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Targeting EZH2 by microRNA-449a inhibits osteosarcoma cell proliferation, invasion and migration *via* regulation of PI3K/AKT signaling pathway and epithelial-mesenchymal transition

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Abstract. – OBJECTIVE: Osteosarcoma (OS) is one common bone malignant tumor prevailing in young adults and children. It is increasingly recognized microRNA 449a (miR 449a) as an anti-tumor factor in various tumours. However, little is known about the biological significance of miR 449a in OS. The intent of our study was to seek the prognostic values of miR-449a in OS.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to examine the level of miR-449a expression in 48 pairs of OS tissues and para-cancerous specimens, and the relationship between miR-449a level and clinical features of OS patient prognosis was analyzed. Moreover, we measured the miR-449a expression levels in OS cells. Transwell assay was further performed to investigate whether miR-449a influenced MG63 cell migration and invasion, which was important for malignant metastases.

RESULTS: Quantitative real-time polymerase chain reaction (qRT-PCR) analysis demonstrated a notable decrease of miR-449a expressions in OS. The declined miR-449a expression was relevant with the poor prognosis and malignant clinicopathologic characteristics of OS patients. Thereafter, the functional assay was performed to determine the role of miR-449a in OS progression. Results of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays and transwell assays indicated that miR-449a overexpression significantly repressed OS cell proliferation, invasion, and migration. Furthermore, luciferase reporter assay showed that enhancer of zeste homolog 2 (EZH2) was a downstream target of miR-449a in OS cells. Additionally, Western blot analysis demonstrated that miR-449a exerted anti-OS functions via the regulation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway and epithelial-mesenchymal transition. We also indicated that miR-449a restoration could inhibit *in vivo* tumor growth.

CONCLUSIONS: These results manifested that miR-449a may thus be used as a therapeutic target in OS treatments.

Key Words:
Osteosarcoma, MiR-449a, EZH2, PI3K/AKT, Epithelial-mesenchymal transition (EMT).

Introduction

Osteosarcoma (OS) is one aggressive and prevalent bone tumor in adolescents and children, resulting in high mortalities[1]. Currently, standard treatments for patients with OS encompass surgery operations, adjuvant chemotherapy, and neoadjuvant[2]. Surgery operations have led to amputation; additionally, adjuvant chemotherapy or neoadjuvant could induce toxic effects which can't be efficiently obviated. Although existing treatments have improved patient survival, overall 5-year survival rates for relapsed and metastatic OS patients remain disappointingly low (less than 30%)[3,4]. Therefore, the metastasis process and progression of OS remains to be fully elucidated, and the exploration of innovative therapeutic targets for OS has become an urgent clinical need.

MicroRNA (MiRNA/MiR) can induce translational silencing or degrade the target mRNA *via* binding to their 3'-UTRs[5]. In tumorigenesis, miRNA plays key roles, whether negatively or positively, in almost all malignant progression of tumors, such as apoptosis, invasion, proliferation, and migration[6,7]. Recently, the aberrant expression of certain miRNA has been confirmed[8] to be closely related to the diagnosis, treatment response and prognosis of various tumors, so miRNA is becom-

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ing a crucial therapy target and prognosis biomarker for tumors. That was the case of Azarbarzin et al[9] who identified miR-383 as a diagnosis and prognosis factor for intestinal-type gastric carcinoma; Chen et al[10] showed that miR-148a inhibited non-small cell lung cancer migration and invasion *via* Wnt1, serving as a prognostic biomarker; Wang et al[11] proposed that miR-191 restoration promoted prostate cancer cell invasion and growth through TIMP3. Among the tumor-associated miRNAs, miR-449a has important physiological significance for OS development; however, the mechanism about the anti-cancer function of miR-449a in OS is still unclear.

Generally, it is acknowledged that the pathogenesis and clinical development of OS are related to multiple genes as well as corresponding pathways. Enhancer of zeste homolog 2 (EZH2) is the core number of polycomb repressive complex-2 (PRC2) and is one of the key epigenetic regulators for gene expressions[12]. Previous scholars[13,14] indicated that EZH2 is one kind of histone methyltransferases which could specifically promote gene silencing in a variety of tumors through induction of Lysine-27 of histone 3 methylation. Overexpressed EZH2 is frequently found in different cancers, and they contributed to the tumor aggressiveness[15]. Rabello et al[16] found that EZH2 overexpression in chronic lymphocytic leukemia indicated a poor prognosis; Murai et al[17] indicated that EZH2 promoted small cell lung cancer progression *via* inhibition of TGF-beta-Smad-ASCL1 pathway; Nienstedt et al[18] demonstrated that EZH2 overexpression was associated with lymph node metastasis in head and neck cancer; studies by Masudo et al[19] showed that EZH2 overexpression in thyroid cancer was a useful prognostic biomarker for malignant behaviours. However, the association between EZH2 expression and miR-449a expression in human OS requires further exploration.

