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Knockdown of ZEB2-AS1 inhibits cell proliferation, invasion and induces apoptosis in osteosarcoma by combining with EZH2

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Abstract. – OBJECTIVE: This study aims to explore whether ZEB2-AS1 can promote the development of osteosarcoma by affecting the proliferation, invasion, and apoptosis of osteosarcoma cells.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to detect the ZEB2-AS1 expression in osteosarcoma tissue specimens and normal bone tissues. After ZEB2-AS1 downregulation, Cell Counting Kit-8 (CCK-8) test, plate cloning assay, 5-Ethynyl-2'-deoxyuridine (EdU) experiment, and flow cytometry were conducted to analyze the changes in cell proliferation and apoptosis. RIP assay was performed to detect the binding of ZEB2-AS1 to EZH2, while Western blot was applied to examine the EZH2 expression after EZH2 was inhibited. Meanwhile, after simultaneously inhibiting ZEB2-AS1 and EZH2, the cell invasiveness was determined by transwell assay.

RESULTS: ZEB2-AS1 was highly expressed in osteosarcoma tissues, especially in advanced and metastatic groups. Interfering with ZEB2-AS1 suppressed cell proliferation and enhanced cell apoptosis. In addition, ZEB2-AS1 was confirmed to be able to combine with EZH2. The knockdown of ZEB2-AS1 attenuated the cell invasion ability, which was further decreased after the simultaneous downregulation of ZEB2-AS1 and EZH2.

CONCLUSIONS: The long non-coding RNA, ZEB2-AS1, enhanced the proliferation and invasion of osteosarcoma cells and inhibited the cell apoptosis by combining with EZH2, and thereby promoted the development of osteosarcoma.

Key Words:
Osteosarcoma, ZEB2-AS1, EZH2.

Introduction

Osteosarcoma is currently the most common primary bone malignancy, which mainly occurred in the metaphyseal region with abundant blood supply, especially in the lower femur and the upper tibia. Pulmonary metastasis is the main cause of death because of its early hematogenous metastasis[1], and there is no reliable therapy at present. With the development of neoadjuvant chemotherapy, the 5-year survival rate of osteosarcoma patients receiving surgery combined with neoadjuvant chemotherapy can reach 60%-70%. However, drug resistance, tumor metastasis, and recurrence are still urgent problems that need to be solved[2]. Therefore, the searches for effective early diagnosis methods, biomarkers, and treatment strategies for osteosarcoma are of great importance for improving patients' survival rate and overall treatment efficiency.

Long non-coding RNAs (lncRNAs) widely distributed in the body plays a wide range of biological roles[3]. A variety of lncRNAs have been found to be closely associated with the progression of osteosarcoma. Notably, lncRNA MALAT1 was upregulated in osteosarcoma tissue[4]. It is also reported[5] that MEG3 expression was negatively correlated with the clinical stage and distant metastasis rate of osteosarcoma, and MEG3 might act as an independent risk factor affecting the prognosis of osteosarcoma patients. LncRNA ZEB2-AS1 was first identified in bladder cancer[6] and was involved in transforming growth factor 1-induced epithelial-mesenchymal transformation (EMT) by reducing the level of ZEB2 protein. However, currently, there are still no reports on the correlation between ZEB2-AS1 and osteosarcoma.

EZH2, located on human chromosome 7q35.7q36, is an important member of the polycomb group (PcG) gene family[7]. EZH2 could methylate nucleosomal histones to inhibit the transcription of tumor suppressor genes[8-10]. EZH2 is highly expressed in various tumors, including TN-

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BC and salivary gland ACC, and indicated poor prognosis. However, no studies on EZH2 related to osteosarcoma have been reported. Whether EZH2 expression could be affected by ZEB2-AS1 has not been reported. To provide new ideas for clinical diagnosis and treatment, we investigated whether ZEB2-AS1 could combine with ZEB2-AS1 to affect the occurrence and development of osteosarcoma.

Patients and Methods

Clinical Specimen Collection and Cell Culture

The human osteosarcoma tissue and the corresponding normal adjacent bone tissue were collected from the surgical resection specimens of patients with osteosarcoma treated by the Department of Orthopaedic Surgery. All clinical specimens included in the study were used only for experimental research and were approved by the Hospital Ethics Committee. All patients signed informed consent before surgery. Fresh specimens were preserved in liquid nitrogen for RNA extraction.

