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Original Article

Reduced expression of miR-26b is associated with poor prognosis of osteosarcoma

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Abstract: Altered expression of microRNAs (miRNAs) has been implicated in the initiation and progression of cancer. MiR-26b acts as a tumor suppressor in many types of cancers including osteosarcoma (OS). However, the clinical significance of miR-26b is poorly known. Real-time PCR was performed to assess the expression levels of tissue/serum miR-26b in patients with OS and their respective controls. The Pearson correlation between tissue and serum miR-26b was calculated and the diagnostic value of serum miR-26b was evaluated by Receiver operating characteristic (ROC) curve analysis. Then Chi square test was used to find out whether there was any association between tissue/serum miR-26b expression level and the clinicopathological parameters. Overall survival (OS) and disease-free survival (DFS) were evaluated using Kaplan-Meier method. The expression level of tissue/serum miR-26b was both significantly downregulated in patients with OS (P<0.01), and tissue and serum miR-26b was highly correlated with each other (r=0.521, P<0.01). Serum miR-26b could discriminate OS patients from normal controls with high accuracy (AUC=0.931, P<0.01) and the expression level of serum miR-26b in OS patients was significantly increased following treatment (P<0.01). In addition, tissue/serum miR-26b expression was associated with various important clinicopathological parameters. Moreover, the OS patients in the low tissue/serum miR-26b expression group had a poorer OS and DFS (P<0.05). Our study demonstrated that tissue/serum miR-26b was downregulated in OS patients and associated with poor clinical outcome of this progressive malignancy, suggesting miR-26b plays a tumor suppressive role in OS development and might be a promising biomarker for OS.

Keywords: Biomarker, miR-26b, osteosarcoma, prognosis

Introduction

Osteosarcoma (OS) is the most frequent primary malignant bone tumor that mainly afflicts the pediatric age group and young adults [1]. Although great progress has been achieved in therapeutic strategies including surgery technique, radiotherapy, and chemotherapy, the clinical prognosis of patients with OS are still unsatisfactory and pulmonary metastasis is the major reason leading to the fatal outcome [2, 3]. Therefore, it is important to explore the molecular mechanisms for the OS initiation and progression.

MicroRNAs (miRNAs) are small, highly conserved non-coding RNA molecules that involved in post-transcriptional gene silencing [4]. miRNA has been demonstrated to play important roles in regulating many biological processes such as proliferation, differentiation, cell survival, apoptosis and development [5]. Alterations in miRNA expression has been implicated in numerous human diseases including cancer [6]. miRNA might function as oncogenes or tumor suppressor genes in the initiation and development of OS. Wang et al showed that the expression level of miR-144 was reduced in OS cell lines and clinical samples. In addition, lower miR-144 expression was associated with distant metastasis and poor prognosis. Moreover, overexpression of miR-144 inhibited the oncogenic behaviors of OS both *in vitro* and *in vivo*, indicating miR-144 acted as a tumor suppressor in the progresion of OS [7]. Salah et al reported that ectopic expression of miR-27a promoted the invasion and metastasis of OS and *vice versa*, indicating miR-27a might be an oncogenic miRNA [8].

Table 1. Association between tissue/serum miR-26b expression levels and clinical parameters of osteosarcoma

Variable	No. of patients	Tissue miR-26b expression		*P*	Serum miR-26b expression		*P*
		High	Low		High	Low	
Gender							
Male	42	20	22	0.757	20	22	0.554
Female	54	24	30		29	25	
Age							
<40	58	25	33	0.507	28	30	0.503
≥40	38	19	19		21	17	
Tumor diameter (cm)							
<5	47	24	23	0.314	26	21	0.412
≥5	49	20	29		23	26	
Metastasis							
No	50	29	21	0.013	31	19	0.025
Yes	46	15	31		18	28	
TNM stage							
I + II	45	28	17	0.003	29	16	0.014
III + IV	51	16	35		20	31	
Differentiation							
Well or moderate	52	28	24	0.087	32	20	0.025
Poor	44	16	28		17	27	

Deregulation of miR-26b has been shown to be involved in the development of many types of cancer such as breast cancer, hepatocellular carcinoma and epithelial ovarian carcinoma and osteosarcoma [9-13]. However, whether the altered expression of miR-26b has any clinical significance is not known. Therefore, the objective of our study is to evaluate the expression level of miR-26b in clinical samples of OS and its potential clinical values.

