Original Article

Hsa-miR-662 as a New Prognostic Biomarker in Patients with Breast Cancer; In-silico and Experimental Study

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Abstract

Background & Objective: Breast cancer (BC) is a complex genetic disease that has an average annual incidence of one million people and is the second leading cause of death among women in the world. Therefore, a better understanding of tumor biology and the determination of biomarkers for early diagnosis of disease is essential. MicroRNAs and long non-coding RNAs are novel gene regulators that play key roles in tumor initiation and progression. The current study was performed to assess the biomarker potential. This study performed a combination of in-silico and experimental investigations of altered miRNAs in BC to assess the use of miRNAs as novel biomarkers for early detection and prognosis prediction of patients with BC.

Materials & Methods: We searched the miRNA expression patterns of BC from three expression arrays (GSE58606, GSE38867, and GSE40525) from the Gene Expression Omnibus (GEO) database to recognize differentially expressed miRNAs (DEMs) between BC tissues and normal adjacent samples. Using "Limma" package's Quantile Normalization function and INMEX bioinformatic tool, hub DEMs were identified. MiRNAs targeted genes were found and visualized via the miRWalk and miRTargetLink databases and their Enrichment analysis was performed for identified genes. Due to more validation of DEGs, GSE70951, an independent expression array dataset, was analyzed. By merging DEMs and DEGs, miRNA-mRNA network was constructed. After elucidation of hub miRNAs, the capacity of detected miRNAs to differentiate BC from adjacent controls was estimated by Kaplan-Meier analysis. Furthermore, RT-qPCR on 100 BC samples and 100 adjacent non-tumor tissues was performed to validate the in-silico results.

Results: According to our study, in BC samples, miR-662 was differentially downregulated in comparison with normal adjacent tissues.

Conclusion: Altogether, miR-662 can be considered as a viable target for BC diagnostics and treatment.

Keywords: Breast Cancer, MicroRNA, Hsa-miR-662, Prognosis

Introduction

After skin cancer, Breast cancer (BC) continues to pose a dangerous threat to the health of females worldwide. In 2022, nearly 300,000 new cases of BC are anticipated to be diagnosed in women in the U.S (1). Despite the fact that there are multiple active research on BC hallmarks like cancer initiation, apoptosis, invasion, metastasis, and recurrence, there is considerable intricacy in this case, that makes it a valuable area for doing various research on the diagnosis and treatment of BC (2). Although BC has been studied from many viewpoints, its specific process is still unknown. The early diagnosis and treatment of BC are made more difficult by the several subtypes of BC and their variability and heterogeneity. Targeting purine metabolism may thus be vital to

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develop anticancer treatments against BC development, which is perhaps one of the distinguishing characteristics of BC (3-5). As a result, the purine axis serves as the main source of the building blocks needed for the formation and proliferation of many cancer cells, notably BC cells. Besides, Cell proliferation can be severely impacted and suppressed by blocking the purine pathway. According to bioinformatics databases, some microRNAs (miRNAs), 22nt non-coding RNAs, have recently been discovered to effectively control the purine metabolism by targeting important enzymes like adenosine deaminase (ADA), which may offer a chance of preventing cancer development (6-8). Moreover, miRNAs are used to target certain mRNAs and regulate a range of physiological activities (9). MiRNAs have been demonstrated to have a significant regulatory function in the growth and spread of cancer, and they could be useful therapeutic targets for BC (10). According to a growing body of research, miRNAs may provide an ideal collection of biomarkers used in the early diagnosis and prognosis of cancer because of their stability in human tissues and peripheral blood as well as disease-specific expression (11, 12). Based on this fact, miR-662 is one of the miRNAs that can affect purine metabolism through mechanisms reported by bioinformatic data and a few experimental investigations. (13). MiR-662 plays an important function in malignancies like lung cancer (14) and other diseases like heart failure (15). However, miR-662 might be investigated as a possible prognostic marker and used to modulate purine metabolism. Because metabolic reprogramming is a typical occurrence in cancer cells and this process considerably differs from normal cells, in the current study, the expression level of miR-662 was assessed in tumor samples and non-tumor adjacent tissues of BC patients.

