Global Journal of Molecular Sciences 14 (1): 01-09, 2019
ISSN 1990-9241

DOI: 10.5829/idosi.gjms.2019.01.09

Analysis of Mir-22 Relative Expression in Breast Cancer Patients

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Abstract: Micrornas (miRs), which are a class of small non-coding RNAs, are key regulators of gene expression via induction of translational repression or mRNA degradation. MicroRNA-22 (miR-22) is a highly-conserved 22-nt miRNA, whose roles in human diseases and normal physiology are just beginning to emerge. Recently, miR-22 has been connected to a great number of activities that encompass tumorigenesis. Aberrant expression of miR-22 has been identified in multiple human diseases including cancer. The aim of the study is to evaluate the prognostic value of miR-22 relative to expression in breast cancer patients and correlate their expression with demographic and clinical parameters of the breast cancer patients before treatment and the healthy control. This study was conducted on 20 patients with breast cancer before treatment and 10 healthy controls using quantitative real time PCR for detection of miRNA-22 expression. Our data showed that there was no significant difference in serum level of miR-22 between the breast cancer patients before treatment and the healthy control. Also there was no significant difference between ALT, AST and Urea in Breast Cancer patients before treatment and the healthy control. Serum bilirubin showed a significant difference between breast cancer patients and the healthy control. Conclusion: miRNA-22 cannot use as a diagnostic biomarker for early-stage of Breast cancer patients.

Key words: Micrornas · Cancer · Apoptosis · Tumor Suppressor · Diagnostic Biomarker

INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy universally, arranging second in cancer-related mortality in women [1]. Among females, the pattern in Lower, Middle and Upper Egypt was dominated by the high rate of breast cancer (33.8%, 26.8% and 38.7% respectively) [2]. During the recent decades, although the death rate of breast cancer has decreased by more than 30% due to the early diagnosis, the prognosis of breast cancer patients at late stage still remains poor [3]. Therefore, it is urgently needed to explore the molecular mechanism underlying its malignant progression, which may help develop effective strategies for breast cancer treatment [4]. Breast cancer is clinically, morphologically and genetically a heterogeneous disease, response to therapy, side effects and the outcome depends on the heterogeneous nature of the disease.

Prognostic and predictive biomarkers must be used to decrease mortality rate and reduce possible side effect. Such potential biomarkers miRNAs are non-coding single-stranded RNAs with a length of approximately 22 nucleotides and they function as key post-transcriptional regulators of eukaryotic gene expression through suppressing translation or targeting mRNAs for degradation [5]. It has been widely established that miRs play important roles in various biological processes, such as cell proliferation, differentiation, apoptosis, migration, angiogenesis, as well as tumorigenesis [6, 7]. Therefore, understanding of the regulatory mechanism of miRs in

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human cancers is beneficial for finding promising therapeutic targets. In recent decade, many miRs have been found to have promoting or suppressive effects on breast cancer, such as miR-33b [8] miR-148a [9] miR-181b [10] miR-200b [11] and miR-492 [12]. Among these miRs, miR-22 has been reported to act as an oncogene or tumor suppressor [13-15]. For instance, miR-22 promotes HBV-related hepatocellular carcinoma development in males, while suppresses lung cancer cell progression through directly targeting ErbB3 [13, 14]. Recently, over expression of miR-22 was found to compromise estrogen signaling by causing a reduction of ER alpha levels, at least in part by inducing mRNA degradation and thus it might have an inhibitory impact on the ER alpha-dependent proliferation of breast cancer cells [16]. Indeed, miR-22 was reported to be down regulated in ER alpha-positive breast cancer tissues and cell lines [17]. Furthermore, miR-22 is a promising prognostic biomarker for breast cancer and ectopic expression of miR-22 inhibits the proliferation and invasion of breast cancer cells by targeting GLUT1[18]. However, whether other targets of miR-22 exist in breast cancer still needs to be studied. Accumulating evidence explains that the putative functions of miRNAs might have important clinical significance. For example, they might be considered as tumor suppressors and/or promoters [19] and their abnormal expression is highly associated with the progression and pathology of breast cancer [20] supporting their diagnostic, prognostic and therapeutic potentials in breast cancer [21].

