A low serum microRNA-497-5p expression level is associated with primary breast cancer among Egyptian female patients

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Background/aim
Circulating forms of micro(mi)RNAs are nowadays increasingly recognized as noninvasive promising biomarkers for early diagnosis and management of breast cancer (BC). Among the numerous miRNAs studied in BC, tissue expressed miR-497-5p and miR-182-5p proved to serve as promising diagnostic, prognostic, and therapeutic target tools in BC; yet little is known about their circulating forms in the peripheral blood of such patients. The study aimed to evaluate serum expression levels of miR-497-5p and miR-182-5p in Egyptian female patients with newly diagnosed BC and their possible association with different clinicopathological features.

Patients and methods
The study was conducted on 50 primary BC patients at the Medical Research Institute, Alexandria, Egypt, in addition to 50 healthy female volunteers as a control group. Preoperative serum samples were taken from all patients and from healthy volunteers. Relative quantifications of serum miR-182-5p and miR-497-5p expression levels were done using a reverse transcription-quantitative real time PCR.

Results
The study showed that the median value for fold change in serum miR-497-5p expression was significantly down regulated in BC patients group compared to the healthy control group. A receiver operating characteristics curve generated a cutoff value of 0.54. In serum miR-497-5p expression level was used to discriminate BC patients from controls with a diagnostic specificity of 88%, a sensitivity of 56%, and an overall test accuracy of 68.8%. However, no statistically significant difference was noted in serum miR-182-5p expression level between BC patients and control group. Nevertheless, its serum expression level was significantly higher in BC patients with lymph node involvement compared with BC patients without nodal involvement.

Conclusion
The downregulated serum miR-497-5p expression in BC patients compared with the healthy control group points to loss of its protective role in such BC patients. Further studies of this miRNA on a larger sample of patients with different molecular subtypes are recommended.

Keywords:
breast cancer, microRNA-182-5p, microRNA-497-5p

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Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and is considered one of the leading causes of cancer-related deaths in women worldwide. Risk factors that initiate the process can be inherent factors such as age, sex, and race [1].

Recently, circulating nucleic acids such as cell-free DNA, messenger RNA, and microRNA (miRNA, miR) have evolved as possible biomarkers for BC diagnosis, prognosis, prediction of clinical management, and monitoring response to anticancer therapy. Circulating miRNA is markedly recognized as a promising biomarker due to its stability and resistance to ribonuclease degradation [2]. Such small noncoding RNA fragments, which can influence the expression of other protein-coding RNAs at post-transcriptional level [3], are released from the cells into the circulatory biofluids, such as the blood, saliva, and urine [4,5].

Approximately 50% of human miRNAs are localized in the chromosomal fragile sites or cancer-associated genomic regions, suggesting that the abnormal

expression of miRNA (miR) is related to cancer pathogenesis, being involved in cancer throughout the various stages starting with tumor origin, to immortalization, metastatic steps, and interactions with the host tissue [6]. Dysregulated miRNAs are either oncogenes or tumor suppressor genes. Oncogenes promote cancer development by downregulating tumor suppressor genes and/or genes that control cell differentiation or apoptosis. Underexpressed miRNAs in cancers function as tumor suppressor genes and may suppress cancer development through the regulation of oncogenes and/or genes that affect cell differentiation or apoptosis [7].

MiR-497-5p, a member of the cluster miR-195/497, is a tumor-suppressive miRNA that inhibits cellular growth, suppresses cellular migration, and invasion, and causes a G1 cell cycle arrest through targeting cyclin E1 [8]. MiR-497-5p was reported to be downregulated in various tumors such as gastric [9] and serous ovarian cancer [10]. Similar results were also reported in BC [11]. Moreover, the expression level of miR-497-5p was significantly downregulated in the BC tissue compared with its level in the adjacent normal tissue as well as to benign breast tumors like fibroadenoma [12].

However, miR-182-5p which is a highly conserved miRNA member of the miR-183/96/182 cluster was found to be increased in several human malignancies, such as BC [13], oral squamous cell carcinoma [14], and mesothelioma [15]. Being an oncogenic miRNA, miR-182-5p directly represses the endogenous expression of Forkhead Box O subfamily of transcription factors1 (*FOXO1*), which is a putative tumor suppressor [15,16]. In addition, breast cancer gene-1 (*BRCA1)*, a critical factor involved in maintaining genomic stability, is downregulated by miR-182-5p leading to the tumorigenic progression of breast and ovarian cancers [17].

Most of the studies involving both miR-497-5p and miR-182-5p were conducted on the breast tissue. The lack of data in the literature discussing the possible relation of their circulating forms to BC made it noteworthy to study the serum expression levels of both miRNAs in Egyptian female BC patients and their possible association with different clinicopathological features.