Materials & Methodes

Microarray Datasets Analysis

GEO database (http://www.ncbi.nlm.nih.gov/geo) was used to screen for differentially expressed miRNAs in three miRNA expression arrays, GSE58606, GSE38867, and GSE4052. The raw data was analyzed by "R" software and then normalized by the "Limma" package's Quantile Normalization function (16). Employing the INMEX bioinformatics tool (http://www.inmex.ca), significantly downregulated and upregulated DEMs from each dataset and were compared and merged by the combined effect size method. Cut-off: ¬ ¬logFC¬ ¬< -0.5 and p-value ≤ 0.05 were used to identify the DEMs in BC samples and non-tumor adjacent samples.

Pinpointing and Validating Core MiRNA Target Genes

MiR-662 target genes were obtained from miRWalk (17) and miRTargetLink 2.0 (18) databases and were validated by the gene expression array dataset, GSE70951, from GEO database.

Enrichment Analysis to Identify Biological Functions and Cellular Pathways

In order to improve the biological processes regulated by miR-662 target genes, the verified genes were submitted to Over-Representation enrichment Analysis (ORA) utilizing WebGestalt's (http://www.webgestalt.org/) webserver and GeneMANIA (https://genemania.org/), which executed gene ontology (GO) annotation analysis. Additionally, using the EnrichR online databases, verified genes have been added to enrich miR-662 dysregulation pathways. The P-value < 0.05 was considered as the statistically significant threshold.

Validating Hub DEM by the Kaplan–Meier Survival Analysis

To assess the prognostic value of identified DEM in BC patients, the Kaplan-Meier plotter (KMplotter) was utilized.

Tissue specimens and study population

200 tumor samples and non-tumor adjacent samples were obtained from BC patients undergoing surgery at Tabriz's Al-Zahra Hospital, and they were immediately frozen at -80°C. The samples came from lumpectomies and mastectomies in which a piece of the normal tissue next to

the tumor was taken away from the malignant tissue. After that, a histological examination was used to investigate the invasion and spread of cancer cells. As controls, tumor margin samples that a pathologist deemed to be healthy were also looked at. Patients who did not receive radiation or chemotherapy prior to surgery met the inclusion criteria. All methods were performed in accordance with the guidelines of the Declaration of Helsinki and was approved by the Research Ethics Committee of Azad University of Medical Sciences, Tabriz, Iran with Ethics code IR.IAU.TABRIZ.REC.1401.103. All patients enrolled signed an informed written consent.

Total RNA Extraction

After being divided into 20 µm-thick pieces, liquid nitrogen was used to homogenize tissue samples. Every sample was immediately soaked in TRIzol reagent (GeneAll, South Korea). The purity of the isolated total RNA was evaluated using the Thermo Scientific (USA) NanoDrop spectrophotometer and gel electrophoresis. The isolated RNA was then rinsed in 50 µL of RNase-free water and stored at -80 °C until use.

cDNA Synthesis and Quantitative Real-time Polymerase Chain Reaction

The stem-loop (STL) primers for miR-662 and U6 snRNA (used as reference RNA) were prepared by Cinnaclone Co. Iran, Tehran. Two specific stem-loop primers for miR-662 and RNU6 (for normalization) were created for this purpose, and the PCR machine was run under the following conditions: 30 min at 16 °C, 30 min at 42 °C, and 5 min at 75 °C. SYBR Green master mix (Amplicon, Denmark), miR-662 specific primer, and dNTP were also used for the Real-time PCR reaction. A miR-662 forward primer and common reverse primer, as well as U6 snRNA forward and reverse primers, were designed. For real-time PCR, these primers, and the Danish amplicon SYBR® Green master mix were used. Real-time reactions were all performed twice. Using the MIC qPCR bimolecular technology, the two-step RT-PCR was carried out as follows: 15 min at 95 °C, 40 cycles of 15 sec at 94 °C and 30 sec at 60 °C. The program mic-PCR v1.4.0 displayed the findings. (Table 1) displays the primers' associated sequences.

Table 1. Real-time PCR primer sequences and cDNA synthesis specific stem-loop primer

	MiRNAs and Their Accession Numbers		Sequences
cDNA Synthesis Reaction	hsa-miR-662	hsa-miR-662 (STL)	5'GTCGTATCCAGTGCAGGGTC-CGAGGTATTCGCACTGGATAC-GACCTGCTG3'
	RNU6 NR_003027.2	U6(STL)	5′GTCGTATCCAGTGCAGGGTC-CGAGGTATTCGCACTGGATACGA-CAAAAATAT3′
Real Time PCR Reaction	hsa-miR-662	hsa-miR-662(F)	5'TCCCACGTTGTGGCCCAG3'
	RNU6 NR_003027.2	U6(F) U6(R)	5′GCTTCGGCAGCACATATACTA-AAAT3′ 5′CGCTTCACGAATTTGCGTGT-CAT3′
	hsa-miR-662	Common(R)	5′GTGCAGGGTCCGAGGT3′

