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MiRNA-708/CUL4B axis contributes into cell proliferation and apoptosis of osteosarcoma

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Abstract. – OBJECTIVE: The functions of miRNA-708 for various diseases have been confirmed. However, its roles in osteosarcoma are unclear. In this study, we aimed to explore the role of miRNA-708 in osteosarcoma.

PATIENTS AND METHODS: Detection of the expression of miRNA-708 and CUL4B was used by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cells were transfected with miRNA-708 mimics (mimics group) and miRNA negative control (NC group). Detection of cell growth curve at 24 h, 48 h, 72 h, and 96 h was made by cell counting kit-8 (CCK-8). Examination of the apoptosis rate was made by flow cytometry. The identification of the regulatory function was made by the luciferase reporter assay. The expression level of CUL4B was detected by Western blot.

RESULTS: MiRNA-708 expression was reduced in the tumor cell lines. Compared with NC group, miRNA-708 expression was up-regulated by transfecting with mimics. Lower proliferation efficiency and higher cell apoptosis were showed in miRNA-708 mimics group relative to NC group. MiRNA-708 could regulate the expression of CUL4B by binding to its 3'UTR area. Furthermore, lower miRNA-708 and higher CUL4B were expressed in tumor tissues. MiRNA-708 expression was lower in tissues with IIB-III stage than that in IA-IIA stage.

CONCLUSIONS: MiRNA-708/CUL4B axis contributes into cell proliferation and apoptosis of osteosarcoma.

Key Words:
Osteosarcoma, miRNA-708, Proliferation, Apoptosis, CUL4B.

Introduction

MicroRNA (miRNA) is a small molecule, single-stranded, non-coding RNA with about 20-25 nucleotides, which often act as post-transcriptional inhibitors[1-3]. MiRNAs are involved in the regulation of cell proliferation and apoptosis, and it have a wide range of gene regulation functions[4,5]. MiRNA-708 could suppress cell proliferation and invasion by targeting Notch1 in gastric cancer[6]. MiRNA-708 targeted BAMBI to regulate cell proliferation and apoptosis in mice with melanoma[7]. MiRNA-708 could enhance phagocytosis to eradicate T-ALL cells through controlling CD47[8]. MiRNA-708 could inhibit JAK1, which promoted SH-SY5Y neuronal cell survival[9]. In lung cancer, miRNA-708 downregulated DNMT3A that repressing stem cell-like phenotypes by inhibiting Wnt/β-catenin[10]. In adult B-acute lymphoblastic leukemia, miRNA-708 was involved in Wnt/β-catenin pathway through activating DKK3 gene[11]. In hepatocellular carcinoma, miRNA-708 was decreased and acted as a tumor suppressor *via* modulating SMAD3 expression[12]. In ovarian cancer, miRNA-708 contributed to the cisplatin resistance through IGF2BP1/Akt pathway[13]. Down-regulation of c-FLIPL mediated by miRNA-708 could enhance the sensitivity of cells to anti-cancer drugs in renal cancer[14]. MiRNA-708 could enhance the steroid-induced osteonecrosis of the femoral head and inhibit the osteogenic differentiation *via* regulating SMAD3 expression[15]. In breast cancer, miRNA-708/LSD1 axis contributed to the proliferation and invasion of cells[16]. MiRNA-708 was reduced in lung cancer and function as a therapeutic agent against metastasis[17]. Downregulation of miRNA-708 in hepatocellular cancer could repress the invasion and migration of tumor cells[18]. In prostate cancer, metformin promotes ER stress-dependent apoptosis *via* miR-708/NNAT pathway[19]. In chronic lymphocytic leukemia, the downregulation of miRNA-708 could promote NF-κB (nuclear factor-kappa

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B) signaling[20]. MiRNA-708 was implicated in the repression of cells metastasis by regulating Rap1B in ovarian cancer[21]. MiRNA-708 was differentially expressed in childhood acute lymphoblastic leukemia[22]. MiRNA-708 could modulate CD38 expression *via* JNK MAP kinase and PTEN/AKT[23].

Therefore, the primary purpose of this study was to investigate the effect of miRNA-708 on proliferation and apoptosis of osteosarcoma cells and its underlying mechanisms.

