**MiR-490-5p Functions as an OncomiR in Breast Cancer by Targeting NFATc4**

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**Abstract**

Breast cancer is a serious health problem worldwide in women. MicroRNAs are small non-coding RNAs of 18–25 nucleotides in length that post-transcriptionally modulate gene expression. MiR-490 has been reported as a tumor suppressor and oncomiR microRNA in breast cancer with two separate targets, NFAT and Rho. NFAT is one of the targets for miR-490 but the relationship between hsa-miR-490 and NFATc3, NFATc4 are not clear yet. Except for NFAT5, the other members of NFAT are activated by Ca²⁺ influx in the cell, either via the PLC-γ pathway or via store-operated Ca²⁺ entry, typically in T lymphoid cells. 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. Gene expression analysis of peripheral blood mononuclear cells (PBMCs) was performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to study the NFATc3, NFATc4, and hsa-miR-490-5p gene expression alterations. As per the obtained results, a significant decrease was observed in the expression level of NFATc4 (P<0.05), while hsa-miR-490-5p expression found to be elevated in PBMCs of breast cancer patient (P<0.05). Expression changes were not significant for NFATc3 gene (P>0.05). Taken together findings of this study indicated that serum hsa-miR-490-5p acts as an oncomiR by direct targeting the NFATc4.

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**Introduction**

Breast cancer (BC) has an incidence of 11.6% among all types of cancer, accounting for 6.5% of mortalities worldwide (Pilevarzadeh et al., 2019; Siegel et al., 2019). In Iran, BC is the fifth most common cause of death related to cancer comprising 24.4% of all cancers with the age-standardized rate (ASR) of 23.1 per 100,000 (Ghosn et al., 2020). Therefore, early diagnosis is important to decrease mortality. There are two ways for early diagnosis, either non-invasive or invasive method. In the non-invasive methods, mammographic image analysis and blood analysis with circulating microRNA in the serum have been using in breast cancer and in the invasive method have been using surgical sampling or thick needle sampling (González-Patiño et al., 2019). MicroRNAs are short non-coding RNA with 18-25 nucleotides that binding to target mRNAs and play a role in post-transcriptional gene regulation (Aminisepehr et al., 2018). Perfect or near-perfect complementary binding of microRNAs to their target mRNAs negatively regulates gene expression in terms of accelerating mRNA degradation or suppressing mRNA translation. Many investigations have reported that miRNAs play crucial roles in numerous biological processes, such as cell cycle, cell proliferation, cell differentiation, apoptosis, metabolism, and cellular signaling (Tafrihi et al., 2019). There are several important microRNAs in cancer that miR-490 is one of them. MiR-490-3p has been verified to suppress several cancers’ proliferation, metastasis, and progression in lung cancer, colorectal cancer, and prostate cancer (Gu et al., 2014; Xu et al., 2015). In endometriai cancer, miR-490-3p acts as a tumor suppressor with two targets that c-Fos and TGFα as a direct...
