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Original Article

Serum level of microRNA-222 acts as a diagnostic and prognostic biomarker for osteosarcoma patients

Qichuan Zhang[1], Sufang Wang[2], Yunfeng Wang[1], Yunfei Du[1], Xinsheng Fu[1]

[1]*Second Department of Orthopaedics,* [2]*Department of Central Sterile Supply, Xinxiang Central Hospital, Xinxiang, Henan, People's Republic of China*

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Abstract: Dysregulated expression profiles of microRNAs (miRNAs) and their roles in diagnosis and prognosis of osteosarcoma patients have attracted much attention. Although miR-222 has been shown to be important in several cancers, its roles in osteosarcoma remain unknown. Hence, in this study, we focused on the expression and further to evaluate the clinicopathological, diagnostic and prognostic value of miR-222 for osteosarcoma patients. The peripheral blood expression of miR-222 in 57 pairs of osteosarcoma patients and corresponding healthy controls were estimated by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), and the associations of miRNAs expression with clinicopathological factors, diagnostic and prognostic values of osteosarcoma patients were also analyzed. miR-222 levels in serum from osteosarcoma patients were significantly higher than those in healthy controls ($P < 0.001$). Importantly, miR-222 could efficiently screen osteosarcoma patients from healthy controls (AUC = 0.811). Then, the up-regulation of miR-222 more frequently occurred in osteosarcoma patients with large tumor size ($P < 0.001$), high TNM stage ($P < 0.001$) and positive distant metastasis ($P < 0.001$). Additionally, patients with high miR-222 expression had shorter overall survival ($P < 0.001$) and disease-specific survival ($P < 0.001$) than those with low expression. Moreover, high expression of miR-222 was an independent and significant prognostic factor for overall survival and disease-specific survival of osteosarcoma patients. Elevated expression of miR-222 in serum may be a novel biomarker for screening osteosarcoma patients and predicting poor prognosis of the disease. Therefore, serum miR-222 expression may have clinical potentials as a non-invasive diagnostic and prognostic biomarker for osteosarcoma patients.

Keywords: Osteosarcoma, miR-222, diagnosis, prognosis, overall survival, disease-specific survival, biomarker

Introduction

Osteosarcoma is a primary malignant bone tumor that most commonly affects children, adolescents and young adults [1]. It is generally locally aggressive and tends to produce early systemic metastases. Currently, almost 40% of patients with primary osteosarcoma who achieved a complete surgical remission can become long-term survivors [2]. However, survival for patients with metastatic or relapsed osteosarcoma has virtually stagnated over the past 30 years, with an overall 5-year survival rate of about 20% [3]. Therefore, new agents need to be rationally investigated to strive for improvement in the survival of patients diagnosed with osteosarcoma.

microRNAs (miRNAs) are short RNA molecules of 19-24 nucleotides in length that primarily function at the posttranscriptional level and aberrant expression of them are associated with cancer onset, growth and progression [4]. These properties make miRNAs ideal biomarkers that could be detected for differential diagnosis of cancers [5, 6]. Early detection and accurate monitoring of biomarkers are vital for treatment and positive prognosis for cancer patients, highlighting biomarker identification particularly significant [7-9]. Recently, miRNAs have been detected in the blood of patients with cancers and their clinical values are attracting considerable attention [10-12].

In general, oncogenic miRNAs are up-regulated in cancer, while miRNAs that serve as tumor suppressors are down-regulated, resulting in decreased expression of tumor suppressors and up-regulated oncogene expression, respectively [13]. A number of expression profiling

Table 1. Association between miR-222 expression and characteristics of patients with osteosarcoma

Variables	No. of cases	Expression of miR-222		*P* value
		Low	High	
Age				0.402
< 55	28	15	13	
≥ 55	29	13	16	
Gender				0.063
Man	26	10	16	
Female	31	18	13	
Tumor size (cm)				< 0.001
< 5	30	22	8	
≥ 5	27	6	21	
Location				0.853
Distal	24	12	12	
Proximal	33	16	17	
Histological type				0.106
Osteoblastic	23	11	12	
Chondroblastic	18	11	7	
Telangiectatic	10	4	6	
Fibroblastic	6	2	4	
TNM stage				< 0.001
I + II	33	23	10	
III + IV	24	5	19	
Distant metastasis				< 0.001
No	33	23	10	
Yes	24	5	19	

studies have shown that abnormal expressions of miRNAs are continually found in osteosarcoma patients [14-16]. Recently, it has been reported that miR-222 is frequently and highly expressed and plays vital roles in tumorigenesis in several cancers, such as colorectal cancer [17], prostate cancer [18] and thyroid cancer [19]. However, the roles of miR-222 and its clinical relevance in osteosarcoma have not yet been investigated so far.

