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Serum miR-16 and CA15.3 combination as early diagnostic biomarkers for breast cancer in Egyptian females

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ABSTRACT

Background: Breast cancer (BC) represents one of the most lethal malignancies among women worldwide. MiRNAs play an important role in BC diagnosis and progression. Inconsistent data about miR-16 expression level was reported in BC, thus this study aimed to assess the expression level of serum miR-16 and explore its association with the clinicopathological features in Egyptian BC patients.

Methods: Expression level of miR-16 in sera was estimated in 100 BC patients, 30 benign breast hyperplasia, and 30 healthy controls using Quantitative real-time-polymerase chain reaction. In addition to the determination of CA15.3 levels by ELISA.

Results: Mir-16 expression level was significantly overexpressed in BC patients compared to patients with benign hyperplasia and healthy control, and was found to be associated with increased risk of BC development. Further, the expression level of miR-16 had a superior diagnostic ability to detect BC. However, the combination of miR-16 with CA15.3 enhanced the efficacy of the latter.

Conclusion: The combination of miR-16 with CA15.3 might be used as potential non-invasive diagnostic biomarkers for breast cancer.

1. Introduction

Breast cancer is one of the major cancers in women worldwide. It was considered the first cause of death in females and the 5th cause in both sexes [1]. In Egypt, breast cancer (BC) represents 32.4% of the reported malignancies in women [2].

Although the incidence rate is not as high as in many other countries, breast cancer's 5-year survival rate is low (28% to 68 %) [3,4]. Many factors contribute to this low survival outcomes, the main cause is that most patients are diagnosed at late stages.

Breast cancer is diagnosed using several imaging techniques including mammography, ultrasound, and magnetic resonance imaging; in addition to a variety of biochemical markers including ER, PR, and HER2, genetic markers like BRCA1 and BRCA2 genes in breast tissues, as well as serum m markers such as cancer antigen 15.3 (CA15.3) [5].

Despite CA15.3 being the most commonly used serum tumor marker in BC, the use of this marker is limited due to its low sensitivity and specificity [6]. In addition, the American Society of Clinical Oncology guidelines no longer recommend using serum CA 15.3 for BC screening, diagnosis, staging, or routine follow-up after primary therapy [7,8]. Accordingly, mammography was considered as the current gold standard for BC screening [9]. However, mammography also has significant limitations such as pain, anxiety, high rates of false positive results, and radiation exposure risks [10]. Therefore, finding non-invasive, safe, and accurate biomarkers for early diagnosis and prognosis of breast cancer remains an important research objective.

MicroRNAs (miRNAs) are a class of short non-coding RNAs of 19-25 nucleotides that regulate gene expression post-transcriptionally *via* direct interaction and degradation of target mRNAs [11,12]. Several studies have demonstrated the association of the aberrant expression of miRNAs with various cancers and the vital role of these miRNAs in improving the diagnosis and prognosis [13-15]. As reported, miRNAs have an impact on several biological processes such as cell differentiation, proliferation, and migration *via* the stimulation of many signaling pathways which reflects their important role in the etiology, progression, and prognosis of cancer [16,17]. MiRNAs act as tumor suppressors or oncogenes in cancer depending on their target genes [18,19].

One of these microRNAs, miR-16 is located on the 13q14 chromosome and is a member of the miR-15 (miR15/16/195/424/497) family. Mir-16 is ubiquitously expressed and one of the first miRNAs that was shown to be linked to human cancers [20]. It can act as either a tumor suppressor miRNA in many types of human cancer such as lung cancer [21] and hepatocellular carcinoma [22], or an oncomiR as in renal cell carcinoma [23].

Several contradictory data were obtained regarding miR-16 in breast cancer. Authors reported that the dysregulated expression of miR-16 suggests its oncogenic or tumor suppressive role [24-27]. Therefore, the present work was designed to assess the serum expression level of miR-16 and compare its clinical utility with that of CA15.3 in early diagnosed untreated breast cancer Egyptian patients.