Cell-cycle arrest is induced by the activation of the cyclin-dependent kinase inhibitor CDKN1A (hereafter referred to as p21), whereas apoptosis is induced by the activation of pro-apoptotic genes, including NOXA [22] PUMA [23] and BAX [24] that encode the organize of intrinsic apoptosis pathways. MiR-22 suppresses p21 expression through the inhibition of protein synthesis and promotion p21 mRNA degradation. Under severe damage conditions, apoptosis may be induced by entry into the cell cycle via direct suppression of p21 by miR-22.

Anti-apoptotic function of p21 has recently attracted attention for its oncogenic action, which is opposed to a traditional tumor suppressor function. Disruption of the p21 gene sensitized cancer cells to apoptosis after treatment with chemotherapeutic agents [25]. Numerous studies have revealed that miR-22 functions as either a tumor suppressor miRNA or an Onco-miRNA to inhibit or promote tumor formation and malignant transformation from genetic to post-transcription level via intricate mechanisms, in which miR-22 could stimulate or turn off different cascades of events concerning pathways. miR-22 may serve as a hopeful therapeutic target for precision treatments in diverse cancers to inhibit proliferation, migration, invasion and metastasis, thus weakening or reversing chemo resistance to anticancer drugs [26].

Since, several miRNA have been shown to be dysregulated in breast cancer tissues when compared with normal tissues. The hypothesis of this study was that miRNA modulation during breast cancer cell death may aid in better understanding of the underlying mechanisms that have a critical role in breast cancer.

MATERIALS AND METHODS

Patients: This study was conducted on patients with breast cancer compared to healthy volunteer subjects. Clinical samples were obtained from patients of breast cancer. All patients were given an informed consent under a protocol approved by Faculty of Medicine Ethical Committee Review Board, Tanta University.

Clinical Sample: This work was carried out on 30 subjects who were recruited from the outpatient clinic of Oncology Department, Tanta University Hospital. They were classified into two groups. Group one included 10 healthy volunteer subjects and group two included 20 breast Cancer patients before the treatment.

Demographic Data: Demographic data included age, performance status and menstrual history of the breast cancer patients before treatment and the healthy control.

Exclusion Criteria for Breast Cancer Group: Patients diagnosed with other type of solid or hematological malignancies, metastatic patients and HCV patients.

Inclusion Criteria for Breast Cancer Groups: Patients diagnosed with Breast cancer only.

Collection of Blood Samples: 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 for liver and kidney functions and 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 expression measured using quantitative real-time PCR and TaqMan probes (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 20 ml. Ribosomal 18s RNA was used as the internal standard. RT-PCR was performed on a Step One real-time PCR system (Applied Biosystems). 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-ddCt method.

TaqMan® Gene Expression Assay: The following TaqMan probes were used in this study: 18S (Cat# Mm03928990_g1) and mir-22 (Cat# PN 4427975 Mm01324120_m1). All the probes were obtained from Applied Biosystems (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 [27, 28]. 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 for Breast Cancer Patients Before Treatment and the Healthy Control Volunteers: The data showed that there was no significant difference in the (age, menstrual history and performance status) between the recruited breast cancer patients before treatment and the healthy control volunteers according to Chi square test and Student t-test (t = 1.071 and *P* value = 0.293) as showed in Table 1.

Complete Blood Count (CBC) Analysis of the Breast Cancer Patients Before Treatment and the Healthy Control Volunteer: No significant difference was detected between breast cancer patients before treatment when compared to the healthy control as regards to a complete blood count (CBC) analysis as shown in Table2.