Patients and Methods

Patients and Tissue Samples

48 OS and adjacent normal tissue specimens were collected by excision biopsy from OS patients who received tumorectomy at the Affiliated Hospital of Youjiang Medical College for Nationalities between May 2013 and July 2018. All patients provided the formal written informed consent. All OS patients enrolled in the current study were not suffering from any other diseases and were not subjected to chemotherapy or radiotherapy. This investigation was approved by the Ethics Committee of the Affiliated Hospital of Youjiang Medical College for Nationalities.

Cell Lines

A human osteoblast cell line hFOB 1.19 and OS cells (U2OS, Saos-2, MG-63, HOS) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientifc, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C in a humidified incubator containing 5% CO_2.

Cell Transfections

The OS cells were transfected with miR-449a mimic, inhibitor or the negative controls by Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). 48 h after the transfections, cells were harvested for the subsequent experiments.

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

OS tissues or cells were lysed in TRIzol (Invitrogen, Carlsbad, CA, USA) for total RNA extraction. RNA was then reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT-PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The SYBR Green PCR Master Mix kit with an ABI 7900 system (Applied Biosystems, Waltham, MA, USA) was used to measure the EZH2 expressions. TaqMan miRNA RT-PCR (Applied Biosystem, Waltham, MA, USA) was applied for the detection of miR-449a expressions. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were internal controls. The $2^{-\Delta\Delta Ct}$ method was used to quantify the expressions. Primer sequences for qRT-PCR are available in Table I.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Cell proliferation ability was examined by MTT assays (Sigma-Aldrich, St. Louis, MO, USA). The transfected OS cells were seeded into 96-well plates. Then, the cells were incubated for 24, 48, and 72 h, and cell growth was measured following the addition of MTT (5 mg/mL). Thereafter, 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to solubilize the MTT-formazan crystals. The optical density $(OD)_{490}$ was measured using a microplate (Bio-Rad Laboratories, Hercules, CA, USA).

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-449a forward	5'- TGCGGTGGCAGTGTATTGTTAGC -3'
miR-449a reverse	5'- CCAGTGCAGGGTCCGAGGT -3'
U6 forward	5'- CTCGCTTCGGCAGCACA-3'
U6 reverse	5'- AACGCTTCACGAATTTGCGT-3'
EZH2 forward	5'- GTGGAGAGATTATTCTCAAGATG -3'
EZH2 reverse	5'- CCGACATACTTCAGGGCATCAGCC -3'
GAPDH forward	5'- CTCATGACCACAGTCCATGCC-3'
GAPDH reverse	5'- GGCATGGACTGTGGTCATGAG-3'

U6: small nuclear RNA, snRNA; EZH2: enhancer of zeste homolog 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Transwell Assays

8-μm pore sized transwell chamber (BD Biosciences, San Jose, CA, USA) was used to assess the regulatory effects of miR-449a on OS cell invasion and migration abilities with or without Matrigel (BD Biosciences, San Jose, CA, USA). Transfected OS cells were suspended in FBS-free DMEM medium and seeded into the top chamber. On the other hand, medium containing 10% FBS was added to bottom chambers. Following incubation for 48 h (for invasion) or 24 h (for migration) at 37°C, the non-invaded or non-migrated cells were carefully scraped off with cotton swabs. In the meantime, invaded or migrated cells were fixed and stained with methanol and crystal violet. The results were analyzed under a light microscope (Olympus, Tokyo, Japan).

Western Blot

The transfected OS cells were solubilized with radioimmunoprecipitation assay (RIPA) buffer (Pierce, Rockford, IL, USA) for protein isolation. The bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was utilized for the examination of protein concentration. Then, equal amounts (50 μg) of protein were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Subsequently, the membrane was blocked with 5% skimmed milk in TBST for 2 h at room temperature, followed by the incubation with specific primary antibodies overnight at 4°C. The primary antibodies were as follows: antibodies against AKT (1:1000, Abcam, Cambridge, MA, USA), p-AKT (1:1000, Abcam, Cambridge, MA, USA), PI3K (1:1000, Abcam, Cambridge, MA, USA), p-PI3K (1:2000, Abcam, Cambridge, MA, USA), E-cadherin (1:2000, Abcam, Cambridge, MA, USA), N-cadherin (1:1000, Abcam, Cambridge, MA, USA), Vimentin (1:1000, Abcam, Cambridge, MA, USA) and GAPDH (1:1000, Abcam, Cambridge, MA, USA). GAPDH was used as an internal control. The membrane was then incubated with HRP-conjugated anti-rabbit IgG (1:2000, Abcam, Cambridge, MA, USA) for 2 h at room temperature. The protein band was visualized with ECL reagent (Beyotime, Shanghai, China).

