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MiRNA-188-5p alleviates the progression of osteosarcoma *via* target degrading CCNT2

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Abstract. – OBJECTIVE: The aim of this study was to investigate the biological role of microRNA-188-5p (miRNA-188-5p) in mediating the progression of osteosarcoma by degrading CCNT2.

PATIENTS AND METHODS: The relative expression levels of miRNA-188-5p and CCNT2 in osteosarcoma tissues and para-cancerous normal tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Meanwhile, their expression levels in osteosarcoma cell lines were examined. The regulatory effects of miRNA-188-5p on the proliferative ability and cell cycle progression of osteosarcoma cells were evaluated by Cell Counting Kit-8 (CCK-8) and flow cytometry, respectively. Dual-Luciferase reporter gene assay was applied to verify the binding relationship between miRNA-188-5p and CCNT2. Furthermore, rescue experiments were conducted to clarify the role of miRNA-188-5p/CCNT2 in mediating the progression of osteosarcoma.

RESULTS: MiRNA-188-5p was lowly expressed in osteosarcoma tissues when compared with paracancerous normal tissues. Overexpression of miRNA-188-5p significantly suppressed the proliferative ability and arrested cell cycle progression of osteosarcoma cells. However, knockdown of miRNA-188-5p obtained the opposite trends. The Dual-Luciferase reporter gene assay verified the binding relationship between miRNA-188-5p and CCNT2. The expression level of CCNT2 in HOS and MG-63 cells was markedly downregulated after transfection of miRNA-188-5p mimics. In addition, overexpression of CCNT2 could partially reverse the inhibitory effect of miRNA-188-5p on the proliferative ability and cell cycle progression of osteosarcoma cells.

CONCLUSIONS: MiRNA-188-5p is downregulated in osteosarcoma. Furthermore, it suppresses the proliferative ability and cell cycle progression of osteosarcoma cells *via* target degrading CCNT2.

Key Words:
Osteosarcoma, MiRNA-188-5p, CCNT2, Cellular functions.

Introduction

Osteosarcoma is the most common bone malignancy occurring in all ages. Children and adolescents are a high-risk population of osteosarcoma[1]. Osteosarcoma frequently affects the metaphysis of long bones, and over 50% of cases are accompanied by knee joint[2]. It is known to all that osteosarcoma is highly malignant. Most patients suffer from distant metastasis before diagnosis, mainly as lung and bone metastasis[3,4]. At present, neoadjuvant chemotherapy, surgery and high-dose chemotherapy are the first choices for the treatment of osteosarcoma. The 5-year survival of non-metastatic osteosarcoma patients is about 65-70%, which is as low as 10-30% in patients with metastases[5]. Hence, it is of clinical significance to search for biological hallmarks and therapeutic targets for osteosarcoma.

MicroRNAs (miRNAs) are a class of single-stranded, non-coding RNA with approximately 18-25 nucleotides in length. They are capable of regulating gene expression at post-transcriptional level[6]. Investigators have shown that over 30% of the human genome can be regulated by miRNAs. Meanwhile, miRNAs are widely involved in physiological and pathological processes, including tumor growth, differentiation, metastasis, apoptosis and angiogenesis[7,8]. For example, miR-422a is downregulated in colorectal cancer and is also associated with local infiltration and lymph node metastasis. *In vitro* experiments have demonstrated that overexpression of miR-422a inhibits cellular behaviors of colorectal cancer cells SW480 and HT-29[9]. MiR-545 is significantly downregulated in lung cancer tissues compared with adjacent non-cancerous tissues. Meanwhile, it arrests the cell cycle progression in the G0/G1 phase and induces apoptosis of lung cancer cells by targeting Cyclin D1 and CDK4[10]. MiRNA-143 has been

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found remarkably downregulated in osteosarcoma tissues and cell lines. The proliferative, invasive and metastatic abilities of osteosarcoma are suppressed after overexpression of miRNA-143. Meanwhile, miRNA-143 is capable of inducing the apoptosis of osteosarcoma cells by downregulating Bcl-2 and activating Caspase 3[11,12]. In addition, miRNA-145 affects the metastasis of osteosarcoma. Through genetic examination, it has been identified that miRNA-145 overexpression markedly suppresses the angiogenesis and local invasive ability of osteosarcoma[13].

