

Gene Panel Testing in Hereditary Breast Cancer

Parvin Rostami, PhD¹; Kazem Zendehdel, MD, PhD^{2,3,4}; Reza Shirkoohi MD, PhD^{3,4}; Elmira Ebrahimi, MSc⁴; Mitra Ataei, MSc¹; Hashem Imanian, MSc⁵; Hossein Najmabadi, PhD^{5,6}; Mohammad Reza Akbari, MD, PhD^{7,8,9}; Mohammad Hossein Sanati, PhD^{1*}

¹National Research Center for Genetic Engineering and Biotechnology, Tehran, Iran

²Breast Disease Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran

³Cancer Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran

⁴Cancer Biology Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran

⁵Kariminejad-Najmabadi Pathology and Genetics Center, Tehran, Iran

⁶Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran

⁷Women's College Research Institute, Women's College Hospital, University of Toronto, Toronto, Ontario, Canada

⁸Institute of Medical Science, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

⁹Dalla Lana School of Public Health, University of Toronto, Toronto, Ontario, Canada

Abstract

Background: Breast cancer (BC) is a highly complex, heterogeneous and multifactorial disease and is the most commonly diagnosed cancer and the leading cause of cancer-related mortality in women worldwide. Family history and genetic mutations are important risk factors for BC. While studies in twins have estimated that about 10%–30% of BC are due to hereditary factors, only 4%–5% of them are due to mutations in BRCA1 or BRCA2 genes. Our aim was to investigate the role of other BC genes in familial BC among the Iranian population.

Methods: We selected 61 BC patients who were wild-type for BRCA1 and BRCA2 mutations but who met the criteria for hereditary BC based on the American College of Medical Genetics and Genomics (ACMG) and the National Comprehensive Cancer Network (NCCN) guidelines. We performed targeted sequencing covering the exons of 130 known cancer susceptibility genes based on the Cancer Gene Census list.

Results: We found seven mutations in seven known BC susceptibility genes (RAD50, PTEN, TP53, POLH, DKK1, WRN and CHEK2) in seven patients including two pathogenic frameshift variants in RAD50 and WRN genes, four pathogenic missense variants in TP53, PTEN, POLH, and DKK1 genes and a pathogenic splice donor variant in the CHEK2 gene. The presence of all these variants was confirmed by Sanger sequencing and Gap reverse transcription-polymerase chain reaction (RT-PCR) for the splice variant. *In silico* analysis of all of these variants predicted them to be pathogenic.

Conclusion: Panel testing of BC patients who met the established criteria for hereditary BC but who were negative for BRCA1/2 mutations provided additional relevant clinical information for approximately 11.5% of the families. Our findings indicate that next generation sequencing (NGS) is a powerful tool to investigative putative mutagenic variants among patients who meet the criteria for hereditary BC, but with negative results on BRCA1/2 testing.

Keywords: Breast neoplasms, Iran, Mutation, Next generation sequencing

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Introduction

Breast cancer (BC, MIM#114480) is the most common malignancy among females and is second only to lung cancer as the leading cause of cancer-related mortality in women worldwide. It accounts for nearly a third of all new cancers in women, and in women under 50 years of age, BC is the leading cause of mortality from cancer.¹ The prevalence of BC in the United States has increased over the past few decades, but mortality from BC appears to be declining,² which seems to be the result of early diagnosis and more effective treatment. The incidence of BC varies by up to a factor of 10 between different countries and is very low in Eastern and Southeast Asia. For example, in Japan, the incidence of BC is about one-fifth of the United States. However, these differences are becoming gradually

smaller with the increasing incidence of BC in Asia. In a study in 2003 conducted in Iran, death rate due to BC in 29 provinces was 2.7 per one hundred thousand females.³ The results of a more recent study showed that the death rate caused by BC had an increasing trend in Iran and the mortality rate of 3.93 per hundred thousand people in 2006 had increased to 4.92 per hundred thousand people in 2010.⁴ The difference in BC rates may have different factors, including hormonal exposure, reproduction, lifestyle and genetic factors. An important reason for low incidence could be lack of early detection services.^{5,6}

The etiology of most BC cases is unknown. However, there are many risk factors for this disease, including gender, age, family history of BC at an early age, premature menarche, late menopause, higher age at first

