Int J Clin Exp Pathol 2015;8(8):9314-9319
www.ijcep.com /ISSN:1936-2625/IJCEP0010759

Original Article

Down-regulation of *microRNA152* is associated with the diagnosis and prognosis of patients with osteosarcoma

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Received May 27, 2015; Accepted June 29, 2015; Epub August 1, 2015; Published August 15, 2015

Abstract: Potential values of *microRNA152* (*miR-152*) as a serum diagnostic and prognostic biomarker have not been determined in human osteosarcoma. By detecting the expression of *miR-152* among 80 osteosarcoma patients, 20 periostitis patients and 20 healthy individuals using qRT-PCR, we aimed to explore the clinical significance of *miR-152* in osteosarcoma patients. The expression of *miR-152* was significantly decreased in patients with osteosarcoma compared to patients with periostitis ($P<0.01$) and healthy controls ($P<0.01$). The relationship between clinicopathologic characteristics and *miR-152* was analyzed by chi-square test. The outcome indicated that *miR-152* might be linked with the development of osteosarcoma. Moreover, the receiver operating characteristic (ROC) curve was performed to estimate the diagnostic value of *miR-152*. The result demonstrated that *miR-152* might be a promising diagnostic marker of osteosarcoma with an AUC of 0.956, combing with 92.5% specificity and 96.2% sensitivity. The relationship between *miR-152* and overall survival of osteosarcoma patients was analyzed by Kaplan-Meier curve and log rank test. As a result, the survival time of patients with low *miR-152* expression was significantly shorter than those with high *miR-152* expression ($P<0.001$). Then Cox regression analysis was used to estimate the prognostic value of *miR-152* in osteosarcoma. The outcomes showed that low *miR-152* expression ($P=0.004$) might be a potential independent prognostic marker for osteosarcoma patients. These findings suggested that down-regulation of *miR-152* could be considered as a predictor for diagnosis and prognosis of osteosarcoma patients.

Keywords: *MiR-152,* osteosarcoma, diagnosis, prognosis

Introduction

Human osteosarcoma is a primary cause of cancer-associated death deriving from the proximal tibia or the distal femur and mostly occurs in children and young adults [1, 2]. The main therapies of osteosarcoma include chemotherapy, radiotherapy and tumor excision strategies. However, there are still a high risk of distant metastasis and local relapse even after complete surgical resection for osteosarcoma patients [3]. It has been reported that 50% patients with osteosarcoma suffer metastasize bringing a low cure rate and a low 5-years' survival rate [4]. During the past decades, the 5-year survival rates have apparently raised to approximately 60%-70% as the development of combined therapies [5]. Although a few molecular targeted drugs have been confirmed to be related to tumor genesis, osteosarcoma treatments have not been well set up. Besides, strategy on the diagnosis and prognosis about osteosarcoma is still poor, and the molecular mechanism of osteosarcoma genesis remains unclear. Therefore, the identification of novel diagnostic and prognostic biomarkers is rather significant for improving the clinical outcome of osteosarcoma patients.

MicroRNAs (miRNAs) are a class of highly conserved, short and small (18-24 nucleotides) non-coding RNAs that play essential roles on the regulation of gene expression. Abnormal expression of miRNAs was observed in human osteosarcoma tumor and was corresponded with cellular processes including apoptosis, invasion, cycling and proliferation [6-9]. Furthermore, remarkably stable expression patterns of miRNAs were found in plasmas and serums, suggesting miRNAs to be potential diagnostic and prognostic implements for human cancers [8, 10, 11]. *MicroRNA-152*

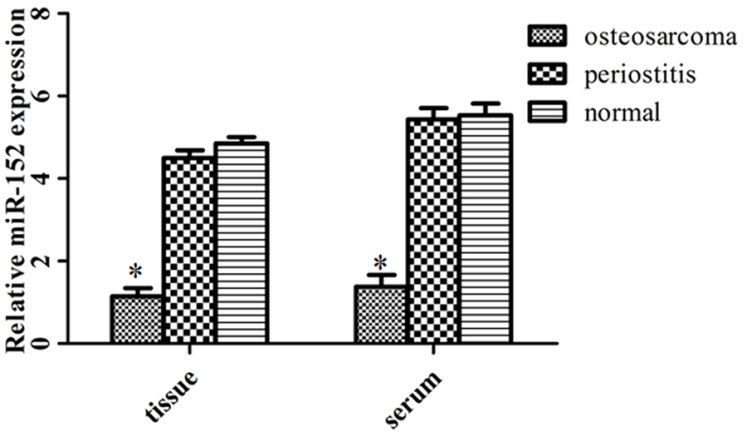

Figure 1. Relative expression levels of *miR-152* in 20 normal controls, 20 periostitis patients and 80 osteosarcoma patients which were examined using qRT-PCR assay. U6 was served as internal controls and all experiments were performed in triplicate.

