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RESEARCH ARTICLE

Evaluation of Salivary *KCNJ3* mRNA Levels in Breast Cancer: A Case–control Study and *in silico* Analysis

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Abstract:

Background:

Breast cancer (BC) is considered the most malignant and central cancer-related death among women worldwide. There is an essential need to discover new methods for developing noninvasive and low-cost diagnoses. The present study examines the expression of *KCNJ3* which acts as a biomarker for detecting BC in the saliva of BC patients compared to controls.

Methods:

The mRNA expression level of *KCNJ3* has been evaluated. Forty-three unstimulated whole saliva samples from BC patients and forty-three salivary samples from healthy controls were collected. The mRNA level was measured using quantitative real-time polymerase chain reaction (qRT-PCR). Furthermore, the protein-protein interaction network in which *KCNJ3* is involved was obtained. *In silico* analysis was applied to predict the possible molecular mechanisms of *KCNJ3* in BC development.

Results:

Differentially expressed *KCNJ3* was statistically significant between BC patients and controls ($p<0.001$). The sensitivity and specificity of *KCNJ3* mRNA in BC detection were 76.74% and 94.95%, respectively. Receiver operating characteristic (ROC) curve analysis of *KCNJ3* mRNA revealed that Area under the curve (AUC) was 0.923 (95% Confidence Interval (CI): 0.866-0.979). AUCs of ROC curve analysis were 0.743 (95% CI: 0.536-0.951), 0.685 (95% CI: 0.445-0.925), and 0.583(95% CI: 0.343-0.823) for differentiation stage I from stage III, stage I to stage II and finally stage II from stage III, respectively. Furthermore, the GABAergic synapse signaling pathway was suggested as a potential pathway involved in BC development.

Conclusion:

Salivary levels of *KCNJ3* could be considered a potential diagnostic biomarker with high sensitivity and specificity for BC detection.

Keywords: Breast cancer, Saliva, *KCNJ3*, Biomarker, Signaling pathway, MRNA.

1. INTRODUCTION

Breast cancer (BC) is considered the most malignant and primary cancer-related death in women worldwide [1]. Based on hormonal factors and molecular classification, BC is divided into three groups: estrogen receptor-positive (ER+), progesterone receptor-positive (PR+), and human epidermal growth factor receptor (HER2), which are the most common categories for the treatment and prediction of outcomes [2]. Genetically, mutations in the *BRCA1* and *BRCA2* genes have been reported in BC patients. In addition, mutations in other tumor suppressor genes, *p53* and *PTEN*, have also been reported. Dysregulation of repair system components such as *ATM, CHEK2, NBN, PALB2*, and *RAD50* can also be considered genetic risk factors for BC [3].

According to the World Health Organization, mammography is the best way to screen BC [4]. It has been

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introduced as an effective and, of course, a high-cost method. Recently, using salivary biomarkers has attracted attention in diagnosing diseases and cancers [5]. Research has been done to diagnose and prognosis using body fluids [6, 7]. Using saliva as a source of diagnostic biomarkers has advantages over blood. First, saliva is readily accessible. In addition, the collection of saliva is noninvasive, less expensive, and does not require trained personnel. Despite these advantages, not all biomarkers are detectable in saliva, primarily because of the difference in biomarkers' concentrations in blood and saliva [8]. Several attempts have differentiated BC patients from healthy control regarding chemical, protein, metabolic, and transcriptomic biomarkers in saliva [9 - 11].

The growth and metastasis of cancer cells require a series of complex signals. These signals are transmitted into the cell by the surface receptors on the membrane. In the past, abnormal ion channel expression has been observed in many cancers [12, 13]. G-Protein-Coupled Inwardly Rectifying Potassium (GIRKs) is a group of k+ channels that connect membranes and extracellular signaling molecules [14]. These channels act as G proteins and regulate cell function by stimulating neurotransmitters and hormones. These channels comprise GRIK1 proteins encoded by the *KCNJ3* gene. Increased expression of the *KCNJ3* gene, which increases *GRIK1* mRNA, is also associated with lung cancer, pancreatic adenocarcinoma, and breast cancer. Up-regulation of the *KCNJ3* gene in breast tissue samples has been reported to cause down-regulation of *p53* and up-regulation of estrogen receptor (ER). Patients with ER+ breast cancer and high *KCNJ3* expression have a worse prognosis and lower survival than ER+ patients with low expression of this gene. Therefore, ER+ patients can be divided into high-risk and low groups based on their expression levels. Based on all this evidence, the study of salivary *KCNJ3* gene expression in BC is valuable for validating this gene as an independent and prognostic biomarker for BC patients [13, 15].