Luciferase Reporter Assay

The wide-type (WT) or mutant-type (MUT) 3'-untranslated region (3'-UTR) sequences of EZH2 containing putative binding sites for miR-449a were cloned into the pGL3 reporter vector (Promega, Madison, WI, USA). OS cells were cotransfected with miR-449a mimics and EZH2-3'UTR-WT or EZH2-3'UTR-MUT. At 48 h post-transfection, cells were harvested, and the luciferase activity was determined with a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA).

Statistical Analysis

All experiments were repeated at least thrice. Statistical analysis was performed with Statistical Product and Service Solutions (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA). The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was made using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). The Kaplan-Meier curve together with the log-rank test was used to analyze the overall survival of OS patients. $p<0.05$ indicated statistically significant differences.

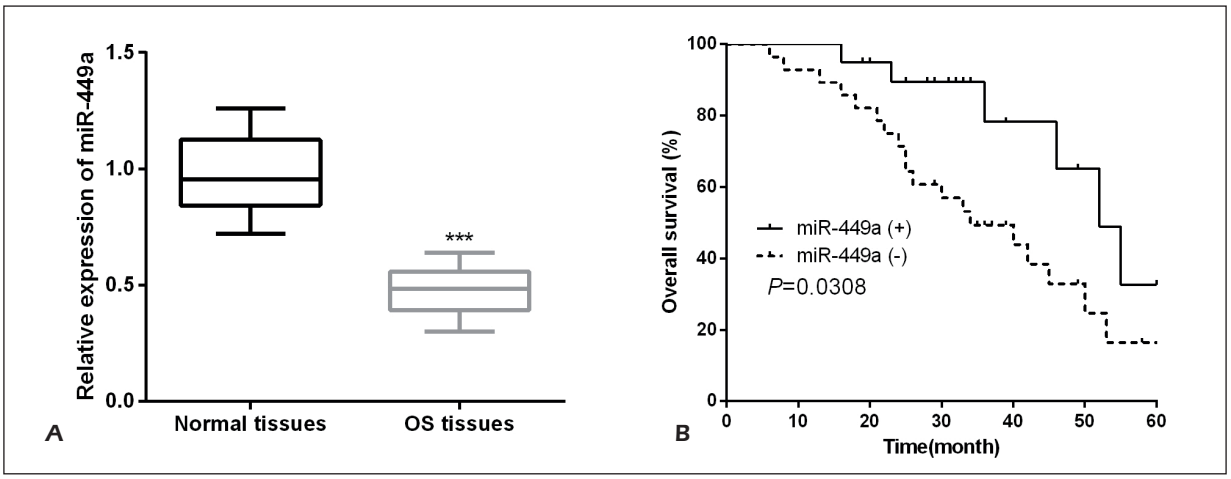

Figure 1. Decreased miR-449a in OS tissue samples indicated poor prognosis of OS patients. **A**, qRT-PCR analysis indicated that miR-449a expressions were reduced in OS tissue samples. **B**, Kaplan-Meier analysis for the overall survival of OS patients with different miR-449a expressions. ***$p<0.001$.

Results

Low MiR-449a Expressions Were Relevant With Clinicopathologic Factors and Prognosis of OS Patient

To clarify the roles of miR-449a in the development of OS, firstly, qRT-PCR was performed to detect miR-449a expressions in OS tissue samples. As shown in Figure 1A, it demonstrated an evident decrease of miR-449a expressions in OS tissues. Subsequently, we performed Kaplan-Meier survival curve analysis to analyze whether miR-449a could serve as a prognostic marker for OS patient, and found that lower miR-449a expression in OS patients presented lower overall survival rates (Figure 1B). In addition, according to the mean level of miR-449a, the OS patients were grouped into high and low miR-449a group. As shown in Table II, the

Table II. Correlation of miR-449a expression with the clinicopathological characteristics of the OS patients.

Clinicopathological features	Cases (n=48)	miR-449a# expression		*p*-value*
		High (n=20)	Low (n=28)	
Age (years)				0.5432
>60	26	11	15	
≤60	22	9	13	
Gender				0.4154
Male	26	10	16	
Female	22	10	12	
Tumor size (cm)				0.3022
≥ 5.0	23	6	17	
<5.0	25	14	11	
Lymph node metastasis				0.0018*
Yes	21	17	4	
No	27	3	24	
TNM stage				0.0021*
I +II	22	15	7	
III+IV	26	5	21	
Distant metastasis				0.0023*
Yes	32	15	17	
No	16	5	11	

OS: Osteosarcoma; TNM: tumor-node-metastasis; #the mean expression level of miR-449a was used as the cutoff. *Statistically significant.

correlation between clinicopathological features and miR-449a expressions were investigated, and low miR-449a expressions betokened malignant clinicopathologic factors.

