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Knockdown of Long Noncoding RNA CAT104 Inhibits the Proliferation, Migration, and Invasion of Human Osteosarcoma Cells by Regulating MicroRNA-381

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Osteosarcoma is the most common primary malignant bone tumor in children and adolescents. This study aimed to explore the effects of long noncoding RNA CAT104 and microRNA-381 (miR-381) on osteosarcoma cell proliferation, migration, invasion, and apoptosis, as well as the underlying potential mechanism. We found that CAT104 was highly expressed in osteosarcoma MG63 and OS-732 cells. Knockdown of CAT104 significantly inhibited OS-732 cell proliferation, migration, and invasion, but promoted cell apoptosis. CAT104 regulated the expression of miR-381, and miR-381 participated in the effects of CAT104 on OS-732 cells. Zinc finger E-box-binding homeobox 1 (ZEB1) was a direct target gene of miR-381, which was involved in the regulatory roles of miR-381 in OS-732 cell proliferation, migration, invasion, and apoptosis, as well as c-Jun N-terminal kinase (JNK) and Wnt/ -catenin pathways. In conclusion, our research verified that suppression of CAT104 exerted significant inhibitory effects on osteosarcoma cell proliferation, migration, and invasion by regulating the expression of miR-381 and downstream ZEB1, as well as JNK and Wnt/ -catenin pathways.

Key words: Osteosarcoma; Long noncoding RNA CAT104; MicroRNA-381; JNK pathway; Zinc-finger E-box-binding homeobox 1 (ZEB1); Wnt/β-catenin pathway

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents, which derives from primitive bone-forming mesenchymal cells[1]. The main clinical symptoms of osteosarcoma are pain, swelling, redness, and dysfunction of bone in localized areas[2,3]. With the development of multiple therapeutic strategies, such as wide tumor resection, adjuvant chemotherapy, and radiotherapy, the 5-year survival rate of nonmetastatic patients has increased from 20% to 70%[4]. However, the prognosis of patients with metastatic tumor is very poor, and the 5-year survival rate is only 20–30%, which has not improved in recent years[5,6]. Considering that the pathogenesis of osteosarcoma is very complex[7,8], it is worth believing that a more clear understanding of the pathogenesis of osteosarcoma will be helpful in defining effective therapeutic targets and strategies for osteosarcoma treatment.

Long noncoding RNAs (lncRNAs) are a class of RNA transcripts in eukaryotic cells with more than 200 nucleotides in length and no protein-coding capacity[9]. Emerging evidence has suggested that lncRNAs can serve as gene regulators capable of regulating the expression of protein coding and noncoding genes[10,11]. Similar to proteins, lncRNAs have important biological functions in the regulation of a variety of cellular functions and disease processes including cell proliferation, cell differentiation, cell apoptosis, neurogenesis, and carcinogenesis[12,13]. For example, Pandey et al. revealed that the risk-associated lncRNA neuroblastoma-associated transcript 1 (NBAT-1) regulated the progression of neuroblastoma by controlling cell proliferation and neuronal differentiation[14]. Cheng et al. indicated that lncRNA homeobox A (HOXA) transcript at the distal tip (HOTTIP) enhanced pancreatic cancer cell proliferation, survival, and migration[15]. Furthermore, Sun et al. demonstrated that downregulation

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of lncRNA maternally expressed gene 3 (MEG3) was associated with poor prognosis in gastric cancer[16]. In terms of osteosarcoma, Uzan et al. pointed out that high expression of lncRNA highly upregulated in liver cancer (HULC) was associated with poor prognosis in osteosarcoma patients[17]. Dong et al. suggested that lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) promoted the proliferation and metastasis of osteosarcoma cells by activating the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway[18]. Zhang et al. reported that downregulation of lncRNA taurine-upregulated 1 (TUG1) inhibited osteosarcoma cell proliferation and promoted cell apoptosis[19]. More experimental research is still needed to further explore the regulatory effects of lncRNAs on osteosarcoma.

A previous study indicated that lncRNA CAT104 was highly expressed in breast cancer and could be used as an independent prognostic biomarker[20]. lncRNAs can regulate the expressions of microRNAs in eukaryotic cells[21,22]. MicroRNAs are another type of noncoding RNAs with 20–24 nucleotides in length[23]. MicroRNA-381 (miR-381) exerts tumor-suppressive effects on colorectal cancer, ovarian cancer, renal cancer, oral squamous cell carcinoma, and breast cancer[24–28]. However, there is no information available about the regulatory effects of CAT104 on osteosarcoma cell proliferation and metastasis, as well as the expression of miR-381 in osteosarcoma cells.

Therefore, in the present study, we aimed to explore the effects of CAT104 on osteosarcoma cell proliferation, migration, invasion, and apoptosis, as well as the expression of miR-381. The possible internal molecular mechanisms and signaling pathways were also investigated. Our findings will be helpful in further understanding the pathogenesis of osteosarcoma and provide a possible therapeutic target for osteosarcoma treatment.

MATERIALS AND METHODS

Cell Lines

Human osteosarcoma cell lines MG63 and OS-732, human osteoblast cell line hFOB1.19, and human embryonic kidney cell line HEK293 were all obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Life Technologies, Carlsbad, CA, USA), 1% penicillin–streptomycin mixture solution (Beyotime Biotechnology, Shanghai, P.R. China), and 1 mM L-glutamine (Sigma-Aldrich). Cells were incubated in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2.

Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA in OS-732 cells was extracted using RiboPure™ RNA Purification Kit (Invitrogen) according to the manufacturer's instruction. The cDNA was reversely transcribed using GoScript™ Reverse Transcriptase System (Promega, Madison, WI, USA) in line with the supplier's instruction. The TaqMan® Noncoding RNA Assay (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR analysis to detect the expression of CAT104. The TaqMan™ OpenArray™ Real-Time PCR Master Mix (Applied Biosystems) was performed to measure the expression of miR-381. For testing the expression of zinc finger E-box-binding homeobox 1 (ZEB1), Power SYBR Green Master Mix (Applied Biosystems) was used. All experiments were performed three times, and the relative expression level was calculated using the $2^{-\ Ct}$ method[29]. The expression of GAPDH acted as the internal control for testing the expressions of CAT104 and ZEB1. The expression of U6 acted as the internal control for testing the expression of miR-381.

Cell Transfection and Generation of Stably Transfected Cell Lines

Short hairpin RNA-targeting CAT104 was ligated into U6/GFP/Neo plasmid (GenePharma, Shanghai, P.R. China) and referred to as sh-CAT104. miR-381 mimic, miR-381 inhibitor, and their respective negative controls (Scramble, NC) were synthesized by Life Technologies Corporation. The full-length ZEB1 sequence was constructed into Pex-2 plasmid (GenePharma) and referred to as pEX-ZEB1. si-ZEB1 was designed and synthesized by Life Technologies Corporation. Cell transfection was performed using Lipofectamine 3000 reagent (Life Technologies Corporation) according to the manufacturer's protocol. Transfection efficiency was detected using RT-qPCR.

Cell Viability Assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; Thermo Fisher Scientific) in line with the manufacturer's instruction. Briefly, transfected or nontransfected OS-732 cells were placed, in triplicate, in a 96-well plate (Corning Incorporated, New York, NY, USA) with a density of 1 10^4 cells/well and cultured at 37°C for 24 h. Then MTT solution (10 µl, 5 mg/ml) was added into each well, and the mixture was incubated at 37°C for 4 h. After that, MTT mixture was removed, and 150 µl of dimethyl sulfoxide (DMSO) was added into each well. Samples were agitated on a shaker for 15 min, and the absorbance at 570 nm was recorded using a microplate reader (Bio-Tek, Winooski, VT, USA). Cell viability (%) was calculated by average

absorbance of transfected group/average absorbance of nontransfected (control) group 100%.

Cell Migration and Invasion Assay

Cell migration was detected using a modified two-chamber migration assay (Corning Incorporated) with a pore size of 8 μm. Briefly, transfected or nontransfected OS-732 cells (1 10^3) were resuspended in 200 μl of serum-free DMEM and added into the upper chamber. Complete DMEM (600 μl) was added into the lower chamber. After incubation at 37°C for 48 h, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) immediately. Nontraversed cells in the upper chamber were removed using a cotton swab, and traversed cells in the lower chamber were counted under a microscope (Nikon, Tokyo, Japan). Cell migration (%) was calculated by traversed cells in transfected group/traversed cells in nontransfected (control) group 100%.

Cell invasion was measured similarly to that for cell migration except that the Transwell membrane was precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

Cell Apoptosis Assay

Cell apoptosis was detected using a Guava Nexin Kit (Guava Technologies, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, transfected or nontransfected OS-732 cells were placed, in triplicate, in a 24-well plate (Corning Incorporated) with a density of 3 10^4 cells/well and cultured at 37°C for 24 h. Then cells were harvested, washed twice with phosphate-buffered saline (PBS), and mixed with 100 μl of Guava solution for 30 min at 37°C in the dark. Cell apoptosis was recorded using a Guava EasyCyte flow cytometer (Guava Technologies). Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA, USA).

Dual-Luciferase Reporter Activity Assay

The 190–195 position of the sequence of CAT104 and 3 -untranslated region (3 -UTR) fragment of ZEB1, containing the predicted miR-381 binding sites, were amplified by PCR and inserted into pmirGLO vectors (Promega) to form the reporter vectors: CAT104-wild type (CAT104-wt) and ZEB1-wt. To mutate the predicted miR-381 binding site in the fragment of CAT104 and 3 -UTR fragment of ZEB1, the predicted binding sites were replaced, amplified, and also inserted into pmirGLO vectors (Promega) to form the reporter vectors: CAT104-mutated type (CAT104-mt) and ZEB1-mt. After that, the reporter vectors and miR-381 mimic were transfected into HEK293 cells simultaneously. The Dual-Luciferase Reporter Assay System (Promega) was used to measure the luciferase activity.

