MicroRNA-21 and microRNA-34a in Relation to other biological markers in Egyptian Breast Cancer Female Patients

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Abstract: Background: Altered microRNAs (miRNAs) expression has an impact on cancer initiation and progression. There is an emerging evidence that circulating miR21 and miR34a may have a potential role in breast cancer (BC) diagnosis and prognosis. **Aim of the work:** The current study aimed at characterizing the expression pattern of miR21 and miR34a in BC female patients pre- and post chemotherapy and delineating their correlation with clinicopathological subtypes and other molecular biomarkers. **Patients and Methods:** Real time quantitative polymerase chain reaction (RQ PCR) was used to assess the relative expression of miR21 and miR34a in sera of 179 BC female patients in relation to 58 healthy females. **Results:** Circulating miR21 and miR34a showed significant upregulation of 5.1 fold change ($p<0.001$) and downregulation of -5.63 fold change, respectively ($p<0.001$). Data showed higher levels of miR21 in triple negative (TN), basal like subtype and stage III BC patients ($p<0.001$). Higher miR34a expression was demonstrated in triple positive, luminal A & B subtypes and stage I & II BC patients ($p<0.001$). MiR21 was directly correlated with Bcl2 level ($p<0.001$). There was direct correlation between miR34a, BRAC1, BRAC2 & p53 ($p<0.001$). MiR21expression decreased significantly postchemotherapy ($p<0.001$). A significant increase in miR34a level was detected after chemotherapy ($p<0.001$). **Conclusion:** Our data suggest a potential role of circulating miR21 and miR34a as molecular biomarkers in BC.
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1. Introduction

Breast cancer (BC) is the most common malignant neoplasm among women and the leading cause of cancer related death in females globally [1]. In 2012, an estimate of 522,000 females died from BC worldwide [2]. An Egyptian study showed that 29% of National Cancer Institute (NCI) patients were diagnosed as BC. Most of them are premenopausal and presented in advanced stage, which may be attributed to the minor role early detection plays in Egypt compared to Europe and North America where screening and early detection are important and have more appreciated role [3].

MicroRNAs (miRNAs) are a class of small noncoding endogenous single stranded RNA molecules, 18-25 nucleotides long, playing an important role in regulating gene expression by pairing with mRNA of protein coding genes [4]. Previous studies have reported that miRNAs dysregulation affects cancer initiation, invasion and metastasis [5, 6]. Circulating miRNAs are very promising biomarkers in cancer diagnosis and prognosis as they are stable, minimally invasive and have high predictive value [7, 8].

The miRNA-21 (miR-21) gene is present on chromosome 17q23.2. This region is often amplified in BC [9]. MiR-21 upregulation may promote tumor initiation and progression through affecting cell cycle differentiation and apoptosis [10]. MiR-21 is considered one of the most important onco-miRNAs. It has a significant role in diagnosis of bronchogenic carcinoma and Gastrointestinal tumors [11, 12]. Previous studies reported miR-21 upregulation in serum of BC female patients [13].

On the other hand, miRNA-34a (miR-34a) is one among a family of three miRNAs; miRNA-34a is expressed in all tissue types except lung, while miR-34b/c are only expressed in lung tissue [14]. MiR-34a is often reported as a tumor suppressor miRNA. It induces apoptosis through p53 dependant tumor suppressor network. Mir-34a is downregulated in most cancer types and it inhibits the expression of Bcl2, various cyclins and cyclin dependant kinases (CDKs) [15].

The current study was undertaken to investigate the potential value of miR-21 and miR-34a expression as diagnostic and prognostic biomarkers in BC. In addition, we investigated the relation between miR-21, miR-34a expression and other biological markers in BC as BRAC1, BRAC2, p53, Bcl2, clinical staging and pathological subtypes.

2. Patients and methods

Patients:

One hundred and seventy nine female patients who presented to Oncology Department, NCI, Cairo. All patients included in this study had a pathologically confirmed diagnosis of primary BC. Median age at diagnosis was 52 years (28.5-75). Fifty eight serum samples were collected from healthy females with a median age of 53.5 years (32-71). The study was approved by the NCI Ethical Committee. Written informed consent was obtained from all participants.

Tissue samples preparation:

Morphological assessment of hematoxylin & eosin stained sections prepared from formalin-fixed paraffin-embedded tissue blocks was performed. Elston and Ellis grading system for invasive carcinoma has been used to evaluate tumor grading [16]. Tumor staging (TNM) was reported according to the World Health Organization (WHO 2003) classification of breast tumors [17].

