

ORIGINAL ARTICLE

MiR-199b-5p promotes malignant progression of osteosarcoma by regulating HER2

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Summary

Purpose: MicroRNAs (miRs) are endogenous, noncoding small RNAs that play a key role in regulating biological and pathological processes. The oncogenic properties of miR-199b-5p have been demonstrated in previous studies but the effect of miR-199b-5p on osteosarcoma (OS) has not yet been clarified. This study aimed to investigate the effect of miR-199b-5p on OS and the relationship between this miR and the pathological parameters and prognosis of OS.

Methods: MiR-199b-5p expression in 57 pairs of OS tissues, corresponding adjacent normal tissues and OS cells was measured by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The relationship between miR-199b-5p and the pathological features and prognosis of OS patients was examined. We constructed small interfering (si) RNA to knock down miR-199b-5p expression in OS cell lines MG63 and U2OS. Cell Counting Kit-8 (CCK-8), cell cloning assay and Transwell cell migration and invasion assay were applied for investigating the biological function of miR-199b-5p, respectively. Finally, western blot was used for exploring its underlying mechanism.

Results: MiR-199b-5p expression in OS was significantly

higher than that of normal tissues. Compared to patients with low expression of miR-199b-5p, patients with high expression level tended to be with younger age, higher incidence of distant metastases and lower overall survival. Compared with interference sequence negative control (si-NC) group, the abilities of proliferation, invasion and metastasis of cells transfected with si-miR-199b-5p were significantly decreased. Western blot analysis indicated that expressions of key proteins related to epithelial to mesenchymal transition (EMT) signaling pathway, including N-cadherin, Vimentin, β -catenin and matrix metalloproteinase-9 (MMP9), were significantly decreased after transfection with si-miR-199b-5p. Furthermore, we found that miR-199b-5p promoted the progression of OS mainly through regulating HER2.

Conclusions: Upregulated miR-199b-5p is significantly related with stage, distant metastasis and poor prognosis of OS. This MiR may promote progression of OS through regulating HER2.

Key words: disease progression, HER2, MiR-199b-5p, osteosarcoma

Introduction

Osteosarcoma (OS), a primary malignant bone tumor, occurs frequently in childhood and adolescence. Of OS cases 80-90% affect the metaphyses of long bones, especially femur, tibia and humerus. The annual prevalence of OS was estimated of about 3/100,000, with male to female ratio 1.5 to 1 [1]. Hematogeneous metastases are common in OS at the early stage, while pulmonary metastases

are very frequently observed [1,2]. OS has become a severe health problem in China due to the increasing incidence every year. With the rapid development of molecular biology, gene diagnosis technology and other disciplines, it is believed that the malignant transformation of cells and irreversible genetic changes induced by long-term interaction of genetic and environmental factors may be

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the underlying pathogenesis of OS. This process mainly corresponds to changes in the activities of oncogenes and tumor suppressors, eventually leading to physiological dysfunctions of cells, such as proliferation, apoptosis, differentiation [1,3,4]. Although great achievements have been made, whether there are other genetic or epigenetic regulation mechanisms involved in this process still need to be further explored. It is of great significance to fully explore OS pathogenesis, so as to provide new directions for standard diagnosis and treatment [4-6].

In recent years, treatment regimens for OS have been attempted and reported in many clinical trials. European Osteosarcoma Intergroup (EOI) tried to adjust the combination of drugs and increase the dose intensity, but it turned out to be disappointing [7,8]. Studies have shown that no significantly prolonged survival rate was observed in those patients who were insensitive to chemotherapy or experienced metastasis after ifosfamide, topotecan and gemcitabine treatment [9,10]. More seriously, chemotherapy was limited by toxic effects of large dose and drug-resistance [11]. Therefore, new treatment protocols are urgently needed for improving the clinical efficacy. Finding out key genes or new immunotherapy targets from the genes which are aberrantly expressed in OS might help find new strategies for the treatment of OS. Taken together, clarifying the molecular mechanism of OS metastasis helps better prediction, diagnosis and prognosis of OS metastasis.

MicroRNAs (miRNAs) exist in eukaryotic organisms with 18-25 nucleotides in length, which help to target mRNA cleavage, inhibitory translation and post-transcriptional regulation of target genes by pairing to target mRNA. MiRNAs regulate their target genes by binding to imperfect complementary sites of their mRNA-targets [12,13]. Hence aberrant expression of miRNAs result in erroneous protein expression [13]. Previous studies proved that unexpected distribution of miRNAs was associated with malignant tumor, indicating miRNAs as tumor markers [14-16]. Different tumors have distinctive patterns of miRNA expression since miRNAs are tissue-specific [15]. MiRNAs exert their pro-oncogenic properties through promoting proliferation, invasion and metastasis of tumor cells [17]. Differentially expressed miRNAs in many tumor tissues, such as OS, breast cancer, etc. provide further evidence that miRNAs are closely related with tumors [18-21].