MiR-449a Overexpression Suppressed OS Cell Proliferation

As miR-449a had been confirmed to be a prognostic biomarker in OS patients, we speculated that miR-449a played anti-tumor functions in OS. To verify the hypothesis, we measured the miR-449a expression levels in OS cells. As expected, miR-449a was also significantly downregulated in the OS cells (Figure 2A). Subsequently, miR-449a mimic or inhibitor was transfected into MG63 cells to upregulate or inhibit miR-449a expressions. As shown in Figure 2B and 2C, miR-449a mimics successfully upregulated whereas miR-449a inhibitor successfully downregulated miR-449a expressions in MG63 cells. After that, MTT assays were carried out to determine the functions of miR-449a in regulating OS cell proliferation. MiR-449a overexpression prominently inhibited MG63 cell proliferation ability while miR-449a silence in MG63 cells significantly facilitated the proliferation capacity (Figure 2D).

MiR-449a Overexpression Inhibited OS Cell Migration and Invasion

Transwell assay was further performed to investigate whether miR-449a influenced MG63 cell migration and invasion, which was important for malignant metastases. As shown in Figure 3A and 3B, MG63 cells transfected with miR-449a mimics presented significant compromised invasion and migration abilities in comparison with the NC-transfected cells. Furthermore, miR-449a

