

Increased expression of microRNA-26a-5p predicted a poor survival outcome in osteosarcoma patients

An observational study

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Abstract

MicroRNA (miR)-26a-5p is an oncogene significantly associated with osteosarcoma. We try to evaluate expression of circulating miR-26a-5p in osteosarcoma patients and evaluate its significance.

A total of 243 consecutive osteosarcoma patients and 96 healthy participates were enrolled. Circulating miR-26a-5p levels were evaluated by using real-time quantitative reverse transcription polymerase chain reactions (RT-PCR). The association between circulating miR-26a-5p level and survival outcomes was evaluated by univariate and multivariate analysis.

Circulating miR-26a-5p levels in osteosarcoma patients was significantly higher than that of healthy volunteers ($P < .05$). Upregulated miR-26a-5p was significantly related to advanced cancer and metastasis (both $P < .05$). Moreover, patients with a high serum miR-26a-5p had a poorer overall survival than those with a low serum miR-26a-5p levels ($P < .05$). Circulating miR-26a-5p level also been showed as independent risk factor for osteosarcoma in multivariate analysis (hazard ratio [HR], 0.38; 95% confidence interval [CI]: 0.11–0.98; $P < .01$).

Circulating miR-26a-5p was significantly upregulated in osteosarcoma patients and remarkably associated with poor prognosis, indicating that circulating miR-26a-5p might serve as a useful diagnostic and prognostic biomarker for osteosarcoma.

Abbreviations: AUC = area under the curve, MiR = MicroRNA, OS = overall survival, RFS = recurrence-free survival, ROC = receiver operating characteristic curve, RT-PCR = real-time quantitative reverse transcription polymerase chain reactions.

Keywords: osteosarcoma, prognosis, serum miR-26a-5p

Editor: Daryle Wane.

No funding was received.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate: All subjects have provided signed informed consent prior to enrollment. This study was also approved by the Research Ethics Committee of Daping Hospital, Army Medical University.

Patient consent for publication: All patients have provided written informed consent for publication.

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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How to cite this article: Xie XY, Chen XM, Shi L, Liu JW. Increased expression of microRNA-26a-5p predicted a poor survival outcome in osteosarcoma patients: an observational study. *Medicine* 2021;100:12(e24765).

Received: 9 May 2020 / Received in final form: 20 October 2020 / Accepted: 26 January 2021

<http://dx.doi.org/10.1097/MD.00000000000024765>

1. Introduction

Osteosarcoma is the most frequent malignant primary sarcoma and the second contributor of cancer related mortality among adolescents.^[1,2] Moreover, most of osteosarcoma cases are confirmed at advanced stage, with a very poor prognosis.^[3,4] Although considerable advancement of diagnostic strategies, surgery and adjuvant treatments during past decades, the long-term prognosis of osteosarcoma patients remains unsatisfactory. Early lung metastasis and recurrences were considered as main contributors of poor prognosis of osteosarcoma.^[5,6] In details, recurrences could be observed in approximately 60% to 80% of osteosarcoma patients received surgery, with a postoperative 5-year survival rate of 15% to 20%.^[7] However, the unclear potential molecular mechanisms underlying the histological heterogeneity, poor treatments response, and recurrent were still bothering orthopedic surgeons. Therefore, furtherly exploring of molecular mechanisms associated with the development, progression, and aggressiveness of osteosarcoma is curial for diagnosis, risk stratification, and individual treatment for osteosarcoma patients.

MicroRNAs (miRNAs) is a novel subset of endogenous small non-coding RNAs, which can specifically regulate post-transcriptional gene expression by inhibiting the translation and/or decreasing of the stability of specific protein-coding gene.^[8] Previous studies showed that dysregulated expression of miRNAs was commonly observed in development of several malignancies.^[9–11] It has been showed that miRNAs can be stably expressed in plasma with an appropriate and measurable

