ORIGINAL ARTICLE

miR-155 rs767649 T>A gene polymorphism is associated with downregulation of *miR-155* expression, suppressor of cytokine signaling-1 overexpression, and low probability of metastatic tumor at the time of breast cancer diagnosis

Sara Iranparast[1,2], Maryam Tahmasebi-Birgani[3,4*], Azim Motamedfar[5,6], Afshin Amari[1,4], Mehri Ghafourian[1,7*]

[1]Department of Immunology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [2]Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [3]Department of Medical Genetics, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [4]Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [5]Assistant professor of Radiology and Fellowship of Interventional Radiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [6]Department of Radiology, Golestan Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [7]Fertility, Infertility and Perinatology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

*These authors equally correspond to this study, however, Mehri Ghafourian is the main correspondence.

Background: *MicroRNA-155* is a key player in inflammatory reactions, carcinogenesis, and tumor development. In this study, polymorphism of *miRNA-155 rs767649 T>A* and its gene and suppressor of cytokine signaling-1 (SOCS-1) expression were investigated in relation to cancer susceptibility and development in breast cancer (BC) patients. **Materials and Methods:** Polymorphism of *miRNA-155 rs767649 T>A* was evaluated between a population of 174 patients with BC and 129 controls using restriction fragment length polymorphism and the expression of *miR-155* and SOCS-1 were examined in peripheral blood mononuclear cells (PBMCs) by real-time polymerase chain reaction. **Results:** TT genotype of *miR-155 rs767649 T>A* was associated with higher level of *miR-155* in PBMCs of BC patients relative to AT and AA genotypes (21.76 ± 4.4, 4.046 ± 1.35, 2.56 ± 0.81, respectively; $P < 0.001$) and increased lymph node metastasis ($r = 0.292$, $P = 0.001$), not BC susceptibility ($P = 0.402$ and $P = 0.535$; respectively). TT genotype of *miR-155 rs767649 T>A* was associated with less gene expression of SOCS-1 in PBMCs of BC patients compared to AT and AA genotypes (1.173 ± 0.57, 0.92 ± 0.827, 5.512 ± 0.92, respectively; $P = 0.003$). **Conclusion:** This study demonstrated for the first time the association between the T allele of the *rs767649 T>A* polymorphism in the *pre-MIR155* gene and higher expression of *miR-155*, lower expression of SOCS-1, and swift latent progression in newly diagnosed BC patients. Thus, *miR-155* may play a critical role in BC pathogenesis.

Key words: Breast cancer, expression, metastasis, *miR*-155, polymorphism

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INTRODUCTION

Breast cancer (BC) is the most common cancer in women worldwide with high mortality.[1,2] Despite various therapeutic plans, due to the progressive course of the disease, there is a possibility of incomplete eradication of malignant breast masses, so researchers are trying to discover new molecules, such as small transcript

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Address for correspondence: Prof. Mehri Ghafourian, Department of Immunology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Golestan Blvd, P.O. Box 6135715794, Ahvaz, Iran.
E-mail: ghafourianbm@gmail.com
Dr. Maryam Tahmasebi-Birgani, Department of Medical Genetics, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
E-mail: Maryam_tahmaseby@yahoo.com
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microRNAs (m*iRs)*, to provide additional therapeutic targeting to optimize detection and treatment methods.[3]

Recently, microRNAs (miRs) have gained attention in the initiation and progression of BC. These small noncoding RNAs regulate a variety of molecular pathways including cell cycle, differentiation, apoptosis, and metabolism.[4] Hence, it is not surprising their altered expressions may be connected with tumorigenesis.[5,6] *MicroRNA-155*, introduced as an oncomiR, is over-expressed in BC patients.[7] It has been well documented that the functional polymorphisms in miRs are the most common form of variation present in the human genome and could affect cancer susceptibility and prognosis.[8,9] However, until now, the role of polymorphisms within noncoding regions of *miR-155* on BC susceptibility remains unknown. Some studies have implied that polymorphisms in miRs may influence the expression of mature miRs by modulating their structure and expression, reflecting the diversities of the susceptible factors for different tumors.[10]

