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Identification of serum exosomal miR-148a as a novel prognostic biomarker for breast cancer

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Abstract. – OBJECTIVE: Breast cancer (BC) is one of the most common malignancies affecting females. Aberrant expression of microRNAs (miRNAs) has been associated with carcinogenesis of BC. The aim of our study was to investigate the correlation of serum exosomal miR-148a expression and the clinical outcome of patients with BC.

PATIENTS AND METHODS: Quantitative Real Time-Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was applied to evaluate the expression level of serum exosomal miR-148a in patients with BC, patients with benign breast tumors, and healthy controls. Then, the clinical value of serum exosomal miR-148a in BC was evaluated.

RESULTS: Serum exosomal miR-148a levels were gradually downregulated from healthy controls patients with benign breast tumors to BC patients. Serum exosomal miR-148a could well distinguish BC patients from healthy volunteers. The expression level of serum exosomal miR-148a in BC patients was significantly upregulated following surgery, while dropped in the cases with disease relapse. A significant association was found between serum exosomal miR-148a levels and the tumor-node-metastasis (TNM) stage, differentiation, and lymph node metastasis in BC. In addition, BC patients with lower expression of serum exosomal miR-148a levels suffered worse overall survival and disease-free survival than those with higher expression of serum exosomal miR-148a levels. Furthermore, serum exosomal miR-148a was an independent risk factor for BC.

CONCLUSIONS: Our data have demonstrated that serum exosomal miR-148a is significantly reduced in patients with BC and downregulation of serum exosomal miR-148a is closely associated with unfavorable clinical outcome of BC, indicating that serum exosomal miR-148a might serve as a promising diagnostic and prognostic biomarker for BC.

Key Words:
Prognosis, MiR-148a, Breast cancer, Serum exosome.

Introduction

Breast cancer (BC) is the most prevalent cancer and the second leading cause of cancer related mortality for women worldwide. In China, BC was reported to account for about 15% of all new female cancer cases in 2015[1,2]. Despite advances in the treatment of BC, the long-term prognosis remains unsatisfactory. One of the major reasons accounting for the poor clinical outcome of BC is that most cases are indicated at the advanced clinical stages[3,4]. Therefore, it is important to elucidate the molecular mechanisms of BC carcinogenesis, as well as identify novel biomarkers for early detection and prognosis prediction of BC.

MicroRNAs (miRNAs) are a class of evolutionarily conserved, small single-stranded molecules with about 19-22 nucleotides in length, which mediate post-transcriptional downregulation of target gene expression[5,6]. MiRNAs have been demonstrated to be associated with a variety of biological processes, such as cell proliferation, differentiation, survival, apoptosis, and migration. Alterations in miRNA expression can significantly influence cellular physiology[7]. Xin et al[8] has reported that miRNAs might act as either tumor suppressors or oncogenes during the initiation and progression of BC. Exosomes are RNA and protein-containing small membrane vesicles with 30-150 nm of diameter. Abnormal expression of exosomal derived contents has been shown to be involved in cancer progression. Exosomal miR-1246 was overexpressed in metastatic BC cells and played a tumor promoting role during the carcinogenesis of BC[9]. More importantly, miRNAs are extremely stable in the bodily fluids, which makes them promising biomarkers for the detection and prognosis prediction of cancer[10,11].

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A total of 68 nucleotide sequences were found in miR-148a, which is located to chromosome 7p15.2. MiR-148a is widely expressed in human tissues and implicated in a series of biological processes, such as cellular differentiation and development[12]. Aberrant expression of miR-148a has been reported in various types of cancers, such as BC, gastric cancer, bladder cancer, pancreatic cancer, and non-small cell lung carcinoma[13-19]. However, the potential clinical significance of serum exosomal miR-148a in BC remains unclear. Here, we first examined the serum exosomal miR-148a levels in patients with BC, and then, evaluated its potential as diagnostic and/or prognostic biomarker for BC.

