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NRF2 MUTATION AT RS6721961 AND ITS ASSOCIATION WITH NRF2 TARGET GENES IN BREAST CANCER PATIENTS WITH NO HISTORY OF CHEMOTHERAPY

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Abstract – *Objective:* *Owing to the putative role of* Nrf2 *in the activation of anti-oxidative genes, the present study aims at investigating the role of* Nrf2 *expression and its mutated form in breast cancer patients with no previous therapy.*

Patients and Methods: *In the case-control study, breast cancer patients and controls from the Western Kurdish population of Iran were selected to evaluate the genotype of* Nrf2 *(rs6721961) by PCR- restriction fragment length polymorphism. In addition, the statistical association between* Nrf2 *(rs6721961) mutation and the expression of* Nrf2*, ER, PR, Her2, SOD, catalase, and other clinicopathological characteristics were analyzed.*

Results: *The mean age of patients and controls were 43.55±1.89 and 46.44±2.16, respectively. In the control group,* Nrf2 *rs6721961 mutation deviated from Hardy-Weinberg Equation (HWE) with a p-value of 0.035 while in the patients' group, SNP exact test for HWE showed a satisfied association with HWE (*p*=0.7). Besides, there was no significant relation between* Nrf2 *expression and catalase activity (*p*= 0.28) while SOD activity had a meaningful association with* Nrf2 *expression (*p*=0.02). There was no meaningful association of SNP with the expression levels of Her2 (*p*=0.30), ER (*p*=0.60) and PR (*p*=0.63). Besides, SNPSTAT showed that the expressions of Her2, ER and PR were in agreement with HWE.*

Conclusions: *The results indicated that* Nrf2 *mutation is an imperative effector in the risk of breast cancer. Besides, the interplay of* Nrf2 *with other main biomarkers of oxidative stress seems to pave the way towards novel and convenient treatment options.*

KEYWORDS: *Breast cancer, Nrf2, Mutation, Oxidative stress.*

LIST OF ABBREVIATIONS: *BC, Breast cancer; CAT, catalase; ER, Estrogen Receptor; HO-1, Heme oxygenase-1; GCL, Glutamylcystien ligase; GPX, glutathione peroxidase; GR, glutathione reductase; HWE, Hardy-Weinberg Equation; INRf2, inhibitor of NRF2; KEAP1, Keltch-like ECH-associated protein1; NRF2, Nuclear factor erythroid2-related factor 2; NQO-1, NAD(P)H: quinone oxidoreductase 1; PCR, Polymerase chain reaction; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; PRDX, Peroxiredoxin; PR, Progesterone Receptor; SOD, Superoxide dismutase; OS, Oxidative Stress; ROS, Reactive Oxygen Species, TXN, thioredoxin; TRXR, thioredoxin reductase.*

INTRODUCTION

Breast Cancer (BC) is one of the most common malignancies and the second leading cause of cancer-related death in women worldwide. Given the high expression of several biological factors including estrogen receptor (ER), progesterone receptor (PR), ErbB2 (Her2/neu) and Ki-67, BC is classified into

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at least 5 main subgroups including luminal A, luminal B, triple-negative, basal-like and normal-like groups[1]. In the light of various biological hallmarks of 5 subtypes, response to the treatment and the clinical outcome are different. It is generally accepted that the heterogenic feature of BC is attributed to the interaction of both genetic alterations and environmental factors[2]. In this line, several mechanisms have been suggested to be involved in the promotion and development of BC. As one of the critical underlying mechanisms of tumorigenesis, oxidative stress (OS) seems to play a causal role in the pathogenesis of BC. Apart from their critical role in normal homeostasis and inflammation, ROS are involved in the initiation and promotion of tumorigenesis through modulating the expression of oncogenes and tumor suppressor genes[3]. Oxidative stress as a consequence of an imbalance between generation and elimination of reactive oxygen species (ROS) is characterized by alterations in intracellular oxidative damage, gene mutation, cellular apoptosis and malignant transformation[4]. It has been suggested that overexpression in the levels of HER2, ER, and/or PR is directly correlated to the ROS production and the stage of breast cancer. As an illustration from the involvement of ROS, estrogen receptor-positive (ER^+) BC patients are commonly identified by upregulation of the oxidative genes, high tumor grade and reduced survival[5]. Furthermore, Her2 targeting by trastuzumab is associated with attenuation of GSH and activation of SOD and catalase[6].

