European Review for Medical and Pharmacological Sciences 2019; 23: 8203-8210

MicroRNA-183-5p suppresses the malignant progression of osteosarcoma *via* binding to AKT

J.-H. SHAO, S. YUAN, Q.-R. QIAN, J. ZHU

Department of Joint Surgery and Orthopedic Medicine, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai, China

Jiahua Shao and *Shuai Yuan* contributed equally to this work

Abstract. – OBJECTIVE: To clarify the role of microRNA-183-5p in the malignant progression of osteosarcoma (OS) and the potential mechanism.

PATIENTS AND METHODS: Relative level of microRNA-183-5p in 40 paired OS tissues and matched normal tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Correlation between microRNA-183-5p level and clinical indexes of OS patients was analyzed. By transfection of microRNA-183-5p mimics in SaOS-2 and MG63 cells, changes in proliferation and migration were evaluated. The potential target of microRNA-183-5p was verified by dual-luciferase reporter gene assay. Finally, the biological function of protein kinase B (AKT) in OS progression mediated by microRNA-183-5p was detected.

RESULTS: MicroRNA-183-5p was downregulated in OS tissues compared to controls. Relative to OS patients with high expression of microRNA-183-5p, those with low expression had a higher rate of distant metastasis and lower overall survival. Transfection of microRNA-183-5p mimics attenuated proliferative and migratory abilities of SaOS-2 and MG63 cells. AKT was upregulated in OS and negatively correlated to microRNA-183-5p. Overexpression of AKT could abolish the inhibitory effect of microRNA-183-5p on proliferative and migratory abilities of OS cells.

CONCLUSIONS: MicroRNA-183-5p is closely related to distant metastasis and poor prognosis of OS. It suppresses the malignant progression of OS by targeting AKT.

Key Words:
MicroRNA-183-5p, AKT, Osteosarcoma, Malignant progression.

Introduction

Osteosarcoma (OS) is a prevalent primary malignancy. In recent years, the incidence of OS is rising, ranking the fifth of all primary malignancies[1-3]. OS is particularly common in children and adolescents aging 15-19 years, with a prevalence of 5% in childhood tumors[4,5]. OS originates from immature tissues, such as osteoid tissues and mesenchymal tissues. Due to the rapid differentiation, proliferation, growth, and invasion of OS cells, normal skeletal tissues are severely attacked by malignant tumor tissues[6]. Early-stage OS has a relatively good prognosis, whereas the clinical outcome of advanced OS is poor. Therefore, development of effective biomarkers and individualized therapy contributes to improving the prognosis of OS[7]. The occurrence of OS is complex involving genetic mutations and pathway alterations. At the genetic level, the interaction between a wide range of unstable genomes and multiple proteins leads to OS[8]. Current researches mainly focus on immune targets, cellular pathways, and drug delivery system of OS[9].

MicroRNAs, small non-coding ribonucleic acids of about 22 nucleotides, are particularly important in tumor progression[10]. They functionally regulate downstream gene expressions by pairing to the 3'-untranslated region (3'-UTR) of target mRNAs, thus participating in cellular behaviors[11,12]. A single microRNA could regulate the biological functions of multiple mRNAs, and conversely, multiple microRNAs could regulate a single mRNA[13]. Alterations in genetic distribution and phenotypes could result in the occurrence and progression of tumors[14,15]. Several studies[16,17] have shown differentially expressed microRNAs in tumors. Dysregulated microRNAs may be related to tumorigenesis and progression. In this paper, protein kinase B (AKT) was predicted to be the downstream gene of microRNA-183-5p through bioinformatics methods. We aim to explore the

Corresponding Authors: Qirong Qian, MD; e-mail: qianqr@smmu.edu.cn
Jun Zhu, MD; e-mail: wietfhz@163.com

biological function of microRNA-183-5p/AKT in the malignant progression of OS.

Patients and Methods

Patients and OS Samples

A total of 40 OS patients were enrolled in this study, including 24 males and 16 females (6-48 years, mean age of 26.24±6.58 years). Meanwhile, 40 cases of normal bone tissues were surgically resected from hip replacement. No differences in gender and age were observed between OS patients and controls. Patients and their families have been fully informed. This investigation was approved by the Ethics Committee of Shanghai Changzheng Hospital, Second Military Medical University.

Cell Culture

Osteoblast cell line hFOB and OS cell lines (HOS, U2OS, SOSP-9607, 143B, SaOS-2, and MG63) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. The medium was replaced every 2-3 days. The cell passage was conducted at 90% of confluence.

Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Cells seeded in the 6-well plates with 70% of confluence were subjected to transfection using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). At 48 h, cells were harvested for subsequent experiments.