Western Blotting

Transfected or nontransfected OS-732 cells were placed, in triplicate, in a six-well plate (Corning Incorporated) with a density of 1 10^5 cells/well and cultured at 37°C for 24 h. Then cells were harvested and washed twice with PBS. Total protein in the cells was isolated using M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). The protein concentrations were quantified using Pierce™ Rapid Gold Protein Assay Kit (Thermo Fisher Scientific). The Western blotting system was established using Bio-Rad Bis-Tris Gel System (Bio-Rad Laboratories, Hercules, CA, USA) in line with the manufacturer's protocol. Proteins in equal concentration were electrophoresed on polyacrylamide gels and transferred onto polyvinylidene difluoride (PDVF) membranes (Millipore, Bedford, MA, USA). After incubation with 5% bovine serum albumin (BSA; Beyotime) for 1 h at room temperature, the PVDF membranes were incubated with primary antibodies overnight. All primary antibodies were diluted in 1% BSA solution at a dilution of 1:1,000. B-cell lymphoma-2 (Bcl-2; #15071), Bcl-2-associated X (Bax; #5023), caspase 3 (#9662), caspase 9 (#9508), c-Jun N-terminal kinase (JNK; #9252), phospho-JNK (p-JNK; #9255), c-Jun (#9165), phospho-c-Jun (p-c-Jun; #9164), Wnt5 (#2530), -catenin (#9562), and GAPDH (#5174) were purchased from Cell Signaling Technology (Beverly, MA, USA). ZEB1 (sc-81428) and Wnt3 (sc-74537) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Subsequently, the PVDF membranes were washed three times with Tris-buffered saline and Tween (TBST; Sigma-Aldrich) and incubated with anti-mouse (rabbit) IgG (H+L) DyLight™ 680 Conjugate (#5470, #5366; Cell Signaling Technology) for 1 h at room temperature. The protein signals were recorded using the Odyssey System (Licor Biosciences, Bad Homburg, Germany).

Statistical Analysis

All the above experiments were repeated three times. Data were expressed as mean±standard deviation (SD). GraphPad 6.0 software (GraphPad, San Diego, CA, USA) was used to determine statistical analysis. Statistical comparisons were calculated using one-way analysis of variance (ANOVA) or Student's test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

CAT104 Was Highly Expressed in Osteosarcoma Cells

The expression levels of CAT104 in human osteoblast hFOB1.19 cells and human osteosarcoma MG63 and OS-732 cells were detected using RT-qPCR. As displayed in Figure 1, CAT104 was highly expressed in

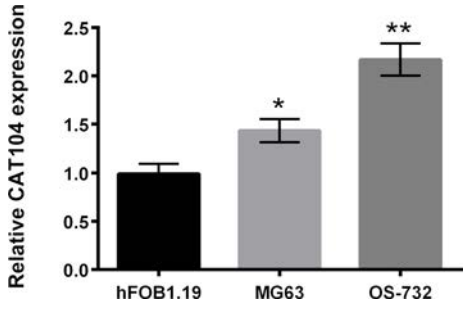

Figure 1. CAT104 was highly expressed in osteosarcoma cells. The relative expression of CAT104 in the human osteoblast cell line hFOB1.19 and the human osteosarcoma cell lines MG63 and OS-732 was detected using RT-qPCR. CAT104, long non-coding RNA CAT104; RT-qPCR, reverse transcription quantitative PCR. *$p<0.05$, **$p<0.01$.

human osteosarcoma MG63 and OS-732 cells compared to human osteoblast hFOB1.19 cells ($p<0.05$ or $p<0.01$). This finding suggested that the upregulated expression of CAT104 might participate in the carcinogenesis of human osteosarcoma cells. Considering that the OS-732 cells had a higher expression of CAT104 than the MG63 cells, OS-732 cells were chosen for subsequent experiments.

Knockdown of CAT104 Inhibited Osteosarcoma Cell Proliferation, Migration, and Invasion, but Promoted Cell Apoptosis

To explore the functional roles of CAT104 in osteosarcoma cell proliferation, migration, invasion, and apoptosis, sh-CAT104 was transfected into OS-732 cells to downregulate the expression of CAT104. Figure 2A