Patients and methods

Patients

This case–control study was conducted at the Medical Research Institute, Alexandria University, Alexandria, Egypt during the years 2018–2020. The present study included 100 Egyptian women in the age range of 45–60 years; 50 participants as the healthy control group and 50 BC patients; breast mass was confirmed by a mammogram. Breast tissue core biopsy was done to that group of patients with the initial diagnosis of BC.

Exclusion criteria for both patients and control groups

Women with any other disease or type of cancer other than BC that might interfere with the study assessments, women with a history of previous BC, and women who were receiving or have received any form of cancer treatment (radiotherapy or chemotherapy or hormonal therapy) were also excluded.

Study design

The present study was conducted on 50 newly diagnosed female BC patients scheduled for breast operation recruited from the Surgery Clinic of the Medical Research Institute, Alexandria University, and another 50 healthy female volunteers of comparable age recruited as a control group from the Internal Medicine Clinic of the Institute. Sample size calculation was done by the Department of Medical Statistics, Medical Research Institute, using PASS program, version 20 A.

Ethical approval

The present study was conducted in context with the Code of Ethics of the World Medical Association, according to the principles that were expressed in the Declaration of Helsinki. This study was approved by the local Ethics Committee of Medical Research Institute, Alexandria, Egypt with approval number IORG0008812, each participant included in this study provided a written informed consent before their participation.

Methods

To all the participating patients, thorough history taking was done stressing on age of first presentation, family history of cancer and drug(s) intake. Physical examination of the breast and draining lymph nodes was done. Mammography/ breast ultrasonography was done whenever possible. Histopathological examination of the breast tumor tissue was done by standard light-microscopic evaluation of sections stained with hematoxylin and eosin. Local tissue receptor status was assessed by immunohistochemistry [estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)]. Tumor, node,

and metastasis (TNM) staging was done with respect to tumor size and location, lymph node involvement, and distant metastasis.

MicroRNA analysis

Peripheral venous blood samples were collected from all participants under strict aseptic techniques in gel separator vacutainer tubes. The serum obtained following tube centrifugation was stored at −80°C till the time of molecular analysis. Total RNA was extracted from serum using Qiagen miRNeasy Mini Kit (Cat. no. 217004; Qiagen GmbH, Hilden, Germany). Total RNA concentration and purity were assessed by a nanodrop spectrophotometer (Thermo Scientific, Thermo Fisher, CA, USA).

Complementary DNA synthesis from RNA was carried out by reverse transcription on a conventional cycler (GeneAmp PCR system 9700; Applied Biosystems, Foster City, California, USA) using TaqMan miRNA reverse transcription master mix components and miRNA-specific RT primers for miR-497-5p, miR-182-5p, and miRNA-16-5p (internal control) (Applied Biosystems, Thermo Fisher Scientific, Baltics, Vilnius, Lithuania) according to the manufacturer's instructions.

Determination of microRNA-497-5p and microRNA-182-5p

Serum expression levels of miR-497-5p and miR-182-5p were determined from the cDNA using real time qPCR on the step-one real-time cycler (Applied Biosystems) with microRNA-16-5p serving as an endogenous control [18]. The TaqMan Universal Master Mix II, no uracil-N-glycoslyase and assays specific for miR-497-5p (ID#001043), miR-182-5p (ID# 002334), and miRNA-16-5p (ID# 000391) were used in this study. Relative expressions of miR-497-5p and miR-182-5p in serum were calculated using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) after normalization for the expression of endogenous control [19–21].

Statistical analysis of the data

Data were fed to a computer and analyzed using IBM SPSS software package, version 20.0 (SPSS ver.20.; IBM Corp., Armonk, New York, USA). Distributions of quantitative variables were tested for normality using the Kolmogorov–Smirnov test. The abnormally distributed data were described using median and range. The normally distributed data were described using mean and SD. Qualitative data were described using number and percent. The statistical tests used were χ^2 test, Student's t test, Mann–Whitney test, Kruskal–Wallis test, and Spearman's correlation coefficient. In all statistical tests, the 5% level of significance was used, below which the results were considered to be statistically significant. In addition, receiver operating characteristic curve (ROC) analysis was done for serum miR-497-5p (using SPSS), where the appropriately deduced cutoff value was used to calculate diagnostic sensitivity and specificity as well as overall test accuracy.

Results

The study was conducted on 50 females with primary operable BC and 50 healthy female volunteers as a control group, from whom serum samples were obtained. The mean age of the patients included in this study was 55.12±10.78 years. No significant differences were noted between the BC patients and controls regarding age and menopausal status (P=0.109 and 1.0, respectively), where the menopausal status in both groups was 48% equally (Table 1).