concentration.^[12,13] Furthermore, various circulating miRNAs have been evaluated as valuable noninvasive biomarkers for cancers.^[14,15] MicroRNA-26a-5p (miR-26a-5p), a tumor-associated miR, has been found to be implicated in the development of various cancers by regulating tumor cell proliferation, invasion, migration, and apoptosis.^[16] Shi et al^[17] found that miR-26a-5p inhibits proliferation, invasion, and metastasis by repressing the expression of Wnt5a in papillary thyroid carcinoma. Huang et al^[18] found that miR-26a-5p inhibits breast cancer cell growth by suppressing RNF6 expression. Moreover, miR-26a-5p has been confirmed to act as a tumor oncogene for osteosarcoma in vitro and in vivo experiments, which can promote proliferation and migration of osteosarcoma by targeting HOXA5.^[19] However, no evidence regarding the value of circulating miR-26a-5p in osteosarcoma has been reported. Therefore, in present study, we try to evaluate expression level of circulating miRNA-26a-5p in osteosarcoma patients and analyze its prognostic value.

2. Materials and methods

2.1. Patients

This study protocol was planned by basing on the relevant guidelines or regulations and conformed to the Declaration of Helsinki. All subjects have provided signed informed consent prior to enrollment. This study was also approved by the Research Ethics Committee of the Daping Hospital, Army Medical University. Total of 243 patients with primary localized osteosarcoma admitted to Department of Orthopaedic Surgery between July 1, 2015 and July 1, 2018 were retrospectively collected. All enrolled patients were diagnosed as osteosarcoma by pathology evaluation. Exclusion was conducted based on following criteria: preoperative comorbidity, relapse and metastasis, incomplete clinical and histopathological data, and life expectancy <4 months. All enrolled patients received neoadjuvant chemotherapy (combination of doxorubicin and methotrexate), surgical resection, and postoperative chemotherapy according to the 2018 European Sarcoma Network Working Group Clinical Practice Guidelines for osteosarcoma.^[20] Total of 96 age and sex-matched healthy participates were recruited as the control group.

All relevant clinical and pathological data of each patient was collected and reconfirmed. The clinical stages were evaluated according to the system for the surgical staging of musculoskeletal sarcoma. All enrolled patients were regularly followed up by outpatient visiting or telephone. Follow-up was ended at June 2019. The median follow-up time was 26.3 months. The primary outcome of interest was survival status. Overall survival (OS) was defined as the interval from the date of enrollment to the date of death or endpoint. Recurrence-free survival (RFS) was defined as from the date of curative treatment to the date of tumor recurrence or last known date alive.

2.2. RNA isolation and quantitative real-time PCR

Total RNA was extracted from 400 mL of serum by using TRIzol Reagent (Invitrogen, Mulgrave, Australia) in accordance with the manufacturer's instructions. The TaqMan miRNA assay kit (Applied Biosystems, Foster City, CA) was used to quantitatively assess the expression levels of miR-26a-5p. LightCycler480 system (Roche, Mannheim, Germany) was used to perform Real-Time PCR with a SYBR Premix Ex TaqTM (Takara, Dalian,

China) according to the manufacturer's instructions. U6 was chosen as the reference gene for normalization. The $2^{-\Delta\Delta Ct}$ method was applied to evaluate the relative expression levels of miR-26a-5p. Each sample was measured in triplicate. The primer sequences used in present study were as follows: miR-26a-5p-forward: 5'-UCCAUAAGUAGGAAACACUACA-3', backward: 5'-CAGUACUUUGUGUAGUACAA-3'; U6-forward: 5'-CTCGCTTCGGCAGCACATATACT-3', backward: 5'-ACGCTTCACGAATTTGCGTGTGTC-3'.^[19]

2.3. Statistical analysis

Statistical analysis was carried out by using the SPSS 20.0 statistical package (IBM, USA). The statistical results were considered as significant while $P < .05$ (2 sided). Continuous variables that expressed as mean \pm SD were compared by using analysis of variance (ANOVA), whereas comparisons of categorical variables were conducted by using chi-square or Fisher exact test, which were presented as frequencies (%). Receiver operating characteristic curve (ROC) analysis was performed to evaluate the prognostic and diagnostic value of circulating miR-26a-5p levels. Kaplan–Meier survival curves for circulating miRNA-26a-5p levels predicting survival were analyzed by log-rank test. Univariate and multivariate Cox hazard regression model was employed to evaluate the prognostic factors for osteosarcoma.