Like other genes, *miR-155* regulates its various target genes by binding to their 3′ untranslated regions. The suppressor of cytokine signaling-1 (SOCS-1) is one of the *miR-155*'s target genes and an essential tumor-suppressor gene that is involved in cytokine signaling and cell proliferation. It has been evidenced that miRNA-155 negatively regulates the expression of the SOCS-1 gene and thereby promotes tumor invasion.[11,12] SOCS-1 gene is the most potent member of the SOCS family that is cytokine-inducible negative regulators of cytokine signaling.[13]

The *rs767649 T>A* polymorphism in the promoter of *miR-155* was reported in many diseases such as lung cancer,[14] cervical cancer (CC),[15] and hepatocellular carcinoma (HCC).[16] We hypothesize that functional polymorphism *rs767649 T>A* located in the flanking region of the *miR-155* gene may contribute to the changes in the expression of *miR-155* and SOCS-1, as well the development of BC. Thus, this study aimed to identify an efficient biomarker for screening and monitoring patients with newly diagnosed BC (NDBCs).

MATERIALS AND METHODS

Patients

This study was performed on 174 patients with NDBC (aged 50.49 ± 12.08 years) recruited from the center of sonography and radiology, Ahvaz, Iran between December 2019 and January 2021. One 129 age-matched healthy controls (51 ± 10.50 years) with a negative history of BC were also enrolled. All subjects gave written informed consent. The inclusion criteria were based on clinical observation, positive family history of BC, breast imaging, and pathological examinations. Excluded subjects were individuals with unrelated pathologic results. All procedures performed in studies involving human participants were in accordance with the ethical standards of Ahvaz Jundishapur University of Medical Sciences (Grant No. CMRC-9814 and code of ethics: IR.AJUMS.REC.1398.690).

Genotyping

Genomic DNA was isolated from peripheral blood using a DNA extraction kit (DNJia Kit, Yazd, Iran) according to the manufacturer's instructions. Specific Single nucleotide polymorphisms (SNPs) were genotyped by restriction fragment length polymorphism (RFLP)-polymerase chain reaction (PCR). The PCR is programmed as follows: initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 35 s, annealing at 56°C for 35 s and extension at 72°C for 35 s and a final extension at 72°C for 5 min. To identify the *miR-155 A/T* polymorphism, the PCR product was digested with the restriction enzyme TSP45I (Thermo Fisher, USA, REF: ER1511). The pattern of RFLP was analyzed through 2% agarose gel electrophoresis [Figure 1].

Isolation of peripheral blood mononuclear cells

Blood samples (5 ml) were obtained from all subjects and collected in Ethylenediaminetetraacetic acid-containing tubes. Peripheral blood mononuclear cells (PBMCs) were obtained from both BC patients and controls using Ficoll-Paque (Lymphodex, Inno-train, Germany) density gradient centrifugation.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from (PBMC using 1 ml TRIzol (Thermo Fisher Scientific, Invitrogen, MA, USA) according to the manufacturer's protocol. The expression of *miR-155* and *U6* (reference gene) was analyzed using universal specific primer sets and BON-miR quantitative polymerase chain reaction Kit (Stem Cell Technology, Tehran, Iran) Two specific primers were used for U6 as follows: (Forward: 5′-TGCGGGTGCTCGCTTCGGCAGC-3′),(Reverse:

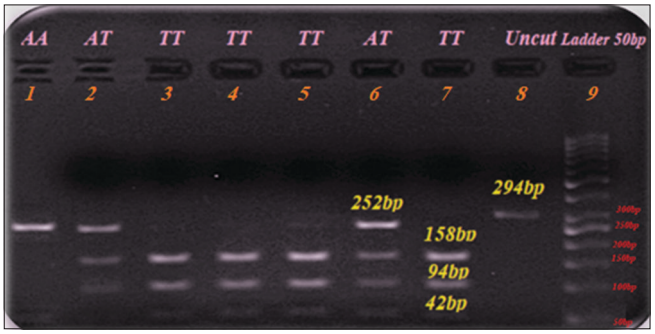

Figure 1: The miR-155 PCR product and its genotypes. Lane 1 shows the AA genotype (bands at 252 and 42bp); lanes 3, 4, 5, and 7 show the TT genotype (bands: 158, 94, and 42bp); lanes 2 and 6 show the AT genotype (bands at 252, 158, 94, 42bp); lane 8 shows uncut PCR product at 294bp; and lane 9 is the DNA ladder-50bp. PCR = Polymerase chain reaction

