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ORIGINAL PAPER

A study of mechanistic mapping of novel SNPs to male breast cancer

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Abstract
Alterations in *BRCA2*, *PALB2*, *CHEK2*, and *p53* genes have been identified for their association with male breast cancer in various studies. The incidence of male breast cancer in India is consistent with its global rate. The present study was carried out with an aim to evaluate the genetic alterations in male breast cancer patients from Malwa region of Punjab, India. Four male breast cancer patients belonging to different families were recruited from Guru Gobind Singh Medical College and Hospital, Faridkot, India. A total of 51 genes reported with implications in the pathogenesis of breast cancer were screened using next generation sequencing. Germline variations were found in *BRCA1*, *BRCA2*, *PMS2*, *p53*, and *PALB2* genes, previously reported to be associated with MBC as well as FBC. In addition to these, 13 novel missense alterations were detected in eight genes including *STK11*, *FZR1*, *PALB2*, *BRCA2*, *NF2*, *BAP1*, *BARD1*, and *CHEK2*. Impact of these missense alterations on structure and function of protein was also analyzed through molecular dynamics simulation. Structural analysis of these single nucleotide polymorphisms (SNPs) revealed significant impact on the encoded protein functioning.

Keywords SNPs · Male breast cancer · Molecular dynamics simulation · Germline variations · Novel missense alterations

Introduction

Male breast cancer (MBC) is a rare/uncommon malignancy representing 1% of all types of cancer and it is increasing at a pace of 1.1% every year [1–3]. The incidence rate of the MBC in Indian population is consistent with the global rate, i.e., one case per 100,000 men/year [4, 5]. The pathogenesis of MBC has been attributed to the genetic, hormonal, and environmental risk factors as in the case of female breast cancer (FBC). Among these, the family history of cancer is considered as the main predisposing factor because the risk of breast cancer increases twofold in case of family history [6]. As far as epidemiological prospective is concerned, MBC resembles post-menopausal FBC. However, it is different from FBC in clinical and pathological characteristics. In addition, the prognosis of MBC is poor in comparison with FBC as the symptoms appear in advanced stage in case of the former [5]. Around 10% of MBC cases are hereditary in nature and are due to germline mutations in tumor suppressor genes including *BRCA2, p53, ATM*, *CHEK2,* and *PALB2*. The mutation 1100delC of *CHEK2* has been linked with tenfold increased risk for MBC, whereas *PALB2* is reported to increase MBC risk by eightfold [7–11]. Genetic profiling of MBC patients can aid in the identification of genetic markers associated with MBC and could potentially aid in the identification of new therapeutic strategies [12].

The present study was taken up with an aim evaluate the incidence, clinical, and genetic profile of MBC patients from the Malwa region of Punjab, India, where cancer is widely feared on account of its steeply rising graph. Genetic alterations reported from this study were also examined using molecular dynamics-based approach to determine their impact on structure and function of protein.

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Materials and methods

Four MBC patients evaluated by an oncologist at Guru Gobind Singh Medical College and Hospital (GGSMCH), Faridkot, India were included in the study. The proposed work was carried out with the approval from the Ethics committees of GGSMCH and Central University of Punjab. Information on demographic profile and breast cancer-associated risk factors was collected by using a structured questionnaire. 5 ml of venous blood was collected from patients in EDTA-coated vacutainers with written informed consent of the patients. DNA was extracted from whole blood by using phenol–chloroform method. After the qualitative and quantitative analysis of DNA, the samples were subjected to next generation sequencing (NGS).

NGS assay

Library preparation for NGS was accomplished using the HaloPlex PCR target enrichment system (Agilent Technologies Inc.) [13, 14]. Using Sure Design studio (Agilent Technologies Inc.), probes were generated to cover the exons of all 51 genes frequently implicated in the pathogenesis of breast cancer. Using this design, libraries from four samples were generated. Design was made for Illumina 150-bp paired-end sequencing.

Library preparation and sequencing

Amplicon libraries were prepared from genomic DNA of all the patients using the HaloPlex PCR target enrichment system according to the manufacturer's recommendations. In brief, 50 ng of DNA was used for restriction reactions and hybridization was performed for 3 h at 54 °C. All the DNA samples were individually indexed. Libraries were quantified using the Qubit fluorometer and DNA concentration was calculated using the formula [1 ng/mL = 3 nmol/L/(library average size in bp/500)], where average fragment length was obtained from bioanalyzer data. Amplicon libraries were diluted to 2 nmol/L and indexed samples were pooled at a final concentration of 6 pmol/L. Sequencing was performed using Next Seq reagent kit 300 cycles on the NextSeq instrument (Illumina Inc.).

