

Serum expression levels of microRNA-382-3p, -598-3p, -1246 and -184 in breast cancer patients

LUN FU¹, ZHAOYUN LI^{1,2}, JIE ZHU², PAN WANG², GUANGMIN FAN², YUECHU DAI²,
ZHIBAO ZHENG² and YANG LIU³

¹College of Laboratory Medicine and Life Science, Wenzhou Medical University,
Wenzhou, Zhejiang 325035; ²Taizhou Central Hospital, Taizhou, Zhejiang 318000;

³The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang 310009, P.R. China

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Abstract. The purpose of the present study was to investigate the serum levels of microRNA (miRNA/miR)-382-3p, -598-3p, -1246 and -184 in breast cancer patients and to assess their feasibility as biomarkers for breast cancer screening. Serum samples were obtained from 100 breast cancer patients and 40 age-matched healthy control subjects in Taizhou Central Hospital (Taizhou, Zhejiang, China) between January 2013 and September 2014. The serum expression levels of miR-382-3p, -598-3p, -1246 and -184 were determined by stem-loop reverse transcription-quantitative polymerase chain reaction. Receiver operating characteristic curves were drawn to evaluate the sensitivity and specificity of the serum miRNA expression levels for the screening of breast cancer. miR-382-3p and -1246 were significantly upregulated in the serum of the breast cancer patients, while miR-598-3p and -184 were significantly downregulated. The sensitivity and specificity to detect breast cancer were as follows: miR-382-3p, 52.0 and 92.5%; miR-598-3p, 95.0 and 85.0%; miR-1246, 93.0 and 75.0%; and miR-184, 87.5 and 71.0%, respectively. The expression levels of the four serum miRNAs were not correlated with the patients' clinical stage. In summary, miR-382-3p, -598-3p, -1246 and -184 are all involved in the development of breast cancer, and are promising biomarkers for breast cancer detection.

Introduction

Breast cancer has become the most common cancer in women in China, accounting for 12.2% of all newly diagnosed breast cancers and 9.6% of all mortalities from breast cancer worldwide (1). Early detection of breast cancer is the key to

successful treatment and patient survival. Mammographies, magnetic resonance imaging and ultrasound are being used for the screening of breast cancer, but these techniques have limitations such as low sensitivity and specificity (2). Finding effective biomarkers is vital for the screening of breast cancer.

MicroRNAs (miRNAs/miRs) are a group of small non-coding RNAs (3), which can bind to the 3' untranslated region (UTR), the 5' UTR (4) or the coding region (5) of target mRNA. miRNAs play important roles in the negative regulation of gene expression in a post-transcriptional manner, and are involved in a number of signal transduction pathways, including cell proliferation, differentiation, apoptosis, immune response and angiogenesis (6). Previous studies have revealed the abnormal expression of miRNAs in the development and progression of breast cancer (7), and miRNAs are closely linked with tumor-associated signal transduction pathways (8). In our previous study, 10 miRNAs that were significantly differentially expressed were found by the second generation of high-throughput sequencing technology, Illumina Hiseq2500 (9). In the present study, 4 miRNAs, miR-382-3p, -598-3p, -1246 and -184, were further investigated. The purpose of this study was to investigate the serum levels of these miRNAs in breast cancer patients and to assess their feasibility as biomarkers for breast cancer screening.

Materials and methods

Subjects. A total of 140 serum samples were obtained in Taizhou Central Hospital (Taizhou, Zhejiang, China) between January 2013 and September 2014, including 100 serum samples from breast cancer patients and 40 samples from 40 age-matched healthy women. The clinical characteristics of the breast cancer patients are listed in Table I. The present study was approved by the Ethics Committee of Taizhou Central Hospital, Taizhou, China.

Reagents and instruments. The QIAzol Lysis Reagent, miRNeasy Serum/Plasma kit, miScript II Reverse Transcription (RT) kit, miScript Primer assays and miScript SYBR Green Polymerase Chain Reaction (PCR) kit were purchased from Qiagen GmbH (Hilden, Germany). Ethanol and chloroform were purchased from Adamas-beta (Shanghai, China).

Correspondence to: Professor Zhaoyun Li, College of Laboratory Medicine and Life Science, Wenzhou Medical University, 268 West University Road, Wenzhou, Zhejiang 325035, P.R. China
E-mail: lzy8151@163.com

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Table I. Clinical features of the breast cancer patients.