2. Subjects and methods

2.1. Study design

This study included 130 patients admitted to the Medical Oncology Department, National Cancer Institute (NCI), Cairo University, Cairo, Egypt. Thirty age-matched females considered as a control group. Patients were classified into 30 with benign breast hyperplasia (BBH) and 100 with breast cancer according to tumor staging, grading, and molecular subtypes which were confirmed with histo-pathological examination. Patients with any other disease or cancers, take any treatment, and underwent chemo-or radiotherapy were excluded.

Clinical data were obtained from the files for each patient. At the beginning, all subjects signed informed written consent and the study followed The Declaration of Helsinki. The work was approved by the Ethics Committee and Institutional Review Board of NCI under IRB number of 202101-04-02001. A volume of four ml of venous blood samples were withdrawn from all participants and centrifuged at 2000 ×g for 10 min to separate sera and stored at -80°C till the analysis.

2.2 Methods

Determination of CA15.3

ELISA kit was purchased from Chemux Bioscience Inc., USA (Cat No. #10104) for the estimation of CA15.3 serum level according to the manufacturer's instructions.

MiR-16 expression analysis

Total RNA including miRNAs was extracted from sera using MiRNeasy Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Extracted RNA was reverse transcripted to synthesize the cDNA using TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher, USA).

TaqMan™ Gene Expression Master Mix (Applied Biosystems) and TaqMan™ miRNA qPCR Assay for miR-16 (assay ID# 000391, Applied Biosystems) were used to conduct the quantitative real-time PCR reactions, and the expression was normalized to miR-484 (assay ID# 00182, Applied Biosystems) [28,29]. The volume of the PCR reaction was 20 µl, consisting of 1 µl of cDNA as a template, 1 µl of 20× TaqMan™ MicroRNA Assay, 10 µl of 2× TaqMan® PCR Master Mix (Applied Biosystems), and finally 8µl of nuclease-free water. PCR reactions were performed using Real-Time PCR System (Applied Biosystems, CA, USA) as follows: 10 min at 95°C for enzyme activation, followed by 45 cycles at 95°C for 15 sec and then 60 sec at 60°C for annealing and extension. At last, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of miR-16 [30].

2.3 Statistical analysis

The normality of data distribution was verified using Shapiro-Wilk test; the mean±SD was used to express the normally distributed data, median and interquartile range (25^{th} and 75^{th} percentile) was used for non-normally distributed data, and categorical variables are expressed as frequencies (percentages). χ2 test was used to compare the differences between categorical variables. One-way ANOVA test followed by Tukey's post hoc or Kruskal Wallis test followed by Dunn test as appropriate were used to compare the difference between groups. The association between the circulating miR-16 level and the risk of BC was investigated using logistic regression analyses. The strength of the association was adjusted for age, the presence of family history and menstruation as potential confounders and their corresponding 95% confidence interval (CI). Receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic value of miR-16. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Baseline characteristics of the studied groups

As shown in Table 1, 100 patients with breast cancer were enrolled in this study with mean age of 39 years and range (26-65 years), in addition to 30 patients with benign hyperplasia (BBH) with mean age of 44 years and range (25-69 years) and 30 healthy donors with mean age of 33 years and range (29-62 years).

72% of BC patients, 46.7% of BBH patients and all healthy donors had no family history of breast cancer, while 28% of BC patients and 53.3% of BBH patients had positive family history. About 68% of BC patients, 70% of BBH patients and 76.7% of healthy donors were pre-menopause, while 32% of BC patients, 30% of BBH patients and 23.3% of healthy donors were post-menopause. There was no significant difference in age and menstruation state among all groups.

The majority of BC patients were grade II (72%), while 22% were grade III, and only 6% were grade I. Most BC patients had lymph node metastasis (84%) while 16% had negative lymph node metastasis. 18% of BC patients fell in stage I, 26% in stage II, 36% in stage III and 20% in stage IV. About 72% of BC patients had estrogen receptor, 75% had progesterone receptor and 69% did not have human epidermal growth factor receptor2. Patients were classified according to molecular subtypes into luminal A (34%), luminal B (35%), HER2 enriched (23%), and triple negative (8%).