Patients and Methods

Patient Recruitment and Sample Collection

This study was approved by the Research Ethics Committee of Shanxi Dayi Hospital. Written informed consent was obtained from all participants. Serum samples were collected from patients who had a confirmed diagnosis of BC based on pathological findings. A cohort of 125 patients with BC and 50 patients with benign breast tumors were recruited in this study. The diagnosis and classification of BC patients were according to the tumor-node-metastasis (TNM) system of American Joint Committee on Cancer (AJCC). The clinicopathological features of the cases were summarized in Table I. The control serum samples were from forty individuals seeking a routine health checkup at our hospital. The healthy controls showed no evidence of cancers or other diseases. Serum was isolated from blood samples for 20 min at 1200 g at room temperature. All serum samples were stored at -80°C for further use.

Exosome Isolation

The exosomes were extracted from serum samples with ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, the serum samples were centrifuged at 12,000 g for 5 min. Then, the supernatant was mixed with ExoQuick™ solution and incubated at 4°C for 30 min, followed by centrifugation at 2000 g for 30 min. The exosome-rich pellets were then washed by being resuspended in phosphate-buffered saline and stored at -80°C for analysis.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from serum samples by using the miRNeasy Mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocols. Cel-miR-39 was used as the spike-in control and was added directly to each sample. The reverse-transcriptase reaction for RNA samples were carried out using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The amplification of miR-148a was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the miScript SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). The relative expression level of serum exosomal miR-148a was calculated using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate.

Statistical Analysis

The Kruskal-Wallis test was used to compare the serum exosomal miR-148a expression among participants in different groups. Receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) were established to analyze the diagnostic value of serum exosomal miR-148a for BC. The associations between serum exosomal miR-148a and various clinicopathological parameters of BC were analyzed by the Chi-square test. The overall survival (OS) and disease-free survival (DFS) analyses were conducted by Kaplan-Meier method, and compared using log-rank test. Multivariate analysis was used to assess the independent prognostic factors of OS in BC patients. Statistical analyses were processed by software GraphPad prism 7.0 (GraphPad Prism Software Inc., La Jolla, CA, USA). *p*-value less than 0.05 was considered to indicate a statistically significant difference.

Results

Serum Exosomal MiR-148a was Downregulated in BC

Quantitative RT-PCR was used to compare serum exosomal miR-148a levels in patients with BC, patients with benign breast tumors and normal controls. Our results showed that serum exosomal miR-148a levels were highest in healthy controls, while progressively reduced in patients with benign breast tumors and BC patients ($p<0.0001$, Figure 1).

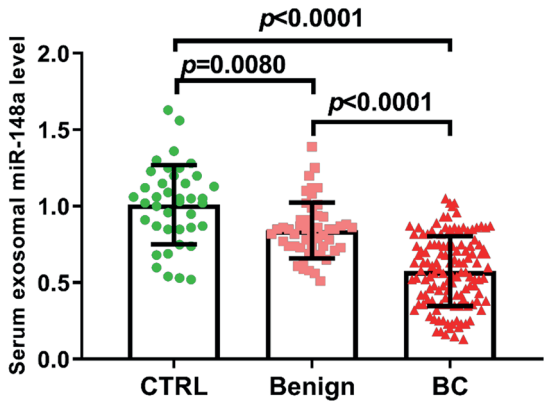

Figure 1. Serum exosomal miR-148a levels were significantly decreased in patients with BC.

The Diagnostic Value of Serum Exosomal MiR-148a Expression in BC

The ROC analysis was used to explore the potential diagnostic value of serum exosomal miR-148a for BC. Notably, serum exosomal miR-148a was powerfully discriminated between patients with BC and normal controls (AUC=0.897; 95% confidence interval=0.840-0.939, specificity=80.0%, sensitivity=84.0%, Figure 2A). In addition, serum exosomal miR-148a showed good performance to identify BC patients and patients with benign breast tumors (AUC=0.806; 95% confidence interval=0.740-0.862, specificity=88.0%, sensitivity=60.8%, Figure 2B).

Relationship of Serum Exosomal MiR-148a Expression and the Clinical Features of BC

A total of 125 patients with BC were stratified into low expression group (n=64) and high expression group (n=61) using the median serum exosomal miR-148a expression level as the cut-off point. The results revealed that serum exosomal miR-148a level was closely correlated with differentiation (p=0.0167), lymph node metastasis (p=0.0011), and TNM stage (p=0.0004). However, it was not associated with age, tumor size, histology, and venous invasion (p>0.05) (Table I).