Therefore, we investigated the serum expression of miR-222 in human osteosarcoma and their associations with clinicopathological features to preliminary assess its clinical application value.

Materials and methods

Patients

Fifty seven osteosarcoma patients and matched (age and sex) healthy controls were included in this study. Patients were consecutively recruited from Xinxiang Central Hospital between 2006 and 2010. Histopathology of the patients was confirmed by surgical resection of the tumors, and tumor stages were determined based on the surgical findings. The peripheral blood samples were obtained from patients prior to surgery. The clinicopathological features of these patients are documented in **Table 1**. The overall survival was defined as the time elapsed from surgery to death or the last follow-up, and disease-specific survival was calculated from the date of operation to the date of tumor related death or the last follow-up. The follow-up information of all participants was updated every 3 months until death or the end of the study period. Patients were followed up after surgical treatment with a median follow-up of 36 months (range from 6 to 70 months). The study was approved by the Research Ethics Committee of Xinxiang Central Hospital. Written informed consent was obtained from all of the patients and healthy controls. All specimens were handled and made anonymous according to the ethical and legal standards.

miRNA quantitative reverse transcriptase PCR (qRT-PCR)

The serum expression of miR-222 in osteosarcoma patients and corresponding healthy controls was determined by qRT-PCR assay. Briefly, total RNA from tissue samples was extracted with TRizol reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. miRNA expression levels were then quantified using the TaqMan miRNA real-time RT-PCR kit (Applied Biosystems) according to the manufacturer's protocol. The universal small nuclear RNA U6 was used as an endogenous control for miRNAs. Each sample was examined in triplicate. The cycle threshold (CT) was calculated. The $2^{-\Delta CT}$ ($\Delta C_T = C_{TmiR-222} - C_{TU6\ RNA}$) method was used to quantify the relative amount of miR-222.

Statistical analysis

The software of SPSS version 18.0 was used for statistical analysis. Continuous variables were expressed as mean ± standard deviation (SD). Mann-Whitney U test was used to com-

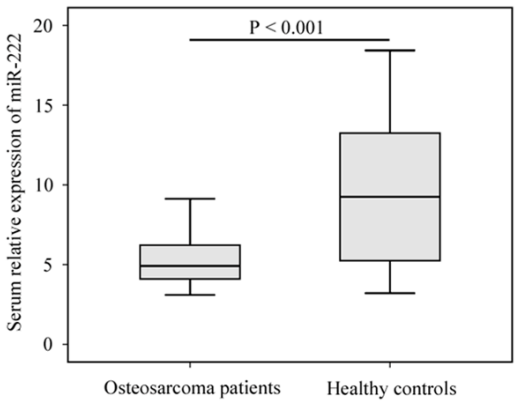

Figure 1. Serum expression levels of miR-222 in human osteosarcoma and healthy control were detected by qRT-PCR assay. The results showed that the expression levels of miR-222 in osteosarcoma serum were significantly higher than those in healthy control ($P < 0.001$).

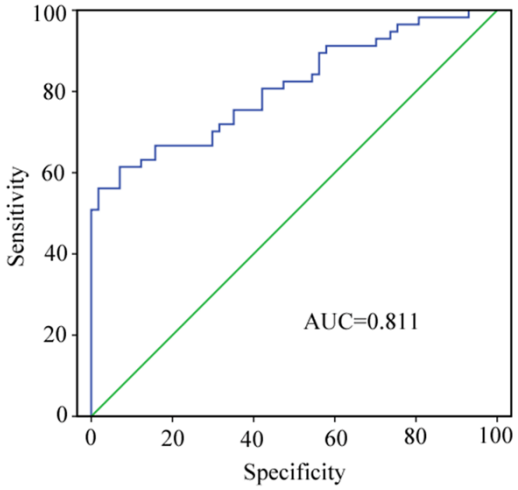

Figure 2. Receiver operating characteristic (ROC) curve analysis of circulating miRNAs. ROC curves were constructed for individual miRNAs in discriminating osteosarcoma patients from controls in the combined two populations (AUC = 0.811).

pare the difference of serum miR-222 expression levels. The receiver operating characteristic (ROC) curve was drawn to evaluate the diagnosis value of serum miR-222 level. Nonparametric test was used to evaluate the correlations of serum miR-222 expression with clinicopathological factors. Patient survival and their differences were determined by Kaplan-Meier method and log-rank test. Cox regression multivariate analysis was used for multivariate analyses of prognostic values. Differences were considered statistically significant when P was < 0.05.