Patients and Methods

Clinical Samples

Osteosarcoma tissues and the matched samples were obtained from patients who underwent surgical resection in our hospital. The Ethics Committee of the Second Affiliated Hospital of Dalian Medical University approved this study. All patients signed the informed consent. All the samples were snap-frozen with liquid nitrogen, and then stored at –80°C condition.

Cell Culture and Transfection

Human osteosarcoma cell lines (U2OS and Saos2) and osteoblast cells (hFOB1.19) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, South Logan, UT, USA) with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) at 37°C 5% CO_2 condition. Oligo miRNA-708 purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China), and transfected into cells by Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) reagent. The sequence of the miRNA-708 mimic was 5'-AAGGAGCUUACAAUCUAGCUGGG-3'. The sequence of NC was 5'-UUCUCCGAACGUGUCACGUTT-3'.

Total RNA Extraction and Reverse Transcription

TRIzol method (Invitrogen, Carlsbad, CA, USA) was conducted to extract RNA. The purity and concentration of total RNA were detected by using external spectrophotometer (TRICOR Systems Inc., Elgin, IL, USA). Then, RNA was reversely transferred into complementary Deoxyribose Nucleic Acid (cDNA) with the reverse transcription kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Detection

Total RNA was extracted from the tissues and cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScriptTM RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$. Primer sequences used in this study were as follows: miRNA-708, F: 5'-GGGGTGTAAACATCCTCGACTG-3', R: 5'-ATTGCGTGTCGTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Detection of Cell Proliferation

After 24 hours of transfection, each group of cells was plated and cultured in 96 well plates (100 μL contains about 3 000 cells). After 24 hours, 48 hours, 72 hours, and 96 hours of incubation, 10 μL Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added into the tested well, and continued to incubate for 2 hours. The optical density (OD) value of each well was detected at a wavelength of 450 nm. Three replicates were set at each point.

Apoptosis Rate Detection

After 24 hours of transfection, each group of cells was collected to make a single cell suspension in 100 μL of binding buffer containing 5 μl of FITC Annexin V and 5 μL of propidium iodide (PI). Flow Cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect cell apoptosis rate. The experiment was repeated three times.

Dual-Luciferase Reporter Assay

According to the protocol, through using Lipofectamine 2000, cells were transfected with miRNA-708 mimics and plasmids containing pMIR-CUL4B-3'UTR wt or pMIR-CUL4B-3'UTR mut. After 48 h of transfection, the activities were detected on a dual-luciferase reporter assay system (Promega Co., Madison, WI, USA). The Renilla luciferase activity was regarded as the internal control.

Protein Extraction and Western Blot

After 72 h of transfection, the protein was obtained from cells. Bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to examine the concentration. 20 μg protein were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel (Beyotime Institute of Biotechnology, Shanghai, China) and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Blocking in 10% milk for 2 h, the membranes were incubated with the CUL4B antibody (1:1500; Abcam, Cambridge, MA, USA) for 4 h. The membranes were washed 3 times with Tris-Buffered Saline-Tween (TBST, Beyotime, Shanghai, China). The analysis was examined by Image J software according to the manufacturer's instructions.

Statistical Analysis

Data analysis was obtained by using Statistical Product and Service Solutions (SPSS) 18.0 software (Chicago, IL, USA). Data were assessed as the mean ± standard deviation (`x ± SD). Differences between groups were carried out by the Student's t-test and the χ^2-test. Differences were considered significant when $p < 0.05$.

Results

MiRNA-708 Expression Was Reduced in Osteosarcoma Cells

To assess the biological functions of miRNA-708 in osteosarcoma, the qRT-PCR method was carried out to measure the different expression of miRNA-708 in U2OS and Saos2 relative to hFOB1.19 cells. Significantly, we found that the expression of miRNA-708 was reduced in U2OS and Saos2 (Figure 1A), indicating miRNA-708 involved in the development of osteosarcoma.

