12]. Chromosomal alterations associated with AML prognosis such as *RUNX1/RUNX1T1* [t(8;21) (q22;q22)], *CBFB/MYH11* [inv(16)(p13q22)], and *PML/RARA* [t(15;17)(q24;q21)] favor prognosis and drug response, *MLLT3/MLL* [t(9;11)(p22;q23)] presents intermediate prognosis, *DEK/NUP214* [t(6;9)(p23;q34)], *RPN1/EVI1* [inv(3)(q21q26)], and *RBM15/MKL1* [t(1;22)(p13;q13)] represent disease aggressiveness with poor prognosis and drug response [12].

Earlier studies have demonstrated the role of various tumor suppressor genes including *BRCA1* in the development of human cancers. *BRCA1* (*BReast CAncer susceptibility gene 1*) is present on the long arm of chromosome 17q21.31, consists of 24 exons, and encodes a tumor suppressor protein with molecular weight of 220 kDa comprised of 1863 amino acids [13].

BRCA1 is a tumor repressor gene involved in the repair of DNA, acts as a protector of the replication fork, and is a regulator of cell cycle and gene transcription [14]. It is known as the DNA damage response (DDR) protein required for activation of cell cycle checkpoint and DNA repair [15]. *BRCA1* along with *BRCA2* plays a vital role in maintaining genomic stability via the DNA double-strand break repair mechanism, such as homologous recombination. *BRCA1* is also involved in the regulation of the nonhomologous end-joining (NHEJ) repair pathway [16]. *BRCA1* has ubiquitin E3 ligase activity, due to which it suppresses gene expression by regulating epigenetic alterations [16].

BRCA1 protein comprises multiple functional domains (Fig. 1) through which it interconnects with regulators of cell cycles and other DNA repair and tumor suppressor proteins performing distinct roles in the BRCA repair pathway [15]. *BRCA1* binds with other tumor suppressor proteins (*MSH2*, *MSH6*, *MLH1*, *ATM*, and *BLM*) and DNA repair proteins (*RAD50–MRE11–NBS1*), forming the *BRCA1*-associated genome surveillance complex (BASC) that functions as a DNA damage sensor, and associated proteins play a role in DNA repair [17]. The ring finger domain (aa 8–96) of *BRCA1* interacts with the ring finger domain of BARD1 (BRCA1-associated RING domain protein 1) to form a *BRCA1–BARD1* complex. The *BARD1–BRCA1* complex stabilizes *BRCA1* and helps to repair double-strand breakage of DNA [18]. Additionally, *BARD1–BRCA1* is a RING-type E3 ubiquitin (Ub) with ligase activity that regulates transcription and maintains genomic stability [19]. RING finger of *BRCA1* catalyzes the distinct ubiquitination on various substrates, depending upon the ubiquitination carrier, E2. Ubiquitination helps to recruit *BRCA1* at DNA damage sites via ubiquitin interacting motifs having RAP80 protein [20]. The CRM1 (chromosome region maintenance protein 1), a type I transmembrane protein, binds to the nuclear export signal (NES, aa 81–99) and mediates the export of *BRCA1* out of the nucleus [21].

The DNA binding domain (aa 452–1079) of the BRCA1 protein is used to interact with its target DNA. The two proteins, *Rad50* and *Rad51*, that are involved in both nonhomologous end joining (NHEJ) and homologous recombination (HR) pathways require *BRCA1* exon 11 amino acids ranging from 341 to 748, indicating the role of *BRCA1* exon 11 in NHEJ and HR [22]. Localization of *BRCA1* protein into the nucleus is regulated through the presence of two nuclear localization signals (NLS) present at aa 503–508 and aa 606–615 [23].

The serine cluster domain (SCD) is located in exons 11–13 and spans a region of 1280–1525 amino acids. It has several phosphorylation sites, and in response to DNA damage, *BRCA1* is phosphorylated by *ATM* (ataxia telangiectasia mutated gene)/*ATR* (ataxia telangiectasia and Rad3-related protein) kinases, resulting in the translocation of *BRCA1* to the damage site [23]. *BRCA1* amino acids ranging from 1364 to 1437 constitute a coiled-coil domain that contains a binding site for *PALB2* (partner and localizer of BRCA2) [22].

There are two BRCT (domains in the *BRCA1* protein, that is, the C-terminal BRCT domain (aa 1640–1729) and the N-terminal BRCT domain (aa 1769–1821). BRCT repeats have a high affinity for phosphorylated peptides. The BRCT–BRCA1 domain recognizes pSer–X–X–Phe for interaction with the other phosphorylated DNA damage-activated phosphoproteins such as BACH1, CtIP, and abraxas [22].

Genetic alteration, i.e., mutation or loss of expression in *BRCA1*, enhanced the risk of developing various cancers including breast, prostate, and ovarian cancer [14]. Meanwhile, germline mutations in *BRCA1* predispose an individual to many other cancers such as colorectal, stomach, pancreatic, and prostate cancers [16]. The BRCA pathway is known to prevent the development of leukemia and lymphoma, which involves gene rearrangement, along with other cancers by regulating error-free DNA repair. Inactivation of BRCA pathway components including *BRCA1* leads to gene rearrangement, which is a hallmark of leukemia and lymphoma, increasing the risk of leukemia up to 2000-fold [24]. *BRCA1* downregulation or deficiency has been associated with primary and therapy-related AML [25].

Earlier studies suggested a parallel link between TP53 and BRCA1. These proteins are phosphoproteins and play a major role in cell cycle arrest and DNA repair mechanisms. *BRCA1* responds to activated *TP53* to maintain genomic integrity by repairing damaged DNA [26].

