Knockdown of lncRNA C5orf66-AS1 inhibits osteosarcoma cell proliferation and invasion via miR-149-5p upregulation

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Abstract. Osteosarcoma (OS) is the most common primary malignant bone tumor in the pediatric age group. Despite the various potential treatments for OS, the cure rate of patients with OS remains very low. An increasing number of long non-coding RNAs (lncRNAs) have been identified as key regulators of the progression of malignant human tumors. However, the biological functions of the lncRNA C5orf66-antisense 1 (C5orf66-AS1) in OS are yet to be fully elucidated. The present study aimed to investigate the functions and underlying mechanisms of C5orf66-AS1 in OS tissues and cell lines. Expression levels of C5orf66-AS1 and microRNA (miRNA/miR)-149-5p in tissues from patients with OS and OS cells lines were evaluated using reverse transcription quantitative (RT-q)PCR. The miRNA target interaction between C5orf66-AS1 and miR-149-5p was predicted and verified using StarBase and dual-luciferase reporter assays. Cell viability, migration, invasion and apoptosis were analyzed using Cell Counting Kit-8, Transwell assays and flow cytometry, respectively. In addition, the expression levels of migration- and apoptosis-associated proteins [matrix metallo-proteinase-9 (MMP-9), Bcl-2 and Bax] were determined using western blotting and RT-qPCR. The results demonstrated that C5orf66-AS1 was significantly upregulated and miR-149-5p was significantly downregulated in OS tissues and cells (MG63 and U2OS). Bioinformatics analysis further confirmed that miR-149-5p could directly bind to C5orf66-AS1. Furthermore, it was revealed that C5orf66-AS1 negatively regulated the expression of miR-149-5p in OS cells, as confirmed by the inhibition of C5orf66-AS1 expression and miR-149-5p upregulation in cells transfected with small interfering (si RNA targeting C5orf66-AS1. In addition, C5orf66-AS1 silencing significantly inhibited the proliferation, invasion and migration of U2OS cells, and stimulated cell apoptosis. These findings were reversed using miR-149-5p inhibitor. Increased Bax expression and decreased Bcl-2 and MMP-9 expression were also observed in C5orf66-AS1-siRNA transfected U2OS cells, compared with the control group. In summary, the results from the present study indicated that C5orf66-AS1 knockdown inhibits OS cell proliferation and invasion via the upregulation of miR-149-5p. This findings may provide a promising novel target for the treatment of OS.

Introduction

Osteosarcoma (OS) accounts for 5% of tumors in children and is the most common bone malignant tumor originating from mesenchymal stem cells (1). Furthermore, OS often occurs in teenagers or children under the age of 20 and is characterized by rapid growth and early metastasis (2). Recently, conventional therapies, including neoadjuvant chemotherapy, surgery and radiotherapy, have been used as treatment strategies for OS, and the 5-year survival rate of patients with osteosarcoma has improved from ~20% before the 1980s to currently ~70% (3,4); However, half of the patients do not survive for longer than 10 year (5,6). In addition, the prognosis of OS remains poor due to drug resistance, tumor recurrence and metastasis (7). It is therefore crucial to better understand the pathogenesis of OS to uncover potential targets of tumor growth and metastasis regulation, which have become essential for OS prevention and treatment in recent years.

Previous studies have demonstrated that long non-coding RNAs (lncRNAs) play significant roles in gene regulation and genomic stability maintenance (8), and that they affect numerous cellular processes, including cell proliferation, migration, invasion, apoptosis and differentiation (9,10). Numerous studies have reported that lncRNAs are involved in the occurrence and development of various types of tumor (11-13). The lncRNA C5orf66-antisense 1 (C5orf66-AS1), a recently discovered lncRNA, has been reported to be associated with the pathogenesis of different types of tumor. For example, Rui *et al* (14) demonstrated that C5orf66-AS1 can promote cervical cancer cell proliferation via the microRNA (miRNA/miR)-637/ring finger protein 1 axis. Lu *et al* (15) demonstrated that C5orf66-AS1 prevents

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oral squamous cell carcinoma cell proliferation and metastasis. However, the role and underlying mechanism of C5orf66-AS1 in OS are yet to be elucidated.

It is well established that lncRNAs participate in the pathogenesis of diseases via interaction with miRNAs (16). miRNAs represent another class of endogenous small non-coding RNAs ~22 nucleotides in length, which can regulate various cell processes, including cell proliferation, apoptosis, invasion and differentiation (17,18). In recent years, certain studies have confirmed that miRNAs serve a key role in tumorigenesis, including in OS (19-21). Previous studies reported that miR-149-5p plays an inhibitory role in numerous types of cancer. For example, Luo *et al* (22) confirmed that miR-149-5p can regulate oral squamous cell carcinoma cell proliferation and invasion by targeting transforming growth factor β2. Furthermore, Ye and Chen (23) demonstrated that miR-149-5p can inhibit medullary thyroid carcinoma cell proliferation and invasion by targeting ARF GTPase-activating protein 1. However, the expression and role of miR-149-5p in OS remains unknown.

The present study aimed to identify the possible roles of C5orf66-AS1 in OS cell proliferation and invasion and to determine its underlying mechanisms.

