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Original Article

Serum *miRNA-34a* serves as a diagnostic and prognostic bio-marker in osteosarcoma

Chunsheng Zhi, Bo Wu

Shenyang Orthopedic Hospital, Shenyang, Liaoning, China

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Abstract: Background: *MicroRNA-34a (miR-34a)* had been reported to be decreased in osteosarcoma, but its effects on diagnosis and prognosis were still unclear. The purpose of this study was to investigate the expression and clinical significance of *miR-34a* in osteosarcoma. Methods: The expression of *miR-34a* in tissues and serum of all patients and controls was detected by quantitative real-time polymerase chain reaction (qRT-PCR) assay, respectively. Besides, the relationship between *miR-34a* expression and clinicopathologic characteristics were analyzed. The association between *miR-34a* expression and overall survival of patients with osteosarcoma was analyzed Kaplan-Meier analysis. The prognostic value of *miR-34a* was estimated via cox regression analysis. Results: The expression level of *miR-34a* was decreased in osteosarcoma tissues and serum compared with the controls. Receiver operating characteristic (ROC) curve demonstrated *miR-34a* could be a diagnostic factor in osteosarcoma with an AUC of corresponding with a sensitivity of and a specificity of. Kaplan-Meier analysis revealed that patients with high *miR-34a* expression had a longer overall survival than those with low expression (Log rank test, $P<0.001$). According to cox regression analysis, *miR-34a* was considered as an independent prognostic marker in osteosarcoma. Conclusion: *miR-34a* expression was down-regulated in osteosarcoma and associated with tumor progression. Moreover, its abnormal expression made it can be an independent diagnostic and prognostic bio-marker for osteosarcoma patients.

Keywords: Osteosarcoma, *miR-34a*, diagnosis, prognosis

Introduction

Osteosarcoma is the most frequent primary malignant bone tumor arising from metaphysis of the long bones of adolescents and young adults, accounting for approximately 5% of all pediatric tumors [1, 2]. About 80% of patients eventually develop recurrent metastatic osteosarcoma following surgical treatment which lead to a low cure rate and the 5-year survival rate is only 50-60% at 20% [3, 4]. Although the treatments of osteosarcoma have achieved great improvement, the prognosis of osteosarcoma is still poor. Moreover, the molecular mechanisms involved in osteosarcoma progress remains poorly understood. It is therefore of critical importance to explore the mechanisms that underlie the pathogenesis, thus shedding light on the complex etiology and developing strategies for the diagnosis, treatment and prognosis of this disease.

MicroRNAs (miRNAs) are a class of endogenous, small, non-coding RNAs with 18-25 nucleotides in length [5]. They are linked to many diseases and regulate multiple physiological processes including cell growth, metastasis and so on [6, 7]. MiRNAs also control the expression of its target gene via specific sites within the 3'-Untranslated Regions (3'-UTR) of a target-mRNA at post transcriptional level [6]. More than 1900 human miRNAs regulating about 60% of the genes in mammals have been identified so far [8]. They have considered as oncogenes or tumor suppressors in several cancers due to their aberrant expression targeting by different genes [9, 10]. MiRNAs not only express in tissues but exhibit aberrant expression profile in tumors which is meaningful in the diagnosis of cancer s [11]. Besides, reports demonstrated that miRNAs had important potential value both in diagnostic and prognostic of cancers as a predictive markers [12]. *miR-34a* is encoded by its own transcript at chromosome 1p36, directly regulated by P53 mutation and expressed very low or undetectable in several human cancers [13, 14]. Previous study

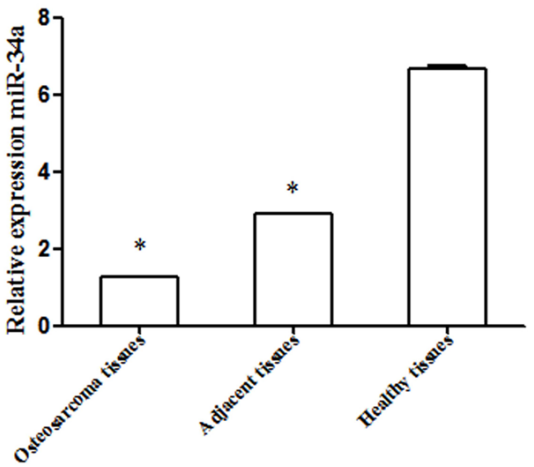

Figure 1. The expression of *miR-34a* in tissues samples. It was significantly lower in osteosarcoma tissues than in adjacent tissues and healthy tissues ($P<0.05$).