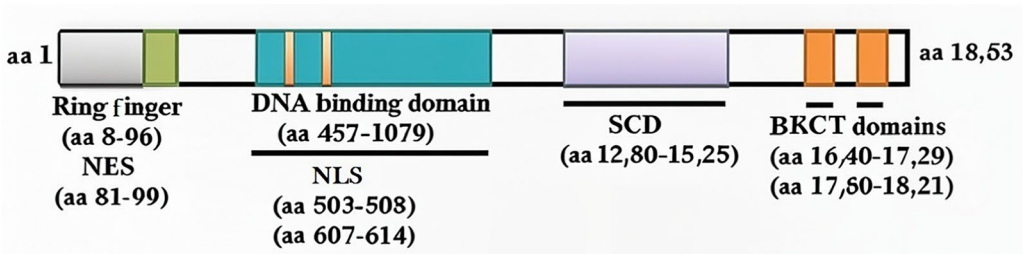


Fig. 1 Architecture of *BRCA1* protein. The ring finger domain, nuclear export signal, DNA binding domain, nuclear localization signals (NLS), serine cluster domain, and two BRCT domains of *BRCA1* protein are shown in this figure. aa = amino acid, NES = nuclear export signal (used to export *BRCA1* protein out of nucleus, NLS = nuclear localization signal (used to transport *BRCA1* inside nucleus), SCD = serine cluster domain (required for phosphorylation of *BRCA1* protein), BRCT = BRC tandem repeats (required for interaction with DNA damage activated phospho proteins). X = stop codon. This figure is a modified version of an earlier reported one [41]

Previously, various mutations were reported in patients with breast cancer (BC) worldwide (Supplementary Fig. 1), and many studies have suggested a role of mutant *BRCA1* in the pathogenesis of BC. However, no data are available regarding the mutational status of *BRCA1* in Pakistani patients with leukemia.

Considering the role of *BRCA1* in other cancers such as BC, this study was initiated to investigate the *BRCA1* mutational spectrum in patients with AML in Bahawalpur, southern Punjab, Pakistan. This information on mutations can be employed for the diagnosis and therapy of patients with AML.

Methodology

The present study involved mutational identification by wet lab experiments and comparative characteristics, functional and structural analysis by dry lab experiments.

Sample collection

Blood samples were collected from patients with AML ($n=13$) and normal individuals ($n=11$) following the standard method in ethylenediaminetetraacetic acid (EDTA)-coated vials. Sample collection was done from a local clinic in Bahawalpur, southern Punjab, Pakistan. All patients consented to participate in the study. The collected blood samples were stored at −70 °C till further analysis.

DNA extraction and amplification by PCR

DNA was extracted from blood samples using an already-reported method [27, 28]. The isolated DNA was quantified by the Nano-drop method (Biotek/Take3) and visualized by 1% agarose gel electrophoresis. The extracted DNA was stored at −80 °C. For mutational analysis, exon 11 and 14 were amplified using four sets of primers (Supplementary Table 1) selected from an earlier study [29]. PCR reaction mixture of 50 µl containing 50–120 ng genomic DNA, 1× PCR buffer, 200 µM dNTPs, 2 mM $MgSO_4$, 20 pmol of both reverse and forward primers, and 1.25 U Taq DNA polymerase (New England BioLabs, cat. no. 0141512) was used. PCR amplification was done using a Mygene TmL series Peltier thermal cycler (UNIEQUIP). For amplification, initial denaturation was carried out at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and final extension of 10 min at 72 °C [29]. PCR amplification was validated by 2% agarose gel electrophoresis. PCR products were purified by Thermofisher purification kit (Cat#T1030S) and then sent to Macrogen, South Korea for Sanger sequence analysis. Owing to financial limitations, only four leukemia samples were subjected to DNA sequence analysis.

Dry lab experiments

Analysis of Sanger sequencing results

Sanger sequencing results were further processed for comparative and mutation analysis. For comparative evaluation, the *BRCA1* wild gene sequence was retrieved from the National Center of Biotechnology (NCBI) available at https://www.ncbi.nlm.nih.gov/. For mutation detection, the sequencing results were compared with the wild *BRCA1* sequence using the Pairwise sequence alignment tool (available at: https://www.ebi.ac.uk/jdispatcher/psa). To determine mutations' effect at the protein level, translation of the retrieved sequence was performed by employing the EXPASY translator tool (https://web.expasy.org/translate/).

Functional and structural analysis of detected mutations

The impact of mutations on the three-dimensional (3D) structure of the resultant proteins was predicted by Phyre2 tool (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The prediction of the subcellular localization of the resultant protein was performed using Protcomp 9.0 (http://www.softberry.com/berry.phtml?topic=protcomp&group=help&subgroup=proloc).

Results

A total of 24 samples, including 11 normal and 13 from patients with leukemia, were analyzed by PCR. The results of PCR exhibited that the positivity ratio of PCR amplification with primer pair F1R1 was higher in cancer samples (100%) than in normal samples (72%). In contrast, PCR amplification with primer pairs F2R2 and F3R3 exhibited a higher positivity ratio in normal samples (72% for F2R2, 63% for F3R3) than in cancer samples (69% for F2R2, 30% for F3R3). All cancer samples were found to be positive for PCR amplification with primer pair F6R6, with a considerable proportion of normal samples (72%) (Fig. 2). PCR-amplified products were resolved on 2% agarose for quality check. DNA sequence analysis revealed missense, nonsense, and silent mutations. For convenience, samples found to be positive for *BRCA1* mutations were categorized as case 1 (LK50), case 2 (LK59), case 3 (LK52), and case 4 (LK53). The encoded proteins are referred to as protein 1, protein 2, protein 3, and protein 4, respectively.