Bioinformatics analysis

The raw data quality was checked using NextSeq Sequencing Analysis Viewer software (Illumina Inc.). The sequences obtained were quality controlled using FastQC software (Babraham Bioinformatics, Cambridge, UK). SAMtools flagstat was used to obtain metrics of the analyzed reads. Raw FASTQ files were first evaluated using quality control checks from FastQC. NGSQC toolkit was then employed for read trimming and filtering. After removing low-quality bases, adapters, and other technical sequences, each library was aligned to the human reference genome (GRCh37) using BWA-mem generating sorted BAM files with SAMtools. Reads from the same libraries were then merged. The regions with < 30 reads of base quality ≥ 30 and mapping quality ≥ 20 were considered as target-sequence gaps. For the identification of germline point mutations, filtering parameters were total read depth of ≥ 6, mutated allele count of ≥ 3, variant frequency of ≥ 0.1, base quality of ≥ 30, mapping quality of ≥ 20. SAMtools was used for conversion of .sam files to .bam files and for generation of .mpileup files. From the .mpileup file, coverage was calculated using a combination of awk and custom scripts in R. Genome Analysis Toolkit (Broad Institute, Cambridge, MA) was used for raw variant calling (UnifiedGenotyper), realignment (RealignerTargetCreator and IndelRealigner), recalibration (BaseRecalibrator and PrintReads), and variant calling (Haplotype caller, Unified Genotyper, Samtools, SNVer and Mutect). Variants were looked for in exons and 20 bp flanking each exon because we wanted to identify variants in splice sites also. Even if flanking regions had not been targeted, this was possible because HaloPlex amplification, as a rule, includes a substantially larger region than the targeted exons, resulting in satisfactory coverage also in the flanking regions. Visual inspection of the alignment was also performed as a final step for all reported variants using Integrative Genomics Viewer. The process of variant calling includes an extra evaluation layer for variants present in provided data sources (COSMIC for somatic and HGMD for germline variants), regardless of the filtering thresholds (including the 0.025 minimum variant frequency otherwise required). Variants called according to these criteria are further evaluated by the filtering thresholds, but instead of being discarded when quality measures are not met, the variant is registered in a different output, facilitating further inspection. The objective of this added flexibility is to identify relevant mutations that would otherwise be missed when cutting thresholds are not met. Variants were annotated using several databases containing functional (Ensembl, CCDS, RefSeq), populational (dbSNP, 1000 Genomes, ESP, ExAC), and disease-related (COSMIC, ICGC, HGMD professional, Clinvar) information, as well as 11 scores from algorithms for prediction of the impact caused by nonsynonymous variants on the structure and function of the protein (SIFT, PolyPhen2, PROVEAN, Mutation Assessor, Mutation Taster, LRT, MetaLR, MetaSVM, FATHMM, and FATHMM-MKL), and to evaluate evolutionary conservation of a particular variant, two tools were used, the Genomic Evolutionary Rate Profiling (GERP) and the PhastCons. The GERP score of > 2.0 and the PhastCons score of > 0.3 indicate a good level of

conservation of the variants and these scores were considered in the screening of variant.

Structural analysis of the novel mutations

Generation of protein models

In order to determine the effect of mutations at the molecular level, SNPs were considered for their structural influence. Since no crystal structure was available for encoded form of genes (i.e., proteins) under study, software tool YASARA (version: 17.11.22, License No.: 476925318) which creates homology models was used [15]. The wild-type protein sequence (i.e., unmutated form) was modeled by YASARA to get the protein model. For mutated proteins, the amino acid corresponding to SNP was swapped with its mutated version in the structural model. Subsequently, the obtained protein models (both unmutated and mutated) were refined using molecular dynamics simulation.

Quality check of the protein models

In order to ascertain the quality of protein models, various checks and validations were performed such as Q mean score [16]. The orientation of residues in protein models was examined using RAMPAGE [17]. All the protein models were reported to be well modeled and did not violate Ramachandran plot.

