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Original Research

Exonic sequencing and MLH3 gene expression analysis of breast cancer patients

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Abstract: Breast cancer is the most common cancer in women worldwide. Detection of breast cancer susceptibility genes is an important issue. Also, MLH3 is a DNA mismatch repair gene and mutation in this gene is harmful in different cancers. This study aimed to use exome sequencing to uncover previously undetected breast cancer-predisposing variants. Also, we investigated the MLH3 gene expression of breast cancer patients which can be a breast cancer susceptibility gene. A total of 80 samples including 40 paired normal and cancer tissue samples were collected at Zheen International Hospital, Erbil, Iraq. Exome sequencing was used to identify mutations. Different in silico tools were used to predict the effect of mutation on the structural features or protein function. Real-time PCR was used for assessing the expression of MLH3 in breast cancer patients. We identified 26 variants in breast cancer patients, 22 inherited variants were found in MLH3, CHECK2, BRCA1, BRCA2, BLM, TP53, MSH6, NBN and PTEN genes and 4 somatic variants were found in PALB2, RAD50 and RBM10 genes. It was found that the expression of the MLH3 gene in tumor samples was significantly down-regulated compared with normal tissues. Statistically, high significance was found. The decreased expression of MLH3 was significant in all ranges of ages and all breast cancer types. Also, the expression of MLH3 decreased significantly in patients with breast cancer grades of II and III. In conclusion, MLH3 can be used as a susceptibility gene especially in grades II and III of breast cancer.

Key words: Breast cancer; Exome sequencing: MLH3; DNA repair.

Introduction

The most common cancer which influences women around the world is breast cancer (1). Many factors such as hormonal or reproductive factors, family history, age, and dietary factors may involve as risk factors for breast cancer (2). Using linkage analysis and positional cloning, the first breast cancer susceptibility genes, breast cancer 1 and 2 (BRCA1 and BRCA2, respectively), were identified. After that, several breast cancer susceptibility genes and alleles have been recognized. Mutations of STK11, PTEN and TP53 have also been associated with a high risk of breast cancer in rare inherited cancer syndromes (3). Moreover, using the candidate gene approach, rare variants in genes such as checkpoint kinase 2 (CHEK2) and partner and localizer of BRCA2 (PALB2), with even higher risk, have been identified (4, 5). Also, a genome-wide association study (GWAS) was used to identify enhancing the number of common low-risk loci (6).

Whole exome sequencing (WES) is a suitable method for identifying causal gene mutations in various diseases (7, 8). This technology has revolutionized genetic studies and made it possible to sequence all nucleotides of the human genome in a relatively short time and low cost. This method is able to identify mutations, whether single-nucleotide mutations or structural arrangements, as well as to study gene expression (9). Genotyping of the identified variants along with exome sequencing is a useful method to identify novel risk alleles in the case-control analysis. It is based on a hypothesis that disease-caused variants are infrequent and mostly located in the protein-coding sites of the genome (10, 11).

It was found that the BRCA1 and BRCA2 genes encoded proteins which are contributed to the repair of DNA breaks, therefore, it was suggested that other DNA repair genes can be considered as susceptibility candidate genes for breast cancer (3). The risk of breast cancer increased because of inappropriate DNA damage response that was due to mutations in the ataxia telangiectasia mutated (ATM) gene (12). Also, mutations in Fanconi anemia (FA) genes involved in DNA repair are associated with an enhanced risk of breast cancer (13).

Loss of mismatch repair (MMR) function predisposes to cancer (14). MLH genes are implicated in maintaining genomic integrity during DNA replication and after meiotic recombination. MLH3 is a defective DNA mismatch repair gene family member that has been investigated in cancers (15, 16). It was shown to have an ability to interact with other MMR genes and repair mismatches in vitro. The Mlh3 gene was first identified as a putative MMR gene in multiple epithelia in 2000. MLH3 is composed of 12 exons and is located on 14q24.3 (14). Initial researches in MLH3 null mice showed that Mlh3 played a limited role in tumor formation (17). It was demonstrated that MLH3 may contribute to carcinogenesis through abnormal interaction with apoptotic pathways (18).