Cell Proliferation Assay

Cells were seeded in the 96-well plate with 2×10^3 cells per well. At the established time points, absorbance (A) at 450 nm of each sample was recorded points using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) to depict the viability curve.

Transwell Migration Assay

Transfected cells for 48 h were adjusted to a dose of 5.0×10^5/mL. 200 μL/well suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA). In the bottom side, 700 μL of medium containing 10% FBS was applied. After 48 h of incubation, migrative cells to the bottom side were subjected to fixation in methanol for 15 min, crystal violet staining for 20 min and cell counting using a microscope. The penetrating cells were counted in 5 randomly selected fields per sample.

Wound Healing Assay

Cells were seeded in a 6-well plate with 5.0×10^5/well. Until 90% of confluence, a 1 mL pipette tip was used to create an artificial wound in the confluent cell monolayer. The percentage of wound closure was calculated at 0 and 24 h, respectively.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified by DNase I treatment. Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). β-actin and U6 were used as internal references. Each sample was performed in triplicate and the relative level calculated by the $2^{-\Delta\Delta Ct}$ method was analyzed by iQ5 2.0 (Bio-Rad, Hercules, CA, USA). Primer sequences were as follows: MicroRNA-183-5p: forward: 5'-TGTGAGTTCTACCATTGCCAA-3'; reverse: 5'-AGTGAATTCTACCAGTGCCAT-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; AKT: forward: 5'-GAACCCAAGGCATCTCCA-3', reverse: 5'-TACCATCAACTCCAACGG-3'; β-actin: forward: 5›-CCTGGCACCCAGCACAAT-3›, reverse: 5›-GCTGATCCACATCTGCTGGAA-3›.

Dual-Luciferase Reporter Gene Assay

Cells were co-transfected with AKT 3'UTR WT/AKT 3'UTR MUT and microRNA-183-5p mimics/microRNA-183-5p inhibitor/NC using Lipofectamine 2000. 24 h later, co-transfected cells were harvested for determining luciferase activity using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. Comparison between multiple groups was done using the One-way ANOVA test followed by Post-Hoc Test (Least Significant

Difference). Kaplan-Meier was introduced for survival analysis and Log-rank test was conducted to compare differences between the two curves. $p<0.05$ was considered as statistically significant.

Results

Downregulated MicroRNA-183-5p in OS

The relative level of microRNA-183-5p was downregulated in OS tissues compared with that of normal controls (Figures 1A, 1B). Identically, the microRNA-183-5p level remained lower in OS cell lines relative to osteoblast cell line hFOB (Figure 1B). Among the six OS cell lines, SaOS-2 and MG63 cells expressed the lowest level of microRNA-183-5p, which were selected for the following *in vitro* experiments (Figure 1C). The Kaplan-Meier curves revealed worse prognosis in OS patients with low expression of microRNA-183-5p than those with high expression (Figure 1D).

Furthermore, we analyzed the correlation between microRNA-183-5p level and clinical indexes of OS patients. As depicted in Table I, the microRNA-183-5p level was negatively correlated to distant metastasis, but not correlated to age, gender, lymph node metastasis and T stage of OS patients. Therefore, microRNA-183-5p was believed to participate in the progression of OS.

Overexpression of MicroRNA-183-5p Inhibited OS Cells to Proliferate and Migrate

To elucidate the potential influence of microRNA-183-5p on OS cell behaviors, microRNA-183-5p mimics was constructed. Transfection of microRNA-183-5p mimics markedly upregulated microRNA-183-5p level in SaOS-2 and MG63 cells (Figure 2A). CCK-8 assay showed that transfection of microRNA-183-5p mimics attenuated viability of OS cells (Figure 2B). Overexpression of microRNA-183-5p in OS cells decreased the number of penetrating cells, indicating the inhibited migratory ability (Figure 2C). Colony formation assay showed the decreased percentage of wound closure in OS cells transfected with microRNA-183-5p mimics (Figure 2D).

