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Original Article

Downregulation of microRNA-26b functions as a potential prognostic marker for osteosarcoma

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Abstract: Objective: In the present study, we aimed to systematically detect the expression of miR-26b in osteosarcoma patients by qRT-PCR. Meanwhile, we intended to analyze the clinical significance and prognosis value of miR-26b in osteosarcoma patients. Methods: Between June 2007 and February 2015, a total of 107 human osteosarcoma samples and the adjacent non-tumor tissues were collected. The expression of miR-26b was estimated by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Log-rank test and Kaplan-Meier method were used to evaluate survival rate in patients with osteosarcoma. Multivariate analysis was performed to evaluate prognostic values using Cox proportional hazards model. Results: The expression of miR-26b was significantly lower in osteosarcoma samples compared to non-cancerous samples ($P<0.001$). Low miR-26b expression level was observed to be closely correlated with distant metastasis ($P=0.004$), response to chemotherapy ($P=0.002$), and clinical stage ($P=0.001$). Kaplan-Meier analysis with the log-rank test indicated that miR-26b expression had a significant impact on overall survival ($P=0.004$). Multivariate analysis revealed that miR-26b expression level was independent prognostic factor for overall survival (HR=2.187, 95% CI: 1.261-8.012; $P=0.019$). Conclusion: Our results suggested that miR-26b expression might be associated with aggressive features of osteosarcoma patients. Detection of tissue miR-26b levels might have clinical potentials as a non-invasive prognostic biomarker for osteosarcoma patients.

Keywords: MicroRNA, miR-26b, prognostic marker, osteosarcoma

Introduction

Osteosarcoma accounts for approximately 20% of primary malignancies of bone and is characterized by high local aggressiveness, rapid growth rate and early metastasis to lungs and distant bones [1]. A large number of patients respond poorly to chemotherapy and patients with osteosarcoma are at a high metastatic risk [2]. Therefore, understanding the molecular pathogenesis and uncovering molecular biomarkers of osteosarcoma would improve the survival of osteosarcoma patients.

MicroRNAs are a class of endogenous noncoding small (18-24 nt) single-stranded RNAs, which can regulate gene expression at the post-transcriptional level. Mechanistically, miRNAs function by binding to the 3'-untranslated region (3'-UTR) of messenger RNA (mRNA), causing translation to be blocked and/or mRNA degradation [3]. Recent researches have indicated that miRNAs served significant functions in almost every biological pathway, and might act as modulators of tumor proliferation, invasion, apoptosis and therapy resistance [4-6].

Previous studies showed that miR-26b was downregulated in several types of cancer, including colorectal cancer, breast cancer, nasopharyngeal carcinoma, lung cancer, and hepatocellular carcinoma [7-12]. In the study by Duan et al, they demonstrated downregulation of miR-26b in osteosarcoma tissues. Furthermore, they found that miR-26b inhibited metastasis of osteosarcoma via targeting connective tissue growth factor (CTGF) and Smad1 [13]. Du et al found that miR-26b inhibited proliferation, migration, invasion and apoptosis by targeting the glycolytic metabolism in osteosarco-

Table 1. Clinicopathological characteristics of 107 patients with osteosarcoma

Characteristics	Cases	Tissue miR-26b level		*P* value
		Low (n=54)	High (n=53)	
Age (years)				
≥25	50	26	24	0.847
<25	57	28	29	
Gender				
Female	39	21	18	0.689
Male	68	33	35	
Tumor size (cm)				
≥8	55	30	25	0.442
<8	52	24	28	
Anatomic location				
Tibia/femur	86	45	41	0.474
Elsewhere	21	9	12	
Distant metastasis				
No	95	43	52	0.004
Yes	12	11	1	
Response to chemotherapy				
Good	33	9	24	0.002
Poor	74	45	29	
Clinical stage				
IIA	59	21	38	0.001
IIB/III	48	33	15	

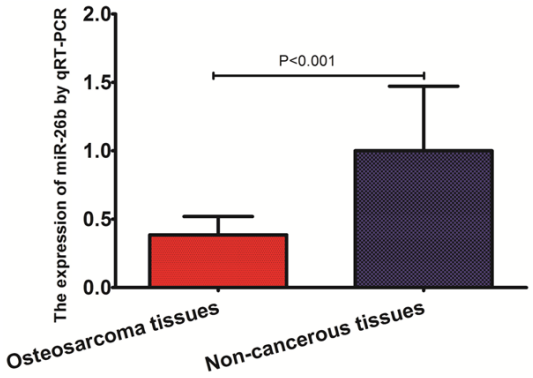

Figure 1. qRT-PCR detection of miR-26b expression in 107 paired of osteosarcoma tissues and the adjacent normal bone tissues.

ma cells [14]. Zheng et al found that miR-26b inhibited osteosarcoma cell proliferation, migration, and invasion by regulating PFKFB3 protein expression [15]. In the present study, we aimed to investigate the clinical significance and prognostic value of miR-26b in patients with osteosarcoma.