Cell Culture

hFOB, Saos-2, U2OS, and MG63 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) complete medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Rockville, MD, USA) at 37°C with 5% carbon dioxide and saturated humidity.

Cell Transfection

Totally 5×10^4 cells in the logarithmic growth phase were plated in 6-well plate and cultured for 24 h. When the cell density reached 70%-80%, sh-ZEB2-AS1 1# sh-ZEB2-AS1 2#, sh-ZEB2-AS1 3#, sh-EZH2, sh-ZEB2-AS1+sh-EZH2 were transfected into human osteosarcoma cell lines, including U20S and MG63 respectively following the procedure of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 6-8 h of incubation in a cell culture incubator, fresh complete medium was added, and incubation was continued for 24 h. (sh-ZEB2-AS1 1 # 5'-GCCTCGAGGATTAGTTTAAAC-3', sh-ZEB2-AS1 2# 5'-GCTCTACTAAATGATC-GTATC-3'; sh-EZH2: 5'-GGAUGGUACUUU-CAUUGAATT-3').

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

Tissue or cell total RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA), and 1 μg of the total RNA was subjected to reverse transcription to obtain complementary deoxyribose nucleic acid (cDNA). Gene expression levels were determined using the SYBR Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan) according to the instructions. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$. The primer sequences are as follows: ZEB2-AS1 F, 5'-ATGAAGAAGCCGCGAAGTGT-3', R, 5'-CACACCCTAATACACATGCCCT-3'; GAPDH F, 5'-ACCACAGTCCATGCCATCAC-3', R, 5'-TCCACCCTGTTGCTGTA-3'.

Cell Counting Kit-8 (CCK-8) Experiment

After transfection, the cells were collected and prepared into suspension with 3×10^4 cells/mL using a complete medium. Then, 100 μL cell suspension/well was added in a 96-well plate. After the routine incubation for 6, 24, 48, 72, and 96 h, 10 μL of CCK-8 reagent (Dojindo, Molecular Technologies, Kumamoto, Japan) was added to each well. The cells were continuously incubated for 2 h at 37°C. The optical density (OD) value of each well was measured at 450 nm by a microplate reader.

Cell Cloning Technology

The transfected cells of each group were seeded in 6-well plates at 500 cells/well. The cells were routinely cultured for 7-10 d, and the culture was terminated when the macroscopic clones appeared. The culture solution was discarded, and cells were fixed by 4% paraformaldehyde solution, and stained with 0.5% crystal violet. Finally, they were counted under an optical microscope.

Flow Cytometry

The collected cells were centrifuged at 1 000 rpm for 5 min, washed twice with phosphate-buffered saline (PBS), and resuspended in 200 μL of 1×binding buffer. A total of 5 μL of Annexin V-FITC (fluorescein isothiocyanate) was added into each sample protecting from light, which was then incubated for 15 min at room temperature. Subsequently, 5 μL of Propidium Iodide (PI) was used to stain cells was added 5 min before detection, and 200 μL of 1× Binding Buffer was added to terminate the reaction.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The cells were seeded in 96-well plates at a density of 300 cells/well and incubated for 48 h in a 37°C incubator. After 24 h, the cells were fixed with 4% formaldehyde for 30 min and permeabilized by treatment with 0.5% Triton X-100 for 10 min at room temperature. Subsequently, the cells were stained with EdU (red) for 1 h, and then counterstained with Hoechst 33342 (blue) for 30 min. After staining, the cells were placed under a fluorescence microscope and five fields were randomly selected to count the EdU-positive cells. The magnification was 100×.

Transwell Assay

Matrigel was melted at 4°C in advance, and 40 μL of diluted Matrigel gel (1:3 ratio of Matrigel to serum-free medium) was then placed on the surface of each polycarbonate microporous membrane and placed in an incubator for 4 h. MG-63 cells in the logarithmic growth phase were cultured in serum-free IMDM culture medium for 24 h. After digested with 0.25% EDTA (ethylenediaminetetraacetic acid) trypsin, the cells were suspended at 4×10^5/mL using a serum-free IM-DM medium. Totally, 200 μL cell suspension was added to the upper chamber with 3 replicate wells set in each group. Another 600 μL of DMEM containing 10% fetal bovine serum was added to the lower chamber, which was placed in an incubator for 24 h. After washing with PBS twice, the cells were fixed with methanol for 20 minutes. After air-dried at room temperature, the cells were further stained with crystal violet for 20 min. Lastly, the cells were observed under an inverted microscope, and the number of cells in the middle of the field and five high (40 times) microscopic fields were counted.