Mutational status of *BRCA1*

In case 1, a novel frameshift mutation c.2339insG (p.E781fs) was noted that altered the downstream amino acid sequence, resulting in the formation of a stop codon (S789X) in the resultant protein (Fig. 3).

Case 2 was found to harbor a total of 14 mutations, including 5 novel insertion mutations (c.2376insC

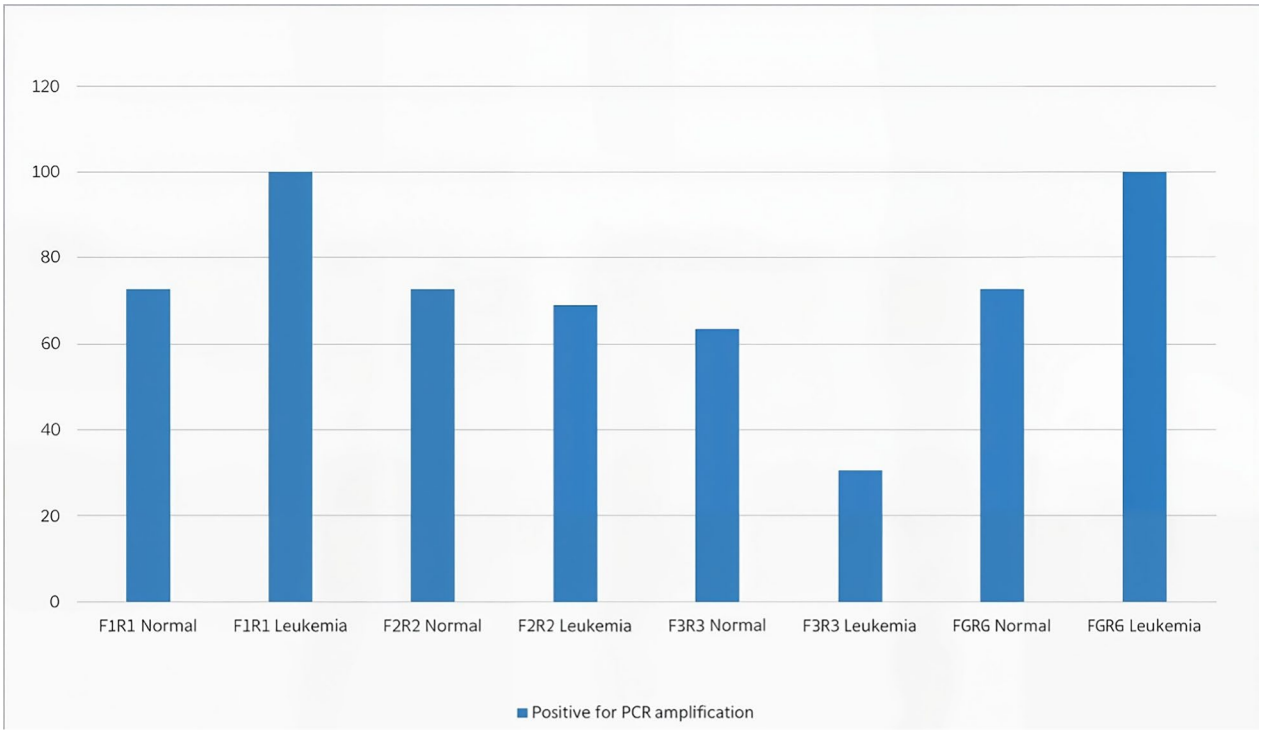


Fig. 2 Percentage positivity ratio of PCR amplification using different pairs of primers (F1R1, F2R2, F3R3, and F6R6)

(p.K793fs), c.2380insT (p.A794fs), c.2443insT (p.I815fs), c.2533insG (p.I845fs), and c.2511insG (p.N838fs)), 2 novel frameshift deletion mutations, namely c.3762delG (p.N1255fs) and c.3830delC (p.A1277fs), and 1 reported deletion mutation c.3621delG (p.Lys1208fs). All these mutations were frameshift variants/mutations causing alteration of amino acid sequence in the downstream open reading frame (ORF) and forming premature termination codon. Further, one nonsense/stop-gain mutation c.2383A > T (p.K795X) and four nonsynonymous missense mutations c.2429A > C (p.N810T), c.2430C > A (p.N810K), c.3644A > T (p.N1215I), and c.3740 T > A (p.V1247D) were identified in this patient.

In case 3, in total, four mutations were observed, including one nonsynonymous mutation c.3577 T > C (p.F1193L) and three synonymous mutations, namely c.3576 T > C (p.P1192P), c.3579C > T (p.F1193F), and c.4311 T > C (p.S1437S), resulting in no change in the protein. In case 4, only one silent mutation c.4252 T > C (p.L1418L) was noted. Details of mutations are provided in Table 1 and Fig. 3.

Functional impact of *BRCA1* indel mutations

Mutational analysis of *BRCA1* helped to identify six insertion and three deletion mutations in AML patients. The c.2339insG (p.E781fs) is the frameshift variant that leads to alteration of the amino acid sequence (E781G, S782K, I783Y, S784L, L785V, L786T, E787G, and V788S) and formation of premature stop codon amber (TAG) at position 789 in the resultant protein 1. This will cause premature termination of translation and formation of truncated protein. This mutation resulted in loss of the DNA binding domain, due to which its ability to bind *RAD51* (758–1064 aa) and *BRCC36* (502–852 aa) will be lost. Further, *BRCA1* phosphorylation by ATM/ATR, formation of BRCA1–BRCA2–PALB2 complex, and binding ability with other phosphoproteins will also be impaired. Thus, truncated and nonfunctional proteins will be synthesized (Fig. 4).