Serum Exosomal MiR-148a Level was Sensitive to Therapeutic Response

Next, we collected blood samples from all BC patients one month after any treatment including surgical operation, chemotherapy and radiotherapy. Moreover, serum samples were obtained from 46 relapsed cases when they have relapsed. For all BC patients, the expression levels of serum exosomal miR-148a were remarkably increased after

Table I. The association between serum exosomal miR-148a level and clinicopathologic features of patients with BC.

Variables	N	Serum exosomal miR-148a level		p
		Low (n=64)	High (n=61)	
Age (y)				
< 50	53	25	28	0.4393
≥ 50	72	39	33	
Tumor size (cm)				
< 3	58	26	32	0.1848
≥ 3	67	38	29	
Histology				
Ductal	94	52	42	0.2465
Lobular	19	8	11	
Others	12	4	8	
Venous invasion				
Negative	81	37	44	0.0938
Positive	44	27	17	
Differentiation				
Well/moderate	79	34	45	**0.0167**
Poor	46	30	16	
Lymph node metastasis				
Negative	85	35	50	**0.0011**
Positive	40	29	11	
TNM stage				
I/II	70	26	44	**0.0004**
III/IV	55	38	17	

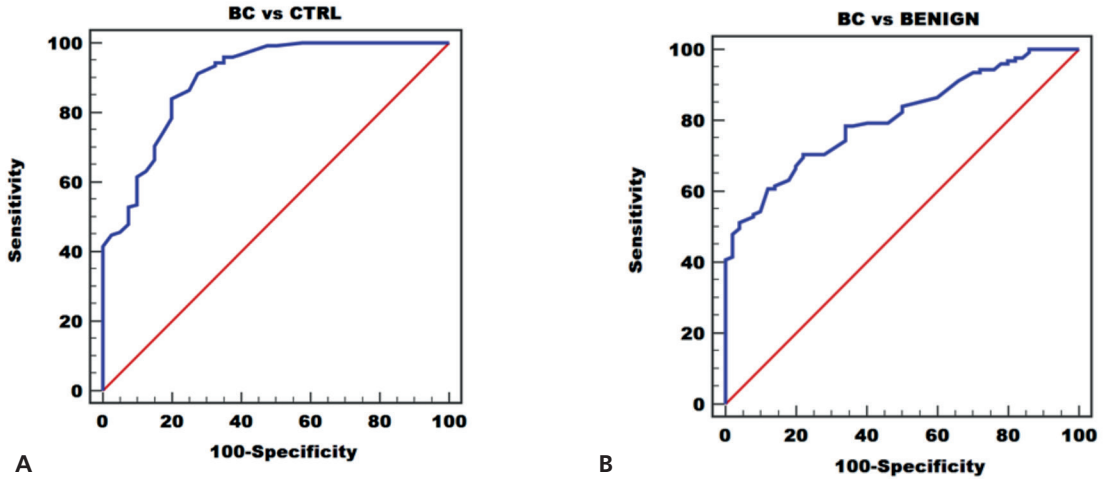

Figure 2. Serum exosomal miR-148a accurately discriminated BC patients from healthy controls (**A**) as well as BC patients from patients with benign breast tumors (**B**).

one month following treatment ($p<0.0001$, Figure 3A). For BC patients that experienced relapse, serum exosomal miR-148 levels dropped significantly in those cases ($p<0.0001$, Figure 3B). Serum exosomal miR-148a in the patients without relapse changed little after one year following treatment (data not shown).

Relationship of Serum Exosomal MiR-148a Expression and the Prognosis of BC

The five-year OS was significantly longer in patients of the high serum exosomal miR-148a group than that of the low serum exosomal miR-148a group ($p=0.0232$, Figure 4A). BC patients with lower level of serum exosomal miR-148a expression had shorter five-year DFS than those with higher level of serum exosomal miR-148a expression ($p=0.0103$, Figure 4B).

