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The Inverse Correlation of MicroRNA-21 and MicroRNA-155 with the Tissue Inhibitor of Metalloproteinase 3 may Foster the Invasiveness of Breast Cancer

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ABSTRACT

Background: *Early detection of breast cancer is paramount for accurate treatment and microRNAs represent the hopeful markers in cancer biology.* ***Aims:*** *To determine the differential co-expression and correlation of tissue and plasma exosomal miRNA-155 and miRNA-21 and their impact on the tissue inhibitor of metalloproteinase 3 (TIMP3) in fostering breast cancer metastasis.* ***Materials and methods:*** *MicroRNA-155 and miRNA-21 were extracted from plasma exosomal and tumor tissues of 60 women with breast tumors, and from plasma of 30 healthy women and miRNA expression was quantified by SYBR Green real-time polymerase chain reaction technique. The results were correlated with TIMP3 protein expression and patients' clinico-pathological profiles.* ***Results:*** *The concomitant significant overexpression of plasma exosomal and tissue miRNA-21 and miRNA-155 was observed in invasive ductal carcinoma more than those of ductal carcinoma in situ and invasive lobular carcinoma. The miRNAs expression is also significantly associated with the advance of TNM staging and high tumor grade with positive correlation of circulating plasma exosomal miRNA with tissue miRNAs in the breast cancer group. The low TIMP3 expression was inversely associated with tissue and exosomal miRNAs expression. The area under curve, sensitivity, and specificity for miRNA-155 were 0.791, 73.3%, 80%, and for miRNA-21 were 0.925, 90%, and 86.7%, respectively.* ***Conclusion:*** *the positive interrelation of plasma exosomal miRNA-21 and 155 and their corresponding tissue miRNAs signifies role of cargos of these miRNAs in tumor metastasis and shed light on the invasive ability of cancer cells through activation of TIMP3/miRNA-21 expression.*

Keywords: MicroRNA-155, MicroRNA-21, MicroRNA-16, TIMP3, Breast cancer

INTRODUCTION

Breast cancer is the most common women malignancy and remains the prime cause of death worldwide from cancer among women. The incidence of breast cancer is low in Asia and Africa, but the discovered cases are steadily rising, and poor survival rates remain a cause of great concern [1].

More than 95% of malignant breast tumors originate from the transformed breast epithelia by genetic and epigenetic factors that generate transcriptional or translational repression of tumor suppressors involved in tumorigenesis and tumor propagation and/or progression [2]. Overexpression of molecular biomarkers of epithelial mesenchymal transition (EMT) in breast cancer biopsies was found to be associated with increased tumor aggressiveness, high recurrence rates, and shorter survival [3]. During oncogenesis, deregulation, or disturbed function of microRNA (miRNA) can result in augmented translation of oncoprotein and/or attenuated translation of tumor suppressor protein [4]. The miRNAs participate in the regulation of breast cancer, for example, miRNA-21, miRNA-10b, miRNA-125b, miRNA-155, miRNA-9, miRNA-497 and miRNA-5003-3p act as inducers or inhibitors of epithelial-mesenchymal transition (EMT); through different mechanisms and signaling pathways.

Tissue inhibitor of matrix metalloproteinase 3 (TIMP3), a member of the metalloproteinase inhibitor family-represents one ECM-binding protein that *in situ* inhibits matrix metalloproteinases (MMPs). The TIMP3 activity suppresses angiogenesis, invasion, and metastasis formation. A recent report has indicated that miRNA-21-5p promotes angiogenesis by targeting TIMP3/MMPs signaling [5].

Exosomes have emerged as a vital communication mechanism between different cell types in the tumor microenvironment. Exosomes range between 30 nm - 100 nm in diameter and contain proteins, nucleic acids and miRNAs [6]. Exosomes have the potential to regulate proliferation, survival in recipient cells. Previous studies have revealed that miRNAs can be released from RBCs during the blood clotting process [7].

Cheng, et al. performed miRNA profiling of plasma from lung cancer cases and controls and found a global increase in abundance of most miRNAs in cases compared to controls, suggesting the presence of a systematic bias [8]. This is due to significant amounts of circulating miRNAs were originated from residual contaminating blood cells [9].

In breast cancer tissues circulating miRNAs may correlate with disease progression, therapeutic responses and patient survival [10], suggesting that the evaluation of miRNAs levels in the blood, either serum or plasma, may be used as non-invasive blood-based biomarkers. But, pre-analytic variables such as differences in sample processing and handling, plasma and serum processing, choice of anticoagulant and hemolysis have been reported to affect miRNA measurement [9].

In this study, to avoid the potential release of miRNA from blood cells, we used an optimized protocol for exosomal miRNA purification from human blood samples and from high fat containing breast tissue. To increase the performance of assays, we used miRNA-16 as an endogenous control for the normalization of miRNA data. Thus, in this context we focused on the miRNA-155, miRNA-21 and its target protein TIMP3 to investigate the true expression levels of these two miRNAs and the strength of interrelation between these miRNAs in both tumor tissues and matched plasma exosome samples with the patient clinicopathological condition and the invasiveness of breast cancer. And to determine the potential of exosomal plasma miRNA-21 and miRNA-155 as surrogate biomarkers in discriminating between breast cancer, benign breast lesions (BBLs) and normal breast tissues. To our knowledge, this is the first study to implicate the role of these miRNAs and TIMP3 in breast tumor patients in Iraq.

METHODS

Study Design

This is a case control study conducted on 60 patients with breast tumors with an age range of 25-67 years and 30 age-matched healthy female volunteers who serve as a control group. Of these 60 patients, 30 were with breast cancer BC) and 30 with benign breast lesions (BBL). Women who received neo-adjuvant chemotherapy or radiation therapies before surgery and/or with recurrent breast cancer are excluded from the study. All participants had signed a consent for their agreement to participate in this study. The research was approved by the Institutional Review Board of college of medicine, Al-Nahrain University. All patients and controls are taken from those who attend the surgical consultation clinic at Al-Imamain alkadhymain Teaching Hospital, Baghdad, Iraq during the period from January 2015 to September 2015.

Blood Sampling

A 5 mL of the venous blood was aspirated into an EDTA tube. The plasma is kept in ice box during transportation and separated from other blood cells within less than 2 h to prevent the release of miRNAs from blood cells during the coagulation process [11]. The plasma was separated from the cell mass by centrifugation at 3500 × g for 5 min. The aspirated plasma was re-centrifuged at 10000 × g to remove the platelets and cell debris then stored in liquid nitrogen container at -190°C for subsequent RNA-miRNA extraction and molecular analyses.

Breast Tissue Specimen Collection

Resected tumors (benign and malignant) and the corresponding apparently normal tissues away from the tumor site were obtained at surgical theatre from those who undergone mastectomy by the Senior Surgeon. Pieces of the fresh tumor specimens were immediately stored in liquid nitrogen for RNA extraction. The diagnosis of breast cancer was

confirmed by the histopathological examination which was carried out on the paraffin embedded tissue blocs and the clinical staging of breast cancer was carried out according to the TNM classification system of the American Joint Committee on Cancer. Paraffin embedded blocks of breast tumors was obtained from the Histopathology Laboratories and sections were taken for the subsequent immunohistochemical analysis of the tissue TIMP3, estrogen receptor (ER), progesterone receptor (PR), and Her2/neu (human epidermal growth factor-2).