Methods

Immunohistochemistry:

Streptavidin/biotin immunoperoxidase technique has been used for immunohistochemichal staining (Dakocytomation, Glostrup, Denmark). Hormone receptors were evaluated using anti-ER (mouse monoclonal IgG, Santa Cruz Biotechnology, CA), anti-PR (rabbit polyclonal IgG, Santa Cruz Biotechnology, CA), anti-HER2 (mouse monoclonal IgG, Santa Cruz Biotechnology, CA) followed by secondary antibodies and Diamino Benzidine (DAB) substrate chromogen (DakoCytomation, Glostrup, Denmark) for visualization.

Serum miRNA assays

RNA extraction:

All blood samples were collected; lymphocytes were separated and suspended in trizol and immediately frozen in liquid nitrogen until use. Total RNA was extracted from blood specimens by trizol RNA isolation protocol. The quality of the RNA samples was determined by Nanodrop, USA. To eliminate genomic DNA contamination, total RNA was treated with DNase I before first strand cDNA synthesis, according to the manufacturer's instructions [18].

Sample lysis was achieved with the addition of 600 uL of lysis-binding solution (Qiagen, GmbH, Hilden, Germany) to the blood pellet, then vortexed vigorously for 30 seconds producing a homogenate lysate. Sixty uL of miRNA homogenate additive (Qiagen, GmbH, Hilden, Germany) was added to the lysate, then vortexed for 10 seconds followed by incubation on ice for 10 minutes. 600 uL of acid-phenol: chloroform (Qiagen, GmbH, Hilden, Germany) was added to the lysate and vortexed for 30 seconds then centrifuged for 5mins at 10000g at Room Temperature (RT).

One-third volume of 100% ethanol was added to the recovered aqueous phase and total volume was passed through a fibre-glass filter cartridge via 15 seconds of centrifugation at 10000g. The filtrate collected containing small RNA molecules whereas the filter cartridge contained the large RNA molecule, each was processed separately [18, 19].

Enrichment for small RNAs/ Collection of small RNAs – filtrate:

Two-third volume of absolute ethanol was added to the collected filtrate, then added to a new fibre-glass filter cartridge and centrifuged for 15 seconds at 10,000 g upon which the filtrate was discarded. The filter cartridge was then subjected to 3 washes (Qiagen, GmbH, Hilden, Germany) in which the flow-through was discarded on each occasion. Finally, 100 uL of Elution Solution, pre-heated at 95 C, was added into the filter cartridge and centrifuged at 13000 g for 30 sec [18, 19].

Collection of large RNAs:

The original filter cartridge was subjected to 3 washes in which the flow-through was discarded on each occasion. Finally 100 uL of elution solution (Qiagen, GmbH, Hilden, Germany), was added to the filter cartridge and centrifuged at 13000 g for 30 seconds [18, 19].

MiRNA expression by Real-Time PCR:

The expression of miRNAs (miR-21 & miR-34a) was evaluated by qRT-PCR analysis, according to the manufacturer's directions. The housekeeping miRNA U6 RNA was used as an endogenous control. For RT-PCR, 5 µl of cDNA template was mixed with 12.5 µl of SYBR Green Master Mix (QIAGEN GmbH, Hilden, Germany), and nuclease free water was added to a final volume of 25 µl and dispensed into a 96-well miScript miRNA PCR array plate and enriched with forward and reverse miRNA specific primers. Real-time PCR was performed using an Applied Biosystems 7500 Real-time PCR System (AB, USA) under the following cyclic conditions: 95°C for 15 min, followed by 40 cycles of amplification at 95°C for 5 s and 60°C for 34 s. The data obtained from the miRNA expression levels were calculated and evaluated by the cycle threshold (Ct) method. The level of miRNA expression was reported as Ct value [20, 21]. Ct was calculated by subtracting the Ct of U6 RNA from the Ct of the miRNA of interest. The Ct was calculated by

subtracting the Ct of the reference sample (normal breast) from the Ct of each sample. Fold change was generated using the equation $2^{-\Delta\Delta Ct}$. A pool of 3 normal whole blood samples was used as reference sample for the Ct. The MicroRNA Assays for U6 RNA (RNU6; Applied Biosystems) was used to normalize the relative abundance of miRNA expression [21].

