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MicroRNA-449b-5p Suppresses Proliferation, Migration, and Invasion of Osteosarcoma by Targeting c-Met

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to clarify the biological function of microRNA-449b-5p in the progression of osteosarcoma (OS) and to define the underlying mechanism.





Material/Methods: Relative levels of microRNA-449b-5p in OS tissues and cell lines was determined by quantitative real-time polymerase chain reaction (qRT-PCR). The correlation between microRNA-449b-5p level and pathological characteristics of OS patients was analyzed by chi-square test. Kaplan-Meier analysis was used for survival analysis of OS patients based on their expression level of microRNA-449b-5p. Regulatory effects of microRNA-449b-5p on cellular behaviors of OS cells were evaluated by cell counting kit-8 (CCK-8) and Transwell assay. The binding relationship between microRNA-449b-5p and c-Met was verified through dual-luciferase reporter gene assay, and their interaction in OS progression was further examined through a series of rescue experiments.

Results: MicroRNA-449b-5p was expressed at low levels in OS. Lower levels of microRNA-449b-5p were seen in OS tissues with worse tumor grade or histological differentiation. OS patients with low levels of microRNA-449b-5p had worse overall survival relative to those with high level of microRNA-449b-5p. Overexpression of microRNA-449b-5p markedly attenuated proliferative, migratory, and invasive abilities of OS cells. C-Met is the downstream target of microRNA-449b-5p, and its level was inhibited in OS cells overexpressing microRNA-449b-5p. Importantly, c-Met partially rescued the inhibitory effects of microRNA-449b-5p on behavior of OS cells.

Conclusions: MicroRNA-449b-5p is downregulated in OS, which alleviates the malignant progression of OS by targeting c-Met.

MeSH Keywords: **Cell Proliferation • MicroRNAs • Osteosarcoma**

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Background

Osteosarcoma (OS) is a highly malignant primary bone tumor that mainly involves the long bones. It accounts for 2.4% of all adolescent malignancies [1,2]. Biological characteristics of OS include rapid growth, strong invasiveness, and high rate of distant metastasis. The prognosis of OS patients is poor, with high mortality and disability rates [1]. It is reported that the 5-year survival of OS is as low as to 30–75%. About 80% of OS patients develop metastases in the lungs or other organs [3,4]. Although great strides have been made in surgical resection, tumor prosthesis replacement, chemotherapy, and radiotherapy, clinical outcomes of OS patients are unsatisfactory [5,6]. Therefore, it is of great importance to discover the pathogenic mechanism of OS.

MicroRNAs (miRNAs) are a class of non-coding, small RNAs that bind to the 3'UTR of their target mRNAs. They are capable of degrading target mRNAs or inhibiting their translation process [7]. miRNAs participate in diverse biological processes [8,9]. Dysregulated miRNAs are reported to be closely related to tumor progression [10–14]. Hence, tumor-related miRNAs could be utilized as diagnostic and therapeutic targets for tumors. Several OS-related miRNAs have been identified. Zhu et al. [15] found that miR-183 downregulates the expression of Ezrin to inhibit the invasive and migratory capacities of OS cells. Novello et al. [16] found that miRNA-34a suppresses the metastasis of OS through directly targeting p53 and c-Met. Cao et al. [17] reported that miR-802 is upregulated in OS and serves as an oncogene to promote cell proliferation by regulating p27. MicroRNA-449b-5p is involved in many types of tumors [18–20]. However, the specific role of microRNA-449b-5p in OS is unclear.

To provide a novel target for clinical treatment of OS, we assessed microRNA-449b-5p levels in OS tissues and cell lines and investigated the potential role of microRNA-449b-5p in the progression of OS.

Material and Methods

OS patients

Tumor tissues and paracancerous tissues were surgically resected from 60 OS patients treated in Weifang People's Hospital from December 2016 to February 2018. They did not receive preoperative anti-tumor therapy and were pathologically diagnosed. All subjects volunteered to participate in the study and signed written informed consent. This study was approved by the Ethics Committee of Weifang People's Hospital (22 Nov 2016).

Cell culture and transfection

Osteoblasts (hFOB1.19) and OS cell lines (Saos2, HOS, U2OS, and MG63) were provided by Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 µg/mL penicillin, and 0.1 mg/mL streptomycin, in a 37°C, 5% CO₂ incubator.