Results

Up-regulation of circulating miR-222 is associated with clinicopathological features

In order to evaluate the association of serum levels of miR-222 with the clinicopathological features of osteosarcoma patients, the median values of miR-222 (9.25) expression in serum of 57 osteosarcoma patients were used as the cutoff points to divide these patients into miR-222-low (n = 28) and miR-222-high (n = 29) expression groups. The correlations between serum miR-222 expression and clinicopathological factors are shown in **Table 1**. As shown in **Figure 1**, the expression level of miR-222 in the osteosarcoma serum was found to be distinctly up-regulated when compared to that in the healthy control ($P < 0.001$). Additionally, these data also indicate that significant up-regulation of miR-222 expression occurred in osteosarcoma was more frequently correlated with the large tumor size, high TNM stage and distant metastasis status.

Diagnostic value of serum miR-222 for osteosarcoma patients

As shown in **Figure 2**, ROC curve analysis illustrated that the circulating miR-222 was a potential biomarker for distinguishing osteosarcoma patients from healthy controls with the area under the ROC curve (AUC) of 0.811, and the serum miR-222 level at 6.77 was the clear cutoff value to distinguish osteosarcoma patients from healthy controls. With this cutoff value, the sensitivity and specificity of serum miR-222 for osteosarcoma patients was 66.7 and 84.2 %, respectively.

Prognosis value of circulating miR-222 for osteosarcoma patients

Univariate survival analysis (log-rank test) showed that tumor size, TNM stage, distant metastasis and miR-222 expression (**Figure 3A**) significantly predicted decreased overall 5-year survival (**Table 2**). Additionally, it also demonstrated that tumor size, TNM stage, distant metastasis and miR-222 expression (**Figure 3B**) significantly predicted decreased disease-specific 5-year survival (**Table 2**). More-

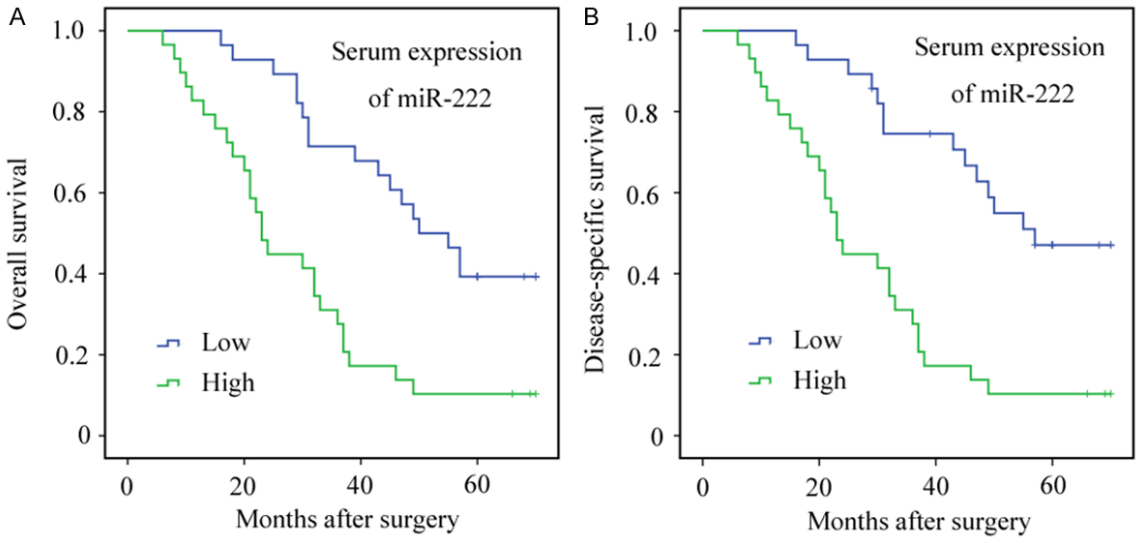

Figure 3. Kaplan-Meier survival curves for osteosarcoma patients according to miR-222 expression. (A) Overall and (B) disease-specific survival curves for two groups of osteosarcoma patients with low and high expression of miR-222.