target in the 3′ UTR (Qu et al., 2017). The expression of miR-490-5p was gradually downregulated and transfection with miR-490-5p lentivirus reversed the differentiation ability of the human adipose-derived stem cells (hADSCs). Thus, miR-490-5p inhibits hADSC differentiation by suppressing bone morphogenetic protein receptor type II (BMPR2) expression (Yang et al., 2015). MiR-490-3p has been validated to act as a regulator of cell proliferation, migration, invasion, or in the EMT in hepatocellular carcinoma cells and vascular smooth muscle cells (Chen et al., 2014). Another recent study shows that the expression of miR-490-5p was significantly down-regulated in neuroblastoma (NB) tissues and cell lines that significantly decreased miR-490-5p levels were correlated with lymph-node metastasis stage and poor survival prognosis in NB patients (Wang et al., 2020). MiR-490-5p has been proven to act as an oncomiR, promotes cell proliferation and inhibits apoptosis in hepatocellular carcinoma by targeting miR-490-5p/SOX2 signaling pathway (Cai et al., 2018). Previous studies have identified various miRNAs functioning as tumor suppressors in bladder cancer, including miR-409-5p that regulate the proliferation, migration, and invasion of bladder cancer cells by downregulating various oncogenes (Liang et al., 2017). The relationship between miR-490-5p and Roundabout homolog 1 (ROBO1) has been verified in Hepatocellular Carcinoma. MiR-490-5p inhibited cell proliferation, migration, and invasion, but promoted cell apoptosis of Hep3B cells by inhibiting ROBO1 (Chen et al., 2019). Another study showed that miR-490-5p inhibits the proliferation of bladder cancer cells by targeting c-Fos (Mao et al., 2015). Evaluation of miR-490 was detected as a biomarker of disease activity among patients with Focal segmental glomerulosclerosis (FSGSclin) (Chung et al., 2017). There are five members for the nuclear factor of activated T-cells (NFAT) family, that two members are very important in cancer and tumor progression (Hoey et al., 1995; Ho et al., 1995). NFAT3(NFATc4) and NFAT4(NFATc3) are activated by Ca^{2+} influx in the cell, either via the phosphoinositide phospholipase C (PLC-γ) pathway or via store-operated Ca^{2+} entry, typically in T lymphoid cells (Luo et al., 1996; Mancini et al., 2009). It has been reported that NFAT isoforms are overexpressed in human solid tumors (Pan et al., 2013). NFAT4 activates transcription of downstream gene targets, thus directly linking calcium signaling to gene expression (Rao et al., 1997). The pro-angiogenic role of NFAT signaling was first demonstrated in NFAT3/NFAT4 null mice and the calcineurin B (CNB1) knockout mice (Graef et al., 2001). Mice lacking CNB1 or both NFAT3/NFAT4 genes die at mid-gestation due to disorganized vasculature and increased and deregulated expression of vascular endothelial growth factor A (VEGFA) (Maillet et al., 2010). NFAT appears to modulate the expression of VEGF by regulating the transcription of VEGF receptor 1 (VEGFR1). VEGF stimulates PLC-γ receptor-mediated activation, increasing intracellular calcium levels that activate calcineurin to cause NFAT nuclear translocation (Schulz et al., 2004). Though NFAT has an inhibitory effect on VEGF expression, VEGF can induce NFAT transcriptional activity by mediating its nuclear translocation (Jinnin et al., 2008). NFAT activation by VEGF in endothelial cells also induces the pro-angiogenic factor granulocyte-macrophage colony-stimulating factor (GM-CSF) (Cockerill et al., 1995). Inhibition of NFAT4 reduces the secreted frizzled-related protein 2 (SFRP2)-stimulated angiogenesis in vitro, and inhibition of calcineurin with tacrolimus also blocks SFRP2-stimulated angiogenesis and angiosarcoma growth (Siamakpour-Reihani et al., 2011). 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-Rodriguez et al., 2004). Thus, this study aimed to evaluate the alteration of hsa-miR-490-5p (mature microRNA), NFATc3 and NFATc4 expression levels in peripheral blood mononuclear cells (PBMCs) obtained from breast cancer patients that relationship between the two groups (miR-490 and NFATc3, and NFATc4) have not been investigated in breast cancer until now.

