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Original Article

Serum microRNA-155 in early diagnosis and prognosis of breast cancer

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Abstract: miR-155 regulates the expression of some 147 targets that are implicated in cancer pathways, and its expression has been shown to be up-regulated in breast cancer. Here, the value of serum expression of miR-155 as a non-invasive biomarker for early diagnosis and prognosis of breast cancer was assessed. RNA was extracted from blood samples from 148 patients with breast cancer and 142 control individuals without cancer, and miR-155 expression was measured via fluorescent real-time quantitative PCR and subsequently correlated with patients' clinical characteristics. Patient follow-up was performed for up to 48 months to identify outcomes including recurrence and death. miR-155 expression was up-regulated 2.62-fold in serum of subjects with breast cancer compared to control subjects (t = 11.338; P < 0.001). Relative serum miR-155 expression significantly differed with cancer stage (F = 68.145; P < 0.001), and expression directly increased with advancing cancer stage. Linear regression analysis indicated that miR-155 expression was linearly related to subjects' number of artificial abortions (r = 0.54; P = 0.01), BMI (r = 0.39; P = 0.03), family history of breast cancer (r = 0.62; P = 0.01), and breast cancer stage (r = 0.48; P = 0.02). Subjects with breast cancer with high serum miR-155 expression had a relatively poor prognosis, and a serum miR-155 concentration of 1.24 U/mL was determined to be the optimal critical point for breast cancer diagnosis. Thus, detection of serum miR-155 expression facilitates early diagnosis and prognosis assessment of breast cancer.

Keywords: microRNA-155, miR-155, breast cancer, early diagnosis, prognosis

Introduction

Breast cancer is one of the most common malignant tumors in women. Statistical data collected in China's major cities, such as Beijing, Shanghai, and Tianjin, indicate that the morbidity rate of breast cancer in China is rising, with an annual increase of up to 3-4%-higher than the worldwide annual increase of 1-2% [1]. Although therapeutic efficacy for breast cancer is improving because of early detection and standardized comprehensive treatment, diagnosis has been largely dependent on clinical imaging and pathology [2]. However, diagnosis is entering a molecular era, with an increasing number of groups studying the utility of biological markers such as serum CA15-13 [3] for earlier diagnosis and more accurate prognostic assessment of breast cancer.

microRNAs (miRNAs) are intimately associated with cancer - miRNA expression has been linked to tumor development, progression, and therapeutic response, and some miRNAs can function as tumor suppressors or oncogenes [4]. Because of these correlations, many studies have explored the ability of miRNAs present in patient serum to function as sensitive diagnostic and predictive biomarkers for various cancers.

miR-155, located on human chromosome 21q21 and was formerly called BIC (B-cell integration Cluster), is a multifunctional miRNA that participates in several pathophysiological processes, including occurrence, development, inflammation, and immunity of tumors [5-8]. Some studies have found overexpression of miR-155 in breast cancer correlates to disease state [9]. A review of published breast cancer studies found that miR-155 has 147 validated target genes related to key cancer pathways, including apoptosis, differentiation,

Table 1. Clinical characteristics of study subjects [n (%)]

Characteristic	Control group	Stage I breast cancer	Stage II breast cancer	Stage III breast cancer
Age				
< 45 years	38 (26.76)	15 (39.47)	13 (20.97)	8 (16.67)
≥ 45 years	104 (73.24)	23 (60.43)	49 (79.03)	40 (83.33)
Age of menarche				
< 13 years	20 (14.08)	12 (31.58)	16 (25.81)	20 (41.67)
≥ 13 years	122 (85.92)	26 (68.42)	46 (74.19)	28 (58.33)
Times of artificial abortions (n)				
0	128 (90.14)	29 (76.32)	44 (70.97)	33 (68.75)
1-2	10 (7.04)	4 (10.53)	10 (16.13)	8 (16.67)
≥ 3	4 (2.82)	5 (13.15)	8 (12.90)	7 (14.58)
BMI				
< 24	39 (27.45)	18 (47.37)	31 (50.00)	32 (66.67)
≥ 24	103 (72.54)	20 (52.63)	31 (50.00)	16 (33.33)
Family history of breast cancer				
Yes	1 (0.70)	2 (5.26)	4 (6.45)	10 (20.83)
No	141 (99.3)	36 (94.74)	58 (93.55)	38 (79.17)
Total	142 (100)	38 (100)	62 (100)	48 (100)