Several studies have examined the effects of potassium channels and ions on various diseases in the saliva and the interaction effects of the disorders and potassium ions [16, 17]. Furthermore, calcium ions are known to play an essential role in the progression of BC [18, 19]. This study aimed to evaluate the salivary *KCNJ3* expression level of BC patients and healthy controls. Furthermore, in combination with *in silico* analysis, this study examined the possible roles of *KCNJ3* during BC development.

2. MATERIALS AND METHODS

2.1. Patient Enrollment and Samples

Forty-three BC women patients in Imam-Khomeini Hospital (Tehran, Iran), and forty-three women were referred to the dental clinic of Imam-Khomeini Hospital for routine oral examinations as controls were enrolled (Fig. **1**). BC in patients was confirmed by histopathological and biopsy results. The demographic characteristics of the enrolled persons in the study are shown in Table **1**.

Table 1. Demographic and clinicopathologic characteristics data of enrolled persons in this study.

-	BC Patients (n=43)	Healthy Volunteer (n=43)
Age, years		
Mean ± standard deviation (SD)	50.26±12.87	49.35±13.88
Range	25-85	21-80
Smoking, n (%)	1 (2.3%)	2 (4.6%)
Alcohol, n (%)	0 (0%)	0(0%)
Stages		
Stage I	11	N/A
Stage II	18	
Stage III	14	
Hormone receptor status		
Estrogen receptor (ER+)	36	N/A
Estrogen receptor (ER-)	7	
progesterone receptor (PR+)	32	
progesterone receptor (PR-)	11	
HER2 status		
Score 0, 1 (Negative)	21	N/A
Score 2 (Borderline)	11	
Score 3 (Positive)	11	
Ki-67 labeling Index		
0%-15%	25	N/A
15%-30%	7	
30%-100%	11	

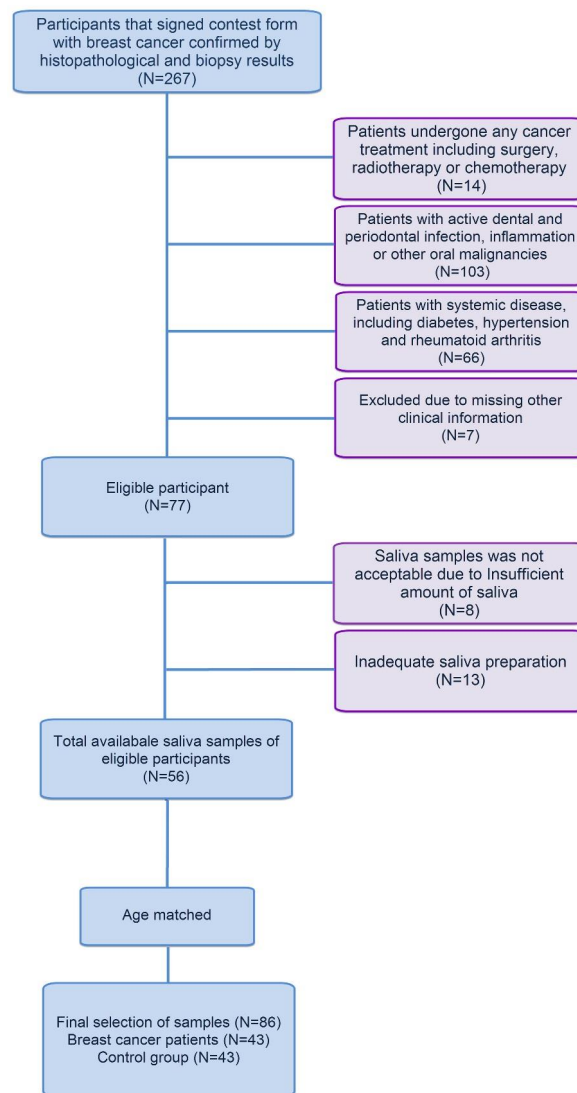| -	BC Patients (n=43)	Healthy Volunteer (n=43)
Subtype		
Invasive ductal carcinoma (IDC)	37	N/A
Invasive lobular carcinoma (ILC)	2	
Ductal carcinoma in situ (DCIS)	4	
IDC/DCIS	5	
Triple-negative	3	N/A

Fig. (1). STARD flow diagram of the participants.

