Journal of Advanced Biomedical Sciences
Homepage: http://jabs.fums.ac.ir
Online ISSN: 2783-1523
Fasa University of Medical Sciences

Investigating The Expression Changes of *miR-93-3p*, *NFATc1* and *NFATc3* Genes in Peripheral Blood Mononuclear Cells (PBMC) of Breast Cancer Patients

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Article Info

Article Type:
Original Article

Article History:
Received
02 Nov 2023
Received in revised form
18 Nov 2023
Accepted
29 Dec 2023
Published online
15 Jan 2024

Publisher:
Fasa University of Medical Sciences

Abstract

Background & Objectives: Breast cancer is the most lethal malignancy in women. miRNAs function as epigenetic regulators and contribute to the pathogenesis of breast cancer. Nuclear factor of activated T-cells, cytoplasmic 1 (*NFATc1*) and 3 (*NFATc3*), are targeted by micro*RNA-93* (miR-93). This study aims to evaluate the expression of these genes in peripheral blood mononuclear cells (PBMCs) of healthy women and women with breast cancer.

Materials & Methods: In this cross-sectional study, blood samples were collected from 20 healthy women and 20 women with early-stage breast cancer. After isolating peripheral blood mononuclear cells (PBMCs), RNA extraction and cDNA synthesis were performed. The expression of the desired genes was then examined by Real-Time Polymerase Chain Reaction (RT-PCR). Statistical analysis was conducted. A p-value less than 0.05 was considered statistically significant, and Student's t-test was used to evaluate the relative changes in gene expression.

Results: The results demonstrated that the expression of *NFATc1* and *NFATc3* genes in peripheral blood mononuclear cells (PBMCs) of breast cancer patients was significantly reduced compared to their expression in healthy individuals. Conversely, the expression of the *miR-93-3p* gene was significantly lower in healthy women than in breast cancer patients ($p < 0.05$).

Conclusions: This study investigated the expression of *miR-93-3p* and its downstream targets, the *NFATc1* and *NFATc3* genes, for the first time in peripheral blood mononuclear cells. The expression levels were shown to be significantly different in patients with breast cancer compared to healthy women.

Keywords: Breast Neoplasms, Real-Time Polymerase Chain Reaction, MicroRNAs

Cite this article: Dastgir M, Rigi G, Ghaedmohammadi S, Tahmasebi S. Investigating The Expression Changes of *miR-93-3p*, *NFATc1* and *NFATc3* Genes in Peripheral Blood Mononuclear Cells (PBMC) of Breast Cancer Patients. JABS.2024; 14(1): 56-66.
DOI: 10.18502/jabs.v14i1.14807

Introduction

Cancer is the second leading cause of death globally, following cardiovascular disease. In developed countries, it remains the second leading cause of death. However, in less developed countries, it ranks third behind cardiovascular disease and accidents (1).

Breast cancer is the most common cancer and deadliest malignancy in women globally, making it a critical public health concern. Women in Asian countries traditionally have a lower prevalence of breast cancer, possibly due to lifestyle factors. In Iran, however, breast cancer has the highest incidence among all cancers.

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[DOI: 10.18502/jabs.v14i1.14807]

Despite extensive research on management and treatment (2-4), it remains the leading cause of death for women in the country. A complex interplay of environmental and hereditary factors contributes to breast cancer, a highly heterogeneous disease. These factors combine to induce epigenetic and genetic changes in breast cancer cells. The specific genes involved in the disease's pathogenesis are crucial for understanding breast cancer (5-9).

