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MiRNA-98-5p inhibits the progression of osteosarcoma by regulating cell cycle *via* targeting CDC25A expression

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Abstract. – OBJECTIVE: The aim of this study was to elucidate the exact role of microRNA-98-5p (miRNA-98-5p) in the progression of osteosarcoma and to explore its potential mechanism.

PATIENTS AND METHODS: The expression levels of miRNA-98-5p and cell division cycle 25 (CDC25A) in osteosarcoma tissues and cell lines were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Meanwhile, the correlation between expressions of miRNA-98-5p and CDC25A and the survival of osteosarcoma patients was analyzed. After altering miRNA-98-5p and CDC25A expressions by liposome transfection, the expression levels of CDC25A, ki67, Cyclin D1, p21, BCL2-Associated X (BAX), B-cell lymphoma-2 (BCL-2) and BCL-XL in osteosarcoma cells were detected. Subsequently, potential binding sites between miRNA-98-5p and CDC25A were predicted and further verified by miRanda and Dual-Luciferase reporter gene assay, respectively. Regulatory effects of miRNA-98-5p and CDC25A on the migratory ability of osteosarcoma cells were evaluated by transwell assay. Moreover, nude mice were subcutaneously implanted with MG-63 cells over-expressing miRNA-98-5p or negative control. In addition, the functions of miRNA-98-5p and CDC25A in tumor-bearing nude mice were explored *in vivo*.

RESULTS: MiRNA-98-5p was lowly expressed in osteosarcoma tissues and cell lines, whereas CDC25A was highly expressed. Survival analysis showed that the survival of osteosarcoma patients with low-level of miRNA-98-5p or high-level of CDC25A was significantly worse. Besides, a negative correlation was identified between miRNA-98-5p and CDC25A. Subsequent experiments revealed that miRNA-98-5p significantly inhibited cell cycle progression and migratory potential, whereas induced the apoptosis of osteosarcoma cells by down-regulating CDC25A.

CONCLUSIONS: MiRNA-98-5p is lowly expressed, while CDC25A is highly expressed in osteosarcoma. Furthermore, miRNA-98-5p regulates cell cycle progression by down-regulating CDC25A, thus inhibiting the progression of osteosarcoma.

Key Words:
MiRNA-98-5p, CDC25A, Osteosarcoma.

Introduction

Osteosarcoma is a malignant mesenchymal tumor. It is also the most common primary malignant bone tumor in pediatrics, posing a huge threat to their families and society[1]. At present, limb salvage combined with neoadjuvant chemotherapy is the major therapeutic approach of osteosarcoma. It can effectively improve the 5-year survival of patients. However, the prognosis of osteosarcoma is still unsatisfactory relative to other malignant tumors[2,3]. Recently, research on improving osteosarcoma prognosis has encountered a bottleneck. Furthermore, in-depth explorations on the pathogenesis of osteosarcoma may provide novel strategies for its clinical treatment.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs with 20 to 24 nucleotides in length. MiRNAs have shown crucial functions in various biological processes and diseases[4,5]. Some authors[6,7] have demonstrated that they are involved in the regulation of cell growth, proliferation, apoptosis, differentiation, migration and metabolism. It is currently believed that miRNA dysfunction is associated with tumor development[8]. Meanwhile, the clarification of miRNA functions in tumor development helps to enhance diagnostic and therapeutic efficacies. Currently, several osteosarcoma-associated miRNAs have been identified in the occurrence and progression of osteosarcoma. For example, miRNA-16,

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miRNA-29b, miRNA-143, miRNA-145 and miRNA-126 are lowly expressed in osteosarcoma, whereas miRNA-21, miRNA-181a, miRNA-9, miRNA-195, miRNA-148a and miRNA-181b are highly expressed[9,10]. Research has indicated that miRNA-98-5p is a stress-specific miRNA participating in cell survival, apoptosis and oxidative stress[11-13]. However, the specific role of miRNA-98-5p in osteosarcoma has not been fully elucidated.