Materials and methods

Patients and tissues

Between June 2007 and February 2015, a total of 107 human osteosarcoma samples and the adjacent non-tumor tissues were collected during surgery at the Department of Orthopedics, Pu Ai Hospital of Tongji Medical College of Huazhong University of Science and Technology. Medical records were used to ascertain patients' medical histories, including age, gender, anatomic location, tumor size, clinical stage, distant metastasis, and response to chemotherapy. All patients' slides were reviewed to confirm the diagnosis and to classify the tumor according to the sixth edition of the tumor node metastases (TNM) classification of the International Union against Cancer (UICC). All specimens were immediately frozen in liquid nitrogen and stored at -70°C until use. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki. All participants have signed informed consent paper. Ethical approval for the use of human subjects was obtained from the research ethics committee of Pu Ai Hospital of Tongji Medical College of Huazhong University of Science and Technology. The clinicopathological features of all 107 patients with osteosarcoma are summarized in **Table 1**.

Isolation of total RNA and real-time PCR

Total miRNA from surgical tissues was extracted using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. cDNA was synthesized using the Taqman miRNA reverse transcription kit (Applied Biosystems), and expression levels of miR-26a were quantified using miRNA-specific TaqMan MicroRNA Assay Kit (Applied Biosystems). Real-time PCR was performed using the Applied Biosystems 7500 Sequence Detection system. The expression of miRNA was defined based on the threshold cycle (Ct), and relative expression levels of miR-26b were calculated as $2^{-[(Ct\ of\ miR\text{-}26b)-(Ct\ of\ RNU6B)]}$ after normalization with reference

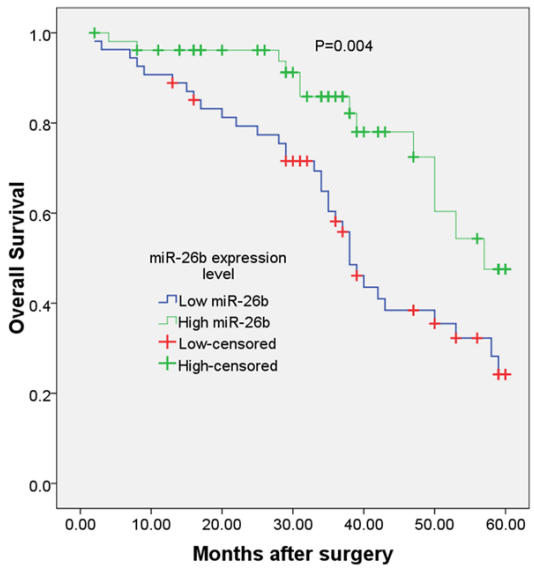

Figure 2. Kaplan-Meier curves showed the relationship between overall survival and miR-26b in osteosarcoma patients. Log rank test was used to compute *P* values.

to expression of RNU6B small nuclear RNA. The primers of miR-26b were as follows: forward primer: 5'-CCCAGTTCAAGTAATTCAGG-3' and reverse primer: 5'-TTTGGCACTAGCACATT-3'.

Statistical analysis

Differences between all variables were analyzed using Student's t test or chi-square test. Log-rank test and Kaplan-Meier method were used to evaluate survival rate in patients with osteosarcoma. Multivariate analysis was performed to evaluate prognostic values using Cox proportional hazards model. A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were performed by the software of SPSS version 18.0 for Windows (SPSS Inc., IL, USA).

Results

Expression level of miR-26b in osteosarcoma

We assessed miR-26b expression in 107 osteosarcoma samples and 107 paired non-cancerous samples. The expression of miR-26b was significantly lower in osteosarcoma samples compared to non-cancerous samples (P<0.001, shown in **Figure 1**). We divided 107 osteosarcoma patients into two groups according to the levels of miR-26b expression. The cut-off point was the median expression level of miR-26b in osteosarcoma samples (low expression group, n=54; high expression group, n=53).

Correlations of miR-26b expression with clinicopathologic features of osteosarcoma patients

The correlations of miR-26b expression with clinicopathologic features of patients with osteosarcoma were statistically analyzed. As shown in **Table 1**, low miR-26b expression level was observed to be closely correlated with distant metastasis (P=0.004), response to chemotherapy (P=0.002), and clinical stage (P=0.001). However, there were no significant correlations between miR-26b expression level and other clinicopathologic factors, including gender (P=0.689), age (P=0.847), tumor size (P=0.442), and anatomic location (P=0.474).

