Evaluation of the Potential Diagnostic Role of the Lnc-MIAT, miR-29a-3p, and FOXO3a ceRNA Networks as Noninvasive Circulatory Bioindicator in Ductal Carcinoma Breast Cancer

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ABSTRACT

BACKGROUND: Over the last few decades, tremendous progress has been achieved in the early detection and treatment of breast cancer (BC). However, the prognosis remains unsatisfactory, and the underlying processes of carcinogenesis are still unclear. The purpose of this research was to find out the relationship between myocardial infarction–associated transcript (*MIAT*), *FOXO3a*, and *miRNA29a-3p* and evaluated the expression levels in patients compare with control and their potential as a noninvasive bioindicator in whole blood in BC.

METHODS: Whole blood and BC tissue are taken from patients before radiotherapy and chemotherapy. Total RNA was extracted from BC tissue and whole blood to synthesize complementary DNA (cDNA). The expression of *MIAT, FOXO3a*, and *miRNA29a-3p* was analyzed by the quantitative reverse transcription-polymerase chain reaction (RT-qPCR) method and the sensitivity and specificity of them were determined by the receiver operating characteristic (ROC) curve. Bioinformatics analysis was used to understand the connections between *MIAT, FOXO3a*, and *miRNA29a-3p* in human BC to develop a ceRNA (competitive endogenous RNA) network.

RESULTS: We identified that in ductal carcinoma BC tissue and whole blood, *MIAT* and *FOXO3a* were more highly expressed, whereas *miRNA29a-3p* was lower compared with those in nontumor samples. There was a positive correlation between the expression levels of *MIAT, FOXO3a*, and *miRNA29a-3p* in BC tissues and whole blood. Our results also proposed *miRNA29a-3p* as a common target between *MIAT* and *FOXO3a*, and we showed them as a ceRNA network.

CONCLUSIONS: This is the first study that indicates *MIAT, FOXO3a*, and *miRNA29a-3p* as a ceRNA network, and their expression was analyzed in both BC tissue and whole blood. As a preliminary assessment, our findings indicate that combined levels of *MIAT, FOXO3a*, and *miR29a-3p* may be considered as potential diagnostic bioindicator for BC.

KEYWORDS: Breast cancer, noninvasive bioindicator, ceRNA network, MIAT, miRNA29a-3p, FOXO3a, bioinformatics

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Introduction

With an estimated 2.3 million new cases of breast cancer (BC) annually (11.7%), it surpassed lung cancer as the most commonly diagnosed cancer in 2020.[1] Both BC incidence and mortality rates are increasing among Iranian women.[2] Iranian women develop this disease at least a decade sooner than women in developed countries and are usually diagnosed with this disease at the age of 40 to 49 years.[3] Overcoming chemoresistance and distant-site metastasis is critical for improving outcomes in BC patients, as these factors continue to pose significant challenges to effective management and treatment of the disease.[4] Despite advances in treatment options, metastatic disease relapses persist and are a leading cause of patient mortality.[5] As a result, increased insight into the molecular mechanisms involved in BC progression could lead to the discovery of more accurate prognostic biomarkers and the development of targeted therapies with enhanced efficacy.

Breast cancer prognosis can be assessed based on classical clinicopathological features including cancer tumor size, histological subtype and grade, lymph node metastases, and lymphovascular invasion, all of which necessitate thorough histological analysis.[6] Although they can provide valuable prognostic information for some BC patients, their utility is limited by their low prognostic capacity and may not be applicable to all individuals.[7] Biopsy-based cancer detection can be uncomfortable, hazardous, costly, time-intensive, and reliant on pathologist proficiency. As such, alternative approaches are noninvasive, pain-free, easy to collect, and potentially cost-effective are gaining increasing attention.[8] Biomarkers are measurable signals that can identify malignancy or provide insight into tumor behavior, prognosis, or response to treatment.[9] The diagnosis of BC relies primarily on imaging, pathology, and serological markers.[10] In spite of their advantages, they do have limitations, including invasiveness, inconvenience, high costs, and a high rate of false-positive results.

Mammography is widely accepted as the most reliable method for diagnosing BC, but it has some limitations, such as the harmful effects of ionizing radiation and low sensitivity in detecting early-stage cancer. Although a needle biopsy or surgical biopsy is commonly used to confirm BC, it is not needed for most benign tumors, and the procedure can be invasive and uncomfortable. The circulating tumor biomarker-based method is a promising alternative to the methods mentioned earlier, as it is simple, convenient, and cost-effective for detecting BC at an early stage and predicting its progression or recurrence.[11-13] Therefore, further research into the molecular etiology of BC is required to identify novel biomarkers and therapeutic targets.

Apoptosis is critical for maintaining a balance between cell death and cell division; thus, deregulation of this pathway leads to uncontrolled cell proliferation, which is involved in many different diseases, such as cancer.[14] The intrinsic pathway of apoptosis is regulated by various factors, such as the *Bcl-2* family and the PI3K/AKT pathway.[15] Numerous studies have shown that the PI3K/AKT pathway is involved in the progression of a variety of tumors through regulating cellular growth and blocking apoptosis.[16] It has been reported that *AKT* interferes with cell death pathways through the phosphorylation of *FOXO3a*.[17,18] *FOXO3a*, a transcription factor of the Forkhead box O (FOXO) family, possesses a vital role in regulating several cellular processes, such as proliferation, apoptosis, DNA damage, and cell-cycle progression.[19] Notably, in the PI3K/AKT pathway, activation of *FOXO3a* induces apoptosis, cell-cycle arrest, and stress resistance in most tissues, whereas *FOXO3a* inactivation triggers cell survival, proliferation, and stress sensitivity.[20] In BC, the deregulation of the PI3K/AKT pathway and its increased activity are associated with reduced diagnosis in patients.[21]

