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Analysis of miRNA-21 Relative Expression in Breast Cancer Patients

[1]Nehal M. El-Mashad, [2]El-Hassan M. Mokhamer, [2,3]Abdel-Aziz A. Zidan,
[2]Mohammed Elgerbed, [4,5]Enas A.Abdul-Baki and [2]Gehad M. Elfarash

[1]Department of Oncology, Faculty of Medicine, Tanta University, Elgharbia, Egypt.
[2]Department of Zoology, Faculty of Science, Damanhour University, Elbehira, Egypt.
[3]Center of Excellence in Cancer Research, Tanta University, Global Educational Hospital, Tanta, Egypt.
[4]Genomic Signature Cancer Center, Tanta University Global Educational Hospital, Tanta, Egypt.
[5]Department of Biology, Faculty of Science, Hafr-Al Batin University, KSA.

Abstract: miRNAs constitute a large family of small, approximately 20–22 nucleotides, non-coding RNAs that regulate the expression of target genes, mainly at the post-transcriptional level. Accumulating lines of evidence have indicated that miRNAs play important roles in the maintenance of biological homeostasis and that aberrant expression levels of miRNAs are associated with the onset of many diseases, including cancer. miR-21 plays a crucial role in a plethora of biological functions and diseases including development, inflammation and cancer, diseases. Aim of the study is to understand the prognostic significance of miR-21 relative to expression in breast cancer patients and correlate their expression with the role of T-cells lymphocytes. The relative expression of microRNAs profiling in 25 patients with breast cancer before the treatment and 5 healthy controls were determined by using real-time PCR. We found that serum levels of miR-21 were non-significantly higher in breast cancer patients before treatment than in healthy control. Our data showed that there was no significant difference in the age and menstrual history between the breast cancer patients before treatment and the healthy control. Red blood cells, hemoglobin, Platelets and White blood cells (Neutrophils, lymphocytes, monocytes, eosinophils and basophils) showed no significant difference in breast cancer patients and in the healthy control, as well as ALT, AST and urea. Bilirubin showed a significant difference between breast cancer patients and the healthy control. Conclusion: analysis ofmiR-21 expression in breast cancer patients has no significant difference between the breast cancer patients before treatment and healthy control in all clinical parameters.

Key words: Breast Cancer · T-cells lymphocytes · miRNAs profile · miRNA-21 · Real-time PCR

INTRODUCTION

Cancer has long been the gravest challenge to human health, which not only reduces the quality of life, but also increases mortality. Breast cancer is a leading cause of cancer death among women in industrialized countries, despite advances in early detection and treatment. The estimate of cancer incidence was 113.1/100, 000 of total population in 2012 and 114.98/100, 000 of total population in 2013. Projections to 2050 estimate the incidence of cancer in Egypt to be 341.169/100, 000 of total population [1]. To develop more effective treatments, it is essential to understand the molecular mechanisms involved in breast tumor development and the acquisition of malignancy. In addition, translational research, which is based on basic cancer research, is required to overcome intractable cancers, such as therapy-resistant and metastatic cancers. MicroRNAs (miRNAs) are a class of 21-25 nucleotide (nt) single-stranded non-coding RNAs [2], which regulate gene expression by base pairing to their target sequences [3]. 60% of human genes are regulated by miRNAs. Previous studies have demonstrated that there are a large number of miRNAs existing in plasma, where they have different expression profiles between breast cancer patients and normal healthy controls. Recent studies have reported that some key miRNAs are frequently dysregulated in breast cancer and contribute to breast cancer development and progression by acting as oncogenes or tumor suppressor genes [4]. Of all the miRNAs known to be associated with

Corresponding Author: El-Hassan M. Mokhamer, Department of Zoology, Faculty of Science, Damanhour University, Elbehira, Egypt. Tel: +201222510555.

breast cancer, miR-21 is among the most commonly up regulated, thus, it currently looks to be among the most promising examples of a miRNA biomarker for both diagnosis and prognosis of breast cancer [5]. Among these potential new diagnostic and therapeutic targets, microRNAs represent a recently uncovered class of small and endogenous non-coding RNAs. MiRNAs play a well conserved and crucial role in normal biological processes, such as cellular differentiation, proliferation and apoptosis through a complicated gene regulation networking [6]. The recent rise of interest in miRNAs is ascribed to the breakthrough of their role in many pathological processes, as well as in malignant transformation [7]. MiRNAs, in fact are implicated in the pathogenesis of different types of cancer, including breast cancer.