*Corresponding Author: Mohammad Hossein Sanati, PhD; National Institute of Genetic Engineering and Biotechnology Shahrokh-e Pajohesh, km 15, Tehran – Karaj Highway, Tehran, Iran, PO Box: 14965/161. Tel: +98-21-44787393; Fax: +98-21-44787395; Email: m-sanati@nigeb.ac.ir

live delivery, prolonged hormone replacement therapy, previous exposure, chest wall radiation therapy, benign proliferative breast disease, and genetic mutations in genes such as BRCA1/2. The most important is family history. Individual risk increases with an increase in the number of relatives with BC and a decrease in the age at diagnosis. While studies in twins have estimated that about 10%–30% of BC are due to hereditary factors, only about 5%–10% of BC patients have a strong mutation in one of the known BC susceptibility genes, of which only 4%–5% are due to mutations in highly intrusive penetrant genes in the autosomal dominant state.⁷ The most important known genes with high penetrance in BC are BRCA1 (MIM#113705) and BRCA2 (MIM#600185), in which the mutation frequency rates are different due to the founder effect throughout the population. Studies have shown that pathogenic variants in these two genes are associated with a 41% to 90% risk of BC. However, more than 50% of individuals with a pathogenic variant who meet the National Comprehensive Cancer Network (NCCN) testing criteria for hereditary breast and ovarian cancer carry mutations in other genes.⁸ A greater extent of BC is produced by a number of medium penetration genes. Germline variants in other susceptibility genes also confer a high risk of BC, including pathogenic variants in ATM (MIM#607585), BARD1 (MIM#601593), BLM (MIM#210900), BRIP1 (MIM# 605882), CDH1 (MIM#192090), CHEK2 (MIM#604373), NBN (MIM#602667), PALB2 (MIM#610355), RAD50 (MIM#604040), PMS2 (MIM#600259), FAM175A (MIM#611143), FANCC/-M (MIM#613899/609644), and RAD51B/-C/D (MIMs#602948/602774/602954) also the more syndromic predisposing genes: TP53 (MIM#113721) (Li–Fraumeni syndrome; MIM#151623), PTEN (MIM#601728) (Cowden's disease; MIM#158350), STK11 (MIM#602216) (Peutz–Jeghers syndrome; MIM#175200), NF1 (MIM#613113) (Neurofibromatosis; MIM#162200), and CDH1 (MIM#192090) (Hereditary diffuse gastric cancer syndrome; MIM#137215).

Until recently, clinical genetic testing has been largely limited to the BRCA1 and BRCA2 genes. Identification of pathogenic BRCA1 and BRCA2 variants is routinely used to predict breast and ovarian cancer risk and guide the use of risk reducing surgery.⁹ Genetic testing of BRCA1 and BRCA2 genes for most women with familial BC is uninformative, because these two genes are not the only causes of BC.¹⁰ Nowadays, large panels of cancer susceptibility genes are available.¹¹ It is often argued that targeted sequencing is preferable if the suspected disease or condition has already been identified, due to higher coverage yield and affordable costs as well as reduced sequencing time.¹²

The current commercial panels include the BC susceptibility genes (in addition to BRCA1 and BRCA2),

and the well-characterized BC predisposition genes (TP53, PALB2, ATM, CHEK2, CDH1, PTEN, STK11 and one recurring mutation in NBN),¹³ to genes where there is only limited evidence that mutations confer an elevated BC risk (e.g., BARD1, BRIP1, MRE11 (MIM#600814), RAD50, RAD51C, RAD51D, CDKN2A (MIM#600160), and XRCC2 (MIM#600375)).¹⁴

Gene panel tests have not yet been implemented in routine oncology practice in Iran. Therefore, we aimed to study the implementation of BC gene panel tests in Iran.

