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Original Article

Serum levels of microRNA-133b and microRNA-206 expression predict prognosis in patients with osteosarcoma

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Abstract: The aim of the present study was to investigate whether the aberrant expression of microRNA (miR)-133b and miR-206 can be used as potential prognostic markers of human osteosarcoma. Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis was performed to detect the expression levels of miR-133b and miR-206 in 100 pairs of osteosarcoma tissues and matched noncancerous bone tissues, and serum samples from 100 patients with osteosarcoma as well as in serum samples from 100 healthy controls. As a result, expression levels of miR-133b and miR-206 were both significantly decreased in osteosarcoma tissues and patients' sera (both $P<0.001$). Then, the downregulation of miR-133b and miR-206 both more frequently occurred in osteosarcoma patients with high tumor grade (both $P=0.01$), positive metastasis (both $P<0.001$) and recurrence (both $P<0.001$). Moreover, the patients with low miR-133b expression and low miR-206 expression both had shorter overall survival (OS, both $P<0.001$) and disease-free survival (DFS, both $P<0.001$) than those with high expressions. Of note, the OS and DFS of patients with combined low expression of miR-133b and miR-206 (miR-133b-low/miR-206-low) were the shortest (both $P<0.001$). Furthermore, low miR-133b expression, low miR-206 expression and conjoined expression of miR-133b/miR-206 were all independent prognostic factors for OS and DFS of osteosarcoma patients. Collectively, the aberrant expression of miR-133b and miR-206 may be implicated in tumorigenesis and tumor progression of osteosarcoma. More interestingly, detection of serum miR-133b and miR-206 expression could be further developed as novel, non-invasive and efficient markers for prognosis in patients with osteosarcomas.

Keywords: Osteosarcoma, microRNA-133b, microRNA-206, clinicopathological features, overall survival, disease-free survival

Introduction

Human osteosarcoma is one of the most common primary sarcomas arising from osteoid tissue and producing immature bone [1]. It has an incidence of 4 to 5 cases per million worldwide and is a leading cause of cancer-related death in children and young adults [2]. With the introduction of combinatorial chemotherapy, the five-year survival rate of patients with osteosarcoma has dramatically improved over the past decades to approximately 60-70% [3]. However, there still are a significant proportion of osteosarcomas patients with poor response to chemotherapy, and they have a high risk of local relapse or distant metastasis even after curative resection of the primary tumor and intensive chemotherapy [4]. Although several molecular targeted drugs have emerged, they have not been well established for the treatment of osteosarcoma. Moreover, the molecular events which initiate and propagate osteosarcomagenesis remain obscure. Therefore, it is of the most important challenge to develop novel and efficient alternative strategies for the management of osteosarcoma.

MicroRNAs (miRNAs) represent small, endogenous, non-coding RNA molecules with highly conserved sequences across species in plants, animals, and DNA viruses [5]. miRNAs can regulate gene expression at the posttranscriptional level by binding to the 3'-untranslated regions of their target mRNAs [6]. It has been estimated

that miRNAs can regulate as much as 60% of the human protein coding genes. miRNAs play crucial roles in a number of biological processes, such as embryogenesis, development, cell maintenance, lineage determination, cell proliferation, apoptosis and differentiation [7]. In addition, accumulating studies also show that miRNAs can be dysregulated in various pathological processes, such as human cancers [8]. They function either as oncogenes or as tumor suppressors depending on the role of their target mRNAs [9]. An increasing number of research have demonstrated that miRNA expression profiles can distinguish tumors from corresponding normal tissues, as well as by their developmental origin and differentiation state [10]. Especially in osteosarcoma, miR-21 was significantly overexpressed in osteosarcoma, and the suppression of miR-21 decreased the invasion and migration in MG-63 OS cell lines [11]; the expression of miR-34 was decreased in osteosarcoma, and miR-34 inhibited the p53-mediated cell cycle arrest and apoptosis in osteosarcoma cells [12]; miR-199a-3p was significantly decreased in the osteosarcoma cell lines as compared to osteoblasts, and overexpression of miR-199a-3p in osteosarcoma cell lines was associated with a significant decrease in cell growth with G1 arrest [13]. Moreover, a large number of miRNAs have also been found to regulate the response to chemotherapy of patients with osteosarcomas. For example, miR-132 had a statistically significant ability to discriminate good responders to ifosfamide from poor responders [14]; miR-140 overexpression caused chemoresistance to methotrexate and 5-Fluorouracil and suppressed cell proliferation, inducing G1 and G2 arrest in osteosarcoma cells [15]. Because of their involvement in osteosaroma, the comprehensive analyses of miRNAs will provide insight into the molecular mechanisms of this disease.