Patient characteristics	Value
Mean age (range), years	50.17 (25-80)
Stages, n	
I	15
II	46
III	24
IV	6
Unknown	9
Lymph nodes, n	
Positive	37
Negative	57
Unknown	6
ER, n	
Positive	54
Negative	30
Unknown	16
PR, n	
Positive	46
Negative	38
Unknown	16
HER2, n	
Positive	56
Negative	26
Unknown	18

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

Primers were purchased from Sangon Biotech (Shanghai, China) and the StepOnePlus PCR thermal cycler was purchased from Applied Biosystems (Foster City, CA, USA).

RNA extraction and purification. Total RNA was isolated from 200 μ l of plasma using the miRNeasy Serum/Plasma kit according to the manufacturer's protocols. RNA was dissolved in RNase-free water. Following the use of QIAzol Lysis Reagent, exogenous cel-miR-39 (Sangon Biotech) was added as an internal standard.

RT-quantitative (q)PCR. RT-qPCR was performed using the ABI StepOne system (Qiagen GmbH). All experiments were performed as specified in the manufacturer's protocols. RT reactions were performed with a reaction mixture that included 4 μ l 5X miScript HiSpec Buffer, 2 μ l 10X miScript Nucleics Mix, 10 μ l RNase-free water, 2 μ l miScript Reverse Transcriptase Mix and 2 μ l RNA. The reaction occurred at 37°C for 60 min and then 95°C for 5 min. The obtained cDNA was stored at -80°C.

Amplifications were performed using the miScript SYBR Green PCR kit (Qiagen GmbH) with a reaction mixture that included 12.5 μ l 2X QuantiTect SYBR Green PCR Master Mix, 2.5 μ l 10X miScript Universal Primer, 2.5 μ l primers, 5 μ l RNase-free water and 2.5 μ l cDNA. The PCR conditions

Table II. Polymerase chain reaction primers.

Products	Primers (5'-3')
miR-382-3p-F	CTGCAATCATTACGGACAAC
miR-598-3p-F	AGCTACGTCATCGTTGTCATC
miR-1246-F	GCCGAATGGATTTTGGAGC
miR-184-F	ATGGACGGAGAACTGATAAGG
Reverse primer	GTGTCTGTCGAGTCGGCAATTC

F, forward primer. The miScript Universal Primer in the miScript SYBR Green PCR kit was used as the reverse primer.

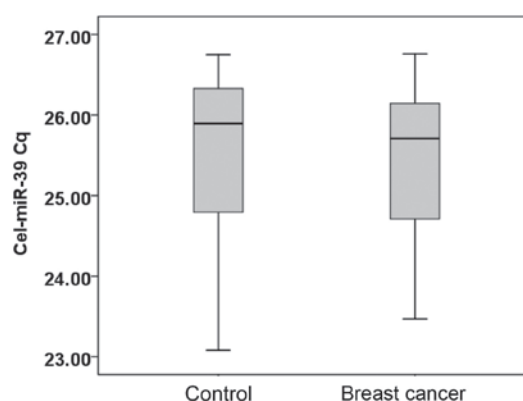


Figure 1. Cel-miR-39 Cq value in healthy controls and breast cancer patients. Cq, quantitation cycle; miR, microRNA.

were as follows: Initial denaturation at 95°C for 15 min; then 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec for 40 cycles. The melting curve was obtained from 65°C to 95°C. A quantitation cycle (Cq) value >35 was regarded as impossible for amplification. qPCR primers are shown in Table II. Each sample was analyzed in triplicate. The relative expression of the miRNA (10) was calculated using the $2^{-\Delta\Delta Cq}$ method as follows: $\Delta\Delta Cq = (Cq_{miRNA} - Cq_{miR-39})_{cancer\ group} - (Cq_{miRNA} - Cq_{miR-39})_{control\ group}$.

Statistical analysis. Statistical analyses were performed using SPSS 17.0. software (SPSS Inc., Chicago, IL, USA). An independent sample t-test, Mann-Whitney U test and Kruskal-Wallis t-test were performed accordingly. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Serum levels of cel-miR-39. The serum levels of cel-miR-39 in 20 randomly selected breast cancer patients and 20 healthy individuals were examined. The median Cq value for cel-miR-39 in the healthy individuals was 25.90 (range, 23.08-26.61), while in patients with breast cancer, the value was 25.71 (range, 23.47-26.76) (Fig. 1).

