

## Circulating MicroRNA-21 As A Promising Marker for Early Detection of Breast Cancer and Disease Progression in Egyptian Females

DOAA M.A. ELZOGHBY, M.D.\*; NERMINE H. MAHMOUD, M.D.\*; HEBA H. ALY, M.D.\* and MOHAMMED MATAR, M.D.\*\*

The Departments of Clinical Pathology\* and General & Laparoscopic Surgery\*\*, Faculty of Medicine, Ain Shams University, Cairo, Egypt

### Abstract

**Background:** The traditional imaging techniques and the currently accepted markers such as serum CA15.3 have limited specificity and sensitivity to identify early stages breast cancer patients. MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level via mRNA degradation and/or translational repression. They can modulate up to 60% of protein-coding genes in the human genome, one of which is miRNA-21. MiRNA-21 and its alterations play critical roles in breast cancer.

**Aim of Study:** The study aimed at assessing the diagnostic and prognostic role of miRNA-21 in breast cancer females.

**Subjects and Methods:** This case-control study was conducted on (150) breast cancer female patients and their age matched (50) healthy controls. All subjects recruited in the present study were subjected to measurement of serum microRNA-21 expression using quantitative real time PCR (qRT-PCR) in comparison to serum CA15.3.

**Results:** Serum miRNA-21 expression is upregulated in breast cancer patients compared to controls ( $Z=4.4, p<0.01$ ), with disease advanced TNMstages ( $H=24, p<0.001$ ) and negative estrogen receptor status ( $Z=2.2, p<0.05$ ). At cut off level 1.07 ( $2^{-\Delta\Delta Ct}$ ), miRNA-21 had 100% sensitivity and 90% specificity in discriminating patients with breast cancer from healthy controls. Meanwhile, CA15.3 had 70% sensitivity and 60% specificity in distinguishing between the two groups. At cut off level 3.33 ( $2^{-\Delta\Delta T}$ ) miRNA-21 expression had 86.7% sensitivity and 100% specificity in discriminating patients with early breast cancer from patients with advanced disease. While serum CA15.3 had 80% sensitivity and 86.7% specificity in discriminating between the same patients groups.

**Conclusion:** Serum miRNA-21 levels are significantly higher in breast cancer patients compared to healthy subjects. Increased miRNA-21 expression levels correlated with disease stages, estrogen receptor presence reflecting prognosis and influencing constructing treatment modalities.

**Key Words:** Breast cancer – miRNA-21 – CA15.3 – qRT-PCR.

### Introduction

**BREAST** cancer is the most common cancer in women and accounts for 29% of all cancers diagnosed every year worldwide. It is the second leading cause of cancer death in women, exceeded only by lung cancer, but it is the first in women under the age of 55 [1]. Therefore, the early diagnosis of breast cancer plays a critical role in its prognosis. Although mammograms are currently the best test for breast cancer screening, yet they have high false positive rates [2]. Hindered by their low sensitivity, the known serum markers such as CA 15.3 and BR27.29 are not used for screening of breast cancer [3]. This implies further investigations using expensive breast imaging and invasive biopsy. Therefore, the development of a more sensitive approach for early breast cancer diagnosis, particularly from benign lesions, is needed to supplement and/or complement existing detection methods [4].

The fundamental proposed regulatory role of miRNAs in a variety of biological processes such as cell proliferation, differentiation, and apoptosis suggests that differential expression of these transcripts may be exploited as a novel source of circulating molecular biomarkers [5]. The levels of circulating miRNAs packaged in exosomes differ between cancer patients and healthy donors and thus measuring circulating miRNAs levels may be useful in detection of early cancer and significantly contribute to treatment success [6]. More importantly, change in contents and amounts of secreted miRNAs are associated with disease stage and

**Correspondence to:** Dr. Doaa M.A. Elzoghby,  
E-Mail: [doaa\\_elzoghby@yahoo.com](mailto:doaa_elzoghby@yahoo.com),  
[Doaa\\_Elzoghby@med.asu.edu.eg](mailto:Doaa_Elzoghby@med.asu.edu.eg)

regulation of the malignant phenotype in many cancers including colorectal cancer [7], lung cancer [8] and breast cancer [9,10].

MiRNA-21 is one of the oncogenic up-regulated miRNAs and its gene is located on chromosome 17q23.1 [11]. Several studies reported its significant up-regulation in breast cancer tissues up to 10-13 folds compared to the normal adjacent tissues and that higher level of exosomal miRNA-21 is significantly associated with the presence of circulating tumor cells [12,13].

In this context, the present study aimed at assessing the possible role of miRNA-21 as a marker for breast cancer and its association with disease progression in Egyptian females.

### Subjects and Methods

#### A- Subjects:

This study was conducted on one hundred and fifty (150) female patients attending the Out-Patient Clinics and Surgery Department at El-Demerdash Hospitals, Ain Shams University in the period from March 2016 till July 2017. Subjects were divided into two groups; Group I (cancer patients) who were further subdivided into two subgroups, Subgroup (IA) early stages breast cancer (stage I and II) including (75) patients and Subgroup (IB) advanced stages breast cancer (stage III and IV) including (75) patients, according to TNM stage classification [14]. While Group II included fifty (50) apparently healthy female subjects serving as a healthy control group.

#### Inclusion criteria:

Confirmed diagnosis of breast cancer by biopsy, histopathological assessment and TNM classification.

#### Exclusion criteria:

- Patients with previous breast cancer treatment who had received chemotherapy, radiotherapy, or operation were excluded from the study groups.
- Patients with benign breast diseases as fibroadenoma, ductal epithelial hyperplasia and fibrocystic disease of the breast.

All studied individuals were subjected to full history taking, clinical examination with special emphasis on breast examination and mammogram. For patients only, radiological investigations as bone scan, CT scan and/or MRI as well as breast biopsy were done for histopathological examination and steroid receptors study.

An informed consent was taken from all subjects included in the study before history taking, physical examination and blood sample withdrawal. The procedures applied in this study were approved by The Ethical Committee of Human Experimentation of Ain Shams University, and are in accordance with the Helsinki Declaration of 1975.