Association of miR-26b expression with prognosis in osteosarcoma patients

Kaplan-Meier analysis with the log-rank test indicated that low miR-26b expression had a significant impact on overall survival (P=0.004; shown in **Figure 2**). Univariate and multivariate analyses were utilized to evaluate whether the miR-26b expression level and various clinicopathological features were independent prognostic parameters of osteosarcoma patient outcomes. Multivariate analysis revealed that miR-26b expression level was independent prognostic factor for overall survival (HR=2.187, 95% CI: 1.261-8.012; P=0.019, shown in **Table 2**).

Discussion

Osteosarcoma derives from primitive bone-forming mesenchymal cells and is the most common primary bone malignancy. Studies to determine the etiology of osteosarcoma involve epidemiologic and environmental factors and genetic impairments. Although currently osteosarcoma patients are routinely treated with combinatorial chemotherapy, curative resection of the primary tumor, and sometimes radiotherapy, which has been shown to improve the 5-year survival rate to approximately 60-70%, a significant proportion of osteosarcoma patients still have a risk of local relapse or distant metastasis even after surgery and intensive chemotherapy [16, 17]. Therefore, it is needed

Table 2. Multivariate analysis for overall survival in 107 patients with osteosarcoma

Factor	Hazard ratio	95% CI	*P* value
Age	0.784	0.337-1.928	0.341
Gender	0.679	0.476-2.448	0.645
Tumor size	2.347	0.798-4.292	0.132
Anatomic location	0.394	0.219-1.283	0.683
Distant metastasis	5.239	2.394-18.993	<0.001
Response to chemotherapy	3.298	1.092-7.115	0.025
Clinical stage	4.465	2.203-15.394	0.007
miR-26b expression	2.187	1.261-8.012	0.019

to identify biomarkers, involved mechanisms, and therapeutic targets for osteosarcoma.

miRNAs play crucial roles in the regulation of diverse target mRNAs at the level of mRNA degradation or translation. Increasingly, evidence suggests that miRNAs are involved in multiple biological processes and have an essential role in the regulation of genes during cancer development, progression, and metastasis [18, 19]. It has been reported that biological activities of various miRNAs contribute to invasion and metastasis in osteosarcoma.

Previous studies showed that miR-26b was downregulated in several types of cancer, including colorectal cancer, breast cancer, nasopharyngeal carcinoma, lung cancer, and hepatocellular carcinoma [7-12]. Furthermore, the clinical significance and prognostic value have been investigated in several cancers. For example, Lin et al found that miR-26b was downregulated in epithelial ovarian carcinoma (EOC) samples, and low expression of miR-26b was associated with advanced FIGO stage, poor differentiation, higher risk of distant metastasis and recurrence. Furthermore, downregulation of miR-26b predicted poor disease-free survival and overall survival in EOC patients [11]. Luo et al found that the expression level of miR-26b in cervical cancer tissues was significantly lower than that in the adjacent normal cervical tissues. Reduced miR-26b was observed to be significantly correlated with advanced FIGO stage, higher incidence of lymph node metastasis and recurrence of cervical cancer patients. In addition, patients with low-miR-26b expression showed poorer RFS and OS than those with high-miR-26b expression. Furthermore, multivariate analyses demonstrated that low miR-26b expression was an independent prognostic factor for predicting the 5-year RFS and OS of cervical cancer patients [20].

Although the expression level and biological mechanism of miR-26b in osteosarcoma have been investigated in previous studies [13-15], the clinical significance and prognostic value have not been evaluated until now. In the present study, we assessed miR-26b expression in 107 osteosarcoma samples and 107 paired non-cancerous samples, and we found that the expression of miR-26b was significantly lower in osteosarcoma samples compared to non-cancerous samples. We then investigated the correlations of miR-26b expression with clinicopathologic features of osteosarcoma patients, and low miR-26b expression level was observed to be closely correlated with distant metastasis, response to chemotherapy, and clinical stage. Furthermore, Kaplan-Meier analysis with the log-rank test indicated that miR-26b expression had a significant impact on overall survival. Multivariate analysis revealed that miR-26b expression level was independent prognostic factor for overall survival.

In conclusion, our results suggested that miR-26b expression might be downregulated in tumor tissues and was associated with aggressive features of this malignancy. Detection of tissue miR-26b levels might have clinical potentials as a non-invasive prognostic biomarker for osteosarcoma patients.

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Disclosure of conflict of interest

None.

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