Table 2. Univariate and multivariate analyses of different prognostic parameters on osteosarcoma patients

Variables	Overall survival			Disease-specific survival		
	Univariate	Multivariate		Univariate	Multivariate	
	P value	*P* value	95% CI	*P* value	*P* value	95% CI
Age	0.890	0.163	0.286-1.234	0.802	0.149	0.265-1.222
Gender	0.800	0.334	0.698-2.878	0.491	0.713	0.556-2.362
Tumor size	0.004	0.231	0.731-3.682	0.003	0.418	0.609-3.305
Location	0.931	0.842	0.492-1.784	0.654	0.672	0.588-2.279
Histological type	0.066	< 0.001	0.360-0.750	0.148	0.002	0.374-0.794
TNM stage	< 0.001	0.175	0.779-3.930	< 0.001	0.251	0.708-3.740
Distant metastasis	0.001	0.134	0.835-3.879	0.001	0.141	0.822-3.955
miR-222 expression	< 0.001	0.008	1.046-1.355	< 0.001	0.003	1.073-1.404

over, Cox regression multivariate analysis highlighted that high expression of miR-222 strengthened its significance as an independent predictor for unfavorable overall survival and disease-specific survival, respectively.

Discussion

Circulating miRNA, a rich source for potential disease biomarkers, fulfill several properties of good clinically useful biomarkers, such as accessibility in various bodily fluids, sequence conservation between human and clinically important animal models, available sensitive measurement methodologies and able to be measured reliably without influence of other factors [20]. Recently, dysregulation expression of serum miRNAs has been demonstrated to be as potential sensitive and accurate biomarkers for the diagnosis and prognosis of osteosarcoma patients [21-23]. Therefore, determination of functional and clinical importance of a specific miRNA may provide effective management of the disease [5, 10, 24]. In the present study, the clinical importance of miR-222 in human osteosarcoma was investigated and its relationship with clinicopathological factors was also evaluated.

Our study suggested that miR-222 expression level in the serum of osteosarcoma patients were significantly higher than that in normal

controls, and its expression was significantly correlated with large tumor size, high TNM stage and distant metastasis, but not with age, gender, location and histological type. This finding provides initial evidence supporting miR-222 as a predictor of poor prognosis in osteosarcoma. Additionally, we also evaluated the diagnostic value of serum miR-222 expression. Our data indicated that miR-222 could efficiently screen osteosarcoma patients from healthy controls (AUC = 0.811). The AUC value is an indicator of the efficacy of the assessment system [25]. The closer to 1.0 the AUC of a test is, the higher the overall efficacy of the test will be. In present study, miR-222 could screen osteosarcoma patients from healthy controls with a relative high AUC value, suggesting that it had a persuasive reason to identify the true osteosarcoma patients. Here, we firstly provide evidence that the significant dysregulation of circulating miR-222 may play as a potentially novel diagnostic biomarker for osteosarcoma patients.

Advanced stages of osteosarcoma are always with corresponding poor prognosis [26-28]. Forecasting the clinical outcomes of osteosarcoma patients will give better treatment stratification and personalized therapeutic regimens [14, 29, 30]. Our results show that patients with miR-222 high expression had significantly poorer overall and disease-specific survival when compared with patients with miR-222 low expression, respectively. Additionally, we also demonstrated that large tumor size, high TNM stage, distant metastasis and miR-222 high expression were independent prognostic factors for poor overall and disease-specific survival of osteosarcoma patients, respectively. Therefore, we have compelling reason to speculate that miR-222 may play a crucial role in tumorigenesis in osteosarcoma and likely would be applied as a novel therapeutic agent in the future.

In conclusion, our results demonstrated that miR-222 is up-regulated in osteosarcoma, and elevated expression of miR-222 more frequently occurs in osteosarcoma samples with adverse clinical stage and poor prognosis of the disease. Therefore, it may have clinical potentials as a non-invasive diagnostic and prognostic biomarker for osteosarcoma patients.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qichuan Zhang, Second Department of Orthopaedics, Xinxiang Central Hospital, Xinxiang 453000, Henan, People's Republic of China. Tel: (86)15637359825; E-mail: zhangqichuandoctor@163.com

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