#### B- Sampling:

Under complete aseptic conditions, five milliliters of venous blood were collected by venipuncture from each subject and were divided into; three milliliters evacuated in sterile EDTA-treated vacutainers then immediately transferred to the laboratory and centrifuged at (1900xg for 10 minutes). Plasma was separated into new aliquote tubes and stored at  $-80^{\circ}\text{C}$  for the subsequent assay of circulating miRNA-21. Two milliliters were withdrawn in a plain tube vacutainer and left to clot for 15 to 20 minutes, then serum was separated by centrifugation (1500xg for 10 minutes), aliquoted and stored at  $-20^{\circ}\text{C}$  for the subsequent assay of CA15.3.

#### C- Methods:

##### 1- Analytical methods:

The assay of serum CA1 5.3 was done on ARCHITECTi2000SR autoanalyzer (Abbott Laboratories Diagnostics, Division Abbott Park, IL 60064 USA) using reagents supplied by the manufacturer based on immunochemiluminescent assay. Assay of miRNA-21 was done by quantitative real time polymerase chain reaction (qRT-PCR).

#### Assay of serum CA15.3:

ARCHITECT CA 15-3 assay is a chemiluminescent microparticle immunoassay for the quantitative determination of DF3 antigen in human serum and plasma on the ARCHITECTi2000SR System using 115D8 and DF3 monoclonal antibodies (mAbs). It is a two-step immunoassay with flexible assay protocols, referred to as Chemiflex. In the first step, sample, wash buffer and mAb (115D8) coated paramagnetic microparticles are combined. DF3 present in the sample binds to the 115D8 coated microparticles. After washing, mAb DF3 acridinium-labeled conjugate is added in the second step. Pre-Trigger and Trigger solutions are then added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amounts of DF3 defined antigen in the sample and the RLUs detected by the ARCHITECTi2000SR optical system [15].

*Assay of serum MicroRNA-21:*

*MiRNA-21 RNA extraction:*

The total RNA, including small RNA, was isolated from plasma using the miRNeasy Mini Kit provided by Qiagen (Qiagen incorporation, 28159 Avenue, Stanford Valencia. CA 91355, USA) following manufacturer protocol.

*Measurement of RNA purity and concentration:*

3µL of RNA solution were added to 72µL of diethylpyrocarbonate (DEPC) water (dilution 1: 25) followed by vortex. Sample was read at 260 nm and 280nm using the Nanodrop spectrophotometer supplied by (Thermo Fisher Scientific, 3411 Silverside Road Wilmington, USA). Protein was detected at 230nm. Samples were considered with good RNA purity if A260/A280 ratio is 1.8-2.

*Reverse transcription:* Following manufacturer protocol, Reverse Transcription (RT) was performed on the extracted RNA prepared in the previous step using TaqMan MicroRNA Reverse Transcription kit. MiRNA-21 specific stem-loop primers and small nuclear miRNA- 16 were used as an endogenous control, all provided by Applied Biosystems (Applied Biosystems, 850 Lincoln Centre Dr Foster City, California, 94404, USA).

*PCR amplification:* Amplification was performed on RT-PCR system using the TaqMan MicroRNA Assays kits provided by Applied Biosystems (Applied Biosystems, 850 Lincoln Centre Dr Foster City, California, 94404, USA) in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines Figs. (1,2) [16].

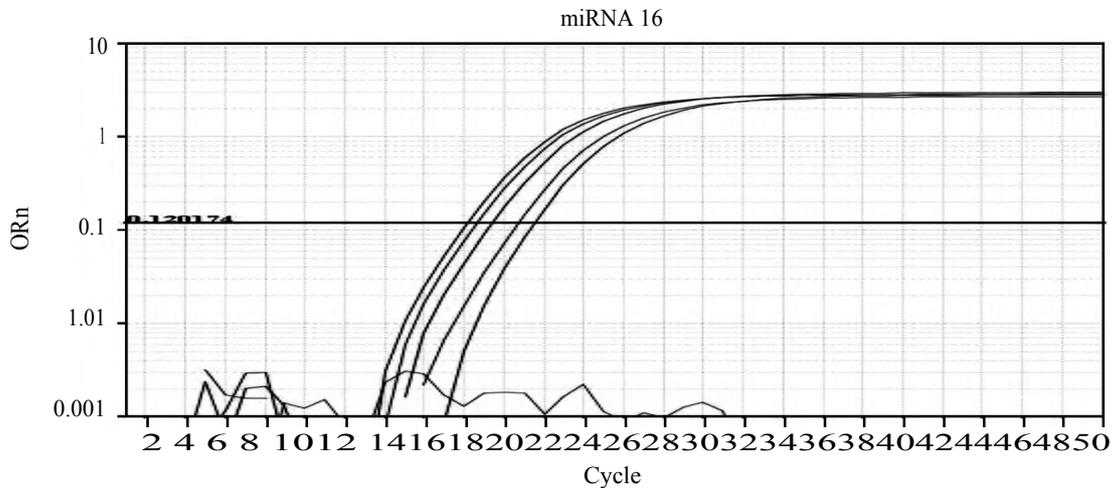


Fig. (1): Amplification Plot for miRNA-16.

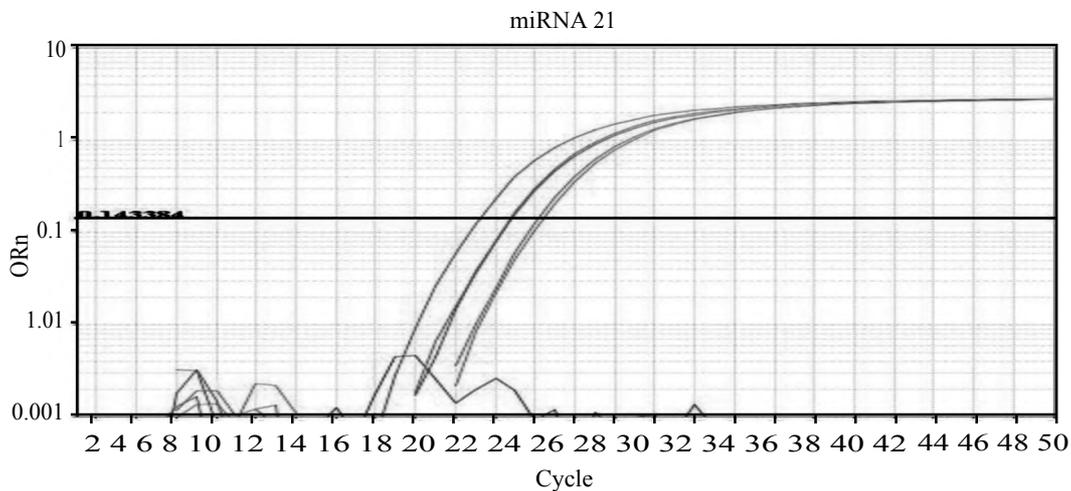


Fig. (2): Amplification Plot for miRNA-21.