In general, mRNA plays a key role in tumor cells through various regulations, such as chromosome recombination, gene imprinting, epigenetic regulation, nucleocytoplasmic transport, mRNA

splicing and translation [16,22]. However, the precise molecular mechanisms by which miRNAs may contribute to tumor development are incompletely understood. Relative studies have pointed out that miRNA-300 and miRNA-20a could promote OS, whereas miRNA-138 and miRNA-497 could inhibit OS development. Multiple signaling pathways have been reported in miRNA regulation, including WNT pathway, EGFR pathway and EMT [23]. However, few studies have explored the role of miRNAs in OS.

We herein detected miR-199b-5p expression in 57 pairs of OS tissues and corresponding normal tissues and analyzed the biological effect of miR-199b-5p on OS. Related studies pointed out that miR-199b-5p participates in tumor development by inhibiting proliferation and metastasis of tumor cells. The present study was to investigate the mechanism underlying the pro-oncogenic property of miR-199b-5p and the specific effect of miR-199b-5p on pathologic parameters and prognosis of OS.

Methods

Patients and OS samples

Tumor and normal adjacent tissues were collected by surgical resection from 57 OS patients without pre-operative chemotherapy and radiotherapy. All patients were classified according to the 8th version of UICC/AJCC osteosarcoma TNM classification criteria. This study was approved by the Ethics Committee of Changzhou No.2 People's Hospital Affiliated to Nanjing Medical University. All patients and/or their families provided signed informed consent.

Cell lines and reagents

Six human-derived OS cell lines (SOSP-9607, U2OS, MG63, HOS, 143B, SaOS-2) and one normal human osteoblast cell line (hFOB) used in the study were purchased from ATCC company (Manassas, VA, USA). Duplecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from American Life Technologies Company (Gaithersburg, MD, USA). Cells were cultured in DMEM containing 10% FBS and incubated in 5% CO₂ incubator at 37°C.

Transfection

The negative control (si-RNA) and the siRNA containing the miR-199b-5p interference sequence (si-miR-199b-5p) were constructed from Shanghai GenePharma Company (Shanghai, China). The cells were seeded into 6-well plates and transfected with siRNA using Lipofectamine 2000 when cell confluence reached 70%. After cell transfection for 48 hrs, cells were collected for qRT-PCR analysis and cell function experiments.

Cell proliferation assay

After transfection for 48hrs, the cells were seeded into the 96-well plates with 2000 cells per well. Ten μ L

of Cell Counting Kit-8 (CCK-8) solution were added in each well after culturing for 6, 24, 48 and 72 hrs, respectively. The optical density (OD) of each well was analyzed with microplate reader at 450 nm.

Colony formation assay

Cells were collected and seeded in a 6-well plate with 200 cells per well. Cells were cultured in complete medium for 2 weeks, and fresh medium was replaced once in the first week and twice in the second week. After 2 weeks, the colonies were formed and washed twice with phosphate buffered saline (PBS). The cells were fixed for 20 min by 2mL of methanol. After washing with PBS, colonies were fixed with 2mL methanol for another 20 min. Then the methanol was removed, and the samples were washed again and stained with 0.1% crystal violet for 20 min, followed by detection of colony formation.

Transwell cell migration and invasion assay

After transfection for 48 hrs, cell suspension at a density of $2.0 \times 10^5/\text{mL}$ was prepared by trypsin digestion and resuspension in serum-free medium. Transwell chambers were firstly placed into 24-well plates. Cell suspension and 500 μL DMEM containing 10% FBS were added into the upper and lower chamber. After cell culture for 48 hrs, chambers were washed with PBS for removing the inner cells. Cells stained on the out layer of basement membrane were observed under microscope and counted in 5 random fields.

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

Total RNA extracted from OS cell lines and tissues by TRIzol reagent was reverse-transcribed into complementary DNA (cDNA) using Prime-script reverse Reagent. qRT-PCR reactions were performed and the primers used in this experiment were as follows: MiR-199b-5p: 5'-CCCAGTGTTTA-GACTATCTGTT-3'; U6: 5'-TGCGGGTGCTCGCTTCG-CAGC-3'; HER2: forward: 5'-CCAGCCTTCGACAACCTC-TATT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'; β -actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'. The ABI Step One software and $2^{-\Delta\Delta\text{Ct}}$ were used for analyzing and calculating the relative expression level of mRNA.

Western blot

The cell lysates prepared by lysis buffer were gently shaken for 30 min and centrifuged at $14000 \times g$ at 4°C for 15 min. Thereafter, the concentration of extracted total protein was calculated by bicinchoninic acid (BCA) protein assay kit. Total protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred it to polyvinylidene difluoride membrane according to the standard procedure. Primary antibodies used in this study were N-cadherin, Vimentin, β -catenin and MMP9 and GAPDH. The corresponding secondary antibodies were anti-mouse and anti-rabbit antibodies (Cell Signaling Technology, Danvers, MA, USA).