Cell division cycle 25 (CDC25A) is a homolog of CDC25 diphosphatase with about 70 kD in size. CDC25A is a phosphatase with dual-properties of tyrosine and threonine, exerting significant roles in regulating cell cycle and apoptosis[14]. CDKs can be regulated through phosphorylation and dephosphorylation of CDC25A. Functionally, CDC25A is capable of abolishing inhibitory phosphatases in CDKs, such as CDK2, CDK4 and CDK6. Meanwhile, it mediates the activities of CDKs that promote cell cycle progression, regulate G1/S and G2/M checkpoints, further precisely controlling cell cycle. Previous studies have indicated that CDC25A is over-expressed in several tumors, including lung cancer[15,16], prostate cancer[17], liver cancer[18] and leukemia[19,20]. Overexpressed CDC25A promotes cancer cells to immediately pass through cell cycle checkpoints, as well as accelerates the proliferative potential. High expression of CDC25A is closely correlated with the progression and prognosis of malignant tumors by serving as an oncogene[21]. However, the relationship between miRNA-98-5p and CDC25A in osteosarcoma has not been fully understood.

The aim of this work was to explore the biological function of miRNA-98-5p in osteosarcoma. Moreover, we also investigated the molecular mechanism of miRNA-98-5p/CDC25A axis in regulating the progression of osteosarcoma.

Patients and Methods

Sample Collection

Osteosarcoma tissues (n=15) and para-cancerous tissues (n=15) were collected during the surgery, and preserved at -80°C for subsequent use. Informed consent was obtained from patients and their families before the study, which was approved by the Ethics Committee of Xi'an Medical University.

Cell Culture and Transfection

Osteosarcoma cell lines (MG-63, U2OS, SAOS-2) and osteoblasts (hFOB) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), and maintained in a 5% CO_2 incubator at 37°C.

MG-63 cells were transfected with NC, miRNA-98-5p mimics, miRNA-98-5p inhibitor, si-CDC25A, pcDNA-CDC25A, si-CDC25A+miRNA-98-5p inhibitor or si-CDC25A+miRNA-98-5p mimics according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced 6 hours later. 48 h after transfection, the cells were harvested for the following experiments.

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

Total RNA in tissues and cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reverse transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Obtained cDNA was then amplified by Real Time-quantitative Polymerase Chain Reaction (qRT-PCR) using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). The relative levels of genes were quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: MiRNA-98-5p, F: 5'-AGATCAGGGTGGCCCCATTT-3', R: 5'-AGATCAGGGTGGCCCCATTT-3'; CDC25A, F: 5'-GAGGAGTCTCCACCTGGAAGTACA-3', R: 5'-GCCATTCAAAACAGATGCCATAA-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5'-GACCTGACCTGCCGTCTA-3', R: 5'-AGGAGTGGGTGTCGCTGT-3'.

Transwell Migration Assay

48 h after transfection, the cells were digested in serum-free medium, and the density of cells was adjusted to 1.0×10^5/mL. 200 μL of cell suspension was added in the upper chamber of a Matrigel-coated transwell chamber (8-μm, Corning Incorporated, Lowell, NY, USA). Meanwhile, 500 μL of medium containing 10% FBS was added in the lower chamber. After 48 h of incubation, invasive cells were fixed with 4% paraformaldehyde and stained with crystal violet. The number of penetrating cells was counted using a microscope. Five fields were randomly selected for each sample.

Western Blot

Proteins were harvested from cell lysis and quantified. 50 μg of protein sample was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies at room temperature for 2 h. Finally, immunoreactive bands were exposed using enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific, Waltham, MA, USA).

Dual-Luciferase Reporter Gene Assay

MG-63 cells were co-transfected with 100 nmol/L miRNA-98-5p mimics/miR-NC and 0.2 μg psiCHECK-2-CDC25A 3' untranslated region (3'UTR)-WT/psiCHECK-2-CDC25A 3'UTR-MUT for 48 h. After washing with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) three times, the cells were lysed for 15 min in the dark. Finally, Luciferase activity was determined.

Subcutaneous Tumorigenesis in Nude Mice

MG-63 cells stably over-expressing miRNA-98-5p or negative controls were first constructed. A total of 2×10^6 cells were subcutaneously implanted into each nude mouse (4-week-old) at the back, with 6 mice in each group. Tumor volume was recorded weekly for 5 weeks.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analyses. Data were expressed as mean ± standard deviation. *t*-test was compared to compare the difference between groups. Chi-square test was applied to compare the differences among multiple groups. $p<0.05$ was considered statistically significant.