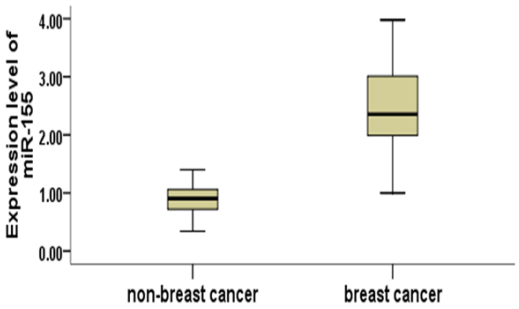

Figure 1. Relative expression of miR-155 in control subjects and subjects with breast cancer.

angiogenesis, proliferation, and epithelial-mesenchymal transition [10].

miR-155 is highly expressed in MCF-7 breast cancer cell lines and promotes proliferation and growth of breast cancer cells through downregulation of target TP53INP1. Estradiol can also upregulate miR-155, again promoting proliferation of breast cancer cells and reducing apoptosis by inhibiting expression of TP53INP1 [11, 12]. In addition, absence of miR-155-mediated C/EBPβ signaling can transform the response of TGF-β signaling to promote epithelial-mesenchymal transition and metastasis of breast cancer cells [13].

miR-155 has been shown to have diagnostic value as a biomarker for esophageal cancer [14] and leukemia [15]. Because miR-155 is present in serum of breast cancer patients and is sensitive to the disease [16], we further investigated the value of miR-155 expression as a biomarker for early diagnosis and prognostic assessment of breast cancer.

Materials and methods

Clinical data

The study enrolled 148 subjects with breast cancer hospitalized at Yancheng Hospital of Traditional Chinese Medicine (Yancheng, China) from June 2012 to June 2013. All subjects were diagnosed and confirmed during the first visit. During first treatment, all subjects were classified according to the 1997 International Union Against Cancer (UICC) Clinical Staging Criteria as stage I, II, or III. Subjects did not receive any surgery, radiotherapy, chemotherapy, or endocrine therapy before blood draws. The study was approved by the ethics committee of Yancheng Hospital of Traditional Chinese Medicine, and all patients provided informed consent.

The study also included 142 control subjects who received physical examination at Yancheng Hospital of Traditional Chinese Medicine during the same time. Females who received examination and did not have tumor histories or clinical

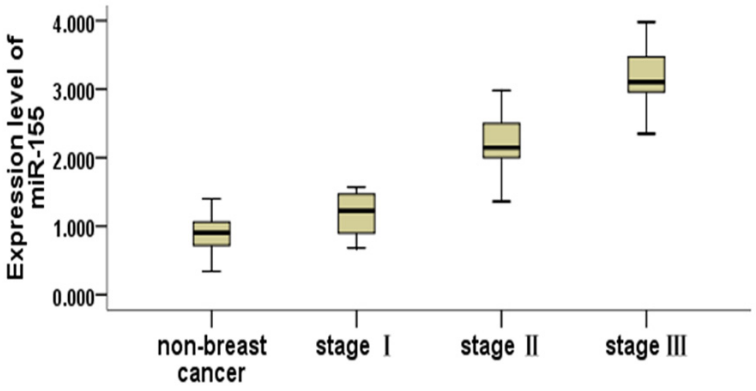

Figure 2. Relative expression of miR-155 in control subjects and subjects with stage I, II, or III breast cancer.

Table 2. miR-155 expression and single-factor and linear regression analyses of clinical factors of breast cancer patients

Characteristic	Cases [n (%)]	miR-155	
		r	*P*
Age			
< 45 years	36 (24.32)	0.01	0.67
≥ 45 years	112 (75.68)		
Age of menarche			
< 13 years	48 (32.43)	0.24	0.04
≥ 13 years	100 (67.57)	-	-
Times of Artificial abortions (n)			
0	106 (71.62)	-	-
1-2	22 (14.86)	0.37	0.02
≥ 3	20 (13.52)	0.54	0.01
BMI			
< 24	81 (54.73)	-	-
≥ 24	67 (45.27)	0.39	0.03
Family history of breast cancer			
No	132 (89.19)	-	-
Yes	16 (10.81)	0.62	0.01
Breast cancer stageing			
Stage I	38 (25.68)	-	-
Stage II	62 (41.89)	0.39	0.03
Stage III	48 (32.43)	0.48	0.02

signs of breast cancer were included in the control group.