Studies in initial colorectal cancers showed that MLH3 mutation plays a minor role in colorectal cancer tumorigenesis (19). A study investigated 12 variants in the MLH3 gene in families with colorectal cancer. Most

mutations showed decreased penetrance which suggests that MLH3 is a low-risk gene in colorectal cancer (20). Also, it was demonstrated that MLH3 mutations are likely to play a role in a subset of endometrial cancers (18). A study on familial gastric cancer demonstrated that Mlh3 can act as a low-risk gene (21). Another study on familial esophageal cancer showed that Mlh3 acts as a low-risk gene in most families. Mutations of Mlh3 accompanied with other genes can result in an enhanced risk of esophageal tumor (22).

In this study, we used exome sequencing to uncover previously undetected breast cancer-predisposing variants. Also, we investigated the MLH3 gene expression of breast cancer patients which can be a breast cancer susceptibility gene.

Materials and Methods

Sample collection

The samples were collected at Zheen International Hospital, Erbil, Iraq. A total of 80 samples was analyzed. The study included 40 paired normal and cancer tissue the sample that was classified according to the types of breast cancer, age and the clinical characteristics of the patients. The tissue samples of the breast were preserved at an RNA stabilizer (RNALater) (ThermoFisher, USA) until further analysis. Participants were given informed consent, and the study was accepted by the Local Ethics Committee. Approval number: 05.01.2020\16.

Mutation analysis

PureLink™ genomic DNA mini kit (ThermoFisher) was used to isolate DNA from peripheral blood, according to the manufacture's instruction. Twist Human Core Exome Enzymatic Fragmentation (EF) Multiplex Complete kit was used for library construction, and MGIEasy FS DNA Library Prep Kit was performed for the library to be ready for sequencing on the MGI system. The library was sequenced on the (MGI-DNBSEQ-G400, China) instrument generating 150 bp paired-end read with 100X mean target coverage. With FastQC, Raw fastq files were quality controlled. Then reads were aligned to the reference human genome (hg19) using Burrows-Wheeler Aligner (BWA). Variants were identified with Genome Analysis Toolkit (GATK). Integrative Genomic Viewer software (IGV) was used for variants visualization.

In silico analysis

Different in silico tools were used to predict the effect of mutation on the structural features or protein function. Polymorphism Phenotyping (PolyPhen-2) (23) and Sorting Intolerant from Tolerant (SIFT) (24) were used to assess the functional effects of variants. MutationTaster was used for the evaluation of mutation effect on protein function and structure (25). Align GVGD was used to compute a biochemical distance score (26, 27).

RNA extraction and cDNA conversion

The RNA samples were extracted from breast tissue using the extraction kit (Bioneer, ExiPrepTM Tissue Full RNA package, Korea) based on the provided procedure by the manufacture. Biophotometer (Eppendorf, Germany) was used to quantify and qualify the total concentration of RNA. In this study protoScript First Strand cDNA Synthesis Kit (BioLabs, England) was utilized. In thermal cycling processes, cDNA was obtained using Master-cycler pro PCR System (Eppendorf, German). Since the quality and quantity of total RNA for all samples were not equal, variable amounts of total RNA were utilized for every individual sample.

RNA expression and RT-PCR

Real-time PCR was performed using Rotor-Gene 6000 RT PCR Machine (Qiagen GmbH, Hilden, Germany) with RT2 SYBR Green ROX FAST Mastermix (Qiagen GmbH, Hilden, Germany) for MLH3 expression. The set of primers for MLH3 was designed considering exon-exon junction and GAPDH was used as an internal control for normalization (housekeeping gene).