Functionally, numerous targets of miR-21 are known and include genes which prevent tumorigenesis, cell invasion and metastasis. The pro-apoptotic protein programmed cell death 4 (PCDC4) is one such target, furnishing miR-21 with a reputation as a potent anti-apoptotic miRNA. Expression of miRNAs is dynamically regulated during activation and differentiation of T-cells [8-10]. Consequently, various effectors T-cell subsets are characterized by distinct miRNA expression profiles [11]. However, the contribution of single miRNAs in the function of individual T-cell subsets is still largely unknown. Several miRNAs are highly expressed in freshly isolated human memory CD4+ T-cells [12, 13]. Amongst these, miR-21 has anti apoptotic properties which have been extensively studied in pathological conditions including cancer, cardiovascular disease and autoimmunity [12, 13]. Indeed, miR-21 was recently shown to suppress apoptosis and induce proliferation of primary murine and human T-cells [14, 15]. In accordance with the pronounced expression in memory T-cells, it has been shown that miR-21 can be induced upon activation of CD2+ Tells [9]. However, the kinetics and degree of miR-21upregulation, as well as the differential functional consequences thereof in naive and memory T-cells remain unknown. In addition to apoptosis-related genes, bioinformatics analysis [16] of putative miR-21 targets relevant for T-cell biology revealed several immune-related genes, including CC chemokine receptor 7 (CCR7), which is substantially expressed on naive T-cells [17, 18]. By binding with its ligands (CCL19 and CCL21) presented on the surface of high endothelial venules, CCR7 enables entry of T-cells into lymph nodes and as such ensures circulation of naive T-cells through the lymphatic system [17, 18]. Late prognosis is one of the most significant reasons for the high mortality rate of breast cancer. Thus, there is a strong need to identify reliable biomarkers in the early detection, precise diagnosis and personalized therapy for breast cancer patients. This study hypothesized that dysfunctional in Cytotoxic T cells may play an important role in the relapse of the patients. As well as, plasma miRNA-21 may be used as a diagnostic biomarker for early-stage of breast cancer.

The aim of this study is to understand the prognostic significance of miRNA-21 relative expression in breast cancer patients before treatment and the healthy control.

MATERIALS AND METHODS

Patients: This study was conducted on patients with breast cancer. Clinical samples were obtained from Patients of breast cancer. All patients were signing an informed consent under a protocol approved by Faculty of Medicine ethical committee review board, Tanta University. This work was carried out on 30 subjects who will be recruited from the outpatient clinic of Oncology Department, Tanta University Hospital.

Work Design: Subjects were classified into two groups; first group included 5 healthy volunteer subjects and Second group included 25 breast cancer patients before the treatment.

Demographic Data: Demographic data age, performance status and menstrual history of the breast cancer patients before treatment and the healthy control were carried out of all subjects form outpatient clinic of Oncology Department, Tanta University Hospital.

Collection of Blood Samples: The blood samples were collected at Oncology unit, Faculty of Medicine, Tanta University. The samples were transferred to the labs of Center of excellence in cancer research, Tanta University Educational Hospital, for further processing, liver and kidney function tests and complete blood count (CBC). The research study was approved by the ethical committee, Faculty of Medicine, Tanta University and informed consent was obtained from all patients before participation.

miRNA Expression Analysis: Total miRNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized from 1 mg of RNA using the Omniscript RT Kit (Qiagen), as per manufacturer's instructions. MiRNA expression was measured using quantitative real-time PCR and TaqMan probes (Applied Bio systems, Foster City, CA, USA) in a final

reaction volume of 20 µl. Ribosomal 18s RNA was used as the internal standard. RT-PCR was performed on a StepOne real-time PCR system (Applied Bio systems). The relative quantification of the target transcripts normalized to the endogenous control was determined by the comparative Ct method. Relative changes in gene expression between samples were analyzed using the $2^{-\Delta\Delta Ct}$ method.

TaqMan® Gene Expression Assay: The following TaqMan probes were used in this study: 18S (Cat# Mm03928990_g1) and mir-21 (Cat# PN4427975). All the probes were obtained from Applied Bio systems (Foster City, CA, USA) and they were used at concentrations recommended by their manufacturers.