Multivariate analysis showed that TNM stage (HR=2.863, 95% CI=1.293-4.822, $p=0.010$), lymph node metastasis (HR=2.051, 95% CI=1.024-3.028, $p=0.019$) and serum exosomal miR-148a (HR=2.460, 95% CI=1.165-3.620, $p=0.015$) were independent prognostic factors for BC (Table II).

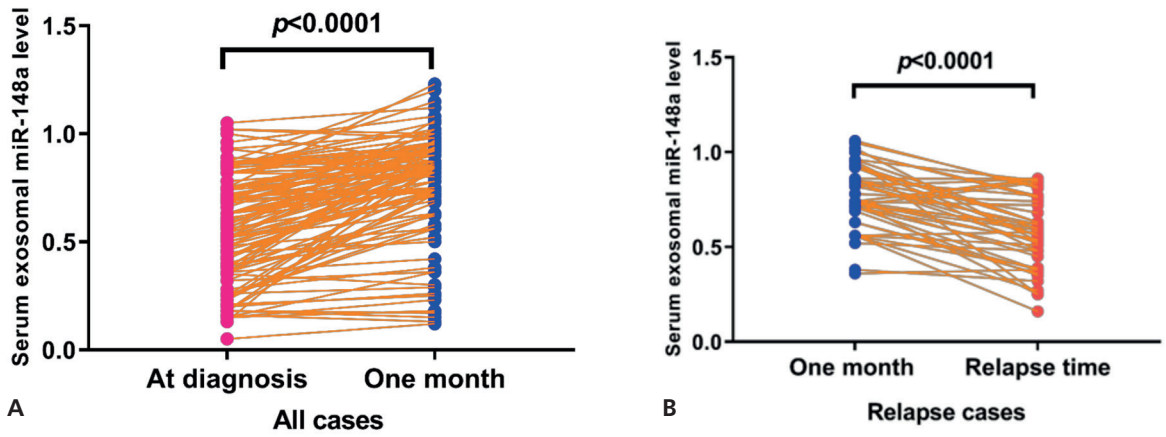

Figure 3. Serum exosomal miR-148a levels were sensitive to therapeutic response. **A,** The expression levels of serum exosomal miR-148a were significantly upregulated in BC cases following treatment. **B,** Serum exosomal miR-148a levels dropped significantly in 46 BC patients with relapse.

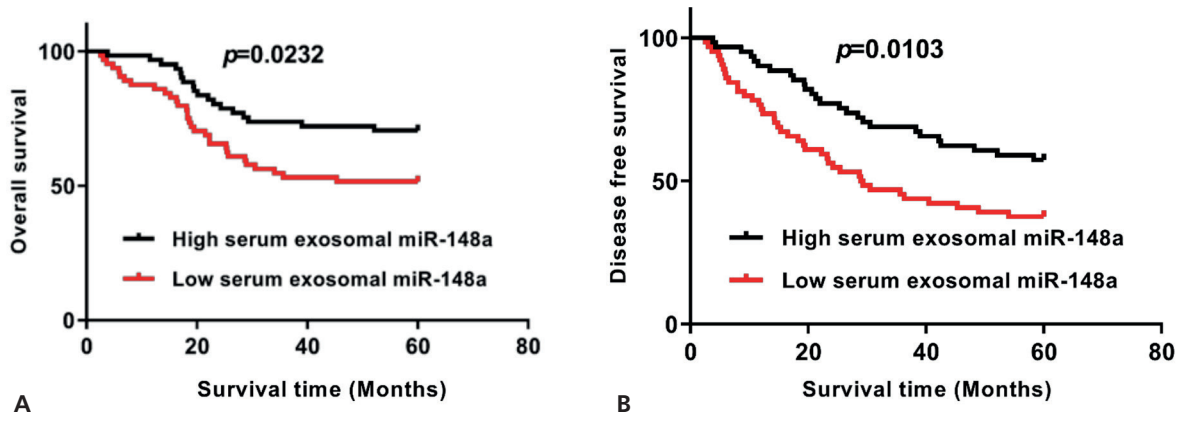

Figure 4. BC patients with lower level of serum exosomal miR-148a expression had shorter five-year OS (**A**) and DFS (**B**) than those with higher level of serum exosomal miR-148a expression.