The Nuclear factor of activated T cells (NFAT) family consists of a group of calcium (Ca^{2+})/ Calcineurin (CN)-dependent transcription factors primarily known as mediators for the activation of cytokine genes in T cells during the immune response. Over three decades after their discovery, NFAT proteins were found to be expressed in many cells other than immune cells and play vital roles during and after embryonic development, including the inflammatory response, heart valve formation, myocardial growth, and other biological processes. Evidence suggests that abnormal expression of NFAT proteins has been implicated in cancer development and progression (10). Elevated intracellular Ca^{2+} levels activate CN, which dephosphorylates NFAT. This promotes the transfer of NFATs to the nucleus, where they generally work with other transcription factors to regulate a set of genes involved in immune function, including Interleukin 26 (IL26) and cyclooxygenase 2 (COX2). Expression of Nuclear factor of activated T-cells, cytoplasmic 1 (*NFATc1*), *NFATc2* and *NFATc3* (but not *NFATc4*) has been detected in normal T cells. The immune regulatory functions of each member are unique (11). The *NFAT* family consists of five members: *NFATc1* (also known as *NFAT2* or *NFATp*), *NFATc2* (*NFAT1* or *NFATc*), *NFATc3* (*NFAT4* or *NFATx*), *NFATc4* (*NFAT3*), and tonicity enhancer binding protein (*NFAT5*). Among these isoforms, *NFATc1* plays a critical role in differentiating T cells into type II helper T cells, effectively activating humoral immunity. *NFATc2* and *NFATc3* collaborate to differentiate T cells into type I helper T cells and activate the cellular immune system (12). *NFATc3*, another family member, possesses anti-tumor effects that depend on the tissue's physiological and cellular characteristics. For instance, *NFATc3*-deficient mice exhibited higher rates of T cell lymphoma and breast cancer, suggesting its anti-tumor potential in both cancers (13). MicroRNAs (miRNAs) can also influence breast cancer pathogenesis, tumor malignancy, and treatment response by targeting various cellular and molecular pathways. Identifying the intrinsic subtype of a primary tumor is crucial in breast cancer. Each intrinsic subtype exhibits a distinct miRNA expression profile. Consequently, evaluating miRNA expression profiles holds promise as a key and effective approach for identifying and diagnosing breast cancer patients (14). Studies have shown that *microRNA-93* (miR-93) expression in human breast cancer cell line Membrane Type 1 (MT-1) leads to tumors with increased blood vessels compared to control cells. miR-93 promotes angiogenesis and metastasis by suppressing Large tumor suppressor kinase 2 (*LATS2*) gene expression. Conversely, inhibiting miR-93 function might be a practical strategy to suppress tumor metastasis (15). Notably, *NFATc1* and *NFATc3* are established targets of miR-93 (16). *miR-93-3p* is a specific subtype of miR-93, with fewer studies investigating its relationship with the genes encoding *NFATc1* and *NFATc3* transcription factors. Given the potential of miR-93 to target *NFATc1* and *NFATc3*, we aimed to investigate their expression in peripheral blood mononuclear cells (PBMCs) of breast cancer patients. This is a novel investigation, as no prior reports exist on this topic. Finally, we evaluated the correlation between *miR-93-3p* expression and the expression of *NFATc1* and *NFATc3*.

Materials and Methods

Sample Collection

This cross-sectional study was conducted in Shahrekord, Iran, between 2021 and 2022. The patient group consisted of 20 women with breast cancer (mean age: 32.25 ± 9.07 years). The sample size was calculated using a formula known

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[DOI: 10.18502/jabs.v14i1.14807]

as Andrew Fisher's Formula, which considers population size (if known), confidence interval, confidence level, and standard deviation. An online tool was used to calculate the sample size (https://www.geopoll.com/blog/sample-size-research/).

The study included patients with triple-negative breast cancer. No mention of chemotherapy, radiation therapy, or immunotherapy was needed as these treatments are typically not administered for this specific type of cancer. Pathological examination by expert oncologists confirmed the presence of tumors in all patients. The control group consisted of 20 healthy individuals (mean age: 36.45 ± 7.89 years) who were randomly selected. All participants were in good health without any acute or chronic diseases. Written informed consent was obtained from all participants before blood sampling. Exclusion criteria included: Patients with advanced or metastatic cancer who had already begun neoadjuvant or any other treatments, presence of a significant clinical disorder, psychiatric drug use within the past 6 months.

Five milliliters of fresh peripheral blood were collected from both healthy women and patients with breast cancer in heparinized tubes. This study was approved by the Ethics Committee of Shahrekord University (Ethics committee approval code: IR.SKU.REC.1401.053).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Five milliliters (mL) of heparinized blood were diluted 1:2 with phosphate-buffered saline (PBS). The diluted blood was then carefully layered onto an equal volume of Ficoll-Lymphodex solution (Innotrain, Germany). The sample was centrifuged at 20°C for 20 minutes at 2,500 rpm. Following centrifugation, the upper layer was discarded. The mononuclear cell layer was carefully transferred to a new 15 mL tube and washed three times with 5 mL of PBS. Finally, the PBMC pellet was resuspended in 1 mL of PBS solution. The isolated cells were then used for RNA extraction and further experiments.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the PBMCs using RNX-plus solution (Sinaclone, Iran), following the manufacturer's guidelines. The quality of the extracted RNA was assessed by agarose gel electrophoresis. The gel contained a safe stain for visualization of nucleic acid bands. RNA purity and quantity were determined by measuring its absorbance at 260 nm and 280 nm using a spectrophotometer. Complementary DNA (cDNA) was then synthesized for NFATc1 and NFATc3 evaluation using a cDNA synthesis kit (Sinaclone, Iran). Specific hairpin loop primers were used to synthesize cDNA specific to the microRNAs of interest. The resulting cDNA was stored at -20°C according to the kit protocol.

