MiR-133a and MiR-155 as Potential Minimally Invasive Biomarkers in Breast Cancer

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Abstract: Breast cancer is one of the most common cancers in women around the world and the second leading cause of cancer death worldwide. MicroRNAs (miRNAs) expression participates in breast cancer progression. The aim of this study is to investigate the expression of miR-133a and miR-155 in breast cancer serum and study their correlation with tumor suppressor protein (p53), carcinoembryonic antigen (CEA) and cancer antigen-15.3 (CA-15.3) concentrations in serum of breast cancer patients and also study their correlations with clinicopathological features. **Methods:** The expression of miR-133a and miR-155 in serum was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR), P53 concentration was measured by enzyme-linked immunosorbent assays (ELISA**)** and CEA and CA-15-3 concentrations were measured using chemiluminescent immunoassay in women with breast cancer (n=60) and controls (n = 20). **Results:** MiRNA-155 was significantly overexpressed (P<0.001) while miR-133a had significant down expression (P<0.001) in the serum of breast cancer patients compared to control serum. CEA and CA-15.3 have significant higher concentrations in the serum of breast cancer patients compared to control serum. A significant association was observed between miR-133a with tumor grade (P=0.045) and miR-155 with lymph node involvement (P=0.024). A significant correlation between miR-155 and CEA (P <0.05) was observed. P53 had no significant correlations with any of the studied miRNAs .There were no correlations between miR-133a and p53, CEA, CA-15.3. **Our Conclusion:** These miRNAs have a significant signature in the pathogenesis of breast cancer and can be used as minimally invasive biomarkers to diagnose breast cancer patients.

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1. Introduction

Breast cancer is one of the most common cancers in women worldwide, and the second cause of death in female cancer patients **[1]**. A total of 1,665,540 new cases and 585, 720 deaths occurred in the USA during the year 2014, according to the American cancer society **[2]**. In Egypt, According to the Egyptian National Cancer Institute (NCI) (2002), breast cancer is the most common type of cancer between Egyptian women representing 18.9% of total cancer cases **[3]**. However in 2014 this percentage was increased, to 38% of reported malignancies between Egyptian women **[4]**. Worldwide Public Health data revealed that more than one million women are diagnosed with breast cancer each year and more than 410,000 women will die from the disease **[5]**. Breast cancer is an heterogeneous disease with numerous morphological appearances, behaviors, molecular features, and response to therapy **[6]**. Treatment of breast cancer is based on the accessibility of strong diagnostic, prognostic, and predictive factors to guide the choice of different treatment options **[7]**.

MicroRNAs are highly conserved noncoding RNA molecules that are approximately 17–25 nucleotides in length. They control gene expression at the posttranscriptional level by interacting with a specific target messenger RNA (mRNA) **[8]**. They also regulate a variety of cellular processes, such as proliferation, differentiation, metabolism, aging، and cell death. As such, the importance of miRNAs is increasingly recognized in almost all fields of biological and biomedical fields **[9]**. Until now, more than 2500 mature miRNAs in human genome have been discovered and registered **[10]**, which regulate approximately 30% of all protein-coding genes **[11]**.

The importance of miRNAs in oncogenesis has also been recognized. Dysregulation of miRNA expression plays an important role in cancer development through various mechanisms, such as deletions, amplifications, epigenetic silencing, or mutations in miRNA loci **[12]**.

The current in vivo diagnostic tools used for the detection of breast cancer at its early stages, e.g., mammography and ultrasound had several limitations, such as breast density or calcification recognition.

Other imaging modalities, e.g., magnetic resonance imaging (MRI), have been proposed as complementary diagnostic modalities, with limited sensitivity **[13]**. Nevertheless, the cost incurred and skill required for a mammogram has hindered a wide acceptance of this method. Thus, there is still need to clarify new mechanisms to develop accurate screening method that can diagnose patients with early cancer or precursor lesions by minimally invasive techniques **[14]**.

The emergence of small non-protein-coding RNAs called miRNAs which are stable in serum and playing important roles in oncogenesis has opened new opportunities for early cancer diagnosis **[15],[16]**. MiRNA has a great potential to be a novel biomarker for breast cancer and holds a potential for individualizing patients' treatment regimens **[17]**. However, there is still restricted awareness on the exact mRNA target of the deregulated miRNAs in breast cancer.

