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Altered Expression Levels of MicroRNA-155 and SOCS-1 in Peripheral Blood Mononuclear Cells of Newly Diagnosed Breast Cancer Patients

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ABSTRACT

MicroRNA-155 (miR-155) has a critical role in pro-inflammatory activation and tumor progression. In addition, miR-155 has various oncogenic effects in the tumor microenvironment by targeting the suppressor gene of cytokine signaling-1(SOCS-1) and interleukin-6 (IL-6). This study investigated the association of inflammatory changes with the variations of miR-155 expression in newly diagnosed breast cancer (NDBC) patients.

Seventy NDBC patients were categorized as lobular and ductal subgroups and forty healthy individuals participated in this study. The expression rate of miR-155 and its downstream target gene, SOCS-1, as well as the plasma levels of IL-6, were evaluated in peripheral blood mononuclear cells of NDBC patients; using real-time PCR and enzyme-linked immunosorbent assay, respectively.

Our results indicated an over-expression of miR-155 in the PBMCs of NDBC patients which was significantly associated with the tumor grade and the type of ductal carcinoma. In contrast, a significant downregulation of *SOCS-1* was observed in NDBC patients compared to the control group, however, there was no significant difference between the two subtypes of BC. Furthermore, a higher concentration of plasma IL-6 was detected in NDBC patients compared to the healthy control group which had an inverse correlation with the *SOCS-1* levels.

According to the potential effects of miR-155 on regulating the expression of SOCS-1 and IL-6, we suggest this small transcript as a promising diagnostic marker for various types of BC patients.

Keywords: Breast cancer; IL-6; MicroRNA; Suppressor of cytokine signaling

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INTRODUCTION

Breast cancer (BC) is known as the most commonly diagnosed malignancy in women worldwide with a relatively high rate of mortality[1] Several factors are involved in increasing the risk of BC including chronic inflammation, family history of BC or certain non-cancerous breast diseases, late-onset menopause, menopause hormone therapy, late pregnancy, and radiation exposure.[2] Different treatment approaches are considered for early-onset diseases such as a combination of surgery, radiation therapy, and chemotherapy [3] or hormone therapy.[4]

Recent enormous investigations on targeted cancer therapy have been conducted based on determining newly or previously identified mediators or molecular pathways involved in emerging the cancer cells or potentiating the malignancies. The growing scientific evidence explained the altered expression levels of microRNAs (miRNAs) in a variety of human cancers; thus the BC is no exception.[5] MicroRNAs are small non-coding RNA molecules (~ 20–25 nucleotides in length) functioning as regulators of genes during the post-transcriptional modifications.[6] In addition, miRNAs regulate more than 30% of the human genome and play important roles in critical activities of the cells such as proliferation, growth, and apoptosis.[6,7] Therefore, altered expression of miRNAs is considerable to be related to abnormal functions of the cells and different types of cancers. Importantly, miRNAs can be detected using quantitative molecular methods in various biological fluids like plasma, saliva, urine, breast milk, and semen.[8] Therefore, these small molecules have been considered as promising prognostic biomarkers and also potential therapeutic targets in different types of cancers particularly BC.[9-11] Several investigators have demonstrated the noticeable roles of miRNAs in BC progression, invasion, and disease onset. Among them, *miR-155*, processed from a 3-exon gene named the *B cell integration cluster (BIC*), is known as an oncogenic transcript mediating inflammatory responses.[7,12-14] Moreover, this molecule negatively regulates the expression of (suppressing or of cytokine signaling 1 (*SOCS-1)* gene, thereby promoting tumor invasion.[15,16] *SOCS-1*, the most potent member of the *SOCS* family, encodes a member of the signal transducer and activator of transcription (*STAT)*-induced *STAT* inhibitor (*SSI*) family genes. *SSI* genes are induced by cytokines and regulate them in a negative feedback loop manner via downregulating the downstream molecules of the cytokine receptors.[17] The SOCS-1 protein interacts with a variety of cellular targets like genes involved in expressing the interleukin (IL)-6 receptor (IL-6R).[18,19] Interleukin-6 has vital roles in regulating the tumor microenvironment[20] and producing the BC stem cell-like cells.[21] In this study, was aimed to examine the expression level of *miR-155* in peripheral blood mononuclear cells (PBMCs) of newly diagnosed breast cancer (NDBC) patients and investigate the probable correlation between *miR-155*expression and its related functional genes like *SOCS-1* and *IL-6*. To the best of our knowledge, this is the first report on evaluating these molecules in the pathogenesis of BC.