Statistical analysis

Relative quantification RT-PCR was performed in triplicate. Values were obtained as the threshold cycle (Ct) for MLH3 and normalized using the housekeeping gene, respectively. The $2^{-\Delta Ct}$ method was used to calculate relative changes (gene expression with respect to the housekeeping gene or internal control) in MLH3 expressions in tumor and normal samples, separately. According to qRT-PCR results, the formula $2-\Delta\Delta Ct$ was used in the statistical comparison between tissues. This value gave information about the expression level of the related gene in a breast cancer tissue based on the control sample, fold change information was obtained. SPSS 22.0 software (SPSS, Chicago, USA) was used for statistical analysis. For evaluation of study data, Student's t-test was used in the comparison of two groups with normal distribution parameters. Statistical significance was considered at $p < 0.05$.

Results

Mutation result

We identified 26 variants in breast cancer patients, 22 inherited variants were found in MLH3, CHECK2, BRCA1, BRCA2, BLM, TP53, MSH6, NBN and PTEN genes and 4 somatic variants were found in PALB2, RAD50 and RBM10 genes. Variants were presented in Table 1. The results of in silico predictions are also presented.

For predicting the effects and potential significance of variants, many in silico tools have been developed. Polymorphism Phenotyping (PolyPhen), Sorting Intolerant from Tolerant (SIFT) and MutationTaster were applied to investigate functional effects of 26 variants.

The PolyPhen gives predictions to find structural features and sequence alignment changes caused by amino acid substitution. PolyPhen predicts the functional significance of an allele substitution. Variants with scores of 0.0 are predicted to be benign, the score 0.15–0.85 is predicted as possibly damaging, the score more than 0.85 are more confidently considered as damaging. Polyphen predicted benign function for a variant of BRCA2, MSH6, BRCA1, BRCA2, and a variant of CHEK2. Also, it predicted probably damaging function for a variant of CHEK2 (NM_001005735.1:c.1286G>T).

SIFT assesses functional effects of the variants by

Table 1. Mutations identified in breast cancer patients.

Gene	Variant Coordinates	Amino Acid change	Zygosity	In-silico Parameters	Mutation Type	Mutation Classification
BRCA1	NM_007300.3:c.3329dup	p.(Gln1111Alafs*4)	heterozygote	PolyPhen: N/A Align-GVGD:N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Frameshift	Pathogenic (class1)
BLM	NM_000057.2:c.2695C>T	p.(Arg899*)	heterozygote	PolyPhen: N/A Align-GVGD:N/A SIFT: N/A MutationTaster: N/A Conservation_nt: weak Conservation_aa: N/A Disease causing	Nonsense	Pathogenic (class1)
BRCA2	NM_000059.3:c.4936_4939del	p.(Glu1646Glnfs*23)	heterozygote	PolyPhen: N/A Align-GVGD:N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Frameshift	Pathogenic (class1)
BRCA2	NM_000059.3:c.5479A>G	p.(IIe1827Val	heterozygote	PolyPhen: Benign Align-GVGD: C15 SIFT: Tolerated MutationTaster: Polymorphism Conservation_nt: weak Conservation_aa: moderate	Missense	Uncertain significant (class3)
BRCA2	NM_000059.3:c.1055dup	p.(Tyr352fs*)	heterozygote	PolyPhen: N/A Align-GVGD:N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Nonsense	Likely pathogenic (class2)
BRCA2	NM_000059.3:c.6029_6030insA	p.(Phe2011Leufs*7)	heterozygote	PolyPhen: N/A Align-GVGD:N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Frameshift	Likely pathogenic (class 2)
PALB2	NM_024675.3:c.406delA	p.(Ser136fs)				Likely pathogenic Tier 2