Molecular Analyses

RNA isolation from plasma and tissue samples

Exosomes were extracted from 600 µL of plasma using miRCURY™ Exosome kit (Exiqon, Denmark) according to the manufacturer's instructions. The total RNA and miRNAs were extracted from the isolated exosomes using the miRCURY™ RNA isolation kit - Biofluids (300112, Exiqon, Denmark) and the purified miRNAs were eluted from the extraction column in a final volume of 15 µL. For optimal isolation of RNA from breast tissues that have high lipid content we used lysis additive solution (300121, Exiqon, Denmark) as suggested by the manufacturer to remove the interfering fat and glycoprotein complexes which are abundant in breast tissue specimens. The lysis solution was prepared freshly prior to tissue homogenization by the addition of 10 µL of β-mercaptoethanol to each of 1 mL lysis solution (biofluids- Exiqon, Denmark) before use. To about 20 mg piece of frozen tissue specimen a 600 µL of lysis solution was added and the tissue was homogenized thoroughly using a pestle mechanical homogenizer for less than one minute. The tissue homogenate was aspirated 5-10 times through a 25-gauge needle attached to a 5-mL syringe to shear the genomic DNA prior to loading to the extraction minicolumns. The total RNA from tissue homogenate was extracted using the miRCURY™ RNA Cell & Plant Isolation Kit (300110, Exiqon, Denmark) and eluted in a total volume of 25 µL. The quality and yield of each RNA sample was measured using a UV Spectrophotometer. The RNA specimens were stored at -80°C until reverse transcription.

Reverse transcription of RNA and real-time PCR

In the reverse transcription (RT) step, the cDNA is synthesized by the reverse transcription of the RNA in the samples (plasma exosomes and breast tumor tissue) using the universal cDNA synthesis kit (303301, Exiqon, Denmark) according to the manufacturer's instructions, after adjustment of each of the template RNA samples to a concentration 5 ng/µL with nuclease free water in a total volume of 10 µL. The RT aliquots were incubated for 60 min at 42°C and 5 min at 95°C, and then maintained at 4°C for real-time qPCR. In the second step RT-qPCR was performed using the ExiLENT SYBR Green master mix (303402, Exiqon no. 203403, Denmark) and LNA™ PCR miRNA-21, miRNA-155 primer sets and miRNA-16 as reference gene (Exiqon, Denmark). Four microliters of cDNA diluted to 1:80 (for tissue) or 1:50 for circulating exosomal miRNA were used as templates in a 10 µL total reaction volume using the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 10s, 60°C for 1 min. Melting curve analysis was carried out at 95°C for 1 min, 55°C for 30s, 95°C for 30s. All reactions were carried out on the Stratagene (Mx3005P) real-time PCR amplification-detection system (Agilent Technology, Germany). The relative circulating miRNA quantity was determined using the comparative ΔΔCt method. For each sample, the miRNAs expression levels were assayed with endogenous control miR-16, also, with a no template negative control reaction. The normalization step was performed according to the according to (Mean ΔCt Target) - (Mean ΔCt endogenous control) method. The fold-change of the relative expression of the microRNAs` genes was calculated by the equation: Fold change = 2 –ΔΔCT [12]. The cycle threshold (Ct) values, the amplification plot and the melting curves of miRNA -155, miRNA -21 and miRNA-16 are shown in Figures 1 and 2 (supplemented files).

Immunohistochemical Analysis

Immunohistochemical staining for TIMP3 was performed on formalin fixed paraffin sections 5 µm thick of the breast tissue specimens mounted on positive charged microscopic slides. Following heating overnight at 37°C, deparaffinization of slides was done in xylene and subsequent rehydration through graded alcohol solution. The slides were then treated for blocking endogenous peroxidase activity and nonspecific binding. Afterwards the sections were incubated with a monoclonal antibody to TIMP3 at a dilution of 1:200 (Abcam Inc. USA) at 4°C for an overnight in a humidified chamber. Universal anti-mouse/anti-rabbit secondary antibody (Abcam Inc. USA) was used for visualization with diaminobenzidine as a chromogen. Finally, the sections were counterstained with hematoxylin.

Normal mouse skeletal muscle tissue was used as a positive control for TIMP3. Negative controls for the specificity of immunohistochemical reactions were performed by replacing the primary antibody with phosphate-buffered saline. Brown color-staining of TIMP3 protein is considered positive. The staining intensity and the number of stained cells for TIMP3 expression were taken into consideration. TIMP3 was strongly expressed in positive controls. The cytoplasmic staining in malignant and the benign breast cells was scored on a scale of 0 to 3 as described previously [13] and as follows: Score 0: no staining, Score +1: weak to moderate staining in less than 10% of cells, Score +2: moderate to strong staining in 10% to 50% of cells, Score +3: strong staining in more than 50% of cells.

Statistical analyses

All statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) version 21.0 statistical software (SPSS Inc., Chicago IL, USA). $P<0.05$ was considered statistically significant. Data were expressed as the mean ± standard error of the mean (SEM). Differences in miRNAs expression levels between groups were compared using the Student's t-test. Comparisons of miRNA levels were performed by Pearson's t-test. The association of miRNA-21 with clinicopathological features used the Mann-Whitney U test for 2 independent groups, and the Kruskal-Wallis H test for 3 independent groups. The diagnostic accuracy and the area under curve for these parameters were calculated using the receiver operating characteristic (ROC) curve.

RESULTS

Patient details, including age, histological type, TNM stage, tumor grade, lymph node metastasis status, and results of ER, PR, and Her2/neu are summarized in Table 1.

Table 1 The clinical data of the studied participants

Variables	BC N=30 (%)	BBL N=30 (%)	Control N=30 (%)
Age/mean ± S.E.M	52.2 ± 2.03 [a*,b*]	37.9 ± 1.62	40 ± 1.74
Age (years) ≤ 55	14 (46.7)	28 (93.3)	27 (90)
> 55	16 (53.3)	2 (6.7)	3 (10)
BMI (Kg/m^2)/ mean ± S.E.M	30.9 ± 1.04 [a*]	29.7 ± 1.02	28.05 ± 1.14
(Normal) < 25	4 (13.3)	4 (13.3)	8 (26.7)
(Overweight) 25-29.9	7 (23.3)	13 (43.3)	13 (43.3)
(Obese) 30-40	19(63.3)	13 (43.3)	9(30)
Menopausal Status			
Post-menopausal	16 (53.3) [N.S]	7 (23.3)	6 (20)
Pre-menopausal	14 (46.7)	23 (76.7)	24 (80)
Family History of BC	-	-	-
Positive	8 (26.7) [N.S]	3 (10)	3 (10)
Negative	22 (73.3)	27	27
History of other malignancies			
Positive	1(3.3) [N.S]	0	0
Negative	29 (96.7)	30 (100)	0

[a] Student's t-test: breast cancer (BC) versus control group *P<0.05; [b] Student's t-test: BC versus benign breast lesion (BBL) group: *P<0.05; [N.S] Not Significant, BMI: Body Mass Index

Table 2 showed that 20/30 (66.7%) of women with breast cancer were having an invasive ductal carcinoma (IDC), 8 (26.7%) with ductal carcinoma *in situ* (DCIS). Invasive lobular carcinoma (ILC) was found in only 2/30 of the examined women. Based on TNM classification of breast cancer, 13/30 (43.3%) of patients were either in the Stage II or III. The TNM Stage I was found in three patients (10%) and only one patient with advanced tumor Stage IV. The histological Grade II was found in 13 out of 30 patients (43.4%) whereas Grade III in 8 (26.7%) of breast cancer. The tumor size larger than 2 cm and lymph node metastasis were recorded in 40% and 33.6% of patients, respectively. Results of the immunohistochemical staining revealed positive expression for estrogen receptors in 43%, for progesterone receptors it was 66.7%, and was 33.3% for the Her2/neu receptor positive expression in the breast cancer specimens (Table 2).