PATIENTS AND METHODS

This cross-sectional study was approved by the ethics committee of the research deputy of Jundishapur University of Medical Sciences, Ahvaz, Iran (Grant No. CMRC-9814 code of ethics: IR.AJUMS.REC.1398.690), and all in vitro experiments were performed at the university immunology department.

Study Design and Population

Newly diagnosed patients (n=70) with BC, before starting the treatment procedure, were recruited for the study between July 2019 and October 2020. All patients enrolled in the study had a histologically confirmed diagnosis of primary BC with a mean age of 51.3077±12.3 years (from 37 to 65 years). The inclusion criteria were tumor-positive nodes in clinical observations, positive family history of BC, a score of breast imaging, reporting &data system (BI-RADS) of 4C or 5, and positive pathological report on fine needle biopsy. Excluded criteria were the participants with an unrelated pathologic result and/or breast cystic symptoms. Demographic and clinicopathological characteristics have been summarized in Table1. Additionally, 40 age and ethnic-matched healthy individuals without a family history of BC or previous immunological disorders, were considered as a healthy control group. Informed consent was signed by all participants before joining the project. A total of 8 mL blood samples were obtained from all participants (5 mL for PBMC isolation and 3mL for cytokine assay).

Isolation of Peripheral Blood Mononuclear Cells

Ethylenediaminetetraacetic acid (EDTA)-blood samples (5 mL) were obtained from all subjects, layered onto the Ficoll-Paque (Lymphodex, Inno-train, Germany) and PBMCs were removed following the density gradient centrifugation. The cells were then washed twice with phosphate-buffered saline (PBS) (Sigma, Germany) and prepared for gene expression.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from PBMC; using 1 mL TRIzol (Thermo Fisher Scientific, Invitrogen, MA, USA) according to the manufacturer's protocol, and the complementary DNA was synthesized by PrimeScript™ RT reagent kit (SinaColon, Tehran, Iran). The list of primers for specific and housekeeping genes has been shown in Table S1. Real-time PCR was performed using the Real Plus2x Master Mix Green with high ROX™ (Amplicon, Stenhuggervej, Denmark) and ABI Step One Real-time PCR. All procedures were repeated in triplicate. Gene expression was normalized to the Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control to evaluate the relative expression; using the $2^{-\triangle\triangle Ct}$ method.

Table 1. The clinicopathological presentation of breast cancer (BC) patients.

Variables	BC Patient(n=70)
Average of Age (years)	51.3077±12.3
BMI	29.1404±4.63151
Tumor histology	
Ductal	81.43% (n=57)
Lobular	18.57% (n=13)
Tumor grade	
G1	50% (n=35)
G2	30% (n=21)
G3	20% (n=14)
Lymph node metastasis	40% (n=28) (+)
	60% (n=42)(-)
History of menstrualirregularities	34.28% (n=24) (+)
	65.71 (n=46) (-)

The expression level of SOCS-1 was normalized to the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) as an internal control gene to evaluate the relative expression using the $2^{-\triangle\triangle Ct}$ method.

***MiRNA* Expression Analysis**

The expressions of *miR-155* and *U6* (reference) genes were done according to the kit's instruction using the universal specific primer sets and BON-miR QPCR Kit (Stem Cell Technology, Tehran, Iran).

Evaluating the Plasma Levels of IL-6

Separately, 3 mL of blood samples were used for plasma isolation and freezing at -20 or -80 for evaluating the IL-6. The concentration of *IL-6* was measured using a commercial ELISA kit (Karmania Pars Gene, Kerman, Iran) based on the manufacturer's instructions. Absorption was measured; using an ELISA reader (Biotech, U.S.A, Vermont) at 450 nm.

Statistical Analysis

All experiments were done in triplicate. SPSS ver. 26.0 (SPSS Inc., Armonk, NY, USA) was used for statistical analysis. One-way analysis of variance was used to compare data expressed as the median±SEM Mann-Whitney U test was applied for non-normally distributed data, and a t-test was conducted for the analysis of normally distributed data. Associations between variables were calculated by Spearman correlation. The *p*-values of <0.05 were considered to be statistically significant.