Liver Function Investigations of the Breast Cancer Patients Before Treatment and the Healthy Control: Liver function analysis showed that there were no significant difference between the breast cancer patients before treatment and the healthy control volunteers in

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

	Control (n= 10)		Cases (n= 20)			
Demographic data	No.	%	No.	%	Test of sig.	P
Sex						
Female	10	100.0	20	100.0	-	-
Age						
Mean ± SD.	54.80±5.93		48.2±13.5		t=1.071	0.293
Performance Status						
0	10	100.0	5	20.0	?'?????? *	Mc p = 0.019 *
1	0	0.0	5	32.0		
2	0	0.0	3	12.0		
3	0	0.0	6	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.	0.25±0.1		1.68±1.25			
Median	0.0		1.0			
Menstrual History						
Premenopausal	4	40.0	9	52.0	?'?0.959	0.707
Postmenopausal	6	60.0	10	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

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

Analysis	Control	Cases	Test of sig.	P
WBCs (10^3/il)	(n= 10)	(n= 20)		
Mean ± SD.	6.68 ± 1.09	7.0 ± 2.70	U=61.50	0.957
RBC (10^6/il)	(n= 10)	(n= 20)	t=1.287	0.209
Mean ± SD.	4.44 ± 0.25	4.1 ± 0.63		
HGB (g/dl)	(n= 10)	(n= 20)		
Mean ± SD.	11.72 ± 0.45	11.2 ± 1.4	t=0.746	0.462
PLT(C.mm) (10^3/il)	(n= 10)	(n= 20)		
	210.0 ± 15.15	267.1 ± 125.8	U=31.0	0.085
NEUT (il)	(n= 10)	(n= 20[#])		
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 (%)	(n= 10)	(n= 20)	U=52.0	0.559
Mean ± SD.	60.2 ± 19	55.4 ± 11. 9		
LYMPH (il)	(n= 10)	(n= 20)		
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 (%)	(n= 10)	(n= 20)		
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 (il)	(n= 10)	(n= 20[#])		
Mean ± SD.	3.4 ± 0.5	4.3 ± 1.1	U=38.5	0.172
ESINO (il)	(n= 10)	(n= 20)		
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 (%)	(n= 10)	(n= 20)		
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 (il)	(n= 10)	(n= 20)		
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 (%)	(n= 10)	(n= 20)		
Min. – Max	0.0 – 0.0	0.0 – 0.4	U=60.0	0.655
Mean ± SD.	0.0 ± 0.0	0.4 ± 0.0		
Median	0.0	0.0		

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

GPT/ALT (U/L) and GOT/AST (U/L) enzymes but there was a significant difference in serum bilirubin level (mg/dL) with U = 7.50[*] according to Mann Whitney test and Student t-test as shown in Fig. 1A, B and C.

Kidney Function Investigations for the Breast Cancer Patients Before Treatment and the Healthy Control Volunteers: Kidney function analysis represented in the creatinine and urea levels (mg/dL) of the breast cancer patients before treatment and the healthy control is shown in Fig. 2 A and B. It showed that there was no significant difference in creatinine level (mg/dL) and urea level (mg/dL) between the breast cancer patients before treatment and the healthy control according to Mann Whitney test and Student t-test.

Correlation Between miRNA-22 with Demographic and Clinical Parameters of the Breast Cancer Patients Before Treatment and the Healthy Control: Table 3 demonstrated that there was no significant difference between miRNA-22 expression and all clinical data in breast cancer patients before treatment and healthy control.