The two frameshift mutations c.2376insC (p.K793fs) and c.2380insT (p.A794fs) change the downstream amino acid sequence (K793Q, A794G, K795K, T796N, E797R, P798T, N799K, K800X) and (A794C, K795K, T796N, E797R, P798T, N799K, K800X), respectively. Owing to these mutations, premature termination codon (ochre TAA) created at position 800 leads to premature translation termination and formation of truncated protein. The encoded truncated protein is an abnormal protein lacking DNA binding domain, important amino acid sequence 1364–1437 (required for BRCA1 and PALB2 interaction), serine cluster domain (required for phosphorylation of *BRCA1* by ATM/ATR), and BRCT repeats (required for interaction of *BRCA1* with other phosphoproteins). Hence, resultant proteins will lose their ability

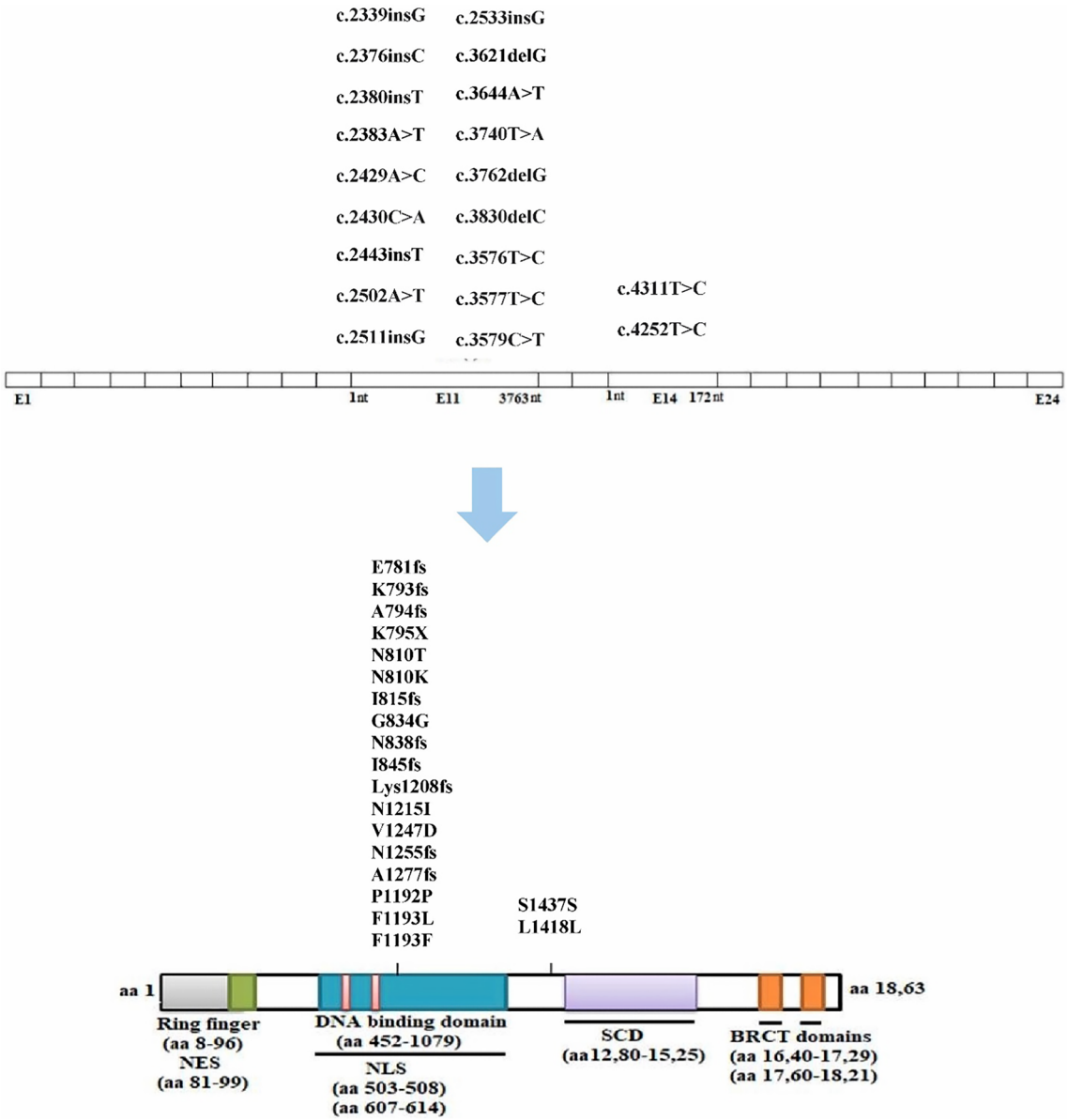


Fig. 3 *BRCA1* mutations observed in the present study and their effect on encoded protein. Observed *BRCA1* mutations in all four cases are mentioned on exon 11 and exon 14 (studied region). To determine the effect of observed mutant, genes were translated into proteins. E = glutamic acid, G = glycine, S = serine, K = lysine, I = isoleucine, Y = tyrosine, A = alanine, V = valine, N = asparagine, T = threonine, R = arginine, P = proline, X = stop codon. fs* = frameshift mutation

to bind with their target DNA, undergo phosphorylation, promote BRCA1–BRCA2–PALB2 complex formation, and interact with other phosphoproteins (Fig. 4).