Real time PCR for gene expression:

Real time PCR for gene expression was performed on (Applied Biosystems 7500) to detect expression of BRCA1, BRCA2, p53, and Bcl-2 genes. All reactions were run in triplicate and included no template and no reverse transcription controls for each gene. Cyclic conditions consisted of 15 s at 95˚C then 40 cycles of 5 sec at 95 ˚C and 1 min at 60˚C.

BRCA1, BRCA2, p53, and Bcl-2 mRNAs expression levels was measured according to Ct method for relative quantitation of gene expression. The Taqman mRNA Assays for GAPDH was used to normalize the relative abundance of BRCA1, BRCA2, p53, and Bcl-2 mRNAs [22].

Statistical analysis

Data was analyzed using IBM SPSS advanced statistics version 22 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between more than two groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc "Schefe test" was used for pair-wise comparison based on Kruskal-Wallis distribution. Spearman-rho method was used to test correlation between numerical variables. Wilcoxon-signed ranks test (non-parametric paired t-test) was used to compare two consecutive measures of numerical variables. All tests were two-tailed. A p-value < 0.05 was considered significant.

3. Results

This study included 179 BC female patients and 58 healthy controls. Both patients and controls were matched for age; median age 52 (28.5- 75) & 53.5 (32-71), respectively.

Characteristics of the patients:

All BC cases had a confirmed pathological diagnosis (31 carcinoma in situ and 148 invasive carcinoma). Table 1 shows patients characteristics.

Median miR-21 was significantly upregulated at time of diagnosis with 5.1 fold change in patients vs. controls (p<0.001). Whereas, median miR-34a showed significant downregulation of -5.63 fold change in patients vs. controls (p<0.001).

Median miR-34a, BRAC1, BRAC2 & p53 fold expression were significantly higher in ER+ve, PR+ve & HER-2 +ve patients vs. ER-ve, PR-ve & HER-2-ve patients pre and post chemotherapy (p <0.001). Median miR-21 & BCL-2 fold expression were significantly lower in ER+ve, PR +ve & HER-2 +ve patients vs. ER-ve, PR-ve & HER-2 -ve patients, respectively pre and post chemotherapy (p <0.001) (Table 2).

Median miR-34a, BRAC1, BRAC2, p53 fold expression pre and post chemotherapy were significantly lower in basal like vs. luminal A & B and HER2/neu subtypes. (p <0.001) whereas median miR21 and BCL2 fold expression pre and post chemotherapy were significantly higher in basal like vs. luminal A & B and HER2/neu subtypes (p <0.001) (Table 3).

Median miR-34a, BRAC1, BRAC2, p53 fold expression pre and post chemotherapy were significantly lower in stage III vs. I/II (p <0.001) whereas median mir21 and bcl2 fold expression pre and post chemotherapy were significantly higher in stage III vs. I/II (p <0.001). From all biological markers studied, only median BRAC1 fold expression pre chemotherapy was significantly higher in stage I vs. stage II (Table 4).

Table (1): Clinicopathological Characteristics of the patients

Characteristics		**Number**	**%**
ER	**Positive**	112	63
	Negative	67	37
PR	**Positive**	106	59
	Negative	73	41
Her2 neu	**Positive**	63	35
	Negative	116	65
Subtypes	**Luminal A**	66	37
	Luminal B	47	26
	Basal Like	50	26
	Her2/neu	16	9
T	**T1**	12	7
	T2	136	76
	T3	31	17
N	**0**	12	7
	1	135	75
	2	18	10
	3	14	8
M	**0**	179	100
	1	0	0
Clinical Stage	**I**	12	7
	II	135	75
	III	32	18

Difference in fold change expression post chemotherapy:

Median miR34a, miR21, BRAC1, BRACA2, P53 and BCL2 difference in fold change expression after therapy (post- pre) were 5.48 (-0.72: 9.38), 5.79 (0.77: 10.5), -5.88 (-8.51: 0.30), 6.25 (-0.77: 8.94), 7.55 (-0.34: 10.14) and -8.47 (-11.19: 1.56), respectively. MiR34a difference in fold change expression was directly correlated with BRAC1, BRAC2 & p53 and inversely correlated with miR21 and Bcl2 (Table 5).

