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Correlation between Expression of *hsa-miR-490-5p* and NFAT5 in Peripheral Blood Mononuclear Cells Obtained from Breast Cancer Patients

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ABSTRACT

Breast cancer is a complex genetic disease that has an average annual incidence of two million people and the second leading cause of death among women all over the world. Micro-RNAs are consistently reported to regulate gene expression in all cancers. The present study, the correlation between the expression of hsa-miR-490-5p and nuclear factor of activated T-cells 5 (*NFAT5*) in breast cancer were investigated. *NFAT5* nuclear accumulation occurs regardless of Wnt/β-catenin activated signaling in a substantial portion of breast cancer. The analysis of prediction target and dual-luciferase reporter assays supported that hsa-miR-490-5p directly targeted *NFAT5* and suppressed the expression of *NFAT5*. In a cross-sectional comparative study, peripheral blood samples were collected from 30 subjects with breast cancer and 30 healthy individuals as a control group. Total mRNA was extracted from peripheral blood mononuclear cells (PBMCs) and cDNA was synthesized to study the *NFAT5* and hsa-miR-490-5p gene expression variations by real-time PCR. A significant decrease was observed in gene expression and sera concentration of *NFAT5*, hsa-miR-490-5p in PBMCs of breast cancer patients. The obtained results indicated that hsa-miR-490-5p acts as oncomir in serum by targeting *NFAT5* direct ($p<0.05$).

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Introduction

Breast cancer is among the most common cancers in the world, contributing 12.3% of the total number of new cancer cases in 2018. It is the second leading cause of death in women, most of whom are over 40 years old (Bray *et al*., 2018). Studies have shown an increase in the prevalence of this cancer in Asian countries, such as Iran, and attributed it to differences in lifestyle, genetics, and geographical area (Hosseinzadeh Colagar *et al*., 2015). There are several ways for early detection of cancer that blood analysis and circulating microRNA in the serum is the easy way (Shimomura *et al*., 2016). MiRNAs are a group of evolutionarily conserved, small single-stranded, noncoding RNA molecules with 20-22 nucleotides that regulate gene expression by targeting mRNAs to trigger either translational repression or degradation of mRNA. Many miRNAs are involved in several human cancers, such as breast cancer (Tafrihi and Hasheminasab, 2019; Babaei *et al*., 2018). Results indicated that miR-490-3p acts as oncosuppressive microRNA and inhibits breast cancer tumorigenesis and progression via direct targeting of RhoA (Zhao *et al*., 2016). Another study showed that hsa-miR-490-3p might be a potential therapeutic solution for patients with CDP-resistant ovarian cancer; in addition, it was revealed that miR-490-3p was down-regulated in CDDP-resistant OVCAR3/CDDP and SKOV3/CDDP cells of the ovarian cancer tissues (Tian *et al*., 2017). Moreover, it was observed that miR-490-3p was essential for TGFβ1-induced tumor cell invasion and migration influenced by CCAT1 (Mu *et al*., 2018). In the renal cell cancer, it was observed that hsa-miR-490-5p was directly bound to 3'UTR of the PIK3CA mRNA and reduced the expression of PIK3CA at both mRNA and