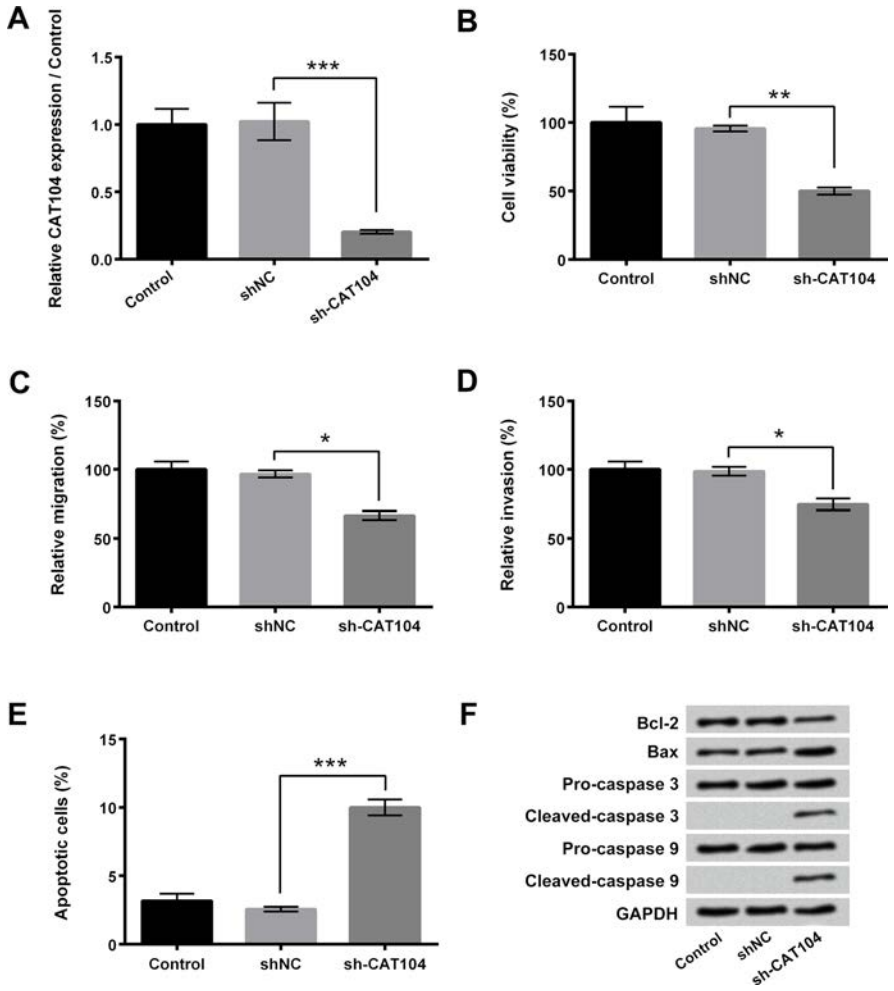

Figure 2. Knockdown of CAT104 inhibited osteosarcoma cell proliferation, migration, and invasion but promoted cell apoptosis. (A) Relative expression of CAT104 in OS-732 cells after transfection with sh-CAT104 was measured using RT-qPCR. (B) Viability, (C) migration, (D) invasion, and (E) apoptosis of OS-732 cells after sh-CAT104 transfection were determined using MTT assay, two-chamber migration invasion assay, and Guava Nexin assay, respectively. (F) Western blotting was performed to analyze the expressions of Bcl-2, Bax, caspase 3, and caspase 9 in OS-732 cells after sh-CAT104 transfection. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; NC, negative control. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

presented that the expression level of CAT104 in OS-732 cells was significantly decreased after sh-CAT104 transfection ($p<0.001$). Figure 2B–D showed that knockdown of CAT104 remarkably inhibited the viability, migration, and invasion of OS-732 cells ($p<0.05$ or $p<0.01$). On the contrary, the rate of apoptotic cells in the sh-CAT104 transfection group was distinctly increased ($p<0.001$) (Fig. 2E). Western blotting displayed that the expression level of Bcl-2 in OS-732 cells after sh-CAT104 transfection was decreased and that the expression levels of Bax, cleaved caspase 3, and cleaved caspase 9 in OS-732 cells after sh-CAT104 transfection were obviously increased (Fig. 2F). The above results indicated that knockdown of CAT104 significantly inhibited osteosarcoma cell proliferation, migration, and invasion, but promoted cell apoptosis.

Knockdown of CAT104 Upregulated the Expression of miR-381 in Osteosarcoma Cells

Bioinformatics analysis found that CAT104 could reversely bind to miR-381. The potential binding sequences are shown in Figure 3A. Results in Figure 3B showed that sh-CAT104 transfection significantly upregulated the expression level of miR-381 in OS-732 cells ($p<0.001$). Moreover, Figure 3C displayed that the relative luciferase activity was remarkably decreased after cotransfection with CAT104-wt and miR-381 mimic ($p<0.01$). These above findings implied that miR-381 might be involved in the regulatory roles of CAT104 in osteosarcoma cell proliferation, migration, invasion, and apoptosis.

Suppression of miR-381 Reversed the Effects of CAT104 Knockdown on Osteosarcoma Cells

To confirm the effects of miR-381 on CAT104 knockdown-induced osteosarcoma cell proliferation, migration, and invasion inhibition, as well as cell apoptosis enhancement, miR-381 inhibitor was transfected into OS-732 cells. Figure 4A showed that miR-381 inhibitor transfection remarkably reversed the sh-CAT104 transfection-induced cell viability decrease ($p<0.05$). Figure 4B and C presented that miR-381 inhibitor transfection significantly reversed the sh-CAT104 transfection-induced cell migration and invasion inhibition ($p<0.05$ or $p<0.01$). Moreover, Figure 4D displayed that miR-381 inhibitor obviously alleviated the sh-CAT104 transfection-induced OS-732 cell apoptosis ($p<0.01$ or $p<0.001$). Compared to the single sh-CAT104 transfection group, the expression level of Bcl-2 in sh-CAT104 + miR-381 inhibitor transfection group was increased, and the expression levels of Bax, cleaved caspase 3, and cleaved caspase 9 were decreased (Fig. 2E). The above results pointed out that miR-381 was involved in the regulatory effects of CAT104 on osteosarcoma cells and that suppression of miR-381 reversed the CAT104 knockdown-induced osteosarcoma cell proliferation, migration, and invasion inhibition, as well as cell apoptosis enhancement.