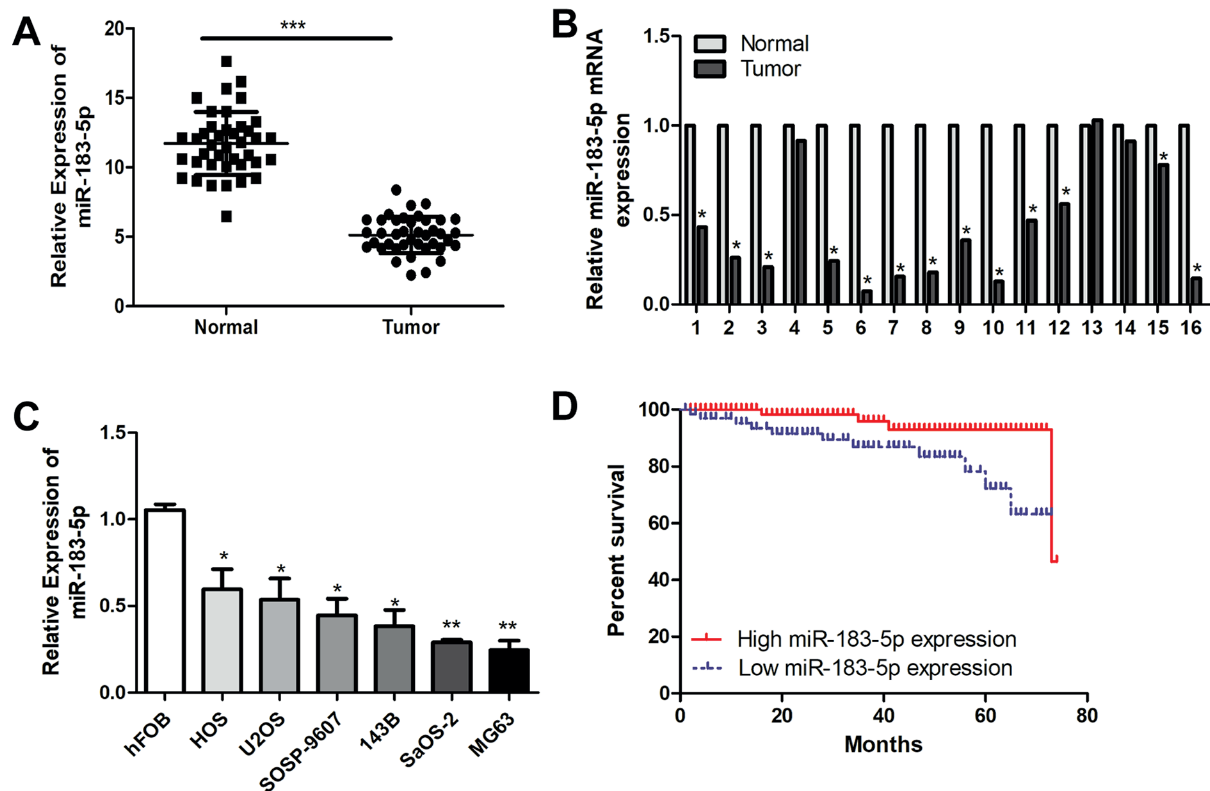

Figure 1. Downregulated miR-183-5p in OS. **A,** Relative level of miR-183-5p in OS tissues and control tissues. **B,** Relative level of miR-183-5p in 16 paired OS tissues and matched normal tissues. **C,** Relative level of miR-183-5p in osteoblast cell line hFOB and OS cell lines (HOS, U2OS, SOSP-9607, 143B, SaOS-2, and MG63). **D,** Kaplan-Meier curves introduced for survival analysis in OS patients with high expression and low expression of miR-183-5p.

Table I. Association of miR-183-5p expression with clinicopathologic characteristics of osteosarcoma.

Parameters	Number of cases	miR-183-5p expression High (%)	miR-183-5p expression Low (%)	*p*-value
Age (years)				0.949
<25	18	10	8	
≥25	22	12	10	
Gender				0.604
Male	24	14	10	
Female	16	8	8	
T stage				0.225
T1-T2	22	14	8	
T3-T4	18	8	10	
Lymph node metastasis				0.071
No	28	18	10	
Yes	12	4	8	
Distance metastasis				0.033
No	27	18	9	
Yes	13	4	9	

Note: Comparison of efficacy. The total effective rate of clinical treatment is 90% in experimental group and 60% in control group. $^*p<0.05$ *vs.* control group