Materials and Methods

Samples collection

Materials and Methods

Samples collection
Venous blood samples (5 ml) were obtained from patients ($n = 30$) with breast cancer and control subjects ($n = 30$). The age of the patients was 29 to 61 years old. All of the healthy controls had no history of breast cancer diseases. 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).

Isolation of peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density-gradient centrifugation (Baharafshan, Tehran, Iran) from the whole blood. The tubes were centrifuged at 800g for 20 min. The PBMCs layer was transferred to a new canonical tube. The cells were washed twice in PBS (for 10 min at 400g followed by 200g for 5 min) and the supernatant was separated. Finally, the mixture was then transferred to a 1.5 ml microcentrifuge tube and kept at -70 °C for further testing.

### RNA extraction and cDNA synthesis

RNA isolation was performed immediately after PBMC preparation. 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 (Fermentase, Germany) according to the manufacturer's instructions, which uses 1 μg of RNA and kept frozen at -20 °C until use.

### Real-time PCR

For real-time PCR, Mic real-time PCR cycler instrument was used. The designed primer pairs for NFATc3, NFATc4, hsa-miR-490-5p, RNU6 (internal control) were utilized (Table 1).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer (5'-3')</th>
<th>Amplicon size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFATc4</td>
<td>F: GCACCGTATCACAGGCAAGATG</td>
<td>131</td>
<td>NM_001136022.2</td>
</tr>
<tr>
<td></td>
<td>R: TCAGGATTCCCGCGCAGTCAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFATc3</td>
<td>F: CGGTTCTGGTGCTGCTCG</td>
<td>246</td>
<td>NM_004555.4</td>
</tr>
<tr>
<td></td>
<td>R: GAAGTCGAGCTCGTCTGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MiR-490-5p</td>
<td>F: TGTTTTTGCCATGGATCTCCAG</td>
<td>74</td>
<td>MIMAT0004764</td>
</tr>
<tr>
<td></td>
<td>R: GTGCAGGGTCCGAGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNU6</td>
<td>F: CTCGCTTCCGGCAAGCACA</td>
<td>94</td>
<td>NR_002439.1</td>
</tr>
<tr>
<td></td>
<td>R: AACGCTTACGAATTTTGCGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cyber green fluorogenic nucleotide (Roche kit, Germany) was used for monitoring the cDNA amplification in the process of real-time PCR. 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 ($NFATc3$), 61 °C ($NFATc4$), 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 amplification specificity of each primer set was also controlled by a melting curve and the amount of mRNA target was assessed via the comparative cycle threshold ($2^{-ΔΔCt}$) method (Fig. 1).

### Statistical analysis

Analysis of variance (ANOVA) test was applied to determine genes that were differentially expressed on one or more of the groups. Real-time PCR data were analyzed by $2^{-ΔΔCt}$ and using Excel (ver.2010), GraphPad PRISM (ver. 5.04) software for the correlation between the changes in NFATc3, NFATc4, and hsa-miR-490-5p expression levels in PBMCs. In the current study, the $p$-value less than 0.05 ($p<0.05$) was statistically considered significant.

### Results

#### Gene expression analysis

Analysis of Real-time PCR data indicated that there are differences in the level of expression of genes among the groups. The expression of NFATc4 in breast cancer patients was lower than the control group, and this was significant ($p<0.05$), but the expression of NFATc3 was not significantly different ($p>0.05$). Also, changes in the expression of hsa-miR-490-5p, in the patients compared to the control group, was significant ($p<0.05$). However, NFATc4 mRNA levels were significantly decreased by about 2-fold in all
patient samples as compared with the control group. The level of NFATc3 did not differ significantly between the control and patient samples. The expression of hsa-miR-490-5p was significantly increased in patient samples as compared with the control group (Fig. 2).