Besides, patients have undergone no surgery, radiotherapy, or chemotherapy, and they were free of any active dental and periodontal infection, inflammation, or other malignancies. The patient also had no systemic diseases, including diabetes, hypertension, and rheumatoid arthritis. The enrolled people in the control group also had no active mouth infections, inflammation, or other malignancies and systemic diseases. Subjects in the case and control groups were matched based on age. After obtaining signed informed consent from the participants, 10 ml of unstimulated whole saliva samples were collected by the spitting method. To prevent the probable effect of circadian rhythm, sampling was done between 8-10 AM. Saliva samples were stored in -80 °C refrigerator. For avoiding detection bias, data analysis was performed in the blinding method. The local Ethics Committee of Tehran University of Medical Sciences approved the study (Ethical code: IR.TUMS.DENTISTRY.REC.1398064).

2.2. RNA Isolation

Total RNA extraction from saliva was performed by using

RiboEX (GeneAll, Seoul, South Korea) RNA Extraction kit according to the manufacturer's protocol. Moreover, the NanoDrop spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was employed to evaluate the purity, quality, and integrity of the extracted RNA. After RNA isolation, the eluted samples were stored at -80 °C.

2.3. cDNA Synthesis And Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

cDNA synthesis was performed by BioFact kit (BioFact, Daejeon, South Korea), according to the manufacturer's protocol. For this purpose, the RNA samples were incubated at room temperature for 5 minutes, 10 minutes at 25 °C, 60 minutes at 42 °C and 10 minutes at 75 °C. For quantitative real-time polymerase chain reaction (qRT-PCR), the Ampliqon SYBR Green master mix (Stenhuggervej, Denmark) and *KCJN3* specific primers were used (Table **2**). All reactions were performed in triplicate. qRT-PCR was performed by Rotor-Gene Q (Qiagen, Germany) as follows; one cycle of 5 minutes at 95 °C, 40 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C, 30 seconds at 72 °C, and one cycle of 5 minutes at 72 °C. The relative expression levels of miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method relative to the actin gene (ACTB).

Table 2. qRT-PCR primers of *KCJN3*.

Primer Sequences	Genes
5'TGAGGGACGGAAAACTCACG3'	*KCNJ3* Forward
5'GGCTGGTTCTCAGACGAGTT3'	*KCNJ3* Reverse
5'GATCAAGATCATTGCTCCTCCTG3'	ACTB Forward
5'CTAGAAGCATTTGCGGTGGAC3'	ACTB Reverse

2.4. Statistical Analysis

SPSS software (version 22; SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.2.1 for Windows (GraphPad Software, San Diego, California) were applied to analyze the qRT-PCR results. Mann-Whitney test was applied to compare *KCJN3* expression levels between case and control groups. Kruskal-Wallis test was used to analyze the expression level in various BC stages. Pearson's test was applied to analyze the correlation between age and gene expression levels. Finally, receiver operating characteristic (ROC) curve analysis was done to evaluate the potential role of *KCJN3* as a diagnostic biomarker in BC patients. All results are presented as mean ± standard deviation (SD), and p≤0.05 was considered statistically significant.

2.5. In Silico Analysis; Protein-protein Interaction (PPI) Network And Enrichment Of KCNJ3-relatEd Signaling Pathways

The string app in Cytoscape v3.7 [20] was employed to obtain the module in which *KCNJ3* is involved (maximum number of interactors=0 and confidence score≥0.9). Next, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [21] was used to identify the signaling pathways in which the module is involved. For this purpose, the human genome was set as the background, and signaling pathways were enriched based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis [22, 23].

3. RESULTS

None of the people in the case and control groups used alcohol, and only one person in the case group and two people in the control group smoked. There were also 11 patients in stage I, 18 patients in stage II, and 14 patients in stage III.