RAD50	NM_005732.3:c.2165delA	p.(Lys722fs)				Likely pathogenic Tier 2
RBM10	NM_001204468.1:c.1058C>G	p.(Ser353*)				Likely pathogenic Tier 2
TP53	NM_000546.4:c.1024C>T	p.(Arg342)	heterozygote	PolyPhen. N/A Align-GVGD:N/A SIFT: N/A MutationTaster: N/A Conservation_nt: weak Conservation _aa: N/A	Nonsense	Pathogenic (class 1)
BRCA1	Chr17(GRCh37):g.41243748A>G NM_007300.3:c.3800T>C	p.(Leu1267Ser)	heterozygote	PolyPhen: Benign Align_GVGD:C0 SIFT: Tolerated MutationTaster: polymorphism Conservation_nt: weak Conservation_aa: moderate	Missense	Uncertain significance (class 3)
MSH6	Chr2(GRCh37):g.48033786T>C NM_000179.2:c.3997T>C	p.(Phe1333Leu)	heterozygote	PolyPhen: Benign Align_GVGD:C15 SIFT: deleterious MutationTaster: disease causing Conservation_nt: moderate Conservation_aa: high	Missense	Uncertain significance (class 3)
MLH3	Chr14(GRCh37):g.75485684C>T NM_001040108.1:c.4091-1G>A		Heterozygote	PolyPhen: N/A Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: high 3/3 likely splice effect	Substitution	Uncertain significance (class 3)
NBN	Chr8(GRCh37):g.90994950_90994961del NM_002485.4:c.163_171+3de		heterozygote	PolyPhen. N/A Align-GVGD:N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Splicing	Likely pathogenic (class 2)
BRCA1	Chr17(GRCh37):g.41223097G>A NM_007300.3:c.4897C>T	p. .(Gln1633*)	heterozygote	PolyPhen: N/A Align-GVGD:N/A SIFT: N/A MutationTaster: - Conservation_nt: no Conservation_aa: N/A	Nonsense	Pathogenic (class 1)

BRCA2	NM_000059.3:c.1055dup	p.(Ty r352f s*)	heterozygous	Poly Phen: N/A Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: Conserv ation_aa:	Nonsense	Likely Pathogenic (class 2)
CHEK2	NM_001005735.1:c.1082G>A	p.(Arg361His)	heterozygous	PolyPhen: Benign Align-GVGD: C0 SIFT: - MutationTaster: Disease causing Conservation_nt: moderate Conserv ation_aa: high	Missense	Uncertain signif icance (class 3)
CHEK2	NM_001005735.1:c.1286G>T	p.(Gly 429Val)	heterozygous	PolyPhen: Probably damaging Align-GVGD: C65 SIFT: Deleterious MutationTaster: Disease causing Conservation_nt: high Conservation_aa: high	Missense	Uncertain signif icance (class 3)
PTEN	NM_000314.4:c.458_459del	p.(Asp153Valf s*26)	heterozygous	Poly Phen: N/A Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservationnt: N/A Conservationaa: N/A	Frameshift	Likely Pathogenic (class 2)
BRCA2	NM_000059.3:c.7435+1G>T		homozygous	Poly Phen: N/A Align-GVGD: N/A SIFT: N/A MutationTaster: Disease causing Conservation_nt: high Conservation_aa: N/A 2/2 likely splice effect	Splicing	Likely Pathogenic (class 2)
BRCA2	NM_000059.3:c.9613_9614delinsCT	p.(Ala3205Leu)	heterozygous	PolyPhen: Benign Align-GVGD: C0 SIFT: Deleterious MutationTaster: - Conservation_nt: N/A Conservation_aa: high	Missense	Uncertain significance (class 3)
BLM	NM_000057.2:c.2695C>T	p.(Arg899*)	heterozygous	PolyPhen: N/A Align-GVGD: N/A SIFT: N/A MutationTaster: Disease causing Conservation_nt: weak Conservation_aa: N/A	Nonsense	Pathogenic (class 1)
BRCA1	NM_007300.3:c.3329dup	p.(Gln1111Alafs*4)	heterozygous	PolyPhen: N/A Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Frameshift	Pathogenic (class 1)
TP53	NM_000546.4: c.1024C>T	p.(Arg342*)	heterozygous	PolyPhen: N/A Align-GVGD: N/A SIFT: N/A MutationTaster: - Conservation_nt: weak Conservation_aa: N/A	Nonsense	Pathogenic (class 1)
RBM10	NM_001204468.1:c.1058C>G	p.(Ser353*)				Tier2
BRCA2	NM_000059.3:c.4936_4939del	p.(Glu1646Glnfs*23)	heterozygous	PolyPhen: N/A Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Frameshift	Pathogenic (class 1)