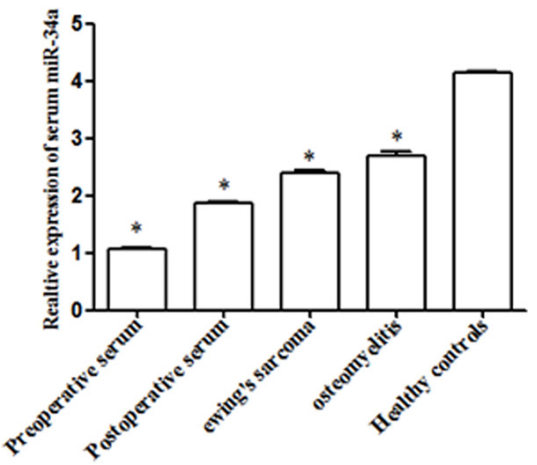

Figure 2. Serum *miR-34a* expression in patients with osteosarcoma and benign diseases controls and healthy controls. It was decreased in preoperative serum compared to postoperative serum, the serum of benign controls and healthy controls ($P<0.05$).

demonstrated that *miR-34a* was decreased in osteosarcoma [15]. However, the clinical significance of *miR-34a* was never reported in osteosarcoma.

In our study, the expression level of *miR-34a* in serum and tissues samples was detected via qRT-PCR analysis, respectively. The relationship between *miR-34a* expression and clinical factors was also analyzed. Through the expression level in serum, we verified its diagnosis value using ROC curve. And then we investigated role of *miR-34a* in prognosis of osteosarcoma by analyzed the association between *miR-34a* and overall survival.

Materials and methods

Patients' samples

50 patients who were diagnosed as osteosarcoma during 2008-2010 in Shenyang Orthopedic Hospital were collected in our study and the study was permitted by the Ethnic Committee of the hospital. Meanwhile, 30 patients including 18 with Ewing's sarcoma and 12 with osteomyelitis were taken as benign diseases controls. Besides, 50 healthy people matching ages with osteosarcoma patients were taken as healthy controls. Written informed consent was obtained from each participant involved in advance. None of the patients had received any chemotherapy or radiotherapy before surgery.

Osteosarcoma tissues, adjacent tissues and healthy tissues were obtained, respectively. Then they were frozen by liquid nitrogen immediately and stored at -80°C for RNA extraction. The serum samples from osteosarcoma patients, benign diseases controls and healthy controls were severally put into blood collection tube of EDTA and stored at -80°C for use. The detailed clinical data including age, sex, site, ALP, histologic type, tumor grade, the status of metastasis and recurrence were collected and stored in a database. A 5-year follow-up was performed for all patients to evaluate its prognostic value. Patients who were died from unexpected events or other diseases were excluded from our study.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from all tissues and serum using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA), respectively. Reverse transcription was conducted by TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) to synthesize the first chain of cDNA. Then RT-PCR reaction was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA). RNU44 was used as internal control. The relative quantity of *miR-34a* was calculated with the comparative cycle threshold (CT) method. Each sample was examined in triplicate.

Table 1. The relationship between *miR-34a* expression and clinical factors of patients with osteosarcoma

Clinicopathological characteristics	n	*miR-34a* expression		*P*
		High	Low	
Sex				0.102
Female	21	6	15	
Male	29	15	14	
Age				0.073
8-11	16	3	13	
12-15	21	11	10	
16-19	13	7	6	
Site				0.497
Femur	7	3	4	
Tibia	14	4	10	
Humeral bone	15	6	9	
Others	14	8	6	
ALP				0.006
<20 ng/mL	11	1	10	
≥20-400 ng/mL	17	7	10	
>400-1000 ng/mL	14	7	7	
>1000 ng/mL	8	6	2	
Histologic type				0.007
Osteoblastic	11	1	10	
Chondroblastic	18	5	13	
Fibroblastic	11	9	2	
Telangiectatic	10	6	4	
Tumor grade				0.000
Low	26	3	23	
High	24	18	6	
Metastasis				0.413
Absent	30	14	16	
Present	20	7	13	
Recurrence				0.493
Absent	21	10	11	
Present	29	11	18	