ZEB1 Was a Target Gene of miR-381 in Osteosarcoma Cells

Bioinformatics analysis found that miR-381 could reversely bind to the 3 -UTR of ZEB1. The potential

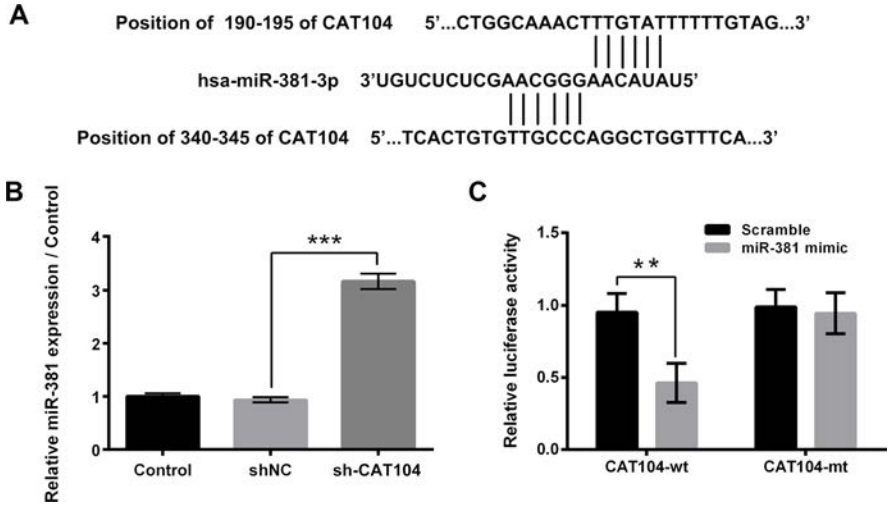

Figure 3. Knockdown of CAT104 upregulated the expression of miR-381 in osteosarcoma cells. (A) Bioinformatics analysis was used to predict the potential binding sequences between CAT104 and miR-381. (B) The relative miR-381 expression in OS-732 cells after sh-CAT104 transfection was measured using RT-qPCR. (C) Dual-luciferase reporter activity was performed to determine the relative luciferase activity after cotransfection with miR-381 mimic and CAT104-wt or CAT104-mt. miR-381, microRNA-381, wt, wild type; mt, mutated type. $^{**}p<0.01$, $^{***}p<0.001$.

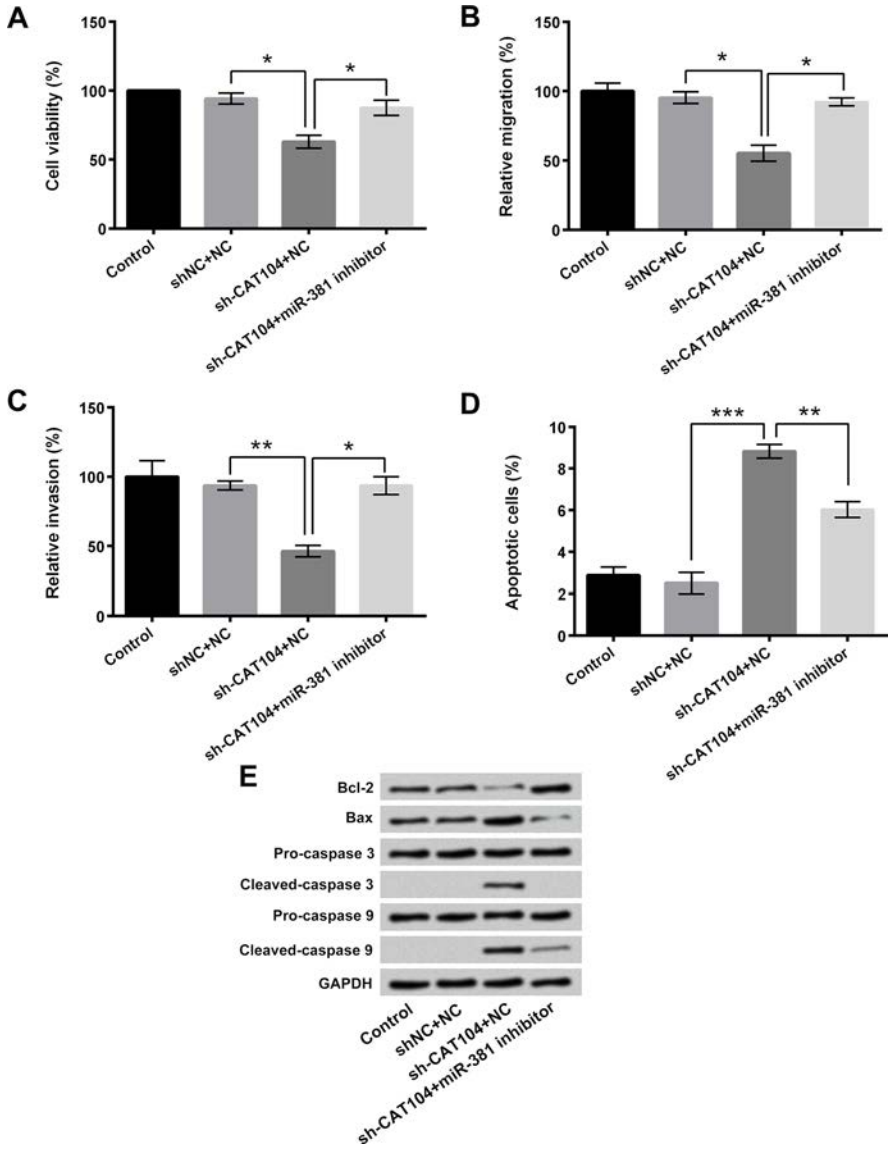

Figure 4. Suppression of miR-381 reversed the effects of CAT104 knockdown on osteosarcoma cells. (A) Viability, (B) migration, (C) invasion, and (D) apoptosis of OS-732 cells after sh-CAT104 and/or miR-381 inhibitor transfection were detected using MTT assay, two-chamber migration (invasion) assay, and Guava Nexin assay, respectively. (E) Western blotting was performed to analyze the expressions of Bcl-2, Bax, caspase 3, and caspase 9 in OS-732 cells after sh-CAT104 and/or miR-381 inhibitor transfection. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