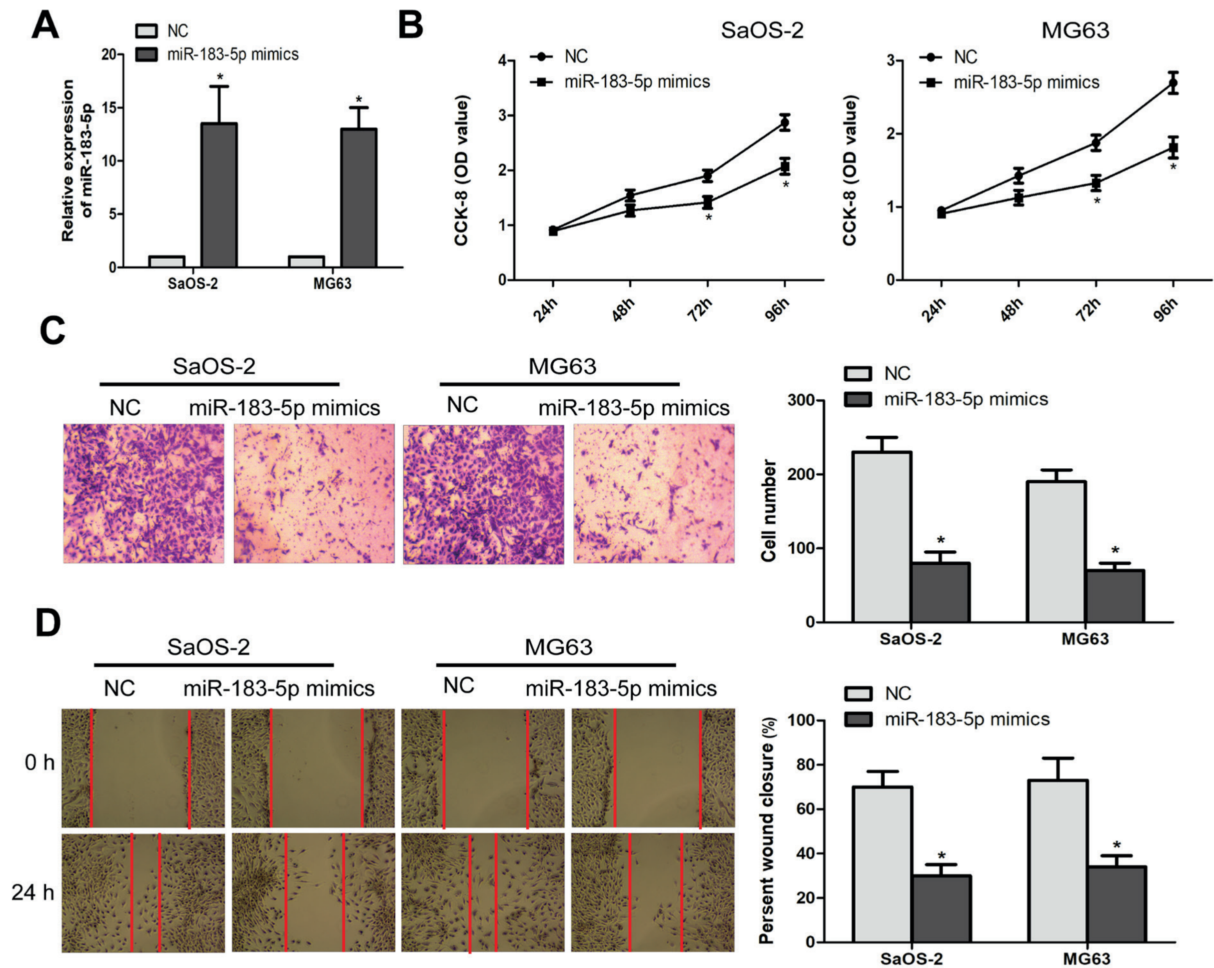

Figure 2. Overexpression of miR-183-5p inhibited OS cells to proliferate and migrate. ***A,*** Transfection efficacy of miR-183-5p in SaOS-2 and MG63 cells. ***B,*** CCK-8 assay showed viability in SaOS-2 and MG63 cells transfected with NC or miR-183-5p mimics. ***C,*** Transwell assay showed migration in SaOS-2 and MG63 cells transfected with NC or miR-183-5p mimics (magnification 20×). ***D,*** Wound healing assay showed the percentage of wound closure in SaOS-2 and MG63 cells transfected with NC or miR-183-5p mimics at 0 and 24 h (magnification 20×).

Upregulated AKT in OS

Transfection of microRNA-183-5p mimics downregulated AKT level in OS cells (Figure 3A). Compared with normal bone tissues, AKT was highly upregulated in OS tissues (Figure 3B). Similarly, AKT was upregulated in OS cell lines (Figure 3C). Correlation analysis revealed a negative relationship between microRNA-183-5p and AKT in 16 selected OS tissues (Figure 3D).

MicroRNA-183-5p Bound to AKT

Based on predicted binding sequences between microRNA-183-5p and AKT, we constructed pmirGLO-microRNA-183-5p-mut and pmirGLO-microRNA-183-5p-wt for dual-luciferase reporter gene assay (Figure 4A). Luciferase activity markedly decreased in OS cells co-transfected with microRNA-183-5p mimics and pmirGLO-microRNA-183-5p-wt; whereas it was elevated after co-transfection of microRNA-183-5p and pmirGLO-microRNA-183-5p-wt (Figure 4B). Hence, AKT was verified to be the downstream gene of microRNA-183-5p.