Discussion

Men can get breast cancer, too, but they account for less than 1% of all breast cancer cases (Becker et al., 2010). Among women, breast cancer is the most second most common cancer diagnosed in women after skin cancer and the second leading cause of cancer deaths after lung cancer (Paluch-Shimon et al., 2020). On average, 1 in 8 women will develop breast cancer in her lifetime. About two-thirds of women with breast cancer are 55 or older. Most of the rest are between 35 and 54. Breast cancer usually begins in a small area of either produce milk (lobular carcinoma) or the ducts (ductal carcinoma), which carry it to the nipple (Wijayabahu et al., 2020). Some women will get breast cancer even without any other risk factors. Most women have some risk factors, but most women do not get breast cancer (Key et al., 2001). 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). There are two important roles in cancer for microRNA, either tumor suppressor or oncomiR. Tumor suppressor miRNAs exhibit their role by inhibiting × the expression of target mRNA (Jiang et al., 2020). One of the microRNAs is miR-490 with an important role in some cancer. The increase of miR-490-5p expression was showed to decrease EGFR expression to suppress bladder cancer (Wu et al., 2019). MiR-490-5p on renal cancer cell was verified to directly bind to the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mRNA and reduce the expression of PIK3CA and inhibits phosphatidylinositol 3-kinase/Akt signaling pathway (Chen et al., 2016). In the case of breast cancer, a significant decrease in NFAT5 gene expression with miR-490-5p was observed and it is concluded that hsa-miR-490-5p acts as oncomir in serum (Nikfarjam et al., 2019) while the relationship between hsa-miR-490-5p and NFATc3, NFATc4 have not studied in breast cancer. NFAT is a family with five members that...
except NFAT5, another member is activated either PLC-γ pathway or via store-operated Ca\(^{2+}\). Phospholipase C gamma (PLC-γ) is one of the important signaling pathways in T-cell. There are several steps for Activate of PLC-γ. The first step is preferentially hydrolyzing of the membrane phospholipid phosphatidylinositol 4, 5-bisphosphate (PIP2) to generate the second messenger’s diacylglycerol and inositol 1, 4, 5-trisphosphate (IP3). Diacylglycerol is retained within membranes where it recruits and activates numerous proteins including conventional isoforms of protein kinase C. In contrast, IP3 diffuses throughout the cytosol where it binds to IP3 receptors embedded in endoplasmic reticulum leading to mobilization of sequestered calcium. PLC-mediated depletion of PIP2 also modulates the activities of several ion channels and proteins with phosphoinositide-binding domains. Thus, the PLCs coordinate fluctuations in PIP2 levels and the bifurcating signaling pathways emanating from PIP2 hydrolysis to regulate numerous cellular processes, including fertilization and embryogenesis, cell proliferation and differentiation, as well as various types of cell migration (Mark et al., 2006). Also, the overall structure is highly electronegative, and this property will inhibit lipase activity by disfavoring interactions with negatively charged membranes. In particular, the overall negative charge of the PH domain indicates that it is unlikely to bind phosphatidylinositol 3, 4, 5-trisphosphate as previously reported. For PLCs to hydrolyze membrane-embedded PIP2, the hydrophobic ridge of the catalytic TIM barrel must insert into lipid bilayers. However, in the case of Phospholipase C-gamma1 (PLC-γ1), the hydrophobic ridge interacts with portions of the serine protease homologs (sPH) domain in the regulatory array; this arrangement is expected to effectively block membrane engagement. The active site sits beneath the hydrophobic ridge and is readily located by the bound Ca\(^{2+}\) cofactor. As implied by the visibility of the Ca\(^{2+}\) cofactor, the active site is fully solvent-exposed and could presumably hydrolyze soluble substrates not embedded in lipid bilayers. Two major interfaces lock the regulatory array on top of the catalytic core. The first is the aforementioned sPH domain interacting with the hydrophobic ridge of the TIM barrel. A second interface is formed between loops of the cSH2 domain and the C\(_2\) domain of the catalytic core. The pinched region of the C\(_2\) domain is an additional membrane anchor point in the Phospholipase C-δ (PLC-δ) isozymes, where Ca\(^{2+}\) mediates between the C\(_2\) domain and negatively charged membranes (Nishimura et al., 2011; Lomasney et al., 2012). Based on sequence conservation and overall charge distribution, this region of the C\(_2\) domain of PLC-g1 also seems likely to interact with Ca\(^{2+}\) and membranes. PLC-g2 is anticipated to engage Ca\(^{2+}\), similarly. Therefore, the PLC-γ pathway and Calcium ion enter the nucleus causes T-cell activation. NFAT acts as a calcium sensor, integrating calcium signaling with other pathways involved in development and growth, immune response, and inflammatory response (Nishida et al., 2003). NFATC4, a member of the nuclear factor of activated T cells (NFAT) family of transcription factors that are involved in immune cell signaling, survival, and angiogenesis (Mancini et al., 2009). NFATc4 is anti-apoptotic and mediates cell survival in some tissues, such as neurons (Benedito et al., 2005). In renal tubular cells, NFATc4 was induced by carboplatin leading to increased apoptosis, which is assumed to mediate carboplatin-induced renal toxicity (Vashishta et al., 2009). Moreover, it may be due to activation of the calcium/calcineurin signaling pathway, which activates NFATc4 and leads to upregulation of FasL and inducing the activation of caspase-8, leading to the activation of caspase-3, -6 and -7, and therefore apoptotic cell death (Kalivendi et al., 2005). Here, our investigation showed significantly high serum levels of hsa-miR-490-5p in breast cancer patients compared to controls. The obtained results illustrated that although hsa-miR-490-5p expression level is reduced, down-regulation was statistically significant. The findings also showed a significantly low serum level NFATc4 in breast cancer patients. NFAT is important for the expression of Interleukin-2 (IL-2) and IL-2 is necessary for activation of T-cell. Therefore, with decrease expression of NFATc4 and disorder PLC-γ, FasL pathways, expression of T-cell, and apoptosis are decreased 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 NFATc4 in the serum of breast cancer patients.

Conflicts of interest

The authors have no competing interests.

References