Statistical Analysis of Data: Statistical analysis was performed using the Student's t-test. Log-rank nonparametric analysis using Graph Pad Prism (Graph Pad Software, Inc.) was used to graph and analyze the survival data. All P values were two sided, with $P \leq 0.05$ considered significant [19, 20]. Cumulative survival was calculated using a Kaplan-Meier curve. The relationship between donor Pmel cells and tumor size was examined by scatter plot analysis and descriptive statistics as well as by fitting a regression model.

RESULTS

Demographic Data of the Breast Cancer Patients Before Treatment and the Healthy Control: Demographic data of the breast cancer patients before treatment and the healthy control are shown in Table 1, the data showed that the age of the breast cancer patients were ranged from (28 to 68) with mean ± SD (48.2 ± 13.5) and the age of the healthy control were ranged from (46 to 60) with mean ± SD (54.80 ± 5.93). The data showed that there was no significant difference in the age between the breast cancer patients before treatment and the healthy control as shown in Table 1. In addition, there was no significant difference in the menstrual history between the breast cancer patients and the healthy control as shown in Table 1.

A complete blood count (CBC) is initial complete blood picture, these data in Table 2 showed that the range of white blood cells (WBCs) counts, the data was (2.9-14.0 *10^3/µl) with a mean (7.0 ± 2.70) in breast cancer patients before treatment and in the healthy control was (5.5 -7.9*10^3/µl) with a mean (6.68 ± 1.09). Red blood cells (RBCs) range for control and breast cancer patients before treatment was (4.1 ± 0.63) and (4.44 ± 0.25), respectively. Platelets count was(plt) 210.0 ± 15.15 in control to 267.1 ± 125.8 in patients. Neutrophils also showed in control mini-max 1760 – 11696 and in breast cancer patients before treatment mini-max 1920 – 7840 with means ± SD 4735.2 ± 4075.6 and 4346.7 ± 1878, respectively. Other parameters as hemoglobin, lymphocytes (Lymph), Lymph%, monocytes (Mono), Eosinophils (Esino), Esino%, Basophils (Baso) and Baso % showed no significance difference[21] as shown in Table 2.

Liver Function Analysis of the Breast Cancer Patients Before Treatment and the Healthy Control Volunteers: Liver function analysis ALT, AST and bilirubin of patients before treatment and healthy control are shown in Figures 1. The activity of ALT (U/L) showed no significant difference between the breast cancer patients before treatment and the healthy control, which was 34.8 ± 12.5 and 42.0 ± 2.55, respectively, as shown in figure 1 A. AST also showed non significance difference between the breast cancer patients before treatment and the healthy control which was 34.8 ± 12.5 and 34.80 ± 3.27, respectively as shown in figure 1 B. Contrariwise, bilirubin levels (mg/dL)showed a significant difference between breast cancer patients before treatment and the healthy control as shown in Figure 1 C.

Kidney Function Analysis of the Breast Cancer Patients Before Treatment and the Healthy Control Volunteers: Kidney function tests represented in the creatinine and urea levels (mg/dL) of the breast cancer patients before treatment and the healthy control are shown in Figure 2 A and B. Creatinine level (mg/dL) showed no significance between breast cancer patients before treatment and the healthy control, the data were1.01 ± 0.15 and 0.92 ±0.24, respectively as shown in Figure 2 A. The mean urea level revealed no significance in the breast cancer patients before treatment and the healthy control which were 42.5 ±4.5 and 31.2 ±3.8, respectively as shown in Figure 2 B.

Associations between serum miRNA-21 expression and clinicopathological features of the breast cancer patients before treatment and the healthy control volunteers.

Results of the MiR-21 expression analysis of breast cancer patients before treatment showed that the miR-21 expression analysis before treatment was 1.15 ± 0.76 compared to1.19 ± 0.10 in healthy control volunteers as shown in Figure 3. These data showed that the miRNA-21 expression analysis of breast cancer patients before treatment has no significant difference with that of the healthy control.

Table 1: Demographic data of the breast cancer patients before treatment and the healthy control volunteers

	Control(n= 5)		Cases (n= 25)			
Demographic data	No.	%	No.	%	Test of sig.	P
Sex						
Female	5	100.0	25	100.0		
Age						
Mean ± SD.	54.80 ± 5.93	48.2 ± 13.5	t=1.071	0.293		
Performance Status						
0	5	100.0	5	20.0	χ^2=9.061*	^{MC}p = 0.019*
1	0	0.0	8	32.0		
2	0	0.0	3	12.0		
3	0	0.0	8	32.0		
4	0	0.0	1	4.0		
Min. – Max.	0.0 – 0.0		0.0 – 4.0		U=12.50*	0.003*
Mean ± SD.	0.0 ± 0.0		1.68 ± 1.25			
Median	0.0		1.0			
Menstrual History						
Premenopausal	2	40.0	13	52.0	χ^2=0.959	0.707
Postmenopausal	3	60.0	11	44.0		
Perimenopausal	0	0.0	1	4.0		