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Statical data analysis

The target DEMs ratio between the BC tissues and non-tumor surrounding tissues was determined by the 2− (ΔΔCT), utilizing quantitative real-time PCR, ΔCTs of the tumor samples, ΔCTs of non-tumor samples, and ΔΔCTs of every sample. Threshold Cycle (CT) pinpointed the amplification cycle when the reaction's real-time fluorescence assertiveness reached the defined threshold, and the amplification was in the logarithmic step. To choose the mean value, each experiment was conducted three times. A P-value of ≤ 0.05 was evaluated as statistically significant when accomplishing the statistical analysis employing the GraphPad Prism (19) and "R" software version 4.3.1.

Result

In-Silico Analyzing and Selecting Differentially expressed MiRNAs

Based on previous studies, the regulation of metabolic pathways of cancer cells were reviewed to determine the most prominent biomarkers like genes and miRNAs. Furthermore, it was revealed that the suppression of purine synthesis may inhibit tumor growth and metastasis in BC. Then, miRNAs targeting purine metabolism were explored by using miRPathDB 2.0 and DIANA mirPath v.3. Among the results of these databases, miR-662 was selected because this miRNA was predicted to take part in purine metabolism. The result was visualized by geneMANIA web server (Figure 1). Understanding p-value ≤ 0.05 and logFC < -0.5 as the cut-off, it was found that miR-662 in GSE58606 (logFC= -0.041504), GSE38867 (logFC= -1.335), and GSE40525 (logFC= -1.044741256) were downregulated in line with the outcomes of our investigation result. The hub DEMs based on their logFC are presented in (Table 2).

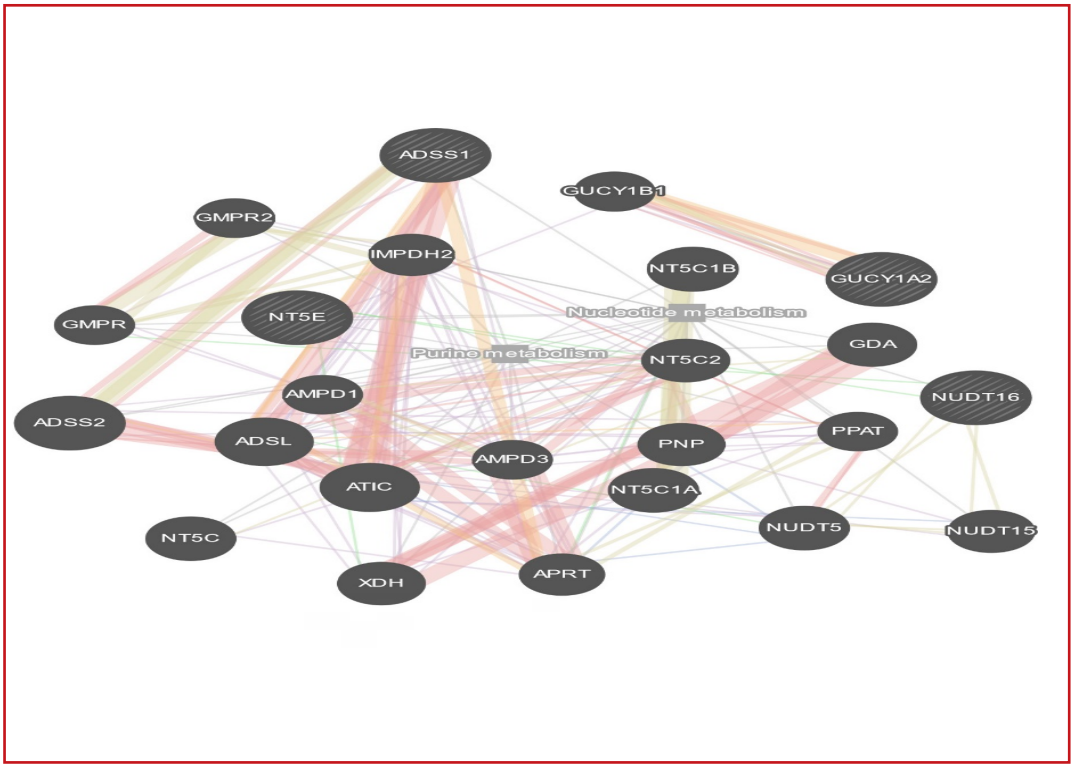

Figure 1. MiR-662 target genes visualization

Table 2. Top Downregulated and Upregulated DEMs of GSE58606, GSE38867, and GSE40525