In response to the high amount of ROS production and/or accumulation, adaptive mechanisms underlying redox regulation have been emerged to counteract deleterious effects of OS. There is a wealth of evidence that the expression of ROS detoxifying system is modulated by nuclear factor erythroid-derived 2-like 2 (NFE2L2) which also known as nuclear factor erythroid2-related factor 2 (NRF2) transcription factor. NRF2 is a key anti-oxidative regulator belongs to the cap'n'collar (CNC) family and/or CNC-bZIP proteins having closely related members including p45-NEF2, NRF1 and NRF3 and two distantly related proteins, Bach1 and Bach2[7,8]. Moreover, another member of Nrf2 family, nuclear factor erythroid-derived 2-like 2 (NFE2L2) participates in the expression of ROS detoxifying system. The various domains of NRF2 contain N-terminal hydrophobic domain followed by the INrf2 (Keap1)-binding domain, transcriptional activation domain, CNC domain and basic/leucine zipper domain (CNC/bZIP) which heterodimerizes with small Maf or Jun proteins and binds to the ARE[9]. In this line, NFκB and NF-E2 related factors (Nrf2 family members) are the main transcription factors responsible for the sensing OS and induction of protective systems to regulate a wide variety of cellular activities including proliferation, immunological reactivates and signal transduction[10]. INrf2, inhibitor of NRF2, which is also named KEAP1 (Keltch-like ECH-associated protein1) is a homodimeric protein, retaining NRF2 in the cytoplasm to render proteasomal degradation. The N-terminal BTB domain of INrf2 through interaction with Rbx1-bound Cul3 and the C-terminal DGR domain of INrf2 modulate the activity of NRF2. This leads to the ubiquitination and degradation of NRF2[11]. Under normal conditions, Nrf2 forms a complex with keltch like Ech-associated protein 1. However, in the event of OS, Nrf2 is separated from the complex and is moved into the nucleus in which forms a heterodimer with Maf and AP1 family proteins and induces the expression of several protective systems including antioxidant enzymes[12]. The antioxidative genes containing ARE sequence induced by Nrf2 include heme oxygenase-1 (*HO-1*), NAD(P)H:quinone oxidoreductase 1 (*NQO-1*), glutamyl cysteine ligase (*GCL*), peroxiredoxin (*PRDX*), superoxide dismutase (*SOD*), catalase (CAT), glutathione reductase (*GR*), thioredoxin (*TXN*), thioredoxin reductase (TRXR) and glutathione peroxidase (GPX)[13].

It has been suggested that a high level of unbounded Nrf2 is associated with chemoresistance in tumor cells[14]. On the other hand, Nrf2 depletion in mice is resulted in the development of tumors and several other diseases[15]. Regarding this evidence, it should be considered that mutation in NFE2L2 and keap1 genes may play an important role in the tumorigenesis. In this line, Keap1 loss of function was associated with the elevated level of Nrf2 and chemoresistance in non-small cell lung cancer (NSCLC). Downregulation of Keap1 through promoter hypermethylation is associated with instability of Nrf2 in BC tumors especially in ER-positive and Her2 negative subtypes[16]. Besides, an identified Keap1 mutation (C23Y) in human BC leads to disruption of Nrf2-Keap1 complex and increases the stability and signaling of Nrf2[17]. The present study aims at investigating the correlation of *Nrf2* mutation at rs6721961 and the expression of target genes including *CAT* and *SOD* in early diagnosed BC patients with no precedent chemotherapy exposure. Results delineated the pivotal role of *Nrf2* mutation at rs6721961 in the development of breast cancer patients at least in Iranian Kurdish populations.

PATIENTS AND METHODS

Patients

This case-control study included 34 BC and hyperplasia patients among 80 suspected BC patients referring to Imam Reza Hospital for the biopsy that have not