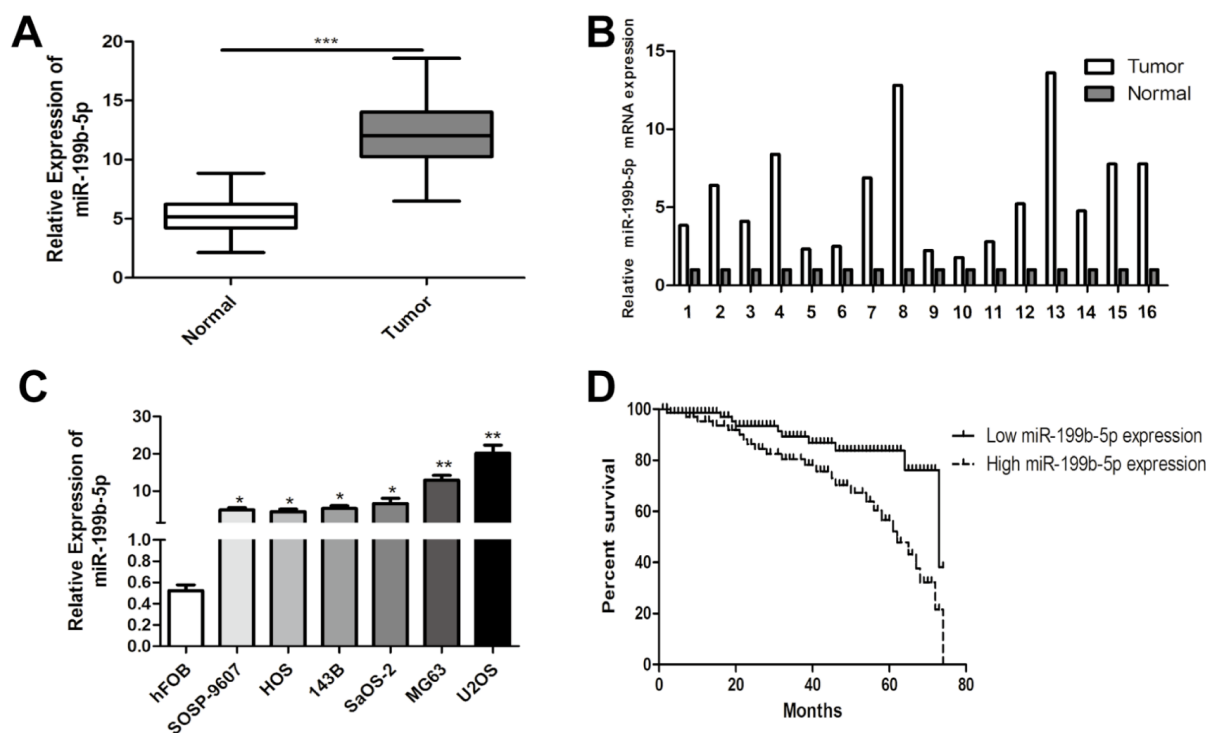


Figure 1. A,B: The expression of miR-199b-5p in 57 pairs of OS tissue was significantly increased. C: Expression levels of miR-199b-5p in 6 OS cell lines (HOS, U2OS, SOSP-9607, MG63, 143B, SaOS-2) and normal skeletogenous cell (hFOB). D: Kaplan-Meier survival curves of patients with OS based on miR-199b-5p expression. Patients in the high expression group had a significantly more unfavorable prognosis than those in low expression group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Statistics

All statistical analyses were conducted using SPSS 22.0 software (Armonk, NY, USA). Quantitative data was expressed as mean \pm standard deviation. Independent-sample t-test was used to compare the differences between two groups and χ^2 test and the Fisher analysis were used for categorical variables. Kaplan-Meier method was performed for evaluating the prognosis and survival and log-rank test was used for comparing the difference between curves. $P < 0.05$ was considered statistically significant.

Results

MiR-199b-5p was overexpressed in OS tissues and cell lines

Compared to the adjacent normal tissues, miR-199b-5p expression in the OS group was significantly increased, and the difference was statistically significant (Figure 1A and 1B). Compared to the normal bone cell line (hFOB), miR-199b-5p expression in OS cells was significantly elevated (Figure 1C). Besides, MG63 and U2OS cells showed the highest expression level of miR-199b-5p, which were selected for the following experiments.

MiR-199b-5p expression was correlated with clinical stage, distant metastasis and overall survival in OS patients

According to miR-199b-5p expression obtained from qRT-PCR, 57 pairs of tissues were divided into high-expression group and low-expression group, and the number of cases of each group was counted.