Materials and methods

Study population

The study was approved by the Research Ethics Committee of The Second Affiliated Hospital of Harbin Medical University and written consent was obtained from the patients and their relatives before sample collection. The study prospectively enrolled 96 OS patients who were treated in our hospital. The group comprised 42 males and 54 females. Paired fresh osteosarcoma specimens and noncancerous bone tissues were collected before any treatment (surgery, radiotherapy and chemotherapy). The clinical features of OS patients was summarized in **Table 1**.

Serum sample collection and storage

Serum samples were obtained from healthy controls and patients with osteosarcoma. Briefly, up to 5 mL of fasting venous blood were withdrawn from all participants. All samples were processed within 1 h after collection and separated by centrifugation at 3000 rpm for 15 min. The separated serum was divided into aliquots and cryopreserved at -80°C until use.

Real-time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Then RNA was converted to first strand cDNA using QuantScript RT Kit (Tiangen Bioech, Beijing, China). PCR was performed using a PCR Master Mix Kit (BioTeke, Beijing, China) in a Light Cycler 480II Real-Time PCR System (Roche, Indianapolis, IN, USA). Each sample was measured in triplicate. The gene expression threshold cycle values of miRNAs were calculated by normalizing with internal control RNU6, and relative quantification values were calculated. The primers for miR-26b and RNU6 were as follows:

miR-26b forward: 5'-CTTCGGCAGCACATATACT-3'; miR-26b reverse: 5'-AAAATATGGAACACTTC-ACG-3'; RNU6 forward: 5'-CTCGCTTCGGCAG-CACATATACT-3'; RNU6 reverse: 5'-ACGCTTCA-CGAATTTGCGTGTC-3'.

Statistical analysis

Mann-Whitney-Wilcoxon test was performed to evaluate the expression level of tissue/serum miR-26b in osteosarcoma patients and the controls. The Pearson correlation coefficient was used to explore the correlation between tissue and serum miR-26b. Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic value of serum miR-26b. Chi square test was