Up-Regulated Mirna-708 Inhibited Cell Proliferation and Induced Cell Apoptosis

MiRNA-708 was up-regulated by transfecting with miRNA-708 mimics (Figure 1B and 1C). The results of CCK8 exhibited that a decreased cell proliferative ability was observed in miRNA-708 over-expression group compared with the NC group (Figure 2A and 2B). Meanwhile,

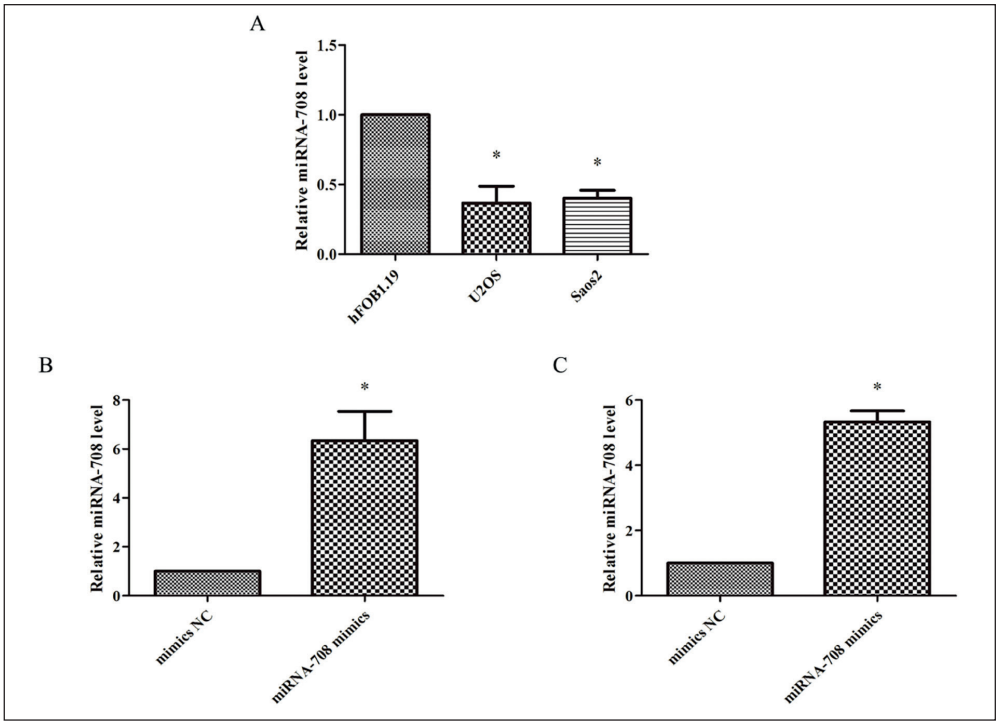

Figure 1. miRNA-708 expression was reduced in osteosarcoma cells. ***A,*** qRT-PCR method was carried out to measure the different expression of miRNA-708 in U2OS and Saos2 relative to hFOB1.19 cells. ***B,*** and ***C,*** miRNA-708 was up-regulated by transfecting with miRNA-708 mimics. $p < 0.05$.