**Detection and calculation of results:** Results were reported in relative quantification. The relative expression level (fold change) for miRNA-21 in each sample was calculated by the comparative cycle threshold ( $2^{-\Delta\Delta C_t}$ ) method using miRNA-16 as an endogenous reference control. The cycle threshold ( $C_t$ ) was determined for (miRNA-21 and miRNA-16) in each experimental sample using Step One Plus™ Software v2.1. The  $\Delta C_t$  in each experimental sample was calculated as  $\Delta C_t = (C_t \text{ miRNA-21} - C_t \text{ miRNA-16})$ . The average (mean)  $C_t$  was calculated for samples of the control samples group. Then ( $\Delta\Delta C_t$ ) was calculated for every sample (malignant and control). Finally, the relative expression level (fold change) for miRNA-21 in each sample was calculated using ( $2^{-\Delta\Delta C_t}$ ) formula  $\Delta\Delta C_t = \Delta C_t$  of every sample-average  $\Delta C_t$  of control samples [17].

**2- Statistical methods:** All statistical analyses were done using software version IBM SPSS (Statistical Package for the Social Sciences) statistics (Version 25.0, IBM Corp., USA, 2017-2018). Descriptive statistics of various studied parameters were expressed as percentage for qualitative data, median (M) and Inter-Quartile Range (IQR) which extends between the 25<sup>th</sup> and 75<sup>th</sup> percentiles (Q1 and Q3, respectively) for quantitative non parametric data. Comparative statistics were done using the Wilcoxon's Rank Sum and the Kruskal-Wallis (KW) tests for non parametric data. Correlation analysis was performed using Spearman's rank correlation coefficient ( $r_s$ ) for non parametric data. Receiver Operator Characteristic (ROC) curve was constructed and optimal cut-off values for plasma miRNA-21 and serum CA15.3 were established and the Area Under the Curve (AUC) was calculated by the best sensitivity and specificity where the right angle at the upper left corner is the best diagnostic threshold (cut-off) of the parameter being varied. In all statistical analyses,  $p$ -value > 0.05: Non significant;  $p$ -value < 0.05: Significant;  $p$ -value < 0.01 or 0.001: Highly significant.

**Results**

Descriptive statistics of the demographic and clinical parameters in Group I (breast cancer patients) and Group II (healthy controls) are shown in Tables (1,2).

Table (2) and Fig. (3) show the comparative statistics of the serum levels of CA 15.3 and miRNA-21 expression levels between the studied Groups I & II. CA 15.3 and miRNA-21 showed highly significant statistical increase in Group I compared to Group II ( $Z=2.3$  and  $4.4$ , respectively with  $p < 0.01$ ).

Table (1): Descriptive data of the demographic and clinical parameters of Group I and Group II.

Parameter	Group I (n=150) Median (Q1-Q3)/ n (%)	Group II (n=50) Median (Q1-Q3)/ n (%)
• Age (years)	50.5 (45.0-62.3)	47.5 (37.5-59.0)
• Age at menarche (years)	14.0 (12.0-14.0)	11.0 (11.0-12.5)
• <i>Marital status:</i>		
Married	145 (80.6%)	35 (19.4%)
Un-married	5 (25%)	15 (75%)
• <i>Family history:</i>		
Negative	140 (75.7%)	45 (24.3%)
Positive	10 (66.7%)	5 (33.3%)
• <i>Breast feeding:</i>		
Yes	145 (80.6%)	35 (19.4%)
No	5 (25%)	15 (75%)
• <i>Administration of oral contraceptives:</i>		
Yes	70 (87.5%)	10 (12.5%)
No	80 (66.7%)	40 (33.3%)
• Positive mammogram finding	100 (100%)	-

Table (2): Descriptive and comparative statistics of the serum levels of CA 15.3 and miRNA-21 expression levels in Group I and Group II using Wilcoxon's Rank Sum Test for non-parametric data.

Parameter	Group I (n=150) Median (Q1-Q3)	Group II (n=50) Median (Q1-Q3)	Z	p
• CA-15.3 (U/mL)	19.7 (9.8-71.3)	11.0 (7.4-16.3)	2.3	<0.01 *
• miRNA-21 ( $2^{-\Delta\Delta C_t}$ )	3.1 (2.2-5.2)	1.0 (0.93-1.06)	4.4	<0.01 *

\* $p < 0.01$ : Highly significant.

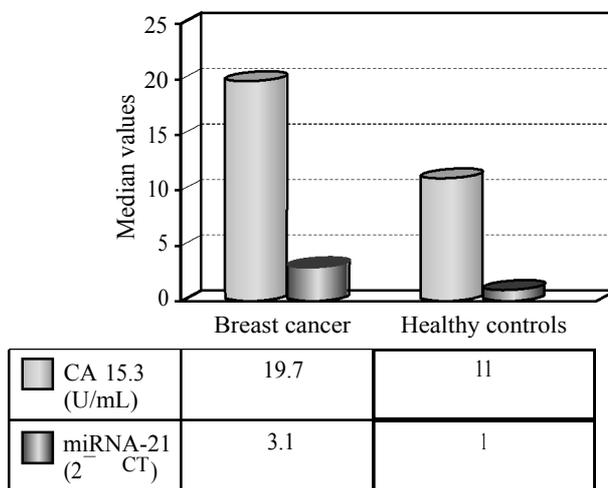


Fig. (3): Median values of CA 15.3 (U/mL) and miRNA-21 ( $2^{-\Delta\Delta C_t}$ ) in breast cancer patients versus healthy controls.

Table (3): Statistical comparison of CA15.3 and miRNA-21 expression levels among the subgroups of breast cancer patients Using Kruskal-Wallis Test for non-parametric data.