binding sequence was displayed in Figure 5A. Thus, in our research, we determined the mRNA and protein levels of ZEB1 in OS-732 cells after miR-381 mimic or miR-381 inhibitor transfection using RT-qPCR and Western blotting, respectively. As shown in Figure 5B, the mRNA and protein expressions of ZEB1 in OS-732 cells were decreased after miR-381 mimic transfection and increased after miR-381 inhibitor transfection. Dual-luciferase reporter activity assay indicated that the relative luciferase activity was significantly decreased after cotransfection with miR-381 mimic and ZEB1-wt ($p<0.05$) (Fig. 5C). The above findings suggested that ZEB1 was a target gene of miR-381 in osteosarcoma cells.

ZEB1 Participated in the Regulatory Effects of miR-381 on Osteosarcoma Cell Proliferation, Migration, Invasion, and Apoptosis

To further explore the regulatory roles of miR-381 and ZEB1 in the proliferation, migration, invasion, and apoptosis of osteosarcoma cells, miR-381 mimic and/or pEX-ZEB1 were also transfected into OS-732 cells. Figure 6A showed that the relative mRNA expression of

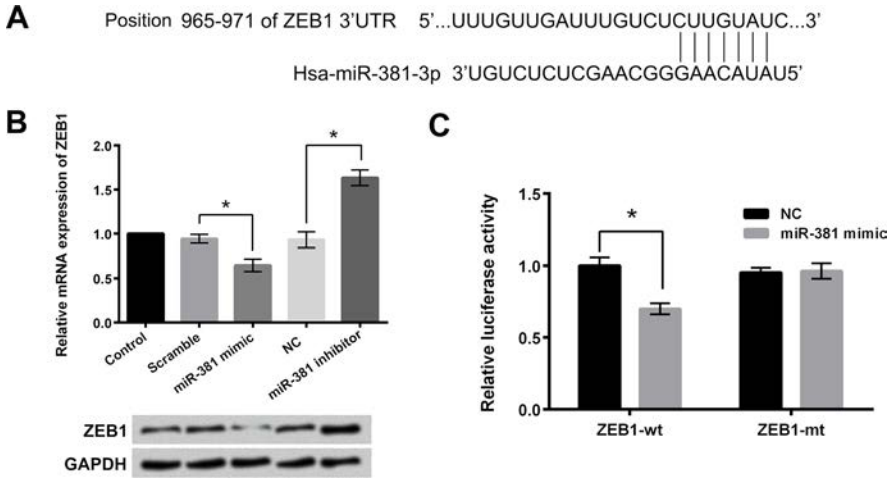

Figure 5. ZEB1 was a target gene of miR-381 in osteosarcoma cells. (A) Bioinformatics analysis was used to predict the potential binding sequence between 3 -UTR of ZEB1 and miR-381. (B) The mRNA and protein levels of ZEB1 in OS-732 cells after miR-381 mimic or miR-381 inhibitor transfection were detected using RT-qPCR and Western blotting, respectively. (C) Dual-luciferase reporter activity assay was used to determine the relative luciferase activity after cotransfection with miR-381 mimic and ZEB1-wt (ZEB-mt). ZEB1, zinc-finger E-box-binding homeobox 1; 3 -UTR, 3 -untranslated region. *$p<0.05$.

ZEB1 in OS-732 cells was significantly decreased after miR-381 mimic transfection and remarkably increased after pEX-ZEB1 transfection ($p<0.05$ or $p<0.01$). pEX-ZEB1 transfection remarkably reversed the miR-381 mimic transfection-induced inhibition of OS-732 cell viability, migration, and invasion ($p<0.05$) (Fig. 6B–D). Moreover, the rate of apoptosis after cotransfection with miR-381 mimic and pEX-ZEB1 was obviously decreased compared to single miR-381 mimic transfection ($p<0.01$ or $p<0.001$) (Fig. 6E). Western blotting displayed that pEX-ZEB1 transfection obviously attenuated the miR-318 mimic transfection-induced upregulation of Bax, cleaved caspase 3, and cleaved caspase 9 as well as the downregulation of Bcl-2 (Fig. 6F). These results indicated that ZEB1 participated in the regulatory effects of miR-381 on osteosarcoma cell proliferation, migration, invasion, and apoptosis.

JNK and Wnt/β-Catenin Pathways Were Involved in the Effects of miR-381 on Osteosarcoma Cells

Western blotting was conducted to analyze the effects of miR-381 and ZEB1 on the activation of the JNK and Wnt/ -catenin pathways in OS-732 cells. Results in Figure 7A presented that miR-381 mimic transfection downregulated the expressions of p-JNK and p-c-Jun in OS-732 cells. On the contrary, miR-381 inhibitor transfection increased the expressions of p-JNK and p-c-Jun in OS-732 cells. In addition, si-ZEB1 transfection alleviated the miR-381 inhibitor transfection-induced upregulation of p-JNK and p-c-Jun. Similar results were found in the Wnt/ -catenin pathway, which displayed that the expressions of Wnt3 , Wnt5 , and -catenin in OS-732 cells were reduced after miR-381 mimic transfection and enhanced after miR-381 inhibitor transfection (Fig. 7B). More importantly, si-ZEB1 transfection alleviated the miR-381 inhibitor transfection-induced upregulation of Wnt3 , Wnt5 , and -catenin in OS-732 cells. The above findings indicated that miR-381 regulated the activation of the JNK and Wnt/ -catenin pathways in OS-732 cells by targeting ZEB1 and implied that the JNK and Wnt/ -catenin pathways might be involved in the effects of miR-381 and ZEB1 on osteosarcoma cells.