Breast cancer patients underwent follow-up visits lasting 12-48 months. Follow-up visits were carried out every three months mainly through family visits, letters, and telephone calls to identify whether patients received timely check-ups and to identify cancer recurrence, metastasis, and cause and time of death, if applicable. Follow-up was completed for all 148 subjects with breast cancer.

Serum RNA extraction

For all study subjects, we extracted 6 mL of peripheral vein blood into tubes without anticoagulant. After standing for 30 minutes, blood was centrifuged at 4,000 rpm for 15-20 minutes. Supernatant was isolated and subpackaged into RNAse-free Eppendorf tubes (300 μL each). We added 1 mL of Trizol reagent (TaKaRa Biotech Co., Dalian, China) to each tube, snap-froze samples in liquid nitrogen, and stored samples at -80°C.

cDNA synthesis of miRNA

As previously report [16], Total serum RNA was extracted with a Trizol one-step method. Dimethyl carbinol precipitation was used to concentrate RNA, and cDNA was synthesized according to a miRNA reverse transcription (RT) system. The components for the miRNA reverse transcription reaction was as follows: 2.5 μL RNA sample, 3 μL of 5×RT primer (500 nM), 1.5 μL of 10× RT Buffer, 1 μL MultiScribe reverse transcriptase, 0.15 μL of dNTPs (10 mM each), 0.19 μL of RNase inhibitor (40 U/μL), 6.66μL of Nuclease-free water, which were then conducted at 16°C for 30 min, 42°C for 60 min and 85°C for 5 min.

Fluorescent real-time quantitative PCR

PCR reactions included cDNA, upstream primer, downstream primer, SYBR green/fluorescein qPCR Master Mix, and ddH_2O. Reactions were amplified with: one cycle of 50.0° for 2 min, 95.0° for 10 min; followed by 40 cycles of 95.0° for 30 s, 60.0° for 30 s.

Data analysis

When PCRs entered the logarithmic phase, quantity of amplified target segments could be approximately considered relevant to initial template concentration and amplification effi-

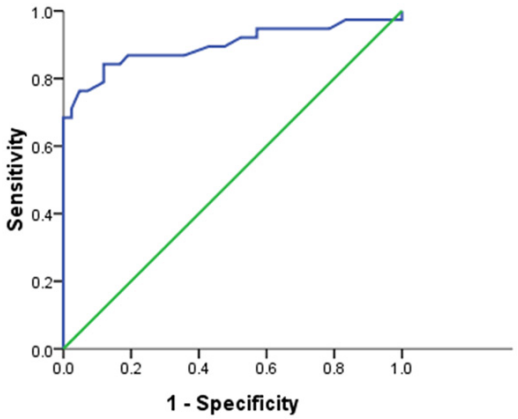

Figure 3. Receiver operating characteristic (ROC) curve of serum miR-155 expression for breast cancer diagnosis.

Table 3. Parameters at critical concentration of serum miR-155 for breast cancer diagnosis

Parameter	Value
miR-155 (U/mL)	1.24
Sensitivity matrix effect (%)	84.2
Specificity matrix effect (%)	88.1
Positive predictive value (%)	87.6
Negative predictive value (%)	84.8
Correct diagnosis index	0.723
Positive likelihood ratio	7.1
Negative likelihood ratio	5.6

ciency. This value was expressed as Ct, the number of cycles required for the fluorescent PCR signal to reach a preset threshold value. The ΔΔCT method was adopted for relative expression (RQ) of target genes, with $RQ = 2^{-\Delta\Delta CT}$. Real-time fluorescent intensity of reactions was calculated over background signal: $\Delta CT_{sample} = CT_{sample} - CT_{U6\ sample}$; $\Delta CT_{control} = CT_{control} - CT_{U6\ control}$; $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{control}$) [17].