miR-133b and miR-206 were both considered to be muscle-specific miRNAs and were demonstrated to play a role in the development of skeletal muscle [16]. In 2012, the miRNA array profiling of Novello et al. identified that miR-133b and miR-206 were the most downregulated miRNAs in osteosarcoma tissues [17]. Then, the research groups of Zhao et al. and Bao et al. in 2013 respectively reported that the downregulation of miR-133b and miR-206 in osteosarcoma cells could inhibit cell proliferation, migration and invasion, and promote apoptosis [18, 19]. On this basis, we hypothesized that the aberrant expression of the two miRNAs might be associated with tumor progression and could be used as potential prognostic markers of human osteosarcoma. To validate this hypothesis, we here detected the expression levels of miR-133b and miR-206 in 100 pairs of osteosarcoma tissues and matched noncancerous bone tissues, and serum samples from 100 patients with osteosarcoma as well as in serum samples from 100 healthy controls by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis. In addition, we further statistically analyzed the associations of miR-133b and miR-206 expressions with clinicopathological parameters and prognosis in patients with osteosarcomas.

Materials and methods

Ethics statement

This study was approved by the Ethical Review Committee of The Second Hospital Affiliated to Xi'an Jiao Tong University School of Medicine, Xi'an, Shaanxi, China. All specimens were handled and made anonymous according to the ethical and legal standards and were obtained with patients' written informed consent.

Patients and tissue samples

In total, we recruited 100 patients with osteosarcomas from Department of Orthopedic Surgery, The Second Hospital Affiliated to Xi'an Jiao Tong University School of Medicine, Xi'an, Shaanxi, China between February 2007 and May 2010. None of the patients enrolled in this study had received any chemotherapy or radiotherapy before surgery. For tissue sample collection, 100 pairs of osteosarcoma tissues and corresponding noncancerous bone tissues were collected from the same patients. The tissues were removed from surgical specimens, immediately transported to the Pathology Laboratory, frozen and stored at -80°C for RNA extraction. In addition, sera from all 100 patients with osteosarcomas and 100 healthy volunteers matched according to sex and age were also collected. All tissue samples were reviewed by two pathologists, and the clinicopathologic data such as age, sex, site, histological type, tumor grade, surgical method, response to chemotherapy, and the status of metastasis and recurrence were retrospectively reviewed and summarized in **Table 1**.

Table 1. Association of miR-133b and miR-206 expression with clinicopathological features of osteosarcoma