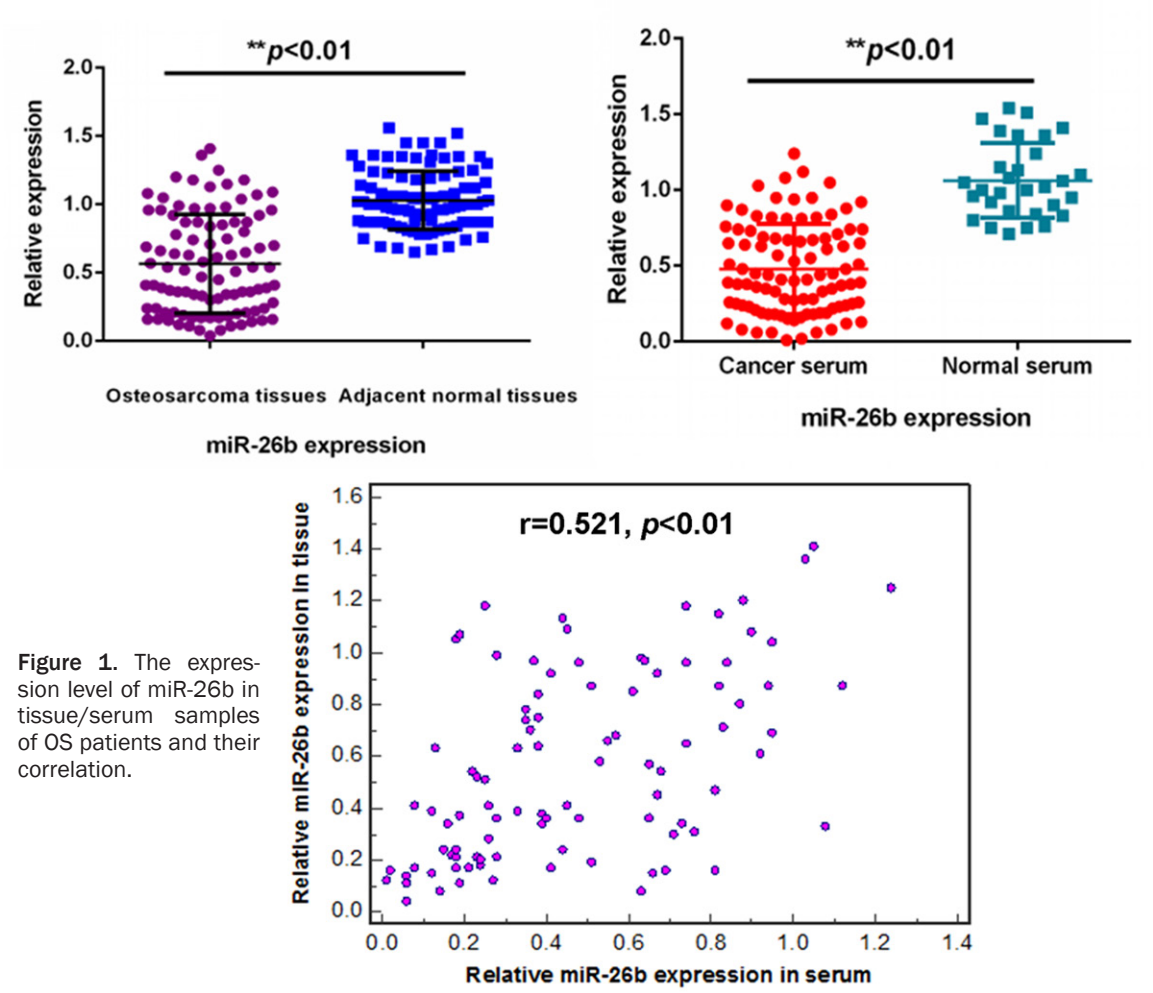

Figure 1. The expression level of miR-26b in tissue/serum samples of OS patients and their correlation.

used to evaluate the tissue/serum miR-26b expression level and clinicopathological parameters. Kaplan-Meier method and log-rank test were conducted to analyze tissue/serum miR-26b expression and OS as well as DFS. All the data were expressed as mean ± SEM and processed using SPSS 21. A *P* value less than 0.05 was considered statistically significant.

Results

The expression level of miR-26b in tissue/serum samples of OS patients

Real-time PCR was performed to compare the expression level of miR-26b in OS tissues and the adjacent normal tissues. The results showed that miR-26b was significantly down-regulated in OS tissues ($P<0.01$). Similarly, the expression level of miR-26b was remarkably reduced in the serum samples derived from OS patients ($P<0.01$). In addition, tissue miR-26b expression level was highly correlated with serum miR-26b expression level ($r=0.521$, $P<0.01$) (**Figure 1**).

Diagnostic value of serum miR-26b

Our results showed that serum miR-26b could distinguished the OS patients from normal controls with high accuracy (AUC=0.931, $P<0.01$) (**Figure 2**). In addition, the expression level of serum miR-26b in OS patients was significantly increased after receiving treatments ($P<0.01$), indicating that serum miR-26b is a sensitive biomarker for monitoring therapeutic responses (**Figure 3**).