evaluating the sequence homology and investigates the conservation degree of amino acid residues among species. SIFT predicts whether an amino acid substitution in a protein will have a phenotypic influence. A score greater than or equal to 0.05 was are predicted to be tolerated and a score less than 0.05 was predicted to be deleterious. SIFT predicted tolerated effects in a variant of BRCA1 and BRCA2. It also predicted deleterious effects in a variant of MSH6, CHEK2 and BRCA2.

MutationTaster evaluates mutation effect on protein function and structure. Also, it considers the effect of mRNA expression or splicing. It predicts the disease potential of an alteration as disease-causing which is probably deleterious, disease-causing automatic which is deleterious, polymorphism which is probably harmless, and polymorphism automatic which is harmless. The results predicted polymorphism in a variant of BRCA1 and BRCA2 and predicted disease-causing in a variant of MSH6, BRCA2 and BLM and two variants of CHEK2.

Align-GVGD computes a biochemical distance score (Grantham difference (GD)) and a conservation score (Grantham variation (GV)). Substitutions are categorized in 7 class based on the scores of GD and GV, from C0 which is least likely to interfere with function to C65 that is most likely to interfere with function. The results predicted C0 class for a variant of BRCA1, BRCA2, and CHEK2. It also predicted C15 class for another variant of BRCA2 and a variant of MSH6 and C65 class for another variant of CHEK2.

Expression result

The mRNA expression rate of the MLH3 gene for 40 cancer patients was conducted for both normal tissues and cancerous tissues. Also, every patient's expression level was distinct, as the comparative among normal controls and tumors indicated in Figure 1.

RT-PCR results

The level of an expression was evaluated by RT-qPCR. It was found that expression of the MLH3 gene in tumor samples was significantly reduced (down-regulated) compared with normal tissues sample. Statistically high significance was found (<0.0001, T-test; $p > 0.05$). Figure 2 reveals statistical results of the level of expression of mRNA in both normal controls and tumors.

The statistical significance

The statistical significance of MLH3 expression was evaluated according to age (Table 2), cancer grade (Table 3) and breast cancer types (Table 4).

The decreased expression of MLH3 was significant in all rang of ages ($P<0.05$). Also, the expression of MLH3 decreased significantly in patients with breast cancer grades of II and III ($P<0.05$). However, this decrease was not significant in patients with a cancer grade of I ($P>0.05$).

In addition, the decreased expression of MLH3 was significant in all breast cancer types including Invasive ductal carcinoma, Carcinoma Medullary like, and Matrix-producing metaplastic ($P<0.05$). Therefore, a significant association with breast cancer was found MLH3

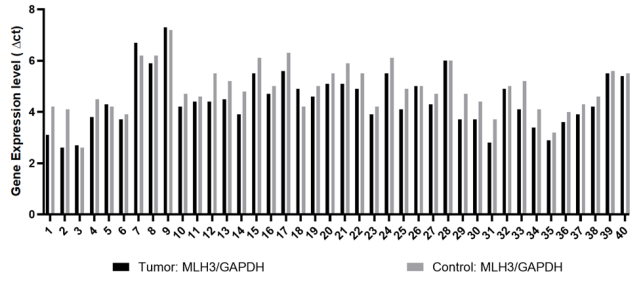

Figure 1. The expression rate of mRNA for both normal controls and tumors according to ΔC_T of *MLH3* gene.

