Research Article

Absence of predisposing germline mutation in Breast cancer candidate genes in a high-risk population, Northeast India

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ABSTRACT

Introduction and aim

Breast cancer is a leading cause of death in Mizoram, Northeast India. Germline mutations in breast cancer patients have been associated with factors such as age, pathology, and ethnicity. This study aimed to screen for germline mutations in select marker genes in a high-risk tribal population to assess their role in breast cancer.

Materials and methods

Fifty unrelated breast cancer patients and fifty healthy controls were screened for mutations using direct Sanger sequencing. This analysis covered the entire coding regions of the BRCA1 gene and the frequently mutated exons of TP53, PTEN, CDH1, CHEK2, and XRCC2 genes. Tools such as Mutation Taster, Align GVGD, PolyPhen-2, and various databases were utilised to determine the pathogenicity of the identified polymorphisms.

Results

Eight polymorphisms were identified in the BRCA1 gene, including a novel exon 15 variant (g.95900A>T: c.4772A>T: p.P1544P). All polymorphisms were of silent mutation with no significant amino acid changes. Additionally, no genetic alterations were detected in the studied exons of the TP53, PTEN, CDH1, CHEK2, and XRCC2 genes.

Conclusion

The absence of pathogenic germline mutations in this high-risk population highlights the unique genetic origins and the need to explore other germline gene variants or novel genes in breast cancer development. Breast cancer is a complex and heterogeneous disease with variable clinical course in patients, and hence understanding of key genes involved is crucial. This study indicates the need to evaluate tumor samples in under-represented populations to better understand gene mutations, which could enhance personalized genetic screening approaches.

Keywords: Germline mutation, BRCA, Cancer susceptibility gene, Breast cancer.

INTRODUCTION

Breast cancer is a multifaceted, complex disease influenced by several variables. It is the common cancer worldwide and the primary cause of mortality among women (1, 2 & 3). The prevalence of breast cancer is considerably higher in North Eastern India than it is in the rest of the country, most likely a result of genotoxic stress brought on by tobacco use (4). As per the study by the National Cancer Registry Program (NCRP), breast cancer is the third most common

malignancy among Mizo women (5). Therefore, there is a necessity for a thorough exploratory investigation on the genetic variants in the patients.

The primary cause of breast cancer is germline mutations in breast cancer susceptibility genes (6). Less than 5% of patients with breast cancer have a germline (inherited) variant (mutation) in either BRCA1 or BRCA2 that is pathogenic or potentially pathogenic (7, 8). The spectrum of BRCA1 gene mutations has been extensively screened in various populations, particularly in Western populations, with most studies focusing on families with hereditary breast and ovarian cancer (9). In white Europeans, germline mutations in the BRCA1 gene account for 5-10% of breast cancer cases, with carriers having an 85% lifetime risk of developing breast cancer by age 70 (10).

Other genes including TP53, PTEN, CDH1, and CHEK2 with lower frequency and penetrance confer breast cancer susceptibility (9, 6). Additionally, a harmful XRCC2 germline mutation has been found in the exome sequencing of patients with familial breast cancer (11, 12). The clinical relevance of genes linked to breast cancer susceptibility and the corresponding mutations is contingent upon the frequency of these genes within a particular population (13). However, such studies remain unexplored in the Mizo population.

This is the first study to examine the potential role of a gene mutation linked to breast cancer risk in the Mizo tribal group. This community is homogeneous in terms of culture and history with high risk of breast cancer, and characterized by unique lifestyle behavior's related to nutrition and tobacco use (14). Breast cancer poses a significant health burden among Mizo women with an Age-Standardized Incidence Rate (ASIR) of 19.9 recorded from 2003 to 2020 resulting in breast cancer as the third most prevalent cancer among women. The impact of breast cancer is further underscored by a concerning mortality rate of 7.1% during the same period, reflecting both the high incidence and the substantial health risks associated with the disease (15). Previous studies have shown excessive use of tobacco products within this population, and the goal of this study was to explore the presence of germline alterations. Therefore, it is valuable to screen breast cancer using the frequently mutated genes and to ascertain the pathogenicity of these mutations.