As regards immunohistochemical markers, all BC patients were ER positive and 96% of them were PR positive. As regards HER2, 32% of BC patients were positive while 68% were negative. The molecular subtypes were luminal A (64%) and luminal B (36%). Most of the BC patients (68%) were positive for lymph node involvement. Patients suffering from vascular invasion represented 76% of all BC patients in this study. Capsular and extranodal fat deposits were detected in 32% of cases. As regards TNM staging, 12% of BC patients were of stage I, 56% of BC patients were of stage II, and 32% were of stage III (Table 2).

Concerning the studied miRNAs, serum miR-497-5p expression level showed a significantly downregulated

Table 1 Descriptive statistics of the two studied groups according to age and menopausal status

	BC patients (N=50)	Controls (N=50)	Test of significance	P value
Age (years)				
Mean±SD	55.12±10.78	50.76±7.88	t=1.632	0.109
Menopausal status				
No	48%	48%	χ^2=0.0	1.000
Yes	52%	52%		

χ^2, χ^2 test; BC, breast cancer; t, Student's t test.

fold change ($2^{\wedge-\Delta\Delta Ct}$) in its median value in the BC patients compared with the control group (U=196, P=0.024). However, in the serum miR-182-5p the fold change in the expression level ($2^{\wedge-\Delta\Delta Ct}$) did not significantly differ between the BC patients and control groups, though approached the level of statistical significance (U=218.5, P=0.068), as shown in Table 3.

In addition, it was found that a significantly higher median value of expression level ($2^{\wedge-\Delta\Delta Ct}$) in serum miR-182-5p was noted in cases with nodal involvement [1.33 (0.19–11.24)] compared with cases without nodal involvement [0.45 (0.00–2.39)] (P=0.027) as shown in Fig. 1.

However, no statistically significant correlations were noted between serum miR-182-5p or serum miR-497 expression levels and age, tumor size and grade, LN involvement, and TNM stage as well as molecular subtypes of the tumor among the patient group (not tabulated).

Table 2 Demographic characteristic of breast cancer patients group according to histopathological findings

Grade II	80%
Grade III	20%
Tumor size (cm) >2 cm	72%
Median tumor size (IQR)	3.0 (2.0–4.0) cm
Minimum–maximum	1.0–6.0 cm
Lymph node involvement	68%
Vascular invasion	76%
Capsular and extra-nodal fat deposits	32%
Stage I	12%
Stage II	56%
Stage III	32%
ER positive	100%
PR positive	96%
HER2 positive	32%
Molecular subtype	
Luminal A	64%
Luminal B	36%

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IQR, interquartile range; PR, progesterone receptor.

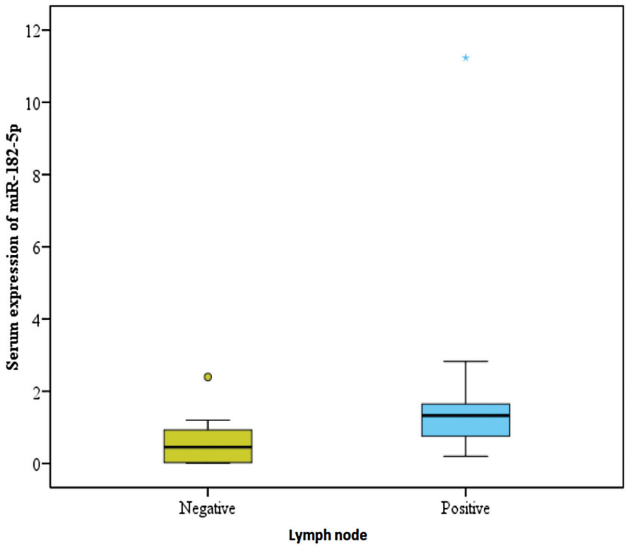
A ROC curve analysis for miR-497-5p serum expression level was done to discriminate BC cases from the control group. It showed an area under the curve of 0.686 (Fig. 2), so a best cutoff value for the miR-497-5p serum level of 0.54 was generated to discriminate BC cases from the control group, resulting in a diagnostic sensitivity of 56%, specificity of 88%, positive predictive value and negative predictive value of 81.2 and 64.7%, respectively, with an overall test accuracy of 68.8% (Table 4).

Discussion

The diagnostic and prognostic roles of microRNAs, particularly their circulating forms, are increasingly recognized nowadays in the management of several types of malignancies, among which is BC [2,22–25].