χ^2: Chi square test, t: Student t-test, P: p value for comparing between the studied groups, *. Statistically significant at p ≤ 0.05.Performance status:0=fully active, 1=Restricted in physically strenuous activity but ambulatory and able to Carry out work of a light or sedentary nature, 2=Ambulatory and Capable of all self-Care but unable to to Carry out any work aCtivites. Up and about 50% of waking hours, 3=Capable of only limited self-Care, Confined to bed or Chair more than 50% of waking hours, 4=completely disabled, Can't Carry on self -Care. Totally Confined to bed or Chair, 5 =Dead.

Table 2: CBC analysis of the breast cancer patients before treatment and the healthy control volunteers

CBC analysis	Control (n=5)	Cases (n=25)	Test of sig.	P
WBCs (10^3/µl)				
Min. – Max	5.50 – 7.90	2.9 – 14.0	U=61.50	0.957
Mean ± SD.	6.68 ± 1.09	7.0 ± 2.70		
Median	6.50	7.0		
RBCs (10^6/µl)				
Mean ± SD.	4.44 ± 0.25	4.1 ± 0.63		
Hg (g/dl)	(n= 10)	(n= 20)		
Mean ± SD.	11.72 ± 0.45	11.2 ± 1.4		
Plt (C.mm) (10^3/µl)				
Mean ± SD.	210.0 ± 15.15	267.1 ± 125.8		
Neut (µl)				
Min. – Max	1760 – 11696	1920 – 7840	U=40.0	0.497
Mean ± SD.	4735.2 ± 4075.6	4346.7 ± 1878		
Median	2680.0	4025.0		
Neut (%)				
Mean ± SD.	60.2 ± 19	55.4 ± 11.9		
Lymph (µl)				
Min. – Max	1080.0 – 1056.0	840.0 – 5980.0	U=39.5	0.201
Mean ± SD.	2031.6 ± 1056	2649 ± 1194.2		
Median	2040.0	2380.0		
Lymph (%)				
Min. – Max	8.0 – 56.0	2.0 – 7.0	U=53.0	0.597
Mean ± SD.	34.4 ± 19.4	4.3 ± 1.4		
Median	30.0	4.0		
Mono (µl)				
Mean ± SD.	3.4 ± 0.5	4.3 ± 1.1		
Esino (µl)				
Min. – Max	80.0 – 272.0	66.0 – 280.0	U=52.0	0.558
Mean ± SD.	143.2 ± 78.8	160.2 ± 66.7		
Median	130.0	150.0		
Esino (%)				
Min. – Max	2.0 – 2.0	1.0 – 6.0	U=60.0	0.871
Mean ± SD.		2 ± 0	2.2 ± 1.1	
Median	2.0	2.0		
Baso (µl)				
Min. – Max	0.0 – 0.0	0.0 – 28.0	U=60.0	0.655
Mean ± SD.	0.0 ± 0.0	1.1 ± 5.6		
Median	0.0	0.0		
Baso (%)				
Mean ± SD.	0.0 ± 0.0	0.4 ± 0.0		

U: Mann Whitney test, t: Student t-test, P: p value for comparing between the studied groups. *: Statistically significant at p ≤ 0.05

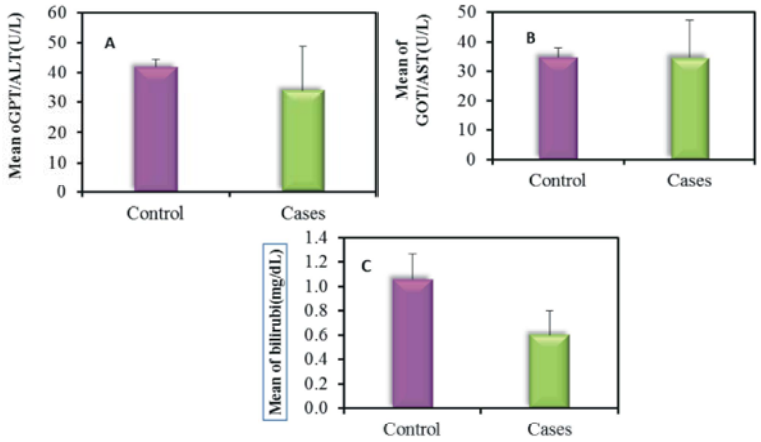

Fig. 1: (A, B, C) Liver function analysis ALT (U/L) (1A), AST (U/L) (1B) and Bilirubin (1C) of the breast cancer patients and the healthy control before treatment volunteers