Table (2): Median Markers Expression in ER, PR, HER2/neu in 179 BC patients pre and post chemotherapy

Table (2a): Median miR-34a, BRACA1, BRCA2 and P53 expression

			miR-34		**BRACA1**		**BRCA2**		**P53**	
			Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
ER	**Positive N=112**	Median	-14.93	-8.28	-16.56	-10.85	-13.18	-7.11	-13.36	-5.21
		Range	-16.56 to -13.83	-14.83 to -7.06	-19.97 to -14.32	-18.64 to -9.25	-16.34 to -12.04	-14.42 to -5.82	-17.63 to -11.71	-13.55 to -4.11
	Negative N=67	Median	-16.0	-13.18	-19.7-	-18.38	-16.56	-14.32	-16.34	-13
		Range	-17.51 to -13.64	-15.67 to -7.01	-21.86 to -15.14	-21.26 to -9.51	-18.38 to -12.55	-16.45 to -6.54	-19.16 to -11.71	-16.34 to -4.17
PR	**Positive N=106**	Median	-14.93	-8.28	-16.51	-10.85	-13.18	-7.11	-13.36	-5.19
		Range	-2.73	-14.83 to -7.06	-19.97 to -14.32	-18.64 to -9.25	-16.34 to -12.04	-14.42 to -5.82	-17.63 to -11.71	-13.55 to -4.11
	Negative N=73	Median	-15.78	-13	-19.43	-18.25	-16.34	-14.22	-16.22	-12.82
		Range	-17.51 to -13.64	-15.67 to -7.01	-21.86 to -15.14	-21.26 to -9.51	-5.83	-16.45 to -6.54	-19.16 to -11.71	-16.34 to -4.17
HER2-neu	**Positive N=63**	Median	-14.93	-8.28	-16.56	-10.85	-13.27	-7.11	-13.36	-5.1
		Range	-16.45 to -13.74	-14.83 to -7.01	-19.97 to -15.14	-18.64 to -9.32	-16.34 to -12.04	-14.42 to -5.82	-17.63 to -11.71	-13.55 to -4.26
	Negative N=116	Median	-15.45	-9.06	-18.38	-11.55	-13.69	-7.36	-14.12	-6.11
		Range	-17.51 to -13.64	-15.67 to -7.26	-21.86 to -14.32	-21.26 to -9.25	-18.38 to -12.04	-16.45 to -5.98	-19.16 to -11.71	-16.34 to -4.11

Table (2b): Median miR-21 and Bcl2 Expression

			miR-21		**Bcl2**	
			Pre	**Post**	**Pre**	**Post**
ER	**Positive N=112**	Median	17.39	11	14.67	5.94
		Range	(15.35 - 22.63)	(9 - 19.29)	(13.09 - 20.25)	(5.13 - 9.06)
	Negative N=67	Median	20.53	19.03	18.13	14.62
		Range	(15.67 - 23.59)	9.00 - 22.16	13.55 - 21.11	5.21 - 17.88
PR	**Positive N=106**	Median	17.39	11	14.62	5.94
		Range	15.35 - 22.63	9.0 - 19.29	13.9 - 20.25	5.13 - 9.6
	Negative N=73	Median	20.53	18.9	18	14.52
		Range	15.67 - 23.59	9.00 - 22.16	13.55 - 21.11	5.21 - 17.88
HER2-neu	**Positive N=63**	Median	17.39	11	14.83	5.86
		Range	15.56 - 22.63	9.0 - 19.29	13.36 - 20.25	5.13 - 9.06
	Negative N=116	Median	18.19	12	15.3	6.36
		Range	15.35 - 23.59	9 - 22.16	13.09 - 21.11	5.17 - 17.88

Table (3): Median Markers Expression in different pathological subtypes and clinical stages in 179 BC patients' pre and post chemotherapy