The c.2443insT (p.I815fs) is also a frameshift mutation that leads to the alteration of amino acids sequence I815Y, H816S, G817W, C818L, S819F, K820Q, D821R, N822X and forms stop codon (ochre TAA) at position 822 of downstream amino acids, resulting in the formation of truncated protein with 822 amino acids, lacking important functional domains, such as a part of DNA binding domain (amino acid sequence 823–1064 required for *RAD51* interaction and amino acid sequence 823–852 site of BRCC36), serine cluster domain (SCD), and *BRCA1* C-terminal (BRCT) domains. Thus, the outcome is the synthesis of nonfunctional protein (Fig. 4).

Another insertion mutation c.2533insG (p.I845fs) switches the sequence of seven downstream amino acids starting at position 845 (I845S, E846R, M847N, E848G, E849R, S850K, E851X) and creates stop codon (TGA, opal) at position 851, thus causing the formation

Table 1 *BRCA1* mutations detected in the AML patients in the present study

Sample no.	Genomic location	Mutation (NM_007294.4)	Protein change	rsID
Case 1	43093191–43093192	c.2339insG	p.E781fs	Nil
Case 2	43093154–43093155	c.2376insC	K793fs	Nil
	43093151–43093152	c.2380insT	p.A794fs	Nil
	43093148	c.2383A > T	p.K795X	rs1567795889
	43093102	c.2429A > C	p.N810T	rs2154377936
	43093101	c.2430C > A	p.N810K	Nil
	43093088–43093089	c.2443insT	p.I815fs	Nil
	43093029	c.2502A > T	p.G834G	rs2154374027
	43093020–43093021	c.2511insG	p.N838fs	Nil
	43092997–43092998	c.2533insG	p.I845fs	Nil
	43091910	c.3621delG	p.Lys1208fs	rs1555587401
	43091887	c.3644A > T	p.N1215I	rs786203310
	43091791	c.3740T > A	p.V1247D	rs2053534509
	43091769	c.3762delG	p.N1255fs	Nil
	43091701	c.3830delC	p.A1277fs	Nil
Case 3	43091955	c.3576 T > C	p.P1192P	rs766447664
	43091954	c.3577 T > C	p.F1193L	rs2154301670
	43091952	c.3579C > T	p.F1193F	rs2053562704
	43082450	c.4311 T > C	p.S1437S	rs1131692092
Case 4	43082509	c.4252 T > C	p.L 1418L	rs2154155348

Nil = not detected or reported previously

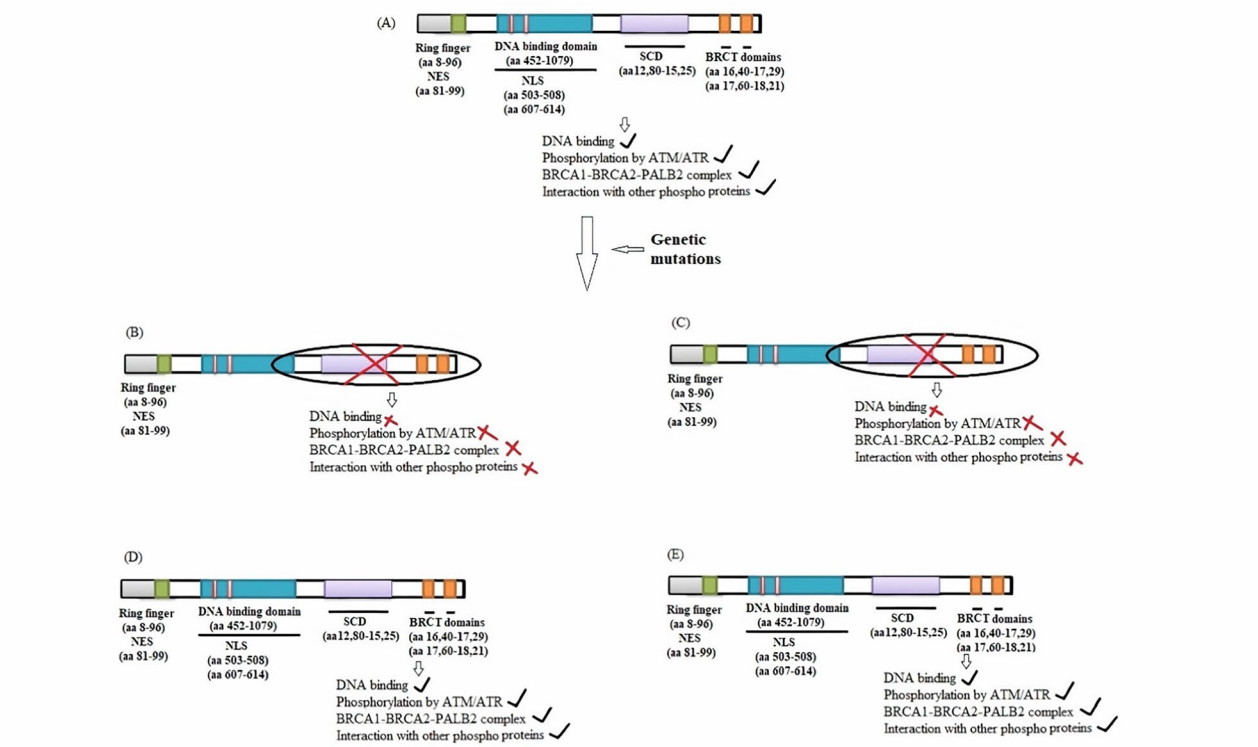


Fig. 4 Functional impact of mutant *BRCA1* proteins as compared with the normal *BRCA1* protein. Mutant protein 1 and 2 differ from normal *BRCA1* protein and lack different functional domains required to perform normal function. No difference was found in mutant proteins 3 and 4. **A** Normal *BRCA1* protein. **B** Protein 1. **C** Protein 2. **D** Protein 3. **E** Protein 4