The present study indicated that serum miR-497-5p expression level in our study was significantly downregulated in BC cases compared with the

Figure 1



Box plot showing serum expression level of miR-182-5p in BC cases with nodal involvement versus cases without lymph node involvement. BC, breast cancer.

Table 3 Fold change in the expression level ($2^{\wedge-\Delta\Delta Ct}$) of microRNA-497-5p and microRNA-182-5p among the studied groups

Serum expression	BC patients (N=50)	Controls (N=50)	U	P value
MiR-497-5p				
Median (IQR)	0.47 (0.17–1.03)	0.91 (0.67–1.53)	196.0	0.024*
Minimum–maximum	0.05–3.73	0.20–7.16		
MiR-182-5p				
Median (IQR)	1.12 (0.49–1.53)	1.46 (0.74–2.64)	218.50	0.068
Minimum–maximum	0.0–11.24	0.01–11.08		

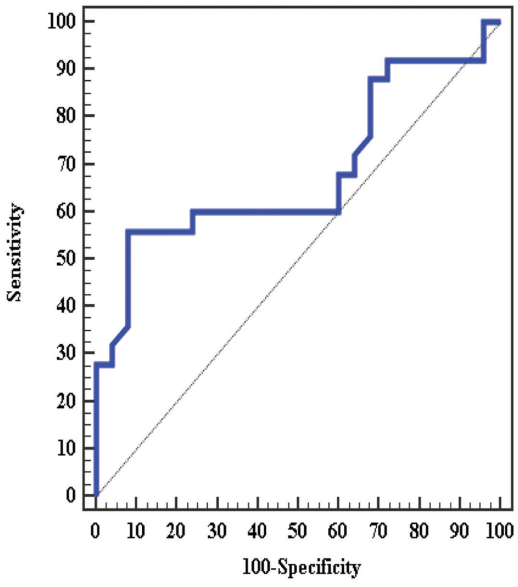
BC, breast cancer; IQR, interquartile range. *Significant difference at P value less than 0.05, using Mann–Whitney test.

control group (P=0.024), with 68% of BC cases showing underexpression of its serum level compared with 32% of cases showing overexpression

There were no statistically significant differences noted in serum miR-497-5p expression level among the different variables namely age, tumor grade, menopausal status, histopathological type, tumor size, lymph node metastasis, vascular invasion, capsular and extranodal fat deposits, TNM staging, HER2 receptors, and molecular subtypes.

The above-mentioned findings were in agreement with several studies. It was reported that there was a significantly reduced miR-497-5p expression in both tumor tissue and circulation of a murine model, 3 weeks following tumor induction. Such a study revealed the tumor suppressor effect of circulating miR-497-5p and the role of its reduced expression in tumor initiation and progression, which was essential for biomarker development [26].

Figure 2



ROC curve for serum expression level of miR-497-5p to discriminate BC cases from the control group. BC, breast cancer; ROC, receiver operating characteristic curve.

Another study demonstrated the protective role of miR-497-5p, being downregulated in BC, where miR-497-5p was one of the miRNA signatures (miR-16, miR-125b, miR-374a, miR-374b, miR-421, miR-655, and miR-497) eligible for predicting distant-disease-free survival in triple-negative breast cancer (TNBC) cases [27].

A third study revealed an underexpression of miR-497-5p in Lebanese BC tissues compared with the normal tissue and that could explain the significant upregulation of *CCND1* gene encoding for cyclin D1, which is a promoter of cell cycle progression [28]. In addition, another study demonstrated that miR-497-5p levels were downregulated in BC tissues compared with the adjacent healthy tissues, where miR-497-5p suppressed BC cell proliferation [11]. They concluded that long noncoding RNA HOXC13 antisense RNA (HOXC13-AS), which was significantly upregulated in BCs, enhanced cell proliferation through the suppressed miR-497-5p. However, a single study revealed that the serum level of miR-497-5p in BC patients were not significantly different from the control group [29].

In this study, the ROC curve analysis for miR-497-5p serum expression to discriminate BC cases from the control group revealed an area under the curve of 0.686, which generated a cutoff value for fold change in miR-497-5p serum expression of less than or equal to 0.54, resulting in a diagnostic sensitivity of 56%, specificity of 88%, positive predictive value and negative predictive value of 81.2 and 64.7%, respectively. This finding was in agreement with a study concerning the diagnostic value of miR-497-5p in BC, and found that the area under the miR-497 ROC curve was 0.719, generated a cutoff value of less than 0.54 giving a sensitivity of 56.84%, a specificity of 84.11%, and the critical value was 1.808, with a 95% confidence interval of 0.647–0.792 [30].