Results

Downregulated MiRNA-98-5p and Upregulated CDC25A in Osteosarcoma

We first analyzed the expression levels of miRNA-98-5p and CDC25A in osteosarcoma tissues and cells by qRT-PCR. MiRNA-98-5p was lowly expressed in osteosarcoma tissues when compared with controls (Figure 1A). Identically, miRNA-98-5p expression in osteosarcoma cell lines was significantly lower than that of normal osteoblasts, especially in MG-63 cells (Figure 1B). Based on the follow-up data of osteosarcoma patients, survival analysis was conducted. Markedly longer survival time (months) was observed in osteosarcoma patients with higher level of miRNA-98-5p than those with lower level (Figure 1C). QRT-PCR data indicated that CDC25A was highly expressed in osteosarcoma as well (Figure 1D, 1E). In particular, MG-63 cells exhibited the highest level of CDC25A. Survival analysis demonstrated the survival time of osteosarcoma patients with higher level of CDC25A was remarkably shorter than those with a lower level (Figure 1F). Moreover, miRNA-98-5p was negatively correlated with CDC25A (Figure 1G). The above results indicated down-regulated miRNA-98-5p and up-regulated CDC25A in osteosarcoma. Furthermore, the poor prognosis was observed in osteosarcoma patients with low expression of miRNA-98-5p or high expression of CDC25A.

MiRNA-98-5p Targeted and Degraded CDC25A in Osteosarcoma Cells

Potential binding sites between miRNA-98-5p and CDC25A were predicted by miRanda (Figure 1A). Subsequently, MG-63 cells were co-transfected with miRNA-98-5p mimics/NC and CDC25A 3'UTR-WT/CDC25A 3'UTR-MUT. Dual-Luciferase reporter gene assay showed that over-expression of miRNA-98-5p significantly quenched the Luciferase activity of wild-type CDC25A. This detected the binding relationship between miRNA-98-5p and CDC25A (Figure 2B). Subsequently, we determined the expression levels of miRNA-98-5p and CDC25A in MG-63 cells with different treatments. As shown by qRT-PCR data, miRNA-98-5p was markedly up-regulated in MG-63 cells transfected with miRNA-98-5p mimics or si-CDC25A+miRNA-98-5p mimics. However, its expression decreased remarkably in cells transfected with miRNA-98-5p inhibitor or si-CDC25A+miRNA-98-5p inhibitor (Figure 1C). Compared with controls, CDC25A expression was significantly up-regulated in MG-63 cells transfected with miRNA-98-5p inhibitor or pcDNA-CDC25A. However, its expression was remarkably down-regulated after transfection of miRNA-98-5p mimics, si-CDC25A or miRNA-98-5p mimics+si-CDC25A. However, no