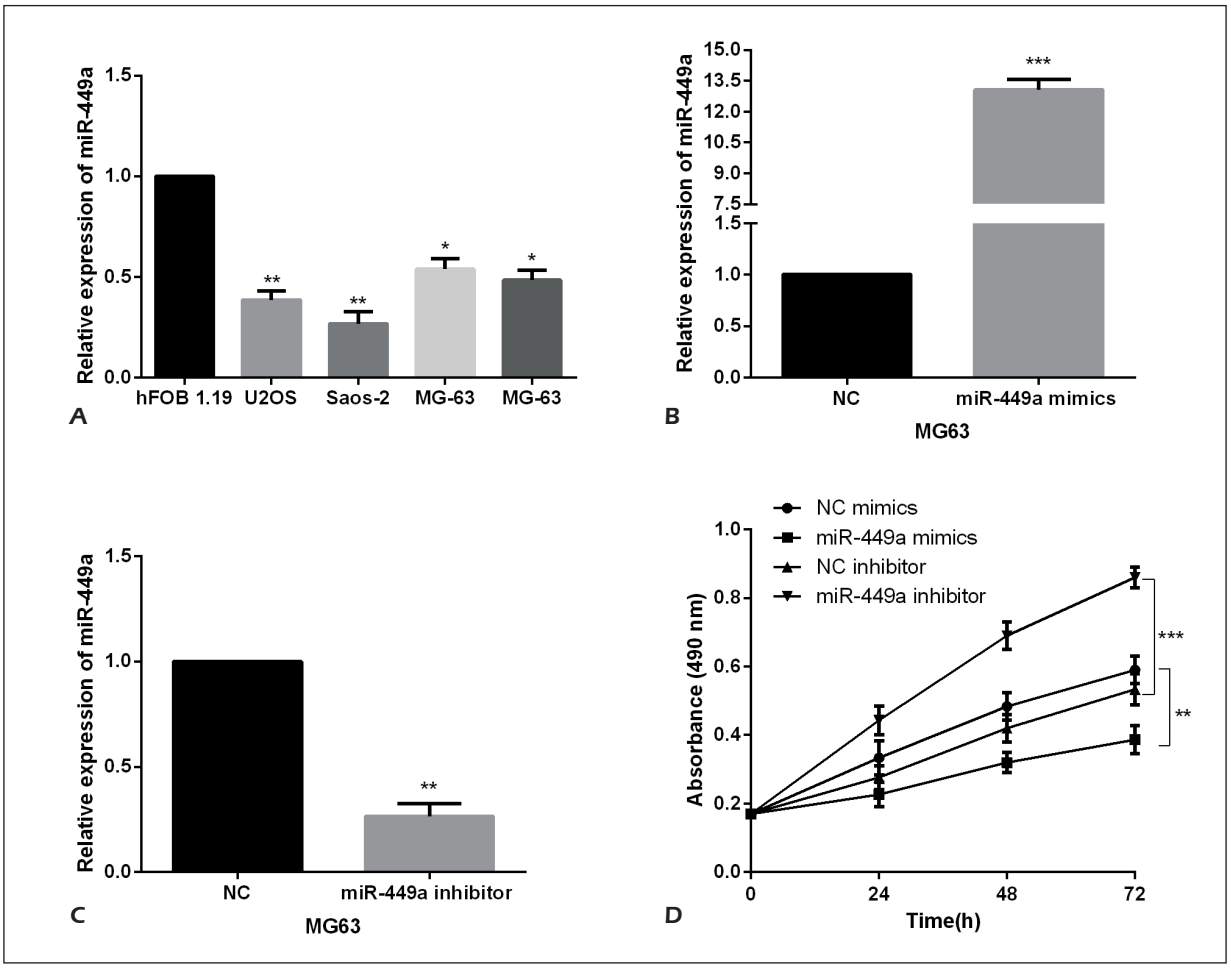

Figure 2. MiR-449a overexpression inhibited OS cell proliferation ability. **A**, MiR-449a expressions were decreased in OS cell lines. **B**-**C**, MiR-449a mimic or inhibitors was transfected into MG63 cells. **D**, MTT assays were carried out to examine the functions of miR-449a in the proliferation capacity of MG63 cells. *p<0.05, **p<0.01, ***p<0.001.

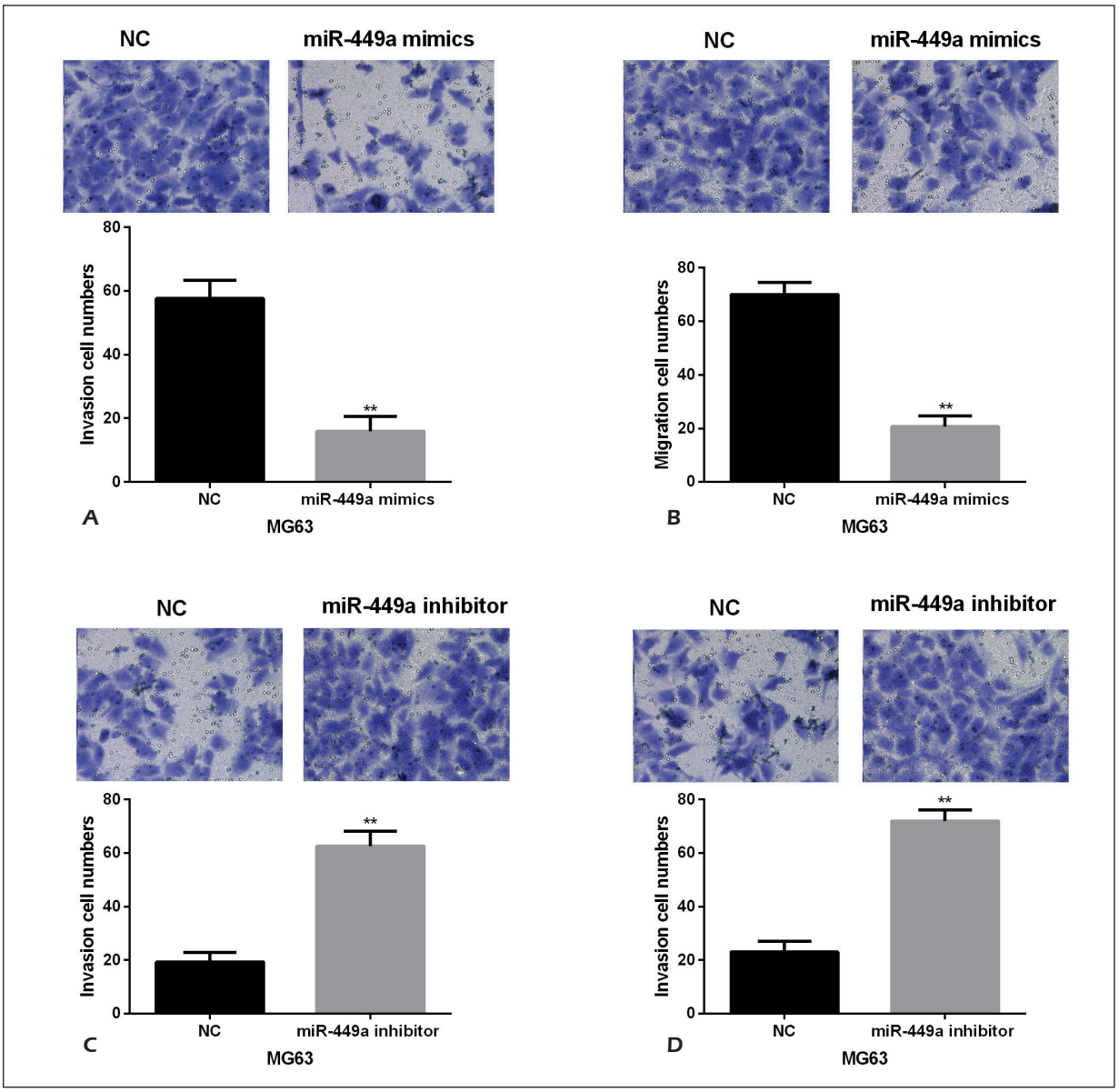

Figure 3. MiR-449a overexpression suppressed MG63 cell migration and invasion. **A-B**, The influence of miR-449a upregulation on MG63 cell migration and invasion was detected by transwell assays (magnification: 40×). **C-D**, MiR-449a inhibition facilitated MG63 cell migration and invasion (magnification: 40×). $^{**}p<0.01$.

inhibitor-transfected MG63 cells exhibited elevated invasion and migration abilities when compared to the NC-transfected cells (Figure 3C and 3D). These findings showed that miR-449a restoration exerted anti-OS functions.