Serum CA15.3 was significantly elevated in BC patients in comparison to the control group (p<0.001). In contrast, patients with benign breast hyperplasia exhibited a borderline significance in serum level of CA15.3 when compared to the control group and a non-significant difference was noticed when compared to BC patients (p=0.06 and p=0.100, respectively)

3.2 Association between the expression level of miR-16 and clinicopathlogical features in BC patients

Fig. 1 showed that miR-16 was significantly over-expressed in BC patients compared to benign hyperplasia and the control groups, with median values and interquartile range of [7.43(4.27-11.26), 2.62(1.31-5.63), and 4.9(0.05-8.54), respectively] (p=0.001 and p=0.002, respectively). While, patients with benign hyperplasia exhibited a non-significant difference in the expression level of miR-16 when compared to the control group (p=1).

BC patients were assigned into two groups based on the median miRNA expression level. Mir-16 did not show any significant associations with family history, menstruation state, histological grade, stage, lymph node metastasis, receptors status, and molecular subtypes as shown in Table 2.

3.3 MiR-16 expression level as risk factor for BC

Table 3 represents the results of logistic regression analyses performed to test the associations of miR-16 serum levels with the risk of breast cancer development. Mir-16 was associated with increased risk of BC development by 100%. This result remained significant after the adjustment of the potential confounders such as age, family history, and menstruation state.

3.3 Relative expression level of miR-16 as a potential diagnostic marker for BC patients

Fig. 2 illustrates the ROC curves of miR-16 relative expression level in addition to CA 15.3 serum levels to discriminate BC patients from the controls.

Mir-16 showed a high diagnostic value for BC at an optimum cut-off point of 2.54 with 98% sensitivity, 47% specificity, and an AUC of 0.71 (95% CI: 0.62–0.80, p<0.001). While, CA 15.3 had lower diagnostic value for BC at an optimum cut-off point of 1.29 with 82% sensitivity, 46% specificity, and an AUC of 0.67 (95% CI: 0.58–0.76, p < 0.001).

After the combination between CA15.3 and miR-16, the combinational ROC analysis showed that the AUC became 0.74 (95% CI: 0.65–0.82, p<0.001) with sensitivity and specificity of (74% and 70%, respectively).

Table 1. Basic characteristics of the studied group

Variables	Control (n=30)	BBH (n=30)	BC (n=100)	*p*-value
Age (years)	46.23±9.14	44.57±15.11	48.02±9.60	**0.277**
Family history, n (%)				
Positive	0(0)	16(53.3)	28(28)	
Negative	30(100)	14(46.7)	72(72)	
Menstruation, n (%)				**0.662**
Pre-menopause	23(76.7)	21(70)	68(68)	
Post- menopause	7(23.3)	9(30)	32(32)	
Histological grade,n (%)				
I			6(6)	
II	-	-	72(72)	
III			22(22)	
Stage, n (%)				
I			18(18)	
II		-	26(26)	
III	-		36(36)	
IV			20(20)	
Lymph nodes, n (%)				
Positive	-	-	84(84)	
Negative			16(16)	
ER status, n (%)				
Positive	-	-	72(72)	
Negative			28(28)	
PR status, n (%)				
Positive	-	-	75(75)	
Negative			25(25)	
HER-2 status, n (%)				
Positive	-	-	31(31)	
Negative			69(69)	
Molecular subtype,n (%)				
Luminal A			34(34)	
Luminal B			35(35)	
HER-2 Enriched	-	-	23(23)	
Triple negative			8(8)	
CA15-3 (U/mL)	8.84(6.80-13.06)	11.98(7.87-19.89)	14.61(9.78- 27) [a]	**<0.001**

Normally distributed variables are expressed as mean ± SD, non-normally distributed variables as median (inter-quartile range), and categorical variables as frequencies (percentages). In multiple comparisons, [a]p<0.05 *vs*. normal control group. ER: estrogen receptor; PR: progesterone receptor; HER-2: human epidermal growth factor receptor-2.