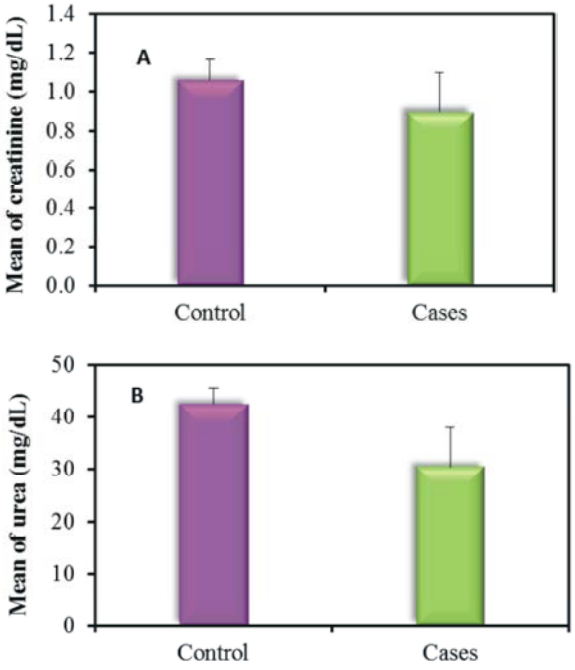

Fig. 2: (A, b) Kidney function analysis of the breast cancer patients before treatment and the healthy control volunteers

Table 3: miRNA-21 Expression analysis of the Breast Cancer patients before treatment and the Healthy control volunteers

miRNA 21	Control (n= 5)	Patients (n= 25)
Min. – Max.	1.09 – 1.33	0.21 – 2.38
Mean ± SD.	1.19 ± 0.10	1.15 ± 0.76
Median	1.16	1.24

DISCUSSION

Despite strong evidence that miRNAs are associated with cancer and are potential biomarkers for outcome, little is known about how they affect the response of a tumor to cytotoxic treatment. To determine whether miRNAs are involved in the cellular response to cytotoxic therapy, we used miRNA microarrays to compare the relative levels of cellular miRNAs breast cancer patients before treatment and the healthy control. In this paper, we obtain a profile of miR-21 expression analysis in breast cancer patients before treatment and the healthy control

volunteers. The cloning method and the qRT-PCR analysis displayed a striking agreement, identifying the miR-21 as differentially expressed between breast cancer patients before treatment and the healthy control.

MiR-21 targets are programmed cell death 4 (Pdcd4) which binds eIF4e to regulate translation, thus acting as a tumor suppressor. There is a relationship between miR-21 and breast cancer, miR-21 is found in breast cancer with high proliferation index, advanced tumor staging, node involvement, aggressive phenotype including pregnancy associated breast cancer [22].

Since miRNAs play important roles in the regulation of tumor initiation and development, modulation of miRNA activity is a promising approach for cancer treatment. Currently, many researchers are attempting to restore the function of tumor suppressor miRNAs using miRNA mimics or expression vectors, or inhibit the function of oncogenic miRNAs using antisense oligonucleotides or expression vectors containing complementary sequences [23].

It was reported that the 20 miRNAs that were most significantly differentially expressed in breast cancer tumors, only seven miRNAs were overexpressed in both tumors and serum and miR-21 was not identified as the most important diagnostic marker [24]. MiR-21 is one of the most frequently studied oncogenic miRNAs (onco-miRNAs). Although a direct correlation between aberrant expression of miR-21 and breast cancer has been previously demonstrated, it is not clear whether suppression of miR-21 alone will affect tumorgenesis [25]. Notably, other study showed that miR-21expression was non-significantly higher in breast cancer tissues of breast cancer before treatment than in normal breast tissues of healthy control volunteers [26].