5′-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGGAAC-3) and miR-155 (Forward: 5′-ACACTCCAGCTGGGTTAATGCTAATCGTGAT-3′) (Stem-loop-primer:5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTG AGACCCCTAT-3′). Optimal reaction conditions included an initial denaturation step at 95°C for 2 min, 40 cycles at 95°C for 5 s, 60°C for 30 s, and a final extension step at 72°C for 10 min.

In silico analysis

In order to investigate the possible effects of the *miR*-155 in the tumor environment, the target genes of the microRNAs were selected at the miRTarBase, mimiRNA, and miRTargetLink Human databases. This study focused only on target genes involved in the immune system and SOCS-1 was selected as a key target gene involved in the regulation of cytokine signaling and cell proliferation.

Quantitative real-time polymerase chain reaction

The expression of SOCS-1 and glyceraldehyde 3-phosphate dehydrogenase (reference gene) was analyzed using universal specific primer sets and cDNA synthesis Kit (SinaClon, Tehran, Iran) Data were normalized based on GAPDH expression as the housekeeping control gene. The primers were as following: (GAPDH; Forward: 5-GTGAACCATGAGAAGTATGACAAC-3, Reverse: 5-CATGAGTCCTTCCACGATACC-3′); (SOCS-1; Forward: 5'-CTGGTGCGCGACAGCCG-3', Revers: 5'-ACGTAGTGCTCCAGCAGCTC-3′) and RealQ Plus2x Master Mix Green with high ROX™ (Amplicon, Stenhuggervej, Denmark). Optimal reaction conditions included an initial denaturation step at 95°C for 15 min, 40 cycles at 95°C for 15 s, 60°C for 60 s, and a final extension step at 72°C for 10 min.

Statistical analysis

All statistical analysis was carried out using SPSS version 26.0 (SPSS Inc., Armonk, NY, USA). The differences in genotype distribution between patients and healthy controls were analyzed by the Chi-square test. The representativeness of the subjects in the current study was evaluated using the Hardy–Weinberg equilibrium (HWE). The difference in gene expressions of *miR-155* and SOCS-1 was assessed by Mann–Whitney *U* and Kruskal–Walis tests. $P \leq 0.05$ were considered statistically significant.

RESULTS

miR-155 *rs767649 T>A* polymorphism is not associated with breast cancer risk

Genotype frequency of *miR-155 rs767649 T>A* in BC patients is summarized in Table 1 and compared with normal counterparts. As indicated TT was a common genotype among BC patients. The same pattern was observed for the control group and no significant difference was observed with the case group ($P = 0.402$ and 0.535–0.592 for alleles and genotypes, respectively) [Table 1]. Distribution of the *rs767649 T>A* genotypes in both the patient ($\chi^2 = 0.173$, $P = 0.677$) and the control ($\chi^2 = 2.842$, $P = 0.0.091$) groups by HWE which indicates randomness of both the patient and control samples.

miR-155 was overexpressed in breast cancer compared with normal ones

As shown in Figure 2a, *miR-155* was markedly expressed in PBMCs of BC patients and the expression level was significantly more than the observed values for control individuals (6.42 ± 2.17 vs. 1.56 ± 0.47; respectively, $P = 0.006$) (Fold change: 4.11 ± 1.4). Additionally, our data indicated that there is a significant discrepancy in the *miR-155* levels of PBMC between TT compared to AT, and AA genotypes (21.76 ± 4.4, 4.046 ± 1.35, 2.56 ± 0.81, respectively; $P < 0.001$) (Fold change AA *vs.* TT: 0.12 ± 0.037) (Fold change AT *vs.* TT: 0.185 ± 0.062). The *miR-155* expression was significantly overexpressed in BC patients with the *A* allele as compared without the *A* allele (2.87 ± 0.69 vs. 21.76 ± 4.40; $P = 0.019$) (Fold change A + vs. A−: 0.134 ± 0.032) [Figure 2b and c].

