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RESEARCH

Increased Expression of microRNA-199b-5p Associates with Poor Prognosis Through Promoting Cell Proliferation, Invasion and Migration Abilities of Human Osteosarcoma

Haibin Zeng · Zhong Zhang · Xiaoming Dai · Yongzhong Chen · Jianhua Ye · Zhixin Jin

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Abstract MicroRNA (miR)-199b-5p has been reported to be upregulated in human osteosarcoma tissues and participate in the Notch signaling in osteosarcoma cells. This study was aimed to investigate the associations of miR-199b-5p expression with tumor progression of primary osteosarcoma, and to deepen the understanding of its involvement in carcinogenesis. Quantitative real-time reverse transcriptase-polymerase chain reaction was performed to detect expression levels of miR-199b-5p in 98 osteosarcoma and corresponding adjacent normal tissues. Then, the correlations of its expression with clinicopathological characteristics and patient prognosis were statistically analyzed. Moreover, in vitro assays were performed to assess the effects of miR-199b-5p on the proliferation, migration and invasion of two human osteosarcoma cell lines MG63 and U2OS. Compared to normal controls, miR-199b-5p expression was significantly upregulated in osteosarcoma tissues ($P<0.001$). In addition, the expression levels of miR-199b-5p in osteosarcoma patients with high tumor grade ($P=0.008$), positive metastasis ($P=0.001$) and positive recurrence ($P=0.001$) were markedly higher than those with low tumor grade, negative metastasis and negative recurrence. Moreover, osteosarcoma patients with high miR-199b-5p expression showed shorter overall survival ($P<.001$) and shorter disease-free survival ($P<0.001$) than those with low expression. Furthermore, the inhibition of miR-199b-5p significantly suppressed cell proliferation, and reduced the migratory and invasive abilities of osteosarcoma cells. This study offer the convincing evidence for the first time that the increased expression of miR-199b-5p may play crucial roles in aggressive progression and poor prognosis of human osteosarcoma. miR-199b-5p may function as an oncogene by positively regulating the malignant potentials of this neoplasm.

Keywords Osteosarcoma · microRNA-199b-5p · Clinicopathological characteristics · Prognosis · Carcinogenesis

H. Zeng (✉) · Z. Zhang · X. Dai · Y. Chen · J. Ye · Z. Jin
Department of Orthopedics, 476 Clinical Division, Fuzhou General Hospital of Nanjing Military Region, Fuzhou 350002, China
e-mail: Zenghaib139@163.com

Introduction

Osteosarcoma is one of the most common primary malignant bone neoplasms affecting rapidly growing bones, particularly in children and adolescents [1]. Since it is characterized by highly malignant and invasive growth, the relative 5-year survival rate of osteosarcoma patients is very low, approximately less than 20 % [2]. Nowadays, surgical resection incorporated with chemotherapy has been standard treatment for osteosarcoma patients. However, if patients become resistant to chemotherapeutic reagents, their clinical outcome can't be significantly improved as a result. Recurrence occurs in 30 to 40 % of osteosarcoma patients and 70 % of patients with recurrence die despite second-line treatment [3]. In clinics, several conventional variables, including tumor site, tumor size, tumor grade, metastasis status and response to chemotherapy, have been extensively used to evaluate prognosis in osteosarcoma patients. However, the sensitivities and specificities of these variables are still unsatisfactory due to the heterogeneity of the patients [4, 5]. Therefore, it is extremely imperative to exploit novel and efficient molecular markers for the prediction of the malignant behavior of osteosarcoma in order to guide clinical treatment and improve prognosis in patients with this disease.