MATERIALS AND METHODS

Sample collection, DNA isolation and gene amplification

Blood samples for this study were collected from 50 unrelated local ethnic breast cancer patients, with or without a family history of breast cancer and 50 healthy controls with no history of any cancer type. Samples were collected with the consent of the patients admitted to Mizoram State Cancer Institute (MSCI), Aizawl, Mizoram and with the approval of the ethical committee of Civil Hospital Aizawl (B.12018/1/13-CH(A)/IEC/33 dt. 15/10/2014). Genomic DNA was isolated from 200 µl of blood using the QIAamp DNA Mini Kit (catalogue: 51306) protocol.

Self-designed specific primers were used for the amplification of the gene of interest (Supplementary Table 1). Polymerase chain reaction (PCR) was carried out using 25 µl total reaction volumes, comprising template DNA (100 ng/µl), dNTPs (10 mM), 10X PCR buffer (2.5 µL), Taq polymerase (1U), forward and reverse primers (10 pM/µl),and MilliQ water (to make up the volume). PCR conditions were maintained at 95°C for 5 minutes for initial denaturation, at 94°C for 30 seconds for denaturation, at 50-63°C for 35 seconds annealing, at 72°C for 35 seconds for extension and a final extension at 72°C for 3 minutes. The PCR products were visualised on 1.2% agarose gel using Bio-Rad Gel documentation system.

Purification, Sequencing and Sequence analysis

The PCR products were purified using a Qiagen purification kit and Exonuclease and shrimp Alkaline phosphatase (Exo-sap) to remove excess salts, primers, and dNTPs. The purified products were then sequenced in both directions for accuracy using the Applied Biosystems 3500 Genetic Analyzer with forward and reverse

primers. The resulting sequence chromatograms were analysed using FinchTV version 1.4.0 and aligned using NCBI BLASTN (www.ncbi.nlm.nih.gov/blast). To ensure correct exon amplification, the sequences were checked against the Ensembl (https://asia.ensembl.org/index.html) and HUGO Gene Nomenclature Committee (HGNC) (https://www.genenames.org) databases. The pathogenicity and novelty of the observed polymorphisms were evaluated using databases such as Mutation Taster (http://www.mutationtaster.org), Breast Cancer Information Core (BIC) BRCA (https://research.nhgri.nih.gov/bic/),ARUP BRCA (http://arup.utah.edu/database/BRCA/Home/BRCA1_landing.php), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Align GVGD (http://agvgd.hci.utah.edu), and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

Supplementary Table 1: List of primers used for gene amplification from the Breast Cancer samples