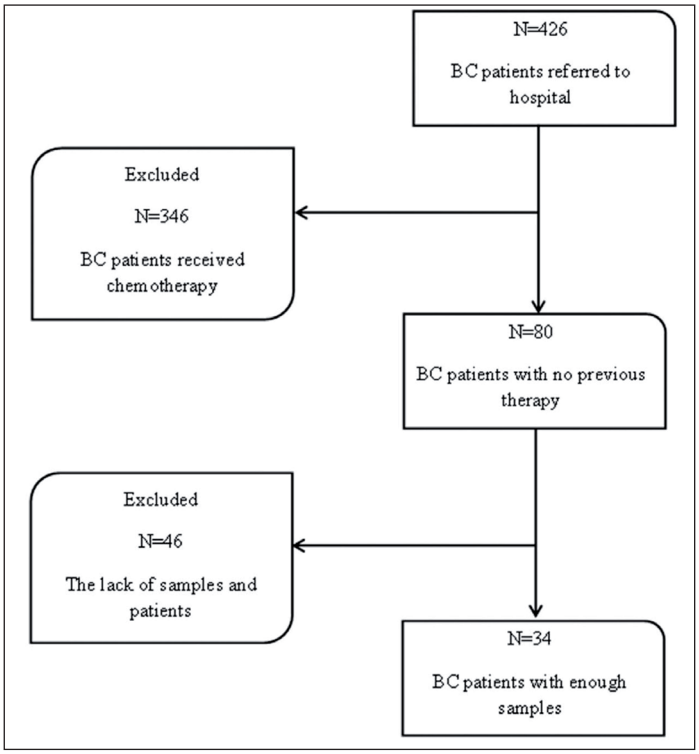

Fig. 1. Flow diagram related to BC participants through the study.

received any chemotherapy (Figure 1) and 80 normal individuals from Kermanshah during one year (2016-2017). Informed consent was taken from patients and involved doctors before blood collection and included the name of the appropriate institutional review board that approved the project. Peripheral blood samples from patients and control subjects were used to extract DNA and RNA. Paraffin-embedded and frozen tissues were prepared from tumor specimens for diagnosis of cancer. Diagnosed tumor tissues were used to staining and classification of BC. In addition, the admission of ethical committee was received from Kermanshah University of Medical Sciences by a code number of KUMS.REC.1395.470 in 2016.

DNA extraction

DNA was extracted from peripheral patients and normal blood samples using the standard protocol. Briefly, DNA was extracted by salting out and chloroform, precipitated by ethanol and then, solvated in ddH_2O. DNA purity and concentration were evaluated by a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Genotyping

Nrf2 (rs6721961) mutation in the promoter region including G to T substitution appears to affect the ARE binding site and possibly gene expression[18]. Genotyping of the *Nrf2* rs6721961 G/T polymorphism was performed using polymerase chain reaction (PCR) from extracted genomic DNA. A 278-bp fragment of *Nrf2* was amplified by PCR using *Nrf2* primers, forward (F) 5'-GAAAGGCGTTGGTGTAGGAG-3' and reverse (R) 5'-GAATGGAGACACGTGGGAGT-3'. PCR condition was included: denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension step of 10 min at 72°C. The amplified fragment was subsequently digested by Ngo-MIV (New England Biolabs, Beverly, MA, USA) in a condition recommended by the supplier. The digested products, 215-bp and 63-bp fragments were then electrophoresed in a 2.5% agarose gel. Results were confirmed by repeating the genotyping for >10% of randomly selected samples, yielding a concordance of 100%.

Quantitative real-time PCR

Total RNA was isolated from the cultured cells using the RiboExLS kit (GeneAll Biotechnology, Songpa-gu, Seoul, Korea) according to the manufacturer's protocol. The RNA concentration and the extent of its purity were determined using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by

measuring the absorbance at 260 nm and by evaluating the A260/A280 ratio, respectively. Then, total RNA (2 µg) in a 20-µl reaction volume was reverse-transcribed using the Prime ScriptT^{MT}RT kit (TaKaRa BioInc., Otsu, Shiga, Japan) in the presence of random hexamer. Quantitative Real-time polymerase chain reaction (RT-qPCR) was performed with a LightCycler instrument (Applied Biosystem, Foster City, CA, USA) using SYBR premix Ex Taq technology (TaKaRa Bio-Inc., Otsu, Shiga, Japan).

The SYBR Green master mix (10 µl) was added to 2 µl of the complementary DNA sample, 0.5 µl of forward and reverse primers (10 pmol) and 7 µl of nuclease-free water to conduct PCR in a 20 µl of the reaction mixture. Thermal cycling conditions involved an initial activation step for 30 s at 95°C followed by 38 cycles, including a denaturation step for 15 s at 60°C. Melting curves were analyzed to validate a single PCR product of each primer.