MicroRNAs (miRNAs), an abundant class of endogenous, small (17–25 nucleotides), noncoding and single-stranded RNAs, regulate gene expression via binding to

the 3′ untranslated regions (3′UTR) of target mRNAs post-transcriptionally [6]. There have been nearly 1000 miRNAs predicted to exist in the human genome, and thousands of human protein-encoding genes are collectively regulated by miRNAs [7]. The relationship between miRNAs and their targets shows combinatorial complication, in terms of both target multiplicity and signal integration. Growing evidence has demonstrated that miRNAs play important roles in many biological processes, including cell proliferation, differentiation, apoptosis, stress resistance, fat metabolism, and development [8, 9]. In recent years, accumulating studies have reported that the degregulation or dysfunction of miRNAs may be implicated in tumorigenesis and tumor progression. They often act either as tumor suppressors or as oncogenes [10]. Especially, an increasing number of miRNAs have been reported to be related to malignant phenotypes of human osteosarcoma. For example, miR-133a is down-regulated in human osteosarcoma, and suppresses proliferation and promotes apoptosis implying its roles in osteosarcoma pathogenesis and its potential in cancer therapy [11]; miR-27a promotes proliferation, migration and invasion by targeting MAP2K4 in human osteosarcoma cells, which may help us understand the molecular mechanism of miR-27a in the tumorigenesis of osteosarcoma and may provide new diagnostic and therapeutic options for the treatment of this neoplasia [12]; miR-1 and miR-133b are both down-regulated in osteosarcoma cell lines compared to normal osteoblasts, and may control cell proliferation and cell cycle through MET protein expression modulation [13]; miR-29b suppressed osteosarcoma cell proliferation and migration via modulation of VEGF [14]; miR-217 might function as a tumor-suppressive miRNA and inhibit the osteosarcoma tumorigenesis through targeting WASF3 [15]; Overexpression of miR-124 attenuated cell proliferation, migration, and invasion and induced apoptosis in osteosarcoma cells in vitro [16]. These findings underscore the regulatory functional importance of miRNAs in osteosarcomas.

miR-199b-5p has been indicated to play crucial roles in various types of human cancers, including chronic myeloid leukemia, medulloblastoma, osteosarcoma, choriocarcinoma, breast cancer, renal cell carcinoma and colorectal cancers [17–23]. Especially, Won et al. [19] in 2012 identified miR-199b-5p as one of upregulated miRNAs in osteosarcoma tissue samples compared with normal controls. They also found that miR-199b-5p may be involved in the regulation of Notch signaling in osteosarcoma. However, its clinical significance and roles in osteosarcoma carcinogenesis remain unknown. Thus, this study was aimed to investigate the associations of miR-199b-5p expression with tumor progression and patients' prognosis of primary osteosarcoma, and to deepen the understanding of its involvement in carcinogenesis of this malignancy.

Materials and Methods

Patients and Tissue Samples

All samples were collected with the written informed consents of the patients. The Institutional Review Board at Fuzhou General Hospital of Nanjing Military Region approved the use of human specimens and all the experiments in this study.

A total of 98 self pairs of osteosarcoma specimens and the corresponding adjacent normal tissues were obtained from 98 patients, with complete histopathology and follow-up information. All patients underwent the same neoadjuvant chemotherapy and 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 [24]. All tissue samples were reviewed by two pathologists, and the clinicopathologic data such as age, sex, site, histologic type, tumor grade, surgical method, response to chemotherapy, and the status of metastasis and recurrence were retrospectively reviewed and summarized in Table 1. To detect the expression levels of miR-199b-5p in osteosarcoma specimens and the corresponding adjacent normal tissues by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), all the tissues removed from surgical specimens were immediately transported to the Pathology Laboratory, frozen and stored at −80 °C for RNA extraction.

All 98 osteosarcoma patients enrolled in this study were received regular follow-ups ranged from 6.2 to 40.5 months (mean 28.3 months) with the survival time, date of death and date of last follow-up being recorded. According to the previous studies, the osteosarcoma patients 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. 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.