RESULTS

Higher Expression of MiR-155 in BC Patients Compared to the Healthy Controls

As indicated in Figure 1A, miR-155 was noticeably expressed in PBMCs of BC patients which were significantly more than that in the healthy controls (16.06±2.87 vs. 5.402±1.02; respectively, $p<0.001$; Fold change: 2.96±8.51).In addition, an increased expression of *miR-155* was detected in patients with ductal carcinoma compared to the control group (16.012±6.09 vs. 6.432±3.28; respectively, $p=0.002$) (Figure 1B). Additionally, as shown in Figure 1C, *miR-155* was upregulated in PBMCs of BC Patients with a high tumor grade (G3), compared to the values in low tumor grade (G2 and G1) (44.150±5.94 vs. 16.93±2. 83 and 2.664±0.38; respectively, $p=0.001$ and $p<0.001$).

Lower Expression of SOCS-1 in BC Patients Compared to the Healthy Controls

In the patient group, the average expression of the *SOCS-1* gene in PBMCs (2.387±0.51) was significantly lower than the expression level in the control group (7.862±1.40) ($p<0.001$; Fold change: 0.281±0.06) (Figure 2A). As indicated in Figure 2B, the relative expression of *SOCS-1* gene in the mononuclear cells of patients with DC form of NDBC was significantly higher than that LC type of ones (2.657±0.565 vs. 0.302±0.035; respectively, $p=0.142$). However, *SOCS-1* expression in both ductal and lobular carcinoma forms was significantly higher than in the control group ($p<0.001$and $p=0.007$; respectively). Additionally, there were no significant changes in the gene expression levels of *SOCS-1* with tumor grade (data not shown).

Higher Plasma Level of IL-6 in BC Patient Compared to the Controls

As illustrated in figure 3A, the plasma level of inflammatory protein IL-6 was elevated in BC patients (37.83±2.09 pg/L while in control individuals this value was in the normal range (12.1979±0.64 pg/mL) ($p<0.001$). Also, no statistical difference was observed in the plasma concentration of IL-6 between ductal BC and lobular cases (37.079±2.35 vs. 41.184±1.58; respectively, $p=0.5373$) (Figure 3B). As presented in Figure 3C, the higher the tumor grades, the higher the serum IL-6 levels (G1 compared to G2 and G3) (35.11±10.66 vs. 36.98±16.20, and 48.81±10.53; respectively, $p=0.59$ and $p<0.001$); Although the variation of IL-6 levels between G1 and G2 ($p=0.59$) was not significant, differences between G1 and G3 ($p<0.001$), and also between G2 and G3 ($p=0.021$) were considerable.

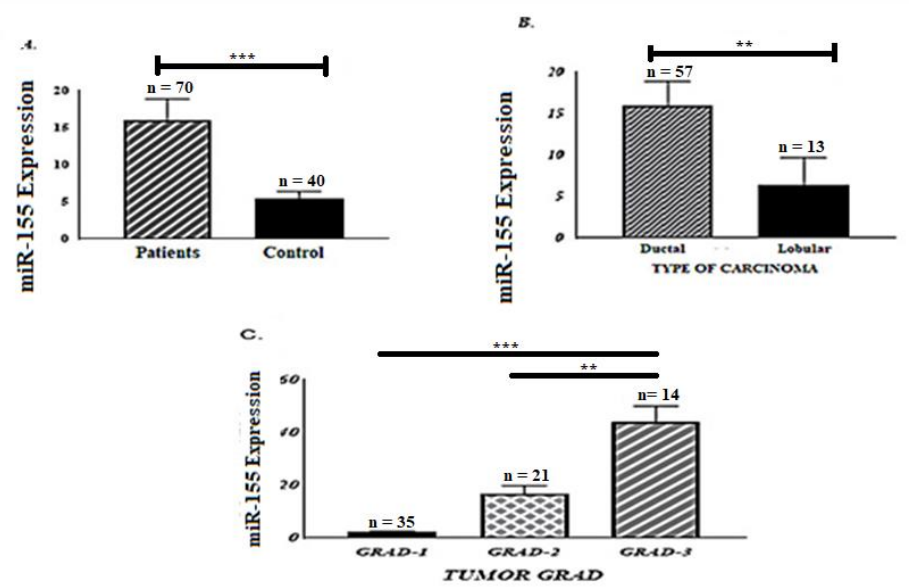

Figure 1. The comparison of the expression level of *miR-155* in BC (BC) patients and control data has been presented as mean±SEM (A). The Association of *mir-155* expression with histological types of BC (B) and tumor grade (C). [$p<0.01$, ***$p<0.001$]**

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Although the expression levels of *miR-155* and *SOCS-1* in BC patients were upregulated and downregulated, respectively, no significant correlation was found between the expression levels of these two genes (r=-0.095, p=0.25). The same result was observed for the expression of *miR-155* and IL-6 (r=0.182, p=0.12). Meanwhile, a significant negative correlation was observed between the *SOCS-1* levels and the plasma concentration of *IL-6* (r=-0.823, p<0.001).