Follow-up visits

Statistical methods analysis: All results were analyzed using SPSS17.0 statistical software (IBM, Armonk, NY, USA). Independent sample t-test and variance analysis methods were used to analyze intergroup differences in serum miR-155 levels. Receiver operating characteristic (ROC) curves were established to evaluate the value of serum miR-155 for diagnosing breast cancer. Kaplan-Meier method was adopted for survival analysis. $P < 0.05$ was considered statistically significant.

Results

Characteristics of clinical study cases subjects

Subjects with breast cancer (n = 148) ranged from 37-68 years old, with a mean age of 49.2 ± 7.0 years (**Table 1**). After assessing clinical stage, 38 subjects had stage I, 62 patients had stage II, and 48 patients had stage III breast cancer. Subjects in the control group (n = 142) ranged from 35-70 years old, with a mean age of 50.4 ± 6.6 years. Age did not significantly differ between the two groups ($P > 0.05$).

Expression level of serum miR-155

Relative expression of serum miR-155 was significantly higher in subjects with breast cancer (2.38 ± 0.67) than control subjects (0.91 ± 0.33) (**Figure 1**). Serum miR-155 expression in subjects with breast cancer was therefore up-regulated 2.62-fold to the expression level of control subjects, a statistically significant difference was observed ($t = 11.338$; $P < 0.001$).

In addition, serum miR-155 expression significantly differed among subjects with breast cancer at different stages and control subjects ($F = 68.145$; $P < 0.001$) (**Figure 2**). Relative expression of serum miR-155 significantly increased with severity of breast cancer as assessed by cancer staging. Considering only subjects with breast cancer at different stages, expression of serum miR-155 in subjects with stage III breast cancer was up-regulated 1.42-fold to that of subjects with stage II breast cancer; up-regulated 2.54-fold to that of subjects with stage I breast cancer; and upregulated 3.43-fold to that of control subjects.

Relationship between expression of miR-155 expression and analysis on clinical pathology of breast cancer clinical factors patients

Single-factor analysis of miR-155 expression among clinical pathologies indicated that miR-155 expression significantly differed among patients according to menarche age ($P = 0.04$), number of artificial abortions ($P = 0.001$), BMI ($P = 0.002$), and family history of breast cancer ($P = 0.001$). Subjects with a menarche age of < 13 years, many artificial abortions, high BMI, and a family history of breast cancer had relatively high miR-155 expression. Linear regres-

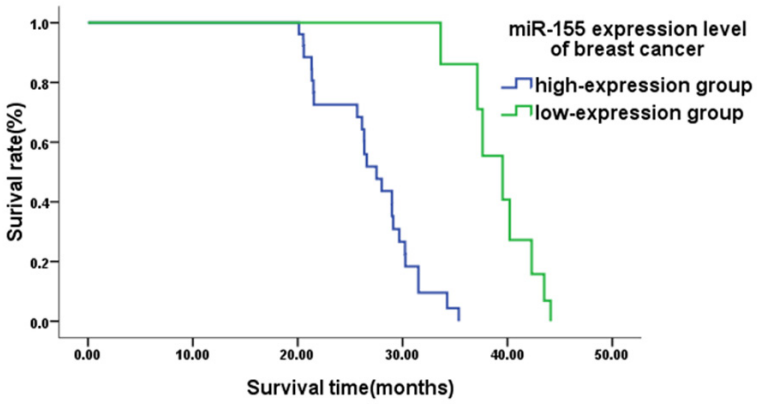

Figure 4. Kaplan-Meier survival curves of mean survival between high and low miR-155 expression groups of all subjects with breast cancer.