Materials and methods

Clinical specimen collection. A total of 20 OS tissues and adjacent non-cancerous tissues (2 cm from the tumor lesion) were collected from patients (age range, 21-68 years; female to male ratio, 12:8) with OS who underwent surgical treatment at the Huangshi Central Hospital (Huangshi, China). All tissues were immediately frozen and stored in liquid nitrogen or at -80˚C. None of the patients received any radiotherapy or chemotherapy prior to the surgery. The study procedures were approved by the Ethics Committee of Huangshi Central Hospital. Written informed consent was obtained from each patient and all patients agreed to the use of their specimens in this study.

Cell culture. The non-cancerous osteoblast hFOB1.19 cell line and the human OS (HOS) cell lines MG63 and U2OS were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 15% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin and placed at 37˚C in a humidified incubator containing 5% CO_2. 293T cells (ATCC) were cultured in DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

Reverse transcription-quantitative (RT-q)PCR. The expression levels of matrix metalloproteinase 9 (MMP-9), Bcl-2, Bax, miR-149-5p and C5orf66-AS1 were evaluated using RT-qPCR. Total RNA was extracted from OS tissues, adjacent non-cancerous tissues and HOS cell lines using an TRIzol® Plus RNA Purification Kit (cat. no. 12183555; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reversed transcribed into cDNA using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol, and

RT-qPCR analysis was conducted using the SYBR PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.) using the ABI 7500 Real-Time PCR system (Agilent Technologies, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 10 min, followed by 37 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for 40 sec and extension at 72˚C for 34 sec. The relative expression levels were normalized to endogenous control GAPDH or U6 (24). The sequences of the primers were as follows: GAPDH forward, 5'-CTTTGGTATCGTGGAAGGA CTC-3' and reverse, 5'-GTAGAGGCA GGGATGATGTTCT-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGA ATTTGCGTGTCAT-3'; miR-149-5p forward, 5'-CCCTCATTCTGTGCCACACTCC AGCTGGG-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'; C5orf66-AS1 forward 5'-GGTCGGGCTTTTTCTTCCCA-3' and reverse, 5'-GCCGCGGGAATGTCTTTATT-3'; MMP-9 forward 5'-AGACCTGGGCAGATTCCAAAC-3' and reverse, 5'-CGGCAAGTCTTCCGAGTAGT-3'; Bcl-2 forward 5'-AGT AAACCTGGACGGTGAAGTGATTG-3' and reverse, 5'-CCA GGTAACAAAACCCCACA-3'; and Bax forward 5'-CAAGAC CAGGGTGGTTGG-3' and reverse, 5'-CACTCCCGCCAC AAAGAT-3'.

Cell transfection. The control-small interfering (si)RNA, C5orf66-AS1-siRNA, inhibitor control (5'-CAGUACUU UUG UGUAGUACAA-3') and miR-149-5p inhibitor (5'-GGGAGUG AAGACACGGAGCCAGA-3') were synthesized by Shanghai GenePharma Co., Ltd. U2OS cells were transfected with 1 μM control-siRNA, 1 μM C5orf66-AS1-siRNA, 100 nM inhibitor control, 100 nM miR-149-5p inhibitor, 1 μM C5orf66-AS1-siRNA + 100 nM inhibitor control, or 1 μM C5orf66-AS1-siRNA + 100 nM miR-149-5p inhibitor at 37˚C for 48 h using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h following transfection, transfection efficiency was determined using RT-qPCR analysis.

Dual-luciferase reporter assay. StarBase (http://starbase.sysu. edu.cn/) was used to identify the relationship between C5orf66-AS1 and miR-149-5p. The 3'-untranslated region (UTR) of C5orf66-AS1 containing the target sequence of miR-149-5p was amplified using RT-PCR and subsequently inserted into a pmirGLO vector (Promega Corporation) to form the reporter vector C5orf66-AS1-wild-type (C5orf66-AS1-WT). An alternative expressing vector named C5orf66-AS1-mutant (C5orf66-AS1-MUT) was also constructed by inserting the mutated binding site into the pmirGLO vector (Promega Corporation). C5orf66-AS1-WT or C5orf66-AS1-MUT and miR-149-5p mimic (sense, 5'-UCUGGCUCCGUGUCUU CACUCCC-3'; anti-sense, 5'-GAGUGAAGACACGGAGCC AGAUU-3'; Shanghai GenePharma Co., Ltd) or mimic control (sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3'; Shanghai GenePharma Co., Ltd.) were co-transfected into 293T cells using Lipofectamine 3000 and incubated for 48 h. The relative luciferase activity was detected by Dual-Luciferase® Reporter assay system (Promega Corporation) following the manufacturer's protocol. All firefly luciferase activities were normalized to *Renilla* luciferase activity.

A

B
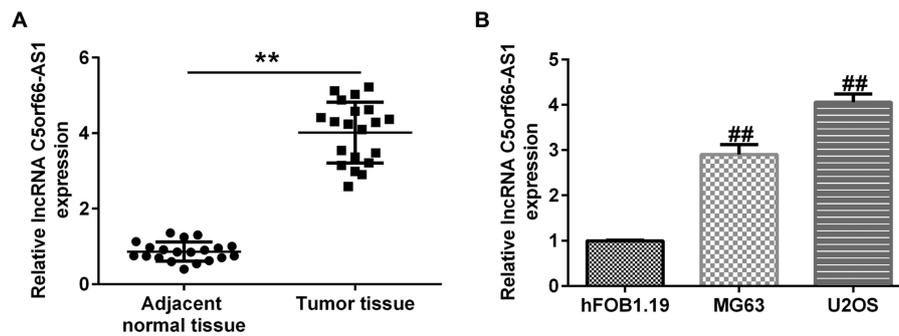
Figure 1. Expression levels of C5orf66-AS1 in OS tissues and cells. (A) Detection of C5orf66-AS1 expression in osteosarcoma and adjacent normal tissues determined by RT-qPCR. (B) RT-qPCR analysis of C5orf66-AS1 in the OS cell lines MG63 and U2OS and the non-cancerous osteoblast cell line hFOB1.19. [**]$P < 0.01$ vs. adjacent normal tissue; [##]$P < 0.01$ vs. hFOB1.19. OS, osteosarcoma; RT-qPCR, reverse transcription-quantitative PCR; C5orf66-AS1, C5orf66-antisense 1; lncRNA, long non-coding RNA.