Materials and Methods

Study Population

In this study, we selected 61 individuals (59 women and 2 men) with BC who were referred to the Cancer Research Center of the Cancer Institute of Iran, Tehran University of Medical Sciences, Iran, during the period 2012–2016. Three control patients with mutations in BRAC1 or BRCA2 genes were also selected. The research was performed under the protocol approved by the National Research Center for Genetic Engineering and Biotechnology and all patients consented to the use of their genomic and clinical data for research purposes. The selection of these patients was based on the American College of Medical Genetics and Genomics (ACMG)¹⁵ and the NCCN¹⁶ guidelines. Clinical information and family history were collected from test requisition forms, and pedigrees provided at the time of testing. All the individuals were checked for any mutation in BRCA1 and BRCA2 genes and apart from the three individuals who acted as controls, the others were wild-type for both genes. In all first- and second-degree family members of the patients, there was at least one patient diagnosed with BC and/or another hereditary cancer before 50 years of age. The three control patients, with mutations in BRAC1 or BRCA2, were used to test the sensitivity of next generation sequencing (NGS) and the BRAC1/2 mutations were successfully re-identified in all of them. The remaining 61 BC patients who were BRCA1/2 mutation-negative were tested to detect mutations in other cancer susceptibility genes.

DNA/RNA Extraction

Genomic DNA was extracted from whole blood of the patients using the QIAamp DNA Mini Kit (ref. 51304) (Qiagen, Hilden, Germany). RNA extraction was performed on whole blood from one patient using the QIAamp RNA Mini Kit (ref. 52304) (Qiagen).

Targeted Sequencing and Variant Analysis

We carried out targeted sequencing covering the coding exons of 130 genes (Table 1). About 3 µg of DNA of our patients were fragmented by ultrasound and prepared for sequencing. Targets were captured using the Agilent SureSelect Human Exome kit (v.4) according to the

Table 1. The 130 Genes Incorporated in the Targeted Sequencing Gene Panel

Gene Names					
ABCB11	CHEK2	FANCF	MSH2	RAD50	SMARCE1
ALK	COL7A1	FANCG	MSH6	RAD51C	SOS1
ALPK2	CYLD	FAS	MTAP	RAD51D	STAT3
APC	DDB2	FH	MUTYH	RB1	STK11
ATM	DICER1	FLCN	NBN	RBM15	SUFU
AXIN1	DIS3L2	GATA2	NF1	RECQL4	TERT
AXIN2	DKC1	GBA	NF2	RET	TGFB1R
BAP1	DOCK8	GJB2	PALB2	RHBDF2	TMEM127
BARD1	EGFR	GPC3	PDGFRA	RUNX1	TP53
BLM	ELANE	HFE	PHOX2B	SBDS	TRIM37
BMPR1A	EPCAM	HMBS	PMS1	SDHA	TSC1
BRCA1	ERCC2	HNF1A	PMS2	SDHAF2	TSC2
BRCA2	ERCC3	HRAS	POLD1	SDHB	TSHZ
BRIP1	ERCC4	ITK	POLE	SDHC	UROD
BUB1B	ERCC5	KCNJ5	POLH	SDHD	VHL
CBL	EXT1	KIT	PPM1D	SERPINA1	WAS
CDC73	EXT2	LMO1	PRF1	SETBP1	WRN
CDH1	FAH	MAX	PRKAR1A	SH2D1A	WT1
CDK4	FANCA	MEN1	PRSS1	SLC25A13	XPA
CDKN1B	FANCC	MET	PTCH1	SMAD4	XPC
CDKN2A	FANCD2	MLH1	PTEN	SMARCA4	
CEBPA	FANCE	MPL	PTPN11	SMARCB1	

manufacturer's instructions (Agilent Technologies, Inc., Santa Clara, CA, USA) and sequenced by 100 bp paired-end reads on an Illumina HiSeq2000 flow-cell platform (Illumina Inc., San Diego, CA, USA). High-quality sequencing data with an average depth of coverage of 265x (range: 91–471x) was obtained. An average of 99.0% (range: 96.1–99.4%) of the targeted regions were covered at 20x or greater depth of coverage. Sequences were aligned to the human reference genome (GRCh37) using the Burrows-Wheeler transform algorithm (<http://picard.sourceforge.net>). Single-nucleotide variants and small indels were called using the Genome Analysis Toolkit (GATK). Analysis was performed on all 130 known cancer susceptibility genes for which the coverage was 97%. The results were compared with the reference data network and a large number of variants were obtained from each patient. ANNOVAR (<http://annovar.openbioinformatics.org>) was used to annotate the functional subsequences of variants, and to retrieve allele alternation from public databases (dbSNP138, 1000 Genomes Project, and NHLBI-ESP 6500 exomes). The frequencies of the detected variants were checked in public databases 1000 Genomes Project, ESP, and ExAC data sites and others. Variants with a minor allele frequency more than 1% were filtered out. For the remaining variants, we focused initially on truncating variants based on CADD score and variants with a