of truncated protein owing to premature termination of translation. The resultant protein lacks essential domains including a few amino acids (852–1064) of the DNA binding domain, which are required for *RAD51* binding, SCD, and BRCT domains. Hence, the encoded protein will lose the ability of poly ADP-ribose (PAR) binding, phosphorylation-independent protein interactions, and DNA binding (Fig. 4).

The mutation c.2511insG (p.N838fs) resulted in the alteration of a single amino acid at position 838 (N838X) to stop codon (TAA, ochre), which leads to premature termination of translation and thus the formation of an abnormal truncated protein lacking many functional domains, including a region of DNA binding domain (*RAD51* and BRCC36 sites), SCD domain, and BRCT domains, being thus unable to form BRCA1/BACH1, BRCA1/RAP80/Abraxas, BRCA1/CtIP, and BRCA1/PALB2/BRCA2 complexes (Fig. 4).

Among the deletion mutations, c.3621delG (p.Lys1208fs) alters the K1208N and forms stop codon L1209X (amber, TAG), terminating translation. The mutation c.3762delG (p.N1255fs) alters the downstream amino acids K1254K, N1255T, T1256Q, E1257R, E1258R, N1259I, L1260Y, L1261Y, S1262H, L1263X and leads to premature truncation of translation at position 1263 (TGA, opal) and synthesis of truncated protein. The c.3830delC (p.A1277fs) is a single base pair deletion mutation at A1277E, resulting in the frameshift of 29 downstream amino acids and creates premature stop codon L1306X (TAG, amber) at position 1306. The encoded protein due to these deletion mutations lacks SCD and BRCT domains and thus loses its ability to bind poly (ADP-ribose) (PAR), *PALB2*, and *BRCA2* and is unable to mediate phosphorylation-dependent interactions with *ATM* and *ATR* proteins and fails to form *BRCA1* complexes with BACH1, PALB2/BRCA2, and CtIP (Fig. 4).

Functional impact of *BRCA1* nonsynonymous mutations

A total of five nonsynonymous mutations were observed in leukemia samples that resulted in the change of a single amino acid. The mutation c.2429A > C (p.N810T) altered asparagine to threonine. No change in function was observed as both are neutral and polar amino acids. Another mutation c.2430C > A (p.N810K) changed asparagine (neutral and polar) to lysine (basic and charged amino acid), thus impacting the protein structure. c.3644A > T (p.N1215I) altered asparagine to hydrophobic isoleucine. c.3740 T > A (p.V1247D) replaced the aliphatic, hydrophobic valine with acidic and charged amino acid aspartate, thus affecting protein stability and structure. c.3577 T > C (p.F1193L) resulted in no functional change in protein, as the nature of the altered amino acid (leucine) is the same (hydrophobic) as the reference amino acid (phenylalanine). The phenylalanine usually remains buried in the core domain, stabilizes the *BRCA1* protein, and does not regulate the interaction of *BRCA1* with various ligands (Fig. 4).

Besides these, a stop-gain mutation c.2383A > T (p.K795X) was also detected, resulting in the termination of translation at position 795 and an encoded truncated protein lacking DNA binding domain, *BRCA2*, *RAD51*, and SCD interacting domain. Moreover, it cannot be phosphorylated by *ATM* and loses its ability for complex formation with BRCA2/PALB2, BACH1, RAP80/Abraxas, and CtIP (Fig. 4).

Mutations' impact on the structure of encoded proteins

For further comparative analysis of mutations in the resultant proteins, pI, molecular weight, subcellular localization, and 3D structures of all encoded proteins were predicted.

Encoded protein 1 (case 1) had a higher pI (6.42) and lower molecular weight (88583.23) as compared with the normal *BRCA1* protein (pI = 5.29, Mw = 2077720.85). The 3D structure of encoded protein 1 lacked various important functional domains. Protein 2 (case 2) contained indel and nonsynonymous mutations, due to which the mutant protein had high pI (6.34) and low molecular weight (89847.63) due to the formation of truncated protein. The 3D structural analysis depicted a loss of functional domains with respect to wild protein. The analysis of protein 3 (case 3) showed a slight variation in the molecular weight of mutant protein 3 (Mw = 207686.83) as compared with the normal *BRCA1* protein. However, the pI (5.29) and 3D structure remained similar to wild-type *BRCA1* protein. In case 4, as a silent mutation was noted to have no impact on protein, thus the pI (5.29), molecular weight (2077720.85), and 3D structure of encoded protein 4 were similar to those of the *BRCA1* wild-type protein (Table 2).