Discussion

In the present study, we have examined serum exosomal miR-148a expression levels obtained from BC patients, patients with benign breast tumors, and healthy individuals. Serum exosomal miR-148a was frequently downregulated in BC and could well differentiate BC patients from healthy controls. In addition, the expression level of serum exosomal miR-148a in most BC patients was increased with different degree following treatment. Moreover, low serum exosomal miR-148a expression was significantly associated with unfavorable clinical parameters and shorter survival in patients with BC. Therefore, we propose that serum exosomal miR-148a serves as a novel biomarker for the diagnosis and prognosis of BC. In line with our findings, Li et al[13] showed that miR-148a expression was reduced in BC tissues, and restoration of miR-148a expression decreased BC cell proliferation and increased cell apoptosis by degrading B-cell lymphoma 2.

Likewise, the downregulation of miR-148a has also been reported in many other types of cancers. Loss of miR-148a was observed in gastric cancer tissues and strongly associated aggressive clinical variables, and miR-148a upregulation or rho-associated kinases 1 knockdown greatly suppressed the carcinogenesis *in vitro* and *in vivo*[14]. In bladder cancer, miR-148a expression was reduced in cancerous tissues and decreased tissue miR-148a level was associated with worse clinical outcome and served as an independent prognostic factor[15]. Reduced expression of miR-148a have also been reported in bladder cancer cell lines. Ectopic expression of miR-148a resulted in reduced cancer cell viability by increasing cell apoptosis, and DNA methyltransferase 1 (DNMT1) was demonstrated to be a downstream target of miR-148a[16]. Feng et al[17] found that miR-148a expression was inversely correlated with Erb-b2 receptor tyrosine kinase 3 (ERBB3) expression in pancreatic cancer (PC), and overexpression of miR-148a restrained PC cell proliferation and migration. Peng et al[18] demonstrated that miR-148a was reduced in PC tissues and cell lines. In addition, miR-148a upregulation suppresses epithelial-mesenchymal transition process, as well as the migration and

Table II. Multivariate analysis of prognostic factors for overall survival.

	Overall survival		
Variables	HR	95% CI	*p*
Differentiation (poor vs. well/moderate)	1.752	0.912-2.502	0.076
TNM stage (III/IV vs. I/II)	2.863	1.293-4.822	0.010
Lymph node metastasis (Yes vs. No)	2.051	1.024-3.028	0.019
Serum exosomal miR-148a (low vs. high)	2.460	1.165-3.620	0.015

invasion of PC cells by targeting Wnt10b and inhibiting the Wnt/β-catenin signaling pathway, suggesting that miR-148a served as a tumor suppressor in PC. Similarly, He et al[19] reported that miR-148a expression was downregulated in non-small cell lung carcinoma (NSCLC) tissues and cell lines. Moreover, enforced miR-148a expression inhibited proliferation, increased apoptotic cell death and suppressed invasion capacity of NSCLC cells *via* regulating of signal transducer and activator of transcription 3 (STAT3). The expression level of serum miR-148a was also found to be significantly reduced in patients with NSCLC[20]. Interestingly, decreased circulating levels of miR-148a was observed in colorectal cancer patients with recurrence[21].

Although most studies suggested that miR-148a suppressed the tumorigenesis process in cancer, it might also function as an oncogene. MiR-148a inhibition suppressed the proliferation of gastric cancer cells by targeting p27 and opposite results were obtained when miR-148a was overexpressed[22]. Kim et al[23] showed that miR-148a was significantly upregulated in human glioblastoma tissues, cell lines and glioblastoma stem cells. In addition, miR-148a overexpression promoted cell growth, survival, migration and invasion by directly regulating mitogen-inducible gene 6 (MIG6). Therefore, the biological function of miR-148a is complicated because it can play an oncogenic or tumor suppressive role in different types of cancers. Further studies are warranted to investigate the molecular mechanisms accounting for the role of miR-148a in cancers.

Conclusions

Taken together, serum exosomal miR-148a level was remarkably reduced in patients with BC and downregulation of serum exosomal miR-148a was associated with worse prognosis of this malignancy. Therefore, serum exosomal miR-148a might be a novel and promising biomarker for the diagnosis and prognosis prediction of BC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgments

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