PHRED score of over 20 were selected. We also predicted the functional effect of missense variants using the following tools: SIFT, PolyPhen 2 HVAR, MutationTaster_Pred, MutationAssessor, FATHMM_Pred, FATHMM_MKL coding. Any nonsynonymous variants that were described as damaging or probably damaging at least by four of these algorithms were regarded as putatively deleterious.

Sanger Sequencing

All detected pathogenic or likely pathogenic variants were confirmed by Sanger sequencing on an Applied Biosystems 3130 Sequencer (Applied Biosystems, Foster City, CA, USA). Specific primers with Primer3 (<http://frodo.wi.mit.edu/primer3/>) were designed to capture each detected mutation. The sequence reads were analyzed by CodonCode Aligner software.

RT-PCR Analysis

Total RNA was extracted from fresh whole blood using the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) and the subsequent cDNA synthesis was performed using M-MLV reverse transcriptase enzyme (Thermo Scientific – EP044). To confirm the effect of potential splicing mutations on the expressed mRNA, the variant located in splice sites was assessed by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers designed to amplify the specified regions on cDNA. Amplified RT-PCR products were analyzed on 2% agarose gel.

Results

In this study, 130 genes that are known to play a role in cancer were investigated in an NGS-targeted panel in 61 Iranian patients with BC and three control patients with known mutations in BRCA1/2. Patient and specimen characteristics are shown in Table 2. All of these patients had been tested previously for mutations in the BRCA1 and BRCA2 genes by targeted NGS and were wild-type. In addition, BRCA1 and BRCA2 genes were included in our panel, to estimate the quality and accuracy of previous NGS results. The targeted NGS tests were performed and the results were analyzed. We found seven putative deleterious mutations including one splice site variant, two protein-truncating variants and four nonsynonymous missense variants, in the known BC susceptibility genes, in seven index cases: four missense variants in POLH (exon 11), PTEN (exon 8), TP53 (exon 7), and DKC1 (exon 9); two frameshift mutations in RAD50 (exon 19) and WRN (exon 19), and a splice site mutation in CHEK2 gene (intron 9) (Table 3). The splice site variant was checked by RT-PCR and was confirmed.

Discussion

We tested 130 known and putative BC susceptibility genes in 61 patients without BRCA1/2 mutations but bearing a

Table 2. Patient and Specimen Characteristics

Variables	Description
Age	
Median age (y)	45 (25-81)
Hormone-receptor, No. (%)	
HR+ and HER2-	27 (-44.3)
HR+ and HER2+	8 (13.1)
HR+ and HER2 unknown	1 (1.7)
HR- and HER2+	7 (11.5)
HR- and HER2-	8 (13.1)
Both unknown	10 (16.4)
Histopathology of breast cancer, No. (%)	
Ductal	48 (78.7)
Lobular	4 (6.6)
Mixed	0 (0)
Other/Unknown	9 (14.7)

HR, hormone receptor; HER2, human epidermal growth factor receptor 2.

strong family history of BC (in addition to three controls who carried the BRCA1 or BRCA2 mutations), which, to our best knowledge, makes it one of the largest studies of these genes in Iranian BC families to date. We found seven variants in seven genes, each in a single BC gene. From these genes, the mutations in RAD50, TP53, CHEK2, and WRN conferred significant risks of BC, and mutations in three genes, PTEN, POLH, and DKK1 were probably pathogenic by *in silico* analysis. Three out of these seven are well-characterized BC predisposition genes (PTEN, TP53, and CHEK2), and the remaining four (POLH, DKK1, RAD50, and WRN) are genes for which there is limited evidence that their mutations may increase BC risk and they are mainly known as susceptibility genes. Next, we shall discuss the seven variants we found in patients with familial BC.