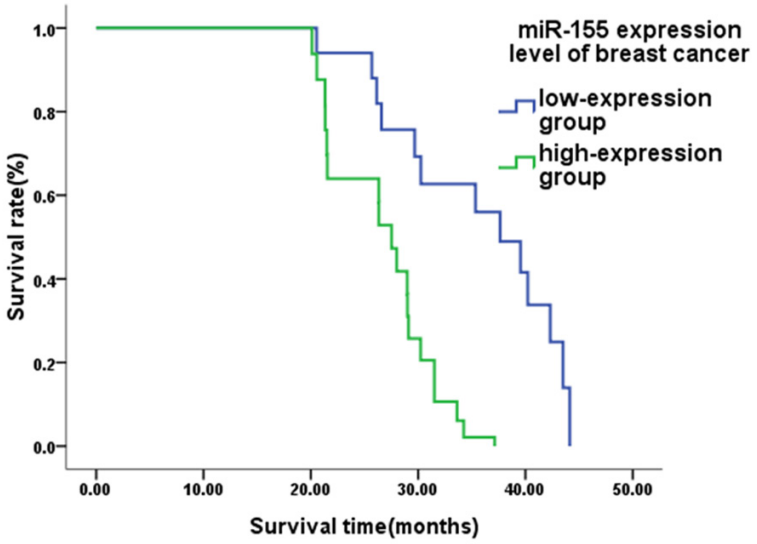

Figure 5. Kaplan-Meier survival curves of mean survival rates between high and low miR-155 expression groups of subjects with breast cancer who received treatment.

sion analysis indicated that miR-155 was linearly related to artificial abortions (r = 0.54; *P* = 0.01), BMI (r = 0.39; *P* = 0.03), family history of breast cancer (r = 0.62; *P* = 0.01), and breast cancer stage (r = 0.48; *P* = 0.02) (**Table 2**).

Value of miR-155 in diagnosing breast cancer diagnosis

We also assessed the value of serum miR-155 in breast cancer diagnosis (**Figure 3**). Correct diagnosis index was largest (0.723) when the area under the ROC curve was 0.879 (95% CI: 0.820-0.868). A serum miR-155 concentration of 1.24 U/mL was the optimal critical point for breast cancer diagnosis. At that concentration, sensitivity was 84.2%, specificity was 88.1%, positive predictive value was 87.6%, negative predictive value was 84.8%, positive likelihood ratio was 7.1, and negative likelihood ratio was 5.6 (**Table 3**).

Relationship between the expression level of serum miR-155 expression and the prognosis of breast cancer prognosispatients

A mean concentration of 1.24 U/mL was set as the critical point for relative serum miR-155 expression of subjects with breast cancer. This value was used to divide the 148 subjects with breast cancer into two groups: high (≥ 1.24) and low (< 1.24) relative miR-155 expression. The low expression group included 54 subjects, 38 of whom received treatment and 16 of whom did not receive treatment. The high expression group included 94 subjects, 67 of whom received treatment and 27 of whom did not receive treatment.

Kaplan-Meier analysis indicated that mean survival of the low miR-155 expression group was 39.77 months, and mean survival of the high miR-155 expression group was 26.81 months. Subjects in the low expression group therefore survived significantly longer than subjects in the high expression group (χ^2 = 38.412; *P* < 0.001) (**Figure 4**). Analyzing only subjects who received treatment, mean survival of treatment-receiving subjects with low and high miR-155 expression was 40.68 and 28.91 months, respectively; again, subjects with low miR-155 expression survived significantly longer (χ^2 = 14.481; *P* = 0.001) (**Figure 5**). Mean survival of subjects who did not receive treatment who had low and high miR-155 expression was 27.21 and 21.98, respectively (χ^2 = 9.692; *P* = 0.007) (**Figure 6**).

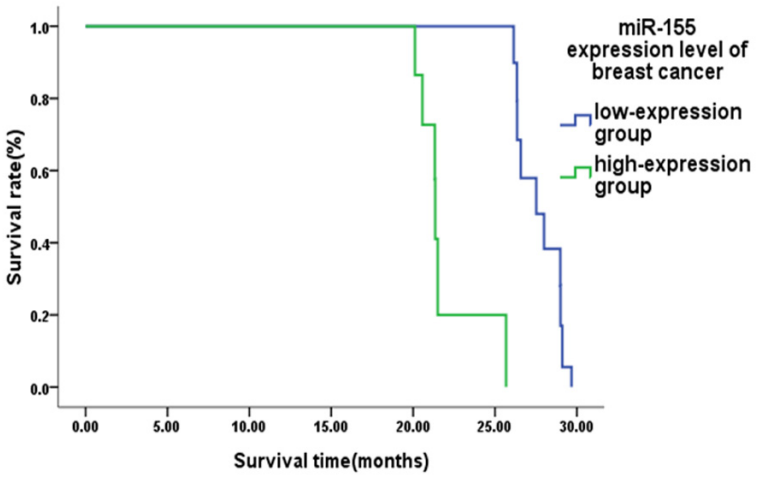

Figure 6. Kaplan-Meier survival curves of mean survival rates between high and low miR-155 expression groups of subjects with breast cancer who did not receive treatment.