Parameter	Subgroup IA		Subgroup IB		H	p
	Stage I (n=30) Median (Q1-Q3)	Stage II (n=45) Median (Q1-Q3)	Stage III (n=35) Median (Q1-Q3)	Stage IV (n=40) Median (Q1-Q3)		
CA-15.3 (U/mL)	16.5 (11.9-31.0)	10.0 (7.5-17.1)	21.0 (8.8-50.0)	218.0 (53.3-556.5)	15.7	<0.001*
miRNA-21 (2 <sup>-ΔΔct</sup> )	1.6 (1.2-1.9)	2.6 (2.3-3.0)	3.5 (3.2-4.8)	7.4 (5.9-8.7)	24.0	<0.001*

\*p<0.001: Highly significant.

As seen in Tables (3,4), a highly significant statistical increase was recorded among patients with different stages of breast cancer regarding CA 15.3 (H=15.7, p<0.001) and miRNA-21 (H=24, p<0.001) using Kruskal-Wallis test for non-parametric data.

As demonstrated in Table (5), a statistically significant increase in miRNA-21 expression levels was found in breast cancer patients with negative ER, compared to breast cancer patients with positive ER, (Z=2.2, p<0.05). On the other hand, no statistically significant difference was found in the expression levels of miRNA-21 between breast cancer patients as regards PR status (Z=1.8, p>0.05). As regards CA 15.3 serum levels, no statistically significant difference was recorded

between breast cancer patients as regards ER and PR status (Z=0.93 and 0.4, respectively with p>0.05).

Table (4): Statistical comparison between CA15.3 and miRNA-21 in patients with different stages of breast cancer.

Parameter	CA 15.3 (U/mL)		miRNA-21 (2 <sup>-ΔΔCt</sup> )	
	Z	p	Z	p
Stage I/Stage II	3.2	<0.01	1.4	>0.05*
Stage II/Stage III	2.8	<0.01	1.5	>0.05*
Stage III/Stage IV	3.2	<0.01	2.6	<0.01**
Stage II/Stage IV	3.5	<0.01	3.5	<0.01**
Stage I/Stage III	3.0	<0.05	0.14	>0.05*
Stage I/Stage IV	3.1	<0.01	2.6	<0.01**

\*p>0.05: Non significant.

\*\* p<0.01: Highly significant.

Table (5): Statistical comparison between CA 15.3 levels and miRNA-21 expression levels in Group I (breast cancer patients) classified according to estrogen/progesterone receptors status.

Parameter	Status	CA 15.3 (U/mL)			miRNA-21 (2 <sup>-ΔΔCt</sup> )		
		M (Q1-Q3)	Z	p	M (Q1-Q3)	Z	p
ER status	Negative (n:60)	41.8 (8.3-153.1)	0.93	>0.05	4.9 (2.7-8.1)	2.2	<0.05*
	Positive (n:90)	17.1 (11.5-23.8)			2.8 (2.0-3.6)		
PR status	Negative (n:85)	21.7 (8.7-149.0)	0.4	>0.05	3.5 (2.7-7.1)	1.8	>0.05**
	Positive (n:65)	19.0 (13.5-27.0)			2.5 (2.0-3.7)		

ER : Estrogen Receptor.

PR : Progesterone Receptor.

\*p>0.05 : Non significant.

\*\*p<0.05: Significant.

Our correlation study between CA 15.3 levels and miRNA-21 expression levels in breast cancer patients (Group I) is shown in Table (6) using Spearman's rank correlation coefficient test. It revealed a highly statistically significant positive correlation between CA 15.3 levels and miRNA-21 expression levels (r<sub>s</sub>=0.56, p<0.001).

As shown in Table (7), serum CA 15.3 at the best chosen cut-off level of 12U/mL had a 70% diagnostic sensitivity, 60% specificity, 84% PPV, 40% NPV and 67.5% total efficacy. At the best

chosen cut-off level of 1.07 (2<sup>-ΔΔCt</sup>) for miRNA-21 expression levels, the marker displayed a 100% sensitivity, 90% specificity, 96.8% PPV, 100% NPV and 97.5% efficacy.

Table (6): Statistical correlation between the various studied parameters in Group I.

Parameter	miRNA-21 (2 <sup>-ΔΔCt</sup> )	
	r <sub>s</sub>	p
CA-15.3 (U/mL)	0.56	<0.01*

\*p<0.01: Highly significant.

Table (7): Diagnostic performance of CA 15.3 and miRNA-21 in discriminating Group I from Group II.

Parameter	Cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficacy (%)
CA 15.3 (U/mL)	12	70	60	84	40	67.5
miRNA-21 ( $2^{-\Delta\Delta Ct}$ )	1.07	100	90	96.8	100	97.5

Table (8) shows the diagnostic performance of CA15.3 and miRNA-21 in discriminating between patients with early breast cancer (Subgroup IA) and patients with advanced breast cancer (Subgroup IB). At cut-off level of 20.3U/mL, serum CA 15.3 had a sensitivity of 80%, 86.7% specificity, 85.7% PPV, 81.3% NPV and 83.3% total efficacy with AUC 0.843. At the best chosen cut-off level of 3.33 ( $2^{-\Delta\Delta Ct}$ ) for miRNA-21 expression levels, the marker displayed a 86.7% sensitivity, 100% specificity, 100% PPV, 88.2% NPV and

93.3% efficacy with AUC 0.973. Multi-ROC curve analysis was constructed for assessment of the diagnostic performance of combination of CA 15.3 at a cut-off level 8.0U/mL and miRNA-21 at a cut-off level of 3.33 ( $2^{-\Delta\Delta Ct}$ ) for discriminating between patients with early breast cancer (Subgroup IA) and patients with advanced breast cancer (Subgroup IB). At these cut-off values the diagnostic sensitivity was 93.3%, specificity 100%, PPV 100%, NPV 93.8% and efficacy 96.7% with AUC 0.992.

Table (8): Diagnostic performance of CA 15.3 and miRNA-21 in Subgroup IA versus Subgroup IB.

Parameter	Cutoff	Diagnostic sensitivity (%)	Diagnostic specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Diagnostic efficacy (%)
CA 15.3 (U/mL)	20.3	80	86.7	85.7	81.3	83.3
miRNA-21 ( $2^{-\Delta\Delta Ct}$ )	3.33	86.7	100	100	88.2	93.3
CA 15.3 (U/mL) and miRNA-21 ( $2^{-\Delta\Delta Ct}$ )	8/3.33	93.3	100	100	93.8	96.7

## Discussion

Clinical trials using circulating miRNAs as cancer biomarkers are being carried out in the United States and other countries [18]. In recent years, with the advent of gene expression profiling technologies, an increasing number of studies have revealed the genetic association between miRNAs and different cancers such as cancer breast [9,10]. Even though there were several reports using circulating miRNA markers for breast cancer detection, they were quite inconsistent [19-24]. In view of the previous observations, our study aimed at assessing the clinical utility of circulating miRNA-21 in breast cancer patients and correlating its level with the routinely used marker CA15.3.