Association between tissue/serum miR-26b expression and clinicopathological parameters of OS

The OS patients were divided into two groups based on the median expression level of tissue

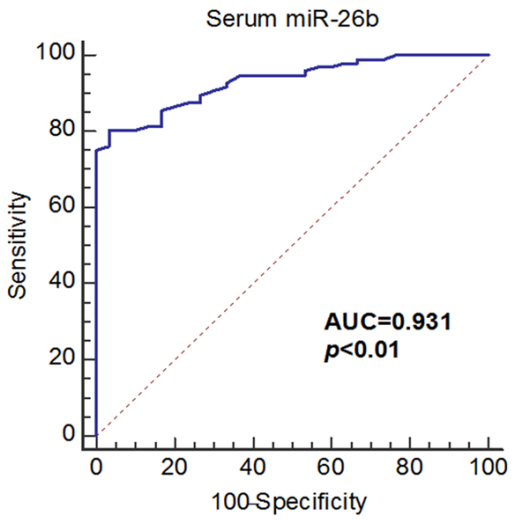

Figure 2. The diagnostic value of serum miR-26b.

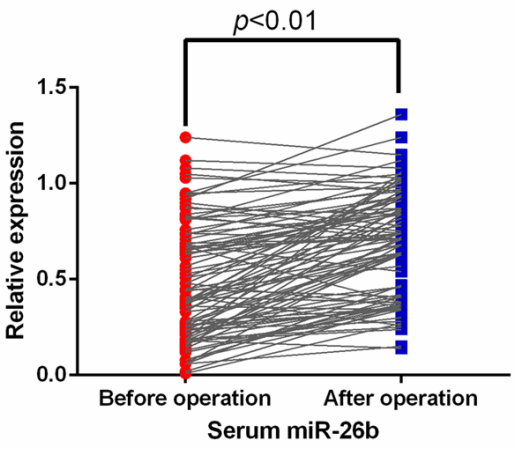

Figure 3. Expression level of serum miR-26b was significantly increased following treatment.

or serum miR-26b respectively. As shown in **Table 1**, tissue miR-26b expression level was associated with various important clinicopathological parameters including metastasis (P=0.013) and TNM stage (P=0.003). Serum miR-26b expression level was correlated with metastasis (P=0.025), TNM stage (P=0.014) and differentiation (P=0.025).

The association between tissue/serum miR-26b expression and OS/DFS

The median overall survival time of OS patients in the low tissue miR-26b group was significant shorter than those in the high tissue miR-26b group (P=0.015). OS patients with low miR-26b expression also had a poorer DFS than the patients with high miR-26b expression (P=0.006). Similarly, the OS patients in the low serum miR-26b group suffered worse OS (P=0.027) and DFS (P=0.042) compared with those in the high serum miR-26b group (**Figure 4**).

Discussion

OS is a significant public health issue worldwide [14]. Therefore, it is urgent and important to fully understand its underlying mechanisms and provide effective therapy for this progressive malignancy. It has been shown that miRNAs play crucial roles in the initiation and development of OS. However, miRNAs that are involved in the progression of OS and their clinical application values still remain poorly known [15].

Our results showed that tissue/serum miR-26b was both significantly downregulated in patients with OS, and tissue and serum miR-26b was highly correlated with each other. Serum miR-26b could discriminate OS patients from normal controls with high accuracy and its expression level was upregulated following treatment. In addition, tissue/serum miR-26b expression was associated with various important clinicopathological parameters. Moreover, the OS patients in the low tissue/serum miR-26b expression group had a poorer OS and DFS. Taken together, our study indicated that miR-26b might function as a tumor suppressor in the progression of OS. Consistent with our findings, recent reports showed that miR-26b was significantly downregulated in OS tissues in comparison with the normal tissues. In addition, ectopic expression of miR-26b inhibited the proliferation, migration and invasion as well as induced apoptosis of osteosarcoma cells by down-regulating 6-phosphofructo-2-kinase/fructose 2, 6-bisphosphatase-3 (PFKFB3) [13, 17]. Duan et al provided both *in vitro* and *in vivo* evidence to demonstrate that overexpression of miR-26b suppressed the migration and metastases of osteosarcoma by targeting connective tissue growth factor (CTGF) and Smad1 [12]. These studies further corroborate our findings that loss of miR-26b contributes to the progression of OS.