In this study, we aimed to explore the efficacy of combining a two of circulating miRNAs (miR133a and miR155) as prospective biomarkers in breast cancer and their relationship with clinicopathological features and concentrations of p53, CEA and CA-15.3. To our knowledge, this is the first study to implicate the role of these miRNAs and p53 in breast cancer patients in Egypt.

2. Patients and methods

Patients:

The study was performed on 80 females, including 60 with newly diagnosed breast cancer at different disease stages and 20 control age corresponding females. Patients with breast lesions were recruited from the surgery department, National Cancer Institute (NCI), Cairo University during the time period from March 2015 to August 2015. The study was permitted by the Institutional Review Board (IRB) of the NCI, Cairo University. It was permitted according to the Helsinki guidelines of studies performed on human beings and a written conversant permission was obtained from all study subjects. All patients were subjected to ordinary biochemical and hematological investigations and imaging diagnoses and chest x-ray for stage IV. Samples were obtained from all patients previous to any therapeutic or surgical intervention. Participant's age showed a mean ± SD of (49.4 ± 7.64) years in breast cancer patients and (43.9 ± 9.45) years in control females. Patients'characteristics is presented in **table (1)**.

Methods:

Seven ml of blood was withdrawn into 2 serum collection tubes, allowed to clot for 30 minutes and centrifuged at 4000 R.P.M for 10 minutes. Yielded serum was divided into 2 micro tubes and stored at -80 ºC till the time of analysis. First tube was used for determination of serum concentration of p53 by ELISA ***using the kit (catalog#: ELH-P53) supplied by Ray Biotech, Inc (Norcross, GA)*** according to the manufacturer's instructions, and for determination of serum concentrations of CA-15.3 and CEA by ***ARCHITECT Immunoasssay Analyser, Abbott, U.S.A***. according to the manufacturer' instruction. The second tube was used for quantification of mature miRNAs (miR-133a and miR-155) in serum by qRT-PCR.

RNA extraction was done using ***the miRNeasy Mini Kit (cat. no. 217004) Qiagen, Germany***, according to the manufacturer's instructions. RNA was isolated from 200 µL of serum and was eluted in 40 µL of RNAse-free water and was stored at −80◦C until qRT-PCR reaction. Concentrations of all RNA samples were measured using Nanodrop 1000 ***(Thermo Scientific, Wilmington, DE, USA)***.

The total RNA (100 ng) was reverse transcribed after thawing using a miScript HiSpec buffer supplied in ***miScript II RT Kit (catalog no. 218161) Qiagen, Germany***, according to the manufacturer's instructions, and complementary DNA (cDNA) synthesis was performed in a thermal cycler ***(IGEM: MIT/2005/Thermo cycler)***, the thermal cycler reaction setting were as follows: incubation at 37°C for 60 min followed by 5 min at 95°C. The cDNA was stored at −20°C until use.

Quantitative Real-time Polymerase Chain Reaction

Quantitative real-time PCR was performed by ***miScript SYBR Green PCR Kit (200) Qiagen, Germany (Catalog no. 218073***) in compliance with the manufacturer's instructions. MicroRNA specific primers were provided by Qiagen/Germany, miR133a/ Hs_miR-133a_2 miScript Primer Assay (MS00031423) and miR-155/ Hs_miR-155_2 miScript Primer Assay (MS000031486). MiRNA expression levels were quantified using Step One ***(Applied Biosystems, USA)***. Cycling program: primary activation step for 15 min at 95 °C to stimulate HotStarTaq DNA polymerase, cycling:(first denaturation for 15 s at 94 °C, annealing for 30 s at 55°C, finally extension for 30 s at 70 °C at which fluorescence data collection were performed) ×40 cycles and endogenous control miScript Primer Assay SNORD68 (Hs_SNORD68_11 miScript Primer Assay (MS00033712) was used to normalize the data **[18]**. The data obtained from the miRNA expression levels were calculated and evaluated by the cycle threshold (Ct) method, which is the number of cycles required for the fluorescent signal to cross the threshold in RT-PCR. The level of miRNA expression was reported as ΔCt value. The ΔCt was calculated by subtracting the Ct value of miRNA SNORD68 from the Ct values of the target miRNAs [mean value Ct (miR-133a, miR-

155) - mean value Ct (housekeeping gene)], the relative expression level of the miRNA of interest corresponded to the $2^{-\Delta Ct}$ value. ΔΔCt was then determined by subtracting the average ΔCt of the control from the ΔCt of cases. The fold change in the miRNA expression level was calculated (fold change = $2^{-\Delta\Delta Ct}$) to determine the relative quantitative levels of individual miRNA **[19]**.

