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Serum MicroRNA-96 as a Potential Diagnostic Marker in Breast Cancer

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ABSTRACT

Breast cancer is the most commonly occurring cancer in women and the fifth leading cause of global cancer mortality. MicroRNAs (miRNAs) are essential regulators in the oncogenesis process and the identification of tumor-specific circulating miRNAs could be used for the early detection of cancer. We aimed to investigate the expression pattern of microRNA 96 (miR-96) and explore its diagnostic potential in breast cancer. This study comprised 30 treatment-naïve female patients diagnosed with primary breast cancer and 20 healthy volunteers as the control group. MicroRNA 96 (miR-96) expression was measured in serum samples using Reverse Transcription Quantitative polymerase chain reaction. The diagnostic value of miR-96 was analyzed with the Receiver-operating characteristics (ROC) curve. Our results show that miR-96 was significantly upregulated in breast cancer cases compared to control subjects ($P<0.05$). MiR-96 showed a significant diagnostic clinical value with the area under the curve (AUC) 0.959, 100% sensitivity, and 95.2% specificity. In conclusion, the current study implies that serum miR-96 may be a valuable and promising diagnostic marker for the early detection of breast cancer. Future studies are needed to investigate the potential role of miR-96 in predicting prognosis and monitoring response to treatment. Additionally, further research is required to study the feasibility of silencing miR-96 using antagomirs for the management of breast cancer.

Keywords: *microRNAs*, *miR-96; breast cancer; diagnosis; liquid biopsy*.

1. INTRODUCTION

Breast cancer is the most common cause of cancer incidence in women; where it now represents 11.7% of cancer cases worldwide, surpassing that of lung cancer [1]. It also became one of the leading causes of cancer mortality worldwide [1]. Therefore, efforts to provide early diagnostic measures and offer timely and optimum cancer care are warranted for cancer control worldwide [2]. Traditional biomarkers like CA15.3 and CA125 exhibit low specificity and sensitivity [3] and so novel biomarkers are urgently needed.

Altered microRNAs (miRNAs) expression has been observed in various human diseases including liver diseases [4], cardiovascular diseases [5], obesity, diabetes [6], and cancer [7]. This altered expression could be related to amplification and mutations in miRNAs, inhibition of miRNA processing, or epigenetic silencing [8]. MiRNAs can function as either oncogenes or tumor suppressors [9]. Oncogenic

miRNAs, also known as oncomiRs, including miR-21, miR-155, and miR-10b, are upregulated in various types of cancers where they promote tumor growth and metastasis [10-12]. On the other hand, miRNAs let-7 and miR-34a are tumor suppressor miRNAs that could prevent tumorigenesis [13, 14]. Dysregulation of many miRNAs has been implicated in the development of breast cancer [9]. Hence, a thorough investigation of the involvement of miRNAs in breast cancer development can provide better insights on tumorigenesis mechanisms and could help in the discovery of novel markers for the early detection of breast cancer.

Numerous studies have identified miRNAs as valuable biomarkers for the diagnosis of human cancers, and as prognostic and therapeutic targets or tools [15]. Circulating miRNAs are stable in circulation, can be easily quantified, and can reflect the underlying biology of the tumor [16]. To date, approximately 4000 miRNAs were identified, and they are estimated to be involved in the regulation of more than 30% of human genes, where they are involved in many biological processes [17]. MiRNAs pair to the complementary sequences located in the 3′-untranslated region (UTR) of the corresponding messenger RNA (mRNA), which leads to either translation inhibition or destabilization [17]. Additionally, miRNAs can regulate gene expression through binding to protein-coding exons [8].

MicroRNA 96 (miR-96) is an oncogenic miRNA that has been involved in various types of cancers including hepatocellular carcinoma, pancreatic cancer, colorectal cancer, and breast cancer [18-21]. However, the expression of serum miR-96 in breast cancer and its potential diagnostic role has not been fully elucidated and its regulatory mechanisms are still unclear. Hence, this study was undertaken to investigate the expression of circulating miR-96 in breast cancer and investigate its utility as a diagnostic biomarker for the early detection of breast cancer.