Apoptosis evaluation by flow cytometry. U2OS cells were transfected with control-siRNA, C5orf66-AS1-siRNA, C5orf66-AS1-siRNA+inhibitor control, or C5orf66-AS1-siRNA+miR-149-5p inhibitor at 37°C for 48 h, and U2OS cell apoptosis was detected using a Annexin-V/PI Apoptosis Detection kit (Beyotime Institute of Biotechnology). Briefly, 200 μl Annexin V-FITC and 10 μl PI were added into the U2OS cell (10^6 cells) suspension for 30 min at 37°C in the dark according to the manufacturer's protocol. The apoptotic cell rate was determined using a flow cytometer (BD Biosciences) and quantified using FlowJo software (version 7.2.4; FlowJo LLC).

Cell Counting Kit-8 (CCK-8) assay. CCK-8 assay (Beyotime Institute of Biotechnology) was used to assess cell proliferation. After transfection, U2OS cells (10^4 cells per well) were seeded in 96-well plates overnight. CCK-8 reagent (10 μl) was added to each well and cells were cultured at 37°C for 2 h. The optical density was measured at a wavelength of 450 nm using a microplate reader following the manufacturer's protocol.

Transwell migration and invasion assay. U2OS cells were transfected with control-siRNA, C5orf66-AS1-siRNA, C5orf66-AS1-siRNA+inhibitor control, or C5orf66-AS1-siRNA+miR-149-5p inhibitor at 37°C for 48 h. U2OS cells ($2x10^4$ cells) were cultured in serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) and seeded into the upper chamber of the Transwell (pore size, 8 μm; Corning, Inc.). RPMI-1640 medium containing 10% FBS was added to the lower chamber. Following incubation at 37°C in a 5% CO_2 atmosphere for 48 h, cells remaining on the upper membrane were scraped with cotton swabs, and the migrated and invaded cells on the lower chamber were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 0.1% crystal violet at room temperature for 30 min. The migratory and invasive capacities of the cells on the lower side of the membrane were visualized and quantified using an inverted microscope (magnification, x100; Nikon Corporation). Transwell chambers were precoated with Matrigel (BD Biosciences) at 37°C for 30 min for the invasion assay only.

Western blotting. U2OS cells were transfected with control-siRNA, C5orf66-AS1-siRNA, C5orf66-AS1-siRNA+

inhibitor control, or C5orf66-AS1-siRNA+miR-149-5p inhibitor at 37°C for 48 h. U2OS cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) at 4°C. Proteins were quantified using a BCA Protein Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Proteins (40 μg per lane) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% skimmed milk in PBS-0.1% supplemented with Tween-20 at room temperature for 1 h, membranes were incubated with primary antibodies against GAPDH (cat. no. ab9485; 1:1,000; Abcam), MMP-9 (cat. no. ab76003; 1:1,000; Abcam), Bcl-2 (cat. no. ab32124; 1:1,000; Abcam) and Bax (cat. no. ab182733; 1:1,000; Abcam) overnight at 4°C. The membranes were washed with PBS-0.1% supplemented with Tween-20 and incubated with a goat anti-rabbit IgG H&L (HRP) pre-adsorbed antibody (cat. no. ab97080; 1:2,000; Abcam) at room temperature for 1 h. Bands were detected using enhanced chemiluminescence substrate (Pierce; Thermo Fisher Scientific, Inc.) and semi-quantified using ImageJ software version 1.46 (National Institutes of Health).

Statistical analysis. Each experiment was performed three times. Data were presented as the means ± standard deviation and analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.). Differences among groups were estimated using paired Student's t-test or one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

C5orf66-AS1 is upregulated in OS tissues and cells. The expression of C5orf66-AS1 was evaluated in OS tissues, adjacent non-cancerous tissues and HOS cells using RT-qPCR. As presented in Fig. 1A, C5orf66-AS1 was upregulated in OS tissues, compared with adjacent normal tissues. Furthermore, a higher expression level of C5orf66-AS1 was reported in U2OS and MG63 cells when compared with hFOB1.19 cells (Fig. 1B). These findings indicated that C5orf66-AS1 may be considered as a regulator of OSCC tumorigenesis.