Quantitative Real-Time PCR Reaction (qRT-PCR)

The expression levels of *miR-93-3p* and its target genes (*NFATc1* and *NFATc3*) were evaluated using a SYBR Green master mix kit (Ampliqon, Korea) on a Real-Time Polymerase Chain Reaction (RT-PCR) instrument (Applied Biosystems, USA). Specific primers are listed in Table 1. The PCR reaction was performed in a 25 µL final volume. The reaction mix contained 12.5 µL of the kit solution, 200 ng of template cDNA, and 2 µL of each forward and reverse primer (10 pmol stock). The PCR program consisted of an initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A negative control lacking cDNA was included for each primer pair to ensure proper reaction performance. Beta-actin was used as the housekeeping gene for both *NFATc1* and *NFATc3* expression normalization. While RNU6 was initially chosen for microRNA normalization, its high Ct values indicated its unsuitability as a control. Beta-actin, a well-established housekeeping gene not targeted by miR-93, served for both analyses. Following RT-PCR, data were analyzed using the ΔΔCt and 2-ΔCt methods. The PCR products were

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then electrophoresed on a 1% agarose gel for visualization.

Table 1. The primer sequences used in the current study

Primer names	F: forward R: Reverse	Primer sequence
Primers for qRT- PCR		
miR-93-3p	F	GTG CAG GGT CCG AGG T
	R	CTT ACT GCT GAG CTA GCA CTT CC
Run6	F	CGC TTC ACG AAT TTG CGT GTC
Run6	R	CGC TTC GGC AGC ACA TAT ACT
NFATc1	F	TGC AGC TGC ATG GCT ACT TG
NFATc1	R	ATC TCC AGG ACT TTG GTG TTG G
NFATc3	F	GTG AAG CTC CTG GGC TAT AAC G
NFATc3	R	TAT CTC TTG GCT TGC AGT AGC G
β -actin	F	AGA CGC AGG ATG GCA TGG G
β -actin	R	GAG ACC TTC AAC ACC CCA GCC
Stem loops for cDNA synthesis of microRNAs		
C miR-93-3p		GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCACTG GAT ACG ACC GGG AAG T
C Run6		GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA AAA ATA T

Statistical Analysis

The $2^{-\Delta\Delta CT}$ and $2^{-\Delta CT}$ methods were used to analyze the qRT-PCR data. Statistical analysis was performed using the SPSS Version 20 statistical software package (SPSS Inc., Chicago, IL, USA). In this study, a p-value less than 0.05 was considered statistically significant. Student's t-test was employed to evaluate the relative changes in microRNA and its target gene expression in PBMCs of the control and target groups.

Results

Demographic characteristics of participants, including age and tumor grade, were collected using a questionnaire provided by the pathology department. These characteristics are shown in Table 2.

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Table 2. Pathological characteristics of patients with breast cancer and healthy participants

Age at diagnosis	(35-45)		(25-35)		(45-55)		(up to 55)		
	n=4		n=12		n=3		n=1		
Marriage	Married n= 16				unmarried n= 4				
First Menstruation (By Age)			mean (n=20)						
			13±1.12						
Menopause	Menopause				Non-menopause				
	n=5				n=15				
Pregnancy			(n=16)						
			mean (2.7±1.7)						
Abortion			(n=6)						
			mean (1.42±0.6)						
Breastfeeding (In Month)			(n=15)						
			mean (18.44±15.3)						
Invasive carcinoma Histology	Invasive ductal carcinoma (IDC)		Invasive lobular carcinoma (ILC)				In situ ductal carcinoma (DCIS)		
	n=15		n=3				n=2		
Tumor grade	grade I		grade II		grade III		not assessed		
	n=4		n=7		n=5		n=2		
Stage	IA	IB	IC	IIA	IIB	IIC	IIIA	B III	C III
	n=4	n=1	n=0	n=9	n=2	n=0	n=2	n=0	n=2
total	n=20								

RNA Extraction

The extracted RNA was loaded onto a 1.5% agarose gel. The presence of bands corresponding to 28S and 18S ribosomal RNAs confirmed the quality of the extracted RNA (data not shown due to the routine nature of the results). The average concentration of the extracted RNA was 1260 ng/µL.

