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Original Article

Over-expression of miR-98 in FFPE tissues might serve as a valuable source for biomarker discovery in breast cancer patients

Zhao-Qun Deng[1,2], Jia-Yu Yin[2], Qin Tang[2], Feng-Qiong Liu[3], Jun Qian[2], Jiang Lin[2], Rui Shao[2], Ming Zhang[2], Li He[1]

[1]School of Basic Medical Science, Wuhan University, Wuhan 430071, People's Republic of China; [2]Affiliated People's Hospital of Jiangsu University, Zhenjiang, 212002, People's Republic of China; [3]Department of Nutrition, School of Public Health, Sun Yat-Sen University, People's Republic of China

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Abstract: The miR-98 is thought to be associated with various cancers. This study was to evaluate the potential predictive value of miR-98 expression in formalin-fixed paraffin-embedded tissue of breast cancer patients. The expression levels of miR-98 were examined in 98 breast cancer patients and 40 cancer-free controls using real-time quantitative PCR. The comparison of miR-98 expression levels between patient and control was performed using the Mann-Whitney test. The miR-98 showed higher expression levels in breast cancer patients compared with cancer free controls ($p<0.01$). The expression levels of miR-98 were highly correlated with miR24/93/378 in breast cancer patients. The miR-98 exhibited great capability of discriminating between cancer patients and controls by the Receiver-operator characteristic (ROC) curve analysis. The miR-98 was found highly correlated with breast cancer by Univariable logistic regression analysis. These results suggest that over-expression of miR-98 in formalin-fixed paraffin-embedded tissues might serve as a valuable source for biomarker discovery in breast cancer patients.

Keywords: Biomarkers, miR-98, breast cancer, miRNA, cancer

Introduction

MiRNAs are short non-coding RNAs that are single-stranded RNAs of 18-25 nucleotides long [1]. MiRNAs play important roles in diverse regulatory pathways, including cell proliferation [2], cell differentiation [3], cell survival [4], cellular self-renewal [5], apoptosis [6, 7], tumor growth [4], cancer development [8, 9], tumor invasion and metastasis [10], control of development [11], protein secretion [12] and viral infection [13].

The miR-98 is thought to be associated with various cancers [14-17]. The miR-98 was also found to be dysregulated in breast cancer [18, 19]. Moreover, it has been observed as a frequent event aberrantly expressed in patients with breast cancer.

Fresh and frozen tissue samples are considered to be a proper source of DNA, RNA and protein for aim of clinic and research. Due to the limitation and inefficiency of prospectively collecting fresh and frozen samples as well as current insufficiency of bio-banking of patient samples, scientists are looking for alternative stocks of archived samples with potential utility for disease analysis such as formalin-fixed paraffin-embedded (FFPE) tissues [20]. RNA was found to be degraded in FFPE tissue [21]. Interestingly, miRNAs have been proved to be stable in FFPE tissues because of their shorter length. The expression of miRNAs in various cancers has been revealed using FFPE tissue [22]. A high correlation between miRNA expression levels derived from fresh-frozen tissues and matched FFPE tissues has been reported [20]. FFPE tissues are thought to be ideal for miRMA analysis and Immunohistochemistry (IHC) which carries abundant pathological data, thus FFPE tissues might be a valuable source for miRNA expression study, biomarker discovery and validation [23].

Emerging evidence has demonstrated that miR-98 is aberrantly expressed in various cancer samples derived from fresh-frozen tissues and

Table 1. Clinicopathological characteristics and expression of miR-98 in breast cancer

characteristics	cases	miR98 expression level		
		Low	high	p
Age (years)				
≤40	18	12	6	0.324
40-55	33	15	18	
>55	47	23	24	
Histological grade				0.599
I	7	3	4	
II	39	18	21	
III	52	29	23	
Tumor stage				0.548
I	18	6	12	
II	11	4	7	
III	5	3	2	
N stage				0.628
0	58	28	30	
1-3	22	12	10	
4-9	9	6	3	
>10	8	3	5	
ER status				0.513
negative	40	22	18	
positive	58	28	30	
PR status				0.529
negative	44	24	20	
positive	54	26	28	

might serve as a potential biomarker for cancer detection. However, little is known about the expression level of miR-98 in FFPE tissue of breast cancer patients and the correlation between miR-98 and breast cancer. In this study, we investigated expression of miR-98 in FFPE tissue of breast cancer patients as well as the correlation between miR-98 and breast cancer. Moreover, we further explored potential predictive value of miR-98 as a novel potential biomarker for breast cancer patients.