miR-149-5p directly interacts with C5orf66-AS1. To investigate the potential mechanisms by which C5orf66-AS1 may
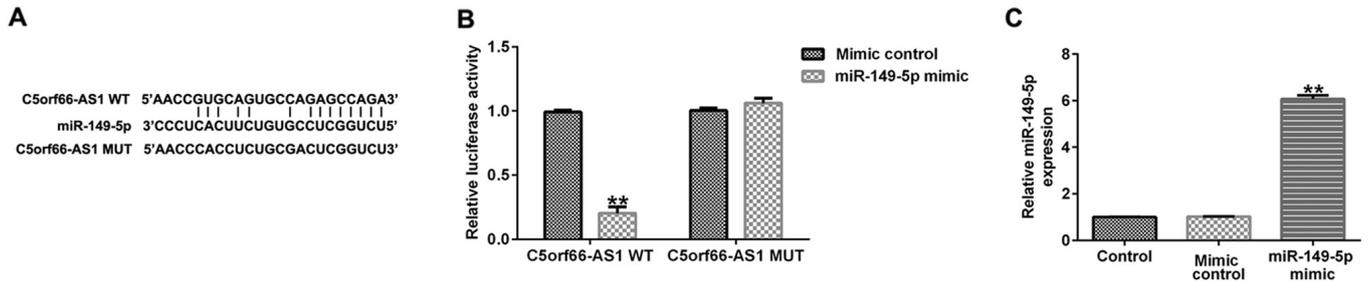
Figure 2. miR-149-5p directly targets C5orf66-AS1. (A) miR-149-5p and C5orf66-AS1 binding sequences and C5orf66-AS1 mutant sequences. (B) Target relationship between miR-149-5p and C5orf66-AS1 was verified using a dual-luciferase reporter assay. (C) Expression of miR-149-5p in 293T cells transfected with mimic control or miR-149-5p mimic was assessed using reverse transcription-quantitative PCR. **P<0.01 vs. mimic control. miR, microRNA; C5orf66-AS1, C5orf66-antisense 1; WT, wild-type; MUT, mutant.
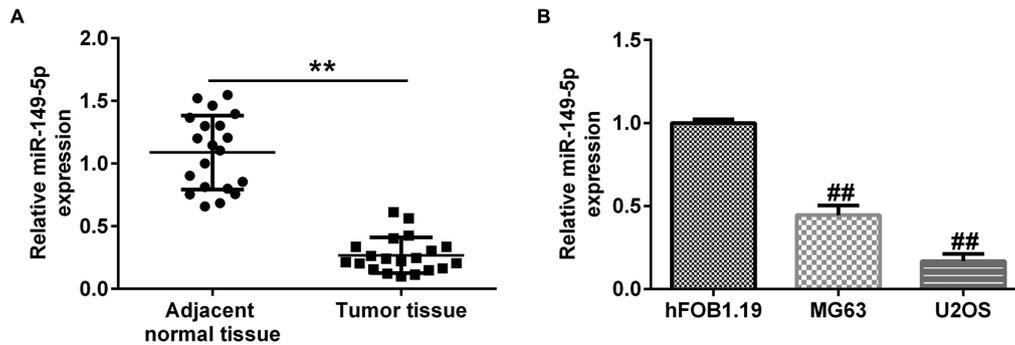
Figure 3. Expression of miR-149-5p in OS tissues and cells. (A) Determination of miR-149-5p expression in OS and adjacent non-cancerous tissues. (B) Expression of miR-149-5p in MG63 and U2OS cells and the non-cancerous osteoblast hFOB1.19 cells. **P<0.01 vs. adjacent non-cancerous tissue; ##P<0.01 vs. hFOB1.19. OS, osteosarcoma; miR, microRNA.

regulate the development of OS, StarBase software was used to determine the target sites of C5orf66-AS1. The results demonstrated that C5orf66-AS1 was a potential target of miR-149-5p (Fig. 2A). In addition, the luciferase reporter assay revealed that miR-149-5p mimic significantly decreased the luciferase activity of the WT C5orf66-AS1 3'-UTR construct, but had no significant effect on the C5orf66-AS1 3'-UTR-MUT reporter (Fig. 2B). Furthermore, miR-149-5p mimic transfection increased miR-149-5p expression in 293T cells, compared with the mimic control group. These findings indicated that C5orf66-AS1 was a direct target of miR-149-5p.

miR-149-5p is downregulated in OS tissues and cells. The expression of miR-149-5p in OS tissues and cells was evaluated using RT-qPCR analysis. The results demonstrated that miR-149-5p expression was significantly lower in OS tissues compared with adjacent normal tissues (Fig. 3A). Furthermore, downregulation of miR-149-5p was observed in MG63 and U2OS cells compared with hFOB1.19 cells (Fig. 3B). All the above results indicated that C5orf66-AS1 may regulate the progression of OS by targeting miR-149-5p.

C5orf66-AS1 negatively regulates the expression of miR-149-5p in OS cells. To further elucidate the regulatory correlation between C5orf66-AS1 and miR-149-5p in OS, control-siRNA, C5orf66-AS1-siRNA, inhibitor control or miR-149-5p inhibitor were transfected into OS cells for 48 h. The results from RT-qPCR demonstrated that C5orf66-AS1-siRNA inhibited

C5orf66-AS1 expression in U2OS cells (Fig. 4A). Furthermore, miR-149-5p expression was downregulated in miR-149-5p inhibitor-transfected cells, compared with the inhibitor control (Fig. 4B). Higher expression level of miR-149-5p was also observed in C5orf66-AS1-siRNA transfected cells compared with that in the control-siRNA group, whereas this high expression level was significantly reversed following transfection with miR-149-5p inhibitor (Fig. 4C). These findings suggested that C5orf66-AS1 negatively regulated the expression of miR-149-5p in OS cells.