TP53 Gene

The missense variant which was observed in the TP53 gene was seen in a male patient who was diagnosed with BC at age 50. TP53 (located on 17p13.1) encodes a transcription factor that regulates several intracellular

pathways involved in cell survival and programmed cell death. TP53 gene is mutated in many human cancers other than breast and ovarian cancer, such as bladder cancer, cholangiocarcinoma, head and neck squamous cell carcinoma, lung cancer, melanoma, and Wilms' tumor. This gene contains 11 exons, 2 transcriptional start sites in exon 1, and alternative splicing occurs in intron 2 and between exons 9 and 10.¹⁷ Most of the mutations reported in this gene are missense.¹⁸ We found the c.725G>A (p.Cys242Tyr) mutation in exon 7 of the TP53 gene. This mutation has been previously reported by Metzger et al in a patient with Li-Fraumeni syndrome.¹⁹ C242Y is located in a highly protected area, which is a DNA binding domain, and C242 is one of the Zn²⁺-binding residues. The mutation in the DNA binding domain reduces the affinity of the TP53 molecule for DNA, but its structure remains intact.¹⁹ It has been noted that loss of function mutations in TP53 are exhibited in only about half of cancers, and ARC (apoptosis repressor with caspase recruitment domain; MIM#605235) inactivate the apoptotic function of wild-type TP53 by binding to it in the nucleus of human cancer cell lines. Approximately all BCs with mutant TP53 lack nuclear ARC, and nuclear ARC is induced in cancer cells and negatively regulates TP53POLH gene.

Another likely pathogenic variant was c.1603A>G (p.Lys535Glu) missense mutation in exon 11 of the POLH gene, which replaced the lysine 535 with glutamic acid. This variation was found in a 35-year-old woman who had an uncle with BC diagnosed at 43 (Figure 1). POLH (DNA Polymerase eta; located on 6p21.1) contains 11 exons, and is a member of the family of damage-bypass replication proteins, which have seven conserved domains. It has been shown that POLH copied damaged DNA with less accuracy dependent on the pattern-template DNA polymerase. POLH does not have proof reading activity and, depending on the inconsistency, has a basic replacement error of about 18 to 380 nucleotides. Therefore, the POLH function should be strictly controlled to prevent the correct synthesis of DNA.^{20,21} The c.1603A>G mutation (K535E) found in our patient was previously reported by Itoh et al in a Japanese patient

Table 3. Identified Variants in 7 out of 61 Familial Breast Cancer Patients

Patients	Coordination	Gene	Exon	Type of Variant	DNA Alteration	cDNA Alteration
Patient 1	Chr5:131945032	RAD50	19	Frame shift	NM_005732.3:c.2980_2983delAAAG	NP_005723.2:p.Glu995fs
Patient 2	Chr8:30969160	WRN	19	Frame shift	NM_000553.4:c.2118_2119delAA	NP_000544.2:p.Ser707fs
Patient 3	Chr6:43581755	POLH	11	Missense	NM_006502.2:c.1603A>G	NP_006493.1:p.lys535Glu
Patient 4	Chr10:89720670	PTEN	8	Missense	NM_000314.4:c.821G>T	NP_000305.3:p.Trp274Leu
Patient 5	Chr17:7577556	TP53	7	Missense	NM_000546.5:c.725G>A	NP_000537.3:p.Cys242Tyr
Patient 6	ChrX:153997508	DKC1	9	Missense	NM_001363.3:c.838A>C	NP_001354.1:p.Ser280Arg
Patient 7	Chr22:29099492	CHEK2	Intron 9	Splice donor	NM_001005735.1:c.1037+1G>T	—