Results of the present study revealed significantly increased levels of serum CA15.3 in breast cancer patients compared to healthy controls. This goes in agreement with Zhang et al., [25], Shao et al., [26] and Fejzić et al., [27], who proved that serum levels of CA 15.3 increased significantly in patients with breast cancer more than healthy controls. They added that, CA 15.3 is expressed on the luminal surface of the normal glandular breast secretory epithelium and its expression and secretion are increased with malignant cell transformation.

Our study also revealed that serum levels of miRNA-21 expression were significantly higher in breast cancer patients than in healthy controls. These results were in agreement with the findings of a study by Gao et al., [12] on 89 breast cancer female patients who had a histologically confirmed diagnosis as compared to 55 healthy controls. The latter research group concluded that miRNA-21 is a useful diagnostic serum marker in breast cancer. Moreover, Zhang et al., [28] measured serum expression levels of miRNA-21 in 106 breast cancer participants whose diagnosis was confirmed by histopathology and they had not received chemotherapy, radiotherapy, or operation. Their results confirmed a statistically significant increase in patients compared to healthy participants. They concluded that elevated serum expression levels of miRNA-21 have great clinical value as a biomarker in breast cancer. Moreover, Si et al., [29] found that the level of miRNA-21 was significantly higher not only in serum samples but also in tissue samples of breast cancer patients compared to their healthy controls.

The underlying mechanism of connection between miRNA-21 and breast cancer is the location of miRNA-21 gene on chromosome 17q23.2. This region is frequently amplified in breast tumors and

that genetic amplification in tumor tissues is correlated with high expression of miRNA-21 [30]. Another possible cause of miRNA-21 up-regulation is due to the presence of a CpG island region, 2 kb upstream the mature miRNA-21 sequence that could be hypomethylated in breast cancer causing up-regulation of its expression [31].

The first study that examined the circulating miRNA-21 expression in an Egyptian population was done by Toraih et al., [32] followed by other studies including Motawi et al., [33] who reported increased expression of serum levels of miRNA-21 in 30 patients with different stages of breast cancer compared to the levels found in healthy control women. The study referred their results to the oncogenicity of miRNA-21 which promoted tumor growth, invasion, angiogenesis, and metastasis by targeting and suppressing several apoptotic and tumor suppressor genes, including PDCD4, TPM1, PTEN and MASPIN.

Our current study showed a highly statistically significant increase in CA15.3 levels among the four different stages of breast cancer in patients group compared to healthy participants. This was similar to results of Nisman et al., [34] and Shao et al., [26], who confirmed the significant association of CA15.3 with TNM stages of breast cancer. Higher serum levels of CA15.3 were more often detected in patients with advanced stage as the elevated levels are related to the tumor burden.

As for the miRNA-21 expression levels, our study marked the highly statistically significant rise in its expression among different TNM stages of the disease. Moreover, miRNA-21 expression levels can successfully differentiate between early stages (I & II) and late stages of breast cancer (III & IV). These results were supported by Toraih et al., [32] and Han et al., [35], who suggested that miRNA-21 is a sensitive and specific non invasive biomarker of breast cancer as well as an indicator for the invasiveness and reported that the serum level of miRNA-21 was significantly high in breast cancer at all TNM stages with significant rise with progression of patients stage. At the same time, Rodríguez-Martínez et al., [13] stated that during neoadjuvant treatment, exosomal miRNA-21 expression levels directly correlated with tumor size and higher levels of exosomal miRNA-21 significantly associated with the presence of circulating tumor cells and thus liquid biopsies based on exosomal miRNA-21 can be a complementary clinical tool for improving breast cancer diagnosis and prognosis.

Another important finding in the current study was the significant high miRNA-21 expression levels in negative ER breast cancer patients, which represents a valuable prognostic and predictive importance in management protocols of these patients. This agrees with a previous research work done by Lee et al., [36] and Wang et al., [37], who reported that ER negativity expression was significantly associated with high miRNA-21 expression. However, in our study, no statistically significant difference was detected between patients with positive and negative PR in the expression of miRNA-21. Similar results were reported by Si et al., [29] and Wang et al., [37], who found no association between high miRNA-21 expression levels and PR status. The high expression of miRNA-21 in tumor stroma is associated with a much poorer clinical outcome in ER and/or PR negative patients. This is attributed to miRNA-21 targeting PTEN in Triple-Negative Breast Cancer (TNBC) tissue which leads to down-regulation of the tumor suppressor gene PTEN. These findings suggested that anti-miRNA-21-based therapies could be a valuable treatment in breast cancer especially in TNBC cases [38].

Few studies examined the relation between CA15.3 and ER/PR status in breast cancer patients to evaluate the prognostic value of CA 15.3 and its correlation with the molecular subtypes of breast cancer [26]. In our study, no statistically significant change was detected in serum levels of CA 15.3 with the ER and PR status. Our results are almost similar to the results shown by Rasmy et al., [39], who measured CA 15.3 serum levels in 280 cancer breast females with 233 ER positive and 196 PR positive patients. They found only a borderline significant correlation between CA 15.3 levels and ER expression. In addition, there was no significant correlation between CA 15.3 levels and PR expression.

On the other hand, Atoum et al., [40] reported that ER positive status (39 cases) was strongly correlated with elevated CA15.3 level among the studied 72 female patients (39/72 ER+/PR+). However, Shao et al., [26] reported that the elevation of CA15.3 levels was significantly greater in patients with ER negative when measured in 432 patients with histopathological molecular subtypes (luminal A, luminal B, Her-2 positive and TNBC). This discrepancy in results can be attributed to the difference in the sample size enrolled in the other study or the histopathological type of the disease.

Our study also revealed that miRNA-21 expression levels are highly significantly positively

correlated with CA 15.3 levels which enforce its possible future role in diagnosis and follow-up of breast cancer patients. This agreed with the results of Han et al., [35], who found positive correlation between the two markers and proves our suggestion for the possible role of miRNA-21 in the disease.