protein levels, resulting in further inhibition of phosphatidylinositol 3-kinase/Akt signaling pathway (Chen *et al*., 2016). In colorectal cancer, overexpression of hsa-miR-490-3p inhibited the cell migration and invasion abilities (Xu *et al*., 2015). Hsa-miR-490-3p overexpression reduced proliferation, promoted G1 arrest and apoptosis, suppressed migration and invasion, and reduced TGFα, NF-kB, cyclin D1, and improved Bax mRNA and protein expression (Sun *et al*., 2016). There are many options for searching miRNA in mirbase Website (www.mirbase.com). One of the predicted targets of hsa-miR-490-5p is the nuclear factor of activated T cells (NFAT). NFAT contains five members, all of which except *NFAT5*, are activated by the Ca^{2+} influx in the cell, thus their insensitivity to calcium and calcineurin depends on extracellular tonicity. (Luo *et al*., 1996). The *NFAT* acts as a multifunctional and powerful regulator of the tumor progression and invasion process in breast cancer and *NFAT5* promotes cell migration (Yoeli-Lerner *et al*., 2005, 2009). In breast cancer, a significant positive correlation was observed between α6β4 integrin expression and NFAT1 and *NFAT5* expressions (Jauliac *et al*., 2002). The α6β4 integrin is released from hemidesmosomes in cancer cells and attaches to the actin cytoskeleton, thereby activating *NFAT5* transcription and facilitating cancer cell metastasis via the activation of downstream targets, such as COX-2 (Yiu and Toker, 2006 and Siamakpour-Reihani *et al*., 2011). Further, the anti-metastatic Wnt ligand WNT5A was observed to block the activation of NFAT in human breast epithelial cells by binding to the NFAT maintenance kinase, CK1 (Dejmek *et al*., 2006 and Foldynová-Trantírková *et al*., 2010). In addition to COX-2, NFATs also induces the transcription of pro-invasive genes, such as autotoxin, in breast epithelial cells. Autotoxin mediates the conversion of lysophosphatidylcholine into lysophosphatidic acid (LPA) which, in turn, promotes invasive and metastatic mammary carcinoma (Chen *et al*., 2005 and Seifert *et al*., 2009). Moreover, proteins belonging to this family play a central role in inducible gene transcription during the immune response and T-cell activation, which are important in breast cancer (López-Rodríguez *et al*., 2004). Thus, the aim of this study was to evaluate the alteration of hsa-miR-490-5p and *NFAT5* in peripheral blood mononuclear cells (PBMCs) obtained from breast cancer patients.

Materials and Methods

Sampling

Blood samples (5 ml) were obtained from 30 patients with breast cancer (31 to 71 years old), 30 without breast cancer as the control group (28 to 61 years old). Patient consent for all samples was obtained (ethics committee: IR.IAU.QOM.REC.1397.011); all pathological information of patients was gathered from the pathology department of the academic Imam Reza Hospital-501(Tehran). All of the samples obtained from patients were without treatment and operation.

Cell isolation

PBMC cell separation was performed using ficoll density gradient centrifugation method. 5 ml sample from the venous vein was taken and in a tube containing heparin was collected. First, blood samples were centrifuged 5 minutes at 800g for serum separation. Then blood samples were diluted with an equal volume of phosphate-buffered saline (PBS). The cell isolate, Centrifugation was used for 25 minutes at 2500 rpm. The cloud layer containing the formed lymphocytes was collected and centrifugation was carried out with 1500 rpm for 10 minutes (Fig. 1). Finally, the obtained pellet with PBS solution was rinsed and kept at -70 $^{\circ C}$ for further testing.

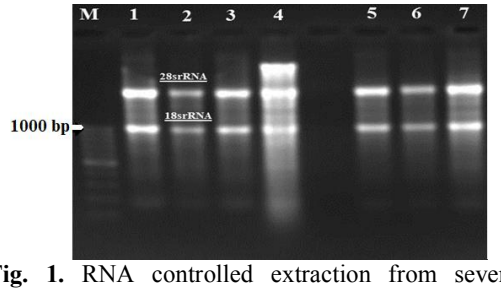

Fig. 1. RNA controlled extraction from seven samples in the 1.5% Agarose gel electrophorese: M= Gene Ruler™ 100bp DNA Ladder (Fermentas, Germany).

Real-time PCR

The mRNA from PBMCs was isolated using the RNA extraction kit (RiboEX Gene All, England) based on the manufacturer's instructions. To synthesize a cDNA from a cDNA synthesis kit (Fermentas, Germany)

according to the manufacturer's instructions, which uses 1µl of RNA. All primers were engineered using the oligo7 software for *NFAT5*, hsa-miR-490-5p, β-actin as a housekeeping gene, and RNU6 as internal control based on Gene Bank sequences.

To investigate the specificity of designed primers, the Blast database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used from NCBI (Table 1).