Energy minimization and molecular dynamics simulation of protein models

GROMACS was used to derive the energy-minimized structure of protein models and their molecular dynamics simulation [18]. As all the proteins under study were water soluble, proteins were simulated in the presence of water. The charge of system was neutralized by adding sufficient number of sodium and chlorine ions. Steepest-descent algorithm, with 50,000 steps and energy step-size of 0.01 kJ/mol/nm, was used for energy minimization. The energy-minimized system was equilibrated with respect to pressure and volume using leap-frog integrator algorithm for 100 ps with a step-size of 2 fs. At last, the equilibrated system was considered for molecular dynamics (MD) simulation using the leap-frog integrator algorithm for 2 ns with a step-size of 2 fs.

Cluster analysis of simulated protein models

The cluster analysis of MD-simulated protein model generated a number of conformational states. To select one conformational state as representative of the protein model, the conformational state with least amount of RMSD with respect to other conformational states was selected and analyzed further.

Neighborhood and structural analysis of wild-type and mutated protein models

The neighborhood (within radius of 5 Å) and structural analysis of wild type and their mutated version of protein models were examined in order to see the changes induced by the mutation at the atomic level.

Results

The age of four patients recruited in the present study was 58, 63, 67, and 72 years at the time of disease diagnosis. The incidence of MBC in this region was observed to be 1.3%, since out of 300 breast cancer patients, only four were found to be MBC cases, the rest were FBC cases. All the patients were positive for ER/PR and one patient was HER2 positive as well. Family history of breast cancer was observed in one patient (Fig. 1b) and rest of the cases were sporadic (Fig. 1a, c, d). Germline missense alterations were detected in *BRCA1* (rs1799966, rs1799967 & rs16942), *BRCA2* (rs45469092), *PMS2* (rs18005321 & rs18005323), *p53* (rs1042522), and *PALB2* (rs152451 & rs45551636) genes previously reported to be associated with MBC and FBC [8, 9]. In addition, the genetic profiling of these four patients has unraveled 13 novel missense alterations in eight genes including *STK11, FZR1, PALB2, BRCA2, NF2, BAP1, BARD1*, and *CHEK2*. These novel alterations were detected in all the four patients.

Two novel mutations Val63Met and His168Asp were detected in STK11 protein. The former was observed in ATP binding domain of the protein, whereas the latter was located in the MO25-binding interface. Structural analysis of H168D revealed changes in interaction network of the amino acids. In the wild-type STK11 protein, Lys175 was observed maintaining hydrogen bonding with Gln214, Thr212, and

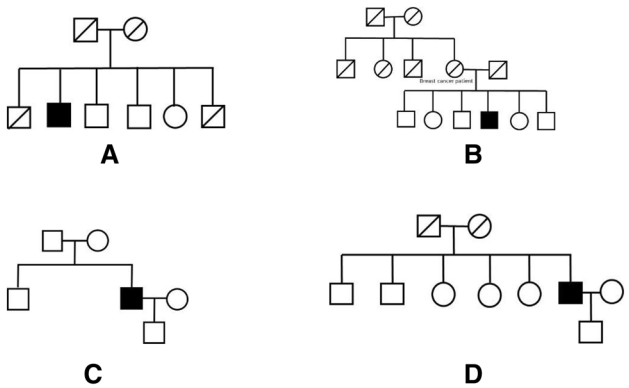

Fig. 1 Pedigree MBC patients

Glu199, whereas the mutation His168Asp in STK11 led to the loss of these interactions. On the other hand, Lys175 was not observed to interact with any residues in the STK11 protein-bearing Val63Met. The hydrogen bonding observed in Cys73 and Thr71 in wild-type STK11 also disappeared in both His168Asp- and Val63Met-mutated STK11 (Supplementary sheet: S1, S2 & S3). These mutations resulted in the local structural configuration modification in the vicinity, thereby probably reducing/altering the ATP and MO25-binding ability.