Table 2 The pathological characteristics of breast cancer patients (N=30)

Histological type	N (%)
Invasive ductal carcinoma (IDC)	20 (66.7)
Ductal carcinoma *in situ* (DCIS)	8 (26.7)
Lobular carcinoma *in situ* (LCIS)	2 (6.7)
TNM Stage	
I	3 (10)
II	13 (43.3)
III	13 (43.3)
Histologic Grade	
I	9 (30)
II	13 (43.4)
III	8 (26.7)
Tumor size	
≥ 2cm	12 (40)
2 cm<	18 (60)
Lymph node metastasis	
yes	10 (33.3)
No	20 (66.7)
Estrogen receptor (ER)	
Positive	13 (43.0)
Negative	17 (56.7)
Progesterone receptor (PR)	
Positive	13 (66.7)
Negative	17 (56.7)
Her2/neu receptor	
Positive	10 (33.3)
Negative	20 (66.7)
ER/PR/EGFR-2 status	
Triple negative breast cancer	18(60%)
other BC subtypes (DNBC, SNBC and TPBC)	12 (40%)
DNBC: ER-; PR-; Her2/neu+; SNBC: ER-; PR+; Her2/neu+; TPBC: ER+; PR+; Her2/neu+	

The mean ± SEM of the threshold cycle of miRNA-21, miRNA-155, and miRNA-16, relative expression and median fold change in the plasma miRNA-21 in the study groups are shown in Table 3. The mean Ct of the circulating miRNA-21 and miRNA-155 in BC were statistically lower (P<0.05) than that of BBL and the control groups. There is highly significant increase in the means of the relative expression of the plasma exosomal miRNA-21 and miRNA-155 in BC group (P<0.001) as compared with BBL and control groups. There were no statistical significant differences (P>0.05) in the relative expression of these plasma exosomal miRNAs in BC group as compared to those of the BBL group.

Table 3 Mean ± SEM of the threshold cycle, relative expression and median of fold change of plasma exosomal miRNA-21 and miRNA-155 in the study groups

Descriptive Statistics of plasma exosomal miRNAs	BC (N=30)	BBLs (N=30)	Control (N=30)
Mean ± SEM			
CtmiR-21	0.30 ± 20.04*	27.54 ± 0.1	27.86 ± 0.12
CtmiR-155	20.76 ± 0.30 *	27.7 ± 0.11	27.51 ± 0.27
CtmiR-16	0.24 ± 23.66	0.12 ± 27.88	0.29 ± 27.97
Relative expression (-ΔCt) = (CtmiRNA-21 – Ct-miRNA-16)	3.63 ± 0.33 [a,b***]	0.33 ± 0.15	0.11 ± 0.30
Relative expression (-ΔCt) = (CtmiRNA-155 – Ct-miRNA-16)	2.9 ± 0.32 [a,b***]	0.16 ± 0.15	0.46 ± 0.46
Median of fold change (2-ΔΔCt)	miRNA-21	miRNA-155	
BC relative to HC	10.7	6.6	
BBL relative to HC	1.01	1.3	
BC relative to BBL	14.9	8.3	

[a] Student's t-test: Breast cancer (BC) group versus control group: ***P<0.001; [b] Student's t-test: BC versus benign breast lesion (BBL) group: ***P<0.001

The fold increase in the relative expression of the plasma exosomal miRNA-21 in BC patients was 10.7 times those of the control group, and 14.9 folds those of the BBL group (P<0.001), whereas the median relative expression of the circulating plasma exosomal miRNA-155 showed 83-fold increase in BC patients when compared with that of BBL and 6.6 times those of the healthy control. The BBL showed a median fold change in the expression of exosomal miR-155 of only 1.34 relative to those of healthy controls (Table 4).

Table 4 Mean ± SEM of the threshold cycle, relative expression and median of fold change of tissue miRNA-21 and miRNA-155 in the study groups

Descriptive Statistics of tissue miRNAs	BC group		BBL group	
Mean± SEM	**disease site**	**normal site**	**disease site**	**normal site**
Ct of tissue miRNA-21	19.44 ± 0.25 [a*]	26.99 ± 0.11	26.3 ± 0.12	26.45 ± 0.16
Ct of tissue miRNA-155	19.96 ± 0.37 [a *]	27.28 ± 0.162	27.09 ± 0.06	27.32 ± 0.05
Ct of tissue miRNA-16	21.68 ± 0.23	26.01 ± 0.181	27.09 ± 0.1	27.5 ± 0.06
Relative expression/miR-21 -ΔCt = (ΔCt disease site – ΔCt normal site)	3.22 ± 0.185 [b***]		1.05 ± 0.183	
Relative expression/miR-55 -ΔCt = (ΔCt disease site – ΔCt normal site)	2.99 ± 0.16 [b***]		0.18 ± 0.12	
Median of fold change (2-ΔΔCt)	miRNA-21		miRNA-155	
BC relative to BBL	11.5		9.8	

[a] Student`s t-test: Breast cancer (BC) group: disease site versus normal site: *P<0.05; b Student`s t-test: Breast cancer (BC) group versus Benign breast lesion (BBL) group: ***P<0.001

The tissue level of miRNA-21 in each sample was normalized to that of miRNA-16 as a reference gene and normalized with the normal tissue site of each excised breast specimen. In Table 4, the mean relative expression level of tissue miRNA-21 was increased in BC patients to 11.5 times those of the BBL group (P<0.001). There was significant increase in the mean relative expression of miRNA-155 gene in the BC compared to BBL groups (P<0.001). The median fold change of tissue miRNA-155(2-ΔΔCt) was 9.8 in the BC group relative to the BBL group. These results signify the overexpression of the plasma exosomal and tissue miRNA-21 and miRNA-155 in women within the BC group.

Relationship between miRNA-21 and miRNA-155 with the clinicopathological features of the patients` groups

The relative concentrations of plasma exosomal and tissue miRNAs (miRNA-21 and miRNA-155) of the 30 breast cancer patients were studied in relation to their clinicopathological data (Table 5). Using Kruskall-Wallis statistical test, twenty of the histologically defined invasive ductal carcinoma (IDC) group showed highly significant increase in the circulating plasma exosomal (P=0.01) and tissue miRNA-155 expression levels (P=0.05) as compared to their levels in DCIS group. Likewise, similar increase in the expression levels of the miRNA-21 was recorded in tissues and plasma exosomes (P=0.05) yet, there was near significance increase in the mean expression level of miRNA-21 in breast tissue (P=0.09). According to the TNM staging of BC, we detected statistical trend towards increased expression of miRNA-21 in plasma exosomes of 26 patients with Stage II, III as compared to those of Stage I. Whereas, the mean expression level of tissue miRNA-21, miRNA-155 (plasma exosomal and tissue) were increased with the advanced TNM Stage. There is consistent increase in plasma exosomal and tissue miRNA-15 in patients with TNM Stage II, and III as compared to miRNA-15 level of expression in those with tumor Stage I (P<0.05). The tissue miRNA-21 showed high significant expression level in TNM stage III more than those of Stage II and Stage I (P<0.01), respectively. Statistical analysis of the concentrations of the studied miRNAs in relation to the tumor size, lymph node metastasis as well as the immunohistochemical presence of ER, and PR did not reach any level of the statistical significance. Whereas, Mann-Whitney test revealed significantly association of the negative HER2/neu with the increase in only the expression level of exosomal miRNA-21 (P<0.05) and exosomal miRNA-155 (P<0.01). In the triple negative type of BC (ER-, PR-, HER2/neu-), there were significant increase (P<0.01) in the level of expression of the tissue and plasma exosomal miRNAs-21and miRNAs-155 as compared to other BC subtypes (ER-/PR+, HER2/neu+; ER-, PR-, HER2/neu+; ER+, PR+, HER2/neu+).