C5orf66-AS1-siRNA inhibits OS cell viability and stimulates OS cell apoptosis by upregulating miR-149-5p. The biological behaviors of U2OS cells co-regulated by C5orf66-AS1 and miR-149-5p were explored. Control-siRNA, C5orf66-AS1-siRNA, inhibitor control or miR-149-5p inhibitor were transfected into U2OS cells for 48 h. The results from CCK-8 assay demonstrated that C5orf66-AS1-siRNA decreased U2OS cell viability (Fig. 5A). Furthermore, an increased apoptotic rate of U2OS cells was observed in the C5orf66-AS1-siRNA group compared with the control-siRNA group (Fig. 5B and C). In addition, the expression levels of apoptosis-related proteins were detected. As presented in Fig. 5D-F, C5orf66-AS1-siRNA enhanced Bax protein and mRNA expression levels (Fig. 5D and E) and decreased Bcl-2 protein and mRNA expression levels (Fig. 5D and F) in U2OS cells compared with the control-siRNA group. However, these observations were reversed following transfection
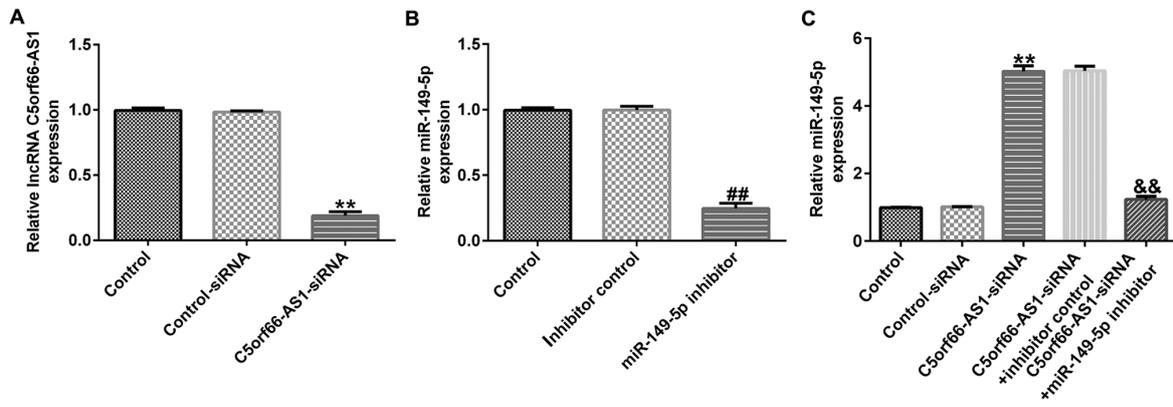
Figure 4. C5orf66-AS1 negatively regulates miR-149-5p in OS cells. Control-siRNA, C5orf66-AS1-siRNA, inhibitor control or miR-149-5p inhibitor were transfected into U2OS cells for 48 h. (A) Relative expression level of C5orf66-AS1 in C5orf66-AS1-siRNA transfected cells. (B) RT-qPCR analysis of miR-149-5p in U2OS cells after miR-149-5p inhibitor