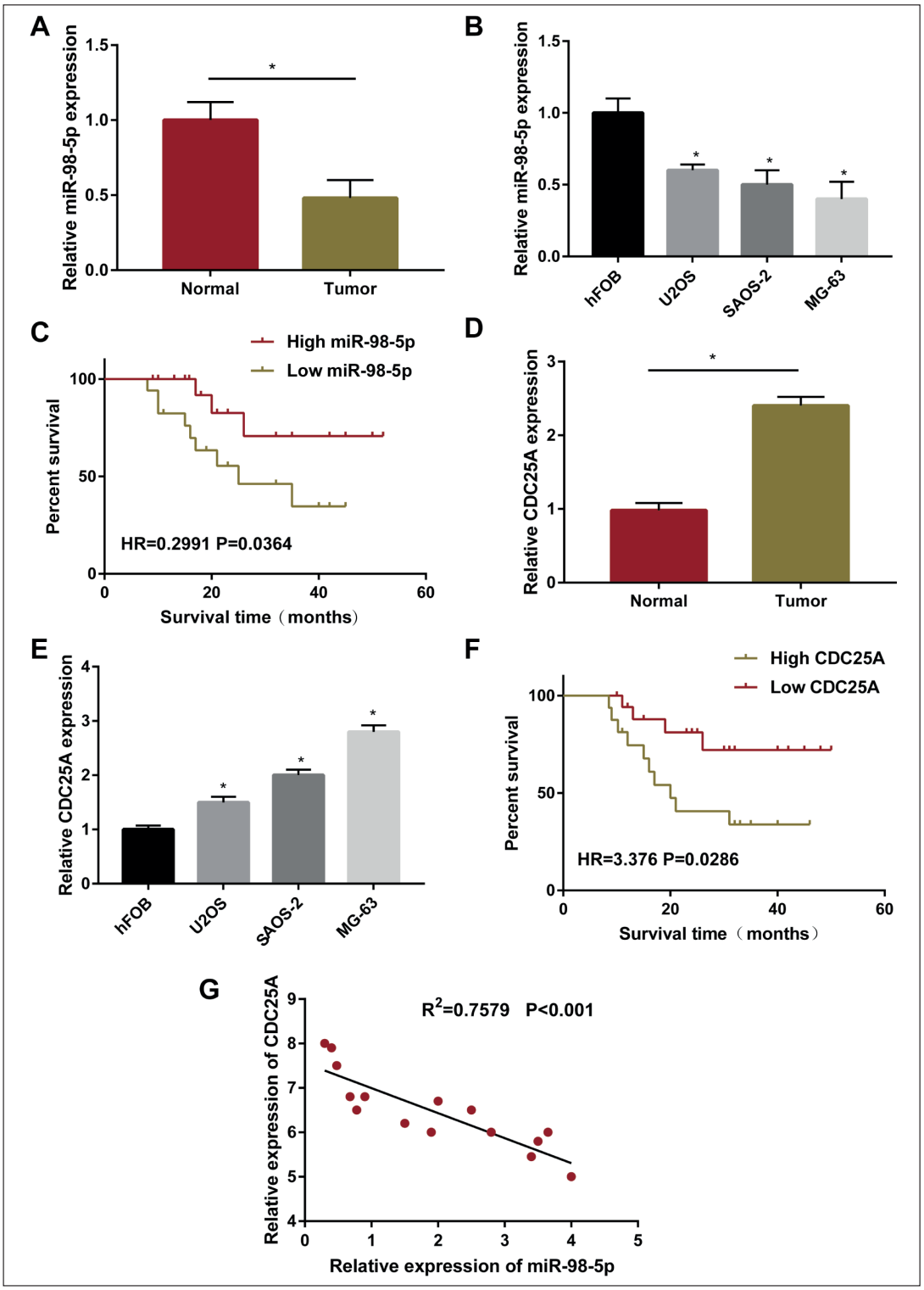

Figure 1. Down-regulated miR-98-5p and up-regulated CDC25A in osteosarcoma. **A,** MiR-98-5p was lowly expressed in osteosarcoma tissues relative to controls. **B,** MiR-98-5p was lowly expressed in osteosarcoma cell lines than that of normal osteoblasts. **C,** Survival analysis showed significantly longer survival time (months) in osteosarcoma patients with higher level of miR-98-5p than those with lower level. **D,** CDC25A was highly expressed in osteosarcoma tissues relative to controls. **E,** CDC25A was highly expressed in osteosarcoma cell lines than that of normal osteoblasts. **F,** Survival analysis showed markedly shorter survival time in osteosarcoma patients with higher level of CDC25A than those with lower level. **G,** A negative correlation was found between miR-98-5p and CDC25A.