Cell Culture

Human osteosarcoma cell lines MG63 and U2OS were obtained from the Cell Bank of Chinese Academy of Sciences and were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10 % heat-inactivated foetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were incubated at

Table 1 Association of microRNA (miR)-199b-5p with clinicopathological characteristics of osteosarcoma

Clinicopathological features	No. of cases	miR-29a-high (n, %)	P
Age			
<20	32 (32.65)	20 (62.50)	NS
≥20	66 (67.35)	38 (57.58)	
Sex			
Male	60 (61.22)	36 (60.00)	NS
Female	38 (38.78)	22 (57.89)	
Tumor site			
Femur	56 (57.14)	33 (58.93)	NS
Tibia	20 (20.41)	12 (60.00)	
Humeral bone	15 (15.31)	9 (60.00)	
Other	7 (7.14)	4 (57.14)	
Histologic type			
Osteoblastic	50 (51.02)	31 (62.00)	NS
Chondroblastic	20 (20.41)	12 (60.00)	
Fibroblastic	18 (18.37)	10 (55.56)	
Telangiectatic	10 (10.20)	5 (50.00)	
Tumor grade			
Low	38 (38.78)	16 (42.11)	0.008
High	60 (61.22)	42 (70.00)	
Metastasis			
Absent	62 (63.27)	28 (45.16)	0.001
Present	36 (36.73)	30 (83.33)	
Response to pre-operative chemotherapy			
Good	68 (69.39)	38 (55.88)	NS
Poor	30 (30.61)	20 (66.67)	
Recurrence			
Absent	62 (63.27)	28 (45.16)	0.001
Present	36 (36.73)	30 (83.33)	

'NS' refers to the difference has no statistical significance

37 °C in a humidified incubator supplemented with 5 % CO_2 and 95 % atmosphere.

RNA Extraction and qRT-PCR

Total RNA was extracted from cell lines and frozen tissues using miRNeasy kit (Qiagen) according to the manufacturer's instructions and stored at −80 °C in RNAsecure reagent (Ambion Inc., Austin, TX, USA). RNA concentration was determined with spectrophotometer and the 260/280 ratio of RNA was 1.8. Purity and quality were identified by a denatured 15 % gel electrophoresis.

To detect miRNA expression in cell lines and frozen tissues, qRT-PCR analysis was performed using LightCycler (Roche) and SYBR RT-PCR kit (Takara) according to the manufacturer's instructions. Quantitative miRNA expression data were acquired and analyzed using an Applied Biosystems 7500 real-time PCR system. U6 was used as an internal control. Primers for detecting miR-199b-5p and U6 expression were described previously [25, 26]. The relative expression level of miR-199b-5p was normalized to that of internal control U6 by using $2^{-\Delta\Delta Ct}$ cycle threshold method [27].

Transfection of miR-199b-5p Inhibitors

MiR-199b-5p inhibitor (anti-miR-199b-5p) and inhibitor negative control (anti-NC, CAG UAC UUU UGU GUA GUA CAA) were purchased from GenePharma (Shanghai, China). Human osteosarcoma cell lines MG63 and U2OS were seeded into 6- or 24-well plates the day before transfection to ensure 50 % cell confluence at the moment of transfection.

Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The oligonucleotides were used at a final concentration of 100 nM. For proliferation assays, cells were trypsinized 24 h after transfection. For migration, invasion, and qRT-PCR assays, cells were collected 48 h after transfection

Cell Proliferation Analysis

The effect of miR-199b-5p on cell proliferation capacity of two human osteosarcoma cell lines MG63 and U2OS was evaluated by a methyl thiazole tetrazolium (MTT) assay. In brief, cells were seeded into 96-well plates at a density of 3000 cells/well and allowed to adhere overnight. Then, cells were transfected with 100nM miR-199b-5p inhibitor or a inhibitor negative control. After 24 h culture, 10 μL of MTT (5 mg/ml) were added, and incubated with transfected cells for another 4 h. Later on, the media was removed and 100 μL DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. Optical density readings were obtained at 450 nm. The experiment was performed in triplicate.