or inhibitor control transfection. (C) mRNA expression of miR-149-5p in C5orf66-AS1-siRNA+inhibitor control or C5orf66-AS1-siRNA+miR-149-5p inhibitor transfected into U2OS cells was evaluated using RT-qPCR. **$P<0.01$ vs. control-siRNA; ##$P<0.01$ vs. inhibitor control; &&$P<0.01$ vs. C5orf66-AS1-siRNA+inhibitor control. miR, microRNA; OS, osteosarcoma; si, small interfering; RT-qPCR, reverse transcription-quantitative PCR; C5orf66-AS1, C5orf66-antisense 1; lncRNA, long non-coding RNA.
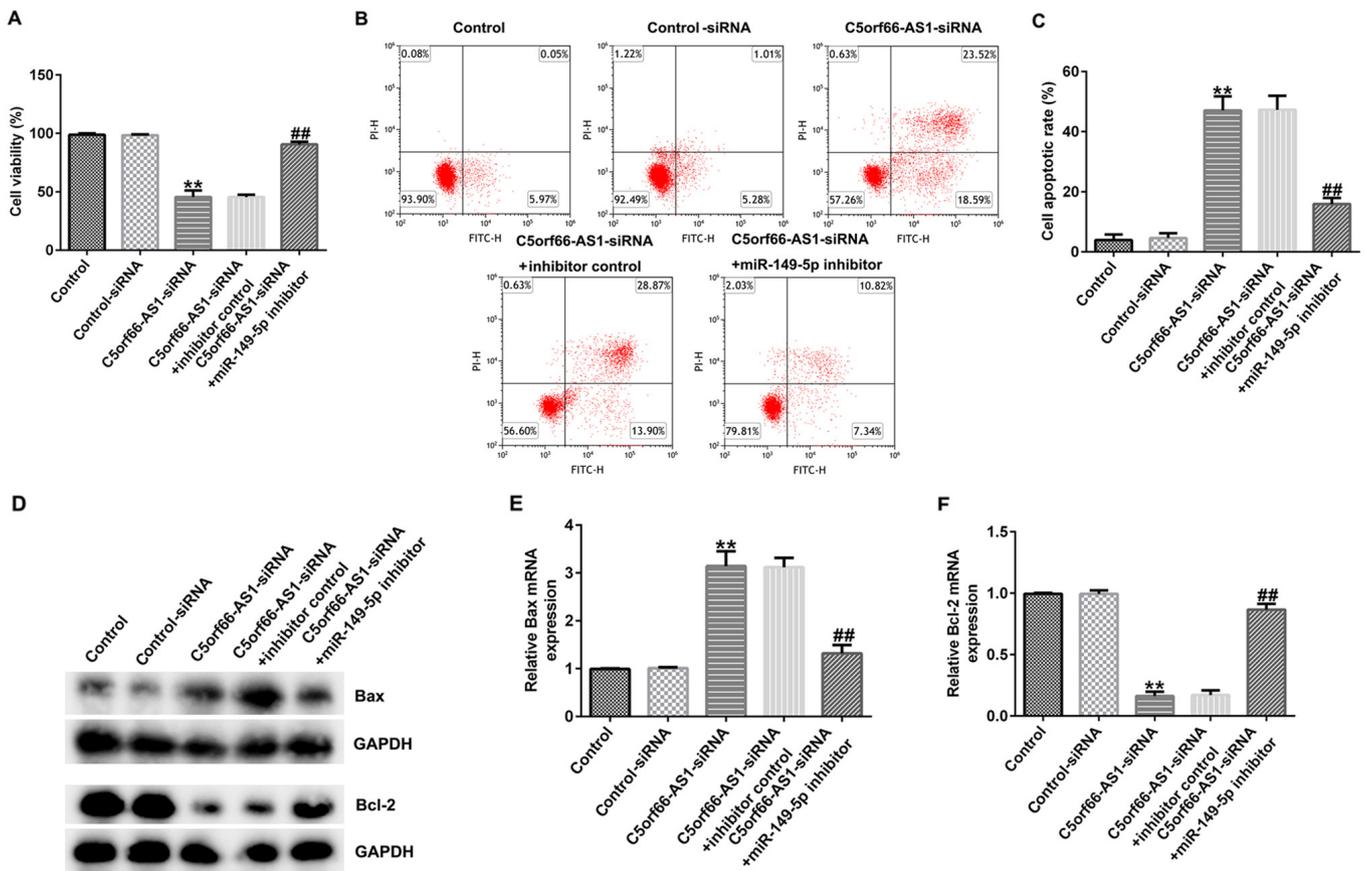
Figure 5. Effects of C5orf66-AS1-siRNA on OS cell proliferation and apoptosis. U2OS cells were transfected with control-siRNA, C5orf66-AS1-siRNA, C5orf66-AS1-siRNA+inhibitor control or C5orf66-AS1-siRNA+miR-149-5p inhibitor for 48 h. (A) Cell Counting Kit-8 assay was used to assess cell viability. (B) Apoptotic rate of U2OS cells was evaluated by flow cytometry. (C) Quantification of the apoptotic cell rate. (D) Western blot analysis of Bax or Bcl-2 protein expression. Expression levels of (E) Bax and (F) Bcl-2 in U2OS cells were determined by RT-qPCR. **$P<0.01$ vs. control-siRNA; ##$P<0.01$ vs. C5orf66-AS1-siRNA+inhibitor control. miR, microRNA; OS, osteosarcoma; si, small interfering; RT-qPCR, reverse transcription-quantitative PCR, CCK-8, Cell Counting Kit-8; C5orf66-AS1, C5orf66-antisense 1.