Zhang et al[14] illustrated that downregulation of miRNA-188-5p inhibits the growth and invasion of prostate cancer *via* degrading LAPTM4B. However, the potential role of miRNA-188-5p in osteosarcoma remains unclear. The aim of this study was to investigate the biological function of miRNA-188-5p in the progression of osteosarcoma and the underlying mechanism.

Patients and Methods

Baseline Characteristics

Osteosarcoma tissues and paracancerous normal tissues (n=48) were harvested from osteosarcoma patients who received treatment in our hospital. Collected tissues samples were immediately placed in liquid nitrogen for use. Patients volunteered to participate in this study, and informed consent was obtained. This study was approved by the Ethics Committee of our hospital.

Cell Culture

Osteoblasts (NHOst) and osteosarcoma cell lines (U-2OS, HOS, Saos-2, MG-63) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin, and maintained in an incubator with 5% CO_2 at 37°C.

Cell Transfection

One day prior to transfection, cells were seeded into plates. Until 70-80% of confluence, cell transfection was conducted according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 4 hours later, fresh medium was replaced for another 24 h of incubation.

RNA Extraction

Tissues (50 mg) or cells (5×10^6 cells) were first lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). After incubation at room temperature for 5 min, 200 μL of chloroform was added, followed by incubation at room temperature for 5 min. The supernatant was transferred into a new RNase-free centrifuge tube after centrifugation at 4°C and 12000 rpm for 15 min. Isopropanol with the same volume of the supernatant was added for harvesting RNA precipitate by centrifugation. Subsequently, extracted RNA was air dried and quantified. RNA samples were dissolved in 10-20 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China).

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

Total RNA in tissues and cells was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration of RNA was measured by an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). After that, extracted RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of PrimeScript™ RT Master-Mix kit (Invitrogen, Carlsbad, CA, USA). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was carried out in accordance with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The total qRT-PCR system was 10 μL. QRT-PCR was performed as follows: pre-denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. The relative expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: MiRNA-188-5p, F: 5′-ACACTCCAGCTGGGCATCCCTTGCATGGT-3′,R:5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCCTCCAC-3′; U6, F: 5′-CTCGCTTCGGCAGCACATATA-3′, R: 5′-AAATATGGAACGCTTCACGA-3′; CCNT2, F: 5′-GGAGTGGAGGCGGATAAAGAG-3′, R: 5′-AGAGACATTGAGACGCTGTCC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5′-CTAAGGCCAACCGTGAAAAG-3′, R: 5-ACCAGAGGCATACAGGGACA-3′.

Dual-Luciferase Reporter Gene Assay

Wild-type (WT) and mutant-type (MUT) CCNT2 sequences containing the binding sites of miRNA-188-5p were inserted into a pGL3 Dual-Luciferase vector to construct CCNT2 WT

and CCNT2 MUT, respectively. Subsequently, the cells were co-transfected with miRNA-188-5p mimics/negative control and CCNT2 WT/ CCNT2 MUT. Transfected cells were lysed 24 h later. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Cell Cycle Assay

Cells were first seeded into 6-well plates and cultured overnight. Then, the cells were fixed with ethanol at 4°C overnight and stained with 1 mL of DNA Staining Solution for 30 min. Finally, cell cycle progression was determined using MACS flow cytometry (Partec AG, Arlesheim, Switzerland).

Cell Counting Kit-8 (CCK-8)

Cells were first seeded into 96-well plates with 1×10^4 cells per well. After cell adherence, 100 μL of the medium containing 10 μL of Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technology, Kumamoto, Japan) was added to per well. Optical density (OD) value at 450 nm was recorded 1 h later.

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) containing phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China). Protein samples were separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The next day, the membranes were washed with Tris-Buffered Saline with Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA), followed by incubation of corresponding secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analyses. Experimental data were expressed as mean ± standard deviation. Intergroup differences were analyzed by *t*-test. $p<0.05$ was considered statistically significant.