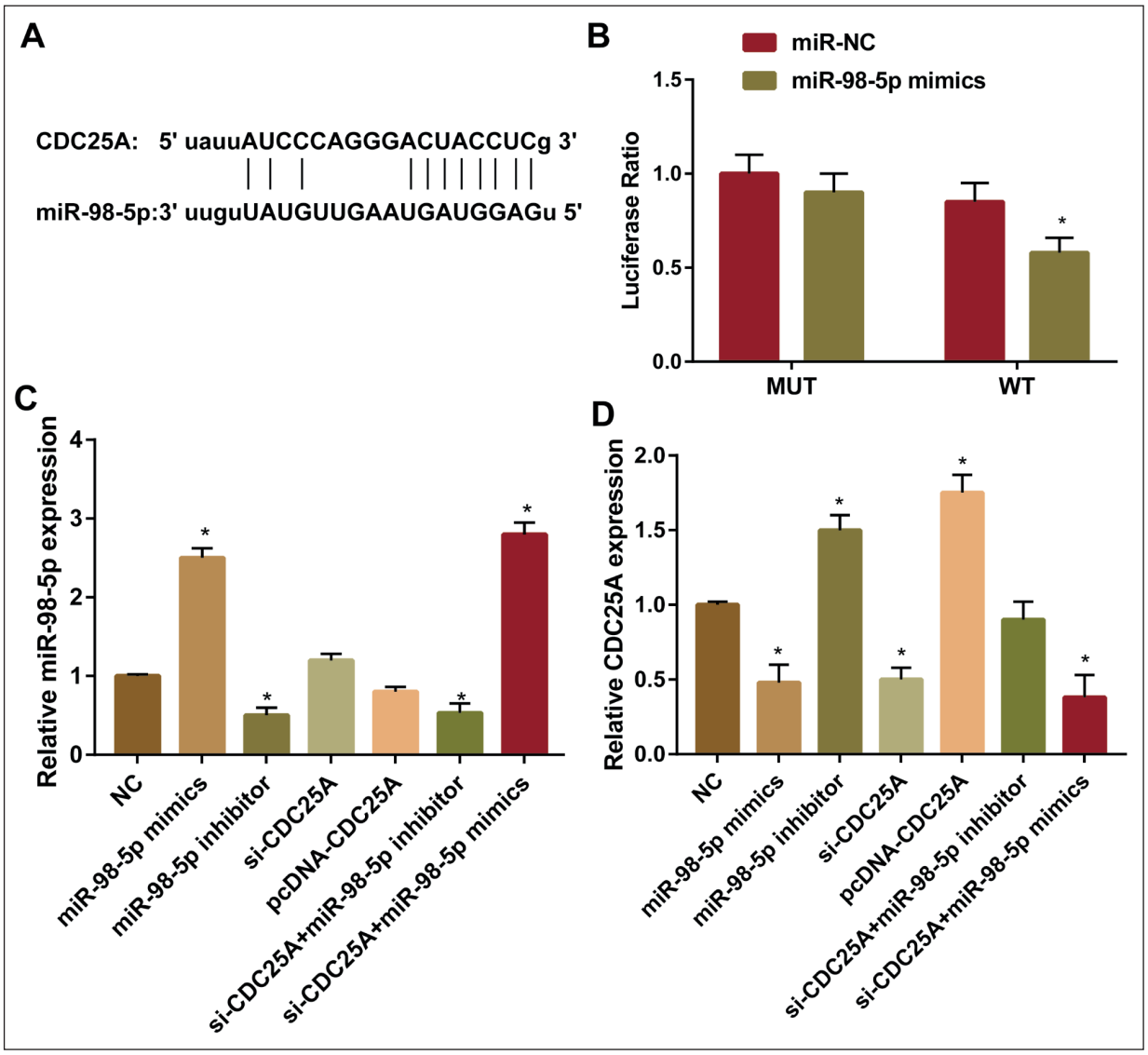

Figure 2. MiR-98-5p targeted CDC25A and inhibited its expression in osteosarcoma cells. **A,** Potential binding sites between miR-98-5p and CDC25A. **B,** MG-63 cells were co-transfected with miR-98-5p mimics/miR-NC and psiCHECK-2-CDC25A 3'UTR-WT/psiCHECK-2-CDC25A 3'UTR-MUT. Dual-Luciferase reporter gene assay found that over-expression of miR-98-5p quenched the Luciferase activity of wild-type CDC25A. MG-63 cells were transfected with NC, miR-98-5p mimics, miR-98-5p inhibitor, si-CDC25A, pcDNA-CDC25A, si-CDC25A+miR-98-5p inhibitor or si-CDC25A+miR-98-5p mimics, respectively. **C, D,** Relative levels of miR-98-5p (**C**) and CDC25A (**D**) in each group.

remarkable changes were found in CDC25A expression in cells transfected with miRNA-98-5p inhibitor+si-CDC25A. The above results indicated that miRNA-98-5p could target CDC25A and exhibited a low expression in osteosarcoma cells.

In Vitro Effects of MiRNA-98-5p and CDC25A on Osteosarcoma Cells

Subsequently, we detected the protein expression levels of apoptosis-related genes by Western blot. The results indicated that the protein level of BAX was significantly up-regulated in MG-63 cells transfected with miRNA-98-5p mimics, si-CDC25A or miRNA-98-5p mimics+si-CDC25A. Conversely, the protein levels of BCL-2 and BCL-XL were markedly down-regulated in these treated groups. In addition, BAX was lowly expressed in cells transfected with miRNA-98-5p inhibitor or pcDNA-CDC25A, whereas BCL-2 and BCL-XL

were up-regulated. However, we did not observe remarkable changes in the protein expressions of these genes after transfection of miRNA-98-5p inhibitor+si-CDC25A (Figure 3A). QRT-PCR showed miRNA-98-5p over-expression or CDC25A knockdown markedly down-regulated the levels of CDC25A, ki67 and Cyclin D1, whereas increased p21 expression. Co-transfection of miRNA-98-5p inhibitor and si-CDC25A did not significantly alter the expression changes in the above genes (Figure 3B). Transfection of miRNA-98-5p mimics, si-CDC25A or miRNA-98-5p mimics+si-CDC25A remarkably decreased the migratory ability of MG-63 cells. On the contrary, miRNA-98-5p knockdown accelerated the migratory potential of cells. The silence of both miRNA-98-5p and CDC25A did not result in changes in the migration of cells (Figure 3C). These results demonstrated that miRNA-98-5p inhibited migration and induced apoptosis of osteosarcoma cells by binding to CDC25A.