The following primers were used for SYBR Green-based real-time PCR: β-actin forward: 5'-TGACCCAGATCATGTTTGAGACC-3' and β-actin reverse: 5'-CTCGTAGATGGG-TACTGTGTGGG-3'. *CAT* forward: 5'- TTTC-CCAGGAAGATCCTGAC -3' and CAT reverse: 5'- ACCTTGGTGAGATCGAAT-GG -3'; *SOD* forward: 5'- AGGGCATCAT-CAATTTCGAG -3' and *SOD* reverse: 5'- TG-CCTCTCTTCATCCTTTGG -3' and *Nrf2* forward: 5'-AGTGGATCTGCCAACTACTC-3' and *Nrf2* reverse: 5'-CATCTACAAACGGGAAT-GTCTG-3'. Comparative quantitation analysis ($2^{-\Delta\Delta Ct}$ method) was used to determine fold change in the gene expression and normalized to the house-keeping gene, β-actin, which has been validated as the housekeeping gene for current studies[19].

Immunohistochemistry

Immunohistological staining was performed on formalin-fixed paraffin-embedded tissue sections using antibodies against Her2. For this aim, 4 µm tissue sections were deparaffinized at 37ºC for 2 h and xylene for 24 h. Then, slides rehydrated in a graded series of ethanol solutions and PBS (phosphate-buffered saline) for about 12 min. To retrieve antigens, slides immersed in the jar containing Tris buffer (pH=9) and heated in the water bath at 95ºC for 20 min followed by washing in PBS solution. To quench the intracellular activity of peroxidases, slides were immersed in a solution of 3% Hydrogen peroxide in methanol for 10 min, washed with PBS and placed in jars containing avidin solution for 5 min. Then, biotin was added to increase the specificity of staining. After washing with PBS, slides were incubated by primary and secondary antibodies for 45°C and 30°C, respectively in a humid and dark place at room temperature.

The slides were washed in PBS and stained with the substrate–chromogen solution known as 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 min. The counterstaining was performed with hematoxylin for 30 s and washed in water. The stained slides immersed in graded series of ethanol and then, xylene to transparency and dehydration of tissues. Then, slides mounted to study under a microscope. Negative controls were exposed to antibody diluent replacing primary antibody.

Statistical analysis

Data were analyzed using the SPSS (V.18; SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to evaluate the normality of data. For normal data, parametric test Pearson correlation coefficient (the relationship between quantitative data) and for non-normal data, Spearman's correlation test, were used. The correlation between qualitative data was assessed with chi-square tests. The association of SNP with other molecular markers was also analyzed with online SNPSTAT software[20]. All differences were considered statistically significant at the level of $p<0.05$.

RESULTS

Characteristics of study subjects

To investigate the role of *Nrf2* mutation in breast cancer patients with no previous treatment, among patients with early diagnosed BC referred to the Imam Reza hospitals, 30 BC and 4 hyperplasia patients who have not received therapies and 80 controls were included in this study. The statistical analysis of clinical characteristics related to patients and controls are shown in Table 1. The mean ages of patients and controls were 43.55±1.89 and 46.44±2.16, respectively. 16 patients and 36 controls were less than 40 old, while 24 patients and 44 controls were more than 40 old years. Age was well matched between patients and controls ($p=0.697$) (Table 1).

Nrf2 (rs6721961) mutation

The frequency of mutations was performed using SNPSTAT software and was analyzed for concordance with Hardy-Weinberg equilibrium (HWE) that satisfied with $p>0.05$. Agarose gel electro-

TABLE 1. The frequency of studied samples based on varieties.

Variables		*Control*	*Patient*	
			Breast cancer	*Hyperplasia*
Age	≤40	36	10	3
	>40	44	19	2
SNP	G/G	2	4	0
	G/T	49	9	2
	T/T	29	16	3
ER expression	Positive	-	21	-
	Negative	-	9	-
PR expression	Positive	-	19	-
	Negative	-	11	-
Her2 expression	Positive	-	24	-
	Negative	-	6	-
Nrf2	<1	1	3	1
	>1	1	27	3
Catalase	<1	1	8	1
	>1	1	22	3
SOD	<1	1	5	2
	>1	1	25	2