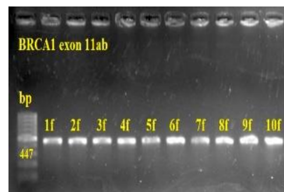
Gene	Exon	Primers (5'-3')	Product size (bp)
BRCA	2	GAAGTTGATCATTTTATAAACCTTT TGTCTTTTCTTCCCTAGTATGT	258
	3	TCCTGACACAGCAGACATTTA TTGGATTTTCGTTCTCACTTA	338
	5	GTTGTGAGATTATCTTTTCATGGC CTTCCAACCTAGCATCATTACCA	208
	6	CTTATTTAGTGTCCTTAAAAGG TTTCATGGACAGCACTTGAGTG	206
	7	CACAACAAAGAGCATACATAGG AGAAGAAGAAGAAAACAAATGG	269
	8	TGTTAGCTGACTGATGATGGT ATCCAGCAATTATTATTAAATAC	267
	9	CCACAGTAGATGCTCAGTAAATA TAGGAAAATACCAGCTTCATAGA	211
	10	TGGTCAGCTTTCTGTAATCG GTATCTACCCACTCTCTTCTTCAG	242
	11AB	TAGCCAGTTGGTTGATTTCC CTCACACAGGGGATCAGCATTC	477
	11C	CAACATAACAGATGGGCTGGAAG ACGTCCAATACATCAGCTACTTTGG	350
	11EF	GGTTCTGATGACTCACATGATGGG TCATCACTTGACCATTCTGCTCC	460
	11G	GAGCCACAGATAATACAAGAGCGTC GCAGATTCTTTTTCGAGTGATTCTATTGGG	272
	11H	ATCAGGGAACTAACCAAACGGAG CGCATGAATATGCCTGGTAGAAG	269
	11J	CTAAAAAGAATAGGCTGAGGAGGAAGT CAGCTCTGGGAAAGTATCGCTG	284
	11K	GCAACTGGAGCCAAGAAGAGTAAC TCTGTGTCATTTCTATTATCTTTGGA	458
	11N	GCACTCTAGGGAAGGCAAAAACAG CATTCCTCTTCTGCATTTCCTGG	280
	11P	GCCAGTCATTTGCTCCGTTTTC CGTTGCCTCTGAACTGAGATGATAG	288
	11Q	TGCAGGCTTTCCTGTGGTTG GGCTAATTGTGCTCACTGTACTTGG	305
	11S	TCAATGTCACCTGAAAGAGAAATGG CAGGATGCTTACAATTACTTCCAGG	301
	11TU	TTGAATGCTATGCTTAGATTAGGGG TTCTGAGGACTCTAATTTCTTGG	402
	11V	GAGTCCTAGCCCTTTCACCCATAC GTGATGTTCCTGAGATGCCTTTG	289
	11WX	CGTTGCTACCGAGTGTCTGTCTAAG GTGCTCCCAAAAGCATAAA	438
	12	GTCCTGCCAATGAGAAGAAA TGTCAGCAAACCTAAGAATGT	265
	13	AATGGAAAGCTTCTCAAAGTA ATGTTGGAGCTAGGTCCTTAC	320
	14	CTAACCTGAATTATCACTATCA GTGTATAAATGCCTGTATGCA	312
	15	TGGCTGCCCAGGAAGTATG AACCAGAATATCTTTATGTAGGA	338
	16	AATTCTTAACAGAGACCAGAAC AAAACTCTTTCCAGAATGTTGT	449
	17	GTGTAGAACGTGCAGGATTG TCGCCTCATGTGGTTTTA	263
	18	GGCTCTTTAGCTTCTTAGGAC GAGACCATTTTCCCAGCATC	351
	19	CTGTCATTCTTCCTGTGCTC CATTGTTAAGGAAAGTGGTGC	249
	20	ATATGACGTGTCTGCTCCACC AATGAAGCGGCCCATCTC	249
	21	AAGCTCTTCCTTTTTGAAAGTC GTAGAGAAATAGAATAGCCTCT	298
	22	TCCCATTGAGAGGTCTTGCT GAGAAGACTTCTGAGGCTAC	297
	23	CAGAGCAAGACCCTGTCTC ACTGTGCTACTCAAGCACCA	255
	24	ATGAATTGACACTAATCTCTGC GTAGCCAGGACAGTAGAAGGA	280
TP53	5.6	CGCTAGTGGGTTGCAGGA CACTGACAACCACCCTTAAC	550
	8.9	GTTGGGAGTAGATGGAGCCT GGCATTTTGAGTGTTAGACTG	455
PTEN	4	CTGTATTAGTGGCATCACAAGTTT TGCACTTTAGTCTTCCTGACAA	527
	5	CCACAGTTGCACAATATCCTTT CCAATAAATTCTCAGATCCAGGAAG	301
CDH1	9	GACACATCTCTTTGCTCTGC GGGACAAGGGTATGAACAGC	269
	12	GTCTGGTGGAAGGCAATGG GAAGCATGGCAGTTGGAGC	345
CHEK2	10	TGTCAACTGTTTGCTTGTCTTAATG GCCAAGAAGAGAACAGCAAAC	341
	12	CTTGGACTGGCAGACTATGTT ATGGTGGTGTGCATCTGTAG	432
XRCC2	2	CAGCACCCAGCCTAAAGTTAT AAGACAGAGGTCAAGGCATATT	426
	3	CAGCAGTCTACTCTGAGGAAATG TGCAGTGAGCCATGATTGT	457

RESULTS

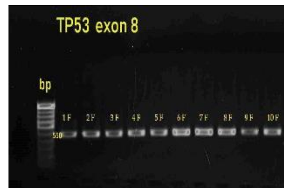
Cases were diagnosed across a range of ages, from 26 to 85 years, with a mean age of 48.14

years. Supplementary fig 1 provides the isolated genomic DNA and PCR-amplified products of the selected exons. Eight polymorphisms, four synonymous and four non-synonymous, were found in exons 11, 13, & 15 of the BRCA1 gene. Notably, among these, it was discovered that exon 15 of the BRCA1 gene's g.95900A>T: c.4772A>T: p.P1544P was novel. These polymorphisms were found exclusively in cases with a family history of breast cancer.