A novel mutation Ser163Ile was detected in the FHA domain of CHEK2 protein. The structural analysis of Ser163Ile showed the change in interactions among various amino acids through disruption in hydrogen bonding and hydrophobic interaction. Arg191 was seen interacting with Asn155 in the wild type but an increase in hydrogen bonding in mutated form allowed Arg191 to interact with an additional residue, i.e., Asp154. Additionally, in mutated CHEK2, Thr164 starts interacting with other residues through hydrogen bonding which could not be observed in wild type. Hydrophobic interactions of Cys164 were also observed in mutated CHEK2. The amino acids displaying hydrophobic interaction were different in both mutated and wild-type CHEK2. The mutation had also changed the orientation of the oxygen atoms of Agr160, Asp161, and Ile163 (Supplementary sheet: S4 & S5), leading to variations in the interacting pattern of the protein.

The mutation Thr2968Asn in the tumor suppressor gene *BRCA2* was detected in its DNA binding domain. The neighborhood analysis revealed that Thr2968Asn posed a significant disturbance in the interacting networks of the residues Trp2830 and Arg2991. Thr2968 did not show any hydrogen bonding with any other residues in the wild type (Supplementary sheet: S6 & S7). However, mutated BRCA2 interacts with Gln2829 and Arg2991 through hydrogen bonding. Strong hydrophobic interactions of Trp2990, absent in wild type, were also observed in the mutated BRCA2.

Two novel mutations Gly158Val and Gly158Arg were observed in BAP1 protein, 10 amino acids prior to the catalytic site. The structural analysis of the Gly158Arg-mutated BAP1 protein revealed an increase in the hydrogen bonding between Glu7 and Arg163. On the other hand, Gly158Val mutation resulted in the loss of original hydrogen bond between Glu7 and Arg163. Additionally, the hydrophobic interactions increased in both of these mutated forms of BAP1 in comparison with the wild type (Supplementary sheet: S8, S9 & S10).

Two mutations Leu299Ile and Leu299Arg in *NF2* gene were detected on its actin binding site. Significant changes were observed in the interaction pattern of amino acids in wild-type and mutated *NF2* gene as the residues Thr71, Glu7, Asp237 in the wild-type NF2 were seen interacting with other residues through hydrogen bonding but the same interactions were missing in NF2 with L299I and L299R mutations. Trp74 interacted with Leu297 via hydrogen bonding in the wild-type NF2 protein. However, Leu299Ile alteration resulted in the disappearance of this interaction, whereas Leu299Arg mutation changed the interacting partner of Trp74 from Leu297 to Glu298. In addition, the amino acids exhibiting the hydrophobic interactions were different in normal and mutated NF2 protein (Supplementary sheet: S11, S12 & S13).

The WD40 domains of *PALB2* and *FZR1* genes were observed to be mutated on account of Lys899Asn and Lys899Ile, and Leu284Met and Leu284Arg, changes, respectively. Alteration Lys670Asn in *BARD1* gene was present on the C-terminal domain (Table 1).

Table 1 List of domain containing novel alterations

Gene	HGVSp	Domain
PALB2	p.Lys899Asn	Partner and localizer of BRCA2 WD40 domain
	p.Lys899Ile	Partner and localizer of BRCA2 WD40 domain
STK11	p.Val63Met	ATP binding; catalytic domain
	p.His168Asp	MO25 interface on conserved domain; catalytic domain
FZR1	p.Leu284Met	WD40 domain
	p.Leu284Arg	WD40 domain
NF2	p.Leu299Ile	FERM domain; actin binding site on conserved domain
	p.Leu299Arg	FERM domain; actin binding site on conserved domain
BAP1	p.Gly158Val	Cysteine peptidase C12 containing ubiquitin carboxyl-terminal hydrolase
	p.Gly158Arg	Cysteine peptidase C12 containing ubiquitin carboxyl-terminal hydrolase
BARD1	p.Lys670Asn	C-terminal domain
CHEK2	p.Ser163Ile	Fork-head associated domain
BRCA2	p.Thr2968Asn	DNA binding domain

Discussion

In the present study, demographic and molecular analyses of four cases of MBC from the Malwa region of Punjab (India) were carried out. To the best of our knowledge, the present study is the first one to analyze the genetic profiling of MBC cases from North India. Further, the study is the first of its kind from Malwa region of Punjab, where breast cancer is the second most common cancer.

MBC incidence in this region was observed to be 1.3%, which was in accordance with the previous reports [5]. A previous study carried out by Chikaraddi et al. reported the frequency of MBC to be 0.4% in Indian populations, whereas another study carried out in the North Indian population reported the MBC incidence to be 0.5% [19]. On the contrary, the incidence of MBC was reported to be 4.1% in the Kashmiri population which is quite high in comparison with the reports from other regions of India as well as other countries [20, 21].