Migration Assay

The effect of miR-199b-5p on cell migration capacity of two human osteosarcoma cell lines MG63 and U2OS was carried out using Transwell Permeable Support (Corning Incorporated, Corning, NY, USA). After transfection, cells were carefully transferred on the top chamber of each transwell apparatus at a density of 1×10^6/mL (100 μL/chamber). Cells were allowed to migrate for 48 h at 37 °C. Cells that had penetrated to the bottom side of the membrane were then fixed in methanol, stained using hematoxylin and counted at microscope. The experiment was performed in triplicate.

Invasion Assay

The effect of miR-199b-5p on cell invasion capacity of two human osteosarcoma cell lines MG63 and U2OS was analyzed by using Cultrex 24 well BME Cell Invasion Assay (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. After transfection, cells were seeded in 100 μL serum-free media into the upper wells previously coated with Matrigel basement extract, and 500 μL of media were added in the bottom wells. After 48 h of CO_2 incubation at 37 °C, the non-invasive cells on the upper surface were removed and the cells migrated to the lower surface were fixed in 500 μL of Cell Dissociation Solution/Calcein-AM for 1 h and quantified by fluorimetric analysis (485 excitation, 520 nm emission). The experiment was performed in triplicate.

Statistical Analysis

The software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. Data were shown as mean ± SD from at least three separate experiments. The Mann–Whitney *U*-test was used for two-group comparisons. Chi-squared test was used to analyze difference of classified variable. Kaplan–Meier method and log-rank test were used for survival analysis. Differences were considered statistically significant when *P* was less than 0.05.

Results

Upregulation of miR-199b-5p in Human Osteosarcoma Tissues

Expression level of miR-199b-5p in osteosarcoma tissues was significantly higher than those in the corresponding adjacent normal tissues (tumor vs. normal: 4.84±1.63 vs. 1.74±0.59, $P<0.001$, Fig. 1).

In order to classify all 98 osteosarcoma patients into high miR-199b-5p expression and low miR-199b-5p expression groups, a cutoff value for miR-199b-5p expression levels was chosen on the basis of a measure of heterogeneity with the log-rank test statistic with respect to overall survival. As a result, an optimal cutoff value (4.35) was identified: the low miR-199b-5p expression group [expression level lower than the cutoff value; mean expression value 3.16, $n=40$, 40.82 %] and the high miR-199b-5p expression group [expression level higher than the cutoff value; mean expression value 6.01, $n=58$, 59.18 %].

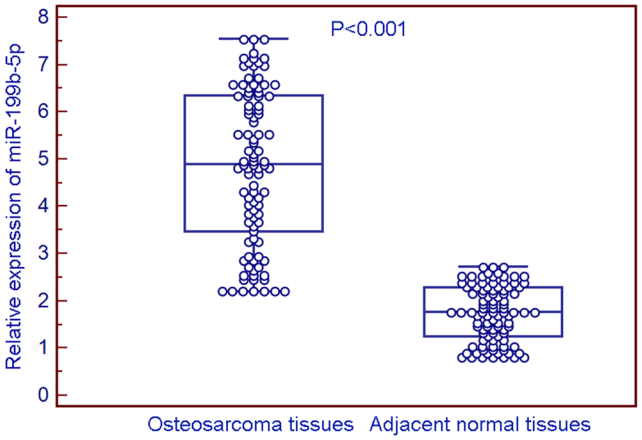

Fig. 1 Expression levels of microRNA (miR)-199b-5p in human osteosarcoma tissues detected by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) assay. Statistical analysis showed that expression level of miR-199b-5p in osteosarcoma tissues was significantly higher than those in the corresponding adjacent normal tissues (tumor vs. normal: 4.84±1.63 vs. 1.74±0.59, $P<0.001$)

Upregulation of miR-199b-5p Associations with Aggressive Tumor Progression of Human Osteosarcoma

As shown in Table 1, the expression levels of miR-199b-5p in osteosarcoma patients with high tumor grade ($P=0.008$), positive metastasis ($P=0.001$) and positive recurrence ($P=0.001$) were markedly higher than those with low tumor grade, negative metastasis and negative recurrence.