Clinicopathological features	No. of cases	miR-133b-low (n, %)	*P*	miR-206-low (n, %)	*P*	miR-133b-low/ miR-206-low (n, %)	P
Age							
<18	32 (32.00)	20 (62.51)	NS	20 (62.51)	NS	10 (31.28)	NS
≥18	68 (68.00)	36 (52.94)		38 (55.88)		28 (41.18)	
Sex							
Male	70 (70.00)	40 (57.14)	NS	40 (57.14)	NS	28 (40.00)	NS
Female	30 (30.00)	16 (53.33)		18 (60.00)		10 (33.33)	
Tumor site							
Femur	58 (58.00)	35 (60.34)	NS	35 (60.34)	NS	21 (36.21)	NS
Tibia	20 (20.00)	10 (50.00)		11 (55.00)		9 (45.00)	
Humeral bone	15 (15.00)	8 (53.33)		8 (53.33)		6 (40.00)	
Other	7 (7.00)	3 (42.86)		4 (57.14)		2 (28.57)	
Histologic type							
Osteoblastic	52 (52.00)	30 (57.69)	NS	32 (61.54)	NS	20 (38.46)	NS
Chondroblastic	18 (18.00)	10 (55.56)		10 (55.56)		6 (33.33)	
Fibroblastic	20 (20.00)	10 (50.00)		10 (50.00)		8 (40.00)	
Telangiectatic	10 (10.00)	6 (60.00)		6 (60.00)		4 (40.00)	
Tumor grade							
Low	38 (38.00)	16 (47.06)	0.01	16 (47.06)	0.01	8 (23.53)	0.006
High	62 (62.00)	40 (64.52)		42 (67.74)		30 (48.38)	
Metastasis							
Absent	60 (60.00)	26 (43.33)	<0.001	26 (43.33)	<0.001	10 (16.67)	<0.001
Present	40 (40.00)	30 (75.00)		32 (80.00)		28 (70.00)	
Recurrence							
Absent	65 (65.00)	26 (40.00)	<0.001	28 (43.08)	<0.001	12 (18.46)	<0.001
Present	35 (35.00)	30 (85.71)		30 (85.71)		26 (74.29)	
Response to pre-operative chemotherapy							
Good	60 (60.00)	30 (50.00)	0.03	32 (53.33)	NS	18 (30.00)	0.01
Poor	40 (40.00)	26 (65.00)		26 (65.00)		20 (50.00)	

Following the diagnosis, all 100 patients with osteosarcomas enrolled in this study were treated with the same neoadjuvant chemotherapy consisting of methotrexate (MTX), doxorubicin (ADM), cisplatin (CDP), and ifosfamide (IFO). All drugs were given intravenously. After that, all the patients underwent wide resection of tumor. Response to chemotherapy was classified as "poor" (<90% tumor necrosis) and "good" (>90% tumor necrosis) through histologic analysis of tumor specimens after surgery [20].

For the survival analysis, all 100 patients with osteosarcoma enrolled in this study received follow-up and were monitored with computed tomography (CT) performed every 3 months during the first 3 years after chemotherapy, every 4 months during years 4 and 5 and every 6 months thereafter. CT scans or magnetic resonance imaging (MRI) were performed to determine the development of local recurrence and distant metastasis. The median follow-up of this cohort was 30.8 months (range: 7.8-39.6 months). In this study, overall survival (OS) was defined as the time interval from the date of diagnosis at our center to the date of death or the last follow-up. Disease-free survival (DFS) was defined as the time interval from diagnosis at our center to progressive disease, death of any other cause than progression, or a second primary cancer.

RNA extraction

Total RNA was isolated from fresh osteosarcoma tissues, corresponding noncancerous tissues, sera of osteosarcoma patients and healthy controls by using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacture's instruction. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA quality was measured using a denaturing 15% polyacrylamide gel.