DISCUSSION

Osteosarcoma has a high degree of incidence, high rate of malignancy, and high frequency of recurrence and metastasis[30]. Both lncRNAs and microRNAs can act as tumor suppressors or oncogenes and play important regulatory roles in carcinogenesis and cancer development[9,31]. In this research, we found that CAT104 was highly expressed in osteosarcoma cells, and knockdown of CAT104 inhibited the proliferation, migration, and invasion of osteosarcoma cells. The expression of miR-381 was increased in osteosarcoma cells after CAT104 knockdown. Moreover, ZEB1 was a direct target gene of miR-381 and participated in the regulatory effects of miR-381 on osteosarcoma cell proliferation, migration, invasion, and apoptosis. Furthermore, miR-381 inhibited the proliferation, migration, and invasion of osteosarcoma cells at least in part through inactivating the JNK and Wnt/ -catenin pathways.

lncRNAs do not encode specific proteins but regulate the gene expression of tumor cells on multiple levels[13].

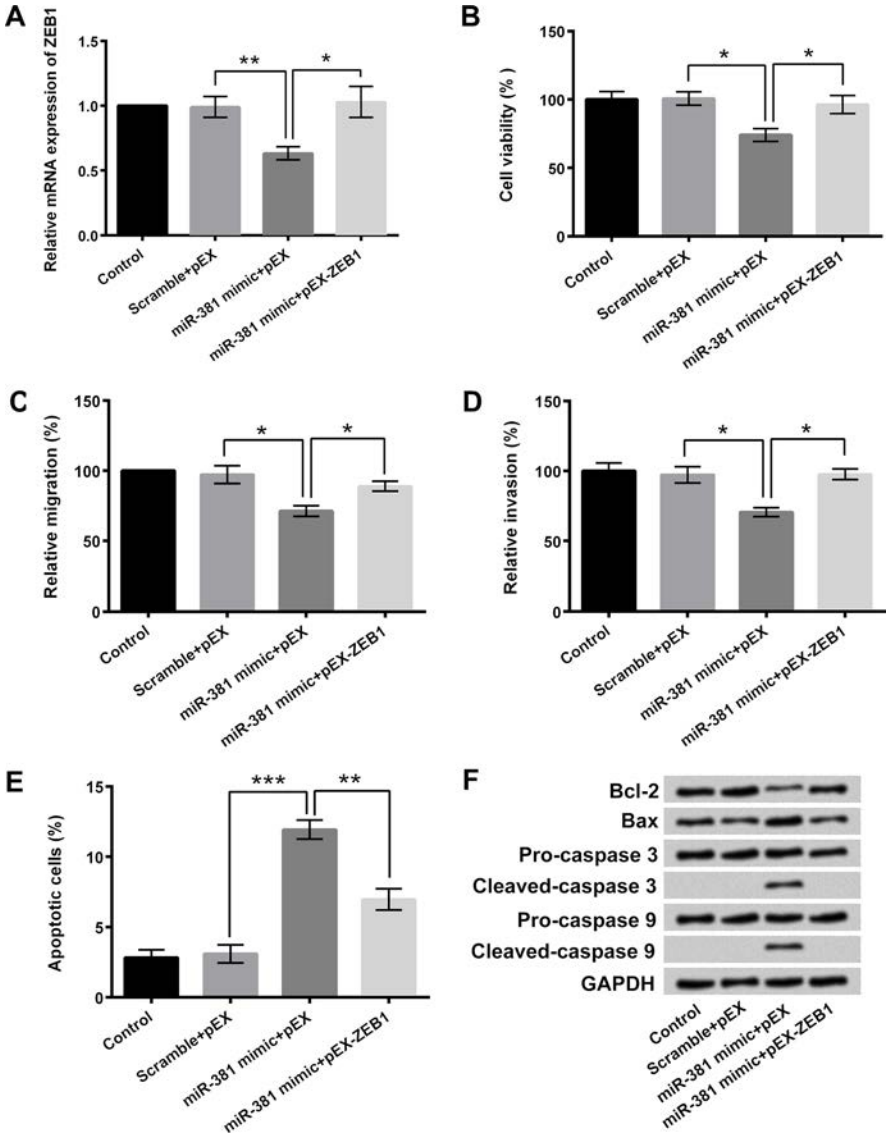

Figure 6. ZEB1 participated in the regulatory effects of miR-381 on osteosarcoma cell proliferation, migration, invasion, and apoptosis. (A) RT-qPCR was performed to measure the relative mRNA expression of ZEB1 in OS-732 cells after miR-381 mimic and/or pEX-ZEB1 transfection. (B) Viability, (C) migration, (D) invasion, and (E) apoptosis of OS-732 cells after miR-381 mimic and/or pEX-ZEB1 transfection were detected using MTT assay, two-chamber migration (invasion) assay, and Guava Nexin assay, respectively. (F) Western blotting was used to analyze the expressions of Bcl-2, Bax, caspase 3, and caspase 9 in OS-732 cells after miR-381 mimic and/or pEX-ZEB1 transfection. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Numerous studies have revealed the oncogenic roles of lncRNAs in tumor cells, due to their contribution to tumor cell proliferation, migration, and invasion[15,18,19]. In the present study, we found that CAT104 had a higher expression in osteosarcoma MG63 and OS-732 cells, compared to osteoblast hFOB1.19 cells. This result was consistent with the previous study, which demonstrated that CAT104 was highly expressed in breast cancer[20]. We also found that suppression of CAT104 reduced the proliferation, migration, and invasion of OS-732 cells, but promoted cell apoptosis. RT-qPCR identified that the expression of miR-381 was increased after CAT104 suppression. Cotransfection with sh-CAT104 and miR-381 inhibitor significantly reversed the inhibition of cell proliferation, migration, and invasion, as well as the enhancement of cell apoptosis, induced by single sh-CAT104 transfection. These findings revealed the critical roles of CAT104 and miR-381 in regulating osteosarcoma cell growth and metastasis and suggested that CAT104 could promote osteosarcoma development by regulating miRNAs.

Osteosarcoma is a tumor with metastatic potential[32]. Thus, inhibition of tumor metastasis is one of the important