Results

MiRNA-188-5p Was Downregulated in Osteosarcoma

The expression level of miRNA-188-5p in osteosarcoma was firstly detected. QRT-PCR results demonstrated that miRNA-188-5p was downregulated in osteosarcoma tissues when compared with para-cancerous normal ones (Figure 1A). Identically, miRNA-188-5p was lowly expressed in osteosarcoma cell lines compared with osteoclasts NHOst (Figure 1B). Subsequently, MG-63 and HOS cell lines were selected for the following experiments. MiRNA-188-5p mimics and inhibitor were constructed and transfected into MG-63 and HOS cells. Transfection efficacy was verified by qRT-PCR (Figure 1C, 1D).

MiRNA-188-5p Suppressed the Proliferative Ability and Cell Cycle of Osteosarcoma

CCK-8 results indicated that overexpression of miRNA-188-5p markedly inhibited the viability of osteosarcoma cells (Figure 2A, 2D). Conversely, transfection of miRNA-188-5p inhibitor remarkably elevated the viability of HOS and MG-63 cells (Figure 2B, 2E). Flow cytometry showed a significantly higher percentage of cells in the G0/G1 phase after miRNA-188-5p overexpression, indicating arrested cell cycle progression. On the contrary, miRNA-188-5p knockdown accelerated cell cycle, manifesting as an increased percentage of cells in the S phase (Figure 2C, 2F).

CCNT2 Was the Target Gene of MiRNA-188-5p

Bioinformatics analysis predicted the potential binding sequences between CCNT2 and miRNA-188-5p (Figure 3A). Based on the binding sequences, CCNT2 WT and CCNT2 MUT were constructed for Dual-Luciferase reporter gene assay. Subsequent results indicated that relative Luciferase activity decreased markedly in osteosarcoma cells co-transfected with CCNT2 WT and miRNA-188-5p mimics. This verified the binding relationship between CCNT2 and miRNA-188-5p (Figure 3B). CCNT2 level in osteosarcoma was examined by qRT-PCR as well. Osteosarcoma

