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Loss of microRNA-132 predicts poor prognosis in patients with primary osteosarcoma

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Abstract MicroRNA-132 (miR-132), an angiogenic growth factor inducible microRNA in the endothelium, facilitates pathological angiogenesis. Previous study showed that miR-132 was downregulated in human osteosarcoma. However, its functional attributes associated with tumor progression of osteosarcoma have not been fully elucidated. The aim of this study was to investigate the clinical significance of miR-132 expression in human osteosarcoma. miR-132 expression was detected by quantitative reverse transcription polymerase chain reaction using 166 pairs of osteosarcoma and noncancerous bone tissues. Then, the association of miR-132 expression with clinicopathological factors or survival of osteosarcoma patients was also evaluated. miR-132 expression was significantly lower in osteosarcoma tissues than that in corresponding noncancerous bone tissues ($P < 0.001$). In addition, miR-132 expression was decreased in the osteosarcoma specimens with advanced clinical stage ($P = 0.009$), positive distant metastasis ($P = 0.006$), and poor response to chemotherapy ($P = 0.009$). Moreover, both the univariate and multivariate analyses showed that osteosarcoma patients with low miR-132 expression had poorer overall and disease-free survival (both $P < 0.001$), and low miR-132 expression was an independent prognostic factor for both overall ($P = 0.001$) and disease-free survival ($P = 0.006$). These findings offer the convinced evidence for the first time that miR-132 may participate in tumor progression of osteosarcoma and loss of miR-132 expression may be a predictor for unfavorable outcome of osteosarcoma patients.

Keywords Osteosarcoma · MicroRNA-132 · Prognosis · Overall survival · Disease-free survival

Introduction

Osteosarcoma represents an aggressive sarcoma and the most common type of primary malignancy deriving from primitive bone-forming mesenchyme [1]. It typically occurs in children, adolescents, and young adults with a peak incidence in the second decade of life. Although the incorporation of chemotherapy into initial treatment significantly improved the clinical outcome, pulmonary metastasis occurs in about 40 % of patients with osteosarcoma and remains a major cause of fatal outcome [2, 3]. The 5-year overall and disease-free survival rates for patients diagnosed with osteosarcoma are around 50–60 % [4]. In recent years, many clinical features, including response to chemotherapy, tumor size, and wide surgical margin, have been useful prognostic factors for osteosarcoma patients. However, they lack sensitivity and specificity, suggesting that within the same clinical stage of tumor, different genetic mechanisms may be operating, altering response to chemotherapy and metastatic

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capability in some tumors [5]. Therefore, it is necessary to identify novel molecular markers for screening osteosarcoma patients with poor prognosis in order to offer them more aggressive therapy at an early stage.

MicroRNA (miRNA) is a novel class of short, endogenous, non-coding RNA with 18–25 nucleotides in length [6]. MiRNAs posttranscriptionally regulate the expression of target genes by binding to the 3′ untranslated regions (3′ UTRs) of their target mRNAs. It has been demonstrated that miRNAs play important roles in many biologic processes, including development, differentiation, cell proliferation, apoptosis, and stress responses [7]. Moreover, a steadily growing number of studies have shown that the dysregulation of miRNAs is essential to keep the malignant phenotype of cancer cells and that they function as tumor suppressors or oncogenes according to the roles of their target genes [8]. Although it is widely recognized that the dysregulation of miRNAs frequently plays a role in oncogenesis, miRNA expression changes in osteosarcoma have not yet been thoroughly examined. Novello et al. [9] assessed specific miRNA profiling deregulation in osteosarcoma clinical samples and suggested that the expression of miR-1 and miR-133b may control cell proliferation and cell cycle through MET (c-Met or MNNG HOS Transforming gene) protein expression modulation. Gougelet et al. [10] measured the miRNA expression in different osteosarcoma samples and identified five discriminating miRNAs in patient tumors, which could be easily transferable to diagnosis. Lulla et al. [11] performed miRNA expression profiling of osteosarcoma cell lines, tumor samples, and normal human osteoblasts and identified four differentially expressed miRNAs in osteosarcoma cells. These findings suggest the involvement of miRNAs in the tumorigenesis of osteosarcoma.