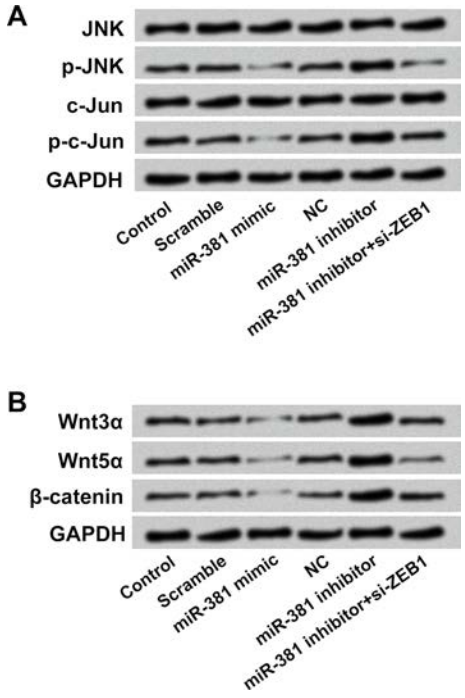

Figure 7. JNK and Wnt/ -catenin pathways were involved in the effects of miR-381 on osteosarcoma cells. (A) The protein levels of JNK, p-JNK, c-Jun, p-c-Jun, (B) Wnt3 , Wnt5 , and -catenin in OS-732 cells after miR-381 mimic, miR-381 inhibitor, or miR-381 inhibitor + si-ZEB1 transfection were measured using Western blotting. JNK, c-Jun N-terminal kinase.

purposes of osteosarcoma treatment[33]. In the process of osteosarcoma metastasis, osteosarcoma cells obtain the abilities of migration and invasion and spread from the primary tumor site to other sites of bone or lung tissue for formation of secondary tumors[34]. ZEB1, a member of the ZEB family, has been recognized as the central inducer of the epithelial-to-mesenchymal transition (EMT) process, which plays a critical role in tumor metastasis[35]. In this research, we defined ZEB1 as the direct target gene of miR-381. The expression of ZEB1 in OS-732 cells was remarkably decreased after miR-381 overexpression and obviously increased after miR-381 suppression. Moreover, we found that pEX-ZEB1 transfection significantly alleviated the miR-381 overexpression-induced inhibition of cell proliferation, migration, and invasion. These above findings indicated that miR-381 played anticancer and antimetastatic effects on osteosarcoma by targeting ZEB1. Moreover, these results were consistent with the previous study, which revealed that miR-381 mimic transfection inhibited the EMT process of lung cancer by downregulating snail and twist[36].

The JNK and Wnt/ -catenin pathways are thought to be important oncogenic signaling pathways in human tumor cells, including osteosarcoma cells[8,37]. Moreover, multiple research studies have demonstrated that the JNK and Wnt/ -catenin pathways were associated with the growth, invasion, and apoptosis of osteosarcoma cells[38,39]. We further explored the effects of miR-381 and ZEB1 on the activation of the JNK and Wnt/ -catenin pathways in OS-732 cells. Our results revealed that miR-381 overexpression inhibited the activation of the JNK and Wnt/ -catenin pathways, and miR-381 suppression promoted the activation of the JNK and Wnt/ -catenin pathways in OS-732 cells. In addition, knockdown of ZEB1 attenuated the effects of miR-381 suppression on the JNK and Wnt/ -catenin pathways. These findings further suggested that miR-381 inhibited the proliferation, migration, and invasion of osteosarcoma cells at least in part through inactivating the JNK and Wnt/ -catenin pathways.

In conclusion, our research verified that suppression of CAT104 exerted significant inhibitory effects on osteosarcoma cell proliferation, migration, and invasion by regulating the expression of miR-381 and downstream ZEB1, as well as the JNK and Wnt/ -catenin pathways. We proposed that suppression of CAT104 might provide a novel therapeutic approach to the inhibition of osteosarcoma growth and reduction of osteosarcoma metastasis.

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