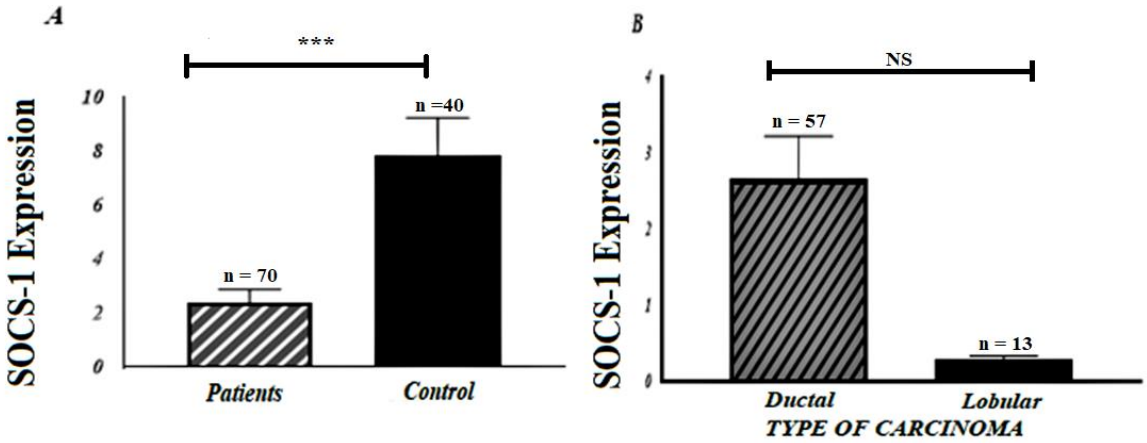

Figure 2. The comparison of the expression level of *SOCS-1* in BC (BC) patients and control data has been presented as mean±SEM (A). The Association of *SOCS-1* expression with histological types of BC (B) [*p<0.001, NS: non-significant]**

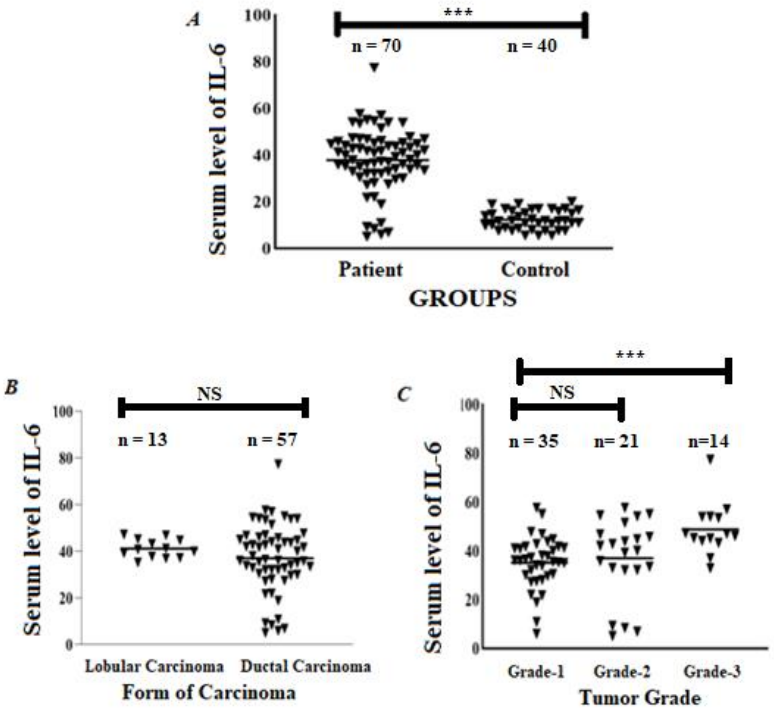

Figure 3. The comparison of the plasma concentration of *IL-6* in BC (BC) patients and control data has been presented as mean±SEM (A). The Association of IL-6 plasma concentration with histological types of BC (B) and tumor grade (C). [*p<0.001, NS : non significant]**