2. SUBJECTS and METHODS

2.1. Subjects

In total, 30 treatment-naïve female patients with histologically proven primary breast cancer were recruited from the Department of Clinical Oncology, Ain Shams University Hospitals, Egypt. The exclusion criteria were: i) Female breast cancer patients receiving anticancer treatment before surgery; ii) Any previous history of malignancy other than breast cancer or evidence of distant metastasis, and iii) Other diseases (including diabetes, liver diseases, and cardiovascular diseases). In addition, 20 healthy volunteers were included representing the control group. Written informed consents were signed and collected from all study participants. The protocol of this study was approved by the Research Ethical Committee, Faculty of Pharmacy, Ain Shams University, and conducted following the regulations and recommendations Helsinki Declaration.

2.2. Methods

2.2.1. Serum samples

Blood samples were collected from the recruited subjects on plain vacutainer tubes. After collection, blood samples were allowed to clot and then centrifuged. The serum samples were then stored at –80 °C.

2.2.2. RNA extraction from serum samples

Extraction of total RNA from serum samples was carried out using miRNeasy Serum/Plasma kit (Qiagen GmbH) following the manufacturer's protocol. The quality of total RNA was measured with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Extracted RNA samples were then stored at −80 °C for subsequent analysis.

2.2.3. Reverse transcription-quantitative PCR analysis

First, synthesis of complementary DNA (cDNA) was carried out using 1 µg RNA with the use of TaqMan[TM] MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California) as stated in the protocol of the manufacturer. RT-qPCR assays were carried out using Rotor-Gene Q Real-Time PCR cycler utilizing specific TaqMan[TM] Universal Master Mix II and specific TaqMan[TM] MicroRNA readymade essay for miR-96 (catalog number 000186). To normalize the expression level of miR-96 in serum samples, we used miR-16 (TaqMan[TM] assay catalog number 000391) as an endogenous control for the assay **[22]**. Relative levels of miR-96 were calculated using the $2^{-\Delta\Delta Ct}$ method **[23]**.

2.2.4. Statistical analysis

Mean and standard deviation (SD) were applied to represent numerical data, and the independent t-test was applied for examining differences between groups. The diagnostic value of miR-96 was evaluated using the receiver operating characteristic (ROC) curve. Specificity and sensitivity were determined using Youden's index according to the equation (sensitivity + specificity-1) **[24]**. Statistical analysis was executed using SPSS software IBM[©] SPSS[©] Statistics version 26 (IBM[©] Corp., Armonk, NY), and statistical significance was set at $P<0.05$.
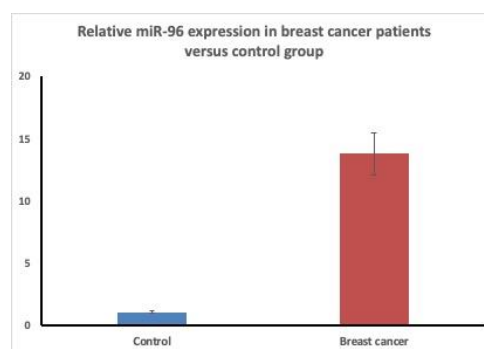
3. RESULTS

3.1. The clinical characteristics of breast cancer patients

The clinical and pathological characteristics of breast cancer patients are shown in **Table 1**. The breast cancer group had a mean age at diagnosis of 43.9 ± 10.3 years, while the mean age of the control group was 35.2 ± 3.1. The predominant hormone receptor status in breast cancer patients was ER, PR positive, and Her2/neu negative. The majority of tumors were T1-T2 (66.67%), and positive lymph node involvement was detected in 73.3% of patients. Positive family history was reported in 14% of cases.