Upregulation of miR-199b-5p Associations with Poor Prognosis in Patients with Osteosarcoma

According to the results of Kaplan–Meier method and log-rank test, the patients with high miR-199b-5p expression had shorter OS ($P<0.001$, Fig. 2a) and DFS ($P<0.001$, Fig. 2b) than those with low miR-199b-5p expression.

Cox proportional hazard model confirmed that tumor grade (for OS: RR 4.19, 95 %CI, 1.36–9.21, $P=0.01$; for DFS: RR 4.93, 95 %CI, 1.71–10.21, $P=0.01$), metastasis status (for OS: RR 5.26, 95 %CI, 1.76–11.02, $P=0.01$; for DFS: RR 5.98, 95 %CI, 1.80–12.19, $P=0.008$), response to pre-operative chemotherapy (for OS: RR 3.01, 95 %CI, 0.78–6.22, $P=0.03$; for DFS: RR 3.26, 95 %CI, 1.01–6.68, $P=0.03$), recurrence status (for OS: RR 3.99, 95 %CI, 0.89–8.03, $P=0.01$; for DFS: RR 4.28, 95 %CI, 1.00–8.99, $P=0.01$) and miR-199b-5p expression (for OS: RR 5.12, 95 %CI, 1.22–11.09, $P=0.008$; for DFS: RR 5.68, 95 %CI, 1.33–12.26, $P=0.008$) were all independent prognostic factors of unfavorable survival in human osteosarcoma (Table 2).

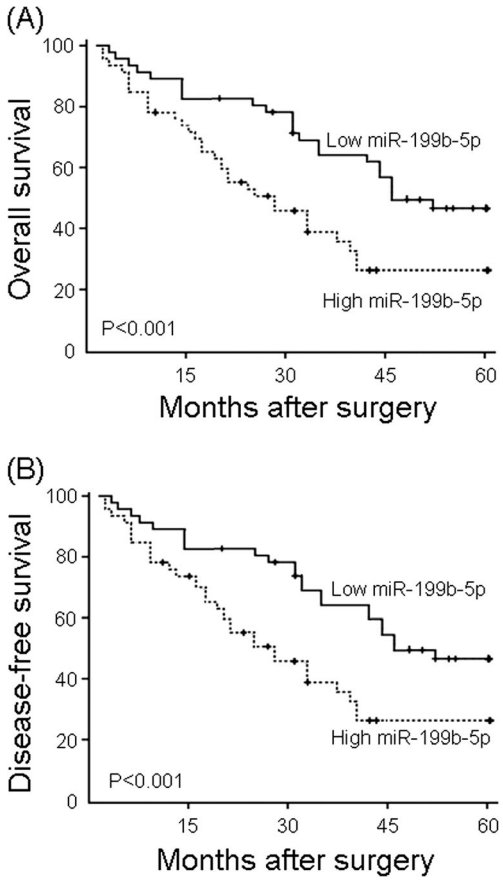

Fig. 2 Kaplan-Meier survival curves for osteosarcoma patients according to microRNA (miR)-199b-5p expression (**a** for overall survival; **b** for disease-free survival)

Inhibition of miR-199b-5p Suppresses Proliferation of Human Osteosarcoma Cells

To investigate the function of miR-199b-5p in osteosarcoma cells, we succeeded establishing two miR-199b-5p-silencing osteosarcoma cell lines: MG63-anti-miR-199b-5p and U2OS-anti-miR-199b-5p. As shown in Fig. 3a and b, the transfection of anti-199b-5p significantly suppressed the intracellular expression of miR-199b-5p in both MG63 and U2OS cells 48~96 h after the transfection with a time-dependent manner. Then, the MTT assay was performed to observe the effect of miR-199b-5p on the proliferation of human osteosarcoma cells 24, 48, 72 and 96 h after the transfection of anti-miR-199b-5p and anti-NC. As a result, the cell proliferation of MG63 and U2OS cells were both significantly lower in anti-miR-199b-5p groups than those in the negative control groups ($P < 0.05$, Fig. 3c and d).