3.1. Expression Analysis of KCNJ3

Using qRT-PCR, the relative expression of *KCNJ3* in 43 saliva samples of BC patients and 43 saliva samples of controls was assessed. Besides, the expression level of *ACTB* in all samples was obtained. Considering *ACTB* as a reference gene, the relative expression of *KCNJ3* was determined. Here, we found that the relative expression of *KCNJ3* was significantly up-regulated in BC patients compared to healthy controls (p<0.001) (Fig. **2**).
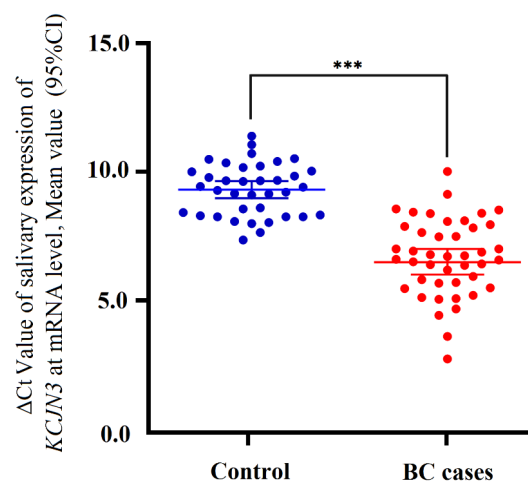

Fig. (2). qRT-PCR analysis of *KCNJ3* in the saliva of BC patients and healthy controls. P-values (p) are calculated using Mann-Whitney test (p=0.0001). *KCNJ3* was significantly up-regulated in BC patients relative to healthy controls. ACTB gene was considered as an endogenous reference to normalize the *KCNJ3* expression level.
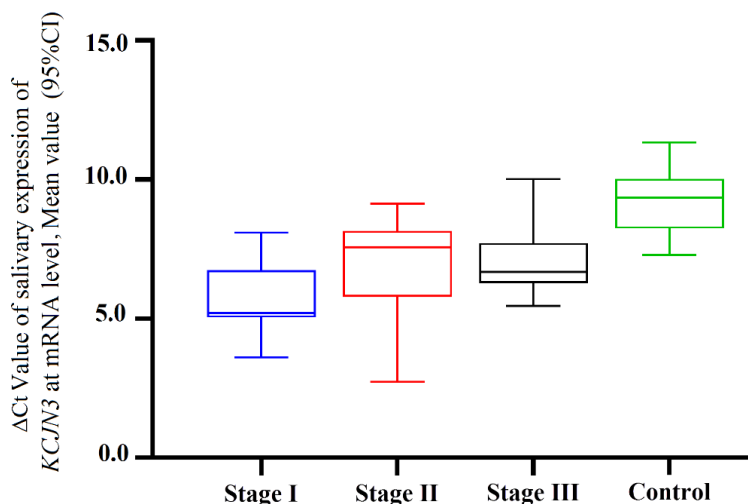
Fig. (3). Comparison of salivary gene expression at different stages of BC and control. Kruskal-Wallis test was applied to analyze the result. Statistically significant expression level alteration was found (p:0.049).

Furthermore, the differentially expressed *KCNJ3* was not statistically significant among various BC stages (p=0.061) (Fig. **2B**).

To determine the linear correlation between age and gene expression, the Pearson correlation analysis was performed. It was determined that there was no correlation between age and the salivary expression of *KCNJ3* in BC patients (p:0.077). Considering the cut-off value of 7.900, the sensitivity and specificity of *KCNJ3* mRNA in BC diagnosis were 76.7% and 94.59%, respectively. The area under the curve (AUC) of ROC curve was 0.923 (95%CI 0.866-0.979) (Fig. **3**). Considering cut-off values of 4.389 for differentiation of stage I *vs*. III, 4.148 for stage I *vs*. II, and 3.885 for stage II *vs*. III, we revealed that the AUC of ROC curves were 0.743 (95% CI:

0.536-0.951), 0.685 (95%CI 0.445-0.925) and 0.583 (95%CI 0.343-0.823) for stage I *vs*. III, stage I *vs*. II and finally stage II *vs*. III, respectively.