Table 1. Primers used in RT-qPCR

Locus	Primer(5'→3')	Amplicon size	Gene AC number
β-actin	F: AGACGCAGGATGGCATGGG R: GAGACCTTCAACACCCCAGCC	161bp	NM_001101.3
NFAT-5	F: AACAACATGACACTGGCGGT R: CTCGAAAAACCAATCTGGCACG	175bp	NM_138714.3
MiR-490-5p	F: TGTTTTTGCCATGGATCTCCAG R: GTGCAGGGTCCGAGGT	74bp	MIMAT0004764
RNU6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT	94bp	NR_002439.1

To evaluate the expression of the *NFAT5* gene in PBMC, a typical PCR was performed for all samples in a volume of 20 ml. The reaction mixture contains 4 µl $MgCl_2$, 2 µl dNTP, 1 µl primer, 1 µl RT, 1 µl Taq DNA polymerase (Sinagene, Iran), 4 µl cDNA and 7 µl deionized water. In order to amplify the *NFAT5* and β-actin genes, the PCR was started at 95 °C for 5 minutes, and amplified during 35 cycles at 95 °C for 1 min, 61 °C (*NFAT5*) and 62 °C (β-actin) for 30 seconds and 72 °C for 30 seconds, the final elongation step was carried out at a temperature of 72 °C for 10 minutes. PCR products were observed in 1.5% agarose gel by electrophoresis. By measuring the intensity of fluorescence and using primer pairs for *NFAT5*, hsa-miR-490-5p, RNU6 was also utilized as the internal control. For real-time used Mic real-time PCR cycler instrument. Cyber green Fluor genic nucleotide (Roche kit, Germany) was used for monitoring the cDNA amplification in the process of real-time PCR. The reaction was conducted in 10 µl of a solution consisting of 2 µl Fast Start Master solutions and 0.3 µM of each primer. A total of 9 µl of the reaction mixture was placed into 0.1 vials, and 1 µl of cDNA was added as a template. Thermal cycling consisted of an initial denaturation step at 95 °C for 10 minutes followed by an amplification program repeated for 45 cycles. The amplification was done at 95 °C for 10 seconds, 61 °C (*NFAT5*), 62 °C (RNU6) and 59°C (hsa-miR-490-5p) for 10 seconds, and 72 °C for 20 seconds with a single fluorescence acquisition at the end of the elongation step. The third step included a melting curve program run by the default program of the real-time PCR. The analysis of the melting curve revealed only one peak for each reaction.

Statistical analysis

The number of samples was determined by Minitab18.1 software. Real-time PCR data were analyzed by ΔΔCt and using Excel (ver.2010), GraphPad PRISM (ver. 5.04) software, the correlation between the changes in *NFAT5*, and hsa-miR-490-5p expression levels in PBMCs, were assessed using the SPSS (ver. 16). In the current study, P-value less than 0.05 ($P<0.05$) was statistically considered significant.

Results

In the present study, changes in the expression of *NFAT5* and hsa-miR-490-5p in PBMCs of breast cancer patients and unconscious individuals as a control group were examined. QRT-PCR results showed that the *NFAT5* gene in PBMC cells expressing breast cancer and healthy individuals (Fig. 2).

Gene expression analysis

In this study, the expression of the *NFAT5* gene and hsa-miR-490-5p in PBMC cells of breast cancer patients and non-infected individuals as a control group was investigated. Analysis of Real-time PCR data indicated that there are differences in the level of expression of genes among the groups. Expression of *NFAT5* in breast cancer patients

was lower than the control group, and this was significant. In addition, changes in the expression of hsa-miR-490-5p in the patients compared to the control group, which was significant in patients and expression was high in patients (Fig. 3). However, the correlation among expressions changes in the two groups was significant between healthy subjects and patients.