Statistical analysis

The SPSS version 13.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. The difference of *miR-34a* expression in different tissues and serum samples were analyzed by one way ANOVA. The difference between two groups was compared by student's t test. ROC curve was built to determine the diagnostic performance of *miR-34a* expression in patients with osteosarcoma. The overall survival of patients with different expression of *miR-34a* was estimated by the Kaplan-Meier analysis. Cox regression analysis was performed to identify the factor with significant influence on overall survival. The differences were considered to be statistically significant when $P<0.05$.

Results

Expression of miR-34a in tissues of patients with osteosarcoma

The expression levels of *miR-34a* were detected by qRT-PCR analysis. The results showed that the relative expression of *miR-34a* in osteosarcoma tissues was obviously lower than in adjacent normal tissue and healthy controls (**Figure 1**, $P<0.05$).

Serum miR-34a in patients with osteosarcoma, benign diseases controls and healthy controls

Serum *miR-34a* expression of preoperative and postoperative as well as benign controls and healthy controls was measured, respectively. As shown in **Figure 2**, serum *miR-34a* expression of preoperative was significantly lower than that of postoperative, benign controls and healthy controls ($P<0.05$). These indicated that serum *miR-34a* expression cloud distinguish osteosarcoma patients from other patients or healthy people.

Relationship between miR-34a expression and clinicopathologic characteristics

To explore whether *miR-34a* was related to the development of osteosarcoma, the relationship between it and clinicopathologic characteristics was analyzed. It was demonstrated that the expression of *miR-34a* was significantly influenced by ALP ($P=0.006$), histologic type ($P=0.007$) and tumor grade ($P=0.000$) which revealed that it was involved in the progression of osteosarcoma (**Table 1**).

Diagnostic value of miR-34a in osteosarcoma

We generated ROC curves to assess the potential value of serum *miR-34a* in the early detection of osteosarcoma. The outcome manifested that serum *miR-34a* expression had a high diagnostic value in discriminating patients with osteosarcoma from control subjects with an AUC value of 0.830 combing with a sensitivity of 86.7% and a specificity of 68.6% (**Figure 3**).

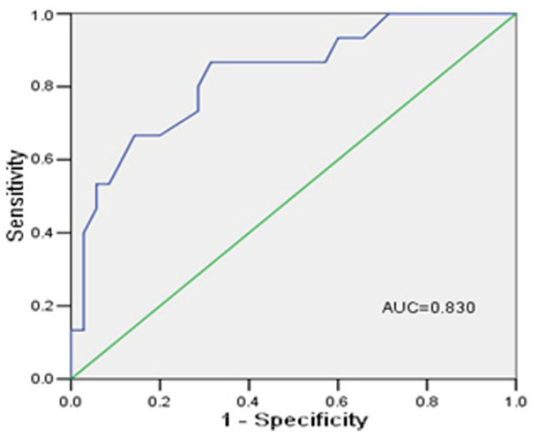

Figure 3. ROC curve for the diagnostic value of *miR-34a* in osteosarcoma. It had a high diagnostic value with an AUC of 0.830 corresponding with a sensitivity of 86.7% and a specificity of 68.6%.

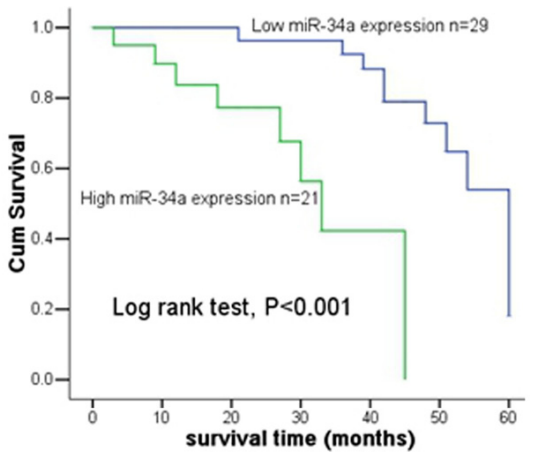

Figure 4. Association between *miR-34a* expression and overall survival of patients with osteosarcoma. Patients with a high *miR-34a* expression had a longer overall survival than those with low *miR-34a* expression (log rank test, *P*<0.001).