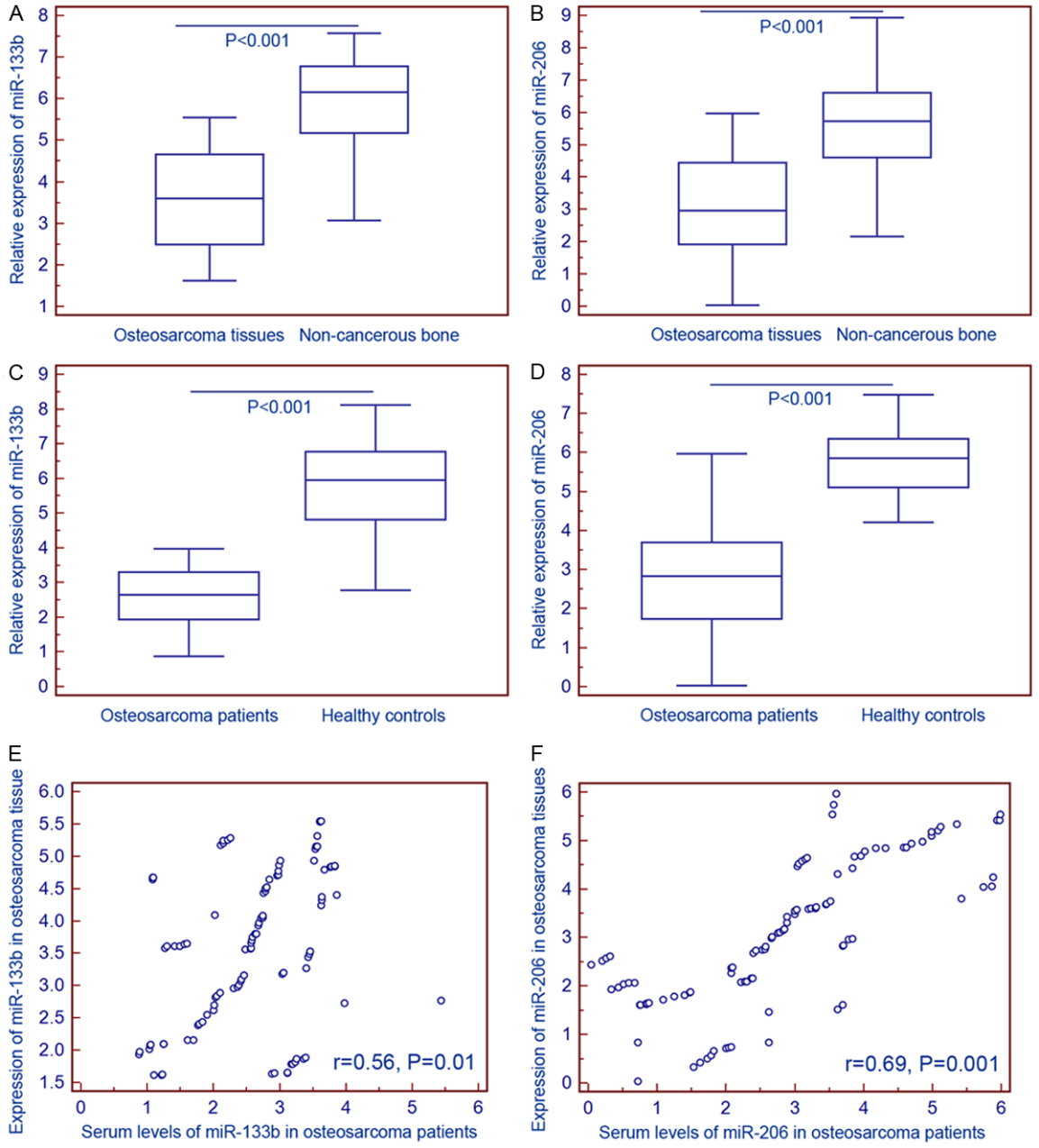

Figure 1. Expression levels of miR-miR-133b and miR-206 in human osteosarcoma tissues and patients' sera detected by qRT-PCR assay. The results showed that the expression levels of miR-133b (A) and miR-206 (B) in osteosarcoma tissues were both significantly lower than those in noncancerous bone tissues (both $P<0.001$). Similarly, the serum levels of miR-133b (C) and miR-206 (D) were also markedly downregulated in patients with osteosarcomas compared with healthy controls (both $P<0.001$). More interestingly, the expression levels of miR-133b and miR-206 in osteosarcoma tissues were both significantly correlated with those in patients' sera (for miR-133b: Spearman's correlation: $r=0.56$, $P=0.01$, E; for miR-206: Spearman's correlation: r=0.69, $P=0.001$, F).