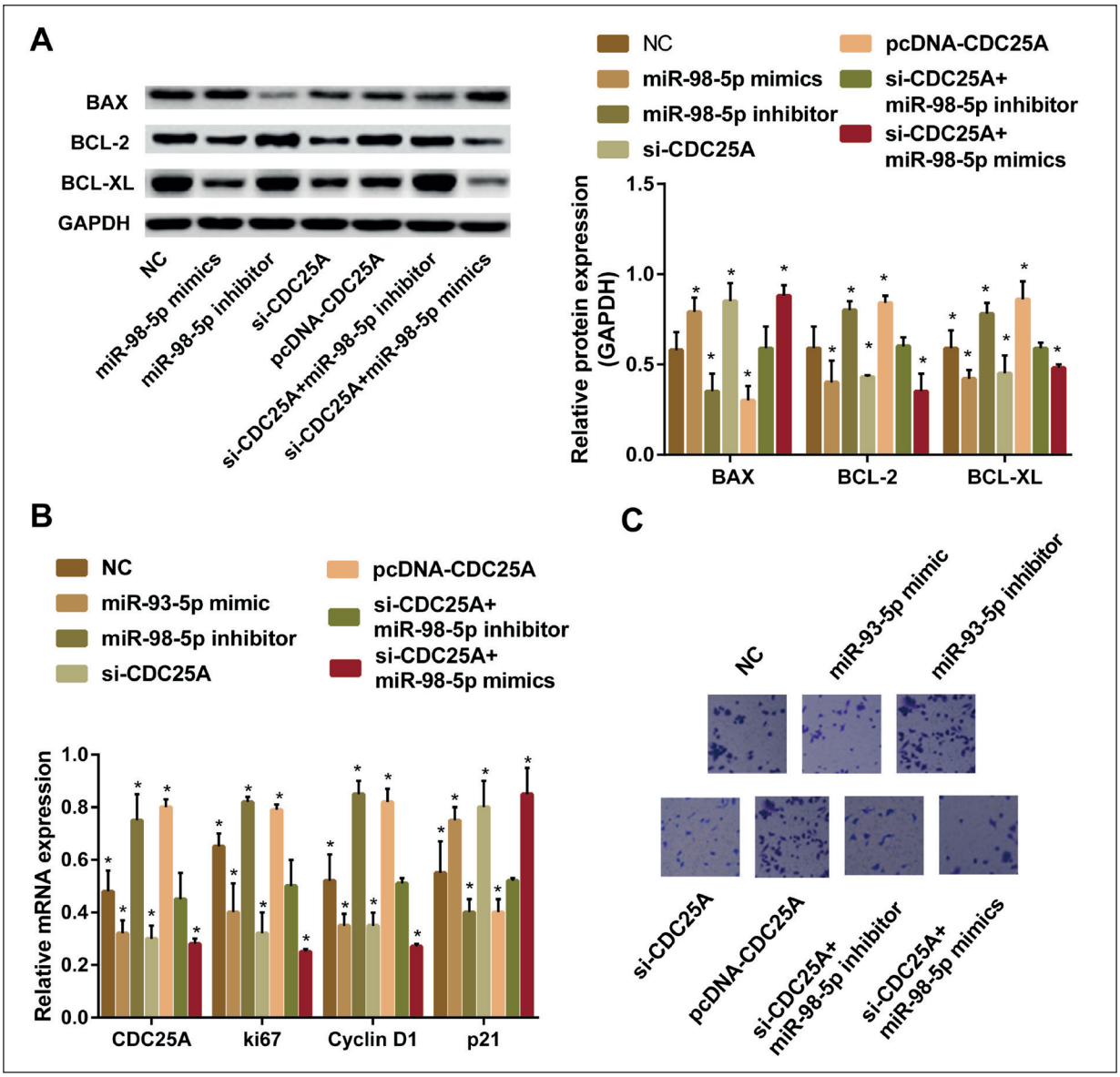

Figure 3. *In vitro* effects of miR-98-5p and CDC25A on osteosarcoma cells. MG-63 cells were transfected with NC, miR-98-5p mimics, miR-98-5p inhibitor, si-CDC25A, pcDNA-CDC25A, si-CDC25A+miR-98-5p inhibitor or si-CDC25A+miR-98-5p mimics, respectively. **A,** Protein levels of BAX, BCL-2 and BCL-XL. **B,** The mRNA levels of CDC25A, ki67, Cyclin D1 and p21. **C,** Invasive ability in each group (Magnification × 40).