Western Blot Assay

The treated adherent cells were washed with PBS, transferred to an Eppendorf (EP, Hamburg, Germany) tube, and centrifuged at 12 000 r/min for 5 min. The collected cells were added to the cell lysate for lysis. The extracted protein was separated by sodium dodecyl sulphate (SDS) electrophoresis before transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked in 5% skim milk for 1 h. The rabbit anti-human Twist (1:750) and polyclonal rabbit anti-human β-actin (1:2 000) were incubated with the membrane at 4°C overnight. The next day, horseradish enzyme-labeled goat anti-rabbit IgG was used to incubate the membrane for 1 h at room temperature. Subsequently, the enhanced chemiluminescence (ECL) indicator was added on the membrane, and the protein blot was developed with an X-ray film.

Statistical Analysis

The data were statistically processed using Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA), and the difference between the two groups was compared by the *t*-test. $p<0.05$ was considered statistically significant: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

High Expression of ZEB2-AS1 in Osteosarcoma

QRT-PCR detection indicated that ZEB2-AS1 was highly expressed in patients with osteosarcoma (Figure 1A). Besides, we found that the expression of ZEB2-AS1 in osteosarcoma

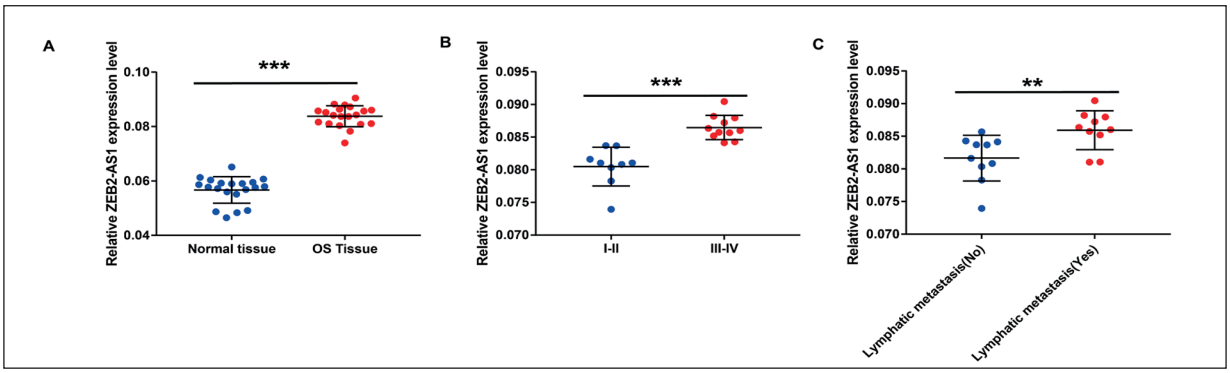

Figure 1. ZEB2-AS1 expression was increased in osteosarcoma tissues. **A,** ZEB2-AS1 was highly expressed in osteosarcoma tissue. **B,** ZEB2-AS1 expression was higher in the advanced phase than in the early group. **C,** ZEB2-AS1 expression was higher in the metastatic group than the non-transfer group.

tissues of stage III/IV was significantly higher than that of stage I/II (Figure 1B). We further detected the expression level of ZEB2-AS1 in tumor tissues of patients with or without metastasis, and it turned out that the ZEB2-AS1 level was significantly higher in the former than in the latter (Figure 1C). These results suggested that the expression level of ZEB2-AS1 might be associated with the development of osteosarcoma.