MiR-132, as a member of the miR-212/132 family, is highly conserved in vertebrates and transcribed from an intergenic region on human chromosome 17 by the transcription factor cAMP response element binding protein (CREB) [12]. Recent studies have demonstrated that miR-132 may modulate the process of tumorigenesis and the behavior of cancer cells by suppressing a series of oncogenes. MiR-132 expression is increased in lung cancer [13], squamous cell carcinoma of the tongue [14], breast cancer [15], and colorectal carcinoma [16, 17]. By contrast, Gougelet et al. [10] indicated that miR-132 expression was decreased in osteosarcoma and that miR-132 showed a statistically significant ability to discriminate good responders to ifosfamide from poor responders. To date, very little is known about the links of miR-132 dysregulation to clinicopathological characteristics of osteosarcoma. To address this issue, we here investigated the clinical significance of miR-132 in this malignancy.

Materials and methods

Patients and tissue samples

This study was approved by the Research Ethics Committee of Shanghai eighth people's hospital, Zhujiang Hospital, and Xuhui central hospital, China. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

For quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis, 166 primary osteosarcoma and corresponding noncancerous bone tissue samples from the same specimens were collected from the Department of Pathology, Shanghai Sixth people's hospital, Shanghai Changhai hospital, Shanghai East hospital, Zhujiang Hospital, and Xuhui central hospital, China, from 1998 to 2008. No patients had received blood transfusion, radiotherapy, or chemotherapy before surgery. Clinical stage of these osteosarcoma patients was classified according to the sixth edition of the tumor node metastases (TNM) classification of the International Union against Cancer (UICC). According to the previous study [18], the response of chemotherapy was considered good if the extent of tumor necrosis was >90 % and poor if it was <90 %. The clinicopathological information of the patients is shown in Table 1.

All 166 osteosarcoma patients received follow-up. The median follow-up was 87 months (range 10–152 months). During the follow-up period, 66 patients (66/166, 39.8 %) died of disease. Distant metastases developed in 42 patients at a mean of 13.8 months (range 3–46 months) after the original diagnosis. Of these patients, 9 had bone metastases and 36 had lung metastases (3 patients had both bone and lung metastases). The median overall and disease-free survival of patients was 31 months (95 % confidence interval [CI] 30.1–42.9 months) and 25 months (95 % CI 23.7–35.2 months), respectively.

qRT-PCR assay

The expression levels of miR-132 in osteosarcoma and corresponding noncancerous tissues were detected by qRT-PCR assay. Briefly, total RNA was extracted from tissues using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Then, miRNA expression levels were quantitated using a MicroRNA Assay Kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's protocol. The two-step protocol involves reverse transcription with a miRNA-specific primer to convert miRNA to complementary DNA, followed by real-time quantitative PCR with TaqMan probes. The universal small nuclear RNA U6 (RNU6B) was used as an endogenous control for miRNAs. The miRNA-specific primer

Table 1 Correlation of miR-132 expression with clinicopathological features of osteosarcoma