The positive likelihood ratio (PLR) for salivary *KCNJ3* was 14.20 (95% CI: 3.65-55.20) and the negative likelihood ratio (NLR) was 0.25 (95% CI: 0.14-0.43). PLR and NLR for discrimination of stage I *vs*. III were 2.077 (95% CI: 0.770-5.600), 0.461 (95% CI: 0.181-1.178). Meanwhile, PLR and NLR for differentiation of stage I *vs*. II: 2.250 (95% CI: 0.844-5.995), 0.375 (95% CI: 0.127-1.108) and PLR and NLR for diagnosis of stage II *vs*. III were 6.461 (95% CI: 0.926-45.094), 0.503 (95% CI: 0.273-0.928) respectively. ROC analysis results suggested that the *KCNJ3* expression level could be considered a diagnostic biomarker when comparing BC patients with healthy controls (Fig. **4**).
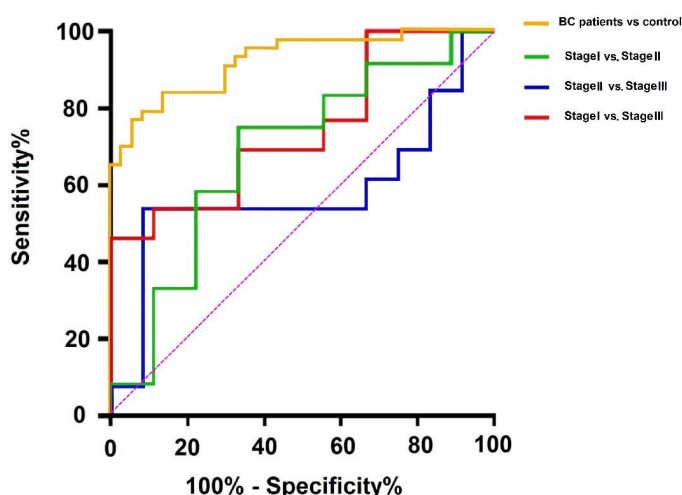

Fig. (4). ROC curve was analyzed to evaluate the value of *KCNJ3* for discrimination of BC cases from healthy control group and ROC cure for differentiation between different stages of BC (stage I *vs*. II, stage II *vs*. II, stage I *vs*. III).

3.2. Signaling Pathway Analysis

The protein-protein interaction (PPI) network in which *KCNJ3* is involved was obtained using the string app in Cytoscape. Except for *KCNJ3*, ten proteins correlate with each other in the main module with a high confidence score (≥0.9) (Fig. **5**).

Furthermore, the DAVID database was applied to enrich module-related signaling pathways. As is evident in Fig. (**5**), Morphine addiction (p=9.89E^{-20}), GABAergic synapse (p=4.31E^{-17}), and circadian entrainment (p=4.32E^{-14}) are the top enriched pathways (Fig. **6**).
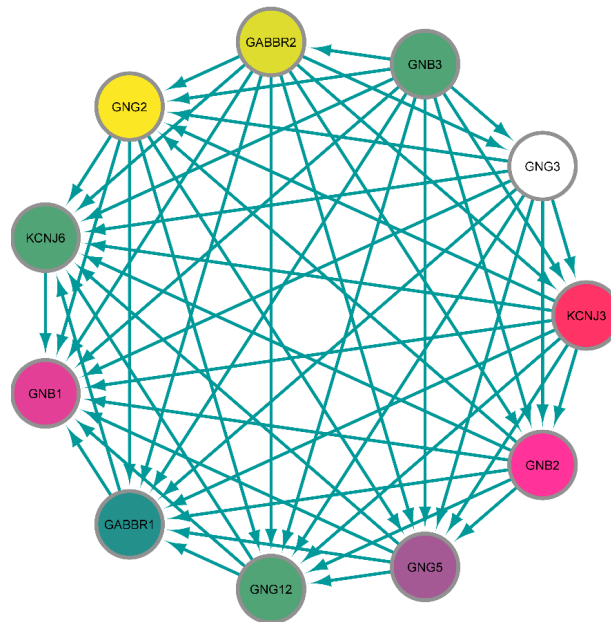