phoresis pattern of some PCR products of *Nrf2* (rs6721961) were shown in Figure 2. In the control group, *Nrf2* rs6721961 mutation deviated from HWE with a *p*-value of 0.035 while among the patients, SNP exact test for HWE showed a satisfied association with HWE (p=0.7) (Tables 2 and 3). The association of SNP with responses was shown in Table 3. In the codominant model, G/T genotype indicated a protective effect (OR=2.32 (1.03-5.9), p=0.05) relative to T/T and/or G/G genotypes. In addition, the protective effect of G/T was demonstrated in the overdominant model, OR (95% CI) = 2.5 (1.14-4.54) p= 0.02) compared to T/T+G/C genotype. Mendelian effect modeling of pair-wise genetic analyzes using SNPSTAT software clearly indicated that the G/T genotype of *Nrf2* rs6721961 mutation is associated with the risk of BC that was comparable with both codominant (p=0.05) and overdominant effect assumptions (p=0.02).

Association between Nrf2 SNP with the expression of Nrf2, SOD and CAT

To evaluate the expression of *Nrf2*, *SOD* and also *CAT* with clinical characteristics of samples, the mRNA levels of *Nrf2*, *SOD* and *CAT* were measured using Real-time PCR. As shown in Table 2,

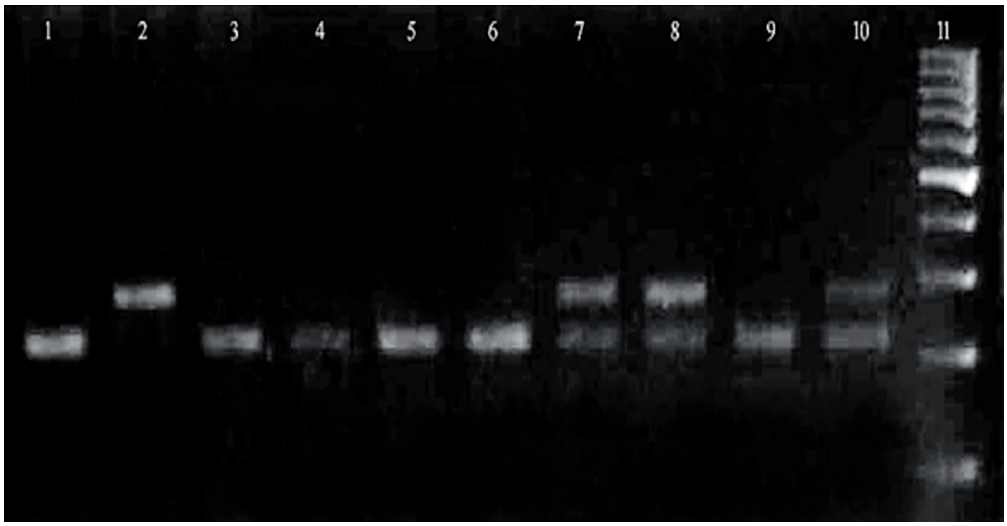

Fig. 2. *Nrf2* G/T products following NgoMIV Restriction enzyme on a 2% agarose gel electrophoresis. Lanes 1, 3, 4, 5, 6 and 9 show 'TT' genotypes Lane 2 shows 'GG' genotype, Lanes 7, 8 and 10 show 'GT' genotype for person C and Lane 7 Shows 100 bp DNA Molecular Markers.

the expression of SOD in line with *Nrf2* was increased in patients, compared to the control group. However, the expression of catalase was decreased in some cases compared to the control group. In addition, statistical analysis showed that there was no significant relation between *Nrf2* expression with catalase (p=0.28) while *SOD* had a meaningful association with *Nrf2* expression (p=0.02). This indicated that despite the causal role of *Nrf2* in the expression of target genes such as *CAT* and *SOD*, the expression of *CAT* is affected by other signaling and/or transcriptional factors.

According to the chi-square test, *Nrf2* SNP did not show a significant association with *Nrf2* and *CAT* expressions (p=0.15 and 0.53) and showed a meaningful relationship with *SOD* expression (p=0.04). However, Montecarlo test indicated that *SOD* also did not have a significant association with SNP (0.35). In addition, T/T genotype had more expression of *Nrf2*, *CAT* and *SOD* than G/T and G/G genotypes. On the other hand, SNPSTAT software showed that the expression of *Nrf2*, *CAT* and *SOD* were in agreement with Hardy-Weinberg equilibrium (p=0.61, 0.63 and 0.24). What is more, there was no difference in the models of codominant, dominant, recessive and overdominant among SNP genotypes (G/G, G/T and T/T) in the expression levels of *Nrf2* (p=0.15, 0.14, 0.33 and 0.07, respectively) and SOD (p=0.7, 0.55, 0.46 and 0.82, respectively). Alternatively, the protective effects of T/T and G/G genotypes were diagnosed in codominant, dominant and overdominant models with p-values of 0.02, 0.01 and 0.005, respectively and OR=1 (Tables 2 and 3).