Long noncoding RNAs (lncRNAs) are a sort of noncoding RNA (ncRNA) that are described as transcripts with a length of more than 200 nucleotides.[22] Recent research has demonstrated that lncRNAs are critical regulators of a variety of biological processes, including innate immunologic responses, genetic expression regulation, post-transcriptional processes, proliferation, invasion, metastasis, and angiogenesis of cancer cells.[23] Accordingly, there is significant evidence suggesting lncRNAs function as competing endogenous RNAs (ceRNAs) to restrict microRNA (miRNA) expression or activity. Thus, lncRNA has the potential to be a sensitive cancer diagnostic biomarker.[24] Myocardial infarction–associated transcript (*MIAT*) is involved in the control of cancer cell apoptosis, cell-cycle regulation, migration, and invasion.[25] In BC cells, knocking down *MIAT* inhibited cell growth and promoted apoptosis. It has been reported that, because of the strong invasive and metastasis ability of MIAT, it has increased in BC cell lines than normal breast cell lines. Moreover, proliferation, migration, invasion, and epithelial-to-mesenchymal transition (EMT) of BC cells were inhibited by the knockdown of MIAT, whereas the rate of apoptosis was promoted. In a xenograft model, the lowest expression of MIAT was associated with decreasing tumor growth and delaying tumor formation, thus indicating *MIAT* acts as an oncogene.[26]

MicroRNAs are classified as noncoding mRNAs with approximately 22 nucleotides in length. Recently, a growing body of research has established that miRNAs exclusively attach to the 3'-untranslated region (3'-UTR) of messenger RNA; hence, gene expression is controlled at the post-transcriptional level.[27] Therefore, changing microRNA expression levels are involved in the onset and progression of a variety of diseases, such as cancer.[28] *miRNA-29a-3p* has a critical role in different biological processes such as proliferation, apoptosis, and cell-cycle regulation. It has been reported that *miRNA-29a-3p* has a significant impact on cancer development by acting as tumor suppressors.[29]

In addition, *miRNA-29a-3p* expression levels have been discovered to be abnormally low in a variety of human malignancies, including papillary thyroid carcinoma (PTC), hepatocellular carcinoma, and gastric cancer.[30] Previous research found that *miRNA-29a-3p* is involved in the progression of BC. Moreover, circRNA *ACAP2* (*circACAP2*) increases BC metastasis and proliferation via sponging *miRNA-29a-3p*.[31]

The purpose of the study was to evaluate the expression levels of *MIAT, FOXO3a*, and *miRNA-29a-3p* as a ceRNA network in the BC tissues compared with the whole blood of BC patients, investigate their relationship with the clinical features of the tumor, and examine their potential as noninvasive biomarkers in BC.

Materials and Methods

Bioinformatics analysis

Protein-protein interaction analysis. The STRING server (https://string-db.org/) is used to investigate protein-protein interaction networks. We searched for *FOXO3a* in the STRING database and found the top 10 proteins that are related to *FOXO3a*. Then, we sorted out these connections based on text mining, experiments, and databases.

Analysis of STRING enrichment. The Gene Ontology (GO) is divided into 3 categories: biological process (BP), molecular function (MF), and cellular component (CC). Along with GO, the Kyoto Encyclopedia of Genes and Genomes (KEGG) is a valuable resource for studying biological pathways. Thus, we used the KEGG and the databases to investigate functions and pathways to determine their biological meaning.

In this study, after finding the protein-protein interaction of *FOXO3a*, GO enrichment and KEGG pathway related to *FOXO3a* protein-protein network from the STRING database were downloaded. They were categorized based on adjusted *P* value, and the top 10 were chosen. Enrichment analysis of *FOXO3a* was visualized in R 4.0.5 software (https://www.r-project.org/) based on the ggplot2 package (https://cran.r-project.org/web/packages/ggplot2/index.html).

The interaction between MIAT, FOXO3a, and miRNA29a-3p. The interaction between *MIAT, FOXO3a*, and *miRNA29a-3p* was determined using TargetScan (http://www.targetscan.org/) and the Starbase database (https://starbase.sysu.edu.cn/).

Construction of the competitive endogenous RNA network. We predicted the miRNAs that interacted with *MIAT* and *FOXO3a* using the miRNet (https://www.mirnet.ca/) and the miRDB (http://mirdb.org/) databases, respectively. Using miRNet, 46 miRNAs were found for MIAT, and using miRDB, 90 miRNAs based on target score were found for *FOXO3a*.

We combined *MIAT*-miRNAs and *FOXO3a*-miRNAs and applied them to Cytoscape software (v3.0.9) (https://cytoscape.org/), and then the ceRNA network was constructed based on the results.

Patients and sample collection

A total of 120 samples were taken from the Imam Khomeini Hospital (Tehran, Iran) among which 70 samples were collected from BC patients (35 blood samples, 35 tissue samples), and 50 cases were collected from healthy women (25 blood samples, 25 normal breast tissue). The mean age of the patients was 47 ± 3.1 years. All patients had neither chemotherapy nor radiotherapy treatment before surgery. The healthy individuals with no family history of any cancers and diseases and no history of alcohol consumption were selected. Also, their age (mean age 45 ± 5.4 years) and sex were consistent with the patient samples. The pathological features of patients were indicated in Supplementary Table 1.

RNA extraction

Total RNA was isolated from whole blood samples of BC patients and healthy controls according to the manufacturer instructions, using Trizol (Cat YT9066-YT9065-YT9064, Yekta Tajhiz Azma, Iran). The xylene-ethanol technique was used to eliminate paraffin from formalin-fixed, paraffin-embedded (FFPE) tissue, and afterward, overall RNA was extracted using Trizol Reagent (Cat YT9066-YT9065-YT9064, Yekta Tajhiz Azma) according to the manufacturer's instructions. RNA quantification and the 260/280 nm ratio were evaluated by NanoDrop NP80 (Implen, Germany). Subsequently, total RNA was treated by RNase-free DNase (Cat MO5401, SinnaClon, Iran) to eliminated genomic DNA.

Quantitative reverse transcription–polymerase chain reaction

High-capacity complementary DNA (cDNA) reverse transcription kit (Cat RP1400, Smobio, Taiwan) was used for cDNA synthesis. Specific primers were designed for *MIAT*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) using oligo primer analysis software (version 0.7.0), a specific primer for *FOXO3a* was used from primer bank Harvard (Gen Bank Accession: NM_001455), and stem-loop RT primer was used for the reverse transcription of *miRNA29a-3p* and is taken from Wang et al.[32] We normalized our data to *U6* expression levels as a reference gene. The primer sequences are shown in Supplementary Table 2.