Fig. (5). Protein-protein interaction network analysis; KCNJ3 protein interacts with ten other proteins with a high-level confidence score (≥0.9), forming a powerful module, suggesting the module's significant role during BC development.
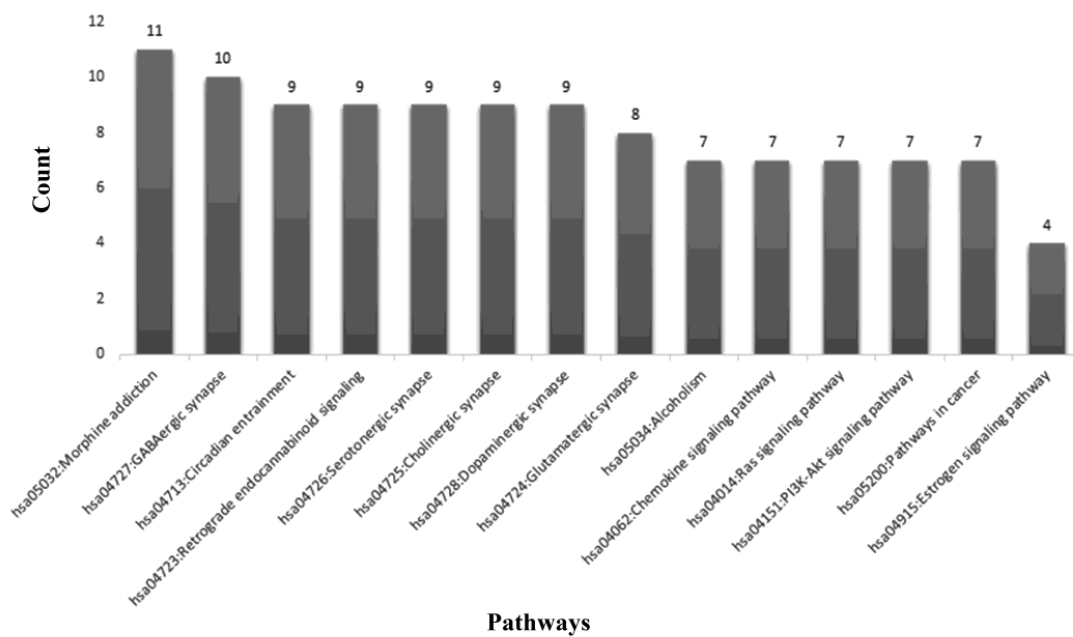

Fig. (6). Enrichment signaling pathways of the module in which *KCNJ3* is involved (p<0.05).

4. DISCUSSION

To date, several studies have been performed to evaluate salivary biomarkers in patients with BC. There are some benefits to using saliva to consider as biomarkers in malignancies. The most crucial advantage of saliva is the noninvasive sampling process and it is more accessible than serum or tissue sampling [24]. Moreover, since saliva sampling does not require special tools and equipment, screening efforts for anomalies like cancer are better than serum [25, 26]. Zhang *et al*. showed that the origin of salivary biomarkers in BC is probably due to the common embryonic origin of salivary gland tissue and breast tissue [9]. Meaning that many of the markers produced by breast tissue are also produced and secreted by the salivary glands. Therefore, common biomarkers have been observed and found in malignancies of both tissues [27]. Zhang *et al*. identified *CSTA, TPT1, S100A8, MDM4, MD6, H6PD, GRIK1*, and *GRM1* mRNAs in BC patients' saliva. According to this study, eight mRNAs have an acceptable potential for BC diagnosis with 83% sensitivity and 97% specificity [9].

For the first time, the mRNA expression level of *KCNJ3* gene in BC patients' saliva relative to healthy controls was evaluated. The results showed significant up-regulation of *KCNJ3* mRNA expression in BC patients' saliva samples compared with the healthy control group. Previous studies have shown that increased expression of *KCNJ3* mRNA is associated with BC metastasis to lymph nodes [28]. This gene is also up-regulated in primary and invasive BC [29]. In a study performed by Kammerer *et al*. on more than 1000 patients with BC showed that the *KCNJ3* gene was up-regulated in breast tissues compared to normal tissues [28]. Rezania *et al*. showed that the expression level of *KCNJ3* in patients with early-stage BC was significantly up-regulated compared to healthy individuals [15], which is consistent with the present study. Based on the ROC curve analysis, it was found that *KCNJ3* expression level could be considered a diagnostic biomarker in BC patients to evaluate this biomarker's sensitivity and specificity in the diagnosis of BC. Relying on its relatively high sensitivity (76.7%), patients can be easily identified as healthy individuals and its high specificity (94.59%) minimizes false positives. Therefore, this gene's salivary value could be considered a potential biomarker for the diagnosis of BC. Positive likelihood ratio higher than 10 and a negative likelihood ratio less than 0.1 results in an acceptable potential of salivary *KCNJ3* in BC diagnosis. *KCNJ3* expression levels were assessed in various disease stages; no statistically significant *KCNJ3* expression alterations were found among the various stages. Therefore, it could be claimed that from the beginning to the final stages, *KCNJ3* expression level is increasing, so it could be considered a diagnostic biomarker for early-stage BC. However, considering the salivary expression levels are not significantly different in the disease stages, this could be due to the study's small sample size. Based on the study by Lopez-Jornet *et al*., it is suggested that CA125 can be a potential diagnostic salivary biomarker besides *KCNJ3* mRNA level with acceptable sensitivity and specificity [30]. Finally, the PPI network analysis determined the leading *KCNJ3* gene network. Ten proteins were determined, including GNG12, GNG5, GNG3, GNB2, GNB3, GABBR2, GNG2,