A: Genomic DNA isolated from the samples.

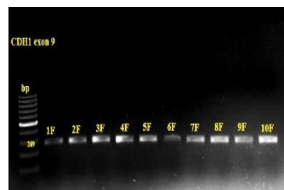


B: BRCA1 amplified products



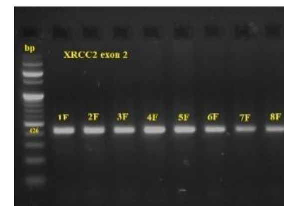
C: TP53 amplified products

D: PTEN amplified products



E: CDH1 amplified products

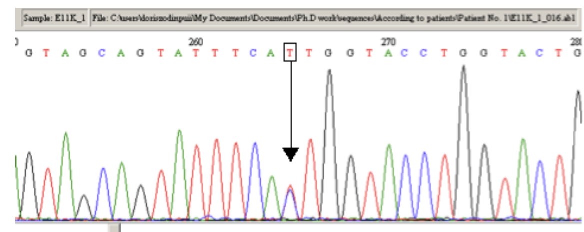
F: CHEK2 amplified products



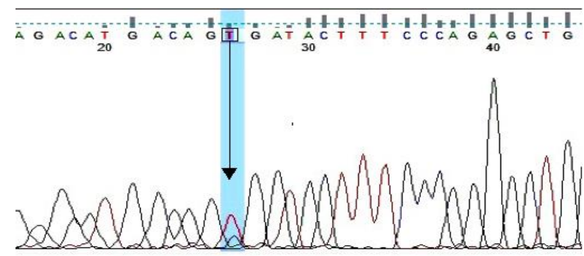
G: XRCC2 amplified products

Supplementary Fig.1: Representative Gel Images for the isolated genomic DNA and the gene amplifications. (1F - represents the first familial cancer sample, and so on)

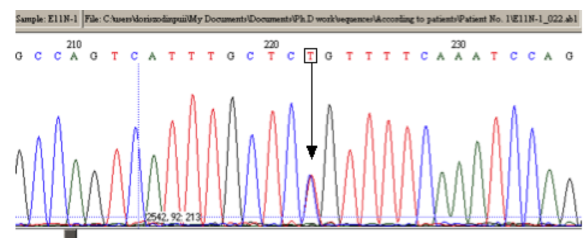
No significant amino acid changes in the BRCA1 gene were observed, as all polymorphisms were silent substitutions. The chromatogram files for each polymorphism are presented in Supplementary Figure 2., and Table 1 lists the polymorphisms in detail along with their estimated scores. Exon 15 of the BRCA1 gene revealed two polymorphisms, p.P1544P and p.D1546Y. Among these, a synonymous mutation (p.P1544P) was identified as novel since it has not been documented in any known breast cancer or other existing databases. There were no polymorphisms observed in TP53, PTEN, CDH1, CHEK2 and XRCC2.