Knock-down of ZEB2-AS1 Inhibited Osteosarcoma Cell Proliferation and Induced Cell Apoptosis

To further explore the association between ZEB2-AS1 and osteosarcoma, we further examined the expression level of ZEB2-AS1 in different osteosarcoma cell lines. ZEB2-AS1 expression in osteosarcoma cell lines (Saos-2, U2OS, and MG63) was significantly higher than that in the normal osteoblast cell line hFOB (Figure 2A). Here, we chose U2OS and MG63 cell lines for subsequent experiments. We constructed a total of three ZEB2-AS1 interference sequences and found that ZEB2-AS1 1# and 2# successfully reduced the ZEB2-AS1 level in MG63 and U2OS (Figure 2B). CCK-8 and cell plate cloning assays results revealed that interference with ZEB2-AS1 significantly inhibited cell proliferation and clonal ability of MG63 and U2OS cell lines (Figures 2C, 2D, 2E). At the same time, flow cytometry results indicated that the downregulation of ZEB2-AS1 could induce cell apoptosis (Figure 2F). The above results suggested that interference with ZEB2-AS1 remarkably inhibited the osteosarcoma cell proliferation while inducing cell apoptosis.

Knockdown of ZEB2-AS1 Inhibited Osteosarcoma Cell Proliferation

To further explore the relationship between ZEB2-AS1 and osteosarcoma cell proliferation, we performed an EdU experiment. After transfecting sh-ZEB2-AS1 in MG63 and U2OS cells, the number of proliferating cells was significantly reduced (Figure 3), indicating that ZEB2-AS1 could promote osteosarcoma development.

ZEB2-AS1 Promoted Osteosarcoma Cell Invasion Via Binding to EZH2

To explore the mechanism of ZEB2-AS1 in promoting the development of osteosarcoma, we performed the RNA Immunoprecipitation (RIP) to confirm the binding relationship between

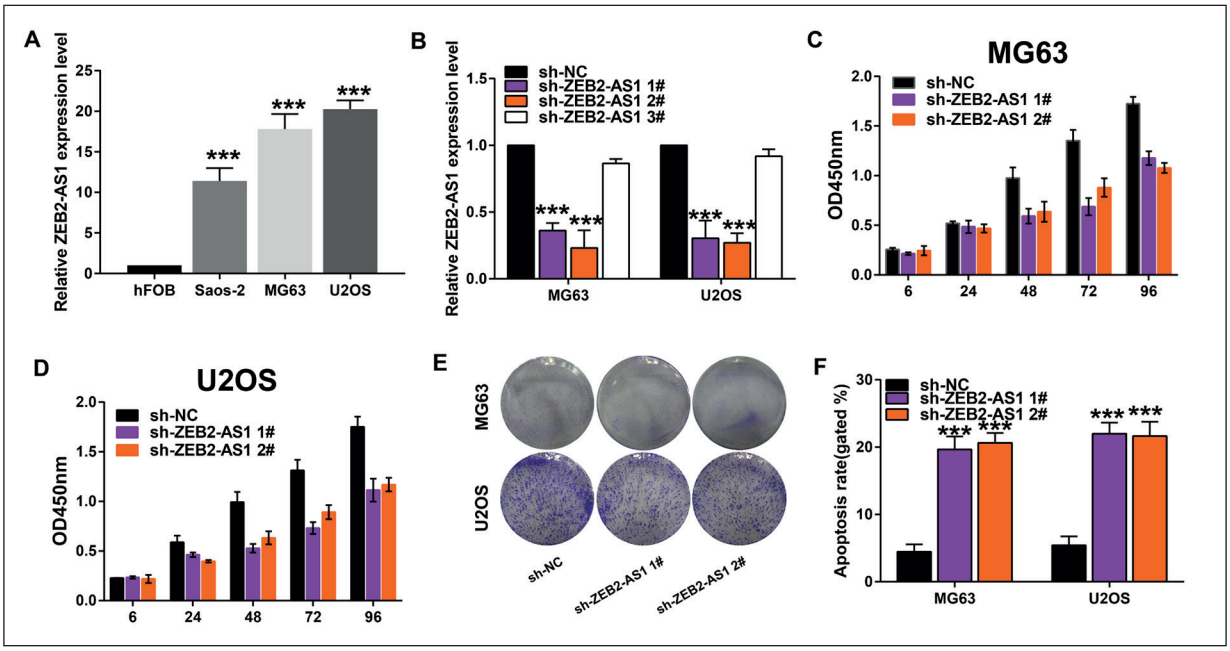

Figure 2. Interference with ZEB2-AS1 inhibited osteosarcoma proliferation and induced apoptosis. **A,** ZEB2-AS1 expression was higher in osteosarcoma cell lines than normal osteoblast lines. **B,** Construction of the ZEB2-AS1 interference sequence. **C,** and **D,** CCK-8 results indicated that interference with ZEB2-AS1 significantly inhibited the proliferation of MG63 and U2OS cells. **E,** Plate cloning results indicated that interference with ZEB2-AS1 significantly inhibited the proliferation of MG63 and U2OS cells (20×). **F,** Interference with ZEB2-AS1 could induce cell apoptosis.