Clinicopathological features	No. of cases	miR-132 expression		*P*
		High (*n* %)	Low (*n* %)	
Age				
<55	72	35 (48.6)	37 (51.4)	NS
≥55	94	41 (43.6)	53 (56.4)	
Gender				
Male	96	46 (47.9)	50 (52.1)	NS
Female	70	30 (42.9)	40 (57.1)	
Tumor size (*cm*)				
>8	88	38 (43.2)	50 (56.8)	NS
≤8	78	38 (48.7)	40 (51.3)	
Anatomic location				
Tibia/femur	103	53 (51.5)	56 (48.5)	NS
Elsewhere	63	29 (46.0)	34 (54.0)	
Serum level of lactate dehydrogenase				
Elevated	90	40 (44.4)	50 (55.6)	NS
Normal	76	36 (47.4)	40 (52.6)	
Serum level of alkaline phosphatase				
Elevated	108	52 (48.1)	56 (51.9)	NS
Normal	58	24 (41.4)	34 (58.6)	
Clinical stage				
IIA	68	48 (70.6)	20 (29.4)	**0.009**
IIB/III	98	28 (28.6)	70 (71.4)	
Distant metastasis				
Absent	124	74 (59.7)	50 (40.3)	**0.006**
Present	42	2 (4.8)	40 (95.2)	
Response to chemotherapy				
Good	68	52 (76.5)	16 (23.5)	**0.009**
Poor	98	24 (24.5)	74 (75.5)	

NS refers to the differences among groups have no statistical significance

P value with bold mark refers to the differences with statistic significance

sequences were 5′-ACGCAAATTCGTGAAGCGTT-3′ for RNU6B and 5′-ACACTCCAGCTGGGTAACAGTC TACAGCCA-3′ for miR-132. Each sample was examined in triplicate and the amounts of the PCR products produced were normalized to RNU6B.

Statistical analysis

The software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. The chi-square test was used to show differences in categorical variables. Patient survival and their differences were determined by Kaplan–Meier method and log-rank test. Cox regression (proportional hazard model) was adopted for multivariate analysis of prognostic factors. Differences were considered statistically significant when P was less than 0.05.

Results

Decreased expression of miR-132 in human osteosarcoma tissues

The expression levels of miR-132 were detected in 166 pairs of osteosarcoma and corresponding noncancerous bone tissues normalized to RNU6B. As shown in Fig. 1a, the expression levels of miR-132 were found to be distinctly decreased in osteosarcoma tissues compared to noncancerous bone tissues. The statistic results showed that the relative level of miR-132 expression in osteosarcoma tissues (mean ± SD 1.6 ± 0.7) was significantly lower than that in corresponding noncancerous bone tissues (mean ± SD 4.3 ± 0.8; $P < 0.001$, Fig. 1b).

Decreased expression of miR-132 associates with advanced clinicopathological features of osteosarcoma

In order to investigate the associations of miR-132 expression with various clinicopathological parameters of osteosarcoma tissues, we divided the patients into two groups according to the expression levels of miR-132 using its median (1.5) as a cutoff: high miR-132 expression group ($n = 76$, mean ± SD 2.2 ± 0.4) and low miR-132 expression group ($n = 90$, mean ± SD 1.1 ± 0.3). As shown in Table 1, miR-132 expression was significantly lower in osteosarcoma patients with advanced clinical stage, positive distant metastasis and poor response to chemotherapy than those with low clinical stage ($P = 0.009$), without distant metastasis ($P = 0.006$) and good response to chemotherapy ($P = 0.009$). However, there were no significant associations between the expression of miR-132 and patients' age, gender, tumor size, anatomic location, serum levels of lactate dehydrogenase, and alkaline phosphatase (all $P > 0.005$).

Decreased expression of miR-132 confers poor prognosis in patients with osteosarcomas

Using Kaplan–Meier method and log-rank test, the overall survival (OS, Fig. 2a, $P < 0.001$) and disease-free survival (DFS, Fig. 2b, $P < 0.001$) of osteosarcoma patients with low miR-132 expression were both significantly shorter than those with high miR-132 expression. Besides, the survival benefits were also found in those with smaller tumor size (both $P = 0.03$, Fig. 2c, d, respectively), higher