Discussion

BIC miR-155 expression is significantly increased in many tumors, including breast cancer [18], where it can function as an oncogene to affect cancer occurrence and development. This study shows that expression of miR-155 in serum of subjects with breast cancer is significantly higher than that in control individuals and is closely related to stage of breast cancer.

Previous studies have identified risk factors for breast cancer. During the early 1990s, a study of familial genes found 3 mutations in *the two genes BRCA1I* and *BRCA2* that indicated a hereditary component to onset of breast cancer and oophoroma, in addition to increased risk of other cancers [19]. According to estimations, 15%-20% of women have a family history of breast cancer, about 5% of individuals with breast cancer carry predisposing genes, and onset risk of breast cancer in individuals with a family history of the disease is 1.8-fold to the risk of those without a family history. In addition, many studies have verified that younger age of menarche and higher frequency of artificial abortions are correlated with higher risk of breast cancer [20, 21]. Regarding young age of menarche, increased risk of breast cancer may be due to higher levels of intracorporeal hormones, which indicate higher exposure to an endogenous estrogen environment. Similarly, this study also shows that miR-155 is linearly related to breast cancer patients' number of artificial abortions, BMI, family history of breast cancer, and cancer stage.

It is generally believed that miRNA in the blood is derived from apoptotic or necrotic cells, active release by cells, and lysis of circulating cells. Recent studies have concluded that miRNA in blood is secreted selectively by tumor cells [22], suggesting that circulating miRNA can be used as a tumor marker. Tumor marker monitoring is used for tumor diagnosis, efficacy judgment, and prognosis evaluation because it is characterized by simplicity, non-invasiveness, objective quantitation, ability for repeated measurement for dynamic monitoring, and relatively low price. Studies have found high miR-155 expression both in cancer tissues and serum of patients, indicating its potential value as a tumor marker. Further, serum miR-155 expression decreases after surgery and chemotherapy, indicating that serum monitoring can be used to characterize therapeutic efficacy [23]. Therefore, serum miR-155 shows great promise as a target marker for breast cancer treatment.

Whether in cell models or nude mouse breast tumor transplantation models, antisense nucleic acid drugs to miR-155 can effectively inhibit proliferation and promote apoptosis of breast cancer cells [24, 25]. In addition, miR-155 could also be an indicator for early diagnosis of breast cancer. ROC curves are comprehensive indicators that reflect continuous variables of sensitivity and specificity and can visually reveal correlations between sensitivity and specificity. ROC curves and area under the curve can serve as indicators to evaluate the accuracy of diagnostic methods. Generally, an area under the curve of 0.5-0.7 indicates relatively low diagnostic accuracy; 0.7-0.9 indicates moderate diagnostic accuracy; and > 0.9 indicates relatively high diagnostic accuracy [26]. According to evidence-based medical principles and ROC curve analysis, this study reveals

that serum miR-155 expression has moderate accuracy in early diagnosis of breast cancer. Specifically, clinical value of miR-155 for breast cancer diagnosis is highest when serum concentration is 1.24 U/mL. miR-155 expression therefore may represent a robust biological marker for early diagnosis and prognostic evaluation of breast cancer. Nonetheless, the restricted sample size and focal location of this study necessitates additional studies for confirmation of these results.

Expression level of some miRNAs-not only in tumor tissues, but also in circulation, such as in plasma-can accurately reflect tumor malignancy [27]. miRNA detection in serum is a more convenient, rapid, and sensitive indicator for preoperative diagnosis. For example, miR-155 expression in plasma of breast cancer patients is five-fold higher than in plasma of control individuals [28]. Analysis of the relation between serum miR-155 expression and prognosis of breast cancer patients showed that survival of breast cancer patients with low miR-155 expression was significantly longer than patients with high expression. These results indicate that serum miR-155 can play a vital role in assessing the prognosis of breast cancer patients.

Disclosure of conflict of interest

None.

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