Expression of Target Genes for *miR-93-3p* were Decreased in PBMCs of Patients with Breast Cancer

Real-time PCR was performed to evaluate the changes in expression levels of *NFATc1*, *NFATc3*, and the housekeeping gene beta-actin. This analysis revealed a significant downregulation of *NFATc1* and *NFATc3* in PBMCs of breast cancer

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patients compared to the control group ($P < 0.05$). The fold change in gene expression is presented in Table 3 and Chart 1.

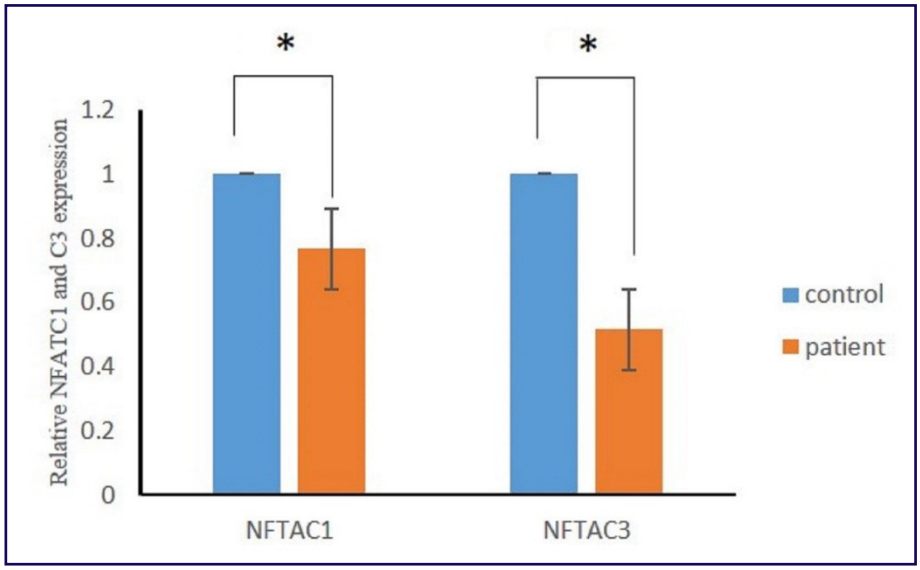


Chart 1. The figure shows the comparison between mRNA expression of *NFATc1* and *NFATc3* in PBMCs from the healthy women and breast cancer patients using real time PCR. The *NFATc1* ($P<0.047$) and *NFATc3* ($P<039$) expression from patients with breast cancer was significantly lower than that of healthy women

miR-93-3p Was Increased in PBMCs of Patients with breast cancer

miR-93-3p expression was significantly upregulated in PBMCs from breast cancer patients compared to the control group ($P < 0.05$). As shown in Table 3 and Chart 2, the fold change in expression was significant. Breast cancer patients exhibited significantly higher *miR-93-3p* expression compared to healthy women.