QRT-PCR for miRNA

Reverse transcription (RT) reaction was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and 10 ng total RNA was utilized in the RT reactions. Following the detection with Applied Biosystems prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), qRT-PCR was performed using specific TaqMan MicroRNA analysis (Applied Biosyst-

ems, Foster City, CA, USA). RNU6 (Applied Biosystems, Foster City, CA, USA) was used as the endogenous control for the expression of miR-133b and miR-206. Real-time PCR reactions for miRNAs were performed in triplicate in 20 μl volumes. The sequences of the primers were as follows: miR-133b forward, 5'-TTT GGT CCC CTT CAA CCA GCT A-3'; reverse, 5'-GTG CAG GGT CCG AGG T-3'; miR-206 forward, 5'-CGT CAG AAG GAA TGA TGC ACA G-3'; reverse, 5'-ACC TGC GTA GGT AGT TTC ATG T-3'; U6 forward 5'-CTC GCT TCG GCA GCA CA-3' and reverse 5'-AAC GCT TCA CGA ATT TGC GT-3'. Quantitative miRNA expression data were acquired and analyzed using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The qRT-PCR assays for a particular gene were undertaken at the same time for all samples under identical conditions, in triplicate. The miRNA relative expression was calculated using a $2^{-\Delta\Delta Ct}$ method [21].

Statistical analysis

Statistical analysis was performed by using the software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA). Continuous variables were expressed as $\bar{x}\pm s$. A paired sample t-test was used to compare differences in miRNA expression between osteosarcoma tissues and noncancerous bone tissues. The correlation of miR-133b or miR-206 expression between osteosarcoma tissues and serum tissues was determined by Spearman Correlation analysis. The statistical significance of the correlation of miR-133b and miR-206 expression with various clinicopathological parameters was evaluated by Fisher's exact test or x^2 test. The Kaplan-Meier test was used to determine the probability of survival and data were analyzed by the log-rank test. Differences were considered statistically significant when *P* was less than 0.05.

Results

Downregulation of miR-133b and miR-206 in human osteosarcoma tissues and patients' sera

Expression levels of miR-133b and miR-206 in osteosarcoma and corresponding noncancerous bone biopsy samples, as well as in patients' sera and healthy controls were detected by qRT-PCR and normalized to RNU6B. As the results, the expression levels of miR-133b and miR-206 in osteosarcoma tissues were both significantly lower than those in noncancerous bone tissues (both $P<0.001$, **Figure 1A**, **1B**). Similarly, the serum levels of the two miRNAs were also markedly downregulated in patients with osteosarcomas compared with healthy controls (both $P<0.001$, **Figure 1C**, **1D**). More interestingly, the expression levels of miR-133b and miR-206 in osteosarcoma tissues were both significantly correlated with those in patients' sera (for miR-133b: Spearman's correlation: $r=0.56$, $P=0.01$, **Figure 1E**; for miR-206: Spearman's correlation: $r=0.69$, $P=0.001$, **Figure 1F**). Hence, we investigated the clinical significance of miR-133b and miR-206 in osteosarcoma using their serum levels in the next sections.

Serum levels of miR-133b and miR-206 associate with clinicopathological features of human osteosarcoma

In order to evaluate the associations of serum levels of miR-133b and miR-206 with the clinicopathological features of osteosarcoma patients, the median values of miR-133b (2.66) and miR-206 (2.84) expression in sera of 100 osteosarcoma patients were used as the cutoff points to divide these patients into miR-133b-low (n=56), miR-133b-high (n=44), miR-206-low (n=58) and miR-206-high (n=42) expression groups. On this basis, 38 (38.00%) cases were both low expression of miR-133b and miR-206, 24 (24.00%) cases were both high expression of miR-133b and miR-206, 18 (18.00%) cases were miR-133b-low and miR-206-high expression, and 20 (20.00%) cases were miR-133b-high and miR-206-low expression.