In Vivo Effect of MiRNA-98-5p on Osteosarcoma Growth by Inhibiting CDC25A

To further validate *in vivo* effects of miRNA-98-5p and CDC25A on osteosarcoma, we established subcutaneous tumorigenesis model in nude mice through injection of MG-63 cells. Mice injected with MG-63 cells over-expressing miRNA-98-5p exhibited significantly smaller tumor volume and lower tumor weight than those of controls (Figure 4A). Subsequently, we collected osteosarcoma tissues from nude mice injected with MG-63 cells over-expressing miRNA-98-5p or NC. The protein levels of relative genes in tumors were determined by Western blot. Consistently with *in vitro* results, miRNA-98-5p over-expression markedly down-regulated the levels of CDC25A, ki67 and Cyclin D1, whereas upregulated p21 level (Figure 4B). Hence, *in vivo* experiments proved that the over-expression of miRNA-98-5p inhibited the growth of osteosarcoma by inhibiting CDC25A expression.

Discussion

Osteosarcoma is a bone malignancy in children and adolescents with 10-20 years[22]. Males are more prone to be affected by osteosarcoma than females[23]. Osteosarcoma is characterized by high malignancy, invasiveness and metastasis. Due to high malignancy, early metastasis and

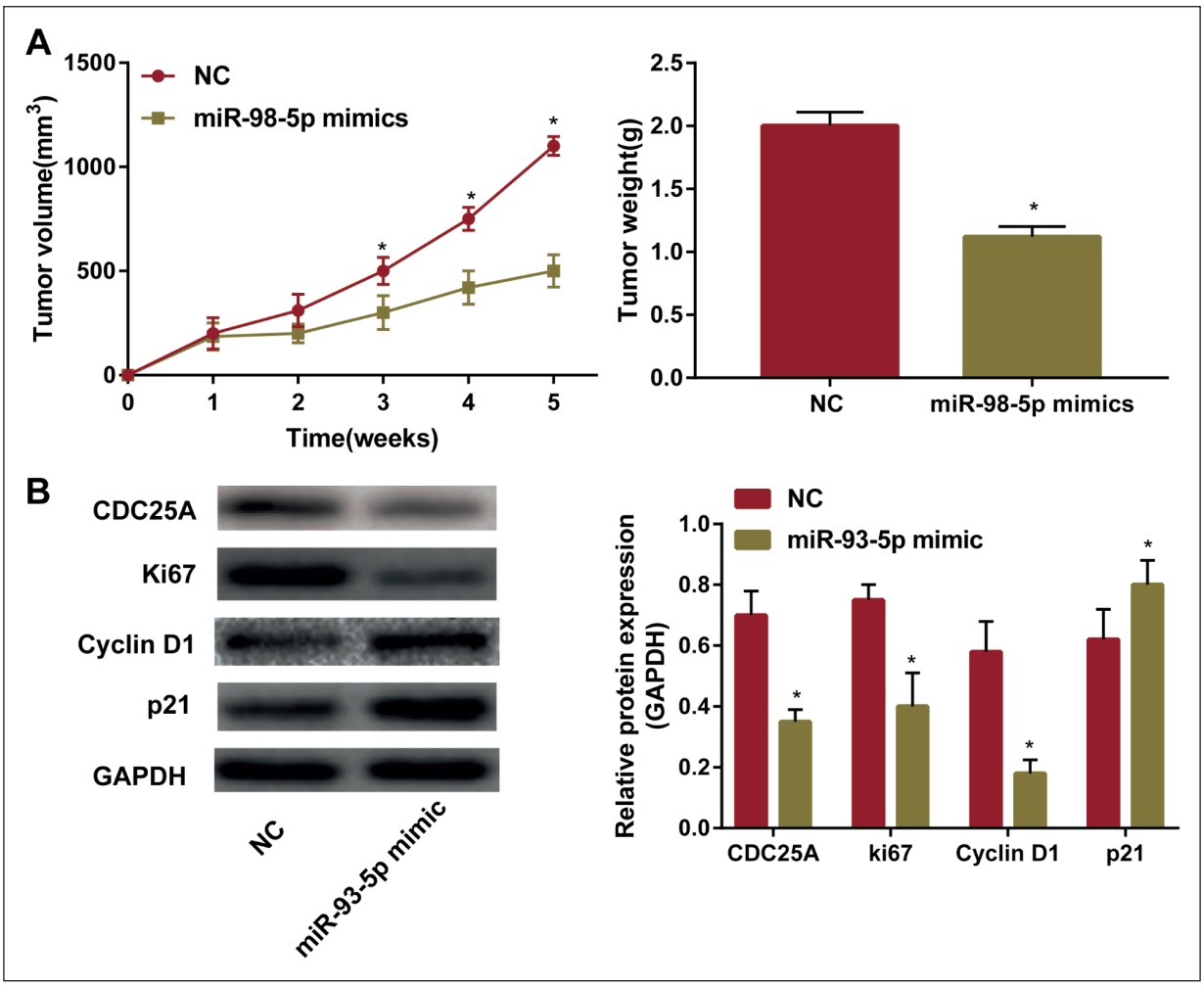

Figure 4. *In vivo* effect of miR-98-5p on osteosarcoma growth by inhibiting CDC25A. **A,** Mice injected with MG-63 cells over-expressing miR-98-5p exhibited significantly smaller tumor volume and weight than those of controls. **B,** MiR-98-5p over-expression markedly down-regulated the levels of CDC25A, Ki-67 and Cyclin D1, whereas upregulated p21 level in tumors harvested from nude mice implanted with MG-63 cells over-expressing miR-98-5p.

drug resistance, osteosarcoma patients are prone to experience local recurrence or distant metastasis even after surgery or high-dose chemotherapy. Meanwhile, the 5-year survival of osteosarcoma patients has not been greatly improved in the past decade[24]. Therefore, it is urgent to develop novel and effective strategies to prolong the survival of osteosarcoma patients. In recent years, relative etiology and molecular biology studies have been conducted.