Cells were inoculated in a 6-well plate and cultured until 80% confluence. Transfection of 50 nmol/L microRNA-449b-5p mimics or NC was conducted using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Cell counting kit-8 (CCK-8)

Cells were inoculated in a 96-well plate (2×10³ cells/well). Absorbance (A) at 450 nm was recorded at predetermined time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) to produce a viability curve.

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

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract cellular RNAs, and they were reversely transcribed into complementary deoxyribose nucleic acid (cDNA). Subsequently, PCR was conducted using the SYBR Green method. Primer sequences were:

MicroRNA-449b-5p: F: 5'-GGGAGGCAGTGTATTGTTA-3',
R: 5'-CAGTGGTGTGCTGGAGT-3';
C-Met: F: 3'-GAGGCAGTGCAGCATGTAGT-5',
R: 3'-GATGATCCCTCGGTCAGAA-5'.

Transwell assay

Diluted Matrigel was used to pre-coat the Transwell chamber overnight at 4°C. Cell density was adjusted to 2×10⁵/mL in serum-free medium. We added 500 µL of medium containing 10% FBS into the basolateral chamber of the 24-well plate. We seeded 200 µL of cell suspension into the apical chamber, and 24 h later, cells were fixed in methanol for 30 min and stained with 0.1% crystal violet for another 30 min. Under an inverted microscope, invasive cells were photographed. Migration assay was conducted following the same procedures except for Matrigel pre-coating (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis

Using radioimmunoprecipitation assay (RIPA), cellular protein was isolated and its concentration was measured by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Electrophoresis was performed and protein was transferred