Materials and methods

Patients and samples

FFPE tissue samples from 98 breast cancer patients in the Affiliated People's Hospital of Jiangsu University were collected in this study. Additionally, FFPE tissue samples collected from 40 cancer-free controls. All samples were obtained according to the guidelines of The Affiliated People's Hospital's protocol including patient consent and specimen collection. The diagnosis and classification of breast cancer patients were based on the Tumor-Node-Metastasis (TNM) system of American Joint Committee on Cancer (AJCC) [24]. All cases were diagnosed with histologically and clinically confirmed stage I, II and III breast cancer. The clinical characteristics of patients were listed in **Table 1**.

RNA extraction and reverse transcription

Total RNA was extracted from 98 FFPE tissue of breast cancer patients and 40 controls using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, catalog no: AM 1975) and reverse transcribed to cDNA using miScript Reverse Transcription Kit (Qiagen, catalog no. 218061).

Real-time quantitative PCR

Real-time quantitative PCR (RQ-PCR) was performed according to the manufacturer's instructions using miScript SYBR green PCR kit (Qiagen, catalog no. 218073) with the manufacturer-provided miScript Universal primer and miRNA-specific forward primer: TGA GGTAGTAAGTTGTATTGT (miR-98). RQ-PCR amplification was described in our previous study [25].

Statistical analyses

All statistical analyses were performed using spss16.0. The comparison of miRNA expression level between patient and control was performed using Mann-Whitney test. Spearman correlation coefficient was used to analyze the correlation between miR-98 and miR-24, miR-93 or miR-378 expression, respectively. The χ^2 test was used to analyze the association of miR-98 expression level with clinicopathological characteristics. Receiver-operator characteristic (ROC) curve analysis was applied to assess the diagnostic significance of miR-98. The correlation between miR-98 and breast cancer was further determined using Univariable logistic regression analysis. All values showed are two-sided, and a p-value<0.05 was considered statistically significant.

Results

Expression of miR-98 with clinicopathological characteristics of breast cancer

To evaluate whether high-expression of miR-98 was related to the clinic progression of breast