Investigation of miRNA–mRNA interactions network

Common miRNA-target pairs deserved more attention due to their prominent role of them in the immune responses. The network consisted of 21 target genes that SOCS-1 was selected to evaluate in the present study.

The expression level of suppressor of cytokine signaling-1 decreased in breast cancer compared with normal ones

As shown in Figure 3a, SOCS-1 was markedly reduced in PBMCs of BC patients and the expression level was significantly less than the observed values for control individuals (3.082 ± 0.50 vs. 7.862 ± 1.40; respectively, $P = 0.006$) (Fold change: 0.326 ± 0.06). Additionally, our data indicated that there is a significant reduction in the SOCS-1 levels of PBMC between TT relative to AT and AA genotypes (1.173 ± 0.57, 0.92 ± 0.827, 5.512 ± 0.92, respectively; $P = 0.003$) (Fold change AA vs. TT: 4.69 ± 0.78) (Fold change AT vs. TT: 0.74 ± 0.7). Besides, SOCS-1 expression was no significant difference in BC patients with *A* allele as compared without *A* allele (4.21 ± 0.75, 1.17 ± 0.57, respectively; $P = 0.248$) (Fold change A + vs. A−: 4.06 ± 0.71) [Figure 3b and c]. However, there was a significant negative correlation between the expression level of *miR-155* and SOCS-1 ($r = -0.234$, $P = 0.032$).

Stratified analysis of the association between *rs767649 T>A* and breast cancer risk by demographic and clinical variables

The relationship between *rs767649 T>A* and invasion of BC was also evaluated. As mentioned in Table 2, A

Table 1: Genotypic and allelic frequencies of miR-155 *rs767649* T>A polymorphisms in newly diagnosed breast cancer and control subjects

Polymorphism	NDBC (*n*=174) subjects, *n* (%)	Control (*n*=129) subjects, *n* (%)	OR (95% CI)	*P*
Allele			-	-
T	299 (86.08)	215 (83.48)	0.817 (0.510-1.310)	0.402
A	49 (13.92)	43 (16.52)		
Co-dominant				
TT	132 (75.95)	93 (72.17)	-	-
AT	35 (20.25)	29 (22.61)	0.851 (0.473-1.533)	0.592
AA	7 (3.80)	7 (5.22)	0.692 (0.216-2.219)	0.535
Dominant				
TT (0)	132 (75.95)	93 (72.17)	-	-
AA + AT (1)	42 (24.05)	36 (27.83)	0.821 (0.475-1.420)	0.481
Recessive				
AT + TT (0)	167 (96.20)	122 (94.78)	-	-
AA (1)	7 (3.80)	7 (5.22)	0.717 (0.225-2.283)	0.574