Cel-miR-39 expression was calculated using the 2^{-Cq} equation (11). The Mann-Whitney U test showed that there was no statistically significant difference between the healthy controls

Table III. Diagnostic performance of serum miR-382-3p, -598-3p, -1246 and -184.

miRNA	AUC	95% CI	Sensitivity, %	Specificity, %	Cut-off value
miR-382-3p	0.740	0.658-0.822	52.0	92.5	1.611
miR-598-3p	0.939	0.902-0.977	95.0	85.0	0.549
miR-1246	0.904	0.849-0.959	93.0	75.0	1.318
miR-184	0.743	0.663-0.824	87.5	71.0	0.484

miR/miRNA, microRNA; AUC, area under receiver operating characteristic curve; CI, confidence interval.

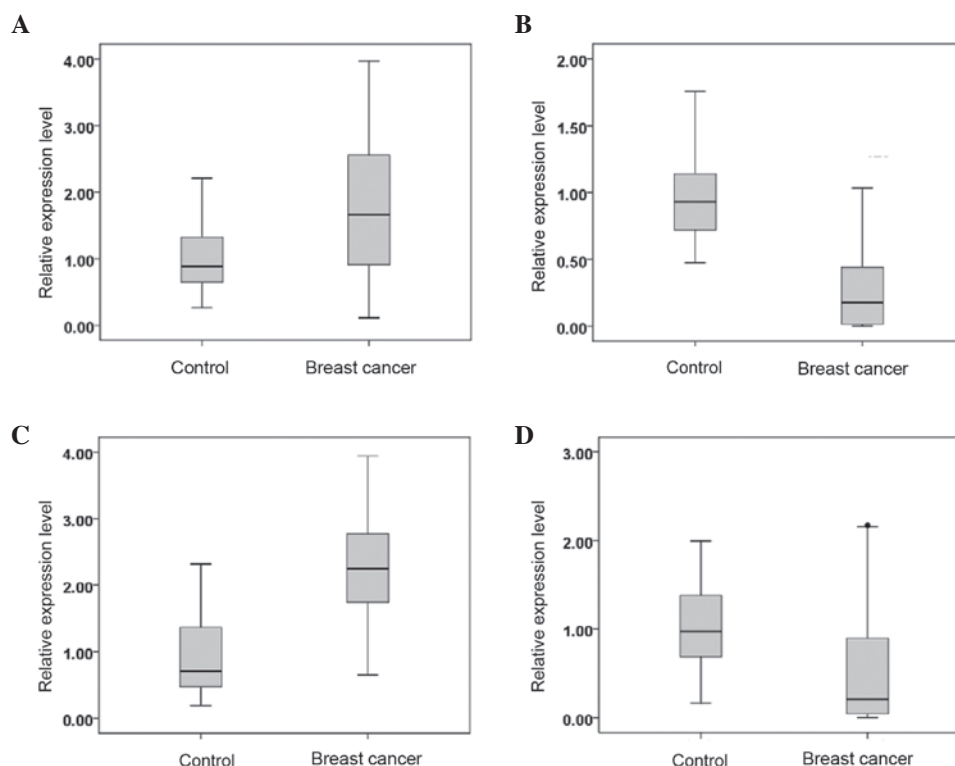


Figure 2. Relative expression levels of (A) miR-382-3p, (B) miR-598-3p, (C) miR-1246 and (D) miR-184 in the serum of breast cancer patients and control subjects. miR, microRNA.

and breast cancer patients ($P=0.543$). Cel-miR-39 expression is stable, and can be used as an exogenous reference in qPCR.

Serum levels of miR-382-3p, -598-3p, -1246 and -184. The serum miR-382-3p, -598-3p, -1246 and -184 expression levels in 100 cases of breast cancer and 40 healthy individuals are shown in Fig. 2. Compared with the serum of the healthy controls, the serum of the breast cancer patients exhibited upregulated levels of miR-382-3p and -1246, while the levels of miR-598-3p and -184 were downregulated ($P<0.05$).

Serum miRNAs in breast cancer patients with different tumor-node-metastasis (TNM) stages. The breast cancer patients were divided into TNM stages I, II, III and IV according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology: Breast Cancer (version 3.2014) (12). The serum miR-382-3p, -598-3p, -1246 and -184 levels were not significantly different between the patients with different TNM stages ($P>0.05$) (Fig. 3).