Table 1. Association of miR-199b-5p expression with clinicopathological characteristics of osteosarcoma

Characteristics	Number of cases	miR-199b-5p expression	
		Low (%)	High (%)
Age (years)			
<21	25	9	16
≥ 21	32	21	11
Gender			
Male	37	18	19
Female	20	9	11
Enneking stage			
IA	7	4	3
IIA	14	5	9
IIB	28	9	19
III	8	3	5
Distant metastasis			
No	32	19	13
Yes	25	8	17

The association of miR-199b-5p expression with age, sex, clinical staging and distant metastasis of OS patients was analyzed by χ^2 test. Our data suggested that miR-199b-5p was positively related to younger age and distant metastasis, but not related to age, sex and Enneking staging of OS patients (Table 1). In addition, in order to explore the relationship between miR-199b-5p expression and the prognosis of OS patients, we collected relevant follow-up data. Kaplan-Meier survival curve showed that miR-199b-5p expression was positively correlated with poor prognosis of OS ($p < 0.05$; Figure 1D). These results indicated that miR-199b-5p may serve as a biological indicator for the prognosis of OS.

Knockdown of miR-199b-5p inhibited cell proliferation

We successfully constructed si-miR-199b-5p and the transfection efficacy was verified by qRT-PCR (Figure 2A and 2B). Proliferation of cells transfected with si-NC or si-miR-199b-5p was then detected by CCK-8 assay. The results showed that cell proliferation rate was significantly decreased after miR-199b-5p knockdown (Figure 2C and 2D). Cell cloning experiment obtained the same results (Figure 2E and 2F).

Knockdown of miR-199b-5p inhibited cell migration and invasion

In MG63 cell line, the number of transmembrane OS cells in membrane chamber decreased significantly after miR-199b-5p knockdown compared with that of the control group (Figure 3A and 3B), revealing the inhibited migration and invasion ability. In the U2OS cell line, the experimental results were in accordance with those of MG63 cells (Figure 3C and 3D).

Knockdown of miR-199b-5p changed the expression of EMT signaling pathway

In order to analyze the potential mechanism underlying how miR-199b-5p promotes cell proliferation, invasion and migration, we detected the expression of N-cadherin, Vimentin, β -catenin and MMP9 in the EMT pathway after miR-199b-5p knockdown. The data showed that the expression levels of the above proteins were significantly changed after the miR-199b-5p knockdown (Figure 4).

HER2 modulated miR-199b-5p expression in human osteosarcoma cells

In order to further explore the pathway by which miR-199b-5p promotes the malignant progression of OS, we noted possible association of

HER2 with miR-199b-5p by bioinformatics analysis. qRT-PCR and western blot were used to estimate the mRNA and protein expressions of HER2 in 57 pairs of tumor and corresponding normal tissues as well as in OS cells lines, respectively. Compared with normal tissues, the protein expressions of HER2 and related genes were significantly

decreased (Figure 5A). The expression of HER2 was found higher in OS cell lines than in FOB, and the difference between the two groups was statistically significant (Figure 5B).

In addition, in order to further explore the interaction between miR-199b-5p and HER2 in OS cells, we found that HER2 increased significantly