The diagnostic performance of CA 15.3 was studied by ROC curve analysis, where at cut-off level of 12U/mL, CA 15.3 is able to differentiate between breast cancer cases and the control group with 70% sensitivity, 60% specificity, 40% NPV, 84% PPV and 67.5% efficacy. These results were comparable to those of Thriveni et al., [41], who stated that at a cut-off 35U/mL, a rather lower diagnostic sensitivity 51.1% but a higher specificity of 100%. In other studies done by Toraih et al., [32] and Han et al., [35], miRNA-21 in breast cancer patients revealed lower diagnostic sensitivity (66.7%) and specificity (86.7% and 88.8%), respectively for the detection of breast cancer patients from healthy controls.

At our best chosen cut off  $1.07 (2^{-\Delta\Delta Ct})$ , miRNA-21 proved an outstanding diagnostic performance for early detection of breast cancer patients with 100% sensitivity, 90% specificity, 96.8% PPV, 100% NPV and 97.5% efficacy providing a new tool for the early effective management of the disease. Zhang et al., [28] observed a diagnostic performance of 77.4% sensitivity, 67.9% specificity at cut off  $0.453 (2^{-\Delta\Delta Ct})$ . ROC curve analysis was constructed to differentiate between patients with early breast cancer and patients with advanced breast cancer. CA 15.3 at cut-off level 20.3U/mL was able to differentiate between patients with early and advanced breast cancer with 80% sensitivity, 86.7% specificity, 85.7% PPV, 81.3% NPV and 83.3% efficacy. At different cut off 25U/mL chosen by Incoronato et al., [42] in their study, the diagnostic sensitivity was 75% and the specificity was 76% which proved low sensitivity of CA 15.3 for early detection of breast cancer.

This difference in diagnostic performance may be explained by the difference in sample size of the study, distribution of study population among different TNM stages of the disease and histopathological subtypes of the disease.

MiRNA-21 expression levels showed better performance in discriminating between patients with earlier stages (TNM stage I and II) of breast cancer and patients with advanced breast cancer (TNM stage III and IV) with 86.7% sensitivity, 100% specificity, 100% PPV, 88.2% NPV and 93.3% efficacy at cut off  $3.33 (2^{-\Delta\Delta Ct})$ . This agreed

with Toraih et al., [32], who proved that miRNA-21 distinguishes patients with advanced breast cancer from patients with earlier stages with 87.5% sensitivity and 92.9% specificity.

In our current study on applying multi-ROC curve analysis for assessment of the diagnostic performance of combination of CA 15.3 at a cut-off level 8.0U/mL and miRNA-21 at a cut-off level of  $3.33 (2^{-\Delta\Delta Ct})$  for discriminating between patients with early breast cancer (TNM stage I and II) and patients with advanced breast cancer (TNM stage III and IV), it revealed a sensitivity of 93.3%, specificity 100%, PPV 100%, NPV 93.8% and efficacy 96.7%. Therefore, the combination showed better diagnostic sensitivity and efficacy than each marker alone.

MiRNA-21 expression levels are significantly higher in breast cancer patients compared to healthy subjects. This finding supports the introduction of microRNA-21 in the panel for screening and early detection of breast cancer. Furthermore, our study proved that increased miRNA-21 expression levels correlated with progression of breast cancer stages. Together with its increased expression levels with negative ER status, this enforces its possible role as a prognostic marker in breast cancer follow-up and disease management protocols.

Our study provided the initial data for Egyptian population which might be further validated on a larger scale by a prospective study in a multicenter clinical trial.

#### Declaration:

All authors declare that there is no financial or institutional conflict of interest. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

#### References

- 1- FAKHRJOU A., NAGHAVI-BEHZAD M, MONTAZERI V., KARKON-SHAYAN F., LEILA L. and PIRI R.: The relationship between histologic grades of invasive carcinoma of breast ducts and mast cell infiltration. *South Asian. J. Cancer*, 5 (1): 5-7, 2016.
- 2- ROSENBERG R.D., YANKASKAS B.C., ABRAHAM L.A., SICKLES E.A., LEHMAN C.D., GELLER B.M., et al.: Performance benchmarks for screening mammography. *Radiology*, 24 (1): 55-66, 2006.
- 3- MOLINA R., BARAK V., VAN DALEN A., DUFFY M.J., EINARSSON R., GION M., et al.: Tumor markers in breast cancer-European group on tumor markers recommendations. *Tumour Biol.*, 26281-93, 2005.
- 4- FANG R., ZHU Y., HU L., KHADKA V.S., AI J., ZOU H., JU D., JIANG B., DENG Y. and HU X.: Plasma microRNA pair panels as novel biomarkers for detection