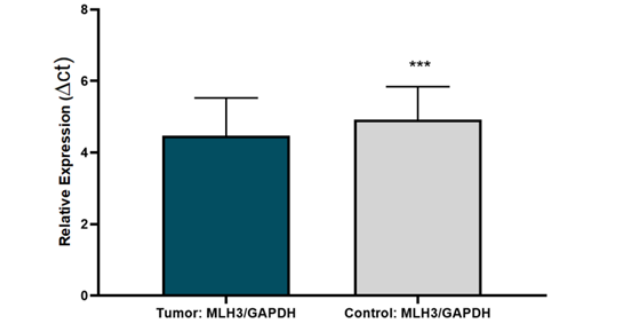

Figure 2. Statistical results of the level of expression of mRNA in both normal controls and tumors.

Table 2. Statistical significance according to ages.

Age	NO. (%)	MLH3 Expression (p-value)	Mean of differences	SD of differences	SEM of differences
<40 years	12 (30)	0.0001	0.5417	0.3288	0.09491
40- 55 Years	19 (47.5)	0.0054	0.4053	0.5592	0.1283
> 56 years	9 (22.5)	0.0064	0.4	0.3279	0.1093

Table 3. Statistical significance according to cancer grade.

Cancer Grade	NO. (%)	MLH3 Expression (p-value)	Mean of differences	SD of differences	SEM of differences
I	8 (20)	0.1462	0.3875	0.6707	0.2371
II	16 (40)	0.0006	0.4563	0.4226	0.1057
III	16 (40)	0.0001	0.4625	0.3631	0.09077

Table 4. Statistical significance according to breast cancer types.

Breast cancer type	NO. (%)	MLH3 Expression (p-value)	Mean of differences	SD of differences	SEM of differences
Invasive ductal carcinoma	21 (52.5)	0.0017	0.4238	0.5375	0.1173
Carcinoma Medullary like	9 (22.5)	0.0064	0.4	0.3279	0.1093
Matrix producing metaplastic	10 (25)	0.0009	0.52	0.3393	0.1073

decreasing expression.

Discussion

Exome sequencing of breast cancer patients was used to identify variants associated with cancer risk in breast cancer susceptibility genes. As a result, 26 variants in breast cancer patients, including 22 inherited variants and 4 somatic variants were found in susceptibility genes. In-silico analysis predicted probably damaging function for a variant of CHEK2 (NM_001005735.1:c.1286G>T). In addition, It was also predicted deleterious effects in a variant of MSH6 (NM_000179.2:c.3997T>C), CHEK2 (NM_001005735.1:c.1286G>T) and BRCA2 (NM_000059.3:c.9613_9614delins CT). Also, disease-causing was predicted in a variant of MSH6 (NM_000179.2:c.3997T>C), BRCA2 (NM_000059.3:c.7435+1G>T) and BLM (NM_000057.2:c.2695C>T) and two variants of CHEK2 (NM_001005735.1:c.1082G>A and NM_001005735.1:c.1286G>T).

MSH6 (MutS Homolog 6) is a protein-coding gene and is a member of the mismatch repair system of DNA. It codes a protein that assists repair DNA damage. The results of a study showed that the MSH6 gene is associated with an enhanced risk for breast cancer (28). Another study investigated the association of rare variants of MSH6 with familial breast cancer. The results showed that none of these variants are pathogenic (29). In addition, whole-exome sequencing of patients with breast cancer in India detected an association between the MSH6 variant and breast cancer (30). In the present study, a novel variant (NM_000179.2:c.3997T>C) of MSH6 was detected and the results of in silico analysis predicted this variant as a disease-causing variant that shows deleterious effects. However, this missense variant was an uncertain significance.