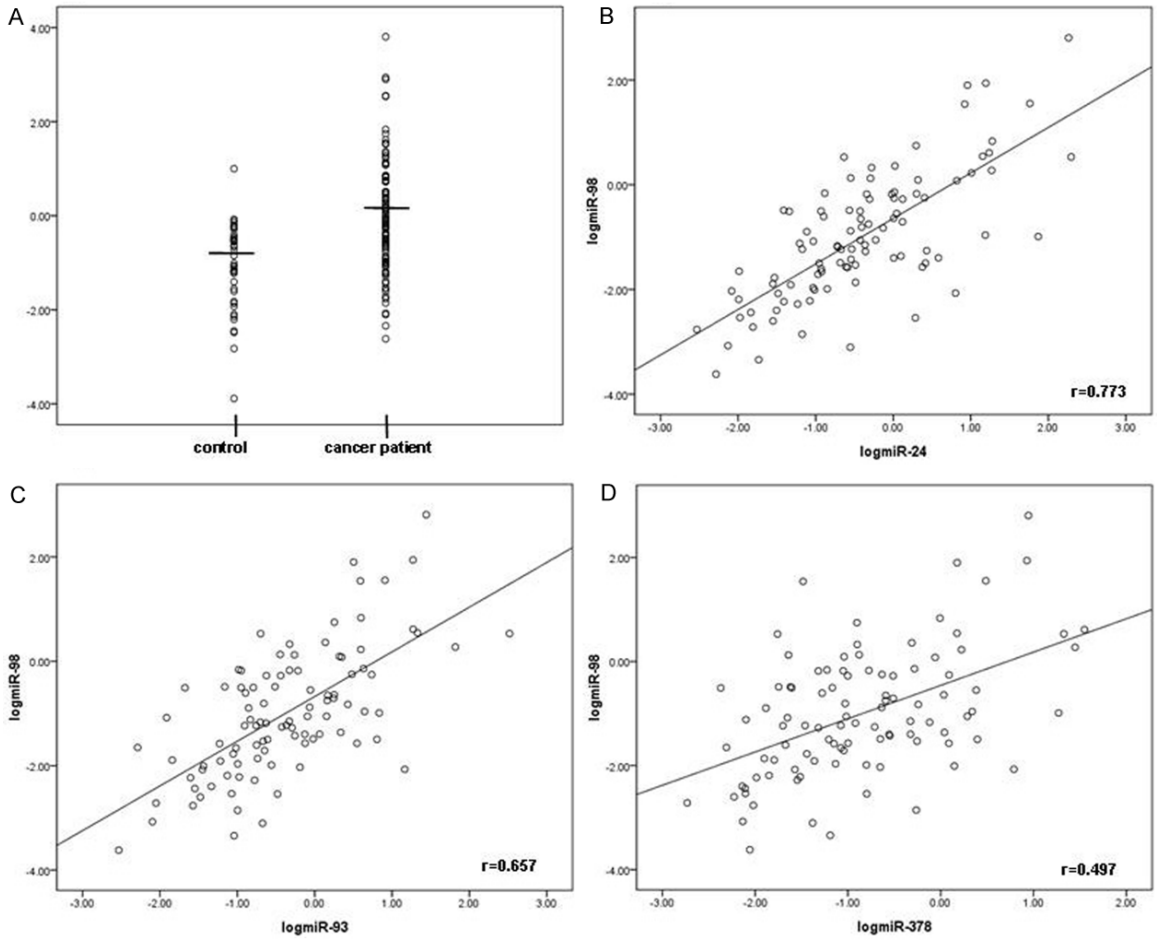

Figure 1. The miR-98 exhibited higher expression levels in breast cancer patients compared with controls (A). Correlation between miR-98 and miR-24/93/378, the spearman correlation scatter plot indicated association between miR-98 and miR-24/93/378, with r=0.773 between miR-98 and miR-24 (B), r=0.657 between miR-98 and miR-93 (C), r=0.497 between miR-98 and miR-378 (D). miRNA expression levels were presented after log10 transformation.

Table 2. Expression level of miR-98 and its diagnostic significance in breast cancer patients

	Median in cancer patients	Median in controls	*P* value	AUC	Cutoffs	Sensitivity (%)	Specificity (%)
miR-98	0.763	0.097	<0.001	0.76	0.084	82.7%	53.8%

cancer, we analyzed the association of miR-98 expression level with the clinicopathological status of patients with breast cancer. Expression of miR-98 had no significant difference in Age, Histological grade, Tumor stage, N (lymph nodes) stage, ER (estrogen receptor) status, and PR (progesterone receptor) status of patients with breast cancer (**Table 1**).

Expression of miR-98 and correlation between miR-98 and miR-24/93/378 in FFPE tissues of breast cancer patients

The miR-98 exhibited higher expression levels in breast cancer patients compared with controls ($p<0.01$) (**Figure 1A**, **Table 2**). Furthermore, the spearman correlation scatter plot indicated high association between miR-98 and miR-24/93/378, with r=0.773 between miR-98 and miR-24 (**Figure 1B**), r=0.657 between miR-98 and miR-93 (**Figure 1C**), r=0.497 between miR-98 and miR-378 (**Figure 1D**).

Highly specificity and sensitivity of miR-98 for determination of breast cancer

ROC analysis then performed to evaluate the capability of miR-98 to discriminate between breast cancer patients and controls. When optimum cutoff value was determined, miR-98