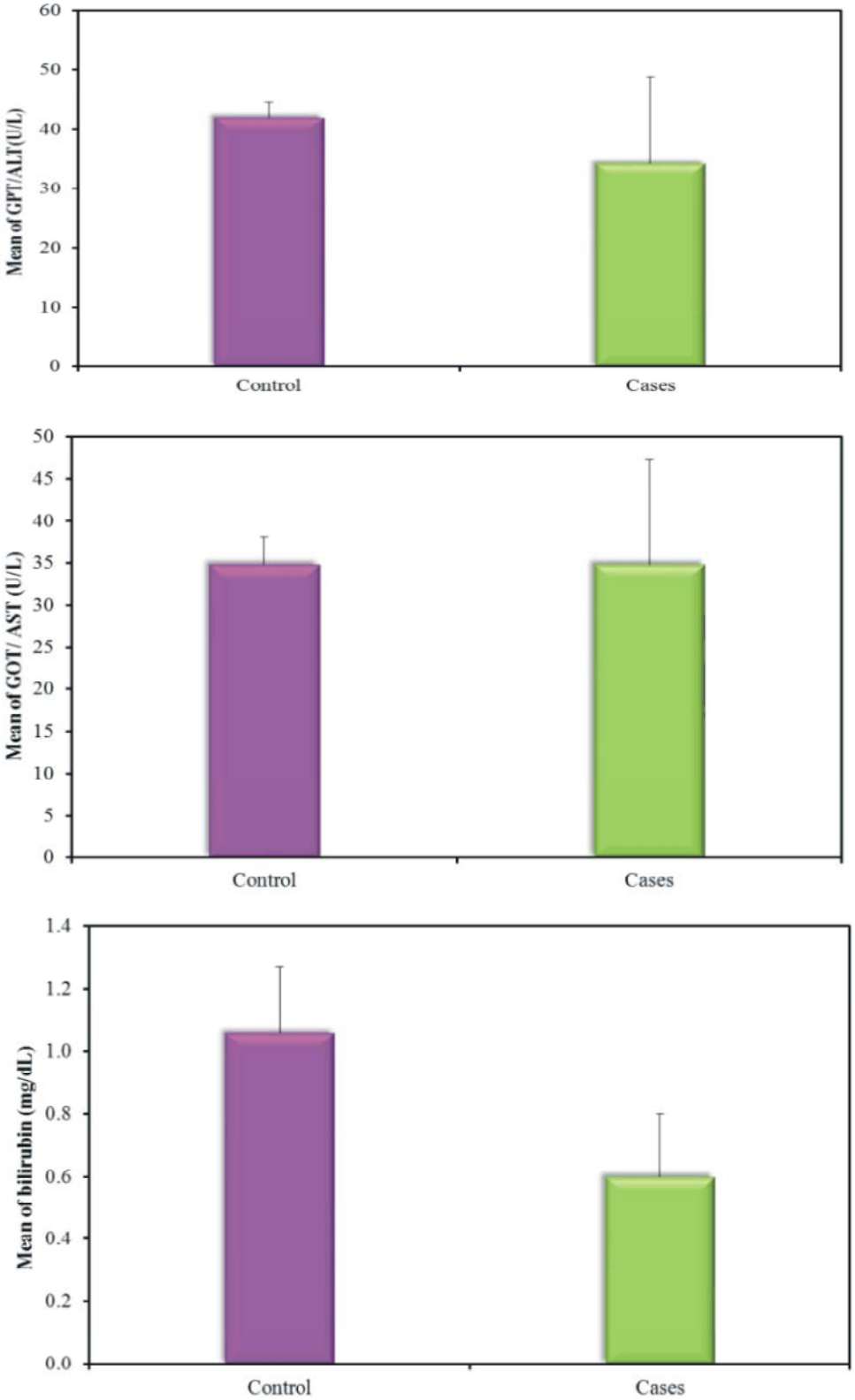

Fig. 1: Liver function analysis ALT (U/L) (A), AST (U/L) (B) and S. Bilirubin (C) of the breast cancer patients before treatment and the healthy control volunteers