with miR-149-5p inhibitor. These results suggested that C5orf66-AS1 knockdown inhibited OS cell viability and promote OS cell apoptosis by regulating miR-149-5p, which may inhibit the development of OS.

C5orf66-AS1-siRNA inhibits U2OS cell migration and invasion via miR-149-5p upregulation. To further determine the role of C5orf66-AS1 in regulating OS cell migration and invasion, Transwell assays were used to evaluate the role
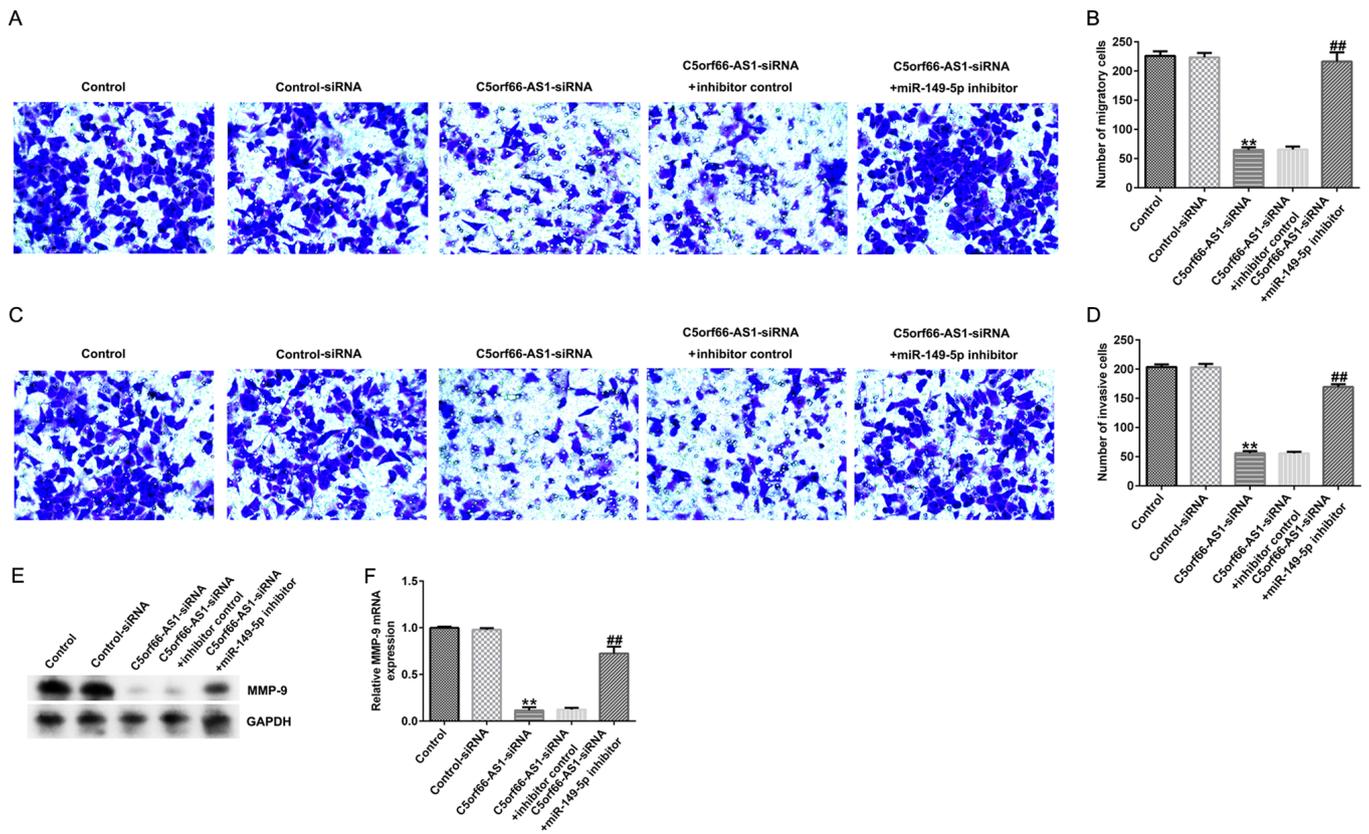
Figure 6. Effects of C5orf66-AS1-siRNA on OS cell migration and invasion. Control-siRNA, C5orf66-AS1-siRNA, C5orf66-AS1-siRNA+inhibitor control or C5orf66-AS1-siRNA+miR-149-5p inhibitor were transfected into U2OS cells for 48 h. U2OS cell (A) migration and (C) invasion were evaluated using Transwell assays. Number of (B) migratory and (D) invasive cells was counted under light microscopy. MMP-9 expression levels were assessed by (E) western blotting and (F) reverse transcription-quantitative PCR. Magnification, x100. **$P<0.01$ vs. control-siRNA; ##$P<0.01$ vs. C5orf66-AS1-siRNA+inhibitor control. si, small interfering; OS, osteosarcoma; miR, microRNA; C5orf66-AS1, C5orf66-antisense 1.

of C5orf66-AS1 on U2OS cell migration and invasion. The results demonstrated that C5orf66-AS1-siRNA decreased U2OS cell migration (Fig. 6A and B) and invasion (Fig. 6C and D) compared with the control-siRNA group. Furthermore, the results from western blotting and RT-qPCR demonstrated that MMP-9 was downregulated in U2OS cells following C5orf66-AS1-siRNA transfection (Fig. 6E and F). These observations were reversed following cell transfection with miR-149-5p inhibitor. These findings suggested that C5orf66-AS1 knockdown may inhibit OS cell proliferation, migration and invasion via miR-149-5p upregulation.

Discussion

OS is the most common primary bone tumor in children and adolescents and accounts for ~20% of all primary bone cancers (1). OS is the second leading cause of cancer-associated mortality in children (25,26). In the recent years, multiple therapies have greatly improved the treatment of OS, including radiopharmaceuticals (27), chemotherapy and radiotherapy (28). However, the prognosis remains poor for patients with OS. Thus, in-depth investigation into the pathogenesis and underlying mechanisms of OS may provide additional clinical evidence for the development of novel treatment methods. The prognostic value of small molecules, including miRNAs and lncRNAs have been demonstrated in OS. Huang et al (29) confirmed that the lncRNA small nucleolar

RNA host gene 4 can promote OS proliferation and migration by sponging miR-377-3p. Furthermore, Yang et al (30) revealed that lncRNA-BC050642 could promote OS cell proliferation, stimulate colony formation and inhibit cell apoptosis.

In the recent years, lncRNAs, which serve vital roles in human diseases, have been widely investigated in association with tumorigenesis and cancer progression. Recently, Zhou et al (31) identified C5orf66-AS1 as a potential biomarker for predicting early gastric cancer and gastric carcinogenesis. Yu et al (32) reported that C5orf66-AS1 inhibits the development and invasion of pituitary null cell adenomas. However, the involvement of C5orf66-AS1 in the tumorigenesis of OS is yet to be fully elucidated. The present study evaluated the expression of C5orf66-AS1 in OS and adjacent non-cancerous tissues and in the HOS cell lines MG63 and U2OS and the non-cancerous osteoblast cell line hFOB1.19. C5orf66-AS1 was upregulated in OS tissues and HOS cell lines, compared with the control groups. These findings indicated that C5orf66-AS1 may be considered as a key factor in OS tumorigenesis.