Four variants were found in the BRCA1 gene and eight variants were found in the BRCA2 gene. These genes are human tumor suppressor genes and are responsible for DNA repair. Mutations in BRCA1 and BRCA2 genes are well-known predispositions for breast cancer (31, 32).

CHEK2 is a tumor suppressor gene that encodes a serine/threonine kinase (33). It is a risk modifier gene in breast cancer families (1). A study suggests that pathogenic variants of CHEK2 carriers have a higher risk for breast cancer (34). Two variants of CHEK2 were identified in the present study. Both of these missense mutations were uncertain significance.

BLM also plays an important role in homologous recombination DNA repair. It is a part of the BRCA1 protein complex (35). Two variants of BLM were identified in the present study. Both of these variants were nonsense mutations and belonged to pathogenic mutation classification. A study investigated the role of a variant of BLM in Russia. The results showed that BLM may be associated with breast cancer risk (36). Also, the association of an inherited variant of BLM with clinical characteristics and risk of breast cancer was investigated. Clinical characteristics of breast cancers in the BLM mutation carriers were as same as non-carriers. Therefore, there is a doubt on considering BLM as a breast cancer susceptibility gene (37).

Four somatic variants were found in RBM10, PALB2, and RAD50. RBM10 is very highly correlated with the Bax expression which is the key gene in the apoptosis pathway in breast cancer. It is also positively correlated with p53 expression (38). An abnormal PALB2 gene results in a higher risk of breast cancer.

TP53 is a tumor suppressor gene and usually is mutated in breast cancer. Any alterations in the gene change the expression of genes that are under the transcriptional control of p53 (39). PTEN is associated with the initiation and progression of breast cancer. Loss of expression of PTEN may be associated with aggressive behavior and worse outcomes in breast cancer patients (40). A study investigated the association of the nibrin gene (NBN) variants with breast cancer and concluded that a variant of the NBN gene may be a genetic risk factor for breast cancer development (41).

The MLH3 protein functions in meiotic recombination and mutations of MLH3 cause meiotic defects. The mechanism of DNA MMR plays a key role in genetic stability, and as a result, MLH3 mutations that interfere with MMR function can be predisposed to cancer. Various mutations of the MLH3 gene were detected (18, 20). A frameshift mutation of the MLH3 gene was found in colorectal cancer patients (14). Mutation of MLH3 was investigated in endometrial cancer and a role for MLH3 in endometrial tumorigenesis was suggested (18). As a result, it was hypothesized that MLH3 might account for cancer susceptibility in breast cancer patients.

Further research is needed to examine genome-wide association studies to determine candidate genes (42) and polymorphisms (43) in different populations.

In this study, one variant of the MLH3 gene (NM_001040108.1:c.4091-1G>A) was found which is associated with breast cancer. The results of RT-qPCR showed that the expression of the MLH3 gene in tumor samples was significantly reduced compared with normal tissues ($P<0.05$). Decreasing the expression of MLH3 in patients with breast cancer shows that mutation of this gene which has a central role in DNA repair can promote breast cancer tumorigenesis through different pathways and mechanisms. Decreasing in the expression MLH3 was significant in all ranges of ages. Therefore, investigating of down-regulation of MLH3 can be used as a biomarker for the prediction of breast cancer occurrences. However, the expression of MLH3 significantly decreased only in patients with breast cancer grades II and III, not in patients with breast cancer grades I. Therefore, this down-regulation usually occurs when the disease is slightly advanced. However, the decreased expression of MLH3 can be a biomarker in grades II and III of breast cancer.

In conclusion, 26 variants in breast cancer patients, 22 inherited variants were found in MLH3, CHECK2, BRCA1, BRCA2, BLM, TP53, MSH6, NBN and PTEN genes and 4 somatic variants were found in PALB2, RAD50 and RBM10 genes. It was found that the expression of the MLH3 gene in tumor samples was significantly down-regulated compared with normal tissues. In conclusion, MLH3 can be used as a susceptibility gene especially in grades II and III of breast cancer.

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