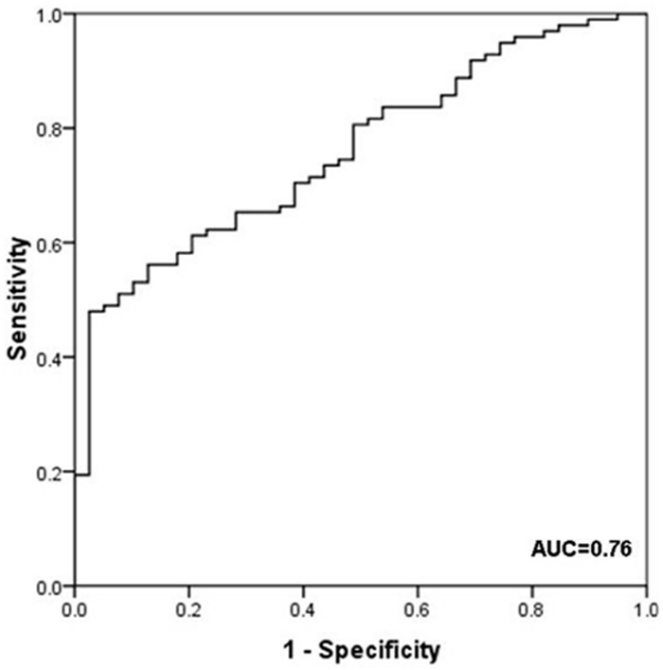

Figure 2. ROC curve analysis of miR-98 expression level in 98 patients diagnosed with breast cancer and 40 controls. The area under the ROC curve (AUC) indicates the accuracy for differentiating breast cancer from patients and controls in terms of sensitivity and specificity. ROC curve of miR-98 showed 0.76 AUC with 82.7% sensitivity and 53.8% specificity.

Table 3. Univariable Logistic regression model of miRNAs

	Regression coefficient (β)	SE	Wald. χ^2	*P* value	Exp (β)
miR-98	1.661	0.433	14.715	<0.01	5.263

showed 0.76 AUC value and yielded 82.7% sensitivity and 53.8% specificity (**Figure 2**). The miR-98 expression level was also found highly correlated with breast cancer by univariate logistic regression analysis (p<0.01, **Table 3**).

Discussion

The correlation between miR-98 expression level and clinicopathological characteristics of breast cancer was also analyzed. We found that expression of miR-98 did not vary with Age, Histological grade, Tumor stage, N stage, ER status, and PR status of patients with breast cancer. It suggested that miR-98 might be a new predictive factor for breast cancer which is independent from other known clinicopathological characteristics.

Aberrant expression patterns of miRNAs were revealed in various cancers and dysregulation of miRNAs were found highly associated with the progression of different cancers [18, 19, 26]. Breast cancer is considered to be a heterogeneous neoplasm, which involves aberrant expression patterns of miRNA and mRNA [27]. Abundant results about the expression of different miRNAs and their function in breast cancer patients were reported [18, 19, 27, 28]. Up-regulation of miR-21 and down-regulation of miR-125b have been found in breast cancer patients [29]. Sun Y et al reported that serum miR-155 was over-expressed in breast cancer patients [30]. Recent studies demonstrated that miRNAs could be good potential candidates for the development of therapeutic targets and novel biomarkers [27]. The miR-98 is thought to be associated with various cancers [14-17]. The miR-98 was also found to be dysregulated in breast cancer [18, 19]. The validation of miR-98 expression in breast cancer patients should be performed with diversity of samples. FFPE tissues could be long-term stored and can permanently preserve the structure of the tissue. MiRNAs were proved to be stable in FFPE tissue because of their shorter length. Recent studies revealed that miRNAs obtained from FFPE tissues showed reliable expression levels as compared with frozen tissues [31-33]. In this study, we analyzed miR-98 expression level in FFPE tissue of breast cancers using RQ-PCR. We have found that miR-98 shows higher expression levels in breast cancer patients compared with controls, which is consistent with Farazi TA' study [18]. Our results validated the high expression of miR-98 in breast cancer patients using FFPE tissues. In order to explore diagnostic ability of miR-98 in FFPE for breast cancer patients, we performed ROC curve analysis. The miR-98 expression in FFPE tissues of breast cancer produced an AUC of 0.76 with sensitivity of 82.7% and specificity of 53.8% in the identification of breast cancer. These results showed that miR-98 had considerable diagnostic power to discriminate between breast cancer patients and controls. The correlation between miR-98 expression level and breast cancer was further confirmed by univariate logistic regression. The miR-24, miR-93 and miR-378 are thought to be onco-miRNAs for their abilities of enhancing tumor

growth. We revealed that miR-24/93/378 were up-regulated in FFPE tissue of breast cancers and the three miRNAs were significantly correlated with each other in breast cancer patients (data not shown). The miR24/93/378 might be good potential candidates for the development of novel biomarkers in breast cancer. In this study, we have found that miR-98 is associated with miR-24/93/378, respectively. The miR-98 might also be considered as a good potential candidate for the development of novel biomarker in breast cancer.

Taken together, we suppose that over-expression of miR-98 in FFPE tissues of breast cancer patients might suggest miR-98 from FFPE tissues can serve as a valuable source for biomarker discovery and validation in breast cancer patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Li He, School of Basic Medical Science, Wuhan University, 185 donghulu, Wuhan 430071, People's Republic of China. Tel: +86 18971062315; E-mail: denghe2015@gmail.com

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