MicroRNA-183-5p Mediated OS Progression by Targeting AKT

Transfection of pcDNA-AKT remarkably upregulated AKT level in OS cells, verifying the sufficient transfection efficacy. Downregulated AKT level in OS cells overexpressing microRNA-183-5p was reversed after overexpression of AKT (Figure 5A). Transfection of pcDNA-AKT elevated the viability of OS cells. Overexpression of AKT reversed the inhibitory effect of microRNA-183-5p on viability and migratory ability of OS cells (Figures 5B-5D). Similar results were obtained through wound healing assay, suggesting the inhibited role of microRNA-183-5p in the proliferation of OS cells was abolished by AKT (Figure 5E).

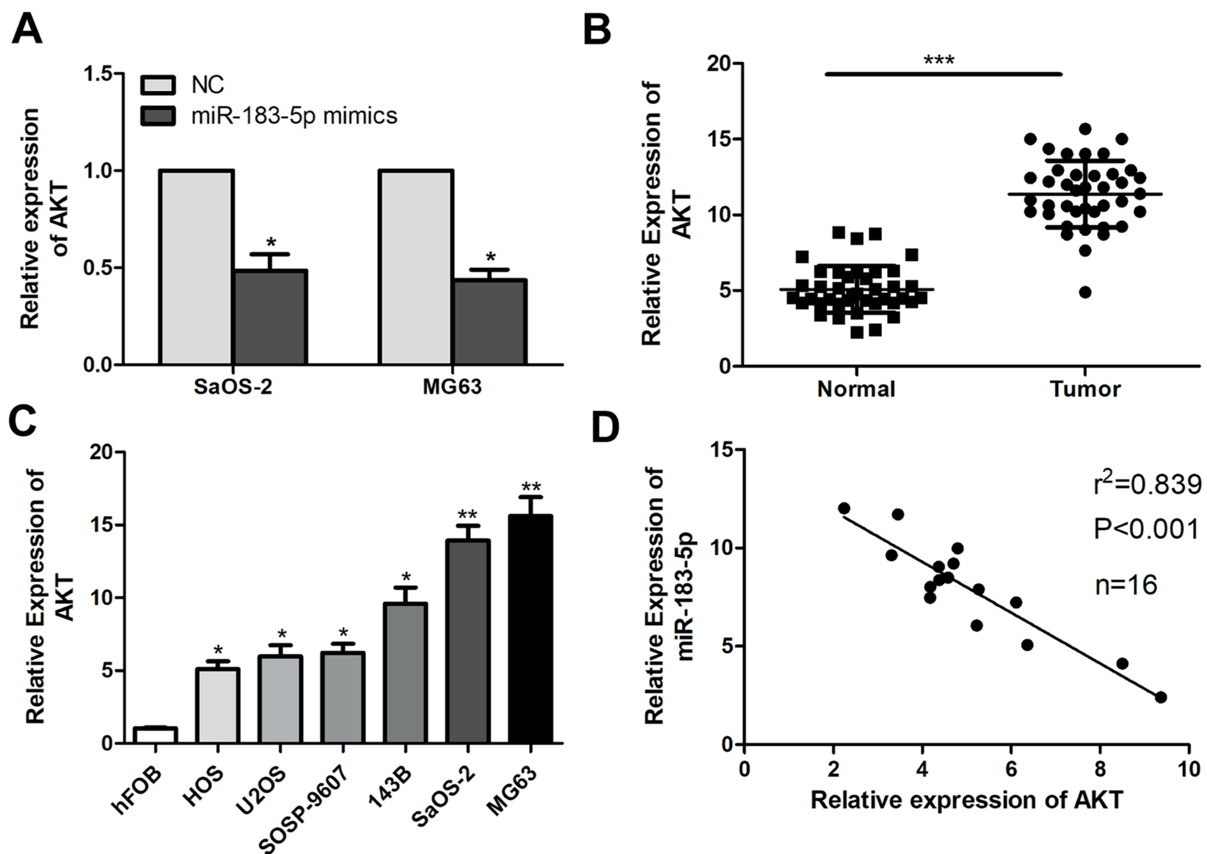

Figure 3. Upregulated AKT in OS. **A,** Relative level of AKT in SaOS-2 and MG63 cells transfected with NC or miR-183-5p mimics. **B,** Relative level of AKT in OS tissues and control tissues. **C,** Relative level of AKT in osteoblast cell line hFOB, and OS cell lines (HOS, U2OS, SOSP-9607, 143B, SaOS-2, and MG63). **D,** Negative correlation between miR-183-5p and AKT in 16 cases of OS tissues.