Table 5 Association of circulating and tissue miRNAs (21 & 155) expression with clinical and pathological features

Variable		Plasma exsosomal miRNA-21 (-ΔCt)	Plasma exsosomal miRNA-155 (-ΔCt)	Tissue miRNA-21(-ΔCt)	Tissue miRNA-155(-ΔCt)
Histological type	**N**	**m ± SEM**	**m ± SEM**	**m ± SEM**	**m ± SEM**
Invasive ductal carcinoma (IDC)	20	4.29 ± 0.33 ^{*c}	3.59 ± 0.33^{**c}	3.39 ± 0.23 ^{*c}	3.25 ± 0.14 ^{*c}
Ductal carcinoma *in situ* (DCIS)	8	2.75 ± 0.60 ^{*c}	1.9 ± 0.52 ^{**c}	3.05 ± 0.34 ^{*c}	2.61 ± 0.35 ^{*c}
TNM Stage					
I	3	0.3 ± 0.29	0.22 ± 0.59	2.06 ± 0.57	1.28 ± 0.37
II	13	3.4 ± 0.35	2.47 ± 0.37 ^{*c}	2.84 ± 0.25 ^{**c}	3.15 ± 0.23 ^{*c}
III	13	3.57 ± 0.44	4.08 ± 0.36 ^{*c}	3.77 ± 0.21 ^{**c}	3.22 ± 0.16 ^{*c}
Histologic Grade ^{NS}					
I	9	3.28 ± 0.71	2.56 ± 0.66	2.73 ± 0.41	2.88 ± 0.43
II	13	4.23 ± 0.46	3.41 ± 0.45	3.56 ± 0.23	2.92 ± 0.22
III	8	3.1 ± 0.59	2.47 ± 0.66	3.08 ± 0.29	3.23 ± 0.15
Tumor size ^{NS}					
≥ 2 cm	12	4.02 ± 0.41	3.34 ± 0.38	3.39 ± 0.26	3.09 ± 0.21
<2 cm	18	3.76 ± 0.365	2.25 ± 0.56	2.97 ± 0.23	2.85 ± 0.25
Lymph node ^{NS}					
Positive	20	3.76 ± 0.37	3.11 ± 0.35	3.30 ± 0.24	3.07 ± 0.19
Negative	10	3.35 ± 0.7	2.49 ± 0.70	3.06 ± 0.29	2.83 ± 0.31
Estrogen receptor ^{NS}					
Positive	17	3. 67 ± 0.38	2.98 ± 0.35	3.04 ± 0.25	2.98 ± 0.23
Negative	13	3.57 ± 0.61	2.81 ± 0.62	3.46 ± 0.27	3.01 ± 0.23
Progesterone receptor ^{NS}					
Positive	17	3.34 ± 0.37	2.72 ± 0.38	3.26 ± 0.28	3.10 ± 0.19
Negative	13	3.99 ± 0.6	3.14 ± 0.59	3.17 ± 0.23	2.85 ± 0.28
Her2/neu receptor					
Positive	10	2.46 ± 0.55	1.59 ± 0.54	3.05 ± 0.3	2.85 ± 0.31
Negative	20	4.21 ± 0.36 ^{*d}	3.56 ± 0.33 ^{**d}	3.31 ± 0.24	3.06 ± 0.19
ER/PR/EGFR-2 status					
Triple negative	10	4.63 ± 0.3 ^{**d}	4.4 ± 0.37 ^{**d}	4.07 ± 0.18 ^{**d}	3.8 ± 0.2 ^{**d}
DNBC, SNBC, TPBC	20	2.7 ± 0.21	2.25 ± 0.31	2.07 ± 0.18	2.6 ± 0.19

DNBC: ER-; PR-; Her2/neu+, SNBC: ER-; PR+; Her2/neu+, TPBC: ER+; PR+; Her2/neu+; ^{c} Kruskal-Wallis Test: *P<0.05, **P<0.01, ^{d} Mann-Whitney Test: *P<0.05, *P<0.01. NS: Not Significant

The diagnostics performance of the miRNA-155 and miRNA-21 in the studied groups

The receiver operator characteristics (ROC) curve analysis revealed that miRNA-155 (plasma and tissue) were effective tests in the BC when compared with BBL group (Figure 1). The sensitivity and specificity of the plasma exosomal miRNA-155 relative to the BBL group were 83.3% and 86.7% at a cut off value of 0.89 and area under curve=0.79 (Figure 1A). The tissue miRNA-155 displayed a sensitivity of 96.7% and a specificity of 96.7 at a cut off value of 0.36 and area under curve of 0.99 relative to the BBL group. Whereas, the plasma exosomal miRNA-155 of BC depicted the lowest diagnostic sensitivity (73.3%) and specificity (80%) when compared with the control group at reading 1.95 and area under curve of 0. 9 (Figure 1B).

In Figure 2A, the sensitivity and specificity of the plasma exosomal miRNA-21 in BC group relative to healthy control were 90% and 86% at a cut off value of 1.2 and area under curve of 0.93 in BC group relative to the BBL group, the plasma exosomal miRNA-21 showed a sensitivity of 93.3% and a specificity of 83.3% at a cut off value of 1.7 and area under curve of 0.94. The tissue miRNA-21 of BC depicted the highest diagnostic sensitivity (100%) and specificity (100%) when compared with the control group at a cut off limit of 0.87 and area under curve of 1 (Figure 2B).