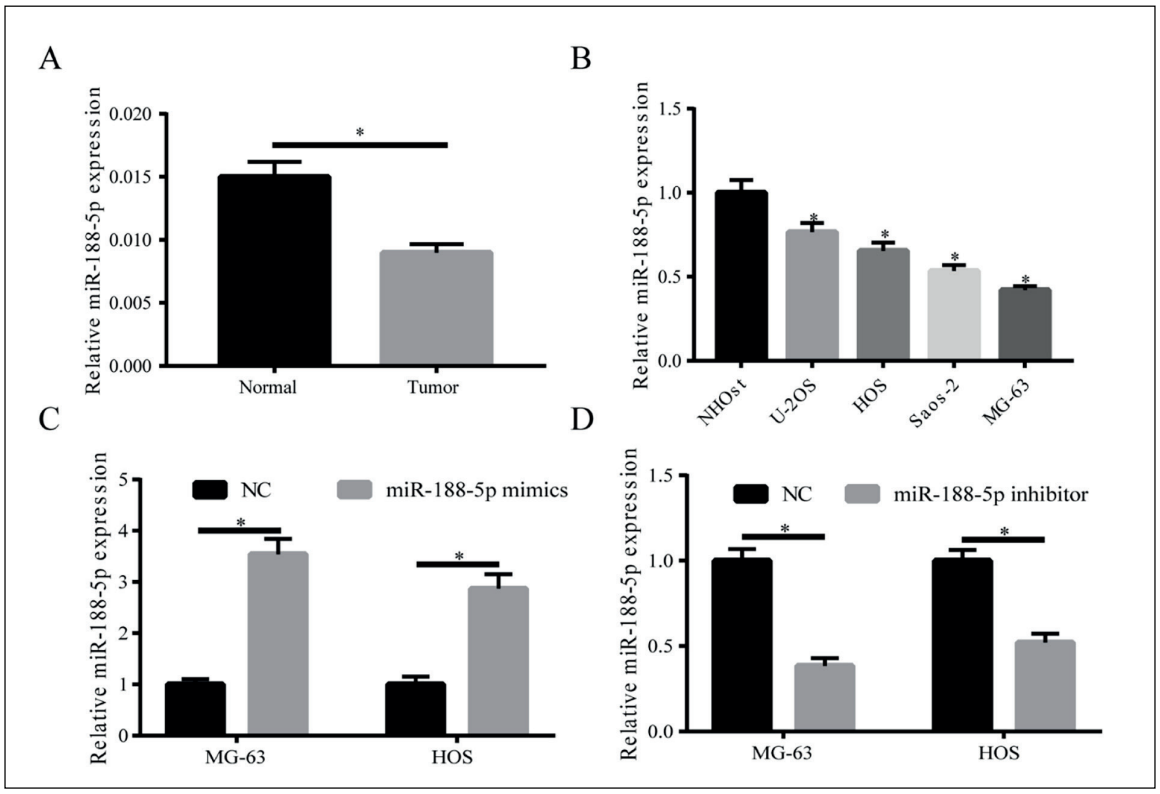

Figure 1. MiR-188-5p was down-regulated in osteosarcoma. **A,** QRT-PCR showed significantly downregulated expression level of miR-188-5p in osteosarcoma tissues relative to paracancerous normal tissues. **B,** MiR-188-5p was down-regulated in osteosarcoma cell lines (U-2OS, HOS, Saos-2 and MG-63) when compared with osteoclasts (NHOst). **C,** Transfection of miR-188-5p mimics upregulated miR-188-5p level in MG-63 and HOS cells. **D,** Transfection of miR-188-5p inhibitor downregulated miR-188-5p level in MG-63 and HOS cells. *$p<0.05$.

tissues presented a significantly higher abundance of CCNT2 relative to controls (Figure 3C). Interestingly, both the mRNA and protein levels of CCNT2 were downregulated in osteosarcoma cells overexpressing miRNA-188-5p (Figure 3D, 3E). The above data showed that CCNT2 was the target gene of miRNA-188-5p, whose expression was negatively regulated by miRNA-188-5p in osteosarcoma.

MiRNA-188-5p Mediated Cellular Behaviors of Osteosarcoma via Degrading CCNT2

To further explore the biological function of miRNA-188-5p/CCNT2 in osteosarcoma, a series of rescue experiments were performed. As CCK-8 assay revealed, suppressed viability of osteosarcoma cells overexpressing miRNA-188-5p was partially reversed by overexpression of CCNT2 (Figure 4A, 4C). Similarly, arrested cell cycle resulted from miRNA-188-5p down-regulation was accelerated by overexpressed CCNT2 (Figure 4B, 4D). The above findings concluded that miRNA-188-5p suppressed the proliferative ability and cell cycle progression by downregulating CCNT2.

Discussion

Osteosarcoma is a common primary bone tumor in children and adolescents. In the past few years, few progress has been made in improving the prognosis of osteosarcoma. With the development researches on genomics and miRNA, the potential role of miRNAs in the occurrence and progression of osteosarcoma has been extensively concerned[15]. Several osteosarcoma-related miRNAs have been currently identified[16]. Gougelet et al[17] have shown that five upregulated miRNAs (miR-92a, miR-193a-5p, miR-99b, miR-132, and miR-422a) serve as oncogenes in osteosarcoma,