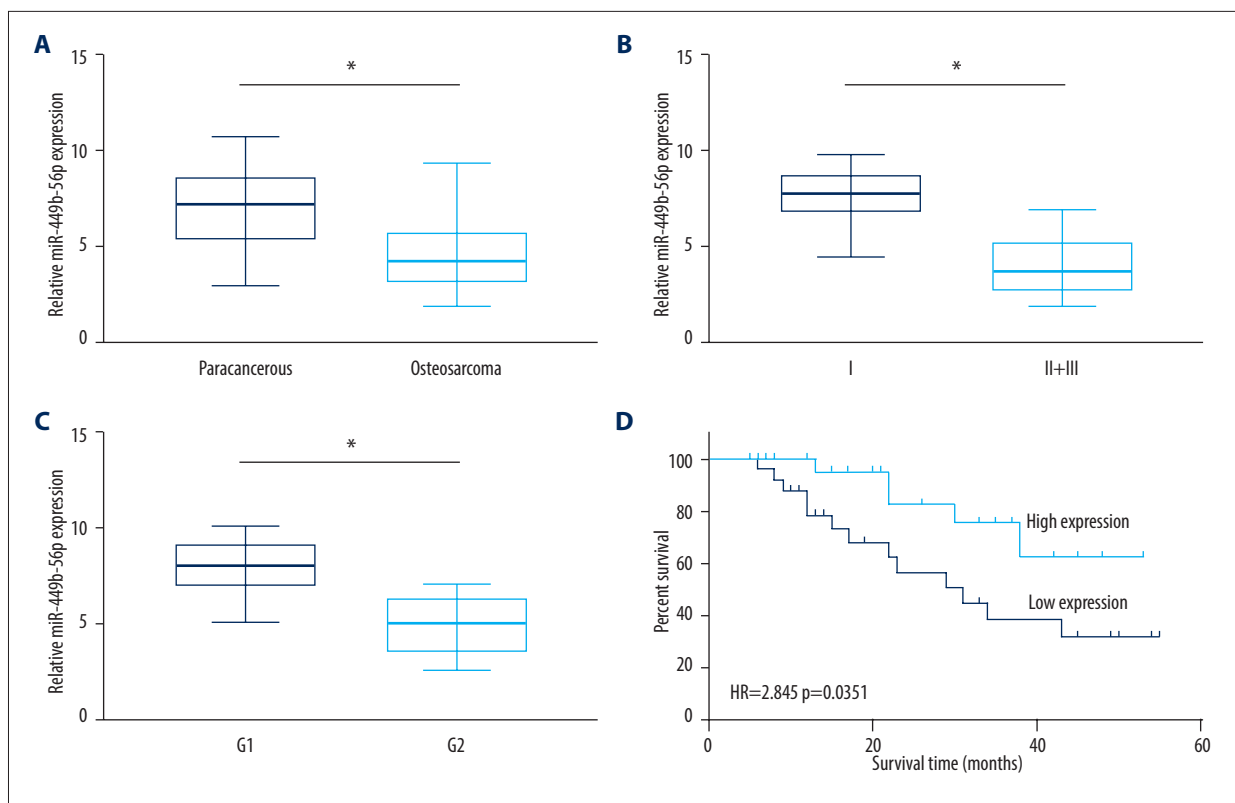


Figure 1. Downregulated miR-449b-5p in OS. (A) Relative levels of miR-449b-5p in OS tissues and paracancerous tissues. (B) Relative levels of miR-449b-5p in OS patients in stage II+III and stage I. (C) Relative levels of miR-449b-5p in OS patients in G1 and G2. (D) Kaplan-Meier curves showing overall survival of OS patients with high and low levels of miR-449b-5p.

on a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After 2-h non-specific antigen blockage in PBS containing 5% skim milk, membranes were reacted with primary and secondary antibodies. Electrochemiluminescence (ECL) was conducted to expose bands, followed by grey-value analysis using Image J Software (NIH, Bethesda, MD, USA).

Dual-luciferase reporter gene assay

Cells seeded into a 24-well plate with 2×10^5 cells per well co-transfected with WT c-Met 3'UTR/MUT c-Met 3'UTR and microRNA-449b-5p mimic/NC. Cells were lysed 48 h later to assess relative luciferase activity (Promega, Madison, WI, USA).

Statistical analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analyses. Data are expressed as mean \pm standard deviation. Differences between 2 groups were analyzed by the *t* test. The chi-square test was performed to assess the correlation between microRNA-449b-5p levels and pathological indexes of OS patients. Survival analysis was carried out using Kaplan-Meier method. $P < 0.05$ was set as the level of statistical significance.

Results

Downregulated microRNA-449b-5p in OS

Compared with paracancerous tissues, microRNA-449b-5p was downregulated in OS tissues (Figure 1A). Furthermore, microRNA-449b-5p levels remained lower in OS patients in stage II+III relative to those in stage I (Figure 1B). Based on the histological differentiation stage, OS patients in G1 presented higher levels of microRNA-449b-5p relative to those in G2 (Figure 1C). Compared with OS patients with high levels of microRNA-449b-5p, those had low levels of microRNA-449b-5p had worse prognosis (Figure 1D). Through analyzing the follow-up data of OS patients, microRNA-449b-5p level was identified to be correlated to tumor grade and histological differentiation of OS patients (Table 1). These results indicate that microRNA-449b-5p may be a prognostic hallmark of OS.

Overexpression of microRNA-449b-5p suppressed the ability of OS cells to proliferate, migrate, and invade

Identical to the expression pattern of microRNA-449b-5p in OS tissues, it was expressed at lower levels in OS cell lines than in osteoblasts (Figure 2A). U2OS and MG63 cells with a

Table 1. Correlation between miR-449b-5p level and pathological indexes of osteosarcoma patients (n=60).

Clinicopathologic features	Number of cases	miR-449b-5p expression		P-value
		Low (n=30)	High (n=30)	
Age (years)				
≤24	27	14	13	0.795
>24	33	16	17	
Gender				
Male	26	11	15	0.297
Female	34	19	15	
Tumor size				
≤5 CM	25	11	14	0.432
>5 CM	35	19	16	
Clinical stage				
I	20	6	14	0.028*
II+III	40	24	16	
Histological differentiation				
G1	23	6	17	0.003*
G2	37	24	13	

relatively low expression of microRNA-449b-5p were selected for the following experiments. The transfection efficacy of microRNA-449b-5p mimic was verified in OS cells (Figure 2B). CCK-8 assay revealed inhibited viability in OS cells overexpressing microRNA-449b-5p (Figure 2C). Moreover, migratory and invasive abilities were attenuated after transfection of microRNA-449b-5p mimic in U2OS and MG63 cells (Figure 2D).