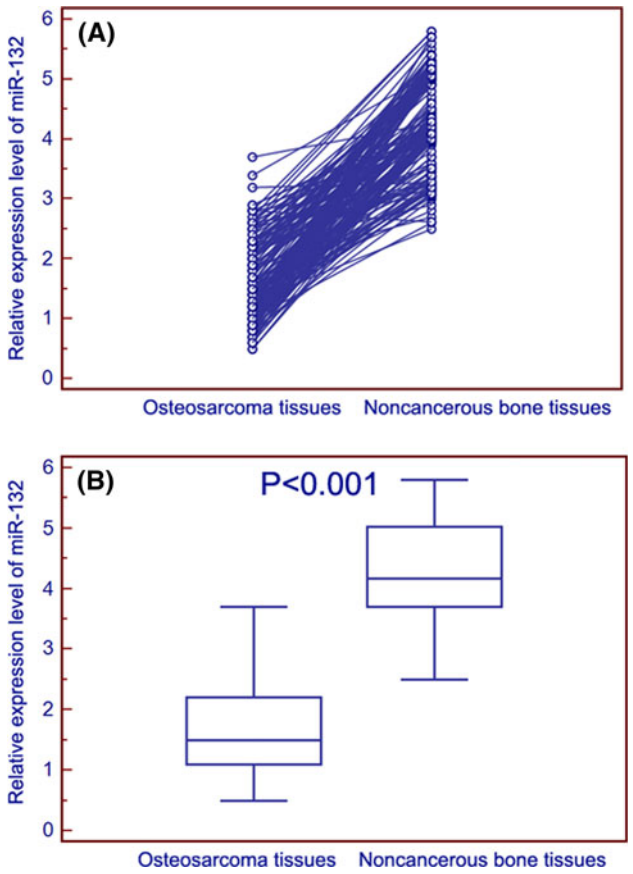

Fig. 1 microRNA-132 (miR-132) expression in 166 osteosarcoma and corresponding noncancerous bone tissues were respectively detected by quantitative real-time reverse transcriptase polymerase chain reaction analysis. **a** Expression levels of miR-132 in osteosarcoma tissues and noncancerous bone tissues. **b** Statistic analysis showed that the relative level of miR-132 expression in osteosarcoma tissues (mean ± SD 1.6 ± 0.7) was significantly lower than that in corresponding noncancerous bone tissues (mean ± SD 4.3 ± 0.8; $P < 0.001$)

clinical stage ($P = 0.01$ and 0.006, Fig. 2e, f, respectively), without distant metastasis ($P = 0.008$ and 0.003, Fig. 2g, h, respectively), and better response to chemotherapy (both $P = 0.02$, Fig. 2i, j, respectively) for OS and DFS.

Multivariate Cox regression analysis enrolling above-mentioned significant parameters revealed that miR-132 expression (RR 4.9, 95 % CI 1.0–10.2, $P = 0.001$), clinical stage (RR 2.3, 95 % CI 1.1–7.0, $P = 0.02$), distant metastasis status (RR 3.7, 95 % CI 1.9–9.8, $P = 0.01$), and response to chemotherapy (RR 2.6, 95 % CI 1.5–8.2, $P = 0.02$) were independent prognostic markers for OS of patients with osteosarcoma (Table 2). Turning to DFS, miR-132 expression (RR 4.1, 95 % CI 0.8–9.7, $P = 0.006$), clinical stage (RR 1.8, 95 % CI 0.7–8.1, $P = 0.03$), and metastasis status (RR 2.9, 95 % CI 1.3–10.6, $P = 0.02$) were also independent prognostic markers for DFS of patients with osteosarcoma (Table 2).

Discussion

Previous studies described the genomic abnormalities in osteosarcoma in great detail and several candidate miRNAs have been proposed, such as miR-145 [19], miR-210 [20], miR-221 [21], miR-1, and miR-133b [9]. Expression profiling study also implicated miR-132 in differential response to chemotherapy [10]. Based on these studies, it is thought that the aberrant regulation of miR-132 may be involved in osteosarcoma. The aim of this study was to validate this hypothesis. There are four points of our findings. Firstly, miR-132 was downregulated in human osteosarcoma tissues compared with noncancerous bone tissues. Secondly, the decreased miR-132 expression in osteosarcoma tissues was significantly correlated with aggressive clinicopathological features including advanced clinical stage, positive distant metastasis, and poor response to chemotherapy. Thirdly, the results of Kaplan–Meier analyses show that osteosarcoma patients with low miR-132 expression tend to have shorter overall and disease-free survival. Finally, the multivariate analysis clearly demonstrated that the loss of miR-132 expression was a statistically significant risk factor affecting both overall and disease-free survival in osteosarcoma patients, suggesting that miR-132 expression could be a valuable marker of tumor progression and prognosis of osteosarcoma. To our knowledge, this is the first study to analyze the expression patterns and the clinical significance of miR-132 in a large number of osteosarcoma patients.