(*miR-152*), a member of *miR-148/152* family that participates into a series of cellular activities such as cell proliferation, invasion and angiogenesis [12, 13]. Down-expression of *miR-152* was related to distant metastasis and survival time in many diseases, including oropharyngeal carcinoma and endometrial serous adenocarcinomas [14, 15]. Yet, the clinical and pathological significance of *miR-152* in human osteosarcoma remains unknown.

In the present study, we aimed to systematically detect the expression of *miR-152* in osteosarcoma patients and control subjects by qRT-PCR. Meanwhile, intended to analyze the diagnosis and prognosis value of *miR-152* in osteosarcoma patients.

Materials and methods

Sample collection

Our study protocol was recognized by Research Ethics Committee in Shenyang Orthopedic Hospital. We obtained written informed consents from each participant. Osteosarcoma samples (tissues and corresponding serums) were collected from 80 patients diagnosed as osteosarcoma in Shenyang Orthopedic Hospital. 20 patients with periostitis and 20 healthy people matching with the ages of osteosarcoma patients were recruited as controls. The selecting of cases was complied with the standard of diagnosis performance. Meanwhile, the patients had never received any chemotherapy or radiotherapy before operation.

The tissues and serum from osteosarcoma patients, periostitis patients and healthy people were extracted, respectively. Then the tissues were frozen in liquid nitrogen and stored at -80°C for RNA extraction. The serum samples were put into blood collection tube of EDTA and stored at -80°C for RNA extraction.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction was performed from fresh serum and tissues following the instructions of a miRcute miRNA isolation kit (Tiangen, Beijing city, China). Then cDNA synthesis kit (Qiagen, Germany) was carried out to conduct reverse transcription. qRT-PCR reaction was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA). The expression of *miR-152* was normalized using an internal standard U6. The $2^{-\Delta\Delta Ct}$ method was used to calculate the quantity of *miR-152*. Moreover, all experiments were operated in triplicate.

Follow-up

A 5-year follow-up was conducted for the osteosarcoma patients. The information of follow-up was gotten by outpatient visits or telephone calls and updated every three months. Patients who died of unexpected occurrences or other diseases were excluded from our study. The overall survival time was defined from the diagnosis day to the time of death.

Statistical analysis

All variables were expressed as mean ± SD. The statistical analyses and the design of figures were executed using SPSS 20.0 (SPSS Inc., Chicago, USA) and GraphPad Software (San Diego, CA, USA). The one-way ANOVA assay was used to compare the variances of *miR-152* expression in osteosarcoma patients, periostitis patients and healthy people. Diagnostic accuracy of *miR-152* was assessed employing receiver operating characteristic (ROC) curve. The relationship between the clini-

Table 1. The relationship between *miR-152* expression and clinicopathologic characteristics in osteosarcoma patients

Parameters	Cases (n)	*miR-152* expression		*P* value
		High-expression (n)	Low-expression (n)	
Gender				0.142
Male	40	19	21	
Female	40	21	19	
Age				0.403
≤ 19	40	20	20	
> 19	40	20	20	
Tumor size				0.741
< 8 cm	41	21	20	
≥ 8 cm	39	19	20	
Tumor location				0.802
Femur	40	21	19	
Tibia	40	19	21	
Enneking				0.000*
I A	17	0	17	
II A	28	17	11	
II B	19	17	2	
III	16	6	10	
Distant metastasis				0.000*
Absent	68	39	29	
Present	12	1	11	

"*" notes statistical significance of *P* values.