There is a large amount of evidence indicating that miR-21 is associated with regulation of proliferation and differentiation during development. Also, it is possible to develop treatment strategies by targeting miR-21 [27]. Serum miRNA levels show a number of small differences in females who later develop cancer versus those who remain cancer-free. Microarray analysis of miRNAs in the serum of breast cancer patients has only recently been reported. Aberrantly expressed miR-21 in the blood of breast cancer patients is occasionally reported and expression of miR-21 in plasma samples from breast cancer patients might be useful for breast cancer diagnosis [28]. Our results showed that expression of serum miR-21 has no significance difference in breast cancer before treatment compared to healthy controls volunteer. Although the impact of miR-21 on breast cancer prognosis remains controversial, a few studies have reported promising associations with miR-21 expression levels that could make this a novel potential biomarker for BC prognosis. Serum miR-21 was a more sensitive breast cancer marker than cancer antigen (CA) 15-3 or carcinoembryonic antigen; in particular, miR-21 was a potential tumor marker for the diagnosis of early-stage breast cancer. When breast cancer is suspected, clinicians may check factors such as CA15-3, Ki-67, cytokeratin 8/18/19, BRCA1/2 and ER/HER2, which are still the main diagnostic biomarkers [28]. The BRCA1 and BRCA2 tumor suppressor genes are strong predictors of breast cancer development; mutations in BRCA 1 and 2 are inherited in an autosomal dominant manner and play important roles in breast cancer risk [29]. Reports have indicated that miR-21 functions as an oncogene and modulates tumorigenesis through regulation of genes such as bcl-2; therefore, miR-21 may serve as a novel therapeutic target [30]. In this study, we examined expression of miR-21 in serum from 25 breast cancer patients before treatment and 5 healthy controls. We found that serum levels of miR-21 had no significant increase in breast cancer before treatment patients in comparison to that of healthy control. This finding is in agreement with the study by Mar-Aguilar that found similar levels of miR-21 expression in breast cancer patients and controls [31]. These findings are in agreement with our data which showed that there was no significant difference in the age and menstrual history between the breast cancer patients before treatment and the healthy control. White blood cells (WBCs) counts Red blood cells (RBCs), Platelets, Neutrophils, hemoglobin [32], lymphocytes (Lymph), Lymph%, monocytes (Mono), Eosinophils (Esino), Esino%, Basophils (Baso) and Baso % showed no significant difference at $p \leq 0.05$ in breast cancer patients before treatment and in the healthy control. The activity of ALT and AST (U/L) showed no significant difference at $p \leq 0.05$ between the breast cancer patients before treatment and the healthy control. Serum bilirubin levels (mg/dL) showed a significant difference at $p \leq 0.05$ between breast cancer patients before treatment and the healthy control. Urea level was no significant at $p \leq 0.05$ of the breast cancer patients before treatment and the healthy control. These data showed that the miRNA 21 expression analysis of breast cancer patients has no significant difference between the breast cancer patients before treatment and all clinical parameters; Also There was no significant difference between the Healthy control and all clinical parameters.

Though new technologies are emerging to improve the specificity, stability and efficiency of miRNA delivery and therapy, the final outcomes of this strategy remain uncertain [33]. Currently, clinicians do not have an effective molecular biology marker for breast cancer. Expression profiles of circulating miRNAs may yield promising biomarkers for diagnosis and assessment of the prognosis of cancer patients. Sensitive techniques allow the expression levels of many miRNAs to be determined and this information can be used for diagnostic purposes[34]. The mechanism underlying miRNA-21 stability is still being investigated for BC detection [35].

The utility of miRNA profiles as potential diagnostic or prognostic markers for breast cancer has been gaining interest [36].Our study had some limitations, including small sample size and a limited ability to generalize our results since all our patients were Egyptian females. Despite these limitations, our study provided initial data about the regulation of miR-21 in breast cancer patients and suggested that miRNA found similar levels of miR-21 expression in breast cancer patients before treatments and healthy control. Serum miR-21 may have clinical utility for monitoring and follow-up of breast cancer patients with metastasis. Clearly, our results should be further validated by a prospective study in a multicenter clinical trial. The ideas in this study should be further explored by studies with larger sample size.

Recent studies have suggested that miRNAs are involved in tumor initiation through the regulation of cancer stem cell (CSC) properties, including self-renewal ability, tumorigenicity and drug resistance In breast cancer, a number of miRNAs have been identified as tumor suppressors or oncogenes and have been characterized as critical regulators of tumor initiation, metastasis and chemoresistance[37].

CONCLUSIONS

In conclusion, we found that serum miR-21 has no significant difference between breast cancer patients before treatment when compared with the healthy control volunteers. That means miR-21 can't serve as a diagnostic marker between breast cancer patients before treatment and the healthy control volunteers.

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