3. Results

3.1. Circulating miR-26a-5p expression in patients with osteosarcoma

Circulating miR-26a-5p level was significantly upregulated in osteosarcoma patients compared with the healthy subjects ($P < .05$, see Fig. 1A). Furthermore, ROC curve analysis demonstrated that circulating miR-26a-5p level could be used as a promising biomarker for distinguishing osteosarcoma patients from healthy subjects, with an area under the curve (AUC) of 0.76 (95% CI: 0.64–0.98), a sensitivity of 69.7% and a specificity of 74.3%, ($P < .05$, Fig. 1B).

3.2. Association of circulating miR-26a-5p and clinicalopathological characteristics

All 243 osteosarcoma patients were divided into a high miR-26a-5p group ($n = 126$) or a low miR-26a-5p group ($n = 117$) by using the median value of serum miR-26a-5p level as the cut-off point. As showed in Table 1, a high serum miR-26a-5p levels was significantly associated with distance metastasis and advanced clinical stage (both $P < .01$, Table 1). However, other clinical variables, including age, sex, tumor site and size, and pathology were not closely associated with serum miR-26a-5p expression (all $P > .05$). (Table 1).

3.3. Prognostic significance of circulating miR-26a-5p level in osteosarcoma patients

According to the results of Kaplan–Meier method and log-rank test, patients with an upregulated expression of circulating miR-26a-5p had a significantly poorer OS than those with a low expression of miR-26a-5p ($P = .02$, Fig. 2). Moreover, a higher circulating miR-26a-5p level was also significantly associated a poorer RFS ($P = .04$, Fig. 3).

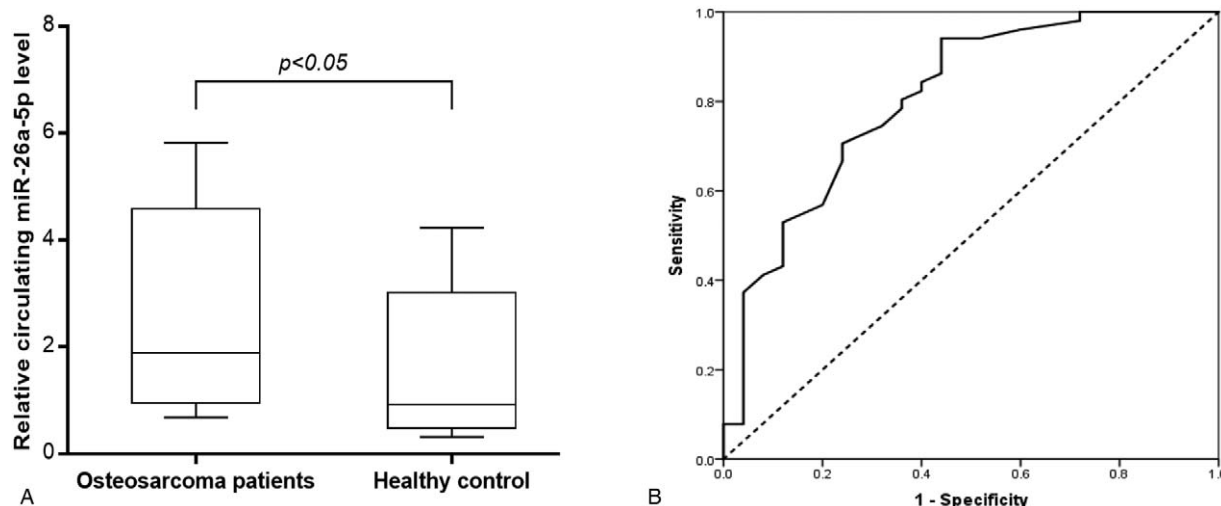


Figure 1. Circulating microRNA-26a-5p (miR-26a-5p) expression in patients with osteosarcoma and its potential value for osteosarcoma diagnosis. Panel A showed that mean circulating miR-26a-5p level of 243 osteosarcoma patients was significant higher than that of 96 age-matched healthy volunteers ($P < .05$). Panel B showed that a AUC of 0.76 for miR-26a-5p with a 95% confidence interval between 0.64 and 0.98 in ROC curve, $P < .05$. AUC=area under the curve, ROC= receiver operating characteristic curve.