Association of Nrf2 SNP with the expression of ER, PR and Her2 in patients

The expressions of ER, PR and Her2 were measured using immunohistochemistry in BC samples (Figure 3). As indicated in Table 2, there was not the meaningful association of*Nrf2* SNP with the expression levels of Her2 (p=0.30), ER (p=0.60) and PR (p=0.63). Besides, SNPSTAT showed that the expressions of Her2, ER and PR were in agreement with Hardy-Weinberg equations (p=1). The association of SNP with the expressions of ER, PR and Her2 in the models of Codominant, dominant, recessive and overdominant was not protective (p>0.05 and OR<1) (Table 3). The results indicated the protective effects of Nrf2 on the levels of ER, PR and Her2 in breast cancer.

DISCUSSION

Given the well-known role of the oxidative system in several disorders including BC, this study was directed to delineate the involvement of *Nrf2* mutation and polymorphism, as a master antioxidant regulator and its association with clinicopathological factors in BC patients who have not received chemotherapy so far. The results clearly indicated the putative role of Nrf2 in the development of breast cancer at least in the Iranian Kurdish populations.

It is well established that antioxidant pathways contribute to the development of carcinogenesis through induction of survival and proliferative factors[21].

TABLE 2. The association statistics between SNP and Variables.

Variable	*SNP*	*G/G*	*G/T*	*T/T*	*p-value (chi-square)*	*p-value (HWE)*
Disease	Breast cancer Hyperplasia Control	4 0 2	9 2 49	17 2 29	0.023	0.003
Age	≤ 40 > 40	2 4	27 33	20 28	0.835	0.027
ER expression	Positive Negative	2 2	7 2	12 5	0.599	0.69
PR expression	Positive Negative	2 2	5 4	12 5	0.654	0.69
Her2 expression	Positive Negative	4 0	8 1	12 5	0.303	0.69
Nrf2 expression	< 1 > 1	0 4	3 8	1 18	0.145	1
SOD expression	< 1 > 1	0 4	5 6	2 17	0.041	1
Catalase expression	< 1 > 1	1 3	4 7	4 15	0.66	1

TABLE 3. The models for inheritance of Nrf2 rs6721961 genotypes based on SNPSAT software (https://www.snpstats.net/start.htm).

Variables	Model	Codominant			Dominant			Recessive			Overdominant		
		G/G	G/T	T/T	G/G	G/T	T/T	G/G	G/T	T/T	G/G	G/T	T/T
Disease	*p*-value	0.05	0.05	0.05	0.14	0.14	0.14	0.09	0.09	0.09	0.5	0.5	0.5
	OR (95% CI)	-0.50	0.30	0.00	0.25	0.25	0.00	-0.7	0.00	0.00	0.00	0.35	0.00
Age	*p*-value	0.49	0.49	0.49	0.52	0.52	0.52	0.27	0.27	0.27	0.85	0.85	0.85
	OR (95% CI)	3.5	1.2	1	1.28	1.28	1	1	3.15	1	1	1.08	1
ER	*p*-value	0.91	0.91	0.91	1	1	1	0	0	0	0.81	0.81	0.81
	OR (95% CI)	0.17	-0.04	0	0	0	0	0.18	0.48	0.48	0.52	-0.06	0.52
PR	*p*-value	0.88	0.88	0.88	0.61	0.61	0.61	0	0	0	0.72	0.72	0.72
	OR (95% CI)	0.17	0.12	0	0.12	0.12	0	0.12	0.55	0.55	0.52	0.09	0.52
Her2	*p*-value	0.53	0.53	0.53	0.44	0.44	0.44	0	0	0	0.86	0.86	0.86
	OR (95% CI)	-0.50	-0.12	0	-0.19	-0.19	0	-0.45	-0.45	0.45	0.43	-0.04	0.43
Nrf2	*p*-value	0.02	0.02	0.02	0.01	0.01	0.01	0.37	0.37	0.37	0.005	0.005	0.005
	OR (95% CI)	1	0	1	0	0	1	NA	1	1	1	0	1
SOD	*p*-value	0.52	0.52	0.52	NA	NA	NA	0.27	0.27	0.27	0.63	0.63	0.63
	OR (95% CI)	NA	0.79	1	1	1	1	NA	1	1	1	0.63	1
Catalase	*p*-value	0.38	0.38	0.38	0.70	0.70	0.70	0.16	0.16	0.16	0.82	0.82	0.82
	OR (95% CI)	NA	1.04	1	1.35	1.35	1	NA	1	1	1	0.83	1