C-Met is the downstream target of microRNA-449b-5p

Through TargetScan prediction, potential binding sequences of c-Met to microRNA-449b-5p were identified (Figure 3A). Luciferase activity was markedly decreased in cells co-transfected with microRNA-449b-5p mimic and WT c-Met 3'UTR, suggesting the binding relationship between microRNA-449b-5p and c-Met (Figure 3B). After transfection of microRNA-449b-5p mimic in U2OS and MG63 cells, the protein level of c-Met was markedly downregulated (Figure 3C, 3D). A negative correlation was observed between expression levels of microRNA-449b-5p and c-Met in OS, further confirming that microRNA-449b-5p can bind to c-Met and negatively regulate its level. By detecting c-Met levels in OS tissues, we found they were higher in OS tissues than in paracancerous tissues (Figure 3E, 3F).

By targeting c-Met, microRNA-449b-5p suppressed the ability of OS cells to proliferate, migrate, and invade

To clarify the involvement of microRNA-449b-5p/c-Met in the progression of OS, rescue experiments were performed. Transfection of microRNA-449b-5p mimic in OS cells markedly downregulated c-Met levels, which was reversed by overexpression of c-Met (Figure 4A, 4B). Overexpression of microRNA-449b-5p inhibited the viability of OS cells, but was reversed by transfection of pcDNA-c-Met (Figure 4C). Similarly, the inhibited migratory and invasive abilities of OS cells overexpressing microRNA-449b-5p were reversed by overexpression of c-Met (Figure 4D). These results suggest that microRNA-449b-5p suppresses the cellular behaviors of OS cells by downregulating c-Met.

Discussion

OS is a malignant tumor that has the potential of unlimited proliferation and evading growth suppressors [21]. OS is prone to metastasis at an early stage and is highly invasive. Prevention and control of proliferation and metastasis of OS are important aspects of OS research. This study identified that microRNA-449b-5p was downregulated in OS. Low levels of microRNA-449b-5p were closely related to advanced tumor stage, histological differentiation, and poor overall survival. Our data suggest the potential of microRNA-449b-5p as a therapeutic target for OS.