MicroRNAs are a class of non-coding RNAs. They can downregulate the expression of target genes by pairing to the 3'UTR of corresponding mRNAs, thus participating in disease progression[25,26]. MiRNA-98-5p is involved in several types of tumors[27,28]. For example, miRNA-98-5p can be used as a prognostic indicator for triple-negative breast cancer. MiRNA-98-5p is markedly associated with the prognosis of gastric cancer. Meanwhile, a correlation between miRNA-98-5p expression with poor prognosis, tumor growth and metastasis of ovarian cancer has been observed. Relative studies have pointed out that miRNA-98-5p can be used as a biomarker for early diagnosis of laryngeal cancer. Chen et al[29] have found that the expression level of miRNA-98-5p in circulating blood of NSCLC patients decreases remarkably, which may be utilized as a biomarker for early screening or prognosis of NSCLC. In addition, miRNA-98-5p significantly reduces cell proliferation and accelerates tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in NSCLC patients[30,31]. These studies all suggest the vital roles of miRNA-98-5p in tumor development. In our research, miRNA-98-5p was lowly expressed in osteosarcoma tissues and cell lines. Subsequently, we analyzed the regulatory influence of miRNA-98-5p on the migratory, apoptosis and cell cycle of osteosarcoma cells. The over-expression of miRNA-98-5p significantly inhibited the expressions of cell cycle-related genes and induced apoptosis of osteosarcoma cells. Moreover, miRNA-98-5p could remarkably reduce the volume and weight of osteosarcoma tissues *in vivo*.

In addition, we investigated the targeting relationship between miRNA-98-5p and CDC25A. Surprisingly, a negative correlation was found between miRNA-98-5p and CDC25A. Research has shown that the vast majority of malignancies present cell cycle disorders or irregularities[32]. Regulation of cell cycle progression depends on three types of proteins, including Cyclins, Cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs)[33]. CDC25A belongs to the Cyclin family, which is overexpressed by activating CDK/Cyclin. It can accelerate cell cycle progression from G2 phase to S phase. Enhanced proliferative rate leads to the imbalance between proliferation and apoptosis, eventually leading to tumorigenesis[34]. In this paper, we found that miRNA-98-5p regulated cell cycle by regulating the expression of CDC25A, thereafter inhibiting the progression of osteosarcoma.

Conclusions

This investigation indicates that miRNA-98-5p is lowly expressed, while CDC25A is highly expressed in osteosarcoma. Moreover, miRNA-98-5p regulates cell cycle progression by down-regulating CDC25A, thus inhibiting the progression of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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