NO	DOWNREGULATED MIRNAS	LOG FC	P.VALUE	ADJ P.VALUE	AVE EXPR
1	hsa-miR-662	-1.9836733	5.71E-24	1.09E-23	8.439505296
2	hsa-miR-760	-1.9612689	1.76E-24	3.65E-22	4.402720424
3	hsa-miR-654-5p	-1.9279773	6.03E-22	9.26E-21	3.428872291
4	hsa-miR-134	-1.890296	8.14E-15	5.07E-14	3.547636596
5	hsa-miR-936	-1.8524093	1.44E-14	8.78E-14	4.848019788
6	hsa-miR-452	-1.8412856	5.20E-14	3.02E-13	5.379960591
7	hsa-miR-601	-1.8364003	3.40E-18	3.26E-17	6.101569863
8	hsa-miR-187*	-1.8320413	4.58E-18	4.24E-17	4.04611926
9	hsa-miR-493	-1.8067488	3.97E-10	1.55E-09	3.542874478
10	hsa-miR-526b	-1.7875484	3.77E-13	1.86E-12	3.537029705
NO	**Upregulated miRNAs**	**Log FC**	**P.value**	**Adj P.value**	**Ave Expr**
1	hsa-miR-146b-5p	1.95664751	7.47E-19	7.96E-18	7.931110488
2	hsa-miR-582-5p	1.9399801	1.49E-13	7.98E-13	3.585938072
3	hsa-miR-142-5p	1.93650048	1.06E-18	1.09E-17	7.859354127
4	hsa-miR-331-3p	1.91949744	2.21E-27	6.51E-26	8.61337161
5	hsa-miR-449a	1.91548429	8.25E-08	2.53E-07	3.118581312
6	hsa-miR-98	1.89018427	1.16E-20	1.43E-19	7.170404517
7	hsa-miR-744	1.87726052	1.31E-23	2.45E-22	3.988588997
8	hsa-miR-24-1*	1.83510161	2.37E-17	1.91E-16	3.245788814
9	hsa-miR-17*	1.80450324	5.52E-18	4.92E-17	5.458289085
10	hsa-miR-362-5p	1.79213329	1.68E-18	1.70E-17	4.732129394

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Functional Enrichment Analysis

Functional enrichment and pathway analysis revealed the biological processes, cellular components, molecular functions, and pathways associated with the miR-662 common target genes between three datasets (Figure 2) and (Table 3). The most enriched biological process was the "Metabolic Process", the most enriched cellular component was "Cytosol", and the most enriched molecular function was "Ion Binding". Moreover, the research also identified important signaling pathways where the targets of miR-662 are presented (Table 4).

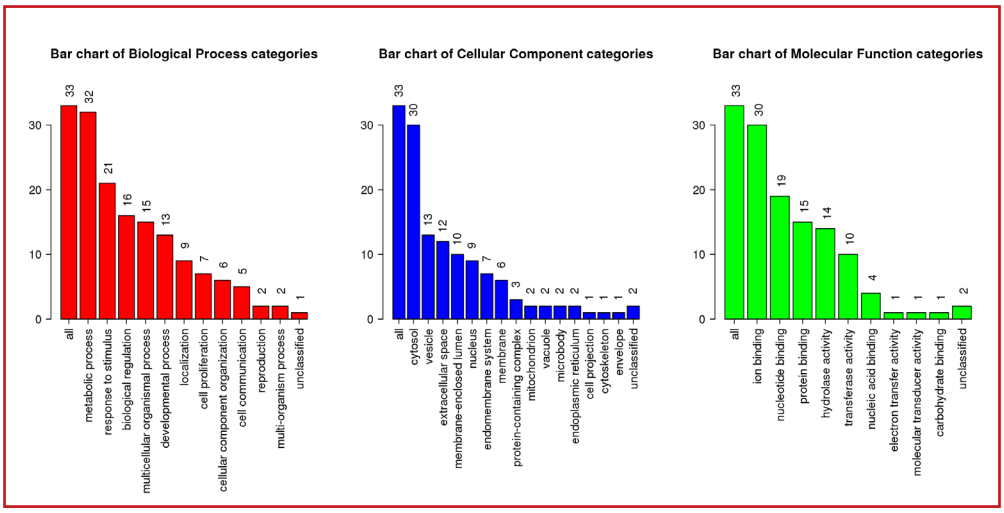

Figure 2. GO analysis of the shared differentially expressed mRNAs

Table 3. Top enriched biological processes among target genes of miR-662

Gene Set	Description	P.value	Adj P.value
(GO:0009167)	Purine ribonucleoside monophosphate metabolic process	1.402e-46	2.117e-44
(GO:0072522)	Purine-containing compound biosynthetic process	3.827e-43	2.889e-41
(GO:0009156)	Ribonucleoside monophosphate biosynthetic process	1.480e-38	7.448e-37