The relative expression was carried out by SYBR Green RealQ_Plus 2× Master Mix Green (CatA325402, Ampliqon, Denmark) using the Applied Biosystems StepOnePlus™ (Waltham, MA, USA). Polymerase chain reaction reactions of *FOXO3a* and *GAPDH* were performed by applying the following thermal protocol: 95°C for 10 minutes, followed by 40 cycles of amplification (95°C for 15 seconds, 60°C for 45 seconds, and 60°C for 60 seconds). Polymerase chain reaction reactions of *miRNA29a-3p*, *MIAT*, and *U6* were incubated at 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and finally 72°C for 30 seconds.

The specificity of our RT-qPCR amplification was validated by melting curves analyses. The *GAPDH* and *U6* levels were used as reference genes as earlier research on *GAPDH* and *U6* expression levels demonstrated consistent expression in multiple organs, good stability, and little fluctuation in circulation. Thus, our data were normalized by *GAPDH* and *U6*. The PCR products were confirmed on 1.5% agarose gel.

Western blot analysis

Total proteins were extracted from tissues by lysis buffer (radioimmunoprecipitation assay buffer [RIPA]) (Cat 89900, Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was detected by the Bicinchoninic Acid Assay (BCA) method (Cat 23225, Thermo Fisher Scientific). 40 µg of lysates protein were separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membranes. These membranes were blocked with 5% skim milk powder for 1 hour and then incubated with rabbit polyclonal antibodies to *FOXO3a* (Cat 720128, Thermo Fisher Scientific) overnight at 4°C. After washing, these members were followed by incubation with horseradish peroxidase (HRP)–conjugated secondary antibody (Cat 15165, Thermo Fisher Scientific) for 2 hours at 4°C. The bound antibodies were detected using an ECL kit (Cat 32106, Thermo Fisher Scientific). Protein bands were visualized and quantified using Quantity-One software (Bio-Rad, Hercules, CA, USA). β-actin, as an internal control, was detected with antibeta-actin monoclonal antibody (1:1000) (Cat AM4302, Thermo Fisher Scientific).

Statistical analysis

All reactions were run in duplicates. The amplification efficiency was analyzed using LinReg PCR software (version 0.1.0). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative level of gene expression. The analytical distinction between the

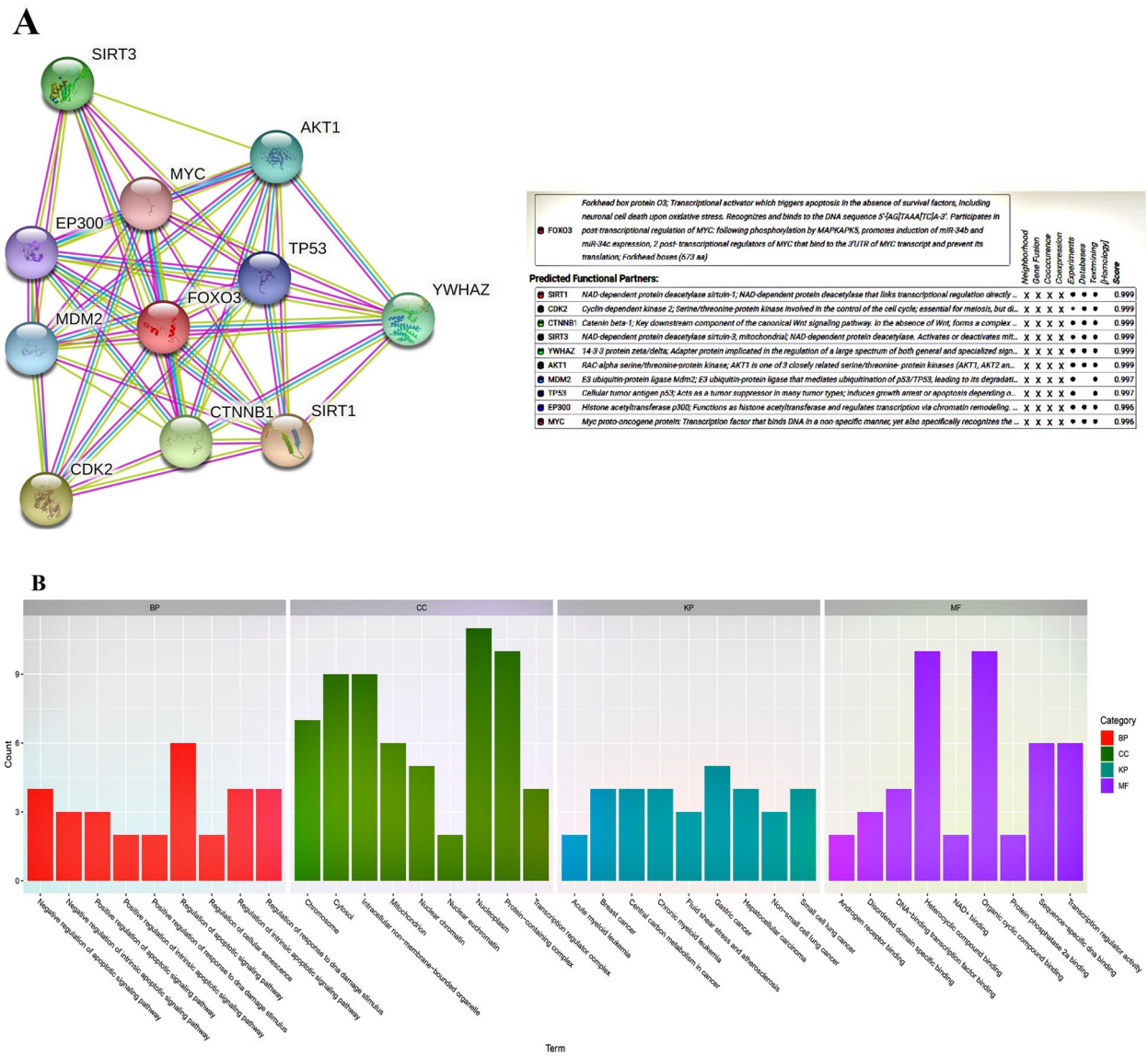

Figure 1. (A) Protein-protein interaction network of *FOXO3a* with 10 partners. (B) Enrichment analysis of *FOXO3a* protein-protein network. BP, indicates biological process; CC, cellular component; MF, molecular function; PK, protein kinases.

groups was evaluated by the 1-way analysis of variance (ANOVA) and independent sample *T*-test, which was executed by the Graph Pad 8.0.2 software (GraphPad Software Inc, San Diego, CA, USA), and P value $< .05$ was considered statistically notable. Indeed, to evaluate the diagnostic ability of *MIAT, FOXO3a*, and *miRNA29a-3p* and in combination receiver operating characteristic (ROC) curve and area under the curve (AUC) were used. The appropriate cut-off values for *MIAT, FOXO3a*, and *miRNA29a-3p* were determined, and based on these cut-off points, the sensitivity and specificity were evaluated.