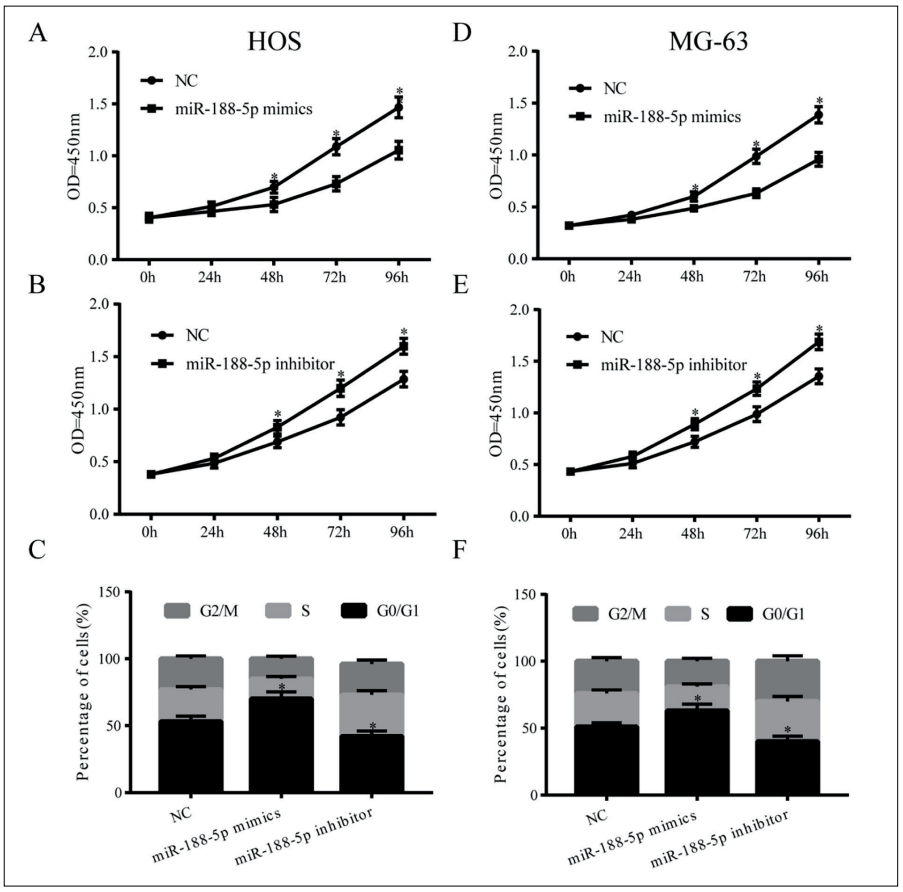

Figure 2. MiR-188-5p suppressed the proliferative ability and cell cycle of osteosarcoma. **A,** CCK-8 results showed that transfection of miR-188-5p mimics decreased the viability of HOS cells. **B,** CCK-8 results showed that transfection of miR-188-5p inhibitor increased the viability of HOS cells. **C,** Flow cytometry showed that transfection of miR-188-5p mimics in HOS cells arrested the cell cycle in the G0/G1 phase. Transfection of miR-188-5p inhibitor in HOS cells accelerated the cell cycle in the S phase. **D,** CCK-8 results showed that transfection of miR-188-5p mimics decreased the viability of MG-63 cells. **E,** CCK-8 results showed that transfection of miR-188-5p inhibitor increased the viability of MG-63 cells. **F,** Flow cytometry showed that transfection of miR-188-5p mimics in MG-63 cells arrested the cell cycle in the G0/G1 phase. Transfection of miR-188-5p inhibitor in MG-63 cells accelerated the cell cycle in the S phase. **p*<0.05.