Discussion

Acute myeloid leukemia (AML) is a heterogeneous disorder comprising of different subtypes based on molecular, morphological, gene expression, and cytogenetic features [30]. Although the molecular basis of AML has been explored extensively, no improvement in the survival of leukemia patients has been achieved yet, indicating the urgency for new diagnostic, prognostic, and therapeutic biomarkers. *BRCA1* is involved in DNA damage repair, and its low expression has been reported in treated patients of AML [25]. The present study was conducted to investigate the mutational status of *BRCA1* in AML patients. PCR amplification of *BRCA1* showed that, except for (F6R6), neither cancerous nor normal blood

Table 2 Comparison of characteristics and structure of mutant protein and normal *BRCA1* protein

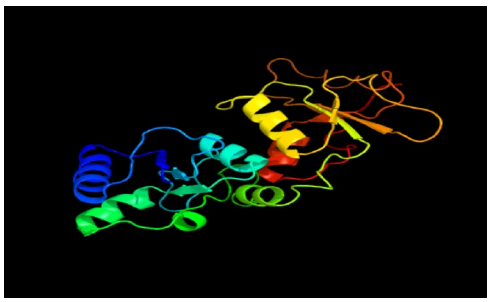
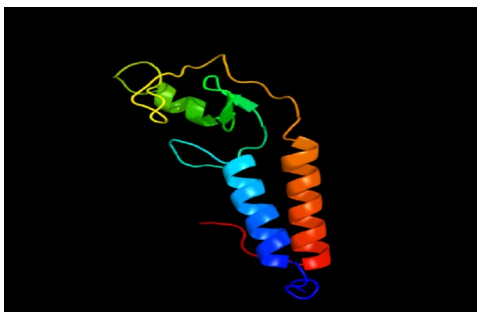
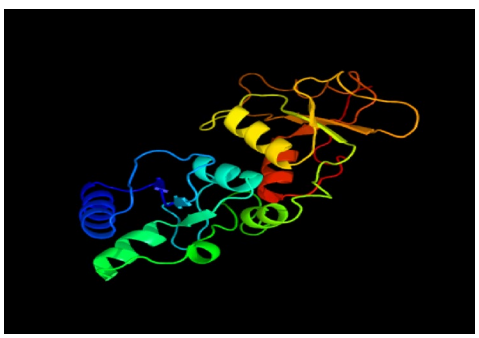
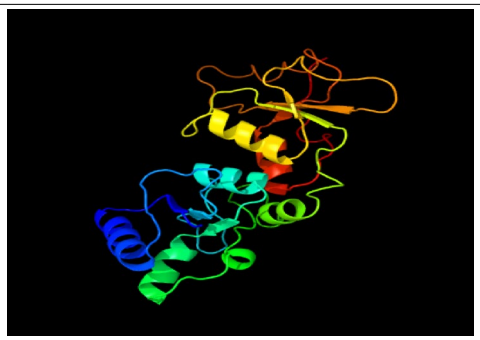
Protein type	pI	Mw (Daltons)	Subcellular localization	3D structure
Normal *BRCA1* protein	5.29	207720.85 (220KDal)	Nuclear	
Protein 1	6.42	88,583.23	Nuclear	
Protein 2	6.34	89,847.63	Nuclear	
Protein 3	5.29	207,686.83	Nuclear	

Table 2 (continued)

Protein type	pI	Mw (Daltons)	Subcellular localization	3D structure
Protein 4	5.29	207,720.85	Nuclear	

samples were found to be positive for PCR amplification with all sets of primers. This absence of amplification with different primers indicates that either the population of Bahawalpur, Southern Punjab, Pakistan possessed unique genetic alteration or the complete absence of highly mutated target regions.

The DNA sequence analysis revealed indel, missense, nonsense, and silent mutations in AML patients. The nature and type of the observed *BRCA1* mutations were similar to already reported *BRCA1* mutations noticed in patients with breast cancer worldwide. In case 1, the only noted frameshift mutation c.2339insG (p.E781fs) was found to be a novel mutation, as this mutation has not been reported before in any literature nor submitted to any database. The premature truncated protein formed had high pI and low molecular weight. Further, this mutation causes loss of DNA binding domain, due to which the encoded protein becomes unable to recruit repair proteins to damaged DNA sites. The DNA binding domain plays an important role in the maintenance of genomic stability. In a previous study, a lack of DBD was noted to be associated with destabilization of DNA replication forks and undermined control of the intra-S-phase checkpoint [31].

Mutational analysis of case 2 depicted novel frameshift insertion mutations, c.2376insC (p.K793fs), c.2380insT (p.A794fs), c.2443insT (p.I815fs), c.2533insG (p.I845fs), and c.2511insG (p.N838fs), and frameshift deletion mutations, c.3762delG (p.N1255fs) and c.3830delC (p.A1277fs), which were not reported previously in any study. These mutations lead to the formation of truncated proteins lacking three functional domains, including DNA binding, BRCT, and SCD. Lack of DNA binding domain leads to the loss of important binding sites of *BRCA1* binding proteins *RAD51* and *PALB2* that are essential for DNA repair. *PALB2* lies in the coiled-coil domain region and plays a role in the homologous recombination during damage repair by forming a complex with *BRCA1* and *BRCA2* [22]. Thus, loss of *PLB2* resulted in impairment of DNA repair mechanism. Previously, mutations (Met1400Val, Met1411Thr, and Leu1407Pro) in the coiled-coil region of *BRCA1* were reported to block *BRCA1* interaction with *PALB2* [32]. Next, the SCD has many phosphorylation sites which upon DNA damage are targeted (phosphorylated) by ATM/ATR kinases, resulting in the translocation of *BRCA1* to the DNA damage site for repair [33]. However, lack of SCD domain due to detected indel mutation resulted in a nonfunctional protein that is unable to be recruited to damage site and participate in the DNA damage repair process suggesting the possible role of mutant *BRCA1* proteins in the pathogenesis of leukemia. Whereas BRCT domain is essential for maintaining genomic stability, protein-protein interactions, cell cycle regulation, and DNA damage response. The BRCT domain recognizes the pSer–X–X–Phe sequence, interacts with CtIP, BACH1, and CCDC98/abraxas, and regulates gene expression of p21 and GADD45 and transcription factors p53 and ER, which altogether promote cell cycle arrest. The *BRCA1*–Abraxas complex is crucial for the recruitment of *BRCA1* to DNA damage site for the repair process [34]. The lack of the BRCT1 domain, occurring owing to the mutations detected in the present study, resulted in the loss of the DNA damage repair ability of *BRCA1* [34]. Mutations in the BRCT domain are known to affect the subcellular localization of *BRCA1* and induce structural and functional alterations promoting protein misfolding, leading to abnormal *BRCA1* protein formation [35]. These mutations have not been reported previously. However, at these mutated positions in dbSNP, different nucleotide