It is common to see the fact that some miRNAs play oncogenic roles in some cancers, while

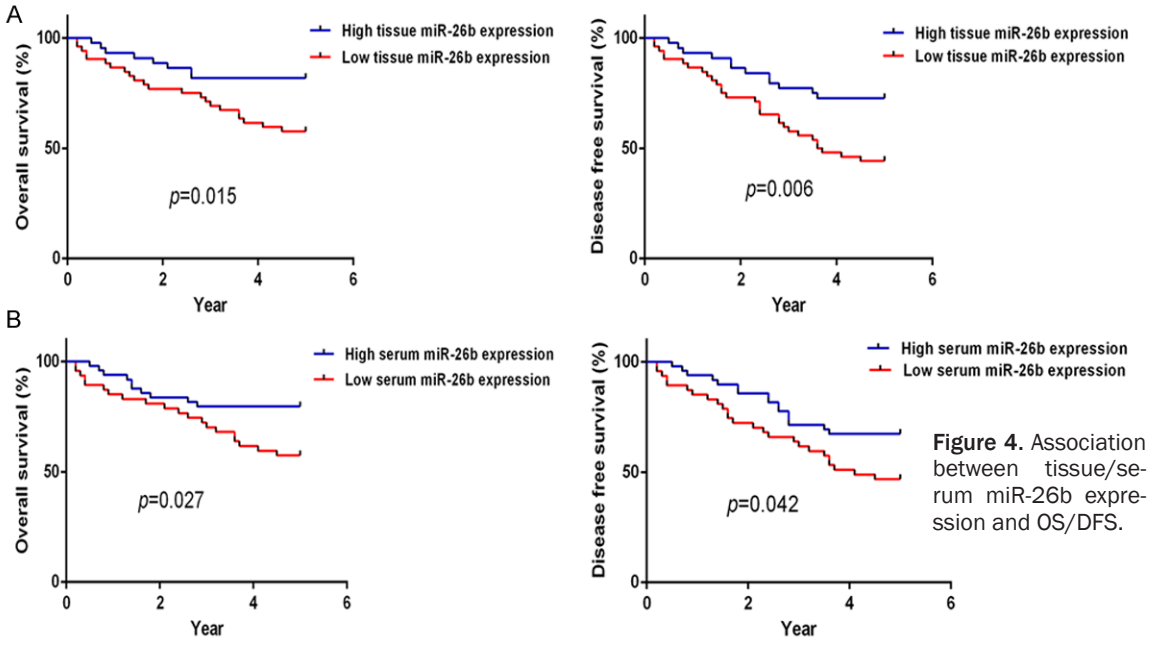

Figure 4. Association between tissue/serum miR-26b expression and OS/DFS.

function as tumor suppressive genes in others [18]. However, to our best knowledge, current available studies support that miR-26b is a tumor suppressor in all types of cancers investigated so far, indicating the biological function of miR-26b is highly conserved and it might play a key role in the initiation and progression of cancer. Liang et al showed that the expression level of miR-26b was significantly downregulated in non-small cell lung cancer (NSCLC) cells and human carcinoma tissues. In addition, miR-26b might regulate NSCLC migration and chemosensitivity through the regulation of PTEN, suggesting miR-26b could be a promising therapeutic target for treating NSCLC. Li et al reported that overexpression of miR-26b inhibited the proliferation, migration, and invasion of hepatocellular carcinoma cells by inhibiting the expression level of EphA2 [19]. Similarly, miR-26b exerts a tumor suppressive role in breast cancer and the miR-26b-mediated growth inhibition is attained through targeting CDK8 [20].

Further studies should be conducted to address the following problems. Firstly, larger clinical trials should be carrier out the confirm the clinical significance of miR-26b for OS. Secondly, detecting the biomarkers in the body fluids is more convenient and might be beneficial to the clinical patients. Therefore, we can also assess the expression level of miR-26b in body fluids such as saliva and urine to determine its potential clinical value. Finally, we should further explore the molecular mechanisms that responsible for the tumor suppressive role of miR-26b in the progression of OS.

In conclusion, our results demonstrated miR-26b might serve as a promising biomarker for OS and its dysregulation is likely to be one of major molecular mechanisms accounting for OS development.

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Disclosure of conflict of interest

None.

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