NDBC=Newly diagnosed breast cancer; OR=Odds ratio; CI=Confidence interval

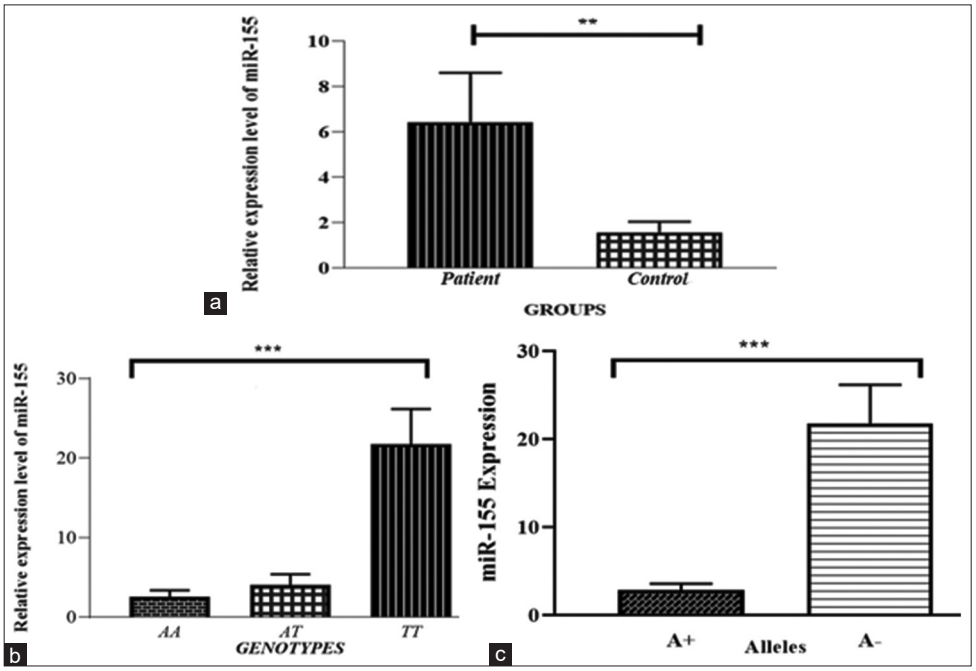

Figure 2: Comparing the expression level of miR-155 in breast cancer patients. A comparison of the expression level of miR-155 in breast cancer patients and control (a). A comparing the miR-155 expression in NDBC patients with different variants of *rs767649 T>A* miR-155 (b). and also with and without A allele (c). NDBC = Newly diagnosed breast cancer. The ** indicates the *P*-value less than 0.01. The *** indicates the *P*-value less than 0.001

allele frequency in *miR-155 rs767649 T>A* variants was significantly associated with lower levels of lymph node metastasis (r = 0.261, P = 0.001) and lower grade of tumor (r = 0.520, P < 0.001) at diagnosis time of NDBC patients in comparison patients without A allele. However, the same data were obtained for a higher Probability of lymph node metastasis and the grade of the tumor was observed in the BC patients studied with the TT genotype, compared to the AA and AT genotypes (r = 0.292, P = 0.001, and r = 0.734, P < 0.001; respectively) [Table 2]. By the way, our analyses showed that the TT was significantly associated with grade III BC, whilst the AT and AA genotypes were observed in patients with grade I and II BC (P < 0.001), implying that the protective effect of the *AA* genotype in *miR-155 rs767649 T>A* on BC risk was exerted mainly among the early stage of patients. Altogether, lacking *A* allele and TT genotype of the *rs767649 T>A* polymorphism in the *pre-MIR155* gene was independently associated with swift latent progression in patients with NDBC.

DISCUSSION

The association between SNP of genes and the risk of BC has been generating great interest in the scientific community. *Rs767649 T>A* polymorphism of *miR-155* located in the promoter of *miR-155* has recently been investigated in different diseases[11-13] and for the first time, we searched its role in BC. In this study, we showed that TT is a common