As shown in **Table 1**, the downregulation of miR-133b and miR-206 both more frequently occurred in osteosarcoma patients with high tumor grade (both $P=0.01$), positive metastasis (both $P<0.001$) and recurrence (both $P<0.001$). Especially, the low expression of miR-133b was also associated with poor response to chemotherapy ($P=0.03$) of osteosarcoma patients. Of note, the combined downregulation of miR-133b and miR-206 was significantly associated with high tumor grade ($P=0.006$), the presence of metastasis ($P<0.001$) and recurrence ($P<0.001$), and the poor response to chemotherapy ($P=0.01$) of patients with osteosarcomas.

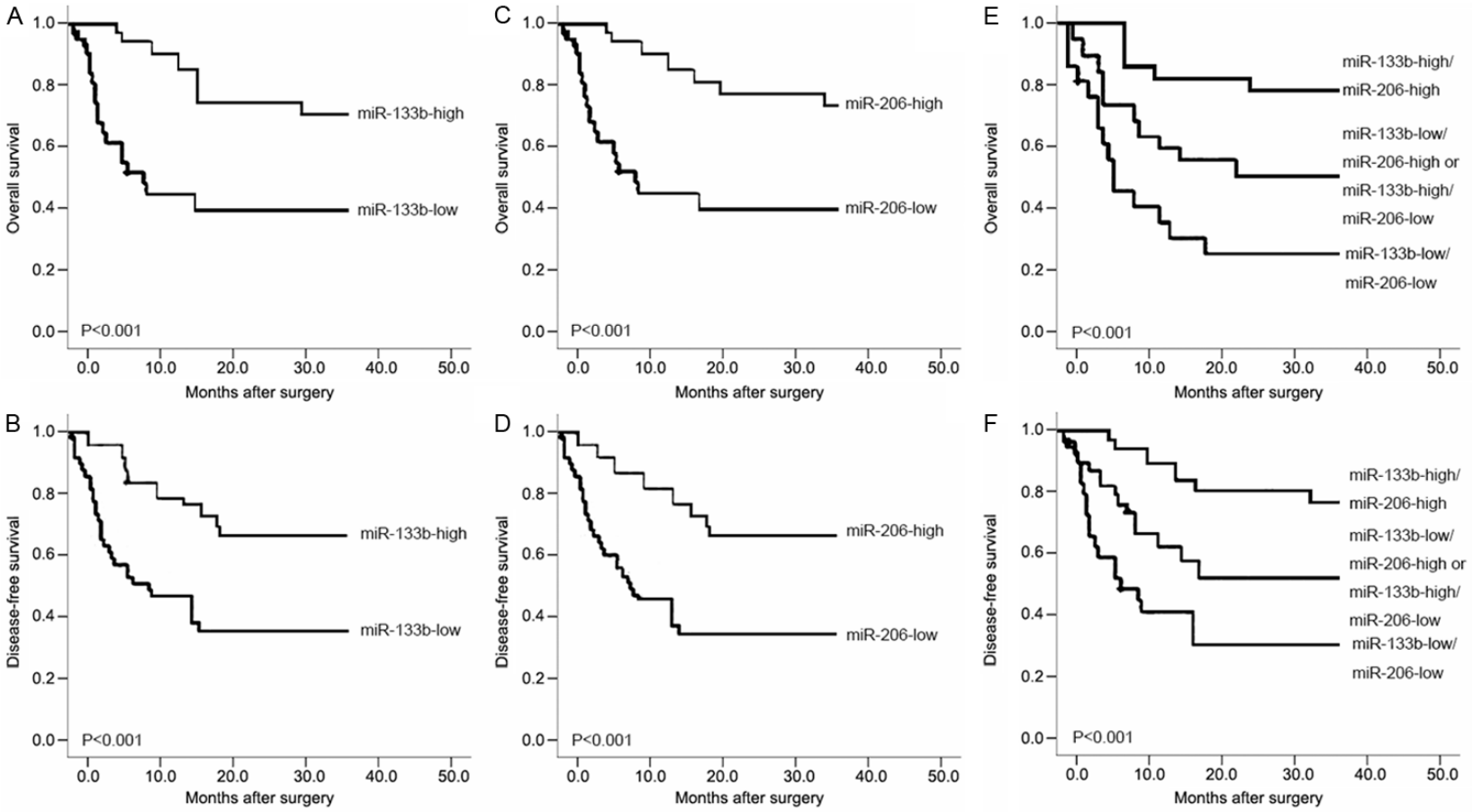

Figure 2. Kaplan-Meier survival curves for osteosarcoma patients according to miR-133b expression (A for overall survival; B for disease-free survival), miR-206 expression (C for overall survival; D for disease-free survival) and concomitant miR-133b and miR-206 expression (miR-133b/miR-206, E for overall survival; F for disease-free survival).

Table 2. Multivariate survival analysis of overall survival (OS) and disease-free survival (DFS) in 100 patients with osteosarcoma

Variables	OS			DFS		
	RR	95% CI	*P*	RR	95% CI	*P*
Tumor grade	6.86	1.69-15.02	0.01	7.37	1.72-16.31	0.008
Response to pre-operative chemotherapy	3.58	0.80-7.21	0.04	4.02	1.01-8.38	0.03
Metastasis status	3.19	0.76-6.82	NS	3.56	0.80-7.19	0.04
Recurrence status	3.48	0.79-7.03	NS	4.00	1.00-8.06	0.03
miR-133b expression	5.36	1.26-11.03	0.02	5.69	1.33-11.26	0.02
miR-206 expression	5.42	1.31-11.28	0.02	5.88	1.56-12.08	0.02