As regards serum miR-182-5p expression level, our study showed that 52% of the BC cases had overexpression of its serum level; yet no statistically significant difference was noted in serum miR-182-5p

Table 4 Diagnostic performance of serum microRNA-497-5p expression level to discriminate breast cancer cases from the control group

Serum expression level (fold change)	AUC	*P* value	95% CI	Cut off value[#]	Sensitivity	Specificity	PPV	NPV	Accuracy
MiR-497-5p	0.686	0.024*	0.535–0.837	≤0.54[#]	56.0%	88.0%	81.2%	64.7%	68.8%

AUC, area under the receiver operating characteristic curve; CI, confidence intervals; NPV, negative predictive value; PPV, positive predictive value. [#]Cutoff was chosen according to Youden index. *Statistically significant at *P* value less than or equal to 0.05.

expression level between BC cases and control group (P=0.068) (Table 1). The only significant difference noted in our study was its upregulation in BC cases with nodal involvement compared with cases without nodal involvement. Controversy existed in several studies concerning the above-mentioned findings. A study showed that not only the serum and tissue miR-182-5p levels in BC patients were significantly upregulated compared with the serum of healthy controls/noncancerous tissue (P<0.01), but also found that serum levels of miR-182-5p were lower in the receptor (ER and PR) positive cases compared with receptor-negative ones [31].

Another study revealed that the expression level of miR-183/182/96 cluster was significantly higher in BC tissues and cell lines. Moreover, miR-182-5p levels were correlated positively with both TNM stage, local relapse, and distant metastasis. In addition [32], a study using a microarray analysis on BC tissue reported a significantly up-regulated miR-183 and miR-182-5p in tumor versus normal adjacent breast tissues among Lebanese BC cases with a fold change of greater than five times [28].

It was reported that the miR-182-5p tissue expression level was significantly higher in cancerous versus normal breast tissues among Algerian patients with BC stages III and IV, thus supporting the notion that miR-182-5p might be a promising future target to inhibit invasiveness and metastasis in advanced BC [33]. Also, it was observed that miR-182-5p and miR-18a overexpression correlated with worse clinical and pathological tumor characteristics in locally advanced triple-negative BC, thus predicting the outcomes and prognosis in such patients. Epigenetically, miR-182-5p underexpression was most commonly associated with tumor size less than 6 cm and a clinical nodal status (N0-N1) [34].

Moreover, it was found that extracellular vesicles secreted by BC cells could carry miR-182-5p to aggravate BC through downregulating CKLF-like MARVEL transmembrane domain-containing 7 (CMTM7) expression and activating the epidermal growth factor receptor/protein kinase B EGFR/AKT signaling pathway [35].

It was reported that the miR-182-5p is significantly upregulated in breast tumors compared with the paired adjacent normal tissues. The higher expression of miR-182-5p in breast tumors reveals a significant association with larger tumors, higher tumor grade, and positive lymph nodes [36].

From the above-mentioned analysis of literature, we may attribute the nonsignificant upregulation in serum miR-182-5p expression level in BC to the fact that apart from a study or two, most studies were conducted on cancerous tissue samples only, and some included TNBC-enriched and HER2-enriched BC in their studies as well as TNM stage IV in another study, which was not the case in our study. Some limitations need to be addressed in this study. First, the relatively small sample size of BC cases that participated in this study as well as the absence of stage IV BC cases suffering from distant metastasis. Second, only luminal A and B molecular subtypes were included in this study with no HER2 positive or TNBC patients. Third, the absence of a group with a benign breast mass to compare serum levels of the two studied microRNAs to BC. Lastly, a multicenter study was lacking to explore the variation among the two studied microRNA expression according to several demographic variables particularly ethnic differences.

Conclusion

In conclusion, the fact that serum miR-497-5p expression level was significantly downregulated in BC cases compared with healthy control group supports the loss of its protective antitumorigenic role in BC. Further studies of this miRNA on a larger sample of BC cases with different molecular subtypes as well as TNM stages III and IV are highly warranted.

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Authors' contributions: Moyassar A. Zaki was responsible for conceptualization, data curation, methodology, review, and editing. Mohamed A. Ahmed practical part concerning microRNA analysis and interpretation, reviewing, and editing. Hazem M. Ehab El Mansy aided in recruiting patients and providing patients data, reviewing, and editing. Sahar M. Omar was responsible for sample collection, data investigation, laboratory investigations, providing reagents in addition to manuscript writing. Marwa A. Mohamed was responsible for conceptualization, data curation, visualization, laboratory analysis, supervision, administration, writing the manuscript, reviewing, and editing in addition to the submission of the manuscript to the journal. All authors read and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

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