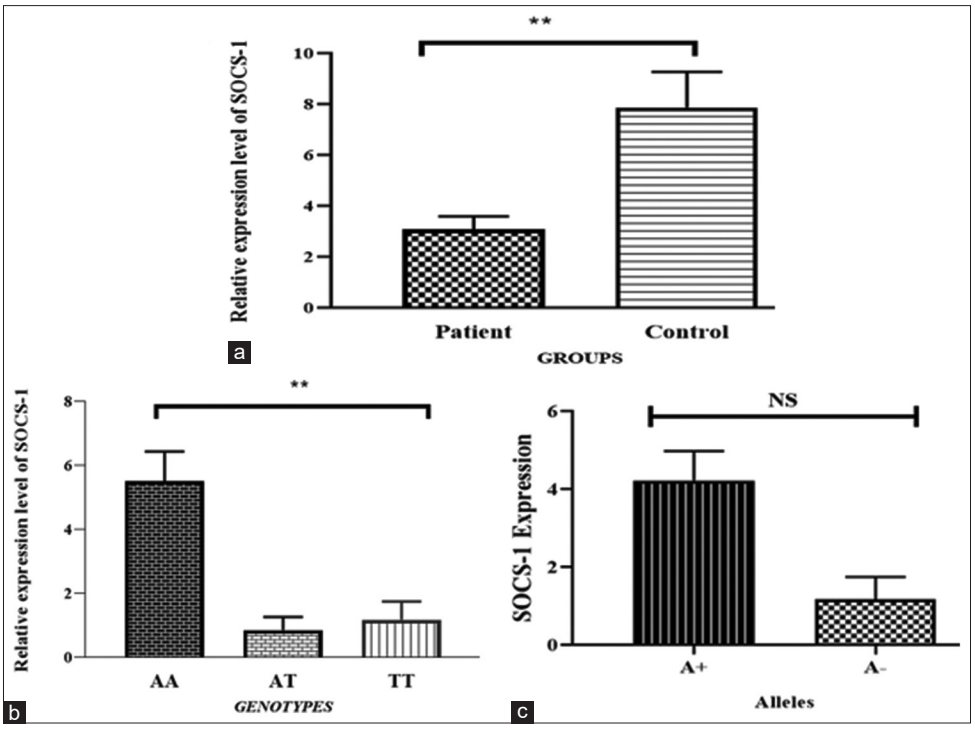

Figure 3: Comparing the expression level of SOCS-1 in breast cancer patients. A comparison of the expression level of SOCS-1 in breast cancer patients and control (a). A comparing the SOCS-1 expression in NDBC patients with different variants of *rs767649 T>A* miR-155 (b). and also with and without A allele (c). SOCS-1 = Suppressor of cytokine signaling-1, NDBC = Newly diagnosed breast cancer, NS = Nonsigficance. The ** indicates the *P*-value less than 0.01

genotype in Iranian NDBC patients and control subjects; our result in this step was in line with the previous studies accomplished on several cancers in Iran and china.[14-16] Additionally, we investigated the association between *miR-155* expression, its functional variant *rs767649 T>A*, cancer susceptibility, and cancer development in the Iranian population using a case-control design. Consistent with our hypotheses, our findings suggested that the functional variant *rs767649 T>A* of *miR-155* located in the regulation region was associated with the swift progression of BC, although there was no association between *miR-155 rs767649 T>A* and BC susceptibility in patients with BC of Iran compared with healthy women. Our results demonstrated although there is no correlation between the presence of either *A* or *T* alleles and the susceptibility to BC, the type of allele expressed of *miR-155* can be correlated with the expression levels of this small transcript and some clinicopathology characteristics of BC. Whilst, to date, only three studies have analyzed the association between *miR-155 rs767649 T>A* polymorphisms and the prognostic outcomes in various carcinomas,[15,16] however, none of them has been performed on BC patients. One of them propounds a hypothesis that the *T* allele of *rs767649 T>A* contributes to the higher risk of HCC.[16] Another *in vitro* study on CC suggests that the transition of the A to T allele might be a causal variant for CC susceptibility through *miR-155* downregulation at the transcriptional level.[15] Meanwhile, a recent study highlighted the risk of NSCLC susceptibility influenced by the *A* allele.[14]

In our study, the analyses of *miR-155* expression showed its diagnostic value to differentiate BC patients from healthy controls. In addition, there were significant differences between different genotypes regarding *miR-155* expression level in BC with a high level in TT genotype, as well as lacking A allele. Furthermore, we found that lacking the *A* allele is associated with an increased expression of *miR-155* in PBMC of BC patients as well with more severe clinical manifestations (higher frequency of lymph node metastasis and higher grade of tumor). Therefore, due to the same frequency of variant TT in BC patients and healthy groups and the significant increase of the expression level of *miR-155* in the patients, especially variant TT, another factor may have affected the change in *miR-155* expression that is present in the tumor environment, not in healthy individuals. The results of the current study may be explained by previous studies which indicated that TT genotype and T allele in *miR-155 rs767649 T>A* were associated with increased risk of HCC, respectively, and T allele in *miR-155 rs767649 T>A* contributed to the higher expression level of *miR-155* in HCC tissues.[16,17] Moreover, Chernyy *et al*. showed evidence that *miR-155* can be defined as molecular markers in regards to BC patients to prognosticate spread to the lymph node.[18] Nina Petrović *et al*. observed that *miR-155* is predominately involved in the early stages of BC formation as well as in tumor spreading to the lymph nodes and that it might be effective for the screening of potential micrometastases that are not detectable with standard diagnostic procedures.[19]

Table 2: The association between alleles and variants frequency of miR-155 *rs767649 T>A* and the demographic data and clinicopathological presentation of breast cancer patients