Table 4. More common pathways among miR-662 target genes

Gene set	P.value	Adj P.value
Purine metabolism	3.489e-72	1.117e-70
Nicotinate and nicotinamide metabolism	2.860e-11	4.576e-10
Pyrimidine metabolism	5.574e-10	5.946e-9
Alanine, aspartate, and glutamate metabolism	4.980e-7	0.000003984
Drug metabolism	0.000001193	0.000007638
Long-term depression	0.004941	0.02259
Renin secretion	0.006485	0.02594

Prognostic Value of miR-662 in BC

To additionally examine the prognostic role of miR-662 in BC, the KM Plotter database, based on TCGA datasets with 1078 samples, was employed to estimate the miR-662 prognostic value. We discovered that high miR-662 expression was associated with better Overall Survival (OS) (HR = 2.23(1.59-2.13), P-value= 2.1e-6) (Fig. 3).

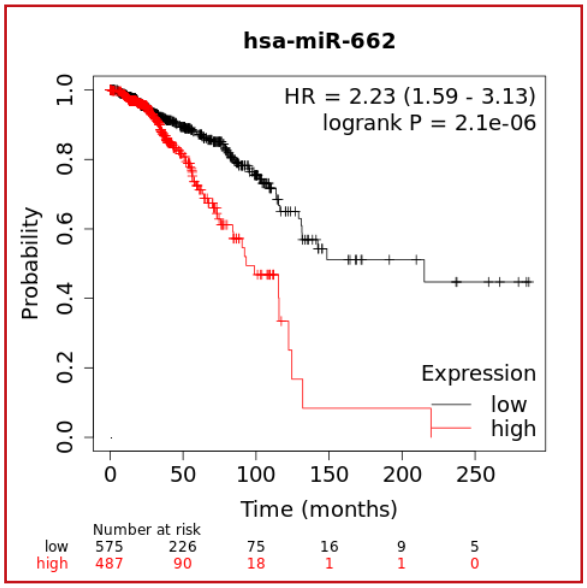

Figure 3. Kaplan-Meier survival curve comparing the high and low expression of miR-662 in BC

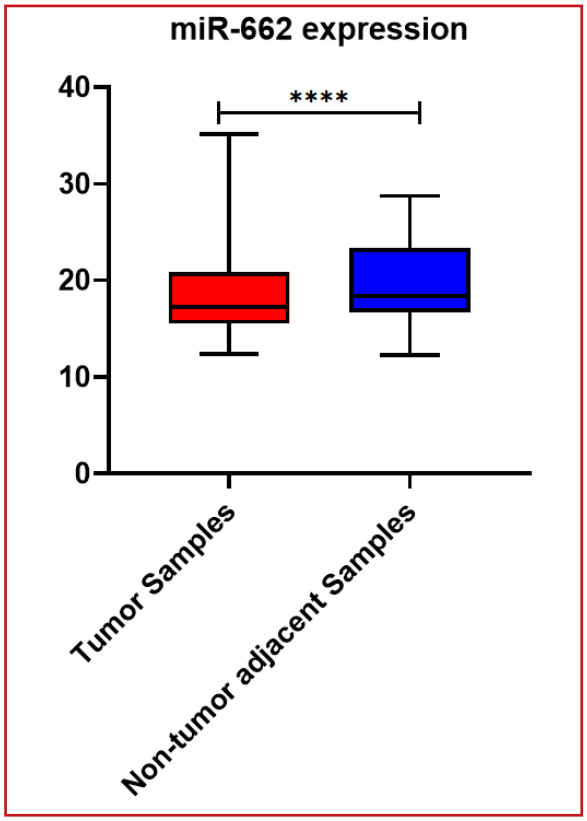

Figure 4. The downregulated expression of miR-662 in BC samples and adjacent non-tumor samples

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Real- time PCR results of the Expression Level of miR-662

The comparative expression amount of the miR-662 in BC samples was matched with those in adjacent non-tumor samples in BC patients. In comparison with adjacent non-tumor tissues, BC tissues have lower levels of miR-662 expression (P-value= 0.0011 and fold change= -1.2097) (Figure 4).