Inhibition of miR-199b-5p Suppresses the Migration and Invasion of Human Osteosarcoma Cells

Transwell migration and invasion assays were performed to determine whether miR-199b-5p was implicated in the migratory and invasive behaviors of human osteosarcoma cells 48 h after the transfection of anti-miR-199b-5p and anti-NC. As shown in Fig. 4, inhibition of miR-199b-5p could dramatically reduced the migration and invasion abilities of both MG63 and U2OS cells.

Discussion

The treatment for osteosarcoma is a formidable challenge. Current therapeutic strategies are not sufficiently effective, therefore, new therapeutic targets are urgently needed. Deregulated miRNAs and their roles in carcinogenesis of human osteosarcoma have attracted much attention. In this study, we found that the expression levels of miR-199b-5p in osteosarcoma tissues were significantly higher than those in the corresponding adjacent normal tissues and miR-199b-5p was upregulated in almost 60 % of all 98 osteosarcoma tissues. In addition, the statistical analysis showed that the high miR-199b-5p expression in osteosarcoma tissues was significantly associated with aggressive tumor progression and poor prognosis in patients with this malignancy. Meanwhile, we used miR-199b-5p inhibitor-induced knockdown of

Table 2 Multivariate survival analysis of overall survival (OS) and disease-free survival (DFS) in 98 patients with osteosarcoma

Variables	OS			DFS		
	RR	95 %CI	P	RR	95 %CI	P
Tumor grade	4.19	1.36–9.21	0.01	4.93	1.71–10.21	0.01
Metastasis status	5.26	1.76–11.02	0.008	5.98	1.80–12.19	0.008
Response to pre-operative chemotherapy	3.01	0.78–6.22	0.03	3.26	1.01–6.68	0.03
Recurrence status	3.99	0.89–8.03	0.01	4.28	1.00–8.99	0.01
miR-199b-5p expression	5.12	1.22–11.09	0.008	5.68	1.33–12.26	0.008