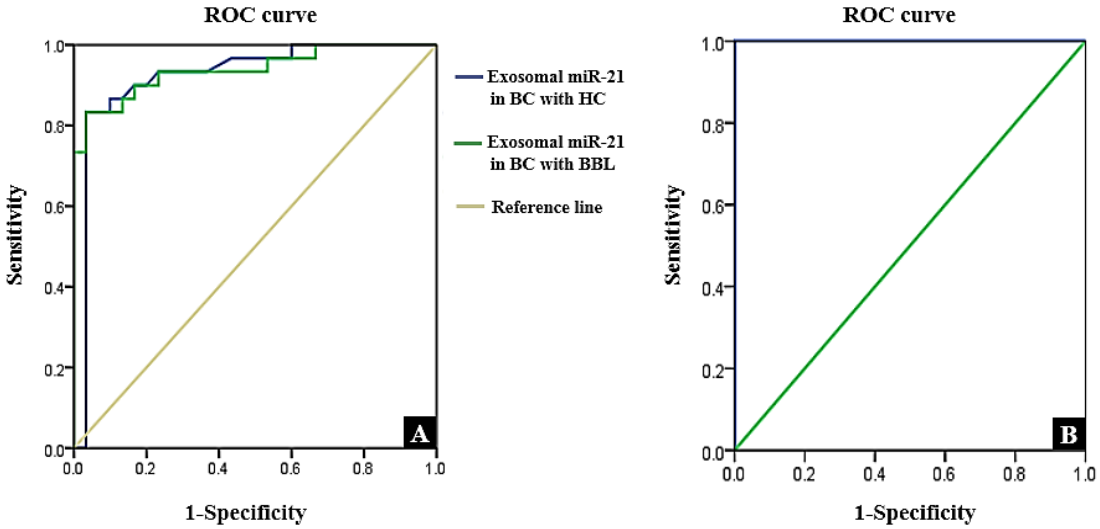

Figure 1 The receiver operator characteristic (ROC) curve for plasma and tissue miRNA-155. (A) the ROC results of the plasma exosomal miRNA-155 in breast cancer (BC) group relative to the healthy control (HC) group (cut off value=1.95, sensitivity=73.3%, specificity=80%, and area under curve=0.9), and in BC group relative to the benign breast lesion (BBL) group (cut off value=0.89, sensitivity=83.3%, specificity= 86.7%, and area under curve=0.79). (B) The ROC results of the tissue miRNA-155 in BC group relative to the BBL group (cut off value=0.36, sensitivity=96.7%, specificity=96.7%, and area under curve=0.99)

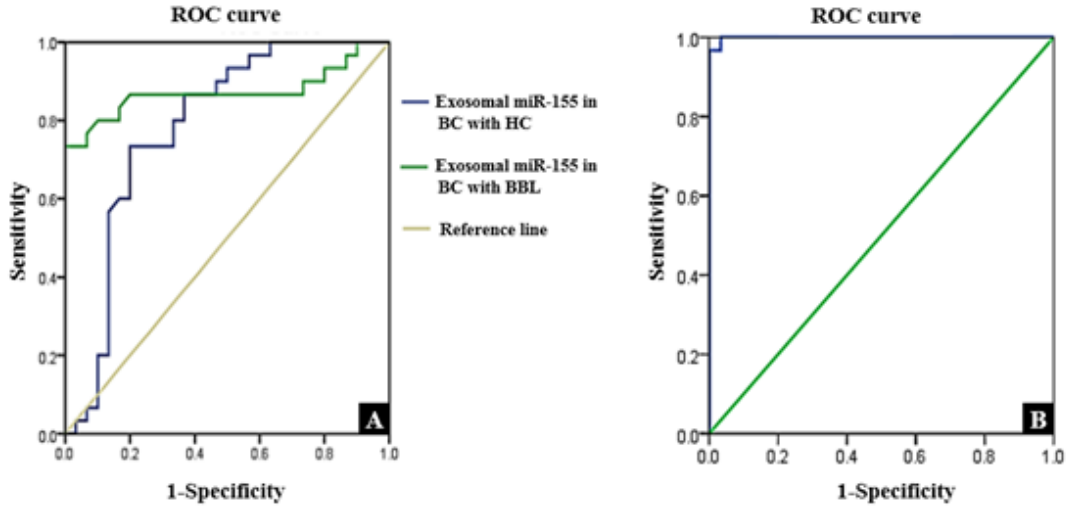

Figure 2 The receiver operator characteristic (ROC) curve for the plasma and tissue miRNA-21. (A) the ROC results of the plasma exosomal miRNA-21 in breast cancer (BC) group relative to the healthy control (HC) group (cut off value=1.2, sensitivity= 90%, specificity=86%, and area under curve =0.93), and in BC group relative to the benign breast lesion (BBL) group (cut off value=1.7, sensitivity=93.3%, specificity=83.3%, and area under curve=0.94). (B) ROC results of tissue miRNA-21 in BC group relative to the BBL group (cut off value=0.87, sensitivity=100%, specificity=100%, and area under curve=1)

Correlation between circulating plasma exosomal and tissue miRNA-155 and miRNA-21 in the studied groups

Application of the Pearson correlation test revealed that the level of TIMP3 protein expression was inversely correlated with plasma exosomal miRNA-21 (r= -0.39, P=0.05) and tissue miRNA-21 gene expression (r= -0.4, P<0.05). The loss or reduction of the TIMP3 expression level was significantly associated with the up regulation of circulating plasma and tissue miRNA-21 in BC patients. We did not observe a significant relationship between TIMP3 protein expression, circulating plasma exosomal miRNA-155, and tissue miRNA-155 in the BC group (Table 6). A highly significant direct correlation (P<0.01) was observed between the relative expression of plasma exosomal miRNA-155

and tissue miRNA-155 levels in BC group (r=0.47). Similar direct correlation was found between tissue miRNA-21 and plasma tissue miRNA-21(r=0.44, P<0.01). There were positive significant correlations between the circulating plasma miRNA-155 with the miRNA-21 in the BC group (r=0.96, P<0.001) on one hand and between the tissue miRNA-155 with the tissue miRNA-21 in women with BC (r=0.33, P<0.05) on the other hand.

Table 6 Correlation between miRNA-21, miRNA-155, and TIMP3 expression

Variable Ct	TIMP3 N=30	Plasma miRNA-155	Tissue miRNA-155	Plasma miRNA- 21
	r	r	r	r
Plasma miRNA-21	-0.39*	0.96***	0.51**	1
Plasma miRNA-155	-0.29	1	0.47**	0.96***
Tissue miRNA-21	-0.4*	0.37*	0.33*	0.44**
Tissue miRNA-155	-0.23	0.47**	1	0.51**
Significant t-test for r: *P<0.05, **P<0.01, *** P<0.001; Pearson correlation coefficient (r). TIMP3: Tissue Inhibitor of Metalloproteinase				

Histological findings of the tissue inhibitor of metalloproteinase 3 (TIMP3)

TIMP-3 protein was immunodetected in the cytoplasm and nucleus of benign breast lesion (Figure 3). All benign breast tissues tested by IHC were positive for TIMP3 30(100%). 5 (16.7%) showed weak intensity, 25(83.3%) of BBL tissues showed moderate to severe intensity (Table 7, Figures 3A and 3B). 4 out 30 of the BC tissues (13.3%) had no detectable TIMP3 protein expression, while 26.7% (8/26) exhibited weak TIMP3 expression with strong staining intensity observed in 10 (33.3%) of BC group. The overall expression of TIMP3 in BC was significantly lower compared with benign breast tumor (P<0.05). The TIMP3 protein expression was visualized at variable intensities in the cytoplasm and nucleus of the malignant cells (Figures 3C-3F).

Table 7 Breast tissue (malignant and benign) patients distributed by the expression of TIMP3 protein

Breast tumor	TIMP3	Total		Reaction Score					
				Weak		Moderate		Strong	
		No.	%	No.	%	No.	%	No.	%
BC (n=30)	Positive	26	86.6	8	26.7	8	26.7	10	33.3
	Negative	4	13.3	---	---	---	---	---	---
BBL (n=30)	Positive	30	100	5	16.7	7	23.3	18	60
	Negative	0	0	---	---	---	---	---	---
TIMP3: Tissue Inhibitor of Metalloproteinase; BC: Breast Cancer; BBL: Benign Breast Lesion									

DISCUSSION

Based on the origin and invasiveness, BC are classified as ductal carcinomas *in situ* (DCIS), invasive ductal carcinomas (IDC), lobular carcinomas *in situ* (LCIS), and invasive lobular carcinomas, ILC. Higher percentages of Iraqi women were presented with an IDC followed by DCIS and most of these patients were either in the TNM stage II or III. Sixty percent of the studied patients with BC [14] were having triple negative breast cancer (TNBC). Triple-negative breast cancer is one of the most recently identified biological variants which comprises 15% of newly diagnosed breast cancer [15]. TNBC do not express receptor for estrogen, progesterone, or HER-2 [16] and has the least desired prognosis among all breast cancer subtypes [17]. TNBC are reported to have a higher histologic grade, diminuted stromal content, high mitotic count, central necrosis, invasion, a stromal lymphocytic response, and multiple apoptotic cells. TNBC present a higher probability of distant metastasis and lack of effective targeted therapy. In US young group of patients, the TNBC was reported to be 35% [18]. Consistent to our findings, other studies found that TNBC were more likely to present at an advanced stage of BC [19]. Contributing factors to these differences are not completely understood. Mostly, BRCA1 carriers are prone to develop breast cancer at a young age with the TNBC subtype.