MiR-449a Directly Targeted EZH2 in OS Cells

Bioinformatics analysis showed that EZH2 was the target gene of miR-449a. The predicted binding sequences were shown in Figure 4A. Then, EZH2-3'UTR-WT or EZH2-3'UTR-MUT luciferase reporter plasmid was constructed for luciferase reporter assays. The luciferase activities of OS cells treated with EZH2-3'UTR-WT was remarkably decreased by miR-449a mimics whereas luciferase activities of OS cells treated with EZH2-3'UTR-MUT had no evident changes (Figure 4B). Moreover, qRT-PCR indicated that miR-449a overexpression inhibited EZH2 expressions while miR-449a knockdown promoted EZH2 expression levels (Figure 4C and 4D). Above results suggested that miR-449a regulated EZH2 expressions in OS cells.

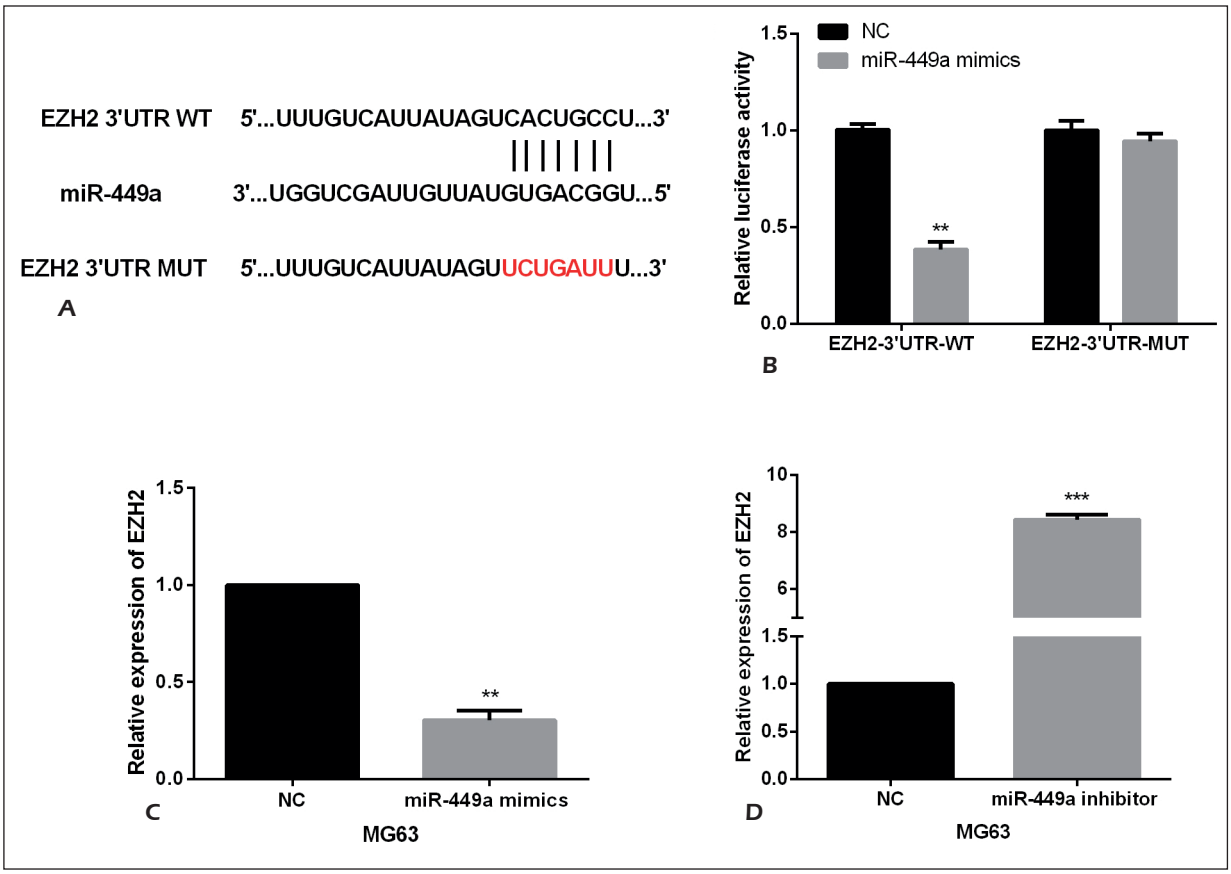

Figure 4. EZH2 was directly targeted by miR-449a in OS cell lines. **A**, The binding sites of miR-449a on the 3'UTRs of EZH2. **B**, Luciferase reporter assays were carried out to confirm the correlation between miR-449a and EZH2. **C-D**, MiR-449a restoration suppressed while miR-449a silence promoted EZH2 expressions. $^{**}p<0.01$, $^{***}p<0.001$.