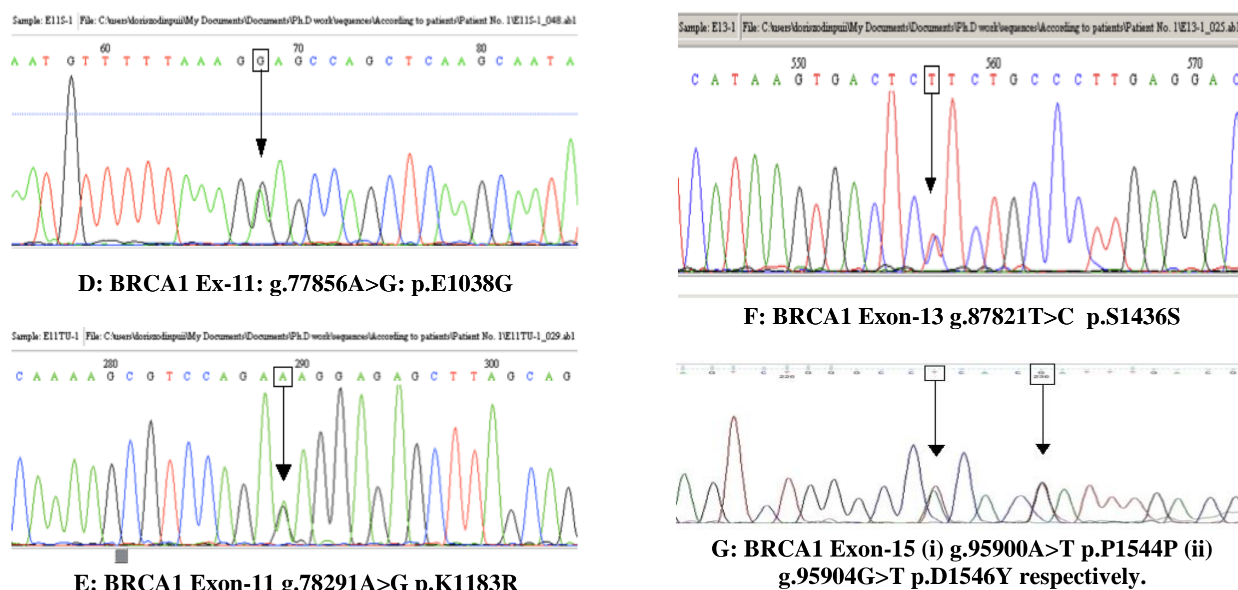
A: BRCA1 Exon -11 g.77054T>C p.L771L



B: BRCA1 Exon 11 g.76825C>T p.S694S



C: BRCA1 Exon-11 g.77355C>T p.P871L



D: BRCA1 Ex-11: g.77856A>G: p.E1038G

E: BRCA1 Exon-11 g.78291A>G p.K1183R

F: BRCA1 Exon-13 g.87821T>C p.S1436S

G: BRCA1 Exon-15 (i) g.95900A>T p.P1544P (ii) g.95904G>T p.D1546Y respectively.

Supplementary Fig 2: Chromatogram showing observed polymorphism in BRCA1 gene.

Table 1: List of variants found in BRCA1 (NM_007294) gene

Exon	Genomic Position	A.A change (HGVS)	rsID	Mutation Taster	Align GVGD Score	Polyphen-2 Score
11	g.77054T>C: c.1423T>C	p.L771L	16940	Polymorphism	-	-
	g.76825C>T: c.1194C>T	p.S694S	1799949		-	-
	g.77355C>T: c.1724C>T	p.P871L	799917		C0	Benign 0
	g.77856A>G: c.2225A>G	p.E1038G	16941		C0	Possibly damaging 0.936
	g.78291A>G: c.2660A>G	p.K1183R	16942		C0	Benign 0
13	g.87821T>C: c.3420T>C	p.S1436S	1060915		-	-
15	g.95900A>T: c.3744A>T	p.P1544P	**Novel (unreported)**		-	-
	g.95904G>T: c.3748G>T	p.D1546Y	28897691		C0	Probably damaging 0.979

DISCUSSION

In this study, the hotspot regions of commonly mutated genes in breast cancer were sequenced from the blood DNA of both familial and non-familial cases from the Mizo ethnic tribe of Northeast India.

The 7 polymorphisms of BRCA1 gene that were observed in this study have been reported by several studies in other populations in databases such as BIC BRCA, ARUP BRCA, dbSNP, and ClinVar (9). In addition, numerous studies have identified p.L771L, p.S694S, p.P871L, p.E1038G, and p.K1183R from exon 11 and p.S1436S from exon 13 of the BRCA1 gene as polymorphisms or variants of unknown importance with minor allele frequency (MAF) more than 25% (16, 17, 18). Age at diagnosis was found to be correlated with p.S694S (20). Most of the observed polymorphisms are in substantial linkage disequilibrium (LD) and are inherited as components of a shared haplotype. Other Indian populations have also reported p.L771L, p.S1436S, p.P871L, p.E1038G, and p.K1183R; among these, p.P871L, p.E1038G, and p.K1183R are mentioned on the top 20 BIC entries.

Studies have suggested that the absence of the BRCA1 p.K1183R polymorphism may increase the risk of breast cancer, implying a potential protective effect of this variant (17, 9). However, in our study, despite the presence of the p.K1183R polymorphism in cases, cancer

occurrence could be influenced by the delicate balance between DNA damage and repair mechanisms, which ultimately determine individual susceptibility to breast cancer. If not fully repaired, environmental factors such as chemical exposure and radiation can disrupt genome stability and contribute to carcinogenesis (20).