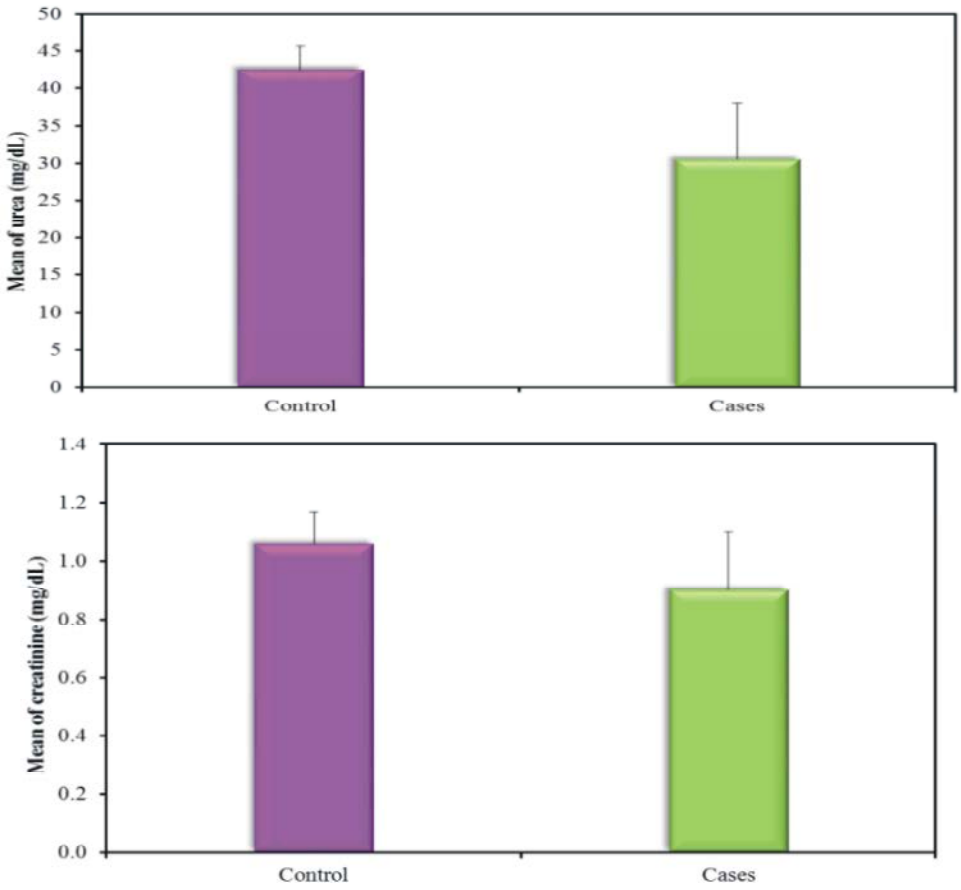

Fig. 2: Kidney function analysis creatinine (mg/dL) (A) and urea (mg/dL) (B) levels of the breast cancer patients before treatment and the the healthy Control

Table 3: Correlation between miRNA-22 expression with demographic and clinical parameters of the breast cancer patients before treatment and the healthy control volunteers

	miRNA 22			
	Control (n= 10)		Patients (n= 20)	
Clinical parameters	r_s	P	r_s	P
Age	0.564	0.322	-0.301	0.369
WBCs (10^3/il)	0.800	0.104	-0.082	0.811
RBC (10^6/il)	0.200	0.747	-0.364	0.272
HGB (g/dl)	-0.527	0.361	-0.600	0.051
PLT(C.mm) (10^3/il)	-0.700	0.188	-0.445	0.170
NEUT (il)	0.500	0.391	-0.333	0.420
NEUT (%)	0.200	0.747	0.118	0.729
LYMPH (il)	0.300	0.624	0.436	0.180
LYMPH (%)	-0.200	0.747	-0.251	0.456
MONO (il)	0.600	0.285	-0.609	0.047
MONO (%)	-0.289	0.638	-0.173	0.610
ESINO (il)	0.718	0.172	0.411	0.209
ESINO (%)	.	.	0.117	0.731
BASO (il)	.	.	-	-
BASO (%)	.	.	.	-
GPT/ALT(U/L)	-0.400	0.505	-0.366	0.298
GOT/AST (U/L)	0.700	0.188	-0.283	0.399
Bilirubin (mg/dL)	-0.500	0.391	-0.092	0.788
Creatinine (mg/dL)	-0.564	0.322	-0.105	0.773
Urea (mg/dL)	-0.800	0.104	-0.320	0.337

r_s: Spearman coefficient

Table 3: miRNA-22 expression analysis for the Breast Cancer patients before treatment and the healthy Control volunteers

miRNA 22	Control (n= 10)	Patients (n= 20)
Min. - Max.	1.06 – 1.70	0.24 – 1.99
Mean ± SD.	1.32 ± 0.30	1.19 ± 0.69
Median	1.15	1.52

MiRNA-22 Expression Analysis for the Breast Cancer Patients Before Treatment and the Healthy Control: MiRNA-22 expression analysis of breast cancer patients before treatment and healthy control was shown in Table 3. The presented data revealed that there was no significant difference between the expression of miRNA-22 in breast cancer patients before treatment and the healthy control.