All the four cases of MBC reported in this study were found to be affected with infiltrating ductal carcinoma. According to the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) database results, more than 90% of the MBC have been reported to be ER positive receptor subtype [22]. All the four MBC patients from the proposed study were ER/PR positive.

Genetic profiling of the patients was carried out by massively parallel sequencing (MPS) of the coding regions of 51 genes which have been reported to be most frequently altered in breast cancer including tumor suppressor, proto-oncogenes, and DNA repair genes. These 51 genes were screened for SNPs and mono/biallelic alterations in *BRCA1, BRCA2, PALB2, PMS2,* and *p53* genes were observed. In addition, 13 novel missense alterations were also detected in 8 genes including *STK11, FZR1, PALB2, BRCA2*, *NF2, BAP1, BARD1,* and *CHEK2*. For their in-depth analysis, these novel alterations were considered for their molecular dynamics simulation-based structural analysis.

According to the previous reports, *BRCA1, BRCA2, ATM, PMS2, p53*, and *PALB2* gene alterations are reported to be associated with FBC as well as MBC. The association of *ATM* gene with MBC has been reported by three previous studies in Greek, Caucasians, and Ashkenazi populations [8, 9]. The alterations found in *ATM* gene are also in consensus with previous studies. A common mutation 1100delC in *CHEK2* gene has been reported to be associated with FBC as well as MBC [9]. However, in the current study, none of the MBC patients was observed to carry this mutation or other mutations reported in the *CHEK2* gene.

CHEK2 gene is responsible for regulation of cell cycle and phosphorylation of genes in response to DNA damage. CHEK2 inhibits the phosphatase activity of CDC25A, CDC25B, and CDC25C. Inhibition of phosphatase activity of CDCs increases the concentration of tyrosine phosphorylated form of CDK-cyclin complexes and thus halts cell cycle progression. It also stimulates transcription of various genes associated with DNA repair including BRCA2 by phosphorylating and activating transcription factor FoxM1 [23]. The CHEK2 protein binds to phosphothreonine, phosphoserine, and sometimes phosphotyrosine with the help of residues Arg160, Leu172, Ser183, Lys184, Gly208, Asn209, and Gly210 of its polypeptide binding site, to carry out various functions. A novel alteration Ser163Ile was observed in the FHA domain of *CHEK2* gene. This domain is responsible for the establishment and maintenance of cell cycle checkpoints [24]. The neighborhood analysis of Ser163Ile revealed the change in interacting network of one of the binding site residues Lys184 which in turn perturbed the interacting network of Arg160, another binding residue of polypeptide binding site of the FHA domain. Therefore, it can be concluded that Ser160Ile has led to disruption in CHEK2 functioning by disturbing the binding residues of FHA domain and interacting pattern among residues which may lead to the loss of function of the other genes or encoded proteins associated with FHA domain.

NF2 gene regulates the hippo signaling pathway, a pathway that plays a pivotal role in tumor suppression by inhibiting cell proliferation and enhances apoptosis. It suppresses cell proliferation and tumorigenesis by inhibiting the ubiquitin–protein ligase complex, CUL4A-RBX1-DDB1-VprBP/DCAF1 E3 [25]. In addition, it also inhibits the activity of PI3kinase as it binds with AGAP2, a factor that stimulates activity of kinase [26]. Two novel alterations Leu299Arg and Leu299Ile observed in MBC patients involved in the current study were located in the actin binding site of FERM domain of *NF2* gene. In addition to actin binding site, FERM domain of *NF2* gene also hosts another three important binding sites: phosphoinositide binding site, peptide binding site, and a homodimer interface. The mutation Leu299Arg was found to reduce the size of interacting network of binding residues of peptide and actin binding sites, and increased the size of interacting network of the binding residues of homodimeric interface. The other mutation Leu299Ile showed reduction in interacting network of binding residues of peptide and actin binding sites of FERM domain. The variability seen in the interaction network of amino acids, because of these mutations, may result in change in structural complexity and thereby affect the functional properties of NF2 protein.