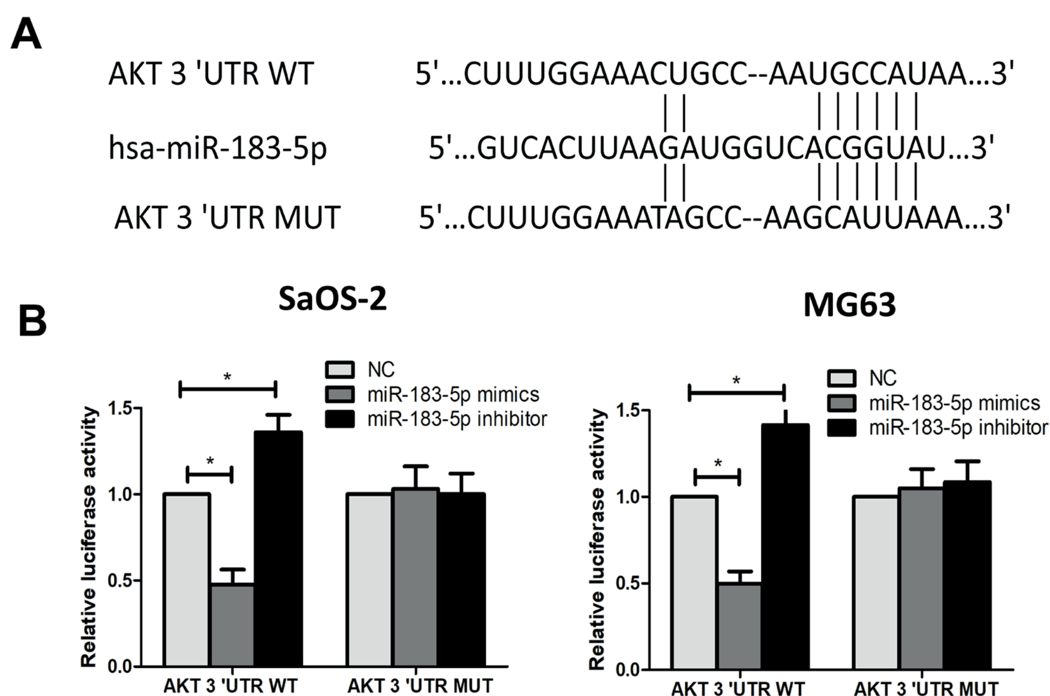

Figure 4. MiR-183-5p bound to AKT. **A,** Predicted binding sequences between miR-183-5p and AKT. **B,** Luciferase activity in SaOS-2 and MG63 cells co-transfected with AKT 3'UTR WT/AKT 3'UTR MUT and miR-183-5p mimics/miR-183-5p inhibitor/NC.

Discussion

OS is a bone tumor that occurs in the proximal end of the long bones and active growing extremities that seriously decreases life quality of affected patients[1-3]. Current strategies on radical resection and postoperative chemotherapy have been greatly advanced. However, the recurrent rate and mortality of OS still remain high, leading to poor prognosis of OS patients[3-5]. The highly malignant OS is prone to distant metastasis at early stage[6]. It is estimated that the postoperative 5-year survival of OS is only 10%. About 40% of OS patients die of lung metastases[7].

Epigenetics is the study of heritable phenotype changes that do not involve alterations in the DNA sequence[9,10]. Histone modification, non-coding RNA, and DNA methylation are the research hot topics, which are extensively involved in biological processes[18]. MicroRNAs are tissue-specific in tumors. Abnormal downregulation or upregulation of tumor-related microRNAs depends on the types of tumors[10]. It is reported that microRNAs could interact with oncogenes or tumor-suppressor genes, thus influencing the occurrence and progression of tumors[19].

Researches on OS-related microRNAs have not been well elucidated[12,13]. These microRNAs are capable of regulating epigenetic characteristics and cellular functions of OS cells *via* mediating downstream genes and various intracellular pathways[14]. Relevant studies[18-20] have identified that microRNA-130b and microRNA-148a are upregulated in OS tissues, which are positively correlated to the malignant progression of OS. MicroRNA-183-5p is found to be crucial in tumor progression. In this work, microRNA-183-5p was downregulated in OS tissues relative to controls. Its level was negatively correlated to distant metastasis of OS patients, suggesting the tumor-suppressor effect on OS. Next, a series of *in vitro* experiments were conducted to explore the biological function of microRNA-183-5p in OS cells. It is suggested that microRNA-183-5p could inhibit the proliferative and migratory abilities of OS cells.