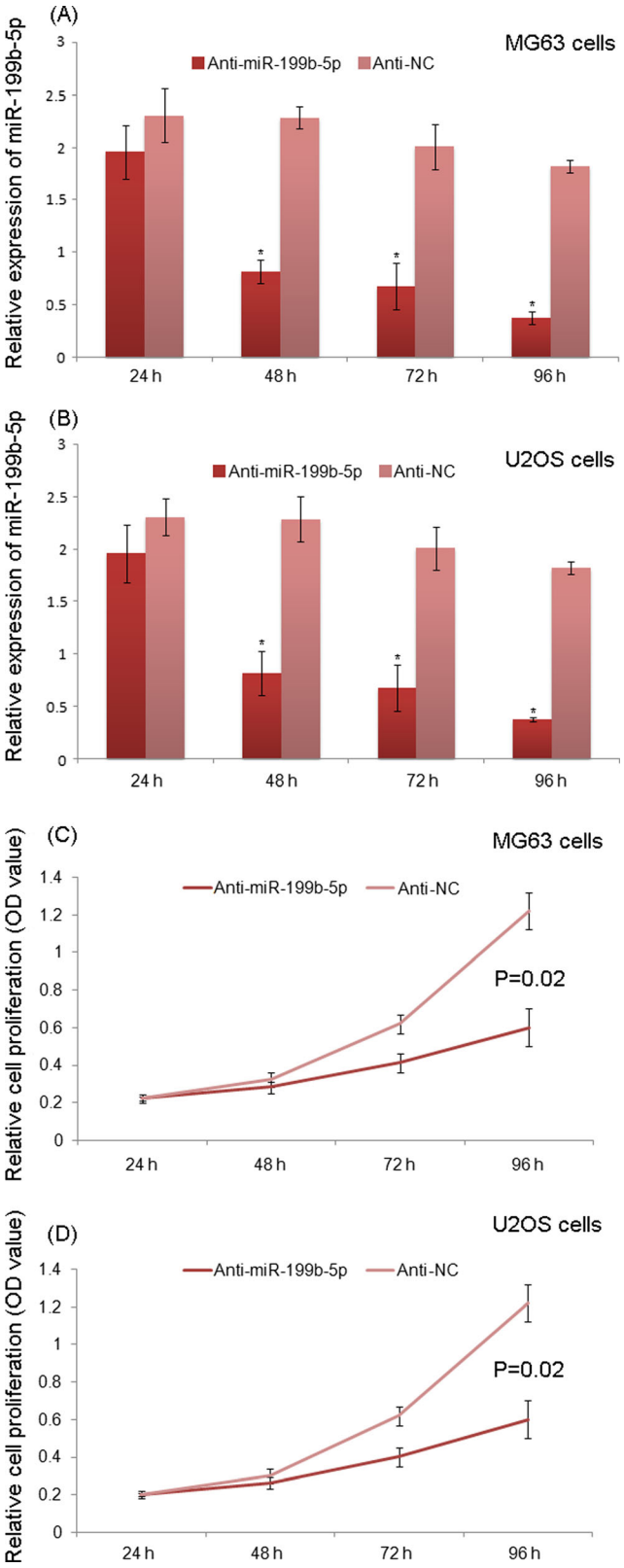

Fig. 3 Inhibition of microRNA (miR)-199b-5p suppresses proliferation of human osteosarcoma cells. **a** and **b** the transfection of anti-199b-5p significantly suppressed the intracellular expression of miR-199b-5p in both MG63 and U2OS cells 48 h after the transfection with an time-dependent manner; '*' $P<0.001$, comparison with anti-NC group. **c** and **d** The cell proliferation of MG63 and U2OS cells were both significantly lower in anti-miR-199b-5p groups than those in the negative control groups (both $P<0.05$)

miR-199b-5p in two human osteosarcoma cell lines to investigate its potential functions in these cells and found that inhibition of miR-199b-5p could result in the suppression of cell proliferation, migration and invasion. These findings suggest that miR-199b-5p may play an oncogenic role in the carcinogenesis and cancer progression of human osteosarcoma.

MiR-199b-5p has been reported to be differentially expressed in normal and cancer cells [17–23]. Functionally, it acts either as an oncogene or a tumor suppressor in various human caner types. For example, miR-199b-5p was firstly indicated to impair cancer stem cells through negative regulation of Hes1 in medulloblastoma in 2009 [18]. The authors identified this miRNA as a regulator of the Notch pathway through its targeting of Hes1 and found that its overexpression could suppress both the proliferation rate and the anchorage-independent growth of medulloblastoma cells in vitro. Then, they also verified the miR-199b-5p regulatory circuit involving Hes1, CD15, and epigenetic modifications in medulloblastoma [28]. In 2013, Fang et al. [20] found miR-199b-5p inhibited HER2 expression by direct targeting its 3′-untranslated region (3′UTR) in breast cancer cells, and miR-199b-5p might have the potential to be a novel important alternative therapeutic target for HER2-positive breast cancer; Liu et al. [21] identified miR-199b-5p as significantly down-regulated in cisplatin-resistant ovarian cancer cells and confirmed that miR-199b-5p may be clinically associated with advanced and poor survival ovarian cancers. In contrast, Li et al. [22] in 2012 performed qRT-PCR and observed the overexpression of miR-199b-5p in human colorectal cancers with brain metastases. Especially, the expression pattern of miR-199b-5p in osteosarcoma cells is still controversial. Won et al. [19] in 2012 reported that miR-199b-5p was upregulated in osteosarcoma tissues compared with normal controls, which was consistent with our findings in the current study. However, Lauvrak et al. [29] in 2013 indicated that the expression level of miR-199b-5p was downregulated in the highly aggressive osteosarcoma cell lines compared with non-aggressive cell lines. These differences might be caused by genetic variation or different pathological states between in vitro and in vivo systems. Moreover, we demonstrated that the expression level of miR-199b-5p in the patients with higher tumor grade, positive metastasis and positive recurrence was markedly higher than those with