insertions and deletions have been reported, including c.2380dupG (rs886037999), c.2443dupA, p.I815N (rs80357598), c.2511delA (rs1555589501), which were classified as pathogenic mutations forming truncated proteins. An already reported (rs1555587401) single deletion mutation c.3621delG (p.Lys1208fs) was also noted. Previously, the role of this mutation in nonsense-mediated decay of mRNA has been reported [36]. Further, the clinical significance of this mutation has been explained by Clinvar. It was reported to be a pathogenic mutation due to the formation of a truncated nonfunctional protein.

A unique (rs1567795889) stop-gain mutation c.2383A > T (p.K795X) also forms a truncated protein. The molecular characteristic of encoded protein 2 was also altered by unique nonsynonymous missense mutations p.N810T (rs2154377936), p.N1215I (rs786203310), and p.V1247D (rs2053534509). In contrast, a different variant (rs1459103756) c.2430C > T was reported in dbSNP at the same position as the detected novel mutation c.2430C > A (p.N810K). The comparative structural analysis of protein 2 harboring these mutations indicated higher pI and low molecular weight due to the premature translation termination. The structural analysis depicted the loss of DNA binding, SCD, and BRCT domain, due to which nonfunctional protein was thus formed, being unable to recruit to the DNA damage site as it lost phosphorylated pSXXF motif recognition and binding affinity for other proteins.

In case 3, the identified missense mutation p.F1193L resulted in a slight decrease in molecular weight. This mutation was also reported in dbSNp (rs2154301670). Further, the silent mutations observed in AML patients in the present study have also been previously reported in dbSNP. Among these mutations, p.P1192P (rs766447664) and p.S1437S (rs1131692092) were classified by Clinvar as a likely benign mutations. These mutations have no functional and structural impact on the encoded protein 3. Case 4 exhibited a silent mutation p.L1418L reported already in dbSNp (rs2154155348), having no impact on the encoded protein 4.

Although the mutations detected in this study have not been reported, various mutations including S1613G, E1038G, S1040N, K1183R (eastern Iran, patients with breast cancer) [37], P2612L, S4837K, C190R in Chinese patients with breast cancer [38], and c.3112G > T and c.845-848dupGGCG (patients with myeloid neoplasm) [39], S1503X, R1835X, 185indelAG, and 185insA (Pakistani population with BC and ovarian cancer) have been documented previously [40].

The findings of the present study strengthened the initial hypothesis, which suggested the occurrence of unique and novel *BRCA1* mutations in Pakistani AML patients.

Conclusion

Mutational analysis of *BRCA1* in AML patients revealed many unique and novel pathogenic mutations that deleteriously impact the encoded *BRCA1* protein, suggesting their role as diagnostic and therapeutic biomarkers of AML. In the future, there is a need to isolate and characterize these mutant *BRCA1* isoforms/variants to design targeted drugs for treatment of AML.

Abbreviations

PCR	Polymerase chain reaction
WBCs	White blood cells
KDa	Kilodalton
BRCA1	BReast CAncer susceptibility gene 1
BASC	BRCA1-associated genome surveillance complex
BARD1	BRCA1-associated RING domain protein 1
PRC1	Protein regulator of cytokinesis 1
PRC2	Protein regulator of cytokinesis 2
CBX4	Chromobox homolog 4
CRIM1	Chromosome region maintenance protein 1
DNA	Deoxyribonucleic acid
NLS	Nuclear localization signal
NES	Nuclear export signal
SCD	Serine cluster domain
BRCT	BRC tandem repeats
ATM	Ataxia telangiectasia mutated gene
ATR	Ataxia telangiectasia and Rad3-related protein
PALB2	Partner and localizer of BRCA2
BACH1	BTB domain and CNC homolog 1
USA	United States of America
CEMB	Center of Excellence in Molecular Biology
HR	Homologous recombination

Supplementary Information

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Supplementary material 1.

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Author contributions

SE designed and supervised the study. AR carried out the lab experiments. YH performed wet and dry lab experiments and wrote the first draft. IA performed mutation data analysis and wrote the manuscript. NI drafted the manuscript. MI and MK participated in the design of the study, and WNM drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data will be made available on reasonable request.

Declarations

Ethics approval and consent to participate

All the described experiments were conducted following Helsinki Convention 1992 guidelines. This study was approved by the Board of Studies (an IRB) of the Department of Biochemistry and Biotechnology, Institute of Biochemistry, Biotechnology, and Bioinformatics, The Islamia University of Bahawalpur. Informed consent was obtained from all participants.

Consent for publication

Written informed consent was obtained from the patients for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Competing interests

The authors do not have any competing interests.

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