The univariate and multivariate analysis enrolled age and sex of patients, tumor size, distant metastasis, clinical stage, histological type and circulating miR-26a-5p level to determine independent prognostic indicator for osteosarcoma patients. After adjustment for potential confounders, an upregulated circulating miR-26a-5p (hazard ratio [HR], 0.38; 95% confidence interval [CI]: 0.11–0.98, $P < .01$) was also an independent risk factor for survival outcome of osteosarcoma patients (Table 2).

Table 1
Correlation between serum miR-217 level and clinicpathologic characteristics of osteosarcoma patients.

Characteristic	Serum miR-217		P
	High (n=77)	Low (n=86)	
Age, y			.63
≥25	36	37	
<25	41	49	
Gender			.26
Male	54	67	
Female	23	19	
Tumor site			.48
Tibia/femur	46	56	
Other	31	30	
Tumor size, cm			.90
≥8	33	36	
<8	44	50	
Distant metastasis			<.01
Yes	18	54	
No	59	32	
Clinical stage			<.01
IIA	54	16	
IIB+III	23	70	
Pathology			.10
Osteogenic or chondrocytic	46	62	
Fibrocytic or mixed	31	24	

4. Discussion

In this study, we demonstrated that circulating miR-26a-5p was significantly upregulated in osteosarcoma patients comparing with healthy participates. Moreover, circulating miR-26a-5p level can be used as a compromising diagnostic biomarker discriminating osteosarcoma patients from healthy subjects. Furthermore, we evaluated the ability of circulating miR-26a-5p in predicting prognosis of osteosarcoma patients, and found that a high circulating miR-26a-5p level was significantly associated with poorer survival in osteosarcoma patients. We also observed an obvious association between circulating miR-26a-5p level and classical unfavorable prognostic markers for osteosarcoma patients. Based on these results, we suggested that miR-26a-5p can be used as a diagnostic biomarker for osteosarcoma patients, which also can serve as a promising prognostic biomarker.

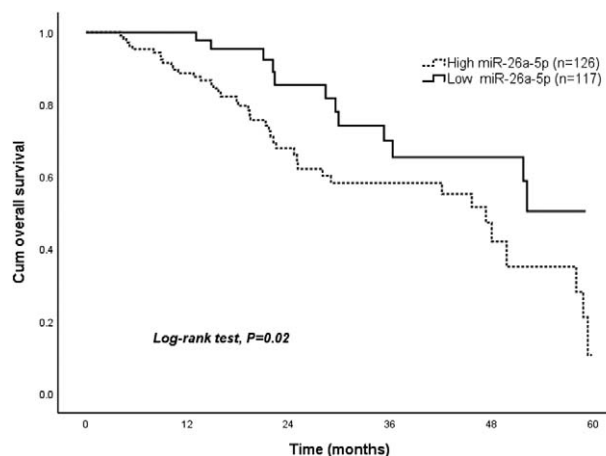


Figure 2. Upregulated circulating miR-26a-5p level was associated with poor prognosis of osteosarcoma. The Log-rank analysis revealed that a high serum miR-26a-5p level was significantly associated with a poor overall survival ($P = .02$).

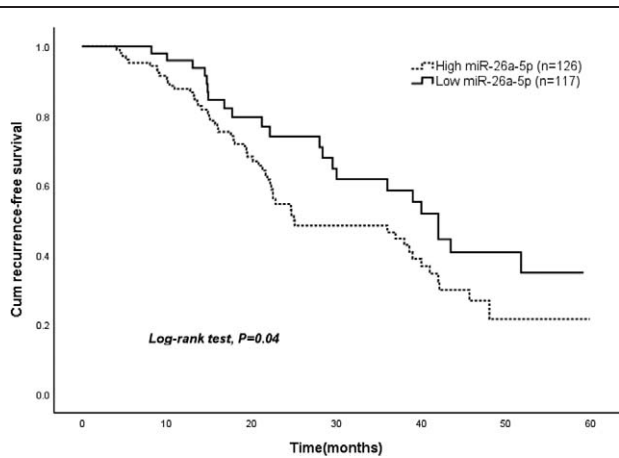


Figure 3. Increased circulating miR-26a-5p level was associated with poor prognosis of osteosarcoma. The Log-rank analysis revealed that a high serum miR-26a-5p level was significantly associated with a recurrence-free survival ($P = .04$).