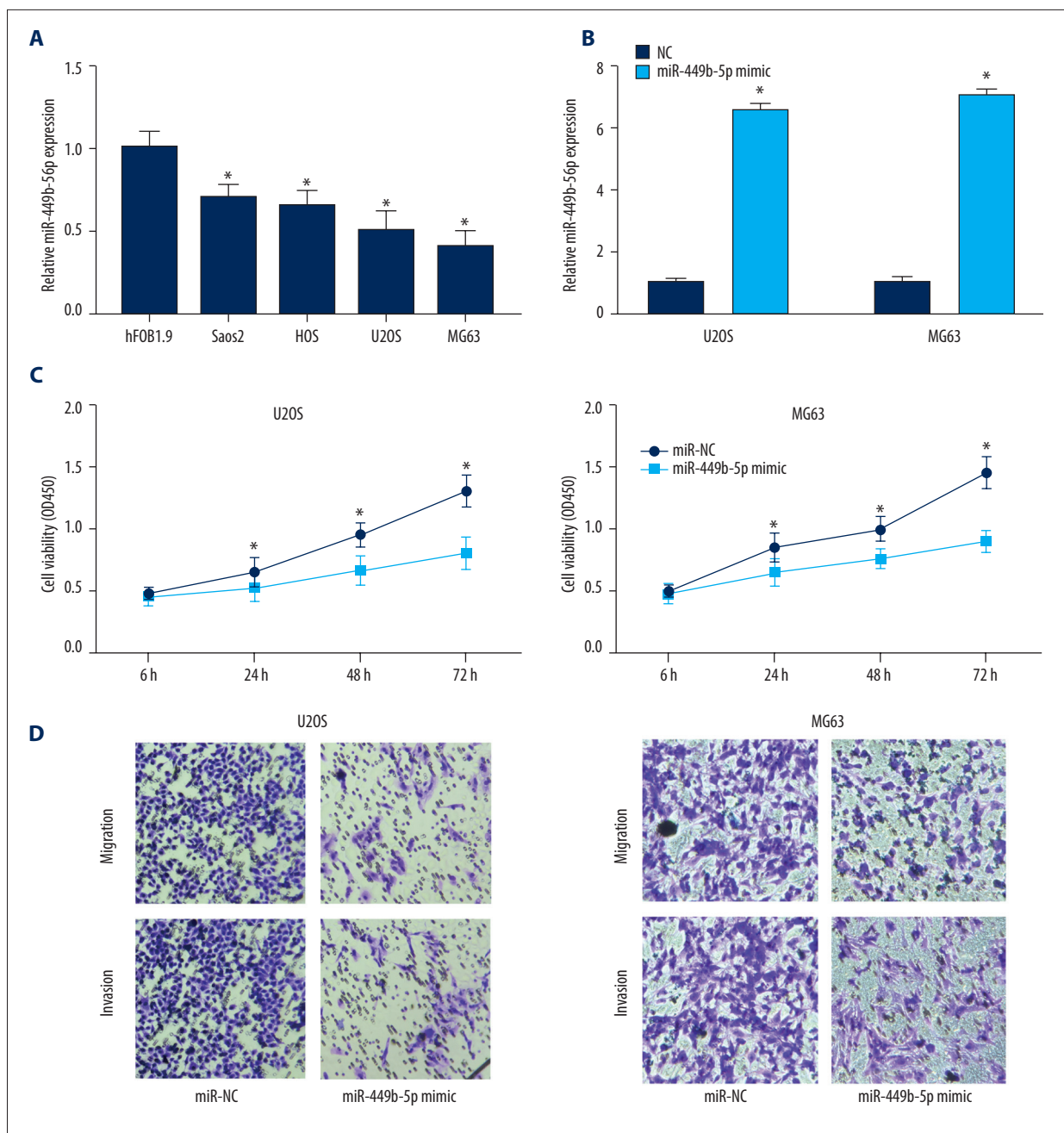


Figure 2. Overexpression of miR-449b-5p suppressed ability of OS cells to proliferate, migrate, and invade. **(A)** Relative levels of miR-449b-5p in osteoblasts and OS cell lines. **(B)** Transfection efficacy of miR-449b-5p mimic in U2OS and MG63 cells. **(C)** CCK-8 assay showed viability in U2OS and MG63 cells transfected with NC or miR-449b-5p mimic. **(D)** Transwell assay showing migration and invasion abilities in U2OS and MG63 cells transfected with NC or miR-449b-5p mimic.

miRNAs are single-stranded, small-molecule RNAs that accounts for about 1% of the human genome. miRNAs are highly conserved and participate in diverse cellular activities [22,23]. The regulatory effects of miRNAs on tumors, serving as oncogenes or tumor suppressors, have been well documented [24]. A growing number of studies have demonstrated the crucial involvement of miRNAs in the progression of OS. Xie et al. [25] found that miR-876-5p can

inhibit proliferation, migration, and invasion abilities of OS cells by targeting c-Met. Fan et al. [26] found that miRNA-145 down-regulates VEGF levels, thereby inhibiting the local invasion and metastasis of OS cells. MicroRNA-449b-5p is reported to exert a carcinogenic role in multiple types of tumors [18]. In this study, overexpression of microRNA-449b-5p in U2OS and MG63 cells attenuated abilities of cells to proliferate, migrate, and invade.