Table (3a): Median miR-34, BRACA1, BRCA2 and P53 expression

			miR-34		BRCA1		BRACA2		P53	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post
Subtypes	Luminal A (N=66)	Median	-14.93	-8.4	-16.45	-10.93	-13.22	-7.06	-13.41	-5.21
		Range	-16.56 to -14.03	-11.08 to -7.26	-18.77 to -14.32	-12.73 to -9.25	-14.22 to -12.04	-8.17 to -5.98	-15.14 to -11.71	-7.31 to -4.11
	Luminal B (N=47)	Median	-14.93	-8.17	-17.15	-10.85	-13	-7.11	-13.36	-5.21
		Range	-16.34 to -13.83	-14.83 to -7.06	-19.97 to -15.35	-18.64 to -9.32	-16.34 to -12.04	-14.42 to -5.82	-17.63 to -11.71	-13.55 to -4.26
	HER2/neu (N=16)	Median	-15.3	-8.31	-16.28	-10.74	-13.55	-7.14	-13.22	-5.01
		Range	-16.45 to -13.74	-10.93 to -7.01	-18.64 to -15.14	-12.73 to -9.85	-14.22 to -12.55	-7.52 to 6.54	-15.03 to -11.71	-6.96 to -4.41
	Basal like (N=50)	Median	-16.22	-13.64	-20.46	-18.77	-17.15	-14.62	-16.8	-13.32
		Range	-17.51 to -13.64	-15.67 to -7.31	-21.86 to -16.22	-21.26 to -9.51	-18.38 to -13.36	-16.45 to -7.01	-19.16 to -11.71	-16.34 to -4.17
Clinical Stages	Stage I (N=12)	Median	-15.09	-8.06	-15.56	-10.74	-13.36	-7.09	-13.88	-4.92
		Range	-16.00 to -13.83	-9.00 to -7.01	-15.78 to -14.32	-11.55 to -9.32	-14.22 to -12.04	-7.36 to -6.63	-15.03 to -12.21	-6.36 to -4.41
	Stage II (N=135)	Median	-15.03	-8.4	-17.75	-10.93	-13.27	-7.16	-13.55	-5.31
		Range	-17.15 to -13.64	-15.67 to -7.16	-20.53 to -15.35	-21.26 to -9.25	-18.38 to -12.04	-15.67 to -5.82	-19.16 to -11.71	-16.00 to -4.11
	Stage III (N=32)	Median	-16	-13.64	-20.98	-19.16	-17.15	-14.42	-16.91	-13.09
		Range	-17.51 to -14.42	-15.03 to -7.89	-21.86 to -19.7	-21.26 to -17.88	-18.13 to -15.78	-16.45 to -7.01	-18.51 to -11.71	-16.34 to -4.17

Table (3b): Median miR-21 and Bcl2 expression

			miR-21		BCL2	
			Pre	Post	Pre	Post
Subtypes	Luminal A (N=66)	Median	17.21	11.04	14.62	5.94
		Range	15.35 – 19.03	9.0 – 13.0	13.09 – 16.11	5.17 – 6.68
	Luminal B (N=47)	Median	17.51	10.85	14.93	5.86
		Range	15.56 – 22.63	9.00 – 19.29	13.36 – 20.25	5.13 – 9.06
	HER2/neu (N=16)	Median	17.09	11.16	14.52	5.88
		Range	15.78 – 18.77	9.25 – 12.55	13.55 – 16.11	5.21 – 6.92
	Basal like (N=50)	Median	21.04	19.7	18.83	15.35
		Range	15.67 – 23.59	9 – 22.16	13.93 – 21.11	5.21 – 17.88
Clinical Stages	Stage I (N=12)	Median	17.39	11	14.57	5.94
		Range	16.11 – 18.64	9.25 – 12.04	13.93 – 15.89	5.21 – 6.36
	Stage II (N=135)	Median	17.51	11.31	14.83	5.98
		Range	15.35 – 22.63	9.00 – 22.16	13.09 – 20.97	5.13 – 17.15
	Stage III (N=32)	Median	20.97	19.7	18.7	15.35
		Range	15.67 – 23.59	9.00 – 21.86	13.93 – 21.11	5.21 – 17.88

Table (4): Correlation between gene fold expression of miR 34a, miR21, BRAC1, BRAC2, p53 and Bcl2 (A) pre-chemotherapy (B) post-chemotherapy

(A) Pre-chemotherapy

	BRAC1	BRAC2	miR-21	miR-34a	P53
BRACA2 r	0.571				
miR-21 r	-0.469	-0.538			
miR34 r	0.432	0.434	-0.489		
P53 r	0.487	0.424	-0.549	0.536	
BCL2 r	-0.439	-0.509	0.930	-0.482	-0.530

(P <0.001)

B) Post-chemotherapy

	BRAC1	BRAC2	miR-21	miR-34a	P53
BRACA2 r	0.608				
miR-21 r	-0.544	-0.512			
miR34 r	0.643	0.514	-0.55		
P53 r	0.547	0.467	-0.536	0.639	
BCL2 r	-0.542	-0.539	0.9	-504	-0.462

(P <0.001)