Results

Bioinformatics analysis

Protein-protein interaction analysis. The interaction analysis revealed that *FOXO3a* protein-protein network is related to nicotine adenine dinucleotide (NAD)–dependent protein deacetylase sirtuin-1 (*SIRT1*), cyclin-dependent kinase 2 (*CDK2*), catenin beta-1 (*CTNNB1*), NAD-dependent protein deacetylase sirtuin-3 (*SIRT3*), 14-3-3 protein zeta/delta (*YWHAZ*), RAC-alpha serine/threonine-protein kinase (*AKT1*), E3 ubiquitin-protein ligase *Mdm2* (*MDM2*), cellular tumor antigen p53 (*TP53*), histone acetyltransferase p300 (*EP300*), and proto-oncogene protein (*MYC*) (Figure 1A). These genes that are involved in protein-protein networks have an important role in the regulation of the apoptotic signaling pathway.

Enrichment analysis based on protein-protein interactions that were constructed in the string database indicated that these genes that are part of protein-protein network in BP in regulation of response to DNA damage stimulus, apoptotic signaling pathway, cellular senescence,

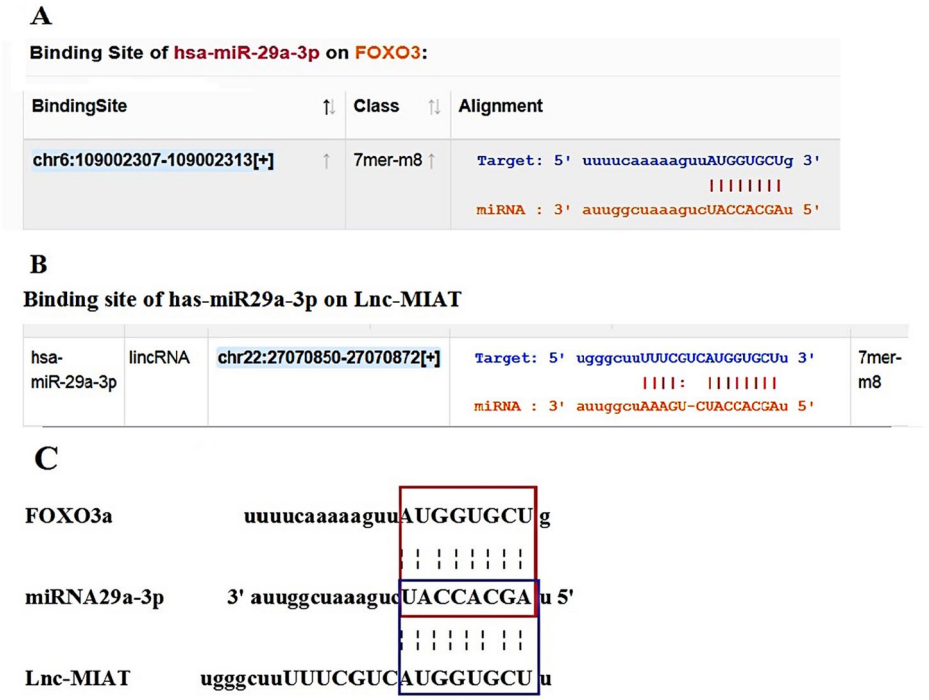

Figure 2. (A) The predicted binding sites of *FOXO3a* to *miRNA-29a-3p* sequence were illustrated. (B) The putative binding sites between *MIAT* and *miRNA-29a-3p* according to the starBase. (C) Bioinformatics analysis revealed that *MIAT* and *FOXO3a* shared a common *miRNA-29a-3p*-binding site. The red box represented the binding site for *miR-29a-3p* on the *FOXO3a* mRNA 3'-UTR, and the blue box represented the binding site for *miR-29a*-3p on *MIAT*.
lnc indicates long noncoding; MIAT, myocardial infarction–associated transcript; miRNA, microRNA; 3'-UTR, 3'-untranslated region.

and according to MF in disorder domain-specific binding, androgen receptor binding, and DNA-binding transcription factor binding are involved. Moreover, CC showed that they are in the cytosol, nucleoplasm, and protein-containing complex. The KEEG pathway has shown the role of theme in various cancer such as BC, gastric cancer, and nonsmall cell lung cancer (Figure 1B).

miR29a-3p has a common binding site with MIAT and FOXO3a. Bioinformatics investigation revealed that *FOXO3a* (Figure 2A) and *MIAT* (Figure 2B) shared a common *miR-29a-3p* binding site. Moreover, both *MIAT* and *FOXO3a* had a conserved *miR-29a-3p* binding sites on their 3'-UTRs (Figure 2C).

The construction of MIAT and FOXO3a competitive endogenous RNA network. Long noncoding RNA can decrease the suppression of mRNA expression by competitively binding to miRNA (23). According to this theory, negative connections exist between miRNAs and lncRNAs or mRNAs, whereas positive correlations exist between mRNAs and lncRNAs.

MIAT and *FOXO3a* in this study were subjected to further bioinformatics analyses to identify the most commonly predicted target miRNAs. The miRNet and miRDB databases were used to predict target miRNAs of *MIAT* and *FOXO3a*, respectively. The combination of miRNAs according to 1 *MIAT* node, 1 gene *FOXO3a* node, 136 miRNA nodes, and 136 edges, which were visualized using Cytoscape. So, as it was demonstrated in Figure 3, *miRNA29a-3p* is only common target between *MIAT* and *FOXO3a*. This finding showed that the *MIAT-miRNA29a-FOXO3a* network acts as a sponge based on competitive endogenous RNA (ceRNA). According to this theory, MIAT can act as ceRNA network to regulate *FOXO3a* expression by sponging *miR-29a-3p* (Figure 3). However, further research is needed in the future.

Differential expression of MIAT, FOXO3a, and miRNA29a-3p in patients' whole blood and healthy blood

Our studies showed that the expression levels of *MIAT* and *FOXO3a* are remarkably higher in the whole blood of BC patients compared with healthy individuals ($P < .04$ and $P < .02$, respectively). In contrast, the expression level of *miRNA29a-3p* ($P < .001$) was substantially lower in the patient group compared with the control groups ($P < .001$) (Figure 4).