Concerning the alteration in the expression of the antioxidant factors such as catalase, SOD and others, it seems that the Nrf2 as a master regulator of antioxidant systems plays an important role in carcinogenesis. Recent studies have unveiled that mutations in the *Nrf2* promoter can affect the expression and activity of Nrf2, susceptibility to several diseases and survival of patients[22]. In this line, the genetic mutation of *Nrf2* with OS-related diseases such as vitiligo showed the protective effect of C allele of *Nrf2* rs35652124 on vitiligo among the Chinese population. However, *Nrf2* SNP at -650 was associated with the progression of vitiligo in China[23]. The involvement of *Nrf2* mutation in the endurance capacity of athletes has also been investigated in several studies[24,25]. Further, the functional mutations of *Nrf2* in the development of acute lung injury (ALI) led to the recognition of multiple SNPs in ethnically diverse individuals, namely, patients with the (-617 C/A) SNP, who showed a higher susceptibility to develop ALI after major trauma[22].

In contrast to the protective effect of Nrf2 in normal cells, depending on the other signaling factors, Nrf2 is able to augment the chemoresistance and radioresistance in tumor cells, indicating the eventual protumorigenic effect of Nrf2 in several cancers[26-28]. For example, the association of *Nrf2* polymorphism with pulmonary diseases has been confirmed[18,22,29]. On contrary, it has been found that there were not an association of *Nrf2* polymorphism with the risk of gastric and breast cancer[30,31].

On the other hand, *Nrf2* polymorphism at rs62721961 in postmenopausal women was administered by estrogen showed a meaningful relationship with the risk of thromboembolism[32].

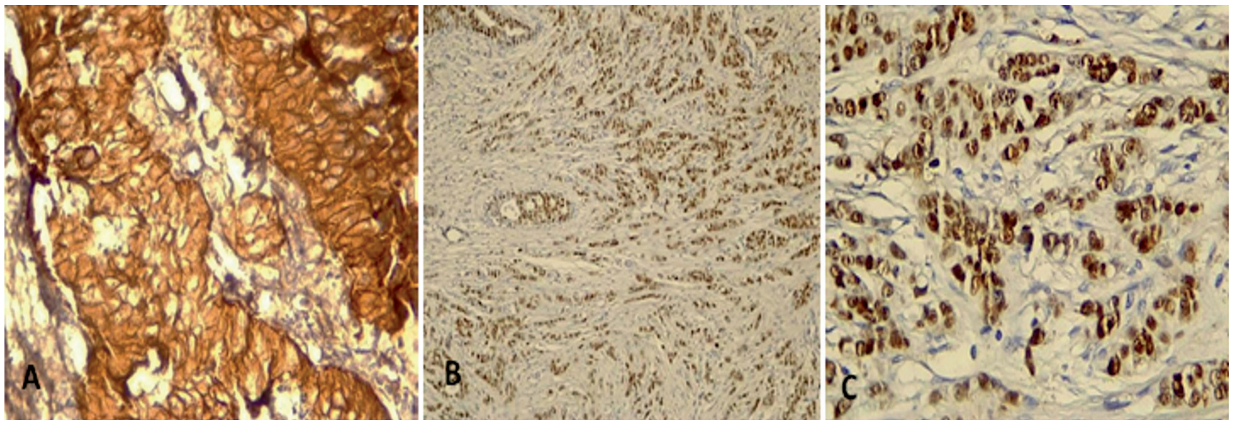

Fig. 3. The photographs related to the expression of ER, PR and HER in breast cancer tissues. ER positive breast cancer (A) PR positive breast cancer (B) and Her2 positive breast cancer (C).