GABBR1, KCNJ6, and GNB1, high correlate with the KCNJ3 protein. Moreover, using *in silico* analysis, the signaling pathway in which *KCNJ3* is involved was enriched (Fig. **5**). Gabaergic synapse signaling was enriched as one of the related pathways. γ-Aminobutyric Acid (GABA) is an inhibitory neurotransmitter, especially in the nervous system. However, the existence of GABA and GABA receptors in various tissues, including lung and oviduct with crucial roles, has been determined. Furthermore, it has been reported that GABA activity is increased in various types of cancers such as colon, gastric, and breast cancer and may affect the proliferation, growth, and metastasis of cancer cells [31, 32]. Zhang *et al*. proved that the GABAerigc signaling system is present in BC cells. GABA$_B$ receptor signaling promotes BC invasion *in vitro* and migration *in vitro* and *in vivo*, mediated by phosphorylation of ERK1/2 and, subsequently, up-regulation of matrix metalloproteinases-2 [33]. However, further studies are needed to clarify the effect of GABA on BC cell proliferation. Estrogen signaling is another pathway in which *KCNJ3* gene is involved. Estrogen receptors belong to the transcription factor family, a key regulator of involved genes in cell proliferation and migration. It has been reported that dysregulation and aberrant expression of ER signaling components lead to BC development and invasion [34]. For future studies, assessing the potential of KCNJ3 mRNA levels for breast cancer screening and the possibility of KCNJ3 protein for breast cancer diagnosis and targeted therapy is suggested.

CONCLUSION

This research was conducted to compare the levels of *KCNJ3* salivary expression in BC patients with healthy individuals. Differentiation criteria based on the salivary expression levels of *KCNJ3* were proposed to distinguish BC patients from healthy controls, using statistical and *in silico* analyses. K *CNJ3* expression at mRNA level in saliva could be a potential biomarker to detect BC from healthy control, especially in the primary stages of BC. Moreover, regarding the enriched signaling pathways such as GABAergic synapse signaling and estrogen signaling, further studies are needed to elucidate the exact role of *KCNJ3* in the development of BC.

LIST OF ABBREVIATIONS

AUC	=	Area under the curve
BC	=	Breast cancer
CI	=	Confidence interval
DCIS	=	Ductal carcinoma *in situ*
ER	=	Estrogen receptor
ER+	=	Estrogen receptor-positive
GABA	=	γ-Aminobutyric Acid
HER2	=	Human epidermal growth factor receptor
IDC	=	Invasive ductal carcinoma
ILC	=	Invasive lobular carcinoma
KEGG	=	Kyoto Encyclopedia of Genes and Genomes
NLR	=	Negative likelihood ratio
PLR	=	Positive likelihood ratio

PR+	=	Progesterone receptor-positive
PPI	=	Protein-protein interaction
qRT-PCR	=	Quantitative real-time polymerase chain reaction
ROC	=	Receiver operating characteristic
SD	=	Standard deviation

AUTHORS' CONTRIBUTIONS

MK and MJ conceived the study idea. MK, MJ, SM, and RM created the study protocol, led the data collection, and wrote the original draft. MK, MJ, and RM contributed to the data analysis/interpretation and preparation of the manuscript. MK and SM led the writing- review and editing. MK and SK interpreted the results. All authors have read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by Tehran University of Medical Science Ethical Committee (ethical code: IR.TUMS.DENTISTRY.REC.1398064).

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the base of this research. All the human procedure were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

After describing the study objectives, written informed consent was obtained from all participants.

STANDARD OF REPORTING

STROBE guidelines were followed.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author [S.M] on reasonable request.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

FUNDING

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