Differential expression of MIAT, FOXO3a, and miRNA29a-3p in ductal carcinoma tissue and normal breast tissue

Our outcomes revealed that the total mean expression levels of *MIAT* and *FOXO3a* were dramatically much higher in

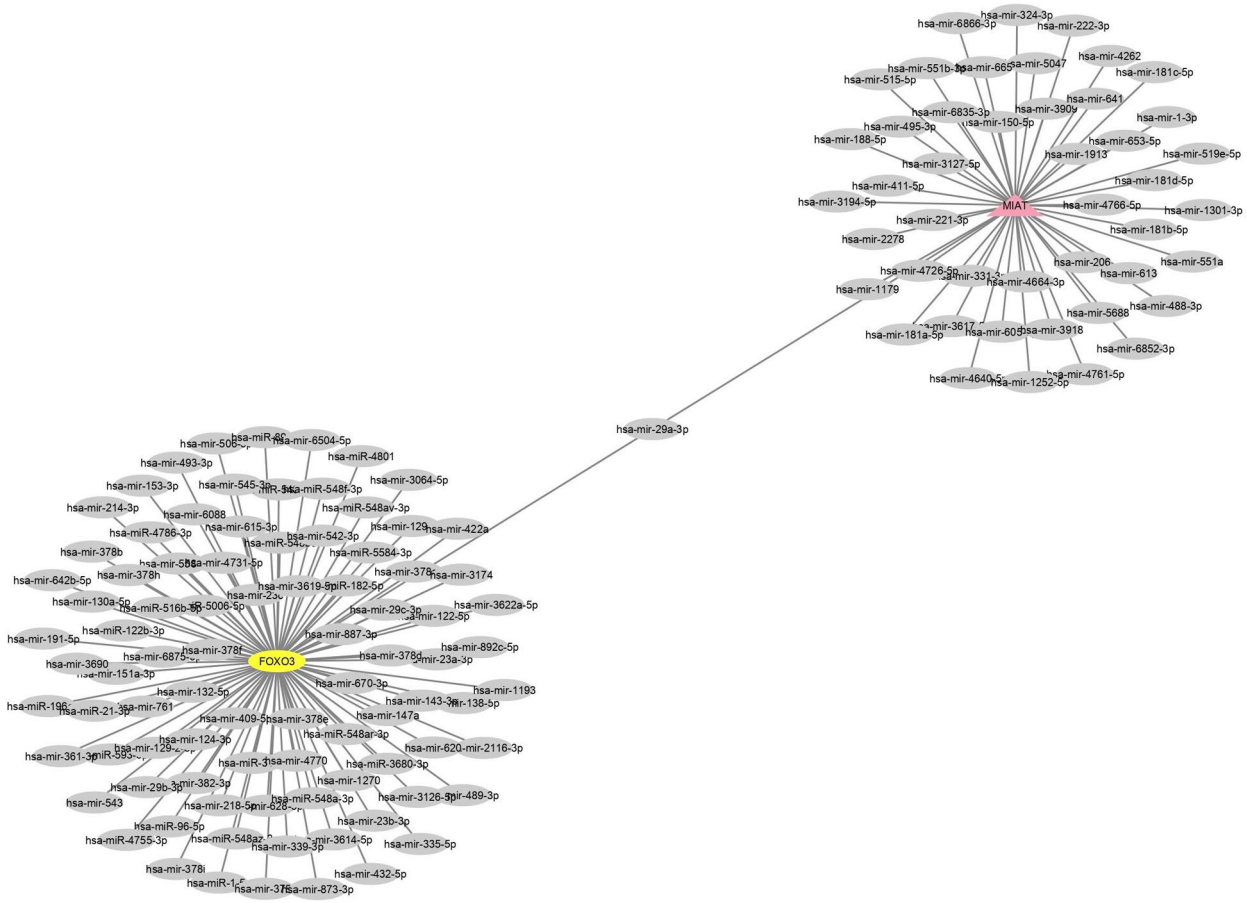

Figure 3. ceRNA network for *MIAT, miRNA29a-3p*, and *FOXO3a* (pink = MIAT, yellow = FOXO3a, and gray = miRNAs).
ceRNA indicates competitive endogenous RNA; MIAT, myocardial infarction–associated transcript; miRNA, microRNA.

BC tissue than normal breast tissue ($P < .03$ and $P < .006$, respectively). In comparison with the control groups, *miRNA29a-3p* expression levels were significantly lower in the patient group ($P < .05$) (Figure 5A).

Western blot analysis of FOXO3a protein in ductal carcinoma breast cancer tissue and normal breast tissue

Our result indicated that the expression level of *FOXO3a* in freshly collected ductal carcinoma BC tissue was significantly higher than normal breast tissue, as shown in Figure 5B. Collectively, these data suggest that the expression of *FOXO3a* might be a frequent event in BC tissues.

Differential expression of MIAT, FOXO3a, and miRNA29a-3p based on clinical features of the tumor in ductal carcinoma breast cancer tissue and whole blood

The result of this study indicated that overexpression of *MIAT* was associated with stage I and stage II breast tumors and lymph node involvement in both BC tissue ($P < .01$ and $P < .03$, respectively) and whole blood ($P < .04$ and $P < .002$, respectively). There was no significant difference in estrogen receptor (ER), human epidermal growth factor receptor 2 (Her2), progesterone receptor (PR) status in BC tissue, and whole blood. However, upregulation of *FOXO3a* was positively associated with ER-positive ($P < .02$), lymph node involvement ($P < .01$), and tumor, node, and metastasis (TNM) stage ($P < .004$) in BC tissue. Moreover, there was a significant difference in the upregulation of *FOXO3a* and ER-positive ($P < .04$), lymph node involvement ($P < .01$), and TNM stage ($P < .007$) in whole blood, but there was no significant difference in Her2, PR status, and ER-negative in BC tissue and whole blood.

In contrast, low expression of *miRNA29a-3p* was clearly associated with ER-positive in both BC tissue ($P < .006$,) and whole blood ($P < .004$), but significant difference has not been seen between downregulation of *miRNA29a-3p* and other features of the tumor.