Receiver operating characteristic (ROC) curve analysis. The area under the ROC curve for miR-382-3p, -598-3p, -1246 and -184 was 0.740, 0.939, 0.904 and 0.743, respectively (Fig. 4). Notably, serum miR-598-3p showed the highest sensitivity and specificity of 95.0 and 85.0%, respectively, while miR-1246 exhibited a lower specificity of 75.0%, and miR-382-3p showed the lowest sensitivity at 52.0% (Table III).

Discussion

The early detection of breast cancer remains a challenge. Mammography X-ray photography is widely used for breast cancer screening and detection, but this technology has limitations, such as irradiation and a high false-positive rate. miRNAs are expressed in the serum and a variety of other bodily fluids (13). The molecules are stable enough for long-term preservation (14) and can tolerate repeated freezing and thawing, which are promising characteristics for tumor markers. A number of studies have shown that serum miRNAs

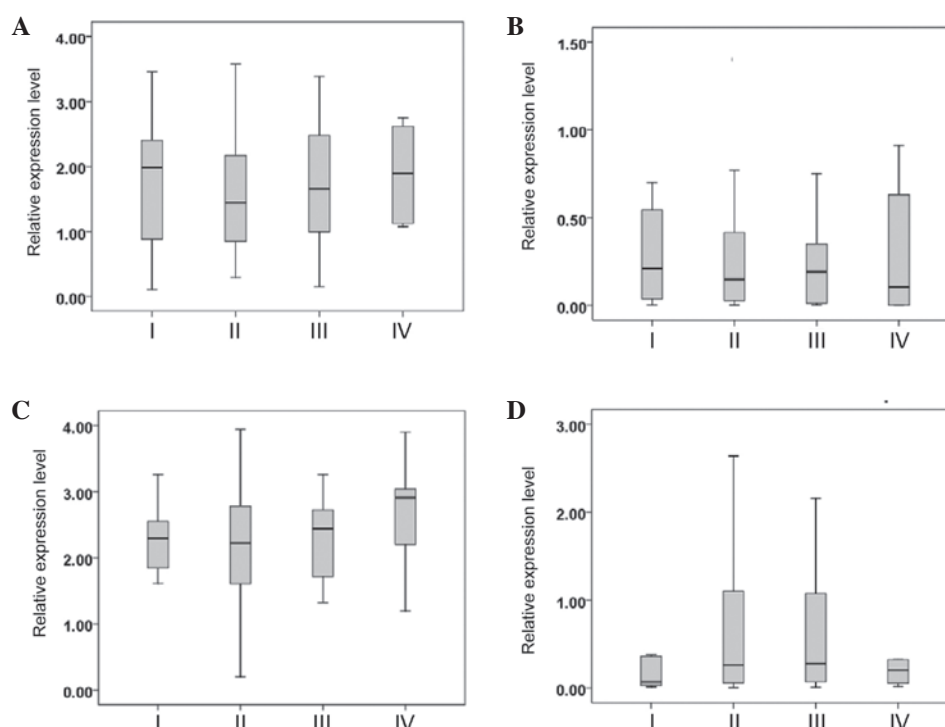


Figure 3. Relative expression of serum (A) miR-382-3p, (B) miR-598-3p, (C) miR-1246 and (D) miR-184 in breast cancer patients with different tumor-node-metastasis stages. miR, microRNA.