MiR-449a Inhibited OS Cell PI3K/AKT Signaling Pathway and Epithelial-Mesenchymal Transition (EMT)

As EZH2 was a direct target for miR-449a, we further detected EZH2 expressions in OS tissues and cells. It demonstrated a significant increase of EZH2 expressions in OS tissues and cells (Figure 5A and 5B). Then, the prognostic value in EZH2 in OS patients was examined by Kaplan-Meier analysis. We found that OS patients with high EZH2 expressions exhibited a shorter survival time than those with low EZH2 expressions (Figure 5C). Next, Western blotting was carried out to ascertain the roles of miR-449a in OS cell PI3K/AKT signaling pathway and EMT by detecting several key molecular. Results demonstrated that the administration of MG63 cells with miR-449a mimics led to a markedly decrease in the phosphorylation level of PI3K and Akt, while there were no evident changes in the total PI3K and Akt levels (Figure 5D). It is reported that EMT is regarded as a crucial representation in cancer metastasis and proved to promote cell growth and survival. Thus, to address the molecular mechanism of miR-449a induced an anti-metastatic effect on OS cells, we detected protein levels closely related with EMT occurrence, such as the mesenchymal markers vimentin and N-cadherin, correspondingly, the epithelial marker E-cadherin. Western blot assay results showed that E-cadherin was evidently upregulated while N-cadherin and vimentin were markedly downregulated in miR-449a groups comparable to NC groups (Figure 5D).

Discussion

OS is an aggressive bone malignancy characterized by high incidence as well as poor prognosis. To decipher the pathogenesis of OS, especially the metastases, could help to improve the survival