Assessment of the diagnostic performance of MIAT, FOXO3a, and miRNA29a-3p in whole blood and ductal carcinoma breast cancer tissue

MIAT, FOXO3a and *miRNA29a-3p* were differently expressed in the whole blood of BC and healthy individuals; hence, ROC

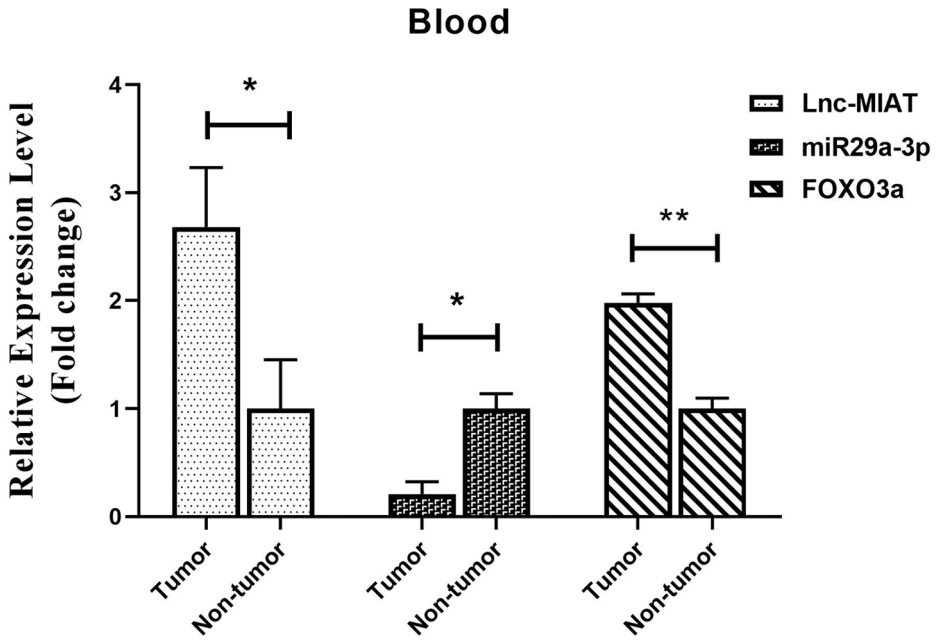

Figure 4. Expression of *MIAT, jFOXO3a*, and *miRNA29a-3p* in the whole blood of breast cancer patients in comparison to the normal control group ($P < .04$, $P < .02$, and $P < .001$, respectively).
lnc indicates long noncoding; MIAT, myocardial infarction–associated transcript; miRNA, microRNA.

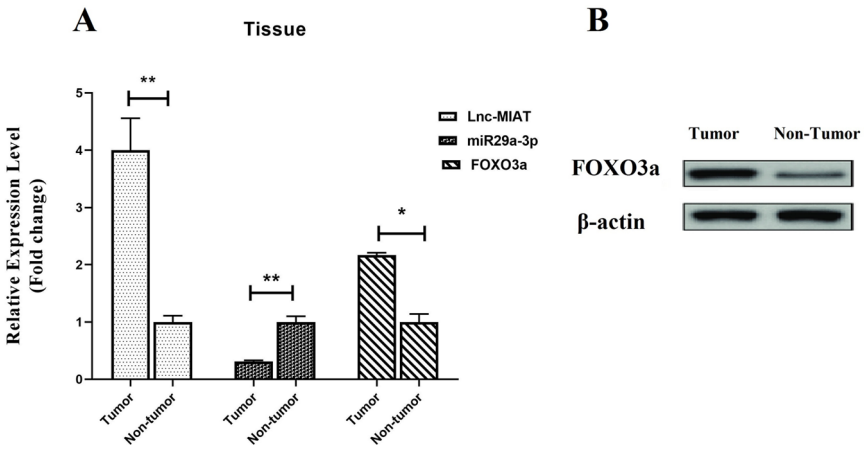

Figure 5. (A) Expression of *MIAT, FOXO3a*, and *miRNA29a-3p* in BC tissue in comparison with normal breast tissue ($P < .03$, $P < .006$, and $P < .05$, respectively). (B) Western blot analysis of *Foxo3a* abundance in BC tissues and normal breast tissue. The total protein extracted from breast tissues was analyzed using a polyclonal antibody against human *FOXO3a*. β-actin was used as a loading control.
BC indicates breast cancer; lnc, long noncoding; MIAT, myocardial infarction–associated transcript; miRNA, microRNA.

curve was applied to predict the potential of these genes in differentiating BC from healthy individuals. *MIAT* at the cut-off 0.8 with a sensitivity of 78%, a specificity of 100%, and AUC values 0.830 (Supplemental Figure 6A), *FOXO3a* at the cut-off 0.9 with a sensitivity of 90%, a specificity of 100%, and AUC values 0.900 (Supplemental Figure 6B), and *miRNA29a-3p* at the cut-off 0.8 with a sensitivity of 100%, a specificity of 80%, and AUC values 0.910 (Supplemental Figure 6C) detect BC from healthy women, respectively.

In addition, ROC curve was used to assess potential of *MIAT, FOXO3a*, and *miRNA29a-3p* to distinguish between BC tissue and normal samples. *MIAT* at the cut-off 0.6 with a sensitivity of 88%, a specificity of 80%, and AUC values 0.880 (Supplemental Figure 6E), *FOXO3a* at the cut-off 0.8 with a sensitivity of 75%, a specificity of 100%, and AUC values 0.853 (Supplemental Figure 6F) and *miRNA29a-3p* at the cut-off 0.8 with a sensitivity of 80%, a specificity of 100%, and AUC values 0.940 (Supplemental Figure 6G) distinguish BC from healthy individuals, respectively.

Furthermore, the diagnostic accuracy of the combination of *MIAT, FOXO3a*, and *miRNA29a-3p* in whole blood and BC tissue was performed. In the whole blood, the combination of

MIAT, FOXO3a, and *miRNA29a-3p* diagnoses BC from healthy women at the cut-off 0.9 with a sensitivity of 95%, a specificity of 90%, and AUC values 0.990 (Supplemental Figure 6D), and in BC tissue, they detect BC from normal samples at the cut-off 0.9 with a sensitivity of 90%, a specificity of 100%, and AUC values 0.100 (Supplemental Figure 6H).