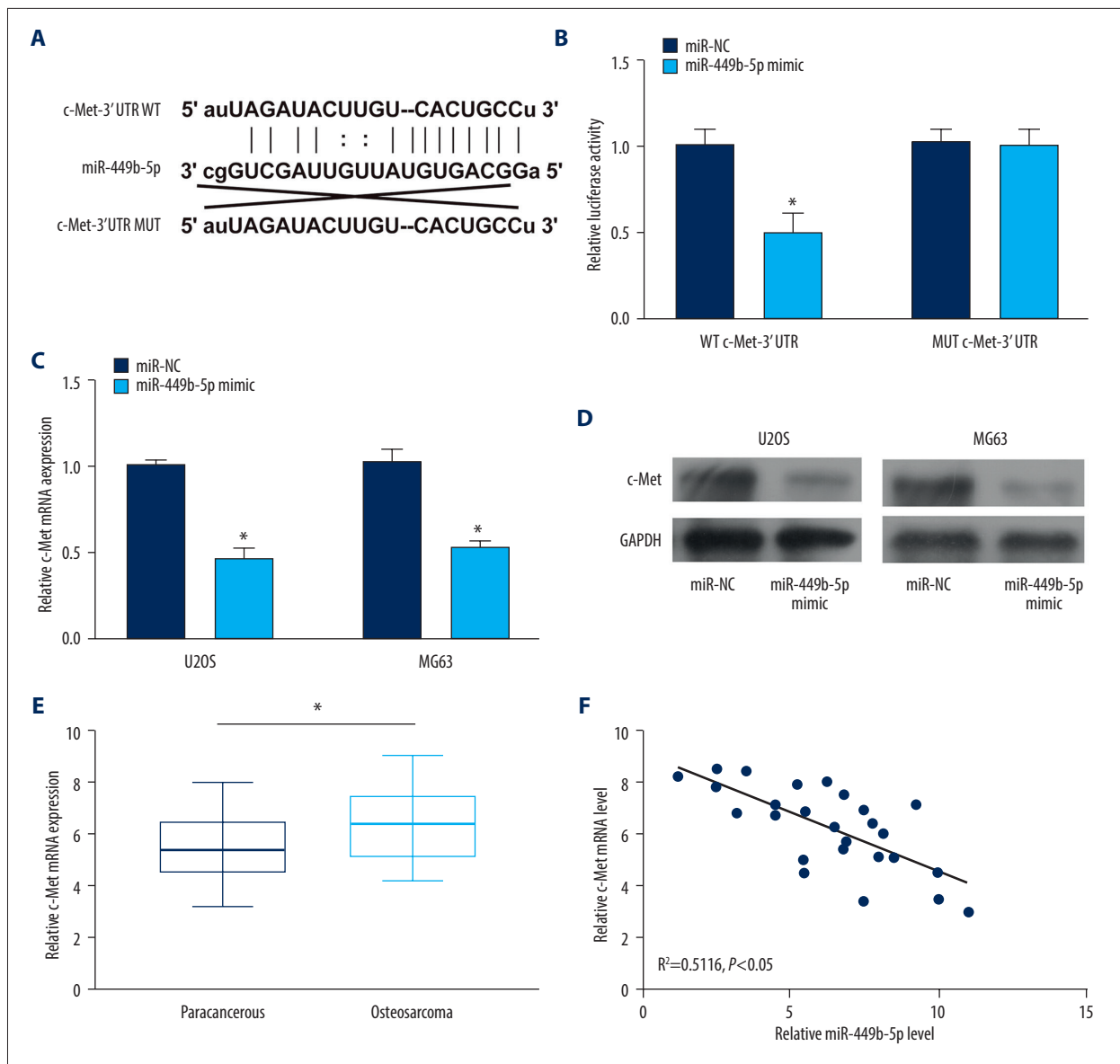


Figure 3. C-Met was the target gene of miR-449b-5p. (A) Potential binding sequences between miR-449b-5p and c-Met. (B) Luciferase activity in cells co-transfected with WT c-Met 3'UTR/MUT c-Met 3'UTR and miR-449b-5p mimic/NC. (C) mRNA levels of c-Met in U2OS and MG63 cells transfected with NC or miR-449b-5p mimic. (D) Protein levels of c-Met in U2OS and MG63 cells transfected with NC or miR-449b-5p mimic. (E) Relative levels of c-Met in OS tissues and paracancerous tissues. (F) Negative correlation between c-Met and miR-449b-5p in OS tissues.

C-Met belongs to the RTKs family, which is mainly expressed in epithelial cells. Serving as an oncogene, c-Met activates multiple pathways and amplifies the transduction cascade to further mediate tumor cell behaviors [27]. C-Met is also considered as predictive marker of prognosis of tumor patients [28–30]. In this study, c-Met was upregulated in OS tissues and cell lines. Through TargetScan prediction and dual-luciferase reporter gene verification, c-Met was identified to be the direct target of microRNA-449b-5p. C-Met was negatively correlated with microRNA-449b-5p in OS. Previous studies have

shown that c-Met regulates the progression of OS by mediating multiple miRNAs [31–33]. In this study, overexpression of c-Met reversed the inhibitory effects of microRNA-449b-5p on behaviors of OS cells. We found that microRNA-449b-5p suppressed proliferative, migratory, and invasive abilities of OS cells by targeting c-Met.