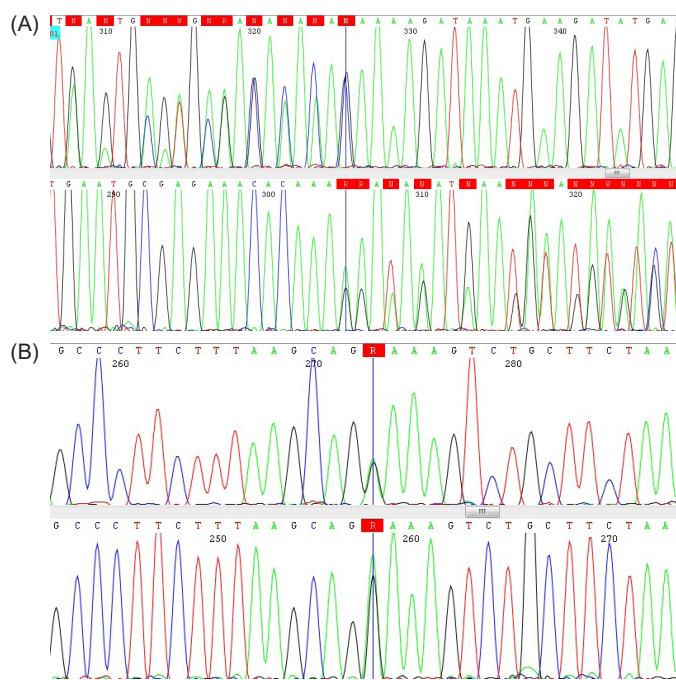


Figure 1. Sanger Sequencing Analysis for RAD50 and POLH Genes by Using the CodonCode Aligner Software. (A) Deletion of four base pairs AAAG (c.2980_2983delAAAAG, p.Glu995fs) in exon 19 of the RAD50 gene, in a family with a history of breast and hepatic cancer and autism spectrum disorder. (B) Missense mutation (c.1603A>G, p.Lys535Glu) in exon 11 of the POLH gene, found in a 35-year-old woman who had an uncle with breast cancer diagnosed at the age of 43.

with xeroderma pigmentosum (MIM#278750).²² Lysine 535 is conserved in human and mouse proteins, and its replacement with glutamic acid is effective in DNA polymerase function and configuration.

PTEN Gene

A likely pathogenic mutation c.821G>T (p.Trp274Leu) in exon 8 of the PTEN gene was found in a female patient with BC diagnosed at age 28 who had two aunts with BC diagnosed at 33 and 47 years of age. In addition, the family had a history of gastric and prostate cancers (Figure 2). In this mutation, the guanine 821 was replaced by thymine, resulting in tryptophan 274 being substituted by leucine. PTEN (located on 10q23.31) is mutated in many human cancers and diseases including Bannayan–Riley–Ruvalcaba syndrome, Cowden syndrome, autism spectrum disorder, head and neck squamous cell carcinoma, lung cancer (MIM#211980), prostate cancer (MIM#176807), etc. PTEN is a tumor suppressor gene that regulates the PI3K signaling pathway through its lipid phosphatase activity and the MAPK pathway through its protein phosphatase activity.²³ Mutations in the PTEN gene are observed in brain, breast, and prostate tumors.²⁴ In total, BC is seen in about 30% of people with Cowden syndrome. In 80% of cases with Cowden syndrome, a PTEN mutation is seen, and somatic mutations in this gene have been identified in sporadic BC^{25,26}; therefore, the PTEN gene is considered to be a potential BC susceptibility gene. Nevertheless, reduced PTEN expression has been detected in 30% to

50% of BC patients, which is associated with poor clinical outcome. Approximately 50% of patients with BC harbor a mutation in PTEN or have lost at least one copy of PTEN.²⁷

The results of other studies have indicated that in the sporadic BC cases, mutations in the PTEN gene are less often observed and PTEN promoter methylation may have the main role in the decreased expression of PTEN. Considering the results of other studies, PTEN is important for the tumorigenesis, development and prognosis of BC.^{28,29} The PTEN 186-351 amino acids are located in the C2 domain of the gene, which lacks Ca²⁺-removal residual and connects to phospholipid membranes independent of calcium. The domains of phosphatase and C2 together form the PTEN catalytic zone.²⁴ PTEN covalent changes in Lys266 in the C2 region increase PTEN binding to the plasma membrane, inhibiting the transfection and progression of the tumor.²⁴ In general, it has been shown that both phosphatase and C2 regions are necessary for tumor suppressive activity.³⁰ Previous studies have shown that the p.R173H mutation disrupts inter-domain hydrogen bonding with W274, causing a side chain near the residue V262³⁰; therefore, it seems that the change we see in our patient (p.Trp274Leu) can break the structure and function of the protein and cause the disease.