- of early stage breast cancer. *Front. Physiol.*, 9: 1879, 2019.
- 5- JUNG H.J. and SUH Y.: Circulating miRNAs in ageing and ageing-related diseases. *J. Genet. Genomics.*, 41 (9): 465-72, 2014.
  - 6- TAKAHASHI R.U., PRIETO-VILA M., HIRONAKA A. and OCHIYA T.: The role of extracellular vesicle microRNAs in cancer biology. *Clin. Chem. Lab. Med.*, 55 (5): 648-56, 2017.
  - 7- SCHETTER A.J. and HARRIS C.C.: Plasma microRNAs: A potential biomarker for colorectal cancer? *Gut.*, 58: 1318-9, 2009.
  - 8- HU L., AI J., LONG H., LIU W., WANG X., ZUO Y., et al.: Integrative microRNA and gene profiling data analysis reveals novel biomarkers and mechanisms for lung cancer. *Oncotarget.*, 78441-8454, 2016.
  - 9- TAKAHASHI R.U., MIYAZAKI H. and OCHIYA T.: The roles of microRNAs in breast cancer. *Cancers (Basel)* 7 (2): 598-616, 2015.
  - 10- KUROZUMI S., YAMAGUCHI Y., KUROSUMI M., OHIRA M., MATSUMOTO H. and HORIGUCHI J.: Recent trends in microRNA research into breast cancer with particular focus on the associations between microRNAs and intrinsic subtypes. *J. Hum. Genet.*, 62: 15-24, 2017.
  - 11- FUJITA S., ITO T., MIZUTANI T., MINOGUCHI S., YAMAMICHI N., SAKURAI K. and IBA H.: miR-21 gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. *J. Mol. Biol.*, 378: 492-504, 2008.
  - 12- GAO J., ZHANG Q., XU J., GUO L. and LI X.: Clinical significance of serum miR-21 in breast cancer compared with CA15-3 and CEA. *Chin. J. Cancer. Res.*, 25 (6): 743-8, 2013.
  - 13- RODRÍGUEZ-MARTÍNEZ A., De MIGUEL-PÉREZ D., ORTEGA F.G., GARCÍA-PUCHE J.L., ROBLES-FERNÁNDEZ I., EXPOSITO J., MARTORELL-MAR-UGAN J., CARMONA-SÁEZ P., GARRIDO-NAVAS M.D.C., ROLFO C., ILYINE H., LORENTE J.A., LEGUEREN M. and SERRANO M.J.: Exosomal miRNA profile as complementary tool in the diagnostic and prediction of treatment response in localized breast cancer under neoadjuvant chemotherapy. *Breast Cancer Res.*, 21 (1): 21, 2019.
  - 14- ANDREOPOULOU E. and HORTOBAGYI G.N.: Prognostic Factors in Metastatic Breast Cancer: Successes and Challenges toward Individualized Therapy. *J. Clin. Oncol.*, 26: 3660-2, 2008.
  - 15- Abbott. <http://www.corelaboratory.abbott/int/en/offerings/brands/architect/architect-i2000SR>, Accessed at 13/1/2018, 9:00 am.
  - 16- BUSTIN S.A., BENES V., GARSON J.A., HELLEMANS J., HUGGETT J., KUBISTA M., MUELLER R., NOLAN T., PFAFFL M.W., SHIPLEY G.L., VANDESOMPELE J. and WITTEWER C.T.: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.*, 55 (4): 611-22, 2009.
  - 17- KUMAR S., KEERTHANA R., PAZHANIMUTHU A. and PERUMAL P.: Overexpression of circulating miRNA-21 and miRNA-146a in plasma samples of breast cancer patients. *Indian J. Biochem. Biophys.*, 50 (3): 210-4, 2013.
  - 18- American Cancer Society. *Cancer Facts and Figures 2017*. Atlanta, GA: American Cancer Society, 2017.
  - 19- ROTH C., RACK B., MULLER V., JANNI W., PANTEL K. and SCHWARZENBACH H.: Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer. *Breast Cancer Res.*, 12, 2010.
  - 20- COOKSON V.J., BENTLEY M.A., HOGAN B.V., HORGAN K., HAYWARD B.E., HAZELWOOD L.D., et al.: Circulating microRNA profiles reflect the presence of breast tumours but not the profiles of microRNAs within the tumours. *Cell. Oncol.*, (35): 301-8, 2012.
  - 21- CHAN M., LIAW C.S., JI S.M., TAN H.H., WONG C.Y., THIKE A.A., et al.: Identification of circulating microRNA signatures for breast cancer detection. *Clin. Cancer Res.*, (19): 4477-87, 2013.
  - 22- CUK K., ZUCKNICK M., HEIL J., MADHAVAN D., SCHOTT S., TURCHINOVICH A., et al.: Circulating microRNAs in plasma as early detection markers for breast cancer. *Int. J. Cancer*, (132): 1602-12, 2013.
  - 23- GUO L., ZHAO Y., YANG S., CAI M., WU Q. and CHEN F.: Genome-wide screen for aberrantly expressed miRNAs reveals miRNA profile signature in breast cancer. *Mol. Biol. Rep.*, (40): 2175-86, 2013.
  - 24- FRERES P., WENRIC S., BOUKERROUCHA M., FASQUELLE C., THIRY J., BOVY N., et al.: Circulating microRNA-based screening tool for breast cancer *Oncotarget.*, (7): 5416-28, 2016.
  - 25- ZHANG S.J., HU Y., QIAN H.L., JIAO S.C., LIU Z.F., TAO H.T. and HAN L.: Expression and significance of ER, PR, VEGF, CA15-3, CA125 and CEA in judging the prognosis of breast cancer. *Asian Pac. J. Cancer Prev.*, 14 (6): 3937-40, 2013.
  - 26- SHAO Y., SU X., HE Y., LIU C. and LIU H.: Elevated Levels of Serum Tumor Markers CEA and CA15-3 Are Prognostic Parameters for Different Molecular Subtypes of Breast Cancer. *PLoS. One*, 10 (7): e0133830, 2015.
  - 27- FEJZIC´ H., MUJAGIC´ S., AZABAGIC S. and BURINA M.: Tumor marker CA 15-3 in breast cancer patients. *Acta. Med. Acad.*, 44 (1): 39-46, 2015.
  - 28- ZHANG J., JIANG C., SHI X., YU H., LIN H. and PENG Y.: Diagnostic value of circulating miR-155, miR-21, and miR-10b as promising biomarkers in human breast cancer. *Int. J. Clin. Exp. Pathol.*, 9 (10): 10258-65, 2016.
  - 29- SI H., SUN X., CHEN Y., CAO Y., CHEN S., WANG H. and HU C.: Circulating microRNA-92a and microRNA-21 as novel minimally invasive biomarkers for primary breast cancer. *J. Cancer Res. Clin. Oncol.*, 139 (2): 223-9, 2013.
  - 30- WANG W., REN F., WU Q., JIANG D., LI H., PENG Z., WANG J. and SHI H.: MicroRNA-497 inhibition of ovarian cancer cell migration and invasion through targeting of SMAD specific E3 ubiquitin protein ligase 1. *Biochem. Biophys. Res. Commun.*, 449 (4): 432-7, 2014.
  - 31- BADR F.M.: Potential role of miRNA-21 in breast cancer diagnosis and therapy. *J.S.M. Biotechnol. Bioeng.*, 3 (5): 1-8, 2016.