Statistical analysis:

In the existing study SPSS software package (version 17 for Windows; SPSS INC., Chicago, IL, USA) was used to perform statistical analysis. To compare the miRNA expression in cancer versus the normal serum; the Wilcoxon's rank sum test for one sample was used. For comparing two different groups such as metastatic and non-metastatic, the Mann-Whitney U nonparametric test was used, while Kruskal-Wallis nonparametric test was used for more than two independent variables. To find a correlation between two variables Spearman's rho (r) was calculated. The Receiver Operating Characteristic (ROC) curve was used to determine the cut-off values of miRNAs and to analyze the diagnostic utility of different markers. A *p*-value of less than 0.05 was considered statistically significant. All *p*-values are two sided.

3. Results

The expression levels of miR-133a and miR-155 were evaluated by qRT-PCR. The expression level of miR-155 was significantly higher in BC serum than in healthy controls ($p < 0.001$), while expression level of miR-133a was significantly lower in breast cancer serum than in healthy controls ($p < 0.001$) as shown in ***Figure 1 (A)***. The serum concentrations of CEA and CA-15.3 were significantly higher in breast cancer than in healthy controls as shown in ***Figure 1 (B)***.

The diagnostic efficacy of miR-133a and miR-155 were evaluated using ROC curve analysis. ROC curve analysis showed that the two miRNAs can significantly differentiate between breast cancer and healthy controls, showing an area under the curve (AUC) of 0.950 for miR-133a (95% CI 0.89-1.00, $p < 0.001$) and AUC 0.767(95% CI 0.66-0.87, $p < 0.001$) for miR-155. The optimal sensitivity and specificity were (95% and 100%) and (76.7% and 100%), respectively. When the diagnostic significance of serum miRNAs was compared in breast cancer patients, the results of the ROC curve suggested that the diagnostic efficacy of serum miR-133a was superior to miR-155 with AUC of 0.950 and 0.767, respectively, and total accuracy were 96.3% and 82.5%, respectively as shown in ***Figure 2 (A)(B) and Table (2)***.

The diagnostic efficacy of p53 was evaluated using ROC curve analysis. All diagnostic efficacies for these markers at the selected cutoff values are shown in ***Table (2).*** But in comparison to studied miRNAs we found that miR-133a is more sensitive than miR-155 and two miRNAs are more sensitive than p53, as shown in ***Table (2)*** and ***Figure (2)***.

Using Spearman's correlation coefficient showed that significant correlation between miR-155 and CEA, also there was a significant correlation between CA-15.3 and CEA, but there was not a significant correlation between two miRNAs and no significant correlation between miR-133a and both of CEA and CA-15.3 as shown in ***Table (3)***.

The relative expression of serum miRNAs of the breast cancer patients were studied in relation to their clinicopathological data. MiR-133a was significantly lower in sera of patients with grade (III) than in those with grade tumors (II) *(P=0.045).* Also miR-155 had a statistically significant association with lymph node involvement (*P*=0.024). No significant differences between miRNAs (miR-133a and miR-155) expression and (age, tumor size, menopausal status, estrogen receptor and progesterone receptor status). The relative concentrations of serum CEA and CA-15.3 of the breast cancer patients were studied in relation to clinicopathological data, which showed that CA-15.3 had a statistically significant association with stages ($p < 0.001$) and HER2 (p=0.036), also CEA had a statistically significant association with stages ($p < 0.001$), and menopausal status (p=0.013) as shown in ***Table (1)***.

P53 concentration:

P53 had statistically significant low concentration in the breast cancer serum range from (5.04-9.27 ng/ml) than control serum ranges from (6.27-19.70 ng/ml) with ($p<0.05$). At the optimal cutoff value of 6.90, the sensitivity and specificity were 55% and 85% respectively ***Figure 2(C)***. Its serum level showed that no significant associations with any of the clinicopathological parameters ***Table (1)***. No significant correlation between p53 and expression of miR-133a and miR-155 was found, no significant correlation between p53 and CEA, CA-15.3 as shown in ***Table (3)***.