miR-133b/miR-206 expression	9.28	2.69-20.79	0.001	9.69	2.80-21.82	0.001

Serum levels of miR-133b and miR-206 predicts prognosis in patients with osteosarcoma

According to the results of Kaplan-Meier method and log-rank test, the patients with low miR-133b expression and low miR-206 expression both had shorter OS (both $P<0.001$, **Figure 2A**, **2C**) and DFS (both $P<0.001$, **Figure 2B**, **2D**) than those with high expressions. Of note, the OS and DFS of patients with combined low expression of miR-133b and miR-206 (miR-133b-low/miR-206-low) were the shortest (both $P<0.001$, **Figure 2E**, **2F**) when compared with patients in other three groups (miR-133b-low/miR-206-high, miR-133b-high/miR-206-low, miR-133b-high/miR-206-high). In addition, the OS and DFS benefits were also found in the patients with low tumor grade ($P=0.006$ and 0.002, respectively), good response to pre-operative chemotherapy (both $P=0.02$), and the absence of metastasis (both $P<0.001$) and recurrence (both $P<0.001$).

Cox proportional hazard model confirmed that miR-133b expression (for OS: RR 5.36, 95% CI, 1.26-11.03, $P=0.02$; for DFS: RR 5.69, 95% CI, 1.33-11.26, $P=0.02$), miR-206 expression (for OS: RR 5.42, 95% CI, 1.31-11.28, $P=0.02$; for DFS: RR 5.88, 95% CI, 1.56-12.08, $P=0.02$) and miR-133b/miR-206 expression (for OS: RR 9.28, 95% CI, 2.69-20.79, $P=0.001$; for DFS: RR 9.69, 95% CI, 2.80-21.82, $P=0.001$) were all independent prognostic factors of unfavorable survival in human osteosarcoma (**Table 2**).