DKC1 Gene

The other probably pathogenic variant found in the present study was the replacement of amino acid serine

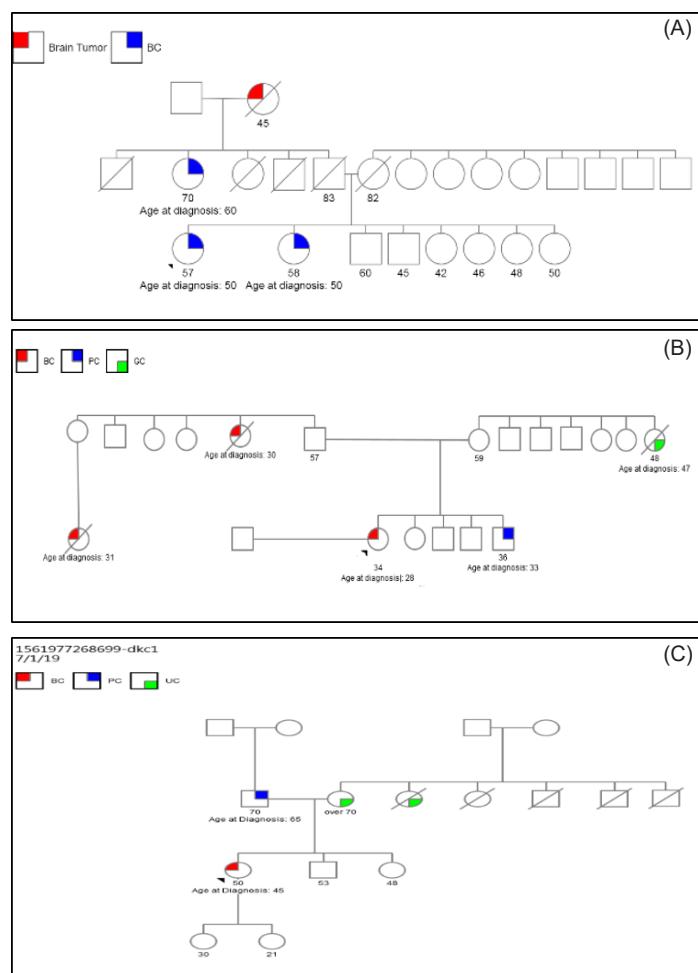


Figure 2. Pedigrees of the Families with Mutations in (A) CHEK2 Gene (c.1037+1G>T) in a Family with a History of Breast and Brain Cancer, (B) PTEN (c.821G>T, p.Trp274Leu) in Exon 8 in a Family with a History of Gastric and Prostate Cancer, and, (C) DKK1 (c.838A>C, p.Ser280Arg) in Exon 9 in a Family with a History of Ovarian and Pancreatic Cancer. BC, Breast cancer; GC, gastric cancer; PC, pancreatic cancer; UC, ovarian cancer.

with arginine in residue 280 of the DKK1 gene (located on Xq28), which is due to the substitution of cytosine instead of adenine at the 838 nucleotide position in exon 9 of the gene (c.838A>C, p.Ser280Arg). We identified this variant in a woman whose cancer was diagnosed at the age of 45. In her family, her mother had ovarian cancer diagnosed at 47 years of age and her father had pancreatic cancer (MIM#613347) (Figure 2). Up to now, there has been no report of DKK1 gene mutations in BC patients and variants in this gene have been only reported for dyskeratosis congenital (MIM#305000) characterized by skin pigmentation, oral leukoplakia, nail dystrophy, different cancers, pulmonary fibrosis as well as aplastic anemia. However, our patient had no clinical characterization of dyskeratosis congenital or any other features of this disease. The DKK1 gene comprises 15 exons spanning at least 16 kb and provides instructions for making a protein called dyskerin, which is involved in maintaining telomere structure. Dyskerin is a nuclear protein that is formed in small particles of nuclear ribonucleoprotein that alter the changes of certain uridine residues from the ribosomal RNA by converting them to