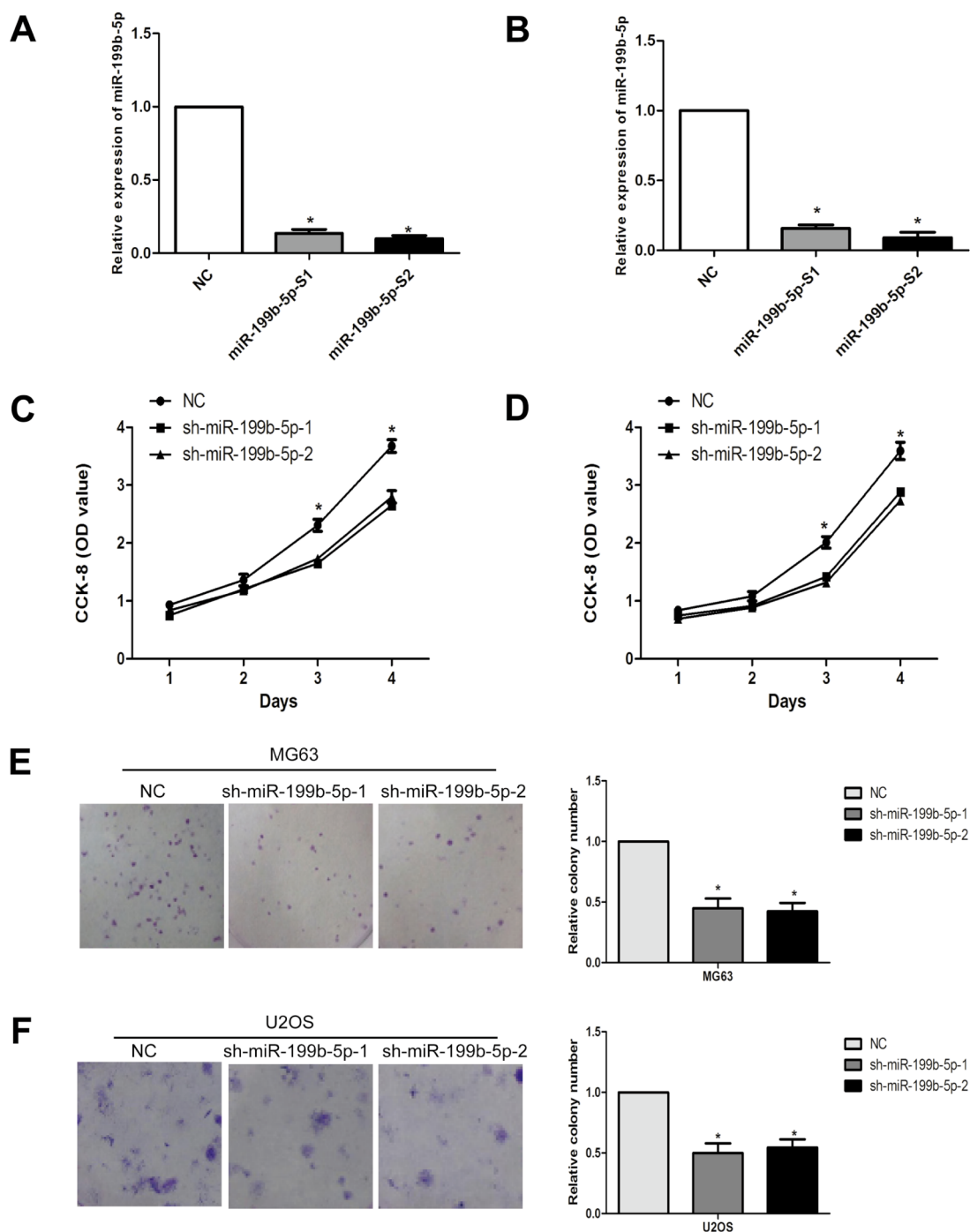


Figure 2. A,B: qRT-PCR was used to verify the efficiency of miR-199b-5p knockdown in MG63 and U2OS cell lines. The miR-199b-5p expression in OS cells transfected with si-miR-199b-5p was significantly decreased. C,D: Growth curve analysis showing the growth of MG63 and U2OS cells with miR-199b-5p knockdown. The results showed that cell proliferation rate was significantly decreased after miR-199b-5p knockdown. E,F: The efficiencies of cell colony formation in MG63 and U2OS cells with miR-199b-5p knockdown. The results showed that the cell proliferation rate was significantly decreased after miR-199b-5p knockdown. * $p < 0.05$.

after miR-199b-5p knockdown in OS cells. Therefore, we selected 16 pairs of samples from the 57 pairs of OS tissues, and evaluated the expressions of miR-199b-5p and HER2 by qRT-PCR. The results showed that miR-199b-5p and HER2 were negatively correlated both in MG63 and U2OS cell lines (Figure 5C). HER2 was downregulated in OS cells compared to FOB cells (Figure 6), and the difference was statistically significant. At the cellular level, we constructed HER2 siRNA, and the transfection

efficiency of HER2 was verified by Western blot. Invasion and metastasis of OS cells transfected with si-miR-199b-5p were decreased after HER2 knockdown.

Discussion

OS is a highly malignant and metastatic primary bone tumor with high mortality, derives from osteoblasts, and is most frequent in adolescence