Variables	Alleles related to miR-155 *rs767649* polymorphism		*P** (correlation coefficient**)
	With A allele (*n*=42), *n* (%)	Without A allele (*n*=132), *n* (%)	
Age	48.66±15.78	51.06±10.63	0.160
BMI	28.26±6.51	27.90±4.38	0.571
Lymph node			
Metastasis	14 (33.33)	83 (62.87)	0.001 (0.261)
Nonmetastasis	28 (66.66)	49 (37.12)	
Side position status			
Left breast	20 (47.61)	71 (53.78)	0.580 (0.042)
Right breast	22 (52.38)	61 (46.21)	
FCC status			
Yes	22 (52.38)	69 (52.27)	0.937 (0.006)
No	20 (47.61)	63 (47.72)	
Menstruation			
Irregular	9 (21.48)	63 (47.72)	0.001 (0.258)
Regular	33 (78.57)	69 (52.27)	
Grad of tumor			
1	27 (64.28)	17 (12.87)	<0.001 (0.520)
2	12 (28.57)	81 (61.36)	
3	3 (7.14)	34 (25.75)	
Variables	**Genotypes of miR-155 rs767649**		***P**** **(correlation coefficient**)**
	AA, *n* (%)	**AT, *n* (%)**	**TT, *n* (%)**
BMI	28.05±4.52	28.03±5.29	30.26±5.79	0.406
Lymph node				
Metastasis	2 (28.57)	20 (57.14)	117 (88.63)	0.001 (0.292)
Nonmetastasis	5 (71.42)	15 (42.85)	15 (11.36)	
Side position status				
Left breast	3 (42.85)	17 (48.57)	103 (78.03)	0.245 (0.127)
Right breast	4 (57.14)	18 (51.42)	29 (21.96)	
FCC status				
Yes	2 (28.57)	14 (40.0)	118 (89.39)	<0.001 (0.390)
No	5 (71.42)	21 (60.0)	14 (10.60)	
Menstruation				
Irregular	2 (28.57)	14 (40.0)	117 (88.63)	<0.001 (0.371)
Regular	5 (71.42)	21 (60.0)	15 (11.36)	
Grad of tumor				
1	4 (57.14)	5 (14.28)	20 (15.15)	<0.001 (0.734)
2	2 (28.57)	25 (71.42)	25 (18.93)	
3	1 (14.28)	5 (14.28)	87 (65.90)	

*A statistically significant test result ($P \leq 0.05$), **Correlation coefficient is a value of between −1 and +1. A −1 means there is a negative correlation and +1 means that there is a positive correlation. BMI=Body mass index; FCC=Federal communications commission

The present study showed that SOCS-1 significantly decrease in patients compared with the controls, whilst the expression level of *miR-155* was elevated in the BC patients. Besides, we observed a significant reduction of SOCS-1 expression in patients with either TT variant compared to the other genotypes, AT and AA. However, a significant negative correlation was observed between the expression level of *miR-155* and SOCS-1. Previous studies have shown that the reduction in SOCS-1 mRNA, especially in high-grade patients, is associated with adverse clinical outcomes and poor prognosis in BC;[20,21] our results suggest that this reduction may be influenced by *miR-155*.

CONCLUSION

The levels of *miR-155* in PBMC were higher, especially in BC with TT genotype. In addition, the wild variant, TT, was associated with less gene expression of SOCS-1 known as a key tumor suppressor. Therefore, the *rs767649 T>A* polymorphism was associated with different levels of *miR-155* and SOCS-1 in PBMC of NDBC. On the other hand, the wild genotype of SNP of *miR-155* investigated in the current study was associated with the hidden progression of BC, higher grade of tumor, and faster lymph node metastasis, not susceptibility to BC and this knowledge may promote our understanding of the molecular mechanisms

underlying the pathogenesis and progression of BC and in the development of improved therapies for the treatment of BC. Due to the same frequency of variant TT in BC patients and healthy groups and the significant increase of the expression level of *miR-155* in the patients, especially variant TT, another factor may have affected the change in *miR-155* expression that is present in the tumor environment, not in healthy individuals. Taken together, these novel findings may be clinically significant and highlight the role of *miR-155* in the pathogenesis of BC, with possible therapeutic implications.

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Conflicts of interest

There are no conflicts of interest.

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