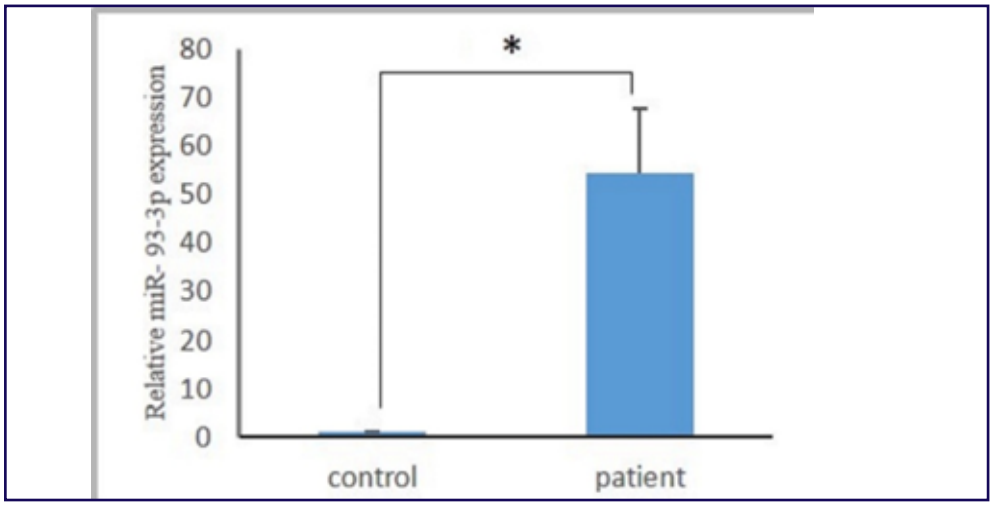


Chart 2. The relative mRNA Expression of *miR-93-3p* in PBMCs from the healthy women and breast cancer patients using real time PCR. The *miR-93-3p* expression from patients with breast cancer was significantly higher than that of healthy women ($P<0.023$)

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Table 3. The fold changes of *miR-93-3p*, *NFATc1* and *NFATc3* in PBMC from Healthy Women and Breast Cancer Patients

Gene names	Significance level	Standard error	P-value	Fold change	Gene expression relative to control
miR-93-3p	P<0.05	± 0.32	0.023	54.21592 ± 13.43	Up
NFATc1	P<0.05	± 0.67	0.047	0.769403± 0.11	Down
NFATc3	P<0.05	± 0.46	0.039	0.516247± 0.017	Down

Discussion

Previous microarray studies have demonstrated that several microRNA levels are elevated in both tumor tissue and plasma of breast cancer patients (18). This study is the first to evaluate the expression of *miR-93-3p* and its downstream target genes, the transcription factors *NFATc1* and *NFATc3*, in peripheral blood mononuclear cells (PBMCs) of healthy women and women with breast cancer. We observed upregulation of *miR-93-3p* and downregulation of *NFATc1* and *NFATc3* in breast cancer patients.

Early diagnosis is crucial for successful breast cancer treatment. Currently, the primary methods for diagnosing and monitoring treatment response involve imaging techniques like mammography, magnetic resonance imaging (MRI), positron emission tomography (PET), computed tomography (CT), and single-photon emission computed tomography (SPECT). However, these techniques have limitations, necessitating the development of new biomarkers. Tumor markers play a vital role in identifying and monitoring breast cancer patients (17). One study (18) demonstrated that miR-182 inhibits NFATs, thereby suppressing T cell activity. Similarly, miR-93 is highly expressed in breast tumor tissue, but its expression in patients' blood mononuclear cells remains unknown. Research suggests that exosomes and microvesicles can transfer miR-93 between tumor cells and immune cells within the tumor microenvironment (19). The concurrent increase of miR-93 in tumor tissue and its high concentration in patient serum imply its potential for transfer to different cell types, particularly immune cells (especially T cells), and subsequent modulation of their expression profiles (10, 20).

T cell activation primarily occurs when T cells bind to peptides presented on major histocompatibility complex (MHC) molecules. This interaction triggers a signaling cascade that leads to T cell differentiation into type 1 helper (TH1) and type 2 helper (TH2) cells. TH1 cells play a critical role in cellular immunity, which is essential for tumor suppression (21-23).

Five NFAT isoforms exist, including *NFATc1* to *NFATc4* and *NFAT5*. Notably, *NFATc1* plays a critical role in differentiating T cells into type 2 helper (Th2) cells and activating humoral immunity. Conversely, *NFATc3* is essential for Th1 cell differentiation and activation of cellular immunity (12). Inhibition of NFAT factors can significantly impair the development of type 1 and type 2 helper T cells, thereby weakening the acquired immune system. This disrupts the expression and activation of NFAT proteins, leading to the downregulation of the FOXP3 transcription factor.

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[DOI: 10.18502/jabs.v14i1.14807]

Consequently, the immune system deviates towards the production of immunosuppressive regulatory T cells (Tregs), which promote breast cancer growth and progression (24).