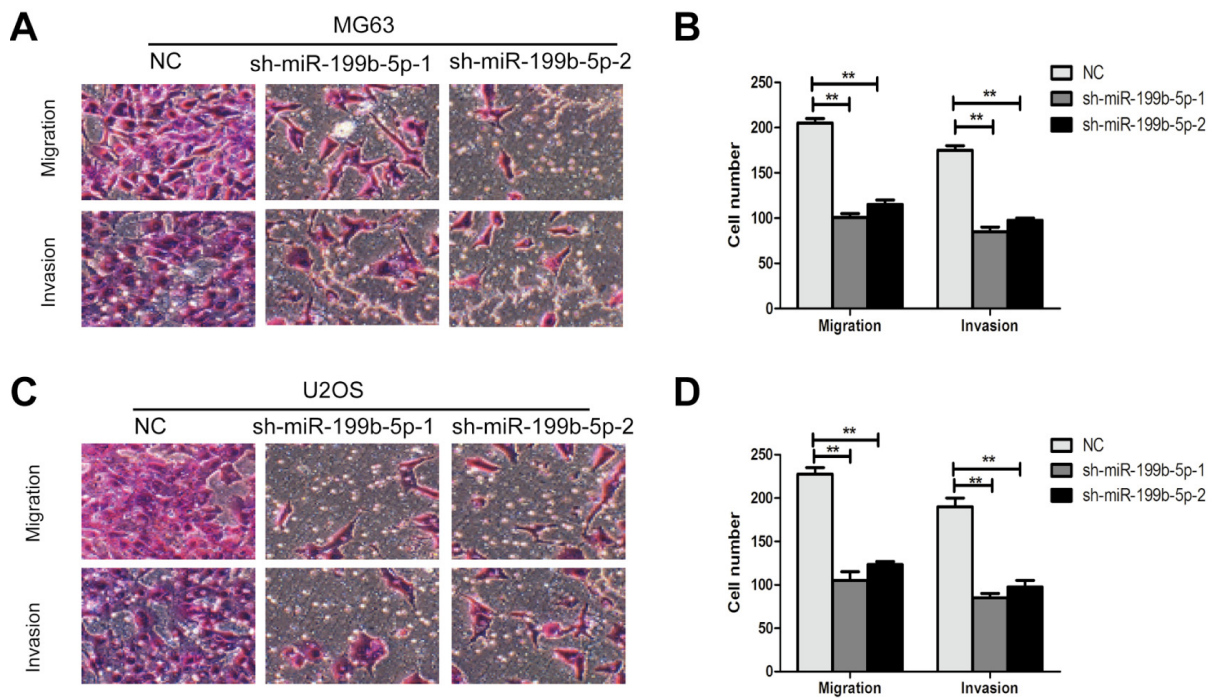


Figure 3. A,B: MG63 cell transfected with si-miR-199b-5p displayed significantly lower migration and invasion capacity. C,D: U2OS cell transfected with si-miR-199b-5p displayed significantly lower migration and invasion capacity. ** $p < 0.01$.

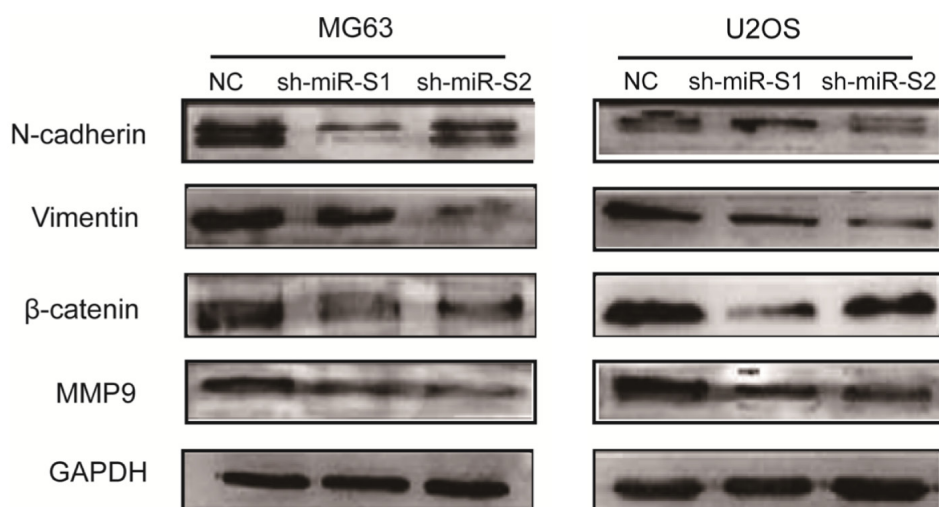


Figure 4. Western Blot Analysis. Knockdown of miR-199b-5p expression significantly decreased the expression of EMT signaling pathway, including N-cadherin, Vimentin, β-catenin and MMP9. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

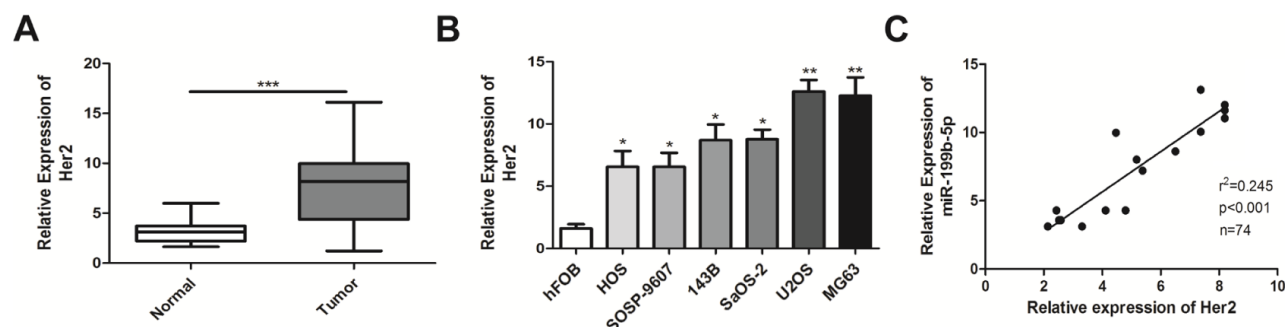


Figure 5. A,B: The mRNA expression level of HER2 relative to GAPDH in human OS tissues and corresponding adjacent tissues, and cell lines were detected by using qRT-PCR. Compared with normal tissues, HER2 protein expressions and related genes were significantly decreased. The expression of HER2 was significantly higher in OS cell lines than that in HFOB. **C:** A significantly negative correlation was found between miR-199b-5p and HER2 in tumor samples. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