Table (5): Correlation between difference in fold change expression of BRAC1, BRAC2, miR21, miR34, p53 and BCL2 (post-pre)

	BRAC1	BRAC2	miR-21	miR-34a	P53
BRACA2 r	0.535				
miR-21 r	-0.463	-0.47			
miR34 r	0.556	0.512	-0.549		
P53 r	0.556	0.483	-0.549	0.609	
BCL2 r	-0.462	-0.444	0.815	-532	-0.509

Median miR-34, BRAC1, BRAC2 & p53 difference in fold change expression were significantly higher in ER+ve, PR+ve & HER-2 +ve patients vs. ER-ve, PR-ve & HER-2-ve patients (p<0.001). Median miR-21 & BCL-2 difference in fold change expression were significantly lower in ER+ve, PR +ve & HER-2 +ve patients vs. ER-ve, PR-ve & HER-2 -ve patients (p<0.001).

Median miR-34, BRAC1, BRAC2 & p53 difference in fold change expression were significantly lower in basal like vs. luminal A & B and HER2/neu subtypes. (p=<0.001) whereas median mir21 and bcl2 difference in fold change expression were significantly higher in basal like vs. luminal A & B and HER2/neu subtypes (p<0.001).

Median miR-34, BRAC1, BRAC2 & p53 difference in fold change expression were significantly lower in stage III vs I/II (p=<0.001) whereas median mir21 and bcl2 difference in fold change expression were significantly higher in stage III vs. I/II (p<0.001). (Table 6).

A statistically significant direct correlation was found between age of the patients and miR34a, miR21, BRAC1, BRAC2, and p53 gene expression fold, whereas an inverse correlation was found between age and miR21 and bcl2 pre, post chemotherapy and fold change (Table 6).

Table (6): Difference in median markers fold change expression (post-pre) in relation to ER, PR, Her2, subtypes and Clinical stages

			mirR-34	BRACA1	BRACA2	P53	miR-21	BCL2
ER	**Positive N=112**	Median	6.63	6.07	6.02	7.88	-6.28	-8.79
		Range	1.51 – 8.94	1.34 – 9.38	1.92 – 7.58	4.08 – 10.14	-8.51 to -3.32	-11.19 to - 6.77
	Negative N=67	Median	3.08	1.89	2.7	4.32	-2.19	-4.08
		Range	0.77 – 8.54	-0.72 – 8.25	0.77– 10.5	-0.34 – 9.62	-7.66 to -0.3	-9.52 to -1.56
PR	**Positive N=106**	Median	6.63	6.07	6.03	7.89	-6.26	-8.79
		Range	1.51– 8.94	1.34 – 9.38	1.92– 7.58	4.08 – 10.14	-8.51 to -3.32	-11.19 to -6.77
	Negative N=73	Median	3.27	2.11	2.93	4.48	-2.57	-4.2
		Range	0.77 – 8.54	-0.72 – 8.25	0.77 – 10.5	-0.34 – 9.65	-7.66 to -0.3	-9.52 to -1.56
HER2-neu	**Positive N=63**	Median	6.57	6.08	6.04	7.93	-6.21	-8.84
		Range	1.51– 8.94	1.34 – 8.04	1.92 – 7.45	4.08 – 9.82	-8.51 to -3.33	-11.19 to -7.42
	Negative N=116	Median	6.07	4.81	5.55	7.27	-5.45	-8.25
		Range	0.77 – 8.72	-0.72to9.38	0.77 – 10.5	-0.34 to 10.14	-8.51 to 0.3	-10.42 to -1.56
Subtypes	**Luminal A (N=66)**	Median	6.63	6.04	6.13	7.87	-6.28	-8.72
		Range	3.96 – 8.72	3.47 – 9.38	4.68 – 7.58	5.56 – 10.14	-8.51 to -3.32	-10.42 to 6.77
	Luminal B (N=47)	Median	6.46	6.24	6	7.93	-6.26	-9.2
		Range	1.51 – 8.94	1.34 – 8.04	1.92 – 7.45	4.08 – 9.82	-8.51 to -3.33	-11.19 to -7.42
	HER2/neu (N=16)	Median	2.76	1.42	2.34	3.62	-1.5	-3.65
		Range	0.77 – 7.41	-0.72 – 8.25	0.77 – 10.5	-0.34 to -8.48	-7.48 to 0.3	-9.37 to -1.56
	Basal like (N=50)	Median	7	5.8	6.43	8.15	-6.06	-8.73
		Range	4.85 – 8.54	4.51 – 7.53	5.32 – 7.06	6.68 – 9.62	-7.66 to -4.54	-9.52 to -7.79
Clinical Stages	**Stage I (N=12)**	Median	6.91	4.72	6.24	8.59	-6.25	-8.82
		Range	5.93 – 8.94	3.47 – 6.24	4.88 – 7.34	7.16-10.11	-8.51 to -5.11	-10.47 to -7.79
	Stage II (N=135)	Median	6.45	6.04	5.95	7.68	-6.03	-8.63
		Range	0.77 – 8.72	-0.72 – 9.38	0.77 – 7.58	1.99 – 10.14	-8.51 to 0.14	-11.19 to -2.75
	Stage III (N=32)	Median	2.81	1.74	2.43	3.94	-1.59	-3.49
		Range	0.97 - 7.04	-0.28 to 33	1.06 – 10.5	-0.34 to 8.48	-7.48 to 0.3	-9.3 to - 1.56