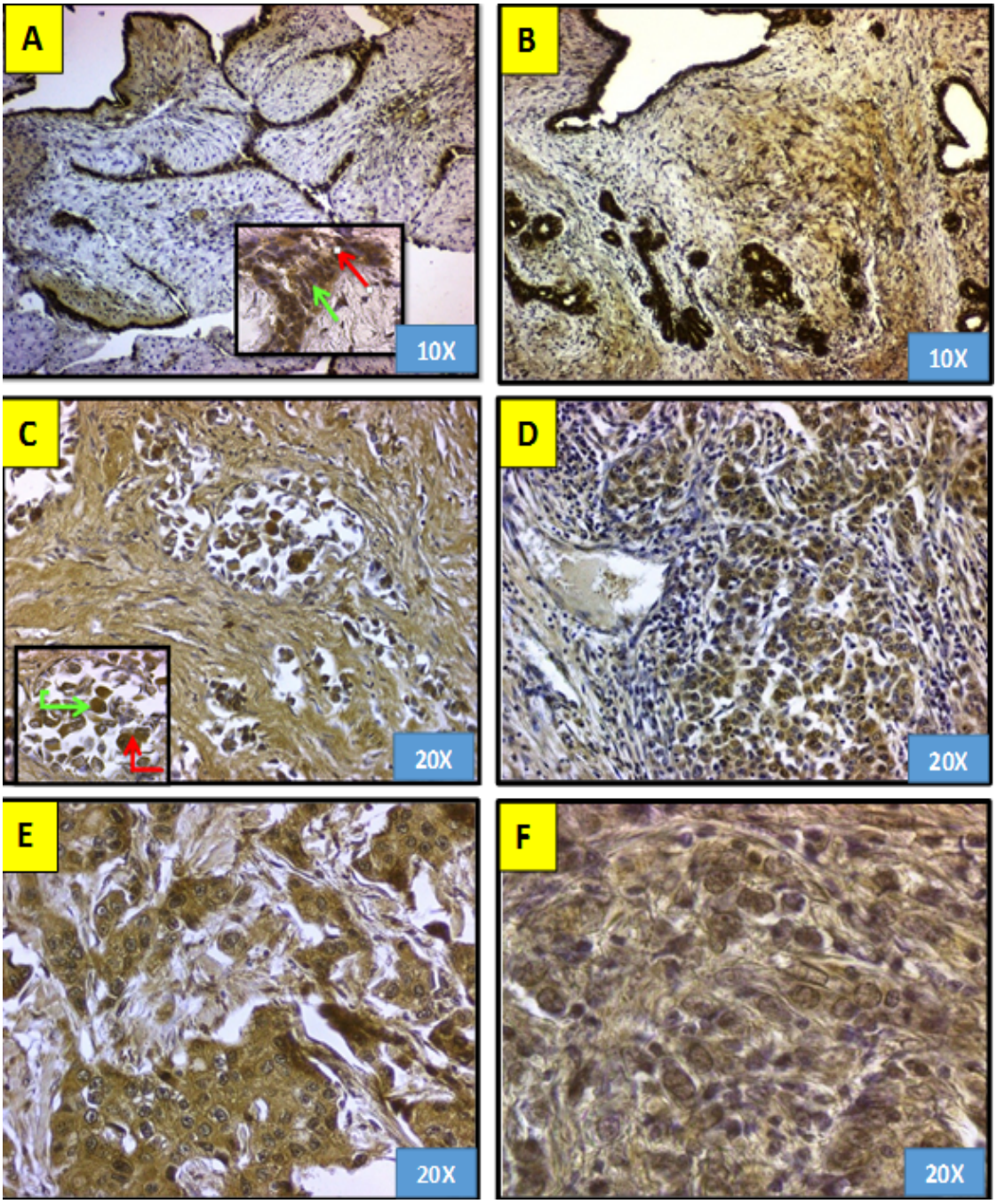

Figure 3 The tissue inhibitor of metalloproteinases (TIMP-3) protein expression in breast cancer and benign breast lesion tissues. The micrographs of the immunohistochemical reactions revealed localization of the TIMP3 in the cytoplasm and nucleus of a fibroadenoma of more than 50% of cells (Score 2), moderate staining intensity 10X (A), fibroadenoma positive reaction of more than 50% of cells (Score 3), strong staining intensity 10X (B), invasive ductal carcinoma positive reaction in the cytoplasm and nucleus of more than 50% of cells (score 3), moderate staining intensity 20X (C), invasive ductal carcinoma positive reaction in the cytoplasm and nucleus of less than 50% of cells (score 2), moderate staining intensity 20X (D), invasive ductal carcinoma positive reaction in the cytoplasm and nucleus of more than 50% of cells (Score 2), moderate staining intensity 20X (E), invasive ductal carcinoma positive reaction in the cytoplasm and nucleus of more than 50% of cells (score 3) strong staining intensity 20X (F)

The process of tumorigenesis is a multifactorial path that differs in each individual. Use of a single serum miRNA measurement as a tumor marker does not provide sufficient specificity. Detecting multiple miRNAs and other tumor

markers, such as protein antigens, may be helpful to improve the accuracy. So, we tested herein the association of miRNAs with tumor characteristics such as TNM grade tumor size, histological grade, lymph node metastasis and ER/PR status. We focused on the miRNAs-155, miRNA-21 and its target protein TIMP3 to investigate the true expression levels of these two miRNAs and the strength of interrelation between these miRNAs in both tumor tissues and matched plasma exosome samples with the patient clinicopathological condition and the invasiveness of breast cancer. Moreover, we determined the potential of plasma exosomal miRNA-21 and miRNA-155 as surrogate biomarkers in discriminating the breast cancer from those of BBLs and normal breast tissues.

Previous studies have involved the use of serum to evaluate the circulating miRNA-155 in patients with breast cancer. However, miRNA can be liberated from blood cells during the process of blood coagulation [7]. To avoid the potential release of miRNA-155 from blood cells, we selected plasma to extract and estimate the circulating exosomal miRNA-155. In addition, there is a lack of endogenous controls for normalization of circulating miRNA-155 data.

Some studies suggested that most circulating miRNAs originate from blood cells, during the coagulation process, and endothelial cells [9], but tumor cells and organs, such as liver, kidney and lung, are also considered to be potential sources of circulating miRNAs, suggesting a complexity of the origin of miRNAs in circulation [20]. In this study, we tried to carefully separate and purify exosomes from patient and control plasma and extract the miRNA from these circulating exosomes and to remove the fatty cells and glycoaggregates from breast tissue specimens during the extraction steps of tissue miRNA using specific kits. This is followed by conducting SYBR Green RT-PCR analysis. The level of circulating plasma exosomal miRNA-21 and miRNA-155, as measured by the Ct value, in BC patient was lower than those in BBL and control groups, which means that the expression level of these miRNAs in plasma BC samples was higher than in other groups (supplemented materials).