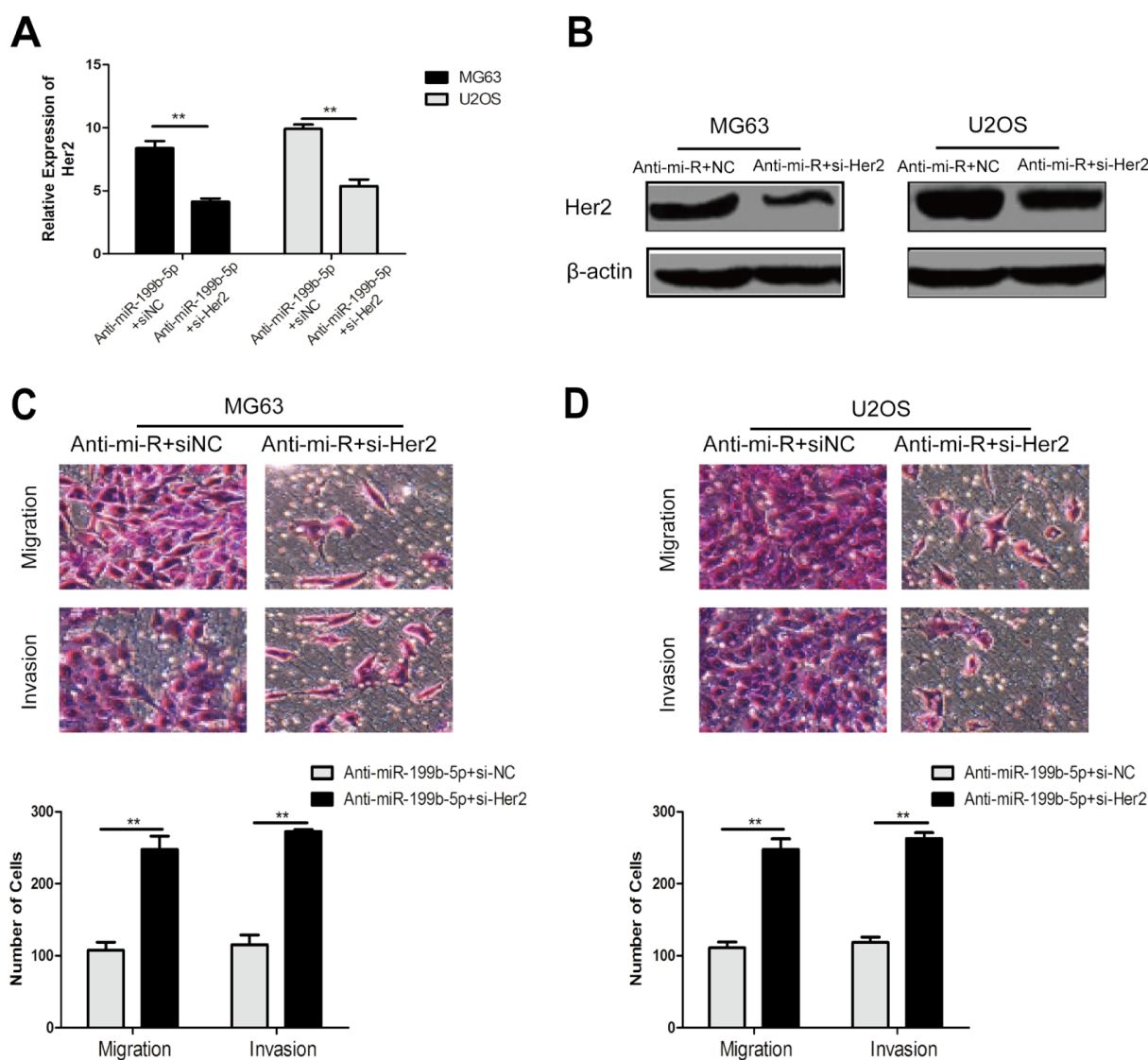


Figure 6. A: The expression of HER2 was verified by qRT-PCR in co-transfected cell lines. The mRNA expression of HER2 in OS cells transfected with si-miR-199b-5p was decreased after HER2 knockdown. **B:** Western blot was used to verify the expression of HER2. The protein expression of HER2 in OS cells transfected with si-miR-199b-5p was decreased after HER2 knockdown. **C,D:** The roles of miR-199b-5p and HER2 in the regulation of OS cell migration and invasion were examined by Transwell assay. Invasion and metastasis of OS cells transfected with si-miR-199b-5p were decreased after HER2 knockdown. A representative data set is displayed as mean \pm SD values. ** $p<0.01$.