($p<0.001$)

Table (6): Correlation between biological markers and age pre, post chemotherapy & difference in fold change expression (post-pre)

		r
BRAC1	Pre	0.585
	Post	0.606
	Difference change	0.543
BRACA2	Pre	0.544
	Post	0.556
	Difference Change	0.515
miR-21	Pre	-0.438
	Post	**-0.456**
	Difference Change	0.461
miR-34a	Pre	0.314
	Post	0.434
	Difference Change	0.485
P53	Pre	0.416
	Post	0.562
	Difference Change	0.558
Bcl2	**Pre**	-0.405
	Post	-0.429
	Difference Change	-0.478

4. Discussion

Evidence proved that several miRNAs exhibit dysregulated expression in BC. In this study, we investigated the relative expression of circulating miR-21 and miR-34a in the serum of 179 BC female patients.

Our study showed a significantly higher relative expression of miR-21 and a remarkably lower relative expression of miR-34a in BC patients compared to normal females. The study of Iorio et al was the first to report miR-21 upregulation in the serum of BC patients in relation to healthy controls [13]. A variation in miR34a expression level in different tumor types has been reported. In consistent with our results Bommer et al and Javeri et al reported that miR-34a was downregulated in different cancer types including GIT tumors, BC and neuroblastomas. [14, 23] On the contrary, Roth et al showed an overexpression of miR-34a in BC patients; we could not find an explanation for this contradiction [24].

We demonstrated in our study that miR34a expression was significantly higher in patients with positive ER, PR and HER-2 and lower in triple negative (TN) patients. Also, Brian et al demonstrated that miR-34a levels were lower in TNBC cell lines [25]. In our study, we found an upregulation of miR21 expression in TNBC patients. Similarly to our results, Hong Fang et al have found that miR-21 was upregulated in TN patients [26].

In this study, miR-21 was inversely correlated with BRAC1, BRAC2 and p53 expression. On the contrary, its expression was significantly correlated with BCL2. However, this correlation was not reported by others.

On the other hand, miR34a expression showed a direct correlation with the tumor suppressor genes BRAC1, BRAC2 and p53 while it showed an inverse correlation with BCL2. Li L. et al stated similar results and concluded that miR-34a is able to inhibit BC cell proliferation and migration through downregulation of Bcl-2 [27].

We found that miR34a was significantly downregulated in stage III versus I and II and in basal cell subtype versus luminal A & B. Our results were in agreement with Peurala and colleagues [28]. We found that miR-21 was upregulated in advanced clinical stages and lymph node metastasis. This finding was also in agreement with Li Xu et al [29].

Our study demonstrated an increased expression of miR-34a post chemotherapy; similar results were reported by Pierre et al [30]. Supporting our results, Gong et al reported a significant downregulation of miR-21 after chemotherapy which showed that it acts as an onco miRNA in BC [31].

Our results support that miR-34a acts as a tumor suppressor miRNA in BC and that it possesses an anti-tumor effect while it suggests that miR21 acts as an onco-miRNa.

In conclusion, miR-21 and miR-34a, as minimally invasive molecular markers, show altered circulating levels in BC patients compared to healthy controls and a remarkable correlation with other biological markers. However, future studies are recommended to confirm and further delineate the potential role of miR-21 and miR-34a as novel diagnostic and prognostic biomarkers in BC.

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