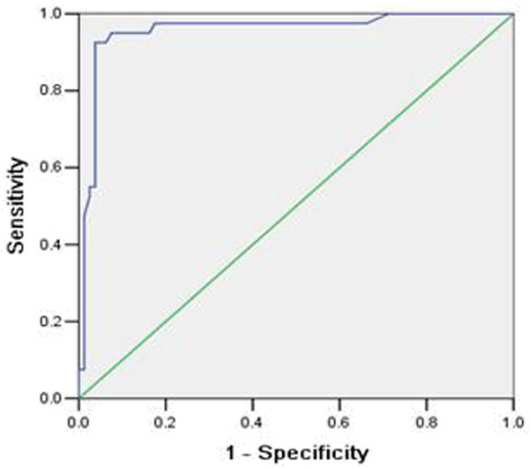

Figure 2. Receiver Operating Characteristic (ROC) was established to analyze the diagnostic value of *miR-152*. The AUC was 0.956 with a specificity of 92.5% and sensitivity of 96.2%.

copathologic characteristics and *miR-152* was estimated by chi-square test. Associations between overall survival and serum *miR-152* expression were evaluated using Kaplan-Meier analysis according to log rank test. Cox regression was carried out to determine the prognostic effects of each clinical characteristic. The value of *P* less than 0.05 was considered to be statistically significant.

Results

miR-152 was low expression in osteosarcoma patients

miR-152 expression levels both in tissues and serum were detected in 120 individuals (including 80 osteosarcoma patients, 20 healthy subjects and 20 periostitis patients) by qRT-PCR. Significant down-expression of *miR-152* was observed in osteosarcoma patients compared with control samples (**Figure 1**).

Relationship between clinicopathologic characteristics and miR-152 expression in osteosarcoma patients

Relative *miR-152* expression in osteosarcoma patients was associated with several clinicopathologic characteristics. We obtained the conclusion that the expression of *miR-152* was influenced by distant metastasis (*P*=0.000) and Enneking (*P*=0.000). Yet, there was no dramaticly correlation between *miR-152* levels and gender, age, tumor size or tumor location of osteosarcoma patients (*P*>0.05) (**Table 1**).

Diagnostic value of serum miR-152 marker

A receiver operating characteristic (ROC) curve was built to estimate the diagnostic value of serum *miR-152*. The AUC of 0.956 was obtained according to ROC assay. Besides, the specificity was 92.5% and the sensitivity was 96.2% with an optimal cut-off value of 3.500 (**Figure 2**).

Association between miR-152 and overall survival time of osteosarcoma patients

The Kaplan-Meier curve was performed for osteosarcoma patients. As displayed in **Figure**

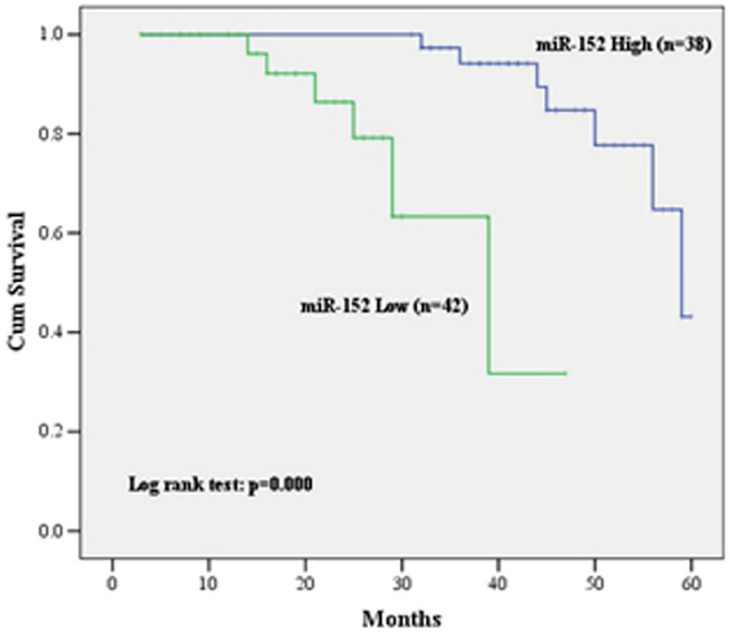

Figure 3. Kaplan-Meier curves showed the relationship between overall survival and *miR-152* in osteosarcoma patients. Log rank test was used to compute *P* values.