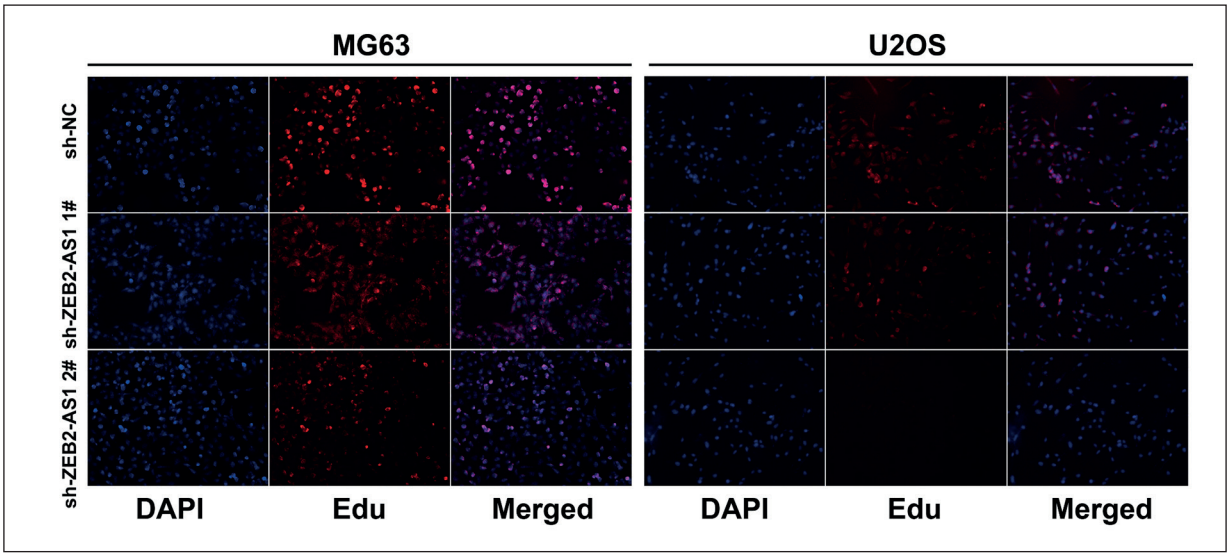

Figure 3. Knockout ZEB2-AS1 inhibited osteosarcoma cell proliferation. The Edu experiment showed that the cell proliferation ability was significantly down-regulated after transfection of sh-ZEB2-AS1 (100×).

ZEB2-AS1 and EZH2 (Figure 4A). The interference efficiency was shown in Figure 4B. The results indicated that the invasive ability of cells was significantly downregulated after ZEB2-AS1 inhibition, and further decreased after co-transfection of sh-ZEB2-AS1 and sh-EZH2 (Figure 4C). These results indicate that EZH2 may play a key regulatory role in the progression of osteosarcoma promoted by ZEB2-AS1.

Discussion

Osteosarcoma, originating from the fibrous malignant bone tumor, can directly or indirectly form tumor osteoid tissue and bone tissue during its development[11]. The early clinical symptoms of osteosarcoma are not typical, and it is easy to be confused with other traumatic pain diseases. In addition, its invasive growth and rapid early proliferation lead to