Discussion

The existing diagnosis approach cannot detect cancer early and it influences the life quality of patients. As a result, it is critical to find a biomarker that would be accessible, cost-effective, and sensitive enough to detect and monitor BC patients. The purpose of this research was to determine the interaction between *MIAT, FOXO3a*, and *miRNA29a-3p* and compare the expression levels of them in BC tissue with whole blood to see if they could be useful bioindicator for ductal carcinoma BC diagnosis and management in clinical practice. This study revealed the overexpression of *MIAT* and *FOXO3a* and the downregulation of *miRNA 29a-3p* in the tissues and whole blood of Iranian women with BC ductal carcinoma (Figures 4 and 5A and B). The AUC values for *MIAT, FOXO3a*, and *miRNA29a-3p* indicated that they are effective candidates with a high degree of specificity and sensitivity for the diagnosis of ductal carcinoma BC.

Moreover, *MIAT-miRNA29a-3p-FOXO3a* have been seen as the ceRNA networks in BC. One of the main strengths of this study is the construction of the ceRNA network using bioinformatics analysis based on *MIAT*-targeting and *FOXO3a*-targeting. According to Figure 3, *miRNA29a-3p* was considered the only common target between *FOXO3a* and *MIAT*.

The *MIAT*/*miRNA-29a-3p*/*FOXO3a* ceRNA network has been described for the first time, which may help us identify a novel ceRNA network involved in the regulation of BC.

The apoptotic pathway is critical for tumor growth and metastasis at all stages. Apoptosis is a BP that contributes significantly to the growth and survival of multicellular organisms by eliminating damaged, old, or autoimmune cells through a regulated cell death mechanism.[33] Apoptosis includes a large number of signaling pathways, which is considered a precise regulatory mechanism.[34] Therefore, any sort of alternation in these pathways leads to tumorigenesis, metastasis, and resistance to anticancer drugs. Thus, cell-cycle control mechanisms have emerged as a potential therapeutic strategy.[35]

In this study, the STRING database and enrichment analysis based on BP and KEEG pathway indicated that *FOXO3a* protein-protein network has a critical role in the regulation of the apoptosis signaling pathway and has both positive and negative effects on the regulation of the intrinsic apoptosis pathway (Figure 1A and B). Among them, *MYC, TP53, CDK2, AKT*, and *MDM2* are important regulators in cell-cycle progress and the apoptosis pathway.

It has been reported that *FOXO3a* acts as a metastasis suppressor because it increases the expression level of E-cadherin and downregulates EMT transcription factors. This resulted in the reversal of the invasive behavior of BC cells.[36] Song et al[37] reported that upregulation of *FOXO3a* substantially inhibited BC cell migration and invasion in vitro. In this study, we have shown significant upregulation of *FOXO3a* in BC tissues and whole blood compared with normal groups. In accordance with our results, the overexpression of *FOXO3a* in breast tumor tissues and cell lines has been shown in some studies.[38] It also has been shown that upregulation of *FOXO3a* promotes growth of cancer cells and tumor progression.[39] However, recent studies have suggested that the low expression of *FOXO3a* induces EMT and subsequently promotes cancer cell invasion and proliferation, which are associated with BC development and poor response to therapy.[40]

Estrogen receptor has a critical role in growth, proliferation, and differentiation in BC. There is abundant evidence to show crosstalk between *FOXO3a* and ER signaling pathways.[41] Sisci et al[42] demonstrated that, in BC cell lines, invasive phenotype of ERα+ had been reversed by activation of *FOXO3a*. Whereas in ERα− cell lines, tumor cell invasion had promoted significantly. Moreover, they proposed that according to functional interaction between *FOXO3a* and ERα, cell migration and invasion had reduced in ERα+ tumors. However, in the absence of receptor, *FOXO3a* triggered many pathways that lead to opposite consequences. Jiang et al[43] established a clear correlation between *FOXO3a* expression and ER-positive in human breast tumors and recognized *FOXO3a* as a promising prognostic marker. They proposed that a high level of *FOXO3a* expression was shown to be strongly associated with long-term survival in ER-positive cell lines. Thus, according to ER status, patients who were identified with FOXO3a+/ER+ had shown better prognoses compare with ER−/FOXO3a+. On the contrary, loss of ER in patients with downregulation of *FOXO3a* has shown that overall survive was being worse compared with ER+ patients. Moreover, they confirmed that *FOXO3a* expression was associated with lymph node involvement and TNM stage. Chen et al[44] observed that upregulation of *FOXO3a* was correlated with increased *AKT* expression and lymph node metastases. Along with these findings, our analysis showed that, overexpression of *FOXO3a* was positively associated with ER-positive, lymph node involvement, and TNM stage in BC tissue and whole blood.

MIAT regulates a variety of signaling pathways in cancer. In the study by Yang et al,[45] it was reported that *MIAT* has been identified as a critical factor in cell invasion, migration, and proliferation through the PI3K/AKT signaling pathway. *MIAT* dramatically increased PI3K and AKT phosphorylation and stimulated the production of *C-MYC* and cyclin D1. Recent literature indicated that the expression level of *MIAT* was higher in BC cells than in normal cell lines, as well as suggesting that *MIAT* may serve as an oncogene in BC, sharing *miRNA-155-5p* response element with *DUSP7* and promoting BC progression.[26] Alipoor et al[46] established for the first time that

MIAT is implicated in the incidence and progression of BC, presenting it as a potential tumor marker for BC detection and therapy. Their findings have suggested *MIAT* was upregulated in BC tissues and cell lines, and this study showed that the expression level of this gene in the whole blood was consistent with its expression in the BC tissues and confirmed the previous data, so it has the potential to be considered as a noninvasive biomarker in the whole blood for BC. They discovered that *MIAT* expression was considerably greater in high-grade breast ductal carcinoma than in surrounding nontumor tissues and was associated with clinic pathological characteristics of tumors, such as the Her2, the *p53* gene, the ER, and the PR. Their findings indicated that the expression level of *MIAT* was significantly increased in ER-positive and PR-positive tumor tissues. Moreover, *MIAT* has recently been found to be overexpressed in *p53*-negative tumor tissues. In addition, it was shown that inhibiting *MIAT* expression resulted in G1-phase arrest and apoptosis in BC cells. These findings suggest that *MIAT* may act as a cell-cycle regulator. Besides, *MIAT* inhibition prevents BC cell migration and decreases the expression of EMT genes. A recent study showed that the expression level of *MIAT* is increased in ER-positive BC tissue and cell lines.[47] It has been reported that *MIAT* is significantly expressed in stage I and stage II breast tumors.[48] In a study by Ye et al,[49] it was shown that higher expression of *MIAT* was positively related to lymph node status and TNM stage in BC, and they proposed that *MIAT* serves as a noninvasive biomarker for the diagnosis of BC. The result of this study indicated that the expression level of *MIAT* was increased in BC tissue and whole blood of patients. Furthermore, overexpression of *MIAT* was clearly associated with stage I and stage II breast tumors and lymph node involvement in both BC tissue ($P < .01$ and $P < .03$, respectively) and whole blood ($P < .04$ and $P < .002$, respectively).