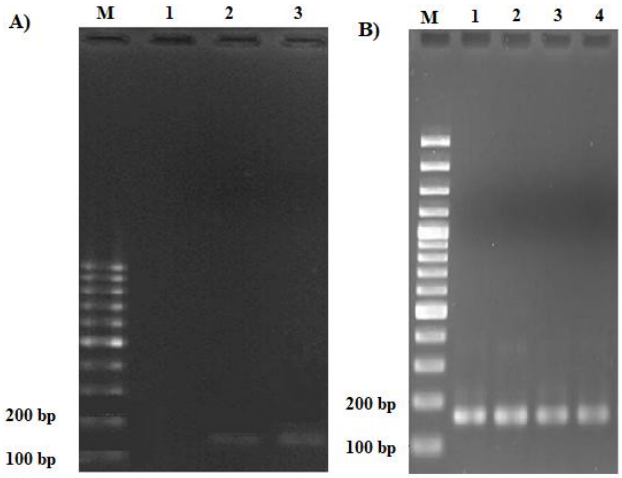

Fig. 2. The pattern of cDNA fragments amplified by the β-actin gene primer (B) and NFAT5 (A); A) NFAT5 gene expression in PBMCs (175 bp). Lane M: 100 bp size ladder (Fermentas, Germany), Lane 1: negative control, Lanes 2 and 3: NFAT-5 expression of the subject group in PBMCs (175 bp); B) β-actin gene Expression in PBMCs (161 bp). Lane M: 100 bp size ladder (Fermentas, Germany), Lanes 1- 4: β-actin gene expression in PBMCs of both subject groups (161 bp).

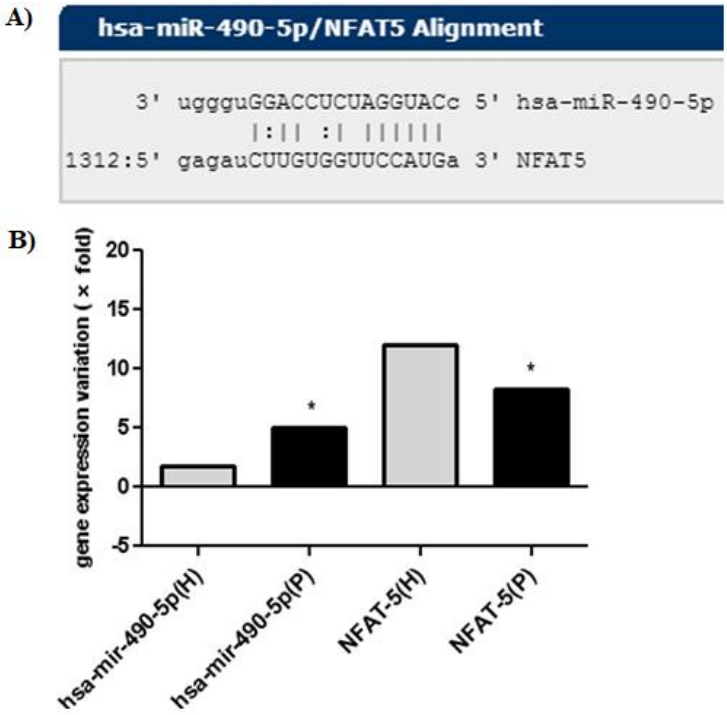

Fig. 3. MiR-490-5p targets NFAT5 and influences NFAT5 downstream factors directly**: A**) NFAT-5 mRNA is supposed to be a target of miR-490-5p with the binding sequence UCCAUG; B) Gene expression of hsa-miR-490-5p and NFAT5 were changed in PBMC. For patient was used (P) and for the control group was used (H). Expression of *NFAT5* in breast cancer patients was lower than the control group, and this was significant. In addition, changes in the expression of hsa-miR-490-5p in the patients compared to the control group, which was significant in patients and expression was high in patients.

Discussion

With the spread of medical knowledge, unfortunately, cancer can be considered the third most common cause of death in Iran, which is different in terms of genders. Women have the highest rates of breast cancer. Most cancers have been cured in case of detecting, and reduce the level of adverse effects of the used drugs. In the late diagnosis of breast cancer in women, including stages 3 and 4, breast and breast surgery, have a very destructive effect on women's spirits. Chemotherapy or even radiotherapy, as well as therapeutic effects, can also cause damage to healthy tissues. There are methods for early cancer diagnosis and also an effective treatment. Other treatments, such as radiation therapy, chemotherapy, and the like, are almost as damaging to the healthy cells and tissues as they have therapeutic effects. Breast cancer is the most common cancer in women, accounting for 32% of women's cancers.