MicroRNAs exert their biological functions through binding to the target genes. Therefore, an accurate identification of microRNAs-targeted mRNAs is the key part in exploring biological mechanism[14-16]. Theoretically, a single microRNA could bind to multiple target mRNAs in an incompletely base-pairing way[14-16,20]. This research

predicted AKT as the downstream target of microRNA-183-5p and further verified by dual-luciferase reporter gene assay. Moreover, a negative correlation was found between expression levels of microRNA-183-5p and AKT in OS tissues. QRT-PCR data showed a higher abundance of AKT in OS tissues and cell lines relative to controls. Notably, the overexpression of AKT could abolish the inhibitory effect of microRNA-183-5p on proliferative and migratory abilities of OS cells. Collectively, microRNA-183-5p/AKT axis influenced the malignant progression of OS.

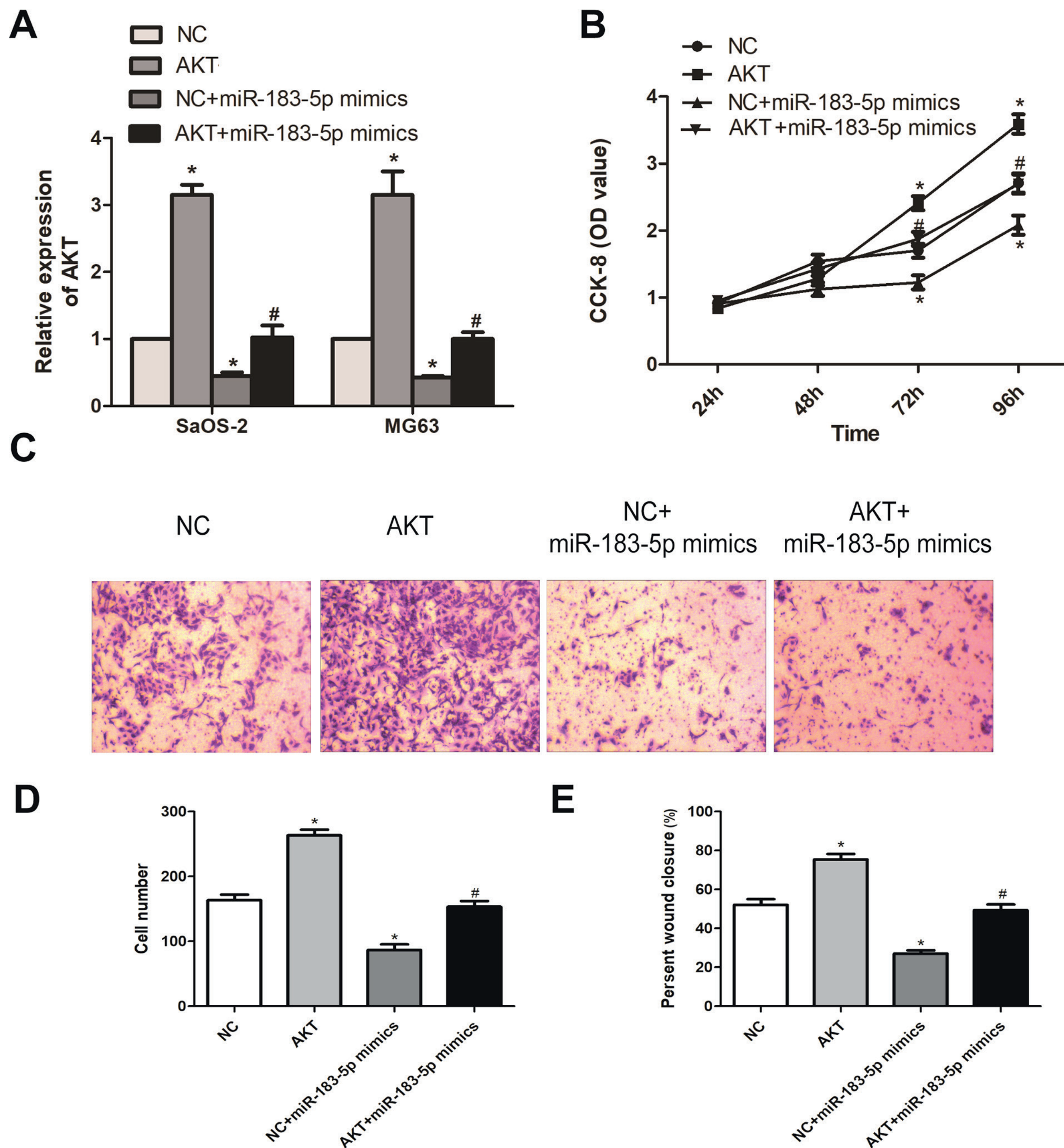

Figure 5. MiR-183-5p mediated OS progression by targeting AKT. SaOS-2 cells were transfected with NC, pcDNA-AKT, NC+miR-183-5p mimics and pcDNA-AKT+miR-183-5p mimics. **A,** Relative level of AKT in each group. **B,** CCK-8 assay showed the viability in each group. **C,** Transwell assay showed the migration in each group (magnification: 40×). **D,** Number of migratory cells in each group. **E,** Percentage of wound healing closure in each group.