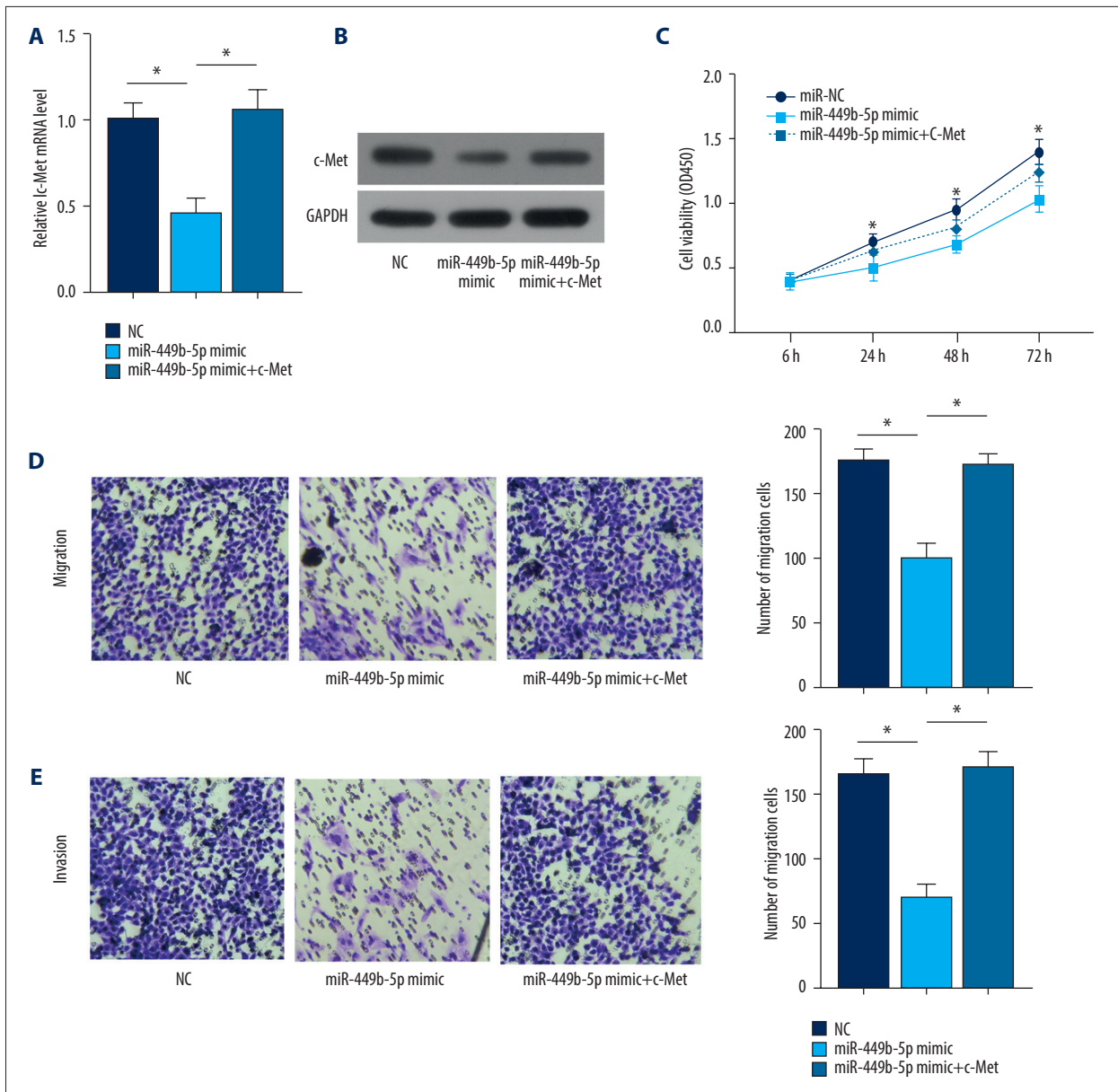


Figure 4. miR-449b-5p suppressed the ability of OS cells to proliferate, migrate, and invade by targeting c-Met. U2OS cells were transfected with NC, miR-449b-5p mimic, or miR-449b-5p mimic + pcDNA-c-Met. **(A)** Relative mRNA levels of c-Met in each group. **(B)** Relative protein levels of c-Met in each group. **(C)** CCK-8 assay showing the viability in each group. **(D)** Transwell assay showing migration and invasion ability in each group.

Conclusions

MicroRNA-449b-5p is downregulated in OS, which attenuates the malignant progression of OS by targeting c-Met.

Conflict of interest

None.

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