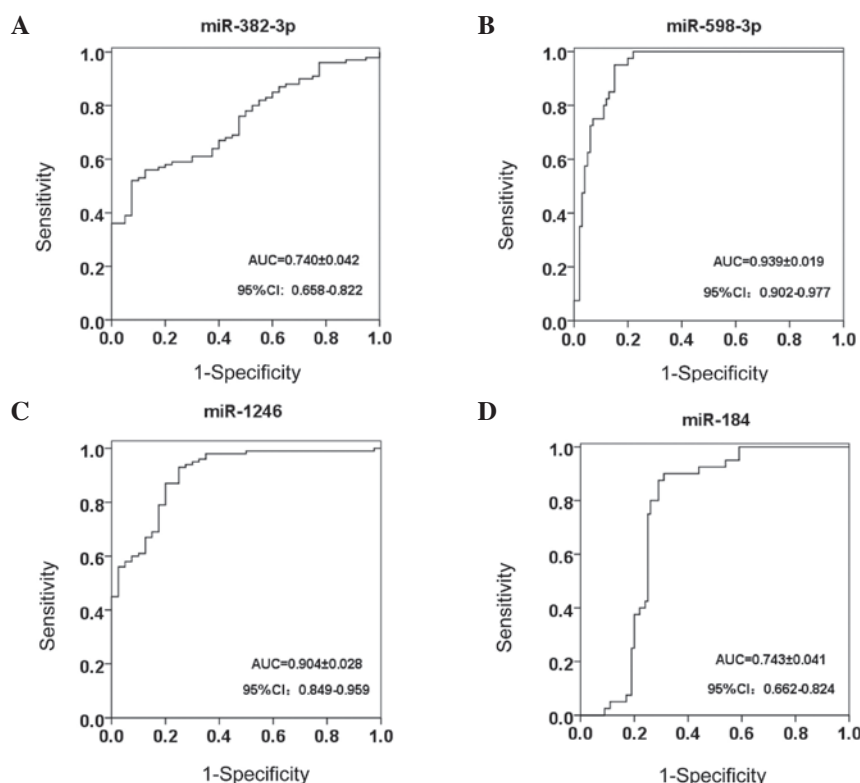


Figure 4. ROCs for (A) miR-382-3p, (B) miR-598-3p, (C) miR-1246 and (D) miR-184. miR, microRNA; ROC, receiver operating curve; AUC, area under the ROC; CI, confidence interval.

are promising biomarkers for cancer detection (15). Sun *et al* showed that serum miRNA-155 can be used as a diagnostic marker for breast cancer (16). It has also been demonstrated that serum miRNA-10b expression is significantly higher

in breast cancer patients with bone metastases, and that it can therefore be used as a tumor marker for breast cancer bone metastasis (17). However, previous studies on serum miRNAs have not provided a standardized internal standard.

A number of studies have used miR-16 as a standardized internal control for serum miRNA experiments (18,19), however, the expression of serum miR-16 is not stable in a number of tumors (20). Furthermore, in miR-16 derived from red blood cells, the occurrence of hemolysis would result in 20-30-fold increase in blood miR-16, which would affect the results (21). In the present study, exogenous RNA cel-miR-39 was added during serum RNA extraction. The results showed that cel-miR-39 expression was stable in the serum of the breast cancer patients and the healthy controls. Therefore, cel-miR-39 can be used as a standard for serum miRNA detection.

The upregulation of miR-598 was previously found in patients with esophageal cancer (22). miR-184 was observed to be highly expressed in prostate cancer, while it was significantly downregulated in renal cell carcinoma patients, and was not correlated with patient age and gender or clinical stage of renal cell carcinoma (23). The study by Mar-Aguilar *et al* confirmed the upregulation of miR-382 in the serum of patients with breast cancer; a sensitivity of 94.40% and a specificity of 90.00% was found for the diagnosis of breast cancer (24). A study by Li *et al* showed that miR-382-5p expression was significantly downregulated in patients with ductal carcinoma *in situ* (25), while miR-1246 was demonstrated to exhibit high expression in colorectal cancer (26), esophageal squamous cell carcinoma (27), cervical carcinoma (28), hepatocellular carcinoma (29) and other cancers. The expression profiles of miRNAs in the serum and tissues are different. miR-1246 is produced by mammary gland epithelial cells and then stored; when the mammary epithelial cells become malignant, miR-1246 is selectively released into the circulating blood, resulted in elevated levels of miR-1246 in the circulation (30).

The results of the present study also showed that miR-382-3p and miR-1246 are highly expressed in the serum of breast cancer patients. To the best of our knowledge, there have been no studies on the serum miR-598 and miR-184 levels in breast cancer. The present study results showed that breast cancer patients had downregulated serum miR-598-3p and miR-184 expression. When using miR-598-3p as a biomarker with a cutoff value of 0.549, the sensitivity and specificity for the detection of breast cancer was 95.0 and 87.5%, respectively, indicating that miR-598-3p is a promising biomarker for breast cancer. Furthermore, the expression levels were not correlated with breast cancer stage.

In summary, in the present study, it was found that the expression of miR-382-3p and -1246 in the serum of breast cancer patients was upregulated, while the expression of miR-598-3p and -184 was downregulated. There were no significant differences in the expression levels among different TNM stages. miR-598-3p was shown to be a promising marker with a high sensitivity and specificity for the diagnosis of breast cancer. This study provides novel insights into serum miRNA expression profiles in breast cancer.

Acknowledgements

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