A previous study demonstrated that lncRNAs regulate gene expression via an interaction with miRNAs in multiple cancer types, including in OS (33). In the present study, Starbase bioinformatics tool and a dual-luciferase reporter assay predicted and further confirmed the target sites of C5orf66-AS1. The results revealed that C5orf66-AS1 directly interacts with miR-149-5p. In addition, miR-149-5p expression was significantly higher in miR-149-5p mimic-transfected 293T cells compared with the

mimic control-transfected cells. miR-149-5p has been found to act as a suppressor in various types of cancer (34,35). In the present study, the expression levels of miR-149-5p in 30 pairs of OS and adjacent non-cancerous tissues, and in HOS and hFOB1.19 cell lines were determined using RT-qPCR. Downregulation of miR-149-5p was observed in OS tissues and HOS cell lines compared with the control groups. These results suggested that C5orf66-AS1 may regulate the progression of OS by targeting miR-149-5p.

To further illustrate the mechanism of C5orf66-AS1 in OS tumorigenesis, control-siRNA, C5orf66-AS1-siRNA, inhibitor control or miR-149-5p inhibitor were transfected into U2OS cells for 48 h. The results suggested that miR-149-5p expression was downregulated in miR-149-5p inhibitor-transfected cells. Furthermore, C5orf66-AS1 silencing increased miR-149-5p expression and decreased C5orf66-AS1 expression levels, and miR-149-5p expression increase was abolished by transfection with a miR-149-5p inhibitor. Therefore, C5orf66-AS1 negatively regulated the expression of miR-149-5p in OS cells.

A previous study reported that dysregulation of oncogenes or tumor inhibitors is crucial in tumorigenesis and cancer development (36). In addition, previous studies demonstrated that lncRNAs regulate the occurrence and development of numerous diseases and are closely associated with tumor cell invasion and proliferation (37,38). The effect of C5orf66-AS1 on HOS cell proliferation and apoptosis was therefore investigated in the present study. Control-siRNA, C5orf66-AS1-siRNA, inhibitor control or miR-149-5p inhibitor were transfected into U2OS cells for 48 h. The results from the CCK-8 assay and flow cytometry indicated that C5orf66-AS1-siRNA inhibited U2OS cell viability and increased U2OS cell apoptosis, respectively, in the C5orf66-AS1-siRNA group, compared with the control-siRNA group, thus demonstrating a potential tumor growth-inhibiting effect. Apoptosis corresponds to the process of cell self-destruction during certain physiological or pathological conditions under the control of multiple genes (39). Bax and Bcl-2 are important mediators of apoptosis and ultimately regulate apoptotic cell death (39). In the present study, apoptosis-related gene expression was determined in U2OS cells using RT-qPCR and western blot analysis. The results demonstrated that C5orf66-AS1-siRNA decreased Bcl-2 expression and promoted Bax expression compared with the control-siRNA group. However, all these effects were reversed by the miR-149-5p inhibitor. These findings suggested that C5orf66-AS1 downregulation regulated OS cell viability and apoptosis by targeting miR-149-5p, which may block the development of OS.

Migration and invasion are indicators of cancer metastasis, including in OS. Zhang et al (40) reported that the lncRNA differentiation antagonizing non-protein coding RNA promotes OS migration and invasion via the miR-149/musashi RNA binding protein 2 axis. Chao et al (41) also demonstrated the promotion of miR-552 in OS cell migration and invasion (41). The effects of C5orf66-AS1 on U2OS cell migration and invasion were therefore investigated in the present study. The results demonstrated that the migration and invasion of U2OS cells were inhibited by C5orf66-AS1-siRNA, while this inhibition was abolished by the miR-149-5p inhibitor. MMP-9, a vital proteolytic enzyme, has been identified in cancer cells during malignant invasion and migration (42). The present study demonstrated that MMP-9 was downregulated in U2OS cells following C5orf66-AS1-siRNA transfection, which was reversed by the miR-149-5p inhibitor.

Taken together, the findings from the present study suggested that C5orf66-AS1 knockdown had the potential to inhibit OS cell proliferation and invasion by regulating miR-149-5p. However, there were certain limitations to the current study. Firstly, only one cell line (U2OS) was used to study the role of C5orf66-AS1 in OS cells, and more OS cell lines should be used in future experiments. Secondly, only one siRNA was used for C5orf66-AS1 silencing; however, to prevent the off-target effect of siRNA, two or more siRNAs for each gene should be used. In addition, this study was just an *in vitro* preliminary study on the role of C5orf66-AS1 in OS cells. To better understand the role of C5orf66-AS1 in OS, further investigation is needed. For example, the correlation between C5orf66-AS1 expression and the clinicopathological characteristics of patients with OS should be determined. In addition, *in vivo* investigation is required to verify the function of C5orf66-AS1 in OS.

In summary, the results from the present study indicated that C5orf66-AS1 expression was upregulated in OS. C5orf66-AS1 silencing may prevent OS progression by inhibiting OS cell proliferation and invasion via the regulation of miR-149-5p, suggesting the anti-tumor function of C5orf66-AS1 in U2OS cells. C5orf66-AS1 may therefore be considered as a potential therapeutic target for the treatment of OS.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HZ contributed to the study design, data collection, statistical analysis, data interpretation and manuscript preparation. JS contributed to data collection, statistical analysis and manuscript preparation. HZ and JS confirm the authenticity of all the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The study procedures were approved by the Ethics Committee of Huangshi Central Hospital (Huangshi, China). Written informed consent was obtained from each patient for the use of their specimens in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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