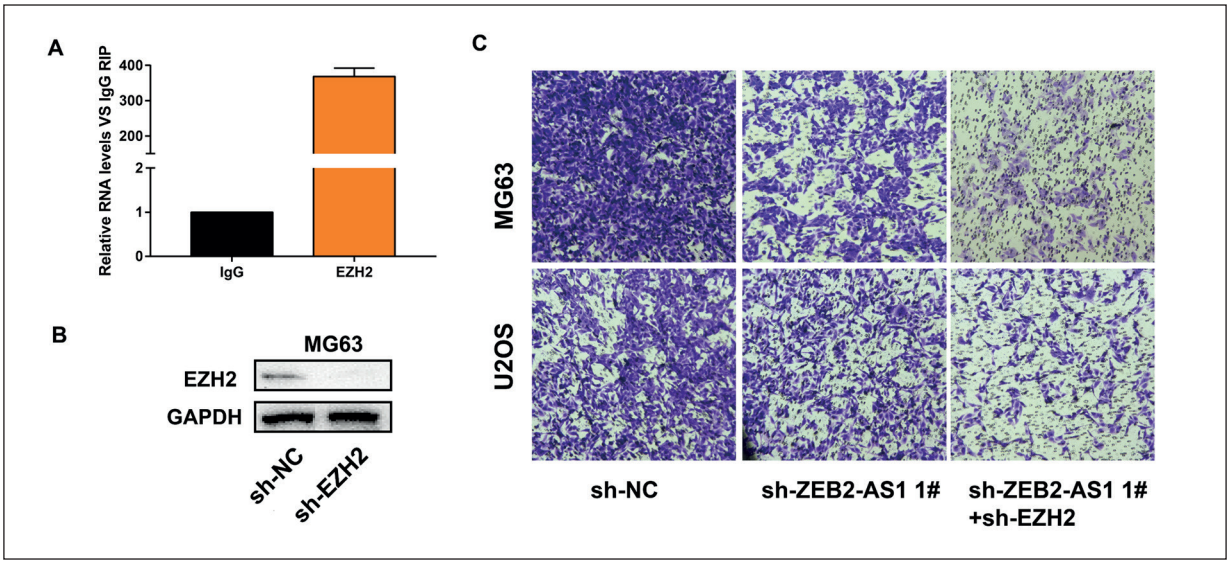

Figure 4. ZEB2-AS1 combined with EZH2 promoted osteosarcoma cell invasion. **A,** The RIP experiment results showed that ZEB2-AS1 could be combined with EZH2. **B,** After interfering with EZH2, its protein level was significantly reduced. **C,** The transwell experiment showed that the invasive ability of cells transfected with sh-ZEB2-AS1 was significantly downregulated, and the cell invasion was further decreased after simultaneous transfection of sh-ZEB2-AS1 and sh-EZH2 (40×).

the poor cure rate and prognosis[12]. The occurrence, development, and biological characteristics of osteosarcoma are the results of the combined action of multiple genes and various abnormal factors[13,14]. Therefore, the study of the gene-level changes is of great significance for us to understand the occurrence and development of osteosarcoma.

Long non-coding RNA refers to a group of transcripts protein-coding ability[15]. With the development and improvement of human genomics, a large number of long-fragment non-coding RNAs have been discovered through deep sequencing and high-throughput chip screening technology. Recently, the role of lncRNAs and their significance in life activities and disease progression have been gradually revealed[16]. Abnormal regulation of lncRNAs could affect epigenetic information, cause abnormal growth of cells, and thereby lead to the occurrence and development of tumors[17]. Lan et al[18] found that lncRNA ZEB2-AS1 was highly expressed in liver cancer tissues and correlated with poor prognosis of tumors. Downregulation of lncRNA ZEB2-AS1 could reduce the proliferation and metastasis of liver cancer cells. All these studies indicated that lncRNA ZEB2-AS1 might have oncogene-like effects. In our study, we also found that ZEB2-AS1 expression was remarkably increased in the osteosarcoma, and ZEB2-AS1 could promote the occurrence and progression of osteosarcoma.

LncRNA can affect the occurrence and development of osteosarcoma in a variety of ways. LncRNA PVT1 negatively regulated microRNA-195 to accelerate the osteosarcoma cell cycle while inhibiting cell apoptosis, and thus mediated the malignant cell proliferation and metastasis[19]. Moreover, lncRNA ZEB2-AS1 can activate ZEB2 through epigenetics to promote the proliferation, metastasis, and epithelial-mesenchymal transformation of breast cancer cells[20]. Consistently, we found that ZEB2-AS1 could bind to ZEB2. Besides, the invasive ability of the cells was further reduced, when ZEB2-AS1 and EZH2 were simultaneously inhibited, which suggested that ZEB2-AS1 could further promote the regulation of osteosarcoma by acting on ZEB2. Our results might provide a new idea and direction for clinical studies of osteosarcoma.

Conclusions

We first detected that ZEB2-AS1 could affect the proliferation, invasion, and apoptosis of osteosarcoma cells by targeting ZEB2, thus promoting the occurrence and development of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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