Table (1): **Association between study markers with different clinicopathological characteristics in breast cancer patients**

		Relative expression* of microRNAs with *p* value in different groups**.				Relative*** concentration with *p* value in different groups**.					
variable	**N (%)**	**miR-133a**	***p* value**	**miR-155**	***p* value**	**p53**	***p* value**	**CA-15.3**	***p* value**	**CEA**	***p* value**
Age											
≤49	36(60)	-1.47	0.561	2.24	0.952	6.58	0.774	28.25	0.428	1.82	**0.085**
>49	24(40)	-1.81		2.64		6.61		32.0		3.26	
Menopausal status											
PRE	35(58.3)	-1.53	0.658	2.32	0.793	6.58	0.893	28.0	0.219	1.8	**0.013**
POST	25(41.7)	-1.87		2.16		6.59		33.9		3.22	
Stages											
I	6(10)	-1.22		1.91		6.57		24.75		2.29	
II	28(46.7)	-1.76	0.228	2.91	0.571	6.59	0.511	23.85	**0.000**	1.76	**0.000**
III	5(8.33)	-0.91		1.14		6.45		21.80		1.71	
IV	21(35)	-2.12		1.63		6.65		39.40		4.41	
Grades											
II	45(75)	-1.41	**0.045**	2.91	0.107	6.58	0.657	29.30	0.813	2.6	0.105
III	15(25)	-2.19		1.21		6.66		28.40		3.28	
HER2(IHC)											
+VE	23(38.3)	-1.37	0.191	2.91	0.134	6.58	0.373	35.21	**0.036**	3.28	0.094
-VE	37(61.7)	-1.88		1.79		6.44		28.10		2.17	
ER(IHC)											
+VE	52(86.7)	-1.78	0.373	2.24	0.811	6.62	0.184	29.90	0.794	2.67	0.853
-VE	8(13.3)	-1.14		2.03		6.47		25.00		2.10	
PR(IHC)											
+VE	53(88.3)	-1.76	0.527	2.16	0.991	6.59	0.375	29.3	0.863	2.61	0.765
-VE	7(11.7)	-1.41		2.63		6.52		28.1		2.04	
Lymphnode involvement											
NO	37(61.7)	-1.87	0.632	1.44	**0.024**	6.64	0.942	29.0	0.824	2.6	0.245
YES	23(38.3)	-1.53		3.45		6.58		31.7		1.85	

* Median of relative expression.
**Mann-Whitney U and Kruskal-Wallis nonparametric test were used for comparing different group.
*** Median of concentration.
IHC: Immuno-histochemistry

Table (2): **Diagnostic efficacy of the studied miRNAs and P53**

	AUC	**95%CI (SE)**	**Sensitivity**	**Specificity**	**Cut-off**	***P* value**	**PPV**	**NPV**	**TA**
miR-133a	0.950	0.89-1.00 (0.028)	95%	100 %	0.940	0.000	100%	87%	96.3%
miR-155	0.767	0.66-0.87 (0.055)	76.7%	100 %	1.006	0.000	100 %	58.8 %	82.5 %
p53	0.731	0.591-0.872 (0.072)	55 %	85 %	6.900	0.002	55%	85%	77.5%

PPV: Positive predictive value
NPV: Negative predictive value
TA: Total accuracy

Table (3): Correlations among studied miRNAs expression and (CEA, CA-15.3 and p53) concentrations in serum of breast cancer patients

	miR-133a	miR-155	CEA	CA-15.3	P53
miR-133a					
CC	1.00	.245	-.131	-.064	-.169
P value	….	.059	.320	.627	.196
miR-155					
CC	.245	1.00	-.295*****	.064	.014
P value	.059		**.022**	.629	.916
CEA (ng/ml)					
CC	-.131	-.295*	1.00	.613**	-.159
P value	.320	**.022**	….	**.000**	.226
CA-15.3 (IU/ml)					
CC	-.064	-.064	.613**	1.00	-.057
P value	.627	.629	**.000**	….	.665
P53 (ng/ml)					
CC	-.169	.014	-.159	-.057	1.00
P value	.196	.916	.226	.665	....