We observed that the increase in the relative expression of the plasma exosomal miRNA-21 and miRNA-155 in BC patients was higher than those of BBL and normal breast tissue. These findings were in line with those of Hafez, et al. [21] who reported that in BC tissues, miRNA-155, miRNA-10, miRNA-21 and miRNA-373 were significantly over-expressed by 14.46, 5.7, 10.69 and 5.87 folds, respectively as compared to normal adjacent tissues. Si, et al. [22] detected significantly higher levels of both circulating and non-circulating miRNA-21 in serum and tissue samples, respectively, compared with samples taken from healthy individuals. In contrast to the present findings, Heneghan, et al. [23] recorded non-significant difference in the level of expression of miRNA-155 between BC patients and healthy controls. Different results for circulating miRNAs levels in previous studies compared to the present study findings in patients with breast cancer, which may be attributed to various intrinsic and extrinsic factors such as methodologies, sample type (serum versus plasma), sample size, endogenous controls and individuals` characteristics (e.g., age, ethnicity, food and medication used). The type of the sample and the normalization method that depends on the endogenous control used are of paramount to the result obtained in each study [7].

The augmented increase in median fold change of tissue miRNA-21 and miRNA- in BC patients relative to those of the BBL group signifies the overexpression of the tissue miRNA-21 and miRNA-155 in women within the BC group. In a study of Gao, et al. [24], the expression of miRNA-155 was reported to be more than 5-fold higher in ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) than in normal breast tissues but it was lower in tumors with brain metastases than in localized breast tumors. In our study, the relative expression of plasma exosomal and tissue miRNAs (miRNA-21 and miRNA-155) of twenty histologically defined invasive ductal carcinoma (IDC) group showed highly significant increase as compared to their levels in both DCIS and ILC groups. Likewise, other studies showed that miRNA-21 was significantly overexpressed in DCIS samples compared with histologically normal samples, and in the samples taken after a reduction mammoplasty. They supposed that miRNA-21 could be involved in the early phases of BC formation [25].

According to the TNM staging of BC, we recorded consistent increase in both plasma exosomal and tissue miRNA-155 in patients with TNM stage II, and III as well as stage IV compared to miRNA-155 level of expression in those with tumor stage I. The tissue miRNA-21 showed high significant expression level in late TNM stage IV and III more than those of stage II and stage. Our data are consistent with other reports indicating that miRNA-21 expression increased with advanced clinical stage [26]. Roth, et al. [27] reported that miRNA-155 expression level was remarkably higher at stages III and IV compared to stages I and II in BC patients. Iorio, et al. [10] found that the miRNA-21 and miRNA-155 were up-regulated in tissue of 76 of BC patients with high tumor stage compared with 10 normal breast

samples and they suggested that deregulation of these two miRNAs may affect the critical molecular events involved in tumor progression. Gao, et al. [24] showed that miRNA-155 in culture medium and exosomes remains constant in FOXP3 Tet-off MCF7 cells through induction of miRNA-155 by FOXP3. Nevertheless, they found that miRNA-155 is elevated in both plasma and blood cells, especially in patients with localized breast cancer, suggesting that the origin of circulating miRNA-155 is from blood cells and not from tumor cells.

No significant association of the increased expressions of the studied miRNA-21 and miRNA-155 with the tumor size, lymph node metastasis as well as the immunohistochemical expression of ER, and PR in BC group. Whereas, Mann-Whitney test revealed significant increase in only the expression level of exosomal miRNA-21 and exosomal miRNA-155 in BC cases with negative HER2/neu expression. Hafez, et al. [21] reported that in large sized tumors, the expression levels of miRNA-21 was 13-fold compared to 9 folds in smaller tumor size. Samples having Grade III, the miRNA-21 expression was two folds more than the samples having grade I or grade II. Han, et al. [28] found that the serum level of miRNA-21 was significantly higher, while miRNA-155 and miRNA-365 was significantly lower in breast cancer than healthy control. Furthermore, low levels of serum miRNA-155 were reported to correlate with increased tumor stages [26]. On the contrary, Gao, et al. [24] indicated that high levels of plasma miRNA-155 were associated with early stage (DCIS and M0) breast cancer, with ER-and PR-negative tumors, or in triple negative BC (TNBCs). The miRNA-155 can act on TGF-β/Smad4 signaling through RhoA, thereby promoting the EMT; in contrast, knockdown of miRNA-155 were found to significantly inhibit the TGF- /Smad4- induced epithelial mesenchymal transition [29].

Hagrass, et al. [30] and Reham, et al. [31] found that the expression of miRNA-155 and miRNA-21 was significantly higher in serum of breast cancer patients with lymph nodes involvement, increasing pathological grade and of size >2 cm than breast cancer patients without lymph nodes involvement, lower tumor grade and smaller tumor size (<2 cm). Yet, Petrovic showed that miRNA-21 was not significantly related to lymph node metastasis [25].

In relation to estrogen/ progesterone receptors, the expression level of miRNA-21 in the BC patients with ER-/PR- was found to be significantly increased but those of miRNA-155 approaches the significance as compared to ER+/ PR+ carriers. Patients with ER-/PR- are poor responders to tamoxifen which is used in treating certain types of BC by halting the estrogen-induced growth. This finding indicates that the high expression of oncogenic miRNA-21 may foster the resistance of cancerous cells to hormonal therapy [32]. Yan, et al. [33] and Petrovic [25] showed that miRNA-21 overexpression in human BC was associated with advanced clinical stage, lymph node metastasis, and poor patient prognosis. They assumed that miRNA-21 levels were related to the later stages of BC progression and invasive abilities and miRNAs can be used as different factors of disease pathology, in various sample types [34]. Elevated miRNA-155 found in Her-2/neu positive or lymph node metastasis positive, or p53 mutant type of BC may have diagnostic value in BC patients as an auxiliary biomarker for different clinicopathological features of BC [35]. Petrovi, et al. suggested that miR-155 might be involved in BC pathogenesis and tumor spreading to the lymph nodes, and this places it as a biomarker for the additional stratification of the invasiveness of breast carcinomas [36].

We recorded consistent significant increase in the level of expression of tissue and plasma exosomal miRNA-21 and miRNA-155 of the TNBC subgroup (the aggressive type of BC), a finding that stresses their biological role in controlling tumor survival and differentiation. Radojicic, et al. [37] demonstrated overexpression of miRNA-21, miRNA-210 and miRNA-221 on one hand and significantly down regulation of miRNA-10b, miRNA-145, miRNA-205, miRNA-122a in TNBC. The oncogenic miRNA-21 is consistently overexpressed in paired tumor tissue (34.64) and sera (13.99) of patients with TNBC in comparison to those of normal breast tissues and other BC subtypes. The expression of miRNA-21 was 2-fold higher in benign breast tumor fibroadenoma as compared to normal adjacent tissue [38].