Recent studies have demonstrated that miR-132 may be involved in neurological development, synaptic transmission, inflammation, and angiogenesis. For example, Wanet et al. [22] suggested that miR-132 expression may be necessary for the proper development, maturation and function of neurons and whose deregulation may be associated with several neurological disorders, such as Alzheimer's disease and tauopathies. Scott et al. [23] demonstrated roles of the CREB-responsive miRNA miR-132 in the regulation of synaptic transmission and in the neuronal mechanisms underlying the formation of short-term recognition memory. Murata et al. [24] found that plasma miR-132 well differentiated health controls from patients with rheumatoid arthritis and osteoarthritis, indicating that this miRNA might be involved in the systematic condition of patients with joint inflammation. Anand et al. [15] indicated that miR-132 may act as an angiogenic switch by suppressing endothelial p120RasGAP expression, leading to Ras activation and the induction of neovascularization, whereas the application of anti-miR-132 could inhibit neovascularization by maintaining vessels in the resting state. As tumors are potent inducers of pathological neovascularization and several targets for miR-132 have been demonstrated to be mediators of angiogenesis, many researchers have tried to investigate the relationship of miR-132 with tumorigenesis of various

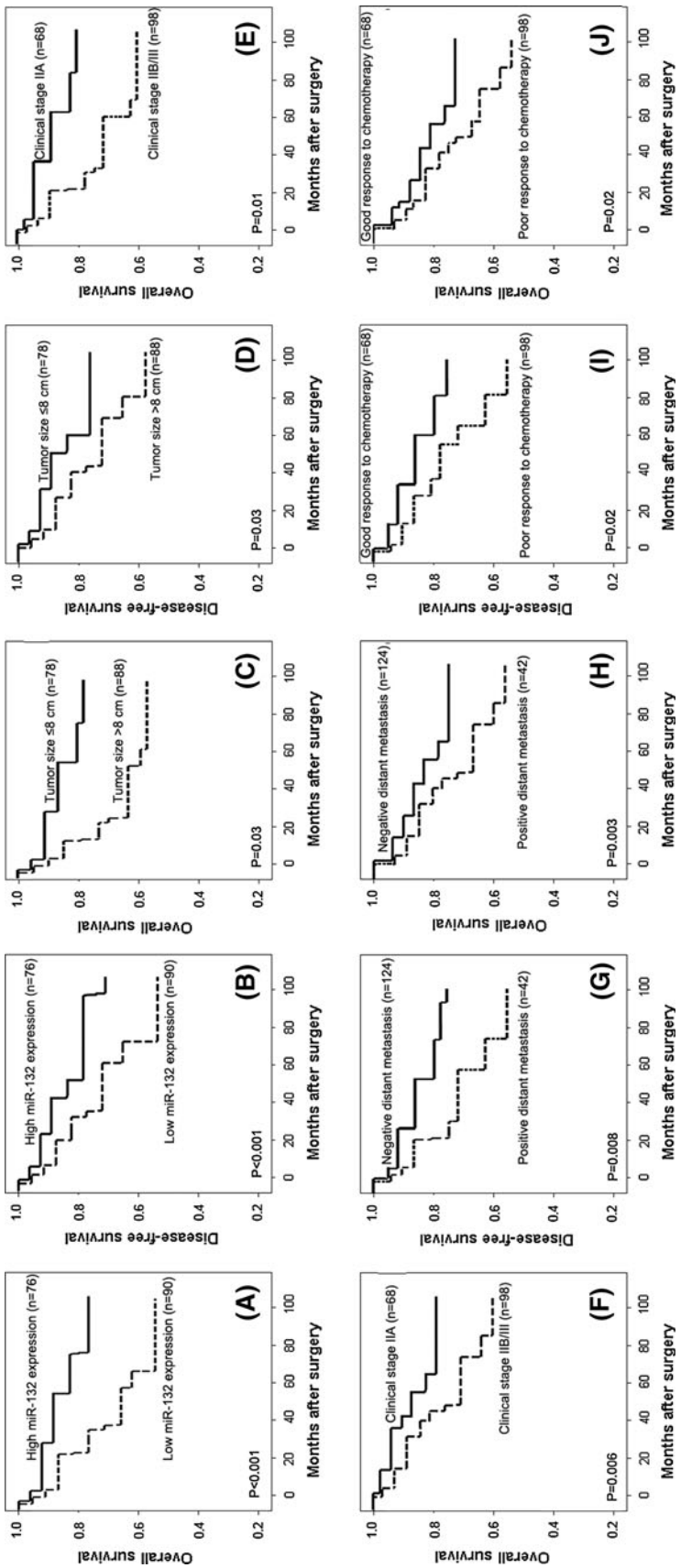

Fig. 2 Overall survival and disease-free survival curves for two groups defined by low and high expression of miR-132 (**a**, **b**), small and large tumor size (**c**, **d**), early and advanced clinical stage (**e**, **f**), negative and positive distant metastasis (**g**, **h**), and by good and poor response to chemotherapy (**i**, **j**) in patients with osteosarcoma

Table 2 Multivariate survival analysis of overall survival and disease-free survival in 166 patients with osteosarcoma

Variables	Overall survival			Disease-free survival		
	RR	95 % CI	*P*	RR	95 % CI	*P*