BRCA2, a tumor suppressor gene, is reported to be frequently altered in familial and sporadic breast cancer. Alterations in the conserved domain might affect the DNA repair function of the protein [27]. The novel mutation Thr2968Asn lies on the most conserved region second

OB fold (OB2) of the 800-amino acid C-terminal ssDNA binding domain (DBD) of the BRCA2. BRCA2 binds to the ssDNA with the help of Ile2828, Gln2829, Trp2830, Met2831, Val2966, Thr2967, Thr2968, Val2969, Trp2970, Ile2989, Trp2990, Arg2991, and Pro2992 residues. Hence, this alteration might affect local orientation of the neighboring residues and thereby disrupt or disturb the functions of BRCA2 encoded protein.

Two missense novel alterations Val63Met and His168Asp were detected in *STK11*, a tumor suppressor gene which activates both AMP-activated protein kinase (AMPK) family proteins and non-AMPK family proteins by phosphorylation [28]. These mutations were found to be located in ATP binding site, MO25 interface, STRAD interface, and active site of the STK11. The residue Val63Met is a binding residue on both ATP binding site and active site of the STK11. It disturbed the interaction network of other binding residues, i.e., Gly58, Lys78, Met129, and Leu183 of ATP binding site and active site domain. The surface analysis of STK11 with respect to Val63Met alteration showed that methionine created an occlusion on the binding site leading to change in geometric complementarity of the site. The other missense alteration His168Asp is located on the MO25 interface of STK11 and results in alteration of interacting network with other binding residues like Glu165, Pro203, Phe234, and Arg301 of MO25 interface. Both the variations changed the interaction network of amino acids resulting in structural and functional variabilities that might abolish the tumor suppressor function of the gene.

BAP1, a BRCA1-associated protein, acts as deubiquitinating enzyme and regulates cellular proliferation, chromatin dynamics, and response to DNA damage [29]. Two missense mutations, Gly158Arg and Gly158Val, were found to be located on ssDNA binding domain of this protein which has been reported to be a cancer-causing mutation site [29]. Gly158Arg and Gly158Val were observed to be exactly ten amino acids upstream to the catalytic domain. Both of these variations altered the neighboring amino acids of Gly158 by 4-6 Å which resulted in decrease in volume of the active site and increased the compactness of protein. So, these changes in structural and interacting network of the protein are likely to cause the loss of tumor suppressor function of BAP1 gene.

PALB2, a partner and localizer of BRCA2 gene, is a tumor suppressor that plays a crucial role in homologous recombination. Heterozygous carriers of germline mutations in *BRCA2*, *PALB2*, and *RAD51C* genes have an increased lifetime risk of developing breast, ovarian, and other cancers [30]. PALB2 interacts with BRCA2 and RAD51D via WD40 domain and recruits the complex at the site of DNA damage. In addition, PALB2 also plays a crucial role in stability and localization of BRCA2. The novel mutations Lys899Ile and Lys899Asn are located on WD40 domain of PALB2 protein. Some malfunctioning may be associated with these alterations. However, based on structural and neighborhood analyses of these mutations, we could not conclude it precisely. Similar difficulties were faced in analyzing two novel variations Leu284Met and Leu284Arg in FZR1 protein and Lys670Asn in BARD1 protein.

In conclusion, we observed the incidence of MBC to be 1.3% in Malwa region of Punjab. Although alterations in various genes including *BRCA1*, *BRCA2*, *PALB2*, *PMS2*, and *p53* genes were observed in MBC patients employed in the current study, we found 13 novel alterations in *STK11*, *FZR1*, *PALB2*, *BRCA2*, *NF2*, *BAP1*, *BARD1*, and *CHEK2* genes. Surprisingly, these alterations were observed in all the patients from this region in MBC cases, which is a rare disease in comparison with FBC. These alterations if confirmed in more MBC patients from this region might guide us in terms of management of the disease in this region. No doubt MBC, like other multifactorial rare diseases, does suffer from the absence of comprehensive studies, thereby restricting the translation of these research finding into a personalized management of the disease. However, studies investigating the appropriate screening and risk management tools for MBC patients might lead to appropriate treatment strategies for this rare disease in future.

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Compliance with ethical standards

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

Ethical approval The blood sample collection of breast cancer patients for the present study was approved by Institutional Ethics Committee (IES) of CUPB.

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