Discussion

Actually, a large number of studies have indicated that miRNAs play important roles as either tumor suppressor genes or oncogenes in several human cancers. However, their involvements in osteosarcomagenesis have not been fully elucidated. In this study, we analyzed the expression of two miRNAs, miR-133b and miR-206, in osteosarcoma tissue and serum samples for association with clinicopathological and survival data from patients. We found that miR-133b and miR-206 expression were both downregulated in osteosarcoma tissues compared to the corresponding noncancerous bone tissues. In addition, their serum levels were also significantly decreased in osteosarcoma patients compared to levels in healthy controls. Moreover, we found that lower serum levels of miR-133b and miR-206 were associated with short OS and short DFS of osteosarcoma patients. Furthermore, the univariate and multivariate analyses showed that serum levels of miR-133b and miR-206, and their combined serum levels were all independent predictors of OS and DFS of osteosarcoma patients.

miR-133b and miR-206, initially known as two muscle-specific miRNAs, form a cluster in chromosomal region 6p12.2 in the human genome [22]. Since it has been demonstrated that miRNAs with a tissue-specific expression manner often play important roles in many aspects of development and physiology, both miR-133b and miR-206 mediate myoblasts proliferation and differentiation [23]. In addition to these functions, miR-133b has been found to be important for heart development, be dysregulated in hypertrophic and failing hearts, and also regulate neuronal differentiation [24]. Pathologically, recent studies have detected the aberrant expression of miR-133b in various cancer types, including head and neck cancer, lung cancer, gastric cancer, colorectal cancer, cervical cancer, bladder cancer, and osteosarcoma [18, 25-29]. It is generally considered as a tumor suppressor miRNA for many human

malignancies by regulating its target genes, such as epidermal growth factor receptor (EGFR), myeloid cell leukemia sequence 1 (MCL-1), fascin homolog 1 (FSCN-1), c-Met, and BCL2-like 2 (BCL2L2) [26-28]. Regarding to miR-206, it also plays a crucial role in several cancers. For example, miR-206 may function as a metastasis suppressor miRNA in human breast cancer, and may serve as a key factor that regulates ERα expression during the development of normal breast epithelium because the expression of miR-206 is under hormonal regulation [30]; miR-206 was also proven to have effects on the invasion of lung cancer [31]; miR-206 has been found downregulated in skeletal musclederived tumors such as rhabdomyosarcomas. Re-expression of miR-206 in these tumors may promote myogenic differentiation and block tumor growth by inhibiting c-met expression [32]. In the current study, we observed the reduced expression of miR-133b and miR-206 in both osteosarcoma tissues and patients' sera, which was in line with the previous findings [17-19]. Then, we also found that the downregulation of miR-133b and miR-206 were both significantly associated with aggressive tumor progression and unfavorable clinical outcome of osteosarcoma patients, suggesting their potential values for diagnosis and prognosis of this disease.

More interestingly, we here also analyzed the association of miR-133b/miR-206 conjoined expression with the prognosis of osteosarcoma. Significant difference of prognosis was found among four different statuses of miR-133b/miR-206 co-expression. The subjects with miR-133b-high/miR-206-high had the best OS and DFS, while the miR-133b-low/miR-206-low had the worst. Multivariate analysis revealed that the miR-133b/miR-206 co-expression profiles were an independent prognostic indicator for osteosarcoma. The advantages of miR-133b/miR-206 co-expression to individual miR-133b or miR-206 in predicting the outcome of osteosarcoma has been shown in our result.

Taken together, these data offer the convincing evidence that the aberrant expression of miR-133b and miR-206 may be implicated in tumorigenesis and tumor progression of osteosarcoma. More interestingly, detection of serum miR-133b and miR-206 expression could be further developed as novel, non-invasive and efficient markers for prognosis in patients with osteosarcomas. Further investigation extended in much more cases is in need to evaluate the potential application value of miR-133b, miR-206, and miR-133b/miR-206 conjoined expression, as dependent or independent prognosis factor/s of osteosarcoma, in clinical setting.

Disclosure of conflict of interest

None.

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