[1]. Changes in the molecular genetics of OS cells, such as the changes in the number of gene copies and the interruption of the coding sequence, are known to greatly affect tumor phenotype [24]. For this reason, a variety of tumor signaling pathways rapidly emerged as a therapeutic target for drug discovery. The prevalence and mortality of OS tend to be increased gradually in China. Clinical analysis of OS showed moderate- or late-stage OS present a lower diagnostic rate [3,6]. Biomarkers for early identification and prognosis definition should be further explored because of its important clinical implications for prompt and standard treatment of OS [1,25].

Genetics, diet, lifestyle and precancerous lesions are closely related with the pathogenesis of OS. Half of OS patients with micro-metastases may experience postoperative recurrence or metastasis [26,27]. Based on this fact, studies on early diagnosis, metastasis, recurrence and adjuvant therapies for postoperative aggressive OS are currently being investigated [4]. Previous studies documented that miRNAs contributed importantly to many diseases, including malignancies. Unexpected distribution of miRNAs in OS also contributed to the diagnosis, treatment and prognosis [3,5]. Therefore, revealing the correlation between abnormal expression of miRNAs and clinical prognosis will help improve the diagnosis, treatment and prognosis of OS patients.

In recent years, many molecular targets regulated by miRNAs have been revealed, increasing evidence elucidates the important role of miRNAs in the biological behavior of OS and the promising future applications of miRNAs in OS therapy [5]. As a small single-stranded non coding RNA of about 22 nucleotides in length, miRNA regulates the transcription of downstream genes through targeted degradation or inhibiting the translation of target mRNA by the combination with 3'UTR of mRNAs [12]. Lots of studies have demonstrated that miRNAs not only influence the proliferation, apoptosis, chemotherapy sensitivity and tumor metastasis, but also define the phenotype of tumor stem cells [18]. For the reasons above, revealing the role and mechanism of miRNAs is helpful to understand the complex molecular mechanism of OS, which could provide target for the development of new anti-OS drugs [24].

This study explored the clinical characteristics of miR-199b-5p in OS, as well as its expression and the role in the progression of this malignancy. Firstly, we validated miR-199b-5p expression in 57 pairs of OS tissues and adjacent tissues. The results showed that miR-199b-5p was upregulated and positively correlated with staging, lymph node

metastasis, distant metastasis and poor prognosis, suggesting its oncogenic potential. In order to further explore the effect of miR-199b-5p on the biological function of OS, we constructed si-miR-199b-5p and CCK-8 cell cloning. Transwell experiments showed that miR-199b-5p promoted the development of OS. However, its specific molecular mechanism on how miR-199b-5p regulates OS remains unclear.

EMT signaling pathway is an important tumor-related signaling pathway [28]. The concept of dynamic transitioning between epithelial to mesenchymal tumor cells has excited particular concern [29]. EMT was demonstrated to play vital role in primary invasion and secondary metastasis in liver cancer, breast cancer, lung cancer etc [30,31]. Therefore, the study of the mechanism on the occurrence and regulation of EMT is of great significance for the treatment of malignant tumors, especially on the metastasis of tumor cells [29], which is expected to promote cancer treatment. In this experiment, we found that the expressions of key proteins in EMT pathway, including N-cadherin, Vimentin, β -catenin and MMP-9 decreased significantly after knockdown of miR-199b-5p, indicating that this miR could promote invasion and metastasis through the EMT signaling pathway in OS.

Epithelial growth factor receptor (EGFR) is mainly expressed on the surface of epithelial cells and belongs to the tyrosine kinase receptor family, including HER1, HER2, HER3 and HER4 [32]. EGFR pathway is important to tumors, while HER2 seems to be tightly associated with cell differentiation, migration and apoptosis. Basic research showed that overexpression of HER2 can promote metastasis of tumor cells by regulating the expressions of adhesion molecules such as cadherin, which also initiates a variety of metastatic mechanisms to accelerate tumor cell metastasis [34]. In this experiment, we found that there were counter-regulatory signals between miR-199b-5p and HER2 through the rescue experiment. HER2 was found to be involved in the occurrence and development of tumors, which could be utilized for better improving tumor treatments. In order to prove whether miR-199b-5p promotes the development of OS through the EMT signaling pathway, we assessed the protein expressions of N-cadherin, Vimentin, β -catenin and MMP-9 after miR-199b-5p knockdown, which suggested that miR-199b-5p promotes the invasion and metastasis of OS through the EMT signaling pathway. In addition, HER2 expression was significantly changed with miR-199b-5p knockdown, indicating that miR-199b-5p might promote the progression of OS through HER2.

Conclusions

In summary, overexpressed miR-199b-5p in OS was significantly correlated with stage, distant metastasis and poor prognosis of OS patients. Moreo-

ver, miR-199b-5p may promote OS progression by regulating HER2.

Conflict of interests

The authors declare no conflict of interests.

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