3.2. MiR-96 expression levels in serum samples of breast cancer patients and controls

As shown in **Fig. 1**, miR-96 expression level was analyzed in serum samples of 30 breast cancer patients and 20 healthy controls. The results of this study showed that the expression of miR-96 in breast cancer serum samples was significantly upregulated (13.7 ± 1.7) in comparison to the control group $(1.05\pm0.08, P<0.05)$.

Fig. 1. Serum miR-96 levels in breast cancer patients versus control group. Data are presented as mean ± SD

3.3. Diagnostic value of miR-16 in serum samples of breast cancer patients and controls

The ROC curve was plotted to explore whether miR-96 serum levels exhibited diagnostic value for early detection of breast cancer; and its area under the curve (AUC), diagnostic specificity, and sensitivity were determined (**Fig. 2**). The present study indicates that miR-96 exhibited high accuracy in discriminating patients with breast cancer from control subjects with AUC, 0.959; sensitivity, 100%; and specificity, 95.2% (**Table 2**).

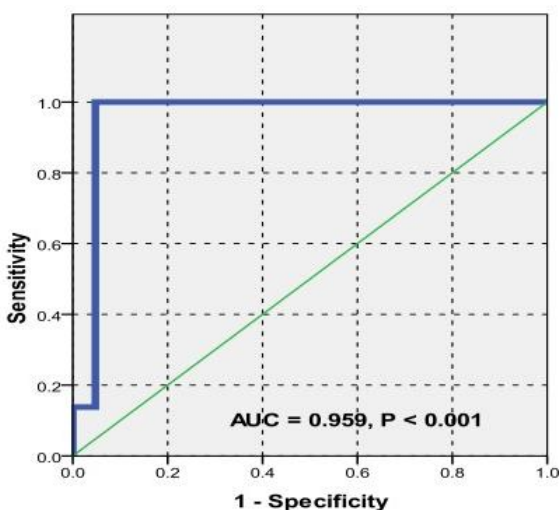**Table1. Clinical Characteristics of breast cancer patients**

Parameter	Mean ± SD/ Count (%)
Age (years)	43.9 ± 10.3
Family history of breast cancer	7 (14.0%)
BIRADS	
BIRADS 4	4 (13.33%)
BIRADS 5	23 (76.67%)
BIRADS 6	3 (10.0%)
ER	
Positive	26 (86.67%)
Negative	4 (13.33 %)
PR	
Positive	27 (90.0%)
Negative	3 (10.0%)
HER2/neu receptor	
Positive	3 (10.0%)
Negative	27 (90.0%)
T stage	
T1-T2	20 (66.67%)
T3-T4	10 (33.33%)
LN stage	
N0	8 (26.6 %)
N1-N2	22 (73.33%)

BIRADS, Breast Imaging Reporting and Database System; LN, lymph node; ER: Estrogen receptor; PR: Progesterone receptor; HER2/neu, human epidermal growth factor receptor; T; tumor size; N, nodal involvement

Table 2. Receiver-operating characteristic (ROC) curve analysis of serum miR-96 for discrimination between breast cancer patients and healthy controls

ROC metric	miR-96
AUC	0.959
SE	0.04
95% CI	0.000 to 1.000
P-value ($AUC_0=0.5$)	<0.0001
Youden index J	0.95
Associated criterion (Cut-off)	5.66
Sensitivity (%)	100
Specificity (%)	95.2

AUC, area under the curve; miR, microRNA; SE, standard error; CI, confidence interval

Fig. 2. Receiver-operating characteristic (ROC) curve for discrimination between cases of breast cancer and controls using miRNA 96. AUC: Area under the curve

4. DISCUSSION

Evidence has shown that miRNAs could reflect the homeostatic processes of the organism, as well as tumor biology [25]. Accordingly, abnormally expressed miRNAs in the serum and/or plasma can serve as potential circulating biomarkers for diagnosis, predicting prognosis, and as tools for personalized treatment approaches for breast cancer patients [8].