Table 2. Association of miR-16 expression level with clinical features in BC patients

	Mir16 expression		
	Low(n=46)	**High(n=54)**	***p*-value**
Family history, n (%)			**0.617**
Positive	14(30.4%)	14(25.9%)	
Negative	32(69.6%)	40(74.1%)	
Menstruation, n (%)			**0.242**
Pre- menopause	34(73.9%)	34(63.0%)	
Post- menopause	12(26.1%)	20(37.0%)	
Histological grade, n (%)			**0.072**
I	4(8.7%)	2(3.7%)	
II	28(60.9%)	44(81.5%)	
III	14(30.4%)	8(14.8%)	
Stage, n (%)			**0.427**
I	6(13.0%)	12(22.2%)	
II	12(26.1%)	14(25.9%)	
III	20(43.5%)	16(29.6%)	
IV	8(17.4%)	12(22.2%)	
Lymph nodes, n (%)			**0.457**
Positive	40(87.0%)	44(81.5%)	
Negative	6(13.0%)	10(18.5%)	
ER status, n (%)			**0.694**
Positive	34(73.9%)	38(70.4%)	
Negative	12(26.1%)	16(29.6%)	
PR status, n (%)			**0.247**
Positive	32(69.6%)	43(79.6%)	
Negative	14(30.4%)	11(20.4%)	
HER-2 status, n (%)			**0.327**
Positive	12(26.1%)	19(35.2%)	
Negative	34(73.9%)	35(64.8%)	
Molecular subtype, n (%)			**0.839**
Luminal A	14(30.4%)	20(37%)	
Luminal B	18(39.1%)	17(31.5%)	
HER-2 Enriched	10(21.7%)	13(24.1%)	
Triple negative	4(8.7%)	4(7.4%)	

ER: estrogen receptor; PR: progesterone receptor; HER-2: human epidermal growth factor receptor-2. Patients were divided according to the median of miR-16

Table 3. Binary logistic regression analysis of miR-16 as risk factor for breast cancer

	OR (95% CI)	**P-value**	**[†]Adjusted OR (95% CI)**	**P-value**
Age	1.02(0.99-1.1)	0.137		
Family history	1.1(0.52-2.19)	0.855		
Menstruation	1.29(0.64-2.63)	0.477		
miR-16 relative expression	1.12(1.05-1.19)	0.001	1.12(1.10-1.20)	0.001

OR: Odd ratio, 95% CI: 95% confidence interval, [†]: adjusted for age, family history and menstruation as potential confounders