And the ideal cut-off value was. The result verified that *miR-34a* could be an independent biomarker for the diagnosis of osteosarcoma.

Association between miR-34a expression and overall survival of patients with osteosarcoma

To further evaluate the association of *miR-34a* expression with overall survival of patients, the osteosarcoma patients were divided into high expression group (high *miR-34a* expression) and low-expression group (low *miR-34a* expression) according to the median expression of *miR-34a* (1.259±0.126). During the follow-up, 18 patients were censored. Kaplan-Meier analysis demonstrated that overall survival of patients with high *miR-34a* expression was longer compared to those with low *miR-34a* expression (**Figure 4**, log rank test, *P*<0.001). Multivariate analysis adjusted for clinical factors and the expression of *miR-34a* using cox regression analysis showed the factors with statistical significance for prognosis of osteosarcoma were the expression of *miR-34a* (**Table 2**, HR=10.134, 95 CI%=2.859-35.916, *P*=0.000) and it might be an independent prognostic marker.

Discussion

The aberrant expression or alterations of miRNAs are closely related to human pathologies such as cardiovascular disorders, inflammatory diseases and cancer [16, 17]. Osteosarcoma is considered to be a differentiation disease caused by genetic changes that interrupt osteoblast differentiation from mesenchymal stem cells [18, 19]. miRNA was verified to regulate specific tissue-lineages during the differentiation of osteosarcoma, but their involvement is still unclear [20-22].

In recent studies, there were multiple miRNAs reported to play roles in osteosarcoma such as miR-31, miR-192, miR-215, miR-140, miR-21, miR-143, and so on. And most of these miRNAs are related to cell proliferation, cell cycle, cell invasion and migration [23-25]. miRNAs may become a new tool for the diagnosis and prognosis of cancers as they can exit in body fluids stably, so study about them is meaningful.

miR-34a is suggested to function as a tumor suppressor gene and contributes to the inhibition of invasion or metastasis in various tumor types. And it has relationship with the progression and poor prognosis of many tumors. *miR-34a* has been confirmed to be down-regulated in many cancers such as human papillomavirus, hepatocellular carcinoma, prostate cancer, colon cancer, non-small cell lung carcinoma and so on [26-30]. However, the prognosis role of *miR-34a* in osteosarcoma was still unclear and need more comprehensive studies.

In this study, we detected the expression level of *miR-34a* in osteosarcoma tissues and serum by QT-PCR. Compared the controls of tissues and serum, *miR-34a* expression was all decr-

Table 2. Cox regression analysis for the prognostic value of *miR-34a* in patients with osteosarcoma

Parameter	Risk ratio	95% confidence interval	*P*
High *miR-34a* expression	-	-	-
Low *miR-34a* expression	10.134	2.859-35.916	0.000

eased in patients with osteosarcoma. Besides, serum *miR-34a* expression of preoperative was lower than that of postoperative, benign diseases controls and healthy controls. These results showed that *miR-34a* might be a tumor suppressor in osteosarcoma. It was consistent with previous study in osteosarcoma [15]. Besides, its expression was considered to be influenced by ALP, histologic type and tumor grade. But the relationship between *miR-34a* expression and progression of osteosarcoma was not clarified in our study. The mechanism of its antitumor and its effect on progression of osteosarcoma are still needs to be further investigated. ROC curve demonstrated that *miR-34a* could be a diagnostic bio-marker for osteosarcoma. Meanwhile, cox regression analysis revealed miR-34a could be an independent prognostic marker in osteosarcoma.

In conclusion, *miR-34a* expression is decreased in osteosarcoma tissues and serum. Its expression is related to ALP, histologic type and tumor grade. *miR-34a* may serve as a diagnostic and prognostic marker in osteosarcoma. Further researches in a large number are warranted to further investigate the significance of *miR-34a* in osteosarcoma.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bo Wu, Shenyang Orthopedic Hospital, 115 Dongbei Road, Shenyang 110044, Liaoning, China. E-mail: wubojaiyoun@sina.com

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