CC: Spearman's correlation coefficient. ***** Correlation is significant at the 0.05 level (2-tailed).
******Correlation is significant at the 0.01 level (2-tailed).

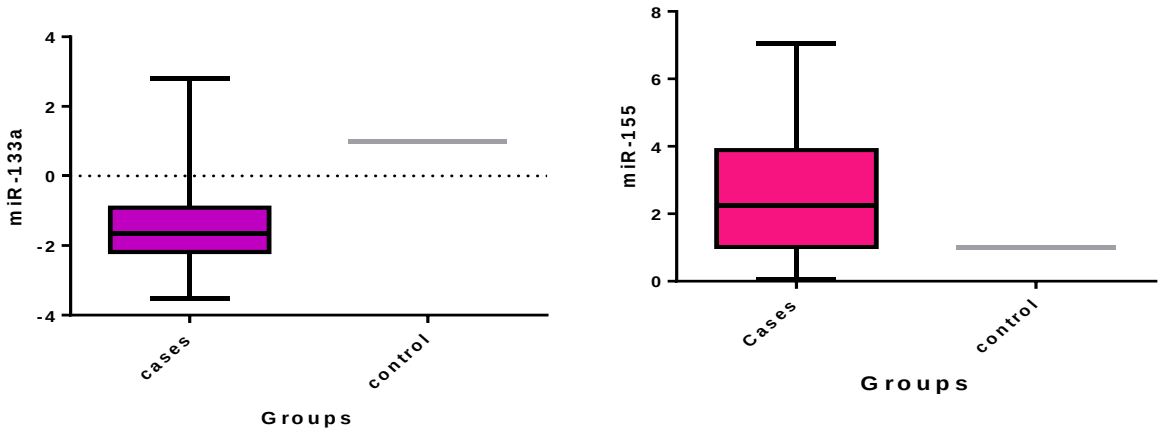

Figure 1(A): Box plots for miR-133a and miR-155 expressions in breast cancer cases versus control.