Conclusions

We found that microRNA-183-5p is closely related to distant metastasis and poor prognosis of OS. It suppresses the malignant progression of OS by targeting AKT.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Simpson S, Dunning MD, de Brot S, Grau-Roma L, Mongan NP, Rutland CS. Comparative review of human and canine osteosarcoma: morphology, epidemiology, prognosis, treatment and genetics. Acta Vet Scand 2017; 59: 71.
2) Moore DD, Luu HH. Osteosarcoma. Cancer Treat Res 2014; 162: 65-92.
3) Chao LM, Sun W, Chen H, Liu BY, Li PF, Zhao DW. MicroRNA-31 inhibits osteosarcoma cell proliferation, migration and invasion by targeting PIK3C2A. Eur Rev Med Pharmacol Sci 2018; 22: 7205-7213.
4) Carina V, Costa V, Sartori M, Bellavia D, De Luca A, Raimondi L, Fini M, Giavaresi G. Adjuvant biophysical therapies in osteosarcoma. Cancers (Basel) 2019; 11. pii: E348.
5) Han X, Wang W, He J, Jiang L, Li X. Osteopontin as a biomarker for osteosarcoma therapy and prognosis. Oncol Lett 2019; 17: 2592-2598.
6) Zamborsky R, Kokavec M, Harsanyi S, Danisovic L. Identification of prognostic and predictive osteosarcoma biomarkers. Med Sci (Basel) 2019; 7. pii: E28.
7) Limaiem F, Khaddour K. Cancer, telangiectatic osteosarcoma. StatPearls Publishing; 2019 Feb 1.
8) Miwa S, Shirai T, Yamamoto N, Hayashi K, Takeuchi A, Igarashi K, Tsuchiya H. Current and emerging targets in immunotherapy for osteosarcoma. J Oncol 2019; 2019: 7035045.
9) Chen R, Wang G, Zheng Y, Hua Y, Cai Z. Drug resistance-related microRNAs in osteosarcoma: translating basic evidence into therapeutic strategies. J Cell Mol Med 2019; 23: 2280-2292.
10) Tutar Y. MiRNA and cancer; computational and experimental approaches. Curr Pharm Biotechnol 2014; 15: 429.
11) Tutar L, Özgür A, Tutar Y. Involvement of miRNAs and pseudogenes in cancer. Methods Mol Biol 2018; 1699: 45-66.
12) Vishnoi A, Rani S. MiRNA Biogenesis and regulation of diseases: an overview. Methods Mol Biol 2017; 1509: 1-10.
13) Andrés-León E, Cases I, Alonso S, Rojas AM. Novel miRNA-mRNA interactions conserved in essential cancer pathways. Sci Rep 2017; 7: 46101.
14) Ors-Kumoglu G, Gulce-Iz S, Biray-Avci C. Therapeutic microRNAs in human cancer. Cytotechnology 2019; 71: 411-425.
15) Aghdam SG, Ebrazeh M, Hemmatzadeh M, Seyfizadeh N, Shabgah AG, Azizi G, Ebrahimi N, Babaie F, Mohammadi H. The role of microRNAs in prostate cancer migration, invasion, and metastasis. J Cell Physiol 2019; 234: 9927-9942.
16) Ghasabi M, Mansoori B, Mohammadi A, Duijf PH, Shomali N, Shirafkan N, Mokhtarzadeh A, Baradaran B. MicroRNAs in cancer drug resistance: basic evidence and clinical applications. J Cell Physiol 2019; 234: 2152-2168.
17) Iqbal MA, Arora S, Prakasam G, Calin GA, Syed MA. MicroRNA in lung cancer: role, mechanisms, pathways and therapeutic relevance. Mol Aspects Med 2018 Aug 17. pii: S0098-2997(18)30065-7. doi: 10.1016/j.mam.2018.07.003. [Epub ahead of print]
18) Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. Cell 2012; 150: 12-27.
19) Si W, Shen J, Zheng H, Fan W. The role and mechanisms of action of microRNAs in cancer drug resistance. Clin Epigenetics 2019; 11: 25.
20) Wang M, Xie R, Si H, Shen B. Integrated bioinformatics analysis of miRNA expression in osteosarcoma. Artif Cells Nanomed Biotechnol 2017; 45: 936-943.