- 32- TORAIH A., MOHAMMED A., FARRAG S., WISSA N. and HOSNY S.: Pilot study of serum MicroRNA-21 as a diagnostic and prognostic biomarker in Egyptian breast cancer patients. *Molecular Diagnosis and Therapy*, 19: 179-90, 2015.
- 33- MOTAWI M.K., SADIK N.A., SHAKER O.G., EL MASRY M.R. and MOHAREB F.: Study of microRNAs-21/221 as potential breast cancer biomarkers in Egyptian women. *Gene*, 590 (2): 210-9, 2016.
- 34- NISMAN B., MAIMON O., ALLWEIS T., KADOURI L., MALY B., HAMBURGER T. and PERETZ T.: The prognostic significance of LIAISON (R) CA15-3 assay in primary breast cancer. *Anticancer Res.*, 33 (1): 293-300, 2013.
- 35- HAN J.G., JIANG Y.D., ZHANG C.H., YANG Y.M., PANG D., SONG Y.N. and ZHANG G.Q.: A novel panel of serum miR-21/miR-155/miR-365 as a potential diagnostic biomarker for breast cancer. *Ann. Surg. Treat. Res.*, 92 (2): 55-66, 2017.
- 36- LEE J.A., LEE H.Y., LEE E.S., KIM I. and BAE J.W.: Prognostic Implications of MicroRNA-21 Overexpression in Invasive Ductal Carcinomas of the Breast. *J. Breast Cancer*, 14 (4): 269-75, 2011.
- 37- WANG Y., ZHANG Y., PAN C., MA F. and ZHANG S.: Prediction of Poor Prognosis in Breast Cancer Patients Based on MicroRNA-21 Expression a meta-analysis. *PLoS One*, 10 (2): 1-13, 2015.
- 38- FANG H., XIE J., ZHANG M., ZHAO Z., WAN Y. and YAO Y.: miRNA-21 promotes proliferation and invasion of triple-negative breast cancer cells through targeting PTEN. *Am. J. Transl. Res.*, 9 (3): 953-61, 2017.
- 39- RAMSY A., ABOZEED W., ELSAMANY S., BAIOMY M.E., NASHWA A., AMRALLAH A., HASAAN E., ALZAHIRANI A., FARIS M., ALSALEH K. and AL-FARAJ A.: Correlation of preoperative Ki67 and serum CA15.3 levels with outcome in early breast cancers a multi-institutional study. *Asian. Pac. J. Cancer Prev.*, 17 (7): 3595-600, 2016.
- 40- ATOUM M., NIMER N., ABDELDAYEM S. and NASR H.: Relationships among serum CA15-3 tumor marker, TNM staging, and estrogen and progesterone receptor expression in benign and malignant breast lesions. *Asian. Pac. J. Cancer Prev.*, 13 (3): 857-60, 2012.
- 41- THRIVENI K., DESHMANE V., RAMASWAMY G. and KRISHNAMOORTHY L.: Diagnostic Significance of CA15-3 with Combination of HER-2/neu Values at 85<sup>th</sup> Percentiles in Breast Cancer. *Indian J. Clin. Biochem.*, 28 (2): 136-40, 2013.
- 42- INCORONATO M., MIRABELLI P., CATALANO O., AIELL M., PARENTE C., SORICELLI A. and NICOLAI E.: CA15-3 is a useful serum tumor marker for diagnostic integration of hybrid positron emission tomography with integrated computed tomography during follow-up of breast cancer patients. *B.M.C. Cancer*, 14 (356): 1-6, 2014.

## الحمض النووي الريبوزي الصغير-٢١ الموجود بالدم كمؤشر حيوي مبشر للكشف المبكر عن سرطان الثدي وتطوير الأمراض في الإناث المصريات

تعد تقنيات التصوير التقليدية ودلالات الأورام المستخدمة حالياً مثل المصل CA15.3 ذات خصوصية وحساسية محدودة لتحديد المراحل المبكرة من مرضى سرطان الثدي. تنظيم الأحماض النووية الريبوزية الصغيرة التعبير الجيني على مستوى ما بعد النسخ عن طريق تكسير الحمض الريبوزي المرسل و/أو تثبيط ترجمته حيث يمكن أن يعدلوا ما يصل إلى ٦٠٪ من جينات ترميز البروتين في الجينوم البشري زو يعد الحمض النووي الريبوزي الصغير-٢١ واحداً منها حيث تلعب وتعديلاته أدواراً حاسمة في سرطان الثدي.

تهدف الدراسة: إلى تقييم الدور التشخيصي والتكهنى للحمض النووي الريبوزي الصغير-٢١ في مرضى سرطان الثدي الإناث. وقد أجريت دراسة الحالات والشواهد على (١٥٠) مريضة بسرطان الثدي و (٥٠) من الإناث بنفس المرحلة العمرية كمجموعة ضابطة. قد تعرض جميع المشاركين في دراستنا لقياس تعبير الحمض النووي الريبوزي الصغير-٢١ الموجود في الدم باستخدام التفاعل التسلسلي البوليمري في الوقت (qRT-PCR) بالمقارنة مع مصل CA15.3.

أظهرت نتائج الدراسة زيادة نسبة الحمض النووي الريبوزي الصغير-٢١ في مرضى سرطان الثدي السيدات بالمقارنة بالمجموعة الضابطة (Z:4.4, p<0.01) ومع مراحل TNM المتقدمة من المرض (H:24, p<0.001) ومع مستقبلات هرمون الأستروجين السلبية (Z:2.2, p<0.05). أظهر الحمض النووي الريبوزي الصغير-٢١ في مرضى سرطان الثدي عند مستوى التعبير (2-ΔΔCt) حساسية ١٠٠٪ وخصوصية ٩٠٪ في تمييز المرضى الذين يعانون من سرطان الثدي من الضوابط الصحية. وفي الوقت نفسه، أظهر CA15.3 عد مستوى التعبير حساسية ٧٠٪ و ٦٠٪ خصوصية في التمييز بين المجموعتين. بينما أظهر الحمض النووي الريبوزي الصغير-٢١ في مرضى سرطان الثدي عند مستوى التعبير (2-ΔΔCt) 3.33 حساسية ٨٦.٧٪ وخصوصية ١٠٠٪ في التمييز المرضى الذين يعانون من سرطان الثدي المبكر من المرضى في مرحلة متقدمة من مرض. بينما كان لدى المصل CA15.3 حساسية ٨٠٪ و٨٦.٧٪ خصوصية في التمييز بين نفس مجموعات المرضى. نستخلص مما سبق أن مستويات الحمض النووي الريبوزي الصغير-٢١ في مرضى سرطان الثدي أعلى بكثير من الأصحاء كما أن زيادة مستويات الحمض النووي الريبوزي الصغير-٢١ في مرضى سرطان الثدي ترتبط بمراحل تقدم المرض كما ترتبط بوجود مستقبلات هرمون الأستروجين التي تعكس التشخيص والتأثير على بناء أساليب العلاج المختلفة.