Discussion

As potential non-invasive markers, miRNAs are better prognosis and diagnosis goals (20). These biomarkers may trigger cancer-associated genes such as tumor suppressor genes and oncogenes or cancer metabolism linked-genes that regulate tumor cells' metabolic evolution (21). The unusual expression of miRNAs influences the mechanisms involved in BC growth, including invasion, metastasis, expanding tissue, enhancing anti-apoptotic effect, drug sensitivity, and metabolic transformation (22-24). Despite recent advancements, the prevalence of BC in women is higher than ever (25). The histopathological and metabolic characteristics of BC are unique, and it also has genetic variations or several carcinogenic pathways (26). As with normal tissues, each tumor tissue has unique expression patterns (27). A biomarker is any of these quantities that connects a biological situation with a possible risk. Biomarkers are becoming more crucial to the therapeutic prevention of recurrent patients with the development of genomic profiling tools and specific molecular targeted medicines (28). Because of their small size and capacity to be quantified in plasma samples, miRNAs exposition of cancer-specific expressions are rapid and affordable to test and are invulnerable to degradation. MiRNAs like miR-662, which have been investigated in this research, are said to be important in purine metabolism by bioinformatics databases. In contrast to healthy adjacent tissues, according to our findings, miR-662 was significantly downregulated in BC tissue samples. Furthermore, to the best of our knowledge, miR-662 has been documented to take part in a few disorders and malignancies. According to the latest study that was done on miR-662, it was declared that abnormal miR-622 expression can accelerate or suppress liver, colorectal, BC, and other tumors, like gliomas (29). However, nucleotide metabolism (purine metabolism) and its dysregulation were confirmed to have a vital role in BC (30, 31). It was shown that the expression of various miRNAs altered in lung cancer in a study published in 2014, and the findings indicated that miR-662 had predictive significance (14). Additionally, in a study conducted by Filipska et al., it was shown that miR-662 enhances invasiveness and chemoresistance in lung cancer and it was shown to have correlation with poor prognosis of patients with early-stage squamous cell lung cancer (32). However, due to three independent studies, miR-662 has vital role in heart failure and disorders. An integrative bioinformatics analysis unveiled that overexpression of miR-662 had an association with the risk of atrial fibrillation (33). Furthermore, miR-662 was found to be differentially expressed in patients with chronic heart failure (15). However, miR-622 was validated through the miR-662/RNF8 axis in BC, due to the Liu et al. study. Their bioinformatics experiment indicated the regulatory mechanism of miR-622 in BC cells' EMT function, viability, and migration in vitro (34). Furthermore, miR-662 was indicated as a novel potential biomarker of BC that could harm the motility of BC cells by triggering NUAK1 kinase (35). Finally, it was declared that the increase in the expression of miR-662 predicts chronic congestive heart failure (36). According to our in-silico study, miR-662 has a tumor-suppressive role in BC. Nevertheless, as compared to non-tumor adjacent tissues, the findings of our investigation revealed that the expression level of miR-662 had significantly changed in BC tissues (Fold change= -1.2097, and P-value= 0.0011).

Conclusion

We examined 200 BC samples notwithstanding all the restrictions on the commission of presented biomarkers. MiR-662 levels were generally lower in BC samples than in nearby normal samples.

confirming the use of a different independent dataset, and real-time PCR. Despite the possibility that this miRNA might serve as a therapeutic target, further research is required to understand the underlying biological processes and confirm the results of bioinformatics analyses.

Ethics approval and consent to participate

All methods were performed in accordance with the guidelines of the Helsinki and was approved by the Research Ethics Committee of Azad University of Medical Sciences, Tabriz, Iran with Ethics code IR.IAU.TABRIZ.REC.1401.103. The patients' written informed permission was gathered before participation.

Availability of data and materials

Please contact the corresponding author for data requests.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Abbreviation

BC: Breast Cancer
DEG: Differentially Expressed Genes
DEM: Differentially Expressed MiRNA
GEO: Gene Expression Omnibus
GO: Gene Ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
MiRNA: MicroRNA
NcRNA: Noncoding RNA
PCR: Polymerase Chain Reaction
ROC: Receiver operating characteristic
RT-PCR: Reverse Transcriptase – Polymerase Chain Reaction

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