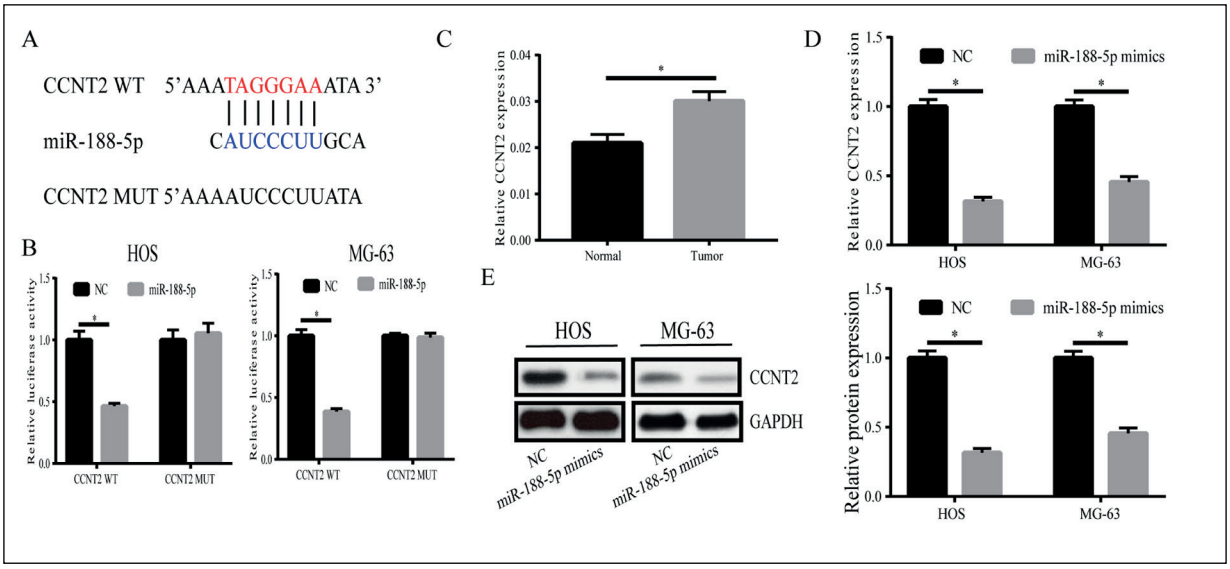

Figure 3. CCNT2 was the target gene of miR-188-5p. **A,** Potential binding sequences between CCNT2 and miR-188-5p. **B,** Relative Luciferase activity markedly decreased in osteosarcoma cells co-transfected with CCNT2 WT and miR-188-5p mimics. **C,** QRT-PCR showed upregulated level of CCNT2 in osteosarcoma tissues relative to paracancerous normal tissues. **D,** Transfection of miR-188-5p mimics downregulated the mRNA level of CCNT2 in HOS and MG-63 cells. **E,** Transfection of miR-188-5p mimics downregulated the protein level of CCNT2 in HOS and MG-63 cells. **p*<0.05.