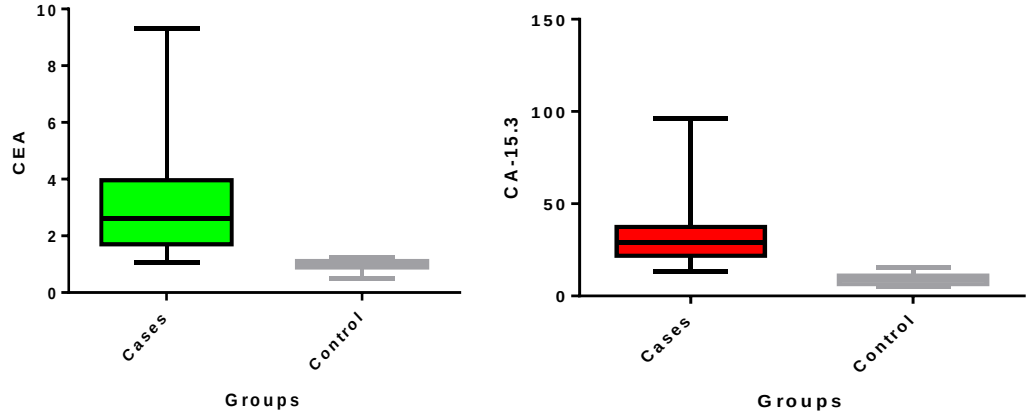

***Figure 1(B)*:** Box plots for CEA and CA-15.3 concentrations in breast cancer cases versus control.

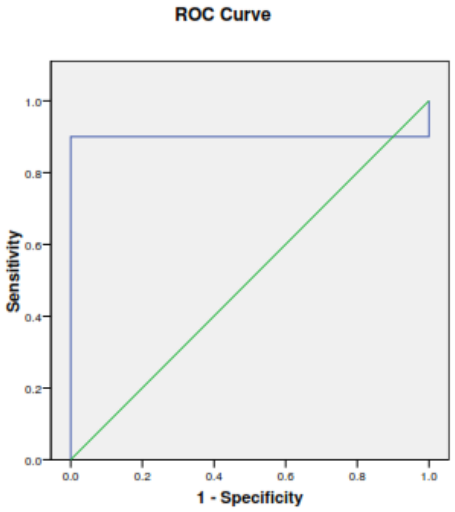

***Figure 2(A)*:** ROC curve of miR-133a.

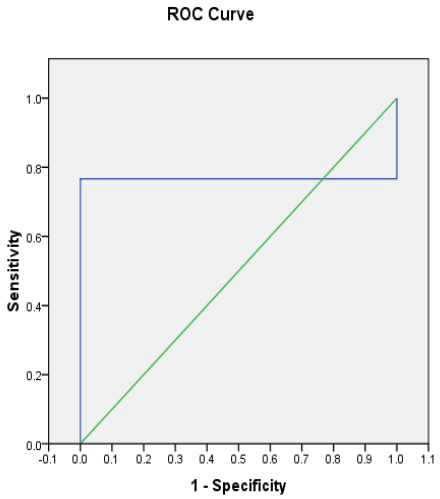

***Figure 2(B)*:** ROC curve of miR-155.

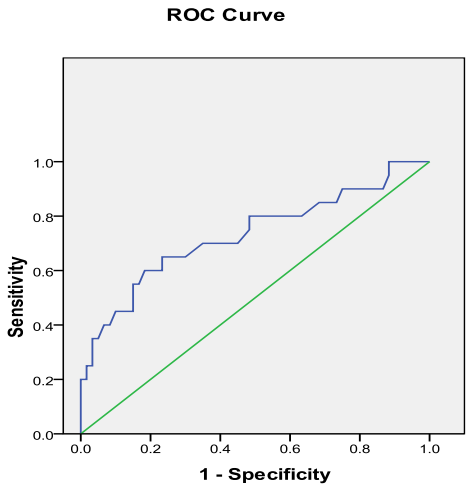

***Figure 2(C)*:** ROC curve of p53.

4. Discussion:

MicroRNAs were identified to play vital roles in the pathogenesis of cancer. The up regulation of oncogenic miRNAs or down regulation of tumor suppressor miRNAs can be implicated in tumorigenesis by varying many pathways, including cell cycle, angiogenesis, invasion and metastasis **[20]**. Thus, there is still a pressing need to elucidate novel mechanism of breast cancer development so as to develop a cost effective and accurate screening method for this cancer. Recently, the emergence of small non protein coding RNAs called microRNAs, playing important roles in oncogenesis, has opened new opportunities for early cancer diagnosis [**16]**.

In this study, we evaluate the expression of miR-133a and miR-155 in serum from 60 breast cancer patients and 20 controls. MiR-133a is one of non-coding RNA which plays an important role in preventing progression of breast cancer, as loss of miR-133a expression resulted in aberrant cell invasion and proliferation associated with poor prognosis in breast cancer **[21]**.

Studies have reported altered expression of miR-133a in several human cancers including esophageal squamous cell carcinoma**,** bladder cancer, and breast cancer **[22]**. MiR-133a affects breast carcinogenesis either through targeting fascin actin-bundling protein 1 (FSCN1) **[23]**, or through regulating the cell cycle and proliferation of breast cancer cells by focusing on the epidermal growth factor receptor (EGFR) through the EGFR/Akt signaling pathway **[21]**. These previous studies are consistent with our current study. We found that the expression of miR-133a was statistically significant lower in breast cancer serum than controls. Hence, supporting the suggestion that miR-133a plays a role as a tumor suppressor gene affecting breast cancer development and progression **[24], [25], [26]**.

Our study data revealed that miR-133a was significantly down regulated in serum of breast cancer than normal control serum which was in agreement with *Kodahl et al* **[22]**, who stated that miR-133a down regulated in breast cancer patients' serum in relation to normal control serum proving that miR-133a is considered a tumor suppressor gene in breast cancer. Our result in contrast to *Chan et al* **[27**], who stated that miR-133a up regulated in serum of female breast cancer versus to control.