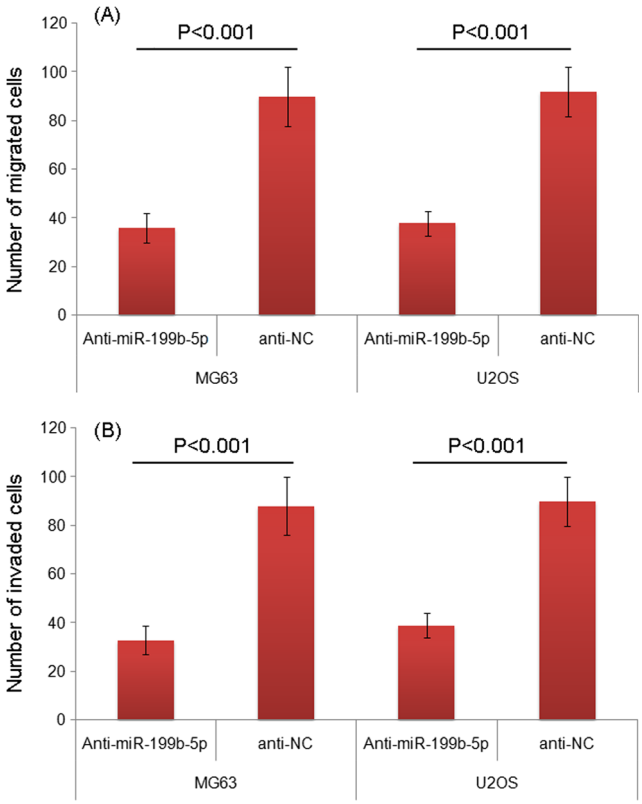

Fig. 4 Inhibition of miR-199b-5p suppresses the migration and invasion of human osteosarcoma cells. **a** and **b** Transfection with miR-199b-5p inhibitor could dramatically reduced the migration and invasion of both MG63 and U2OS cells

lower tumor grade, negative metastasis and negative recurrence. According to the survival analysis of 98 osteosarcoma patients, cases in the miR-199b-5p-high expression group showed both shorter OS and DFS. miR-199b-5p was an independent prognostic factor for both OS and DFS of osteosarcoma patients.

To explore the role of miR-199b-5p in human osteosarcoma cells, we knocked down this miRNA in MG63 and U2OS cells using miR-199b-5p inhibitor and assessed cellular proliferation, migration and invasion. Compared to the control group, highly efficient gene silencing was observed in both MG63 and U2OS cells after transfection with the miR-199b-5p inhibitor. Our data also showed that miR-199b-5p silencing correlated with the decreased proliferation, migration and invasiveness of both MG63 and U2OS cells, suggesting an oncogenic role of miR-199b-5p in osteosarcoma.

In conclusion, this study offer the convincing evidence for the first time that the increased expression of miR-199b-5p may play crucial roles in aggressive progression and poor prognosis of human osteosarcoma. miR-199b-5p may function as an oncogene by positively regulating the malignant potentials of this neoplasm. Further studies should be performed to identify potential target genes of miR-199b-5p in order to uncover the underlying molecular mechanisms of this miRNA in human osteosarcoma.

Conflict of Interest None.

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