A substantial amount of experimental data have demonstrated that miRNAs play a critical role in cancer cell death regulation. Numerous strategies have been developed to either inhibit the expression of oncomiRs or to increase the expression of tumor suppressor miRNAs in an attempt to re-establish miRNA activity in apoptotic pathways.[50] In BC cells, *miRNA-29a-3p* has been demonstrated to have a tumor suppressor function by interrupting the cell cycle during the G0/G1 phase via the negative regulation of the expression of *CDC42*.[51]

In the study by Li et al,[52] it was shown that *miRNA-29a-3p* mimic promoted the proliferation of BC cell lines (MCF-7 and T47D). The inhibition of *miRNA-29a-3p* was shown to suppress the proliferation of these cell lines. It has been reported that *miRNA29a-3p* has a negative effect on *N-MYC*, which leads to upregulation of the mesenchymal phenotype and promotes tumor invasion in BC cells.[53] Pei et al[54] proposed that higher *miRNA-29a-3p* expression increased cell proliferation, whereas decreased *miRNA-29a-3p* expression suppressed cell growth. Wu et al[55] found that in BC cells, the expression level of *miRNA29a-3p* was decreased. Moreover, *miRNA-29a-3p* inhibited cells in the G0/G1 phase and restricted tumor development through decreasing the expression of *B-MYB*. Yan et al[56] revealed that *miRNA29* family members (*miR-29a, miR-29b*, and *miR-29c*) increase *p53* levels and trigger apoptosis in a *p53* pathway. In this research, *miRNA-29a-3p* was downregulated in BC tissue and whole blood of patients compared with control groups. In assessing the relationship between *miRNA29a-3p* expression and clinical features of tumor, we found that overexpression of *miRNA29a-3p* was clearly associated with ER-positive in both BC tissue ($P < .006$) and whole blood ($P < .004$).

Due to advancements in high-throughput sequencing and novel computing technologies, lncRNA has been recognized as a key molecule in the regulation of gene expression at the post-transcriptional level in recent years.[57] Increasing evidence indicates that lncRNA functions as a ceRNA, inhibiting the expression or activity of miRNA. Micro RNA has been recognized as a critical regulatory element in the ceRNA network and has a negative impact on regulating RNA gene expression by interacting with the target region of mRNA 3'-UTR, causing adenosine acidification, decreasing mRNA stability, and limiting translation.[58] The bioinformatics analysis of this study suggested that *miRNA29a-3p* has a common binding site with *MIAT* and *FOXO3a* (Figure 2). Microarray research identified a binding site between *miRNA-29a-3p* and *FOXO3a*, showing a similar targeting connection between *miRNA-29a-3p* and *FOXO3a*. Growing evidence is emerging to connect *miRNA-29a-3p* downregulation to *FOXO3a* overexpression.[59] In another similar study, dual-luciferase demonstrated *miRNA-29a-3p* has a targeting relationship with *FOXO3a* in OC (ovarian cancer) and, according to western blot, overexpression of *miRNA-29a-3p* inhibited the expression of *FOXO3a* and downregulation of *miRNA-29a-3p* elevated the expression of *FOXO3a*. Based on these results, they showed that *FOXO3*a could be targeted by *miRNA-29a-3p*.[60]

In addition, it has been reported that *MIAT* has complementary base pairing sites with *miRNA-29a-3p*, and it may function as an endogenous miRNA sponge to inhibit the expression of *miRNA-29a-3p* in gastric cancer.[61] However, further research is needed to determine the exact mechanism of *miRNA29a-3p* activity in BC. In this study, we showed that the expression levels of *MIAT* and *miRNA29a-3p* in whole blood and ductal carcinoma BC tissues have a negative correlation. In fact, overexpression of *MIAT* is associated with decreased *miRNA29a-3p* and increased *FOXO3a* expression. Following these data, it can be concluded that *MIAT, miRNA-29a-3p*, and *FOXO3a* levels have a significant relationship with the pathogenesis of ductal carcinoma BC.

To confirm the endogenous connection between *miRNA-29a-3p, MIAT*, and *FOXO3a* in BC, we propose that future investigations use a dual-luciferase test.

Considering the fact that an ideal biomarker should have high sensitivity and specificity, this study confirmed that *MIAT,*

FOXO3a, and *miRNA29a-3p*, as well as the combination of them, have shown high sensitivity and specificity in BC tissue and whole blood compared with healthy individuals. Together, these findings indicate that they can be potential bioindicator for BC patients in the whole blood with better sensitivity and specificity.

In summary, it is appealing to demonstrate that *MIAT* and *FOXO3a* with high expression and *miR-29a-3p*, with down expression as a ceRNA network, can be potentially effective bioindicators for the detection of BC in clinical practice. The crosstalk between ncRNAs may provide hope for an accurate diagnosis of BC in the future.

Declarations

Ethical Approval and Consent to Participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Islamic Azad Tehran Medical Sciences University (code: IR.IAU.PS.REC.1398.317 dated on January 21, 2020). Informed consent was obtained from all individual participants included in the study.

Consent for publication

The authors affirm that written informed consent was obtained from all individual participants to publish this article.

Author Contributions

Shokufeh Razi: Conceptualization; Data curation; Formal analysis; Investigation; Software; Validation; Visualization; Writing – original draft; Writing – review & editing.
Hossein Mozdarani: Editing & final manuscript approval; Project administration; Review; Supervision; Visualization.
Roudabeh Behzadi Andouhjerdi: Validation; Visualization.

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Availability of Data and Materials

The data sets generated or analyzed during this study are available from the corresponding author on reasonable request.

Supplemental material

Supplemental material for this article is available online.

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