MiR-26a-5p, as a novel tumor biomarker, plays critical roles in biological process of cancer development.^[21,22] The miR-26a-5p has been confirmed as a potential oncogene in many malignancies including osteosarcoma.^[21,23] Song et al^[24] showed that miR-26a-5p potentiated lung cancer cell metastasis via JAK2/STAT3 pathway by targeting ITGβ8. Yu et al^[19] reported that highly expressed miR-26a-5p in osteosarcoma cells and promotes proliferation and migration by targeting HOXA5. In our study, miR-26a-5p was remarkably upregulated in osteosarcoma patients and significantly correlated with poor prognosis, which are consistent with the previous studies mentioned above.

However, the underlying function and origin of serum miR-26a-5p in malignancy have not yet been fully understood. Several potential mechanisms for circulating miRNAs releasing have been reported, including passive leakage from cells in setting of chronic inflammation or injury, active secretion, complex formation with lipoproteins or RNA binding proteins.^[25–27] Wang et al^[23] reported that miR-26a-5p was plasma miR-26a-5p was significantly increased in bladder cancer patients, which was significantly negatively correlated with survival of bladder cancer patients. It has been reported that low expressions of certain miRNAs were remarkably associated with advanced cancer stage.^[28] In this study, we also found that low serum level of miR-26a-5p was significantly associated with clinical stage and metastasis of osteosarcoma patients. It has been reported that miR-26a-5p inhibitor down-regulated cell proliferation and

migration, but significantly improved cell apoptosis.^[19] These findings confirmed that miR-26a-5p positively correlated with osteosarcoma growth and development.

Our study firstly reported that the upregulation of serum miR-26a-5p level in a considerable scale osteosarcoma patients group, which can serve as a serum diagnostic and prognostic biomarker, as well as a novel therapeutic target for osteosarcoma. However, there were several limitations in this study. One limitation was a single center, small sample size, and retrospective design of study. A large-scale, prospective, and multicenter study is required to furtherly reevaluate such results. Furthermore, the underlying roles and mechanisms of miR-26a-5p in development of osteosarcoma have not yet been fully evaluated. Future experiments need to be performed to elucidate the mechanisms of circulating miR-26a-5p in carcinogenesis.

5. Conclusions

In this study, we found that circulating miR-26a-5p levels were upregulated in osteosarcoma patients. Moreover, upregulated circulating miR-26a-5p level was significantly associated with poor survival of osteosarcoma patients, indicating that miR-26a-5p might not only serve as a diagnostic and prognostic biomarker for osteosarcoma, but also a potential novel treatment target.

Author contributions

- Conceptualization:** Xiao-Yu Xie, Junwei Liu.
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- Writing – original draft:** Xiao-Yu Xie.
- Writing – review & editing:** Xiao-Yu Xie, Junwei Liu.

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Table 2

The prognostic factor of osteosarcoma patients.

	Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P
Age, y	1.22	0.52–2.45	.48			
Gender	1.13	0.36–2.49	.89			
Tumor site	1.01	0.53–1.44	.85			
Tumor size	1.38	0.69–4.57	.14			
Distant metastasis	0.42	0.12–0.98	.02	0.56	0.23–0.98	<.01
Clinical stage	1.76	1.01–6.78	.01	1.16	1.01–4.58	<.01
Pathology	1.12	0.73–3.26	.54			
MIR-26a-5p	0.45	0.13–0.99	.02	0.38	0.11–0.98	<.01

CI = confidence interval, HR = hazard ratio, miR-26a-5p = micro RNA-26a-5p.

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