Since the NFAT family exhibits pleiotropic functions in various cancers and tumor environments, isoform-specific or tumor-specific NFAT-based therapies could be effective. This concept suggests that NFAT or NFAT-binding molecules might be suitable drug targets. Several compounds with these properties have already been reported, including digitoxin, zoledronic acid, and genistein. However, the molecular mechanisms of genistein's anti-neoplastic effects across different tumor types remain poorly understood (10). Our study found that *NFATc1* and *NFATc3* gene expression in PBMCs from women with breast cancer was significantly lower compared to healthy controls. Real-time PCR analysis of *miR-93-3p* in PBMCs from breast cancer patients revealed a significant increase in its expression compared to the control group. Consistent with these findings, *NFATc1*-deficient mice are reported to be more susceptible to chemically induced tumors, suggesting a tumor suppressor role for the *NFATc1* gene (17).

Impaired regulation of *NFAT* gene expression contributes to the expression of various target genes involved in cancer. However, *NFAT* isoforms have a variety of functions in the tumor formation process, such as *NFAT2* activation inhibiting fibroblast cell differentiation and stimulating malignancy induction. In contrast, activation of *NFAT1* inhibits cell cycle and apoptosis, and inhibits H-rasV12-induced transformation. These results suggest that NFAT1 is a tumor suppressor and *NFAT2* is an oncogene (25, 26). Sustained *NFATc1* activity can alter the mechanisms that regulate normal cell growth and differentiation in 3T3-L1 cells. This can be sufficient to induce these mature cells to acquire an altered cellular phenotype, potentially leading to oncogenic transformation (27). *NFATc1* and *NFATc3* expression levels vary across different studies. These discrepancies likely arise because different cell types express distinct primary NFAT isoforms. For example, human keratinocytes primarily express *NFATc1*, while fibroblasts mainly express *NFATc2* (10). Notably, *NFATc1*-deficient mice exhibit a hyperproliferative phenotype (27). Our study supports the hypothesis that *miR-93-3p* may suppress the immune system and promote cancer development. Conversely, *NFATc1* and *NFATc3* appear to have tumor suppressive functions. In other words, a decrease in *NFATc1* and *NFATc3* expression, coupled with an increase in *miR-93-3p*, might contribute to cancer development. This suggests that *miR-93-3p* could promote cancer by downregulating *NFATc1* and *NFATc3*. This study is the first to investigate the importance of miR-93-3p and its downstream targets, the *NFATc1* and *NFATc3* genes, in peripheral blood mononuclear cells (PBMCs). While limitations exist, including a small sample size, recruitment from a single center, and the lack of evaluation for PBMC subtypes (Th17 or Treg) and inflammatory/anti-inflammatory cytokines (IL-17 and IL-10, respectively), this research lays the groundwork for future studies. These future studies can inform the development of appropriate preventive, diagnostic, and therapeutic measures with potentially reduced side effects and treatment costs. This, in turn, could improve the physical and mental health outcomes of breast cancer patients.

Conclusion

In conclusion, our findings suggest that miR-93-3p may decrease the activity of *NFATc1* and *NFATc3*. The observed overexpression of *miR-93-3p* and the corresponding suppression of its target genes, *NFATc1* and *NFATc3*, in PBMCs of breast cancer patients, warrant further investigation for their potential as minimally invasive diagnostic markers.

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[DOI: 10.18502/jabs.v14i1.14807]

Acknowledgements

The authors gratefully acknowledge the Faculty of Basic Science and the Research Institute of Biotechnology at Shahrekord University for providing the necessary equipment.

Conflict of Interest

No conflict of interest was declared by the authors.

Funding

No funding was received.

Ethical Considerations

Human-derived blood samples were collected from healthy individuals and breast cancer patients at the Breast Disease Research Center, Shiraz University of Medical Sciences, after written informed consent was obtained in accordance with guidelines approved by the Shiraz University of Medical Sciences Ethical Committee. All experimental methods adhered to the Declaration of Helsinki. All protocols were additionally approved by an Ethics Committee of professionals at Shahrekord University (code: IR.SKU.REC.1401.053).

Code of Ethics

Ethics committee approval code: IR.SKU.REC.1401.053

Authors' Contributions

MD, GR, SG, and ST contributed to the conception and design of the work, interpretation of the data, and drafting of the article. All authors approved the submitted manuscript and agreed to be accountable for all aspects of the work. They ensure that any questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Data Availability Statement

All data are mentioned in the article.

List of Abbreviations

Nuclear factor of activated T-cells, cytoplasmic 1 (*NFATc1*)

Nuclear factor of activated T-cells, cytoplasmic 3 (*NFATc3*)

microRNA-93 (miR-93)

peripheral blood mononuclear cells (PBMC)

Real-Time Polymerase Chain Reaction (Real-Time PCR)

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