Table 2. Multivariate analyses for prognostic factors in patients with osteosarcoma

Characteristic	HR	95% CI	*P*
Enneking	7.767	1.326-45.493	0.023
miR-152	0.126	0.023-0.701	0.004

3, the survival time of patients with low *miR-152* expression was shorter than those with high *miR-152* expression. The prognostic roles of *miR-152* and clinicopathologic characteristics were analyzed through Cox regression analysis. Enneking stage (HR=7.767; *P*=0.023) and serum *miR-152* levels (HR=0.126; *P*=0.004) (**Table 2**) were verified to be important prognostic factors and they might be as independent biomarkers in osteosarcoma patients.

Discussion

Osteosarcoma is a differentiation disease caused by genetic changes that interrupt osteoblast differentiation from mesenchymal stem cells [16]. The cure rate of osteosarcoma is less than 65% for localized osteosarcoma patients, but it often happens with metastases when osteosarcoma is diagnosed which leads to a poor prognosis [17]. Therefore, the discovery of appropriate biomarkers for the diagnosis and prognosis of osteosarcoma is significant. Many molecular markers have proven diagnostic or prognostic value for osteosarcoma.

With the widely use of various detecting techniques and further research of miRNAs, the relationship between miRNAs and the occurrence as well as development of multiplicate tumors were received more and more attention. In addition, *miR-152* as a member of miRNAs has been reported with aberrant expression levels in different malignant tumors. For example, *miR-152* was found to be over-expressed in neuroblastoma cells [18], but down-expression of *miR-152* was examined in bladder cancer [19], prostate cancer [20], ovarian cancer [21] and supragalottic laryngeal carcinoma [22]. Thus, the role of *miR-152* differs between an oncogene or a tumor suppressor according to various tumor types. Although, there were a variety of miRNAs have been confirmed to be related to osteosarcoma such as *miR-145*, *miR-133b*, *miR-21*, *miR-9*, *miR-206* and so on [9, 23-26], the effects of *miR-152* on osteosarcoma was rarely researched.

In our study, we for the first time determined the expression pattern and clinical significance of *miR-152* in patients with osteosarcoma. The expression levels of *miR-152* in osteosarcoma patients were decreased significantly compared with healthy control and periostitis patients. This might demonstrate that *miR-152* was a tumor suppressor in the development of osteosarcoma. Moreover, significant concordance of *miR-152* expression variation was observed in serums and tissues which verified the specific expression of *miR-152* in osteosarcoma. Besides, the expression of *miR-152* was influenced by Ennking and distant metastasis according to the analysis of the relationship between *miR-152* and clinicopathologic characteristics.

The previous studies have demonstrated that miRNAs play important roles in occurrence and development of many diseases. As their abnormal expression, they can act as oncogene or tumor suppressor in different cancers which

make them to be important markers in the diagnosis or prognosis of cancers. *MiR-152* had been considered as a diagnostic or prognostic biomarker in oropharyngeal carcinoma, bladder cancer and non-small cell lung carcinoma (NSCLC) [14, 27, 28]. Thus, we estimated the diagnostic and prognostic value of *miR-152* in osteosarcoma via ROC curve, Kaplan-Meier and Cox regression analysis. The results including AUC values of 0.956 with 92.5% sensitivity and 96.2% specificity revealed that *miR-152* could be served as a promising noninvasive marker for early detection of osteosarcoma. In addition, the association between overall survival and *miR-152* was performed by Kaplan-Meier analysis which showed the patients with high expression of *miR-152* lived longer than those with low expression. Furthermore, the Cox regression analysis indicated *miR-152* could be an independent prognostic marker as well as the Ennking. Thus, we provided evidences that highlighted the significant down-regulation and potentially novel diagnostic and prognostic value of the *miR-152* expression in osteosarcoma patients. However the detailed molecular mechanisms of *miR-152* down-regulation in osteosarcoma remains to be further studied.

In conclusion, the expression of *miR-152* decreases in osteosarcoma patients compared with controls and is influenced by Enneking and distant metastasis. Besides, *miR-152* may be an independent diagnostic and prognostic maker in osteosarcoma. Our findings provide convincing evidence for the potential application of *miR-152* as a diagnostic and prognostic indicator and the study is expected to present a new therapy for the diagnosis and treatment of osteosarcoma.

Disclosure of conflict of interest

None.

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