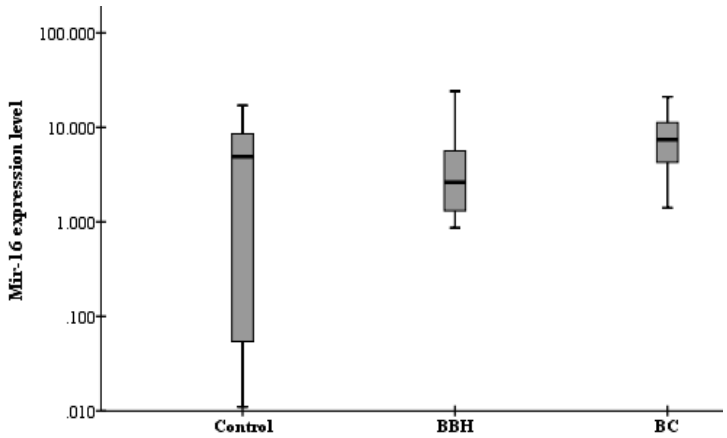
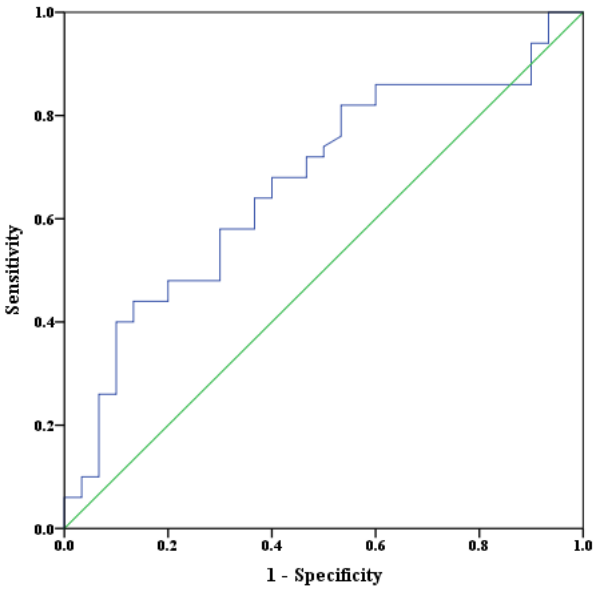
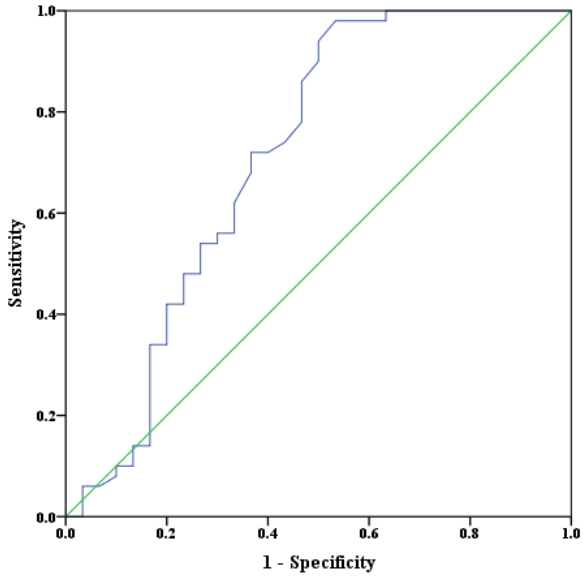


Fig. 1 Relative expression level miR-16 in the studied groups.
BBH: benign breast hyperplasia; BC: breast cancer. Statistically significant differences were determined using Kruskal Wallis test followed by Dunn, [a] p<0.05 *vs* control, and [b] p<0.05 *vs* BBH. The line inside the boxes represents the median value.

a)



b)



c)

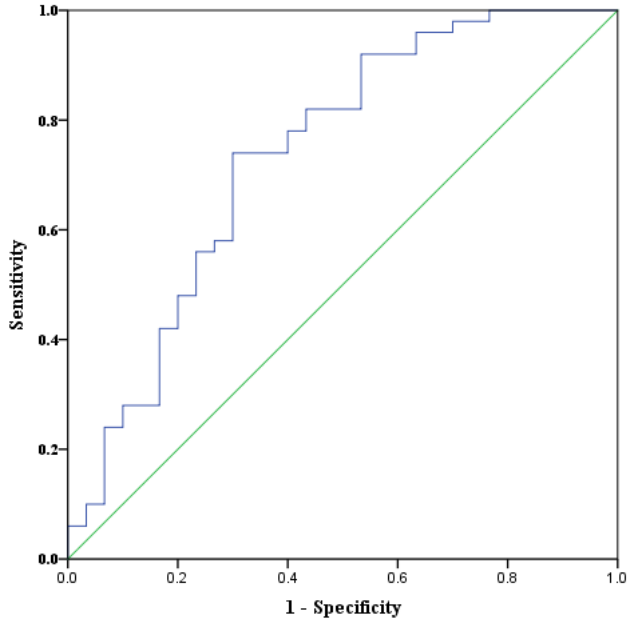


Fig. 2 ROC curves of **(a)** CA15.3 concentration, **(b)** miR-16 expression level, **(c)** combinations of miR-16 and CA15.3 for discriminating BC patients from healthy controls

4. Discussion

Breast cancer is one of the most three commonly diagnosed cancers in women. It is considered as the first leading cause of cancer related deaths between females [1]. The poor response of breast cancer to chemotherapeutic agents may be due to the late discovery of the disease. The diagnosis of BC at an early stage plays a vital role in decreasing the associated mortality rate and improving the survival and treatment outcomes of patients [31]. Despite mammography, ultrasound, and tumor markers are the currently used screening tool, the cost incurred and limited sensitivity and specificity have hampered the wide application of these tools [31]. Thus, there is still a pressing need to develop a cost-effective, accurate, and non-invasive markers for BC detection.