MiRNAs are either inhibitor or augmentative in terms of cancer, which sometimes exhibit different effects with unobtrusive settings. For example, it may be that a tumor suppressor miRNA, with an overlapping configuration of the target gene, is in an increasing state cancer, which involves a change in cellular messaging. The translation inhibits the process by microRNA can be reversible. The complexity of these processes is a reason for the wide-ranging regulation of microRNAs. MicroRNA can target several different mRNAs. Therefore, to determine the performance of microRNA, identification of microRNA target molecules is important. MicroRNA expression is associated with clinical and biological features of the tumor, such as tissue type, differentiation, invasion and response to treatment. The use of microRNA as diagnostic markers is possible through serum or human plasma testing. Therefore, we can detect cancerous microRNAs and tumor cells present in serum or plasma without any invasive technique. In these cases, microRNAs that correlate with malignant phenotypes are very useful as diagnostic markers for the diagnosis of the disease in its early stages, indicating that their expression in the blood increases or decreases. Lin Zhao *et al*. in 2016 showed that overexpression of hsa-mir-490-3p inhibits tumorigenesis and progression in breast cancer. Weiqing Chen *et al*. in 2017 showed that miR-490-5p inhibits Hepatocellular carcinoma cell proliferation, migration, and invasion by directly regulating ROBO1. Based on mirbase website, *NFAT5* is one of the predicted targets for hsa-mir-490-5p. The *NFAT5*, as a transcription factor causes transcription of various genes, including cytokines. Accordingly, the majority of *NFAT5*-positive breast cancer samples revealed an aberrant nuclear expression in comparison with control samples. *NFAT5* nuclear accumulation occurs regardless of WNT/β-catenin activated signaling in a substantial portion of breast cancer, and NFAT5 pathway activation may have a relevant role in breast cancer pathogenesis**.** Wnt is a proto-oncogene. In the absence of Wnt, the TCF transcription factor is linked to the promoter of genes, but when Groucho is binding to TCF, it inhibits the activity of the gene. β-catenin is continuous phosphorylation until complex with Axin, CK1, and GSK3. TrCP ubiquitin ligase destroys the β-catenin in proteasome with binding to the phosphate of β-catenin. As β-catenin is binding to its receptor (Frizzled), destroys Axin, APC, CK1 and GSK3 complex and β-catenin increases accumulation in the cell. Inactivation of Groucho leading to entering β-catenin which finally leads to target genes activation. Inappropriate activation of the Wnt pathway increases β-catenin in the cell, which is a sign of many cancers (Wang *et al*., 2019). The roles of NFAT transcription factors have been extensively studied in the immune system but their impact in human cancer remains poorly understood. Ubiquitous expression of NFAT isoforms in mammalian tissues has been described, and mainly two isoforms, NFAT1 and *NFAT5*, have been reported as overexpressed in human invasive ductal breast carcinomas. Overall, the contribution of specific NFAT isoforms in distinct breast cancer is still unknown. In the basal state, two kinases - tyrosine phosphorylation-regulated kinase2 (DYRK2) and casein kinase 1 (CK1)-phosphorylate NFAT TFs, maintaining them localized to the cytoplasm in an inactive conformation: the nuclear translocation and transcriptional activation of NFAT in cancer cells lead to the induction of genes that promote tumor progression, migration and invasion (Jauliac *et al*., 2002). Here, our investigation showed significantly high serum level of hsa-miR-490-5p in breast cancer

patients compared to controls (P<0.05). The obtained illustrated that although hsa-miR-490-5p expression level is reduced, down-regulation was statistically significant. Based on the result of this study, the hsa-miR-490-5p expression is associated with breast cancer initiation. The findings also showed a significantly low serum level *NFAT5* in breast cancer patients (P<0.05). With decrease expression of *NFAT5* and disorder Wnt pathway, β-catenin is increased in the cell leading to promoting tumor progression, migration. Nevertheless, this is a preliminary report demonstrating the release of hsa-miR-490-5p and its correlation with *NFAT5* in the serum of breast cancer patients. It can be suggested that more samples will be collected in other investigation the potential role of hsa-miR-490-5p in breast cancer.

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