Tumor growth is dependent on the availability of nutrients and oxygen. Initially, the growth of a tumor is supported by nearby blood vessels [39]. However, the tumor volume is restricted only by the consumption of nutrients and oxygen [40]. The formation of new blood vessels (angiogenesis) is necessary for tumor progression. Angiogenesis provides the necessary nourishment and oxygen for tumor cells, but it acts as a route for tumor cell metastasis to distant organs. It is evident that miRNAs mediate the cell fate of TNBC by regulating cell differentiation, and cell survival [37]. This is confirmed by finding of Yuan, et al. [41] of up regulation of TIMP3 between TNBC tissue and the luminal subtype of breast cancer tissue. Moreover, miRNA-155 plays a crucial role in regulating homeostasis in the immune system in cancer patients as it activates the tumor-associated macrophages in breast cancer [42].

In TNBC, miRNA expressions are found to be associated with BRCA mutations and epithelial-mesenchymal transition [43]. On the other hand, the BRCA1/2 gene is a cancer susceptibility gene that is associated with hereditary susceptibility to breast and ovarian cancer. The BRCA1 plays a role in the epigenetic control of the oncogenic miRNA-155 [44].

The ROC curve analysis revealed that plasma exosomal miRNA-155 and miRNA-21 showed high sensitivity and specificity. These results were consistent to those reported by other researchers [24,32]. The tissue miRNA-21 of BC group depicted the highest diagnostic sensitivity and specificity (100%, 100%). In contrast to these findings, Han, et al. [28] revealed that the miRNA-21 and miRNA-155 displayed significantly higher AUCs with maximal values of sensitivity plus specificity for miRNA-21 (66.67% and 88.89%, respectively). The strong association between the circulating exosomal and tissue miRNA-21 and miRNA-155 and the high diagnostic sensitivity and specificity points out to their use as prognostic biomarker for tracing those with future relapse or non-responders to hormonal manipulation in breast cancer. Further studies on a larger sample size are suggested to clarify this effect.

Majority of BBL tissues showed moderate to severe intensity of the TIMP3 protein expression. The overall expression of TIMP3 in BC was significantly lower compared with benign breast tumor. The decreased expression of TIMP3 protein within malignant breast cancer cells seems to be associated with an aggressive tumor phenotype through its correlations with nuclear and histological grade, expression. Reduced expression of TIMP3 was found to be a significant prognostic indicator of low survival rate in the lymph-node-positive and mutant-p53-negative patients, indicating that immunodetection of TIMP3 protein could improve the prediction of disease recurrence in some subgroups of patients with invasive breast cancer [45]. Other studies have revealed no correlation with any of these clinicopathological parameters [46]. This discrepancy from our findings may be due to the different methodology used. Specifically, both groups of authors used polymerase chain reaction to assess TIMP3 mRNA expression levels in tumors.

Pearson correlation test revealed that reduction of the TIMP3 expression level was significantly and inversely associated with the increased expression of circulating plasma exosomal and breast tumor tissue miRNA-21 in BC patients. Similar observation was reported by Song, et al. [47] who recorded that in cell lines with high relative miRNA-21 expression (MDA-MB-435 and MDA-MB-231), a low amount of TIMP3 protein was observed, whereas cell lines with low relative miRNA-21 expression (BCAP-37 and MCF-7) displayed increased concentration of TIMP3 protein, resulting in a significant inverse correlation between miRNA-21 expression and TIMP3 protein content. However, they did not detect significant correlation between miRNA-21 and TIMP3 mRNA in the four cell lines. On the other side, we did not observe a significant relationship between TIMP3 protein expression, circulating plasma exosomal miRNA-155, and tissue miRNA-155 in the BC group. This may rule out the direct influence of the miRNA-155 on the TIMP3 protein expression in malignant breast tumors. A recent report has indicated that miRNA-21-5p promotes angiogenesis by targeting TIMP3/MMPs signaling by directly binding to the 3'UTR of TIMP3 mRNA and inhibiting its expression [5,48]. Accumulating evidence has indicated that miRNAs can regulate tumor angiogenesis by targeting angiogenic factors and protein kinases through multiple signaling [49]. Although miRNA-21 levels are usually increased in invasive breast carcinomas and in carcinomas with poor prognosis, it would not be unusual to sometimes detect very high miRNA-21 expression levels (compared with normal tissue) in *in situ* carcinomas. Overexpression of miRNA-21 could silence its targets, such as TIMP3 [25], or SMAD-7 (miRNA-21 promotes the transforming growth factor-β pathway by silencing SMAD-7), which could initiate epithelial–mesenchymal transformation and invasion. Furthermore, miRNA-21 can trigger the IL-6/STAT3/ NF-κβ-mediated signaling network in human epidermal growth factor receptor 2 (HER2)-positive breast cancer cells, maintain the EMT phenotype of cancer cells, and build an immune microenvironment suitable for the growth of cancer cells. In some cases, increased miRNA-21 levels in precancerous tissue might trigger some mechanisms for malignant transformation via silencing PTEN or PDCD4 [50]. So, targeting these oncomirs may lead to more successful anticancer therapies [51].

To determine the potential cell origin of plasma miRNA-155 and miRNA-21, the levels of these miRNAs were measured in circulating plasma exosomes from all patients with BC and BBLs and correlate them with the breast tumor tissue miRNA levels from these patients. A highly significant direct correlation was observed herein between the relative expression of plasma exosomal miRNA-155 with tissue miRNA-155 and of circulating plasma exosomal with tissue miRNA-21 levels in BC group. Likewise, there were positive significant cross correlation between the plasma exosomal and tissue levels of the miRNA-155 with miRNA-21 in the BC group. This significant association

between the expressions of these two miRNAs underpins their synergistic effect on the promotion of oncogenesis in these patients through their effects via different targets from the same signaling pathway or targets from other pathways [36]. The co-correlation of exosomal miRNA-21 with tissue miRNA-21 on one hand and tissue low expression of TIMP3 on the other hand in these patients points to the fact these isolated exosomes are extruded from the malignant BC cells in tempting to hematogenous spreading and measurement of plasma purified exosomal miRNA can be used as an indirect test to delineate the metastatic potential of these malignant cells. Our findings confirmed those of Gao, et al. who reported higher levels of miR-155 in patients with localized tumors than patients with distant metastases or for healthy female controls. Furthermore, they isolate exosomes from culture medium and measured the levels of exosomal miRNA-155 and they suggested that the plasma miRNA-155 in patients with breast cancer is derived from blood cells and not from tumor cells [24]. On the contrary, Mishra, et al. [52] could not find any significant correlation in miRNA expression profile between peripheral blood mononuclear cells, plasma and tumor tissues of breast cancer patients when compared to controls. They suggest that this is perhaps because of selective secretion of specific miRNAs in peripheral blood when compared to that in plasma or tumor tissue. Utmost caution should be taken upon comparing the miRNA profiles derived from these sources. Although the concentration of miRNA is often higher in serum than plasma, the difference between serum and plasma miRNA concentration showed some associations with miRNA from platelets, which may indicate that the coagulation process may affect the spectrum of extracellular miRNA in blood [11]. The important limitations of this study are the small sample size and so an extensive study on large sample size for extended duration is suggested to clarify the expression level of these miRNAs in different tumor histological stages, tumor grades and other tumor characteristics.

In conclusion, in women breast cancer, the significant consistent intercorrelation of tumor tissue and circulating miRNAs may pinpoint the role of these exosomal miRNAs in promoting tumor spread. Evaluation of miRNAs levels in the circulating plasma exosomes could be used as beneficial noninvasive blood-based biomarkers in the prediction of cancer invasiveness and as prognostic biomarker to assess response to therapy in these patients.

DECLARATIONS

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Conflicts of Interest

Author declared that there are no conflicts of interest.

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