A meta-analysis encompassing 24 studies, with 13,762 cases and 22,388 controls, found no link between the BRCA1 p.P871L variant and breast cancer risk. This variant causes a substitution of proline with leucine at position 871, which is within the interaction region for RAD51, a crucial protein involved in homologous recombination and cancer susceptibility. However, Miao et al. (2017) reported a significant association between this variant and a decreased risk of overall cancer, whether in a homozygous or heterozygous state (20).

There have been inconsistent findings regarding the association of the p.E1038G variant with sporadic breast cancer risk. A study by Ricks-Santi et al. (2013) genotyped 1,005 cases and 1,765 controls, finding no independent link between p.E1038G genotypes and breast cancer risk in either pre- or postmenopausal women. However, an interaction was noted among pre-menopausal breast cancer cases with the p.E1038G polymorphism, where smokers with the TT genotype were at a higher risk (19).

The p.D1546Y variant has been documented exclusively in databases for the European (Non-Finnish) population, exhibiting a very low minor allele frequency (MAF > 0.00003295). It is documented only by genetics clinics in databases such as BIC BRCA, ExAC, and ClinVar.

In our study, neither the cases nor the controls exhibited any mutations in the non-BRCA genes analyzed. The CHECK2 c.1100delC variant, a common founder pathogenic variant with a low penetrance susceptibility allele, has been reported in 0.2% to 0.6% of patients with Northern and Eastern European descent (21). However, our findings of no polymorphisms in CHECK2 are consistent with previous studies in other Asian populations (22), Colombian (23), and in individuals of Spanish ethnicity (24). The absence of CHEK2 variants in a study highlights that CHEK2 mutations are rare events suggesting a no correlation between the germline mutations and breast cancer risk in Rwanda (25).

The prevalence of germline TP53 mutations across different types of breast cancer remains largely unknown. In a large cohort study of unselected breast cancer patients, only 0.5% were found to carry pathogenic germline TP53 mutations (26). However, in cases of very early onset breast cancer, the prevalence of germline TP53 mutations may range from 3% to 8% (27). Given that TP53 germline mutations are rare and associated with poorer survival and earlier onset, our study, with a mean age of cases at 48.14 years, may have been insufficiently sensitive to detect TP53 germline variants. An absence of p53 mutations predicts prolonged disease-free (DFS) and overall survival (OS) following initial therapy in breast cancer research (28). The absence of germline mutations in our study as well as the results of the previous study indicates that *XRCC2* and *PTEN* are not the major mutated breast cancer genes (29, 30).

Our study suggests that there may be variations in the type and frequency of gene polymorphisms across different ethnic groups, highlighting the importance of replicating results in diverse populations and developing population-specific gene panels. Although our exon selection was based on codons where breast cancer-related mutations are frequently reported, the relatively small sample size in our study may have contributed to the absence of observed variants, both known and novel. While the polymorphisms observed in the BRCA1 gene in our study do not directly contribute significantly to breast cancer development in the populations studied, they provide insights into their presence and uniqueness compared to other populations worldwide. This underscores the need for further research to understand the coexistence or distinctiveness of these variants in different ethnic and geographic contexts.

To our knowledge, this study represents the first comprehensive analysis of the complete BRCA1 coding region and other susceptibility genes through direct sequencing in the Mizo

population. Our findings also indicate the absence of tobacco-related mutations in blood cells. While we identified eight non-pathogenic polymorphisms in the BRCA1 gene, including one novel variant, we believe further investigation using tumor samples is warranted to establish the frequency, penetrance, and significance of these candidate gene biomarkers in breast cancer progression.

CONCLUSION

To our knowledge, the present study is the first time to analyze the entire coding regions of BRCA1 and other breast cancer susceptibility genes using direct sequencing in the Mizo population. The outcome of this study also reflects the non-prevalence of tobacco-related mutations in the blood cells. Although we observed only eight non-pathogenic polymorphisms in the BRCA1 gene of which one is a novel variant, we believe that this calls for the need to perform with tumor samples to establish the frequency, penetrance, and significance of the candidate gene biomarkers for their contribution towards breast cancer progression.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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