DISCUSSION

The relationship between inflammation and malignancies was discovered as early as 1863 by Rudolf Virchow. Although the critical association between chronic inflammation and malignancies has been discussed in several reports, underlying mechanisms are discovered gradually in different types of cancers especially in BC. In the present study, three important members of the inflammatory pathways including two genes (*miR-155* and *SOCS-1)* and one cytokine (plasma levels of IL-6) were evaluated in PBMCs and plasma of BC patients (ductal and lobular carcinoma), respectively. Increased level of *miR-155* in BC tissues and its potential role as a diagnostic marker was shown by Sun Y and et al.[22] According to our results, *miR-155 is* markedly expressed in PBMCs of BC patients in the early BC, especially high-grade patients concluding that the increased expression level of miR-155 may involve in the initiation of BC. Additionally, we found that the expression level of *miR-155* in ductal carcinoma is relatively higher than that in the lobular form of carcinoma. This part of the results indicates that activated mononuclear cells could be introduced as one of the main sources of circulating *miR-155* in patients with newly diagnosed BC. As the higher *miR-155* expression in BC has been observed in the higher tumor grade and advanced tumor stage highlighting the expression status of *miR-155* can be involved in tumor progression and is promising as a clinical prognostic biomarker in BC.[23] Moreover, following the report of Fuksiewicz et al, we found elevated plasma levels of IL-6 in BC patients compared to the healthy controls and its significant correlation with the tumor stage.[24] IL-6 is a multifaceted cytokine with a pro-inflammatory nature that can enforce proliferation and anti-apoptotic effects in tumor cells. Several published papers have revealed its oncogenic feature via activating the signaling pathways such as JAK/STAT3, Ras/MAPK, PI3K–PKB/Akt after the IL-6/IL-6R ligation, regulation of CD4+ T cells, and triggering the production of vascular endothelial growth factor (VEGF).[25-28]

Cardoso et al. revealed that *miR-155* promotes cytokine expression in microglia by directly targeting and inhibiting the expression of S*OCS1*, as a target of *miR-155.*[29] Although we observed a reduction of *SOCS-1* expression in PBMCs of BC patients compared to the healthy controls, no significant correlation was found between the expressions of *miR-155* and *SOCS-1*.

However, a substantial negative correlation between the expression of *SOCS-1* and the plasma levels of IL-6 was obtained in BC patients. A principal mechanism of STAT3 activation in BC is through the IL-6/gp130/JAK pathway.[30] STAT3 is tyrosine-phosphorylated through the interleukin-6/glycoprotein 130/Janus kinase pathway in breast cancer.[30] As those breast cancer-derived cell lines expressing high levels of p STAT3 contained IL-6 and were capable of stimulating STAT3 phosphorylation. They found IL-6 levels in primary breast tumors and found a positive correlation between p STAT3 and IL-6 expression.[30] STAT3 activation through the gp130 receptor is regulated by both positive effectors such as IL-6 and negative regulators, including suppressors of cytokine signaling (SOCS) 1 and SOCS3, which inhibit Jak activity.[31] *SOCS-1* gene encodes a tumor-suppressor protein which acts through different mechanisms like inhibiting the IL-6-mediated JAK activation directly through its kinase inhibitory region.[17] In line with our findings on the negative correlation between SOCS-1 and IL-6, Sullivan et al. indicated that loss of SOCS-1 was associated with cancer initiation and progression.[32] In contrast with the results above, another study has been shown that IL-6 and IL-10 are associated with a good prognosis in early-stage invasive breast cancer patients. As IL-6 was associated with improved survival rate in non-basal, estrogen receptor-positive, and non- triple-negative breast cancer (non-TN BC).[33] Additionally, It has been reported that a closely associated with the patient's clinical condition and independent of the cancer histology, the increased IL-6 serum level uniformly appears to correlate with survival as a paraneoplastic condition in later cancer stages independent of the cancer type.[34] Meanwhile, our research limitations include the small sample size needing to be repeated in a study with more cases. This limitation may influence the statistical analysis outputs like the correlation between miR-155 and SOCS-1. However, one of the strengths of this study is to evaluate the newly diagnosed patients without any medication or surgical procedures which is not commonly considered in most BC biomarker research. We also excluded the patients having a history of inflammatory diseases.

In conclusion, there was an increase in the expression levels of miR-155 and serum levels of IL-6 in primary breast cancer patients, especially those with a high grade of the tumor. In addition, a decrease in the miR-155 target gene, SOCS-1, was observed in primary breast cancer patients. Therefore, it can be suggested that *miR-155* may act as a pro-inflammatory mediator in BC patients and promote tumor initiation and progression However, further functional analysis of this microRNA is required to take a broad view of these findings to BC patients.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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