DISCUSSION

The expression of miR-22 was found to be down regulated in gastric cancer [29] MiRNAs play an important role in cancer development and progression in many types of cancers [30]. Previous reports suggested that miR-22 was down-regulated in various cancers including breast cancer [17] colon cancer [31] pancreatic cancer [32] and cervical cancer [33]. However, miR-22 is apparently up-regulated in prostate cancer, thus potentiating host oncogene activation [34]. These controversial results of miR-22 in cancer development may reflect the diverse roles of miR-22 in different types of cancer. Studies have focused on cancer-specific miRNAs and associated target genes to elucidate biological mechanisms [35, 36] such as acute myeloid leukemia (AML) [37] and esophageal squamous cell carcinoma [38]. The mechanisms under the loss of miR-22 are still poorly understood. It was reported that the down regulation of miR-22 in acute myeloid leukemia was caused by TET1/GFI1/EZH2/SIN3A mediated epigenetic repression and/or DNA copy-number loss [37].

These finding in agreement with our data showed that there was no significant difference in the age and menstrual history between the breast cancer patients and the healthy control. White blood cells (WBCs) counts Red blood cells (RBCs), Platelets, Neutrophil, hemoglobin (Hg), lymphocytes (Lymph), Lymph%, monocytes (Mono), Eosinophils (Esino), Esino%, Basophils (Baso) and Baso % showed no significance difference in breast cancer patients and in the healthy control. The activity of ALT and AST (U/L) showed no significant difference between the breast cancer patients and the healthy control. Bilirubin levels (mg/dL) showed a significant difference at between breast cancer patients and the healthy control in Bilirubin levels. Urea level was not significant at the breast cancer patients before treatment and the healthy control. These data showed that the miRNA 22 expression analysis of breast cancer patients has no significant difference between the breast cancer patients and all clinical parameters, Also There was no significant difference between the healthy control and all clinical parameters.

Increasing findings have documented a fascinating and normally ignored mechanism of miR-22 with reference to the regulation of cancer proliferation and epithelial mesenchymal transition (EMT). However, conflicting results have been reported. For example, miR-22 promoted breast cancer proliferation, migration and invasion by silencing acetylase TIP60 [39]. Also miR-22 as a crucial epigenetic modifier and promoter of EMT and breast cancer stemness towards metastasis[40].However, miR-22 was found to induce p53 expression and concurrently target SIRT1, CDK6 and Sp1 to activate pRb signaling pathway; thereby hastening senescence, inhibiting cellular growth, invasion and metastasis in cervical cancer and breast cancer. Moreover, miR-22 suppressed EMT process and cancer distant metastasis by directly targeting TIAM1 (T-cell lymphoma invasion and metastasis 1) and SIRT1 in colorectal cancer and renal cell carcinoma, respectively [41]. Given the fact that miR-22 could directly target either proliferation or EMT-associated tumor suppressors or oncogenes to suppress or induce proliferation and metastasis, it is important to clarify the accurate expression and mechanistic function of miR-22 in different cancer types. In summary, down-regulation of miR-22 was found in breast cancer tissues. This molecule acts as tumor suppressor by inhibiting proliferation and migration and inducing apoptosis of cervical cancer cells. HDAC6 was identified as a direct downstream target of miR22: an inverse correlation of these two molecules was found. It seems that miR-22 dysregulation may impact on HDAC6 induction, possibly promoting cervical carcinogenesis.

CONCLUSIONS

The molecular mechanism of miR-22 underlying the malignant progression of breast cancer remains to be elucidated. In conclusion, our data demonstrated that serum miR-22 has no significant difference between breast

cancer patients before treatment and the healthy control that indicate it cannot expected to serve as a prognostic biomarker for breast cancer patients.

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