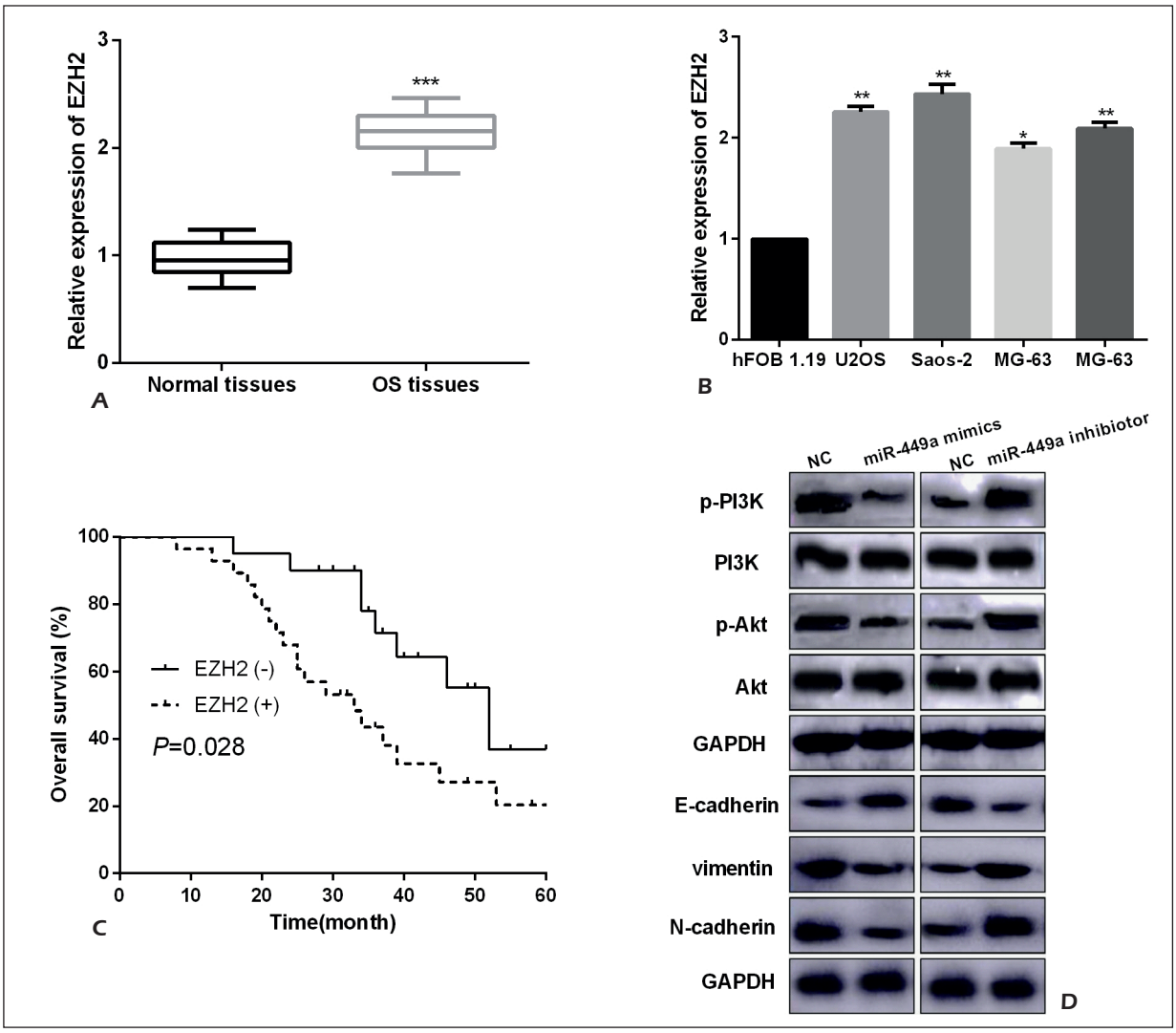

Figure 5. MiR-449a upregulation inactivated Wnt/β-catenin and EMT in OS cell lines. **A**-**B**, EZH2 expressions in OS tissues and cells were detected by qRT-PCR. **C**, High EZH2 expressions presented shorter overall survival of OS patients. **D**, MiR-449a overexpression suppressed Wnt/β-catenin and EMT in OS cell lines. **p*<0.05, ***p*<0.01, ****p*<0.001.

rates of the adolescent preferential malignancy[20]. It is a practical way to explore the mechanism by identifying differentially expressed genes. MiRNA plays pivotal roles in tumor migration, invasion, and differentiation, functioning as a key regulatory factor of cancers[21]. Many miRNAs have considered as targets for OS treatments[22]. Rongxin et al[23] found that miR-340-5p suppressed OS progression *via* binding to STAT3 through the Wnt/β-catenin; Jiashi et al[24] proposed that miR-506-3p suppressed OS cell metastasis and proliferation by downregulation of RAB3D expressions; Jianwei et al[25] reported that miR-181a suppressed apoptosis and improved viability and invasion of OS cells. Here, miR-449a was confirmed as a tumor inhibitor, being involved in OS cell proliferation, invasion, and migration.

Accumulating evidence has demonstrated that downregulated miR-449a functioned as a tumor repressor in various tumors. However, whether miR-449a plays crucial roles in OS still needs further exploration. We demonstrated significant downregulation of miR-449a in OS, and the decreased miR-449a expression indicated poor prognosis of OS patients. We also found that miR-449a levels were negatively correlated with the clinicopathologic features. Additionally, results of functional assays showed that miR-449a prominently suppressed OS cell proliferation, invasion, and migration abilities. Next, we examined the molec-

ular mechanism about the function of miR-449a and demonstrated that EZH2 was a target for miR-449a in OS cells. Previous research[26-29] showed that EZH2 acted as an important regulator in cell differentiation, cell cycle, proliferation, and migration. Data in the current study showed that EZH2 was a functional target of miR449a in OS cells and participated in the roles of miR-449a. We also found that upregulated EZH2 in OS was associated with shorter overall survival of OS patients.

EMT is one of the key steps for early-phase tumors to be transformed into aggressive malignancies, and EMT is closely related to tumor metastasis[30,31]. Characteristic reductions of E-cadherin and increase of N-cadherin are key manifestations of EMT[32]. Additionally, vimentin is also considered as one pivotal hallmark for EMT initiation [33]. Abnormal PI3K/Akt activation is recognized as a key regulator in tumor cell apoptosis and proliferation[34,35]. Actually, PI3K/Akt is frequently activated in different tumors, including gastric cancer[36], breast cancer[37], and thyroid cancer[38]. Recent studies have shown that excessive activation of PI3K/AKT could facilitate OS tumorigenesis[39]. Based on these data and theories, we measured the protein levels by Western blot, and results were consistent with the above evidence. All the results indicated that EMT and PI3K/AKT were involved in the anti-metastatic and anti-proliferative characteristics of miR-449a in OS cells.

Conclusions

Collectively, downregulation of miR-449a in OS indicated a shorter overall survival and adverse clinicopathologic characteristics of OS patients. Moreover, miR-449a upregulation significantly inhibited OS cell proliferation, invasion, and migration *via* regulating PI3K/AKT and EMT. Furthermore, EZH2 was also confirmed to be involved in the functions of miR-449a in OS cells. We also found that miR-449a restoration could suppress the *in vivo* tumor growth. So, the findings of our investigation suggested that miR-449a/EZH2 may be a therapeutic strategy for OS management.

Acknowledgments

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Conflict of Interests

The authors declare that they have no conflict of interests.

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