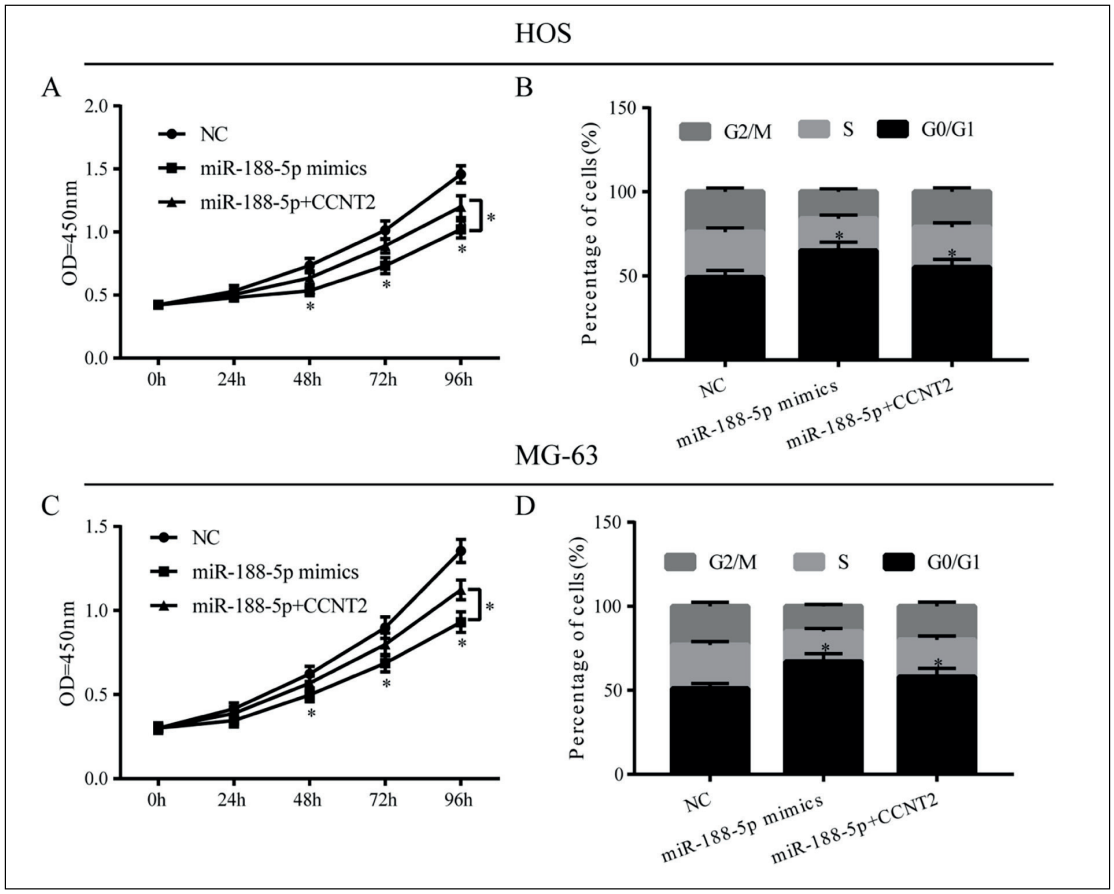

Figure 4. MiR-188-5p mediated the cellular behaviors of osteosarcoma *via* degrading CCNT2. HOS cells were transfected with NC, miR-188-5p mimics or miR-188-5p mimics + pcDNA-CCNT2, respectively. **A,** CCK-8 assay showed cell viability in each group. **B,** Flow cytometry showed the percentage of cells in the G2/M, S and G0/G1 phase. MG-63 cells were transfected with NC, miR-188-5p mimics or miR-188-5p mimics + pcDNA-CCNT2, respectively. **C,** CCK-8 assay showed cell viability in each group. **D,** Flow cytometry showed the percentage of cells in the G2/M, S and G0/G1 phase. **p*<0.05.

which may be utilized as tumor biomarkers. Moreover, miR-802 is upregulated in osteosarcoma[18] and can mediate its progression *via* targeting p27.

In this work, we first examined the expression level of miRNA-188-5p in osteosarcoma. The results showed that miRNA-188-5p was downregulated in tumor tissues when compared with para-cancerous normal tissues. Identically, miRNA-188-5p was lowly expressed in osteosarcoma cell lines, indicating the potential role of miRNA-188-5p in osteosarcoma progression. To further clarify its biological functions, HOS and MG-63 cells were chosen. Subsequent results indicated that overexpression of miRNA-188-5p markedly suppressed the proliferative ability and arrested the cell cycle progression. Opposite trends were observed in osteosarcoma cells with miRNA-188-5p knockdown. Therefore, we speculated that miRNA-188-5p mediated the progression of osteosarcoma by regulating cell proliferation and cell cycle.

Bartel[19] has referred that miRNAs exert their functions by degrading target genes. Here, the bioinformatics method was used to search for the downstream target of miRNA-188-5p. CCNT2 (Cyclin T2) was finally screened out. Dual-Luciferase reporter gene assay confirmed the binding relationship between miRNA-188-5p and CCNT2. Moreover, the relative level of CCNT2 was negatively regulated by miRNA-188-5p in osteosarcoma cells. As a cell cycle-associated protein, CCNT2 is important for the development and progression of early embryos[20]. Meanwhile, it is also involved in the differentiation of various types of cells[20]. In acute leukemia, miR-192 is able to mediate cell proliferation and cell cycle progression by

degrading CCNT2[21]. Therefore, we speculated whether CCNT2 was involved in the progression of osteosarcoma. QRT-PCR data revealed that CCNT2 was highly expressed in osteosarcoma cells. Notably, overexpression of CCNT2 could partially reverse the inhibitory effect of miRNA-188-5p on the proliferative ability and cell cycle progression of osteosarcoma cells. MiRNA-188-5p exerted its regulatory role in osteosarcoma by downregulating CCNT2.

Conclusions

MiRNA-188-5p was downregulated in osteosarcoma, which suppressed the proliferative ability and cell cycle progression of osteosarcoma cells *via* target degrading CCNT2. Furthermore, miRNA-188-5p could be used as a promising target for clinical treatment of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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