miR-132 expression	4.9	1.0–10.2	0.001	4.1	0.8–9.7	0.006
Clinical stage	2.3	1.1–7.0	0.02	1.8	0.7–8.1	0.03
Distant metastasis status	3.7	1.9–9.8	0.01	2.9	1.3–10.6	0.02
Response to chemotherapy	2.6	1.5–8.2	0.02	0.9	0.3–1.7	0.6

human malignancies. Park et al. [25] reported that the overexpression of miR-132 may result in reduced pRb protein in pancreatic cancer cells and that the increase in cell proliferation from the overexpression of this miRNA may be likely due to increased expression of several E2F target genes. Wei et al. [26] suggested that miR-132 may be a promising biochemical marker for hepatocellular carcinoma and may have therapeutic applications in this cancer. Li et al. [27] showed that miR-132 was significantly deregulated in ductal carcinoma in situ of breast and overexpression of miR-132 could inhibit the proliferation and the colony formation of breast cancer cell line. Formosa et al. [28] pointed to miR-132 as a methylation-silenced miRNA with an anti-metastatic role in prostate cancer controlling cellular adhesion. Wong et al. [14] detected the upregulation of miR-132 in squamous cell carcinoma of tongue. In the present study, our data also showed the downregulation of miR-132 in osteosarcoma and its association with advanced tumor progression and poor prognosis in this cancer. These findings suggested that miR-132 plays different roles in various human malignancies because the involvement of this miRNA should be tumor-specific and possibly dependent on its targets in different cancer types.

In conclusion, our findings offer the convinced evidence for the first time that miR-132 may participate in tumor progression of osteosarcoma and loss of miR-132 expression may be a predictor for unfavorable outcome of osteosarcoma patients. Moreover, the molecular mechanisms and direct functional targets of miR-132 in osteosarcoma are still unknown and need further exploration. This study is hypothesis generating, and that further prospective analysis should be worth doing.

Conflict of interest None.

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