The role of *Nrf2* polymorphism in pre- and post-menopausal Egyptian BC patients showed that *Nrf2* (rs6721961) AA genotype is more common in pre-menopausal patients than the other one, implying the implication of *Nrf2* mutation in early BC onset[33]. In a recent study, the role of Nrf2 and its protective target gene (*SRXN1*) polymorphisms has evaluated in the risk, survival and development of BC. In this study, *Nrf2* rs6721961 and rs2706110 as well as *SRXN* rs60350666 were associated with increased risk of BC progression. Furthermore, another study on cardiovascular disease showed an association between the polymorphism of *Nrf2* (rs6721961, rs1962142, rs270611) and *SRXN1* rs6053666 with the risk of cardiovascular diseases[34]. One possible explanation for these observations is the utmost indispensable role of three SNPs of *Nrf2* rs6721961, rs1962142, rs270611 in development of diseases specially BC that should be investigated in different populations. Here, we reported that *Nrf2* promoter polymorphism at rs6721961 interplay with the risk of BC, uncovering the predisposed role of *Nrf2* mutation in the development of BC.

The association of *Nrf2* with the molecular signature of BC including Her2, ER and PR seems to be important for better evaluation of therapeutic strategies. It has been demonstrated that estrogen through PI3K/GSK3β signaling pathway can increase the level and the activity of Nrf2, indicating the important role of hormones in regulation of Nrf2 in BC cells[35,36]. On the other hand, an inhibitory effect of estrogen receptor signaling pathway has been evaluated on Nrf2-dependent enzymes. This suggests that anti-estrogen therapies seem to invert the inhibitory effects of estrogen and suppress BC development through promotion of chemopreventive Nrf2-responsive genes in estrogen-dependent BC diseases (inhibition of estrogen signaling). In addition, a large body of evidence indicates that Her2 overexpression confers drug resistance in BC cells[37]. It has been shown that there is a direct interplay between Nrf2 and Her2 expression with drug resistant through activation of Her2-mediated induction of Nrf2 transcriptional activity in BC cells[38]. On the other hand, a reciprocal link between Nrf2 and Her2 has demonstrated that Nrf2 inhibition led to the suppression of Her2 signaling pathway in SCOV3 ovarian cancer cells[39]. In this study, Nrf2 rs6721961 mutation did not have any association with the expression of ER and PR. Furthermore, the expression of Nrf2 also did not show a meaningful association with the expression of ER and PR.

There is a growing body of evidence that the expression of antioxidant systems such as *SOD* and *CAT* involving in modulation of BC development. As the target genes of *Nrf2*, it seems that mutation of *Nrf2* in BC leads to altered expression of *SOD* and *CAT* in breast cancer. Therefore, the expression of ER and PR and its association with *Nrf2* mutation were evaluated in our study. Our results showed that in contrast to the positive correlation between SNP at *Nrf2* rs6721961 and the expression of *CAT* and *Nrf2,* there is no meaningful association with *SOD* expression in breast cancer patients. In addition, the expression of *Nrf2* had a significant association with the expression of *SOD* but not with catalase expression, suggesting the close relation of *SOD* expression with *Nrf2* in BC patients while the expression of catalase is affected by other signaling factors. It should be mentioned that despite the potential role of SNP at *Nrf2* rs6721961, we proposed that the number of patients should be increased for coming a conclusion that *Nrf2* mutation plays fundamental role in development of breast cancer.

CONCLUSIONS

To sum up, the *Nrf2* promoter mutation at rs6721961 was associated with the risk of BC whereas there was no significant association with the expression of ER, PR and catalase. Also, there was a meaningful association between mutations at *Nrf2* promoter with the expression of SOD among patients. In addition, it seems that despite geographical and racial differences, the mutation of some biomarkers such *Nrf2* and its association with other clinical biomarkers are the imperative effectors in the development and the progression of breast cancer that would be considered in the selection of suitable therapeutic strategies.

Contribution:
Soraya Sajadimajd, Mozafar Khazaei; Contributed to conception and design. Soraya Sajadimajd; Contributed to all experimental work, data and statistical analysis, and interpretation of data. Soraya Sajadimajd, Sedigheh Khazaei; was responsible to prepare and collect the blood samples. Soraya Sajadimajd, Mozafar Khazaei; were responsible for overall supervision. Soraya Sajadimajd; Drafted the manuscript, which was revised by Soraya Sajadimajd and Mozafar Khazaei. All authors read and approved the final manuscript.

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Conflict of interest:
The authors declare no conflict of interest, financial or otherwise.

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