MiR-96 belongs to the miR-183-96-182 cluster that was found to be located on human chromosome 7q32.2 within a 5-kb region [26]. Differentially expressed miR-183-96-182 cluster was observed in different types of cancers, although the exact role of this miRNA cluster remains unclear, where it can function as either tumor suppressor gene or an oncogene, depending on tumor stage, type, and location [27-29]. However, MiR-96 was found to function as an oncogene during tumorigenesis [30].

Although evidence from preclinical studies demonstrated the oncogenic role of miR-96 in various types of cancers, only a few clinical studies have explored its oncogenic role. The current study investigated the expression of circulating miR-96 in breast cancer patients and its potential diagnostic role for early breast cancer detection. The results of our study revealed that miR-96 expression was significantly upregulated in serum samples of breast cancer patients in comparison to healthy controls, which is in alignment with a previous study by Xie *et al* [31]. The oncogenic role of miR-96 was previously studied using miRNA mimics in breast cancer cell lines and its downregulation by inhibitors was shown to decrease cell migration [32]. MiR-96 could promote invasiveness and migration of breast cancer cells through the downregulation of metastasis suppressor-1 (MTSS1) gene expression [31]. Additionally, miR-96 was shown to enhance tumor growth through the inhibition of protein tyrosine phosphatase, non-receptor type 9 (PTPN9) expressions in breast cancer xenograft mouse model, enabling proliferation and invasion of breast tumor cells [30].

Previous studies have demonstrated that the upregulation of the expression of miR-96 contributes to the proliferation of hepatoma cells and breast cancer cells through targeting the transcriptional factor, forkhead box protein FOXO3a [19, 21]. Moreover, the overexpression of miR-96 was also shown to downregulate transcriptional factor FOXO1 [33]. The oncogenic role of miR-96 was also observed in prostate cancer cells and thyroid carcinoma through inhibition of expression of FOXO1 [34, 35]. Xu *et al* also suggested that miR-96 inhibitor treatment could partially suppress the invasiveness of prostate cancer cells [18]. MiR-96 could promote the proliferation of esophageal cancer by down-regulating RECK [36] and could induce proliferation and invasiveness of hepatocellular carcinoma via targeting EphrinA5 [37]. A previous study also showed that miR-96

promotes chemoresistance in non-small cell lung cancer cells through downregulating the expression of SAMD9 **[38]**.

The present study also revealed that miR-96 possesses significant diagnostic value in breast cancer with AUC 0.959, 100% sensitivity, and 95.2% specificity, highlighting its potential value in the discrimination of breast cancer patients from healthy subjects. This finding could be of great clinical value through easy and rapid access to samples, which can allow early diagnosis and of breast cancer.

In conclusion, this study implies that miR-96 is overexpressed in serum samples of breast cancer patients and may be involved in the development of breast carcinogenesis. MiR-96 may be a promising and valuable diagnostic marker for the early detection of breast cancer and can contribute to the optimization of breast cancer management. Future studies involving a larger cohort of patients are demanded to confirm our results and to explore the potential role of miR-96 in predicting prognosis and monitoring response to treatment. Additionally, further research is required to study the feasibility of silencing miR-96 using antagomirs for the management of breast cancer.

List of Abbreviations

miRNA, MicroRNA; miR-96, MicroRNA 96; ROC, cDNA, Complementary DNA; Receiver-operating characteristics; AUC, the area under the curve; 3′-UTR, 3′-untranslated region; PR: Progesterone receptor; SD, standard deviation; CI, confidence interval; LN, lymph node, RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ER: Estrogen receptor; BI-RADS, Breast Imaging Reporting, and Database System; HER2/neu, human epidermal growth factor receptor.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Research Ethical Committee, Faculty of Pharmacy, Ain Shams University, and conducted according to the regulations and recommendations of the Declaration of Helsinki. Written informed consents were signed and collected from all study participants.

Consent to publish

All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Data analyzed during this study are all included in the main manuscript.

Competing interests

No competing interests were declared by the authors.

Funding statement

No funding source was received

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