Concerning another non coding RNA (miR-155), we found that miR-155 had statistically significant higher expression in breast cancer serum than healthy control serum. Our data was in harmony with *Hagrass et al* **[28]**, who found that miR-155 was over expressed in serum of breast cancer patients compared to normal control serum. *Mattiske et al* **[29]** stated that miR-155 was over expressed in serum breast cancer than normal control serum, also miR-155 was up regulated in breast cancer tissue than normal tissue and higher expression of miR-155 in breast cancer cell line when compared to normal cell line all these previous results in harmony with our results in which miR-155 over expressed. Our result is in agreement with *Zhang et al* **[30]**, who stated that miR-155 was over expressed in breast cancer cell line when compared to normal cell line.

In regarding the relation of miRNAs (miR-133a and miR-155) with clinicopathological features of breast cancer, our results clearly showed statistically significant difference down expression of miR-133a in grade III more than grade II in serum of breast cancer. In contrast to our results *Kodahl et al* [**22**] & *Wu et al* **[23]** who stated that there is no association between miR-133a expression and tumor grade of breast cancer tissue and cell line. *Wu et al* **[31]** stated that there was no association between miR-133a expression and patient age, tumor size, or estrogen receptor and progesterone receptor status these results symmetric with our result.

Our results clearly showed statistically significant high expression of miR-155 with lymph nodes involvement. In the same line with our results, *Hagrass et al* **[28]** who found that the expression of miR-155 was significantly higher in serum of breast cancer patients with lymph nodes involvement than breast cancer patients without lymph nodes involvement. On the contrary to our result, the up regulation of miR-155 was not statistically significant to lymph nodes involvement *Nassar et al* **[32]**.

Our result showed significantly higher serum concentrations of CEA and CA-15.3 in breast cancer patients compared to normal control. This was in harmony with *Wu et al* **[31]** & *Shao et al* **[33]** who stated that serum levels of CEA and CA-15.3 were elevated in preoperative breast cancer patients. There was controversy regarding the use of CEA and CA-15.3 in the diagnosis of breast cancer. The European Society for Medical Oncology (ESMO) and the European Group on Tumor Markers (EGTM) suggested that routine measurement of tumor markers such as CEA and CA-15.3 should be performed in patients with breast cancer [**34], [35]**.

However, the American Society of Clinical Oncology (ASCO) does not recommend routine measurement of CEA, CA-15.3 or other tumor markers for patients with breast cancer **[36]**. A report suggested that tumor markers including CEA and CA-15.3 should not be routinely measured in patients with early stage breast cancer **[37]**. Although the limitation of low sensitivity and specificity preclude the use of serum tumor marker CEA and CA-15.3 for the detection of early breast cancer, elevated preoperative tumor marker levels at initial presentation may predict poor outcome **[38]**.

The American Society of Clinical Oncology and the National Comprehensive Cancer Network (NCCN) guidelines do not currently recommend the use of serum CA-15.3 and CEA for breast cancer screening and directing treatment or a routine surveillance tool or for therapeutic response monitoring due to inconsistent findings of their sensitivity and specificity **[39], [33], [40]**. CEA and CA-15.3 serum levels may be increased in other benign conditions such as gastritis, gastric ulcer, bronchitis, cholangitis, and liver abscess in case of CEA and chronic hepatitis, liver cirrhosis, tuberculosis in case of CA-15.3 **[41]**.

In our study we have suggested that there could be a relationship between the expression level of

studied miRNAs and p53 level, based on that miRNAs act as oncogenes or tumor suppressor, but our results revealed that no significant correlation between the studied miRNAs expression level and the p53 serum level. This may be due to the miRNAs not directly targeting p53 as miR-133a act by targeting EGFR **[21**] and finally miR-155 act by negatively regulating a tumor suppressor gene known as suppressor of cytokine signaling 1 (SOCS1) **[31]**.

Regarding their diagnostic efficacy, miR-133a was reported the best sensitivity, specificity, PPV, NPV and TA followed by miR-155 then p53 and finally the routine tumor markers (CA-15.3 and CEA) indicating usefulness of miRNAs as molecular markers for diagnosing of breast cancer. Circulating miRNAs association with some clinicopathological parameters have been reported in other studies **[42]** suggesting their clinical prognostic value and carrying the possibility of a serologic test that can augment the histologic information of a tumor without the need for biopsy.

In conclusion

Due to the fact that miRNAs can exist stable in circulating blood with an easy extraction and quantification methods, these serum miRNA-133a and miR-155 can be used as potential minimally invasive biomarkers for breast cancer diagnosis.

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