The emergence of microRNAs playing vital roles in oncogenesis has opened new opportunities for early cancer diagnosis. MiR-16 is a multifaceted miRNA that could function as an oncomiR or tumor suppressor in BC. Thus, its expression level was controversial [24, 26]. Therefore, the current study aimed to assess the expression level of miR-16 in sera of Egyptian BC patients and explore its clinical utility as a diagnostic marker.

In the current work, serum miR-16 was over-expressed in breast cancer patients when compared to control and benign hyperplasia groups. Consistent with our results, Fan et al. reported that serum level of miR-16 was higher in early stage of BC as well as in several breast cancer molecular subtypes when compared to healthy controls [26]. In addition, Stückrath et al. found that the plasma expression level of miR-16 was significantly upregulated in breast cancer patients compared to healthy individuals [32]. They also found that miR-16 was significantly increased in lymph-node negative patients compared to patients with lymph-node metastasis. According to Ni et al. findings, the level of miR-16 was higher in plasma exosomes of breast cancer and ductal carcinoma in situ (DCIS) patients compared to healthy controls and it is associated with estrogen and progesterone receptor status [33].

On the other hand, some studies have reported that the expression level of miR-16 was downregulated in breast cancer. Shin et al. showed that miR-16 was downregulated in the plasma samples, as well as in cancerous tissues of triple negative breast cancer (TNBC) patients compared to healthy controls [24].

Moreover, Feliciano et al. observed that miR-16 level was downregulated in serum of breast cancer patients compared to healthy controls [27]. Although several researches have supported the implication of miR-16 in various cancers by targeting different genes involved in tumor progression, its potential role in breast cancer remains elusive. Mir-16 was found to have a tumor suppressor role in breast cancer as mentioned in several studies [34,35]. Ruan and Qian found that miR-16 could inhibit the NF-κB pathway and decrease the *AKT3* gene in MCF-7 and BT-549 cell lines, so they deduced that miR-16 may suppress the development of breast cancer. Additionally, it was reported that miR-16 decreases cell proliferation and invasion by affecting the cell cycle as well as enhancing apoptosis through downregulating *ANLN* in breast cancer cell lines [34,35]. Moreover, Mobarra et al. found that the overexpression of miR-16 downregulates the expression of Bcl-2 in MCF-7 cell line [36]. However, other studies reported that miR-16 plays an oncogenic role in breast cancer where it acts as an oncomiR which is consistent with the results of this study [32,33]. Further, the results of our study was supported by the results obtained by Chen et al. who found that miR-16 was elevated in renal cell carcinoma where it acts as an oncogene by accelerating cellular proliferation and migration, and by reducing levels of apoptosis [23]. Furthermore, Liu et al. showed that the plasma level of miR-16 was significantly overexpressed in pancreatic cancer compared with normal controls [37]. Functional studies illustrated that up-regulation of miR-16 could result in the decline of dendritic cells and thus reduce immune responses, which led to immune escape and limitless proliferation of cancer cells [38]. These findings suggest that miR-16 is involved in numerous biological processes in tumor progression.

Herein, ROC curve analysis was performed to show the diagnostic ability of miR-16 serum levels to discriminate BC patients from controls. The result of this study displayed that miR-16 has a better diagnostic impact than CA15.3. Moreover, the combinational analysis of miR-16 with CA15.3 enhanced the diagnostic performance of the latter and gave the best diagnostic value compared to the miRNA alone.

In conclusion, the present study showed an over-expression level of miR-16 in the BC patients compared to controls. Moreover, the results revealed that an up-regulated level of miR-16 is associated with a higher risk of BC.

Thus, the miR-16 may be used as a novel target for BC therapy; further studies are required on a larger sample size. Finally, miR-16 appears to be a good candidate as a non-invasive biomarker for the early detection of BC, especially when combined with CA15.3.

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