pseudouridine.³¹

RAD50 and WRN Genes

In addition, in our study, two variants of nucleotide elimination were found, both of which altered the reading frame, supporting the total loss of function of RAD50 and WRN genes in these patients. The first was the removal of AAAG (c.2980_2983delAAAG, p.Glu995fs), found in exon 19 of the RAD50 gene in a 38-year-old female patient whose father had hepatic cancer and her sister was diagnosed with BC at the age of 43 (Figure 1). The RAD50 gene (located on 5q31.1) encodes a protein that is essential for double-strand DNA repair and comprises 25 exons. Until now, there has been no report of RAD50 gene mutations in BC patients and mutations in RAD50 are the cause of Nijmegen breakage syndrome-like disorder (MIM#613078). However, our patient has no clinical characterization of this disorder. RAD50 is necessary as a component of the multiprotein MRN complex of MRE11, RAD50, and NBS1 (MIM#602667) which plays a central role in double-strand break repair, meiotic recombination, and telomere maintenance. The MRN

complex is probably required for DNA damage signaling via activation of the ATM kinase.³² BRCA1 interacts *in vitro* and *in vivo* with RAD50.

We also found a two-nucleotide deletion in exon 19 of the WRN gene in a 28-year-old patient who had an aunt and a cousin with BC (c.2118_2119delAA, p.Ser707fs); a frame shift mutation resulting in a truncated protein. The mutation in the WRN gene (located on 8p12) is known to cause Werner syndrome (MIM#277700), as well as prostate and other cancers. In this mutation, the 707 serine is converted to phenylalanine and it creates an immature stop codon at position 740. It is known that the WRN protein with its helicase property is involved in the replication, repair, recombination, transcription, and maintenance of telomeres. Friedrich et al showed that most of the pathogenic mutations of this gene are from chains that break down the protein.³³ In addition, the 707 serine is located at the center of the second helicase of this gene, and so this mutation can destroy the function of the protein and cause disease.

CHEK2 Gene

The splice donor site variant c.1037+1G>T was identified in a 57-year-old patient with BC, who had a sister and an aunt diagnosed with BC at the age of 50. Brain tumor was also present in this family (Figure 2). This variant was a cytosine substitute for adenine, which occurred in the first nucleotide of intron 9–10 of the CHEK2 gene. This change led to the removal of two downstream exons. The CHEK2 gene (located on 22q12.1) provides instructions for making protein checkpoint kinase 2 (CHK2), which acts as a tumor suppressor. The CHK2 protein is activated when DNA is damaged or when DNA strands are broken. Previously, CHEK2 gene linearity changes have been shown to increase the risk of developing solid tumors in humans. CHEK2 is mutated in many human cancers and diseases including breast and colorectal cancer (MIM#604373), ovarian cancer (MIM#167000), familial prostate cancer, Li-Fraumeni syndrome, and other cancers. The change we found in our patient has already been identified by Staalesen et al³⁴ in a Norwegian population.

For TP53, and PTEN, detection of a mutation in a family with Li-Fraumeni syndrome or Cowden syndrome, respectively, provides a syndrome-associated mutation where the risk of BC is well established and predictive genetic testing usually proceeds within that family. However, we should note that the TP53 and PTEN mutations we report are only as predictive pathogenicity by *in silico* analysis; functional study would be required to confirm this.

In our study, we found WRN and DKC1 genes to be susceptibility genes for familial BC in Iranian patients.

NGS is a high-throughput, cost-effective method for detection of candidate genes that are responsible for hereditary BC in the Iranian population. Since there

is little research on the genetics of BC in the Iranian population, this technique could be helpful for detection of new susceptibility cancer genes in Iranian patients. Early detection of the underlying cause of the disease and carrier screening can lead to quick treatment and even preventing cancer, and may impose less cost on families to treat the disease.

In summary, we report mutations in seven genes, causing exon skipping and loss of function in Iranian patients with hereditary BC. Targeted NGS analysis can effectively improve the detection rate of deleterious mutated genes of BC. Its application is an important supplement to BC research after whole genome and exome sequencing.

Authors' Contribution

PR designed and performed experiments. KZ and RS as advisors, co-wrote the paper. EE, HI and MA co-wrote the paper. HN supervised the research. MRA as advisor, performed bioinformatic analyses. MHS as corresponding author, supervised the research.

Conflict of Interest Disclosures

The authors declare no competing financial interests.

Ethical Statement

This study has the approval from the National Research Ethics Committee (Code: IRAN.REC.1392.71).

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