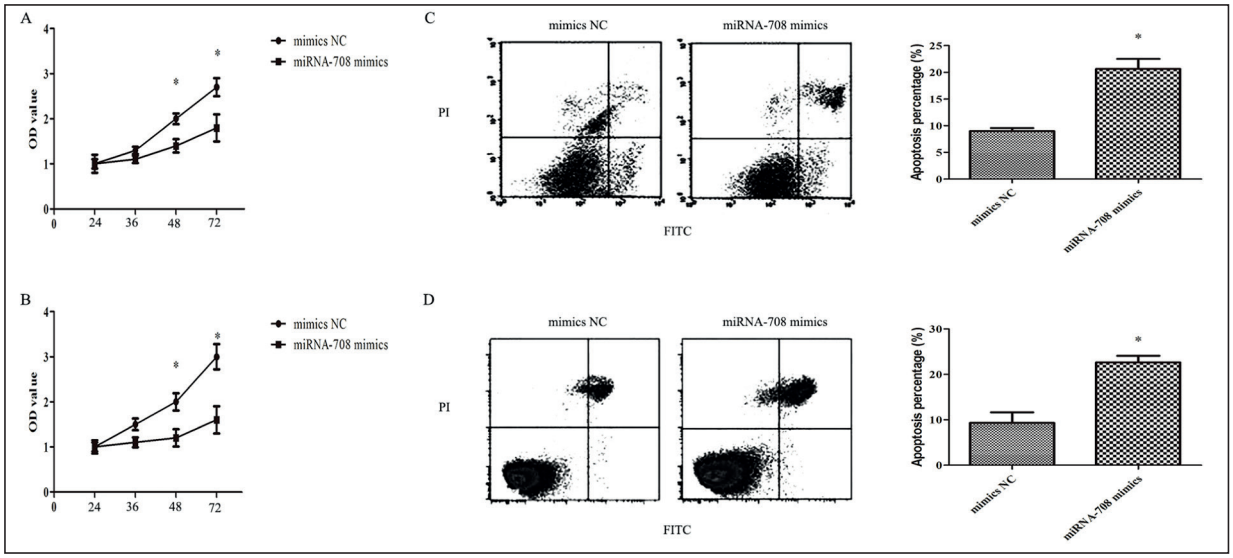

Figure 2. Up-regulated miRNA-708 inhibited cell proliferation and induced cell apoptosis. ***A,*** and ***B,*** The CCK8 analysis for cell proliferation exhibited that decreased cell proliferative ability was observed in miRNA-708 over-expression group as compared with the NC group. ***C,*** and ***D,*** The result of cell apoptosis assay found that induction of cell apoptosis was presented in miRNA-708 over-expression group as compared with the NC group. $p < 0.05$.

the result of cell apoptosis assay indicated that induction of cell apoptosis was presented in miRNA-708 over-expression group compared with the NC group (Figure 2C and 2D).

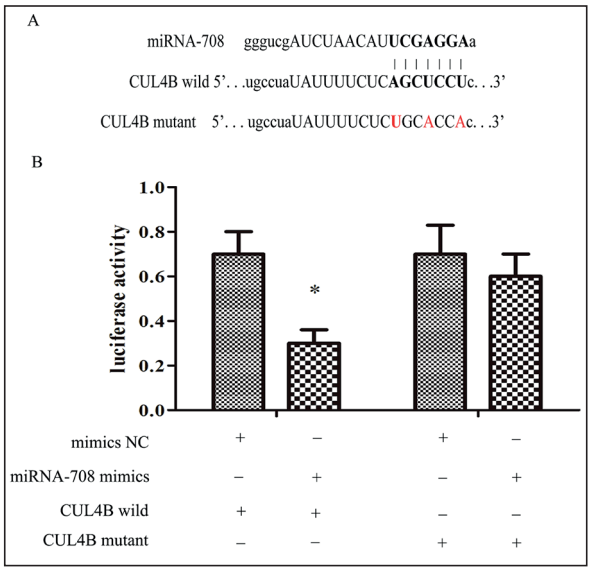

Figure 3. miRNA-708 could directly bind to CUL4B 3'UTR area. ***A,*** The software online predicted that miRNA-708 might be bound to CUL4B 3'UTR. ***B,*** Inhibition of the luciferase activity was obtained in cells were transfected with miRNA-708 mimics and plasmids containing pMIR-CUL4B-3'UTR wild, but no change in cells were transfected with miRNA-708 mimics and plasmids containing pMIR-CUL4B-3'UTR mut.

MiRNA-708 Could Directly Bind to CUL4B 3`UTR area

The software online predicted that miRNA-708 might be bound to CUL4B 3'UTR (Figure 3A). We confirmed this predicting result by using a luciferase reporter. The results of luciferase reporter showed inhibition of the luciferase activity was obtained in cells transfected with miRNA-708 mimics or with plasmids containing pMIR-CUL4B-3'UTR wild-type (wt), but no change was observed in cells transfected with miRNA-708 mimics or with plasmids containing pMIR-CUL4B-3'UTR mut (Figure 3B). This data indicated that miRNA-708 could directly bind to CUL4B 3'UTR area.

CUL4B Expression Was Negatively Regulated by MiRNA-708

To further find the relationship between miRNA-708 expression and CUL4B, we applied qRT-PCR and Western blot methods. The results of qRT-PCR and Western blot methods showed the mRNA (Figure 4A and 4B) and protein (Figure 4C and 4D) expression levels of CUL4B were down-regulated in miRNA-708 over-expression group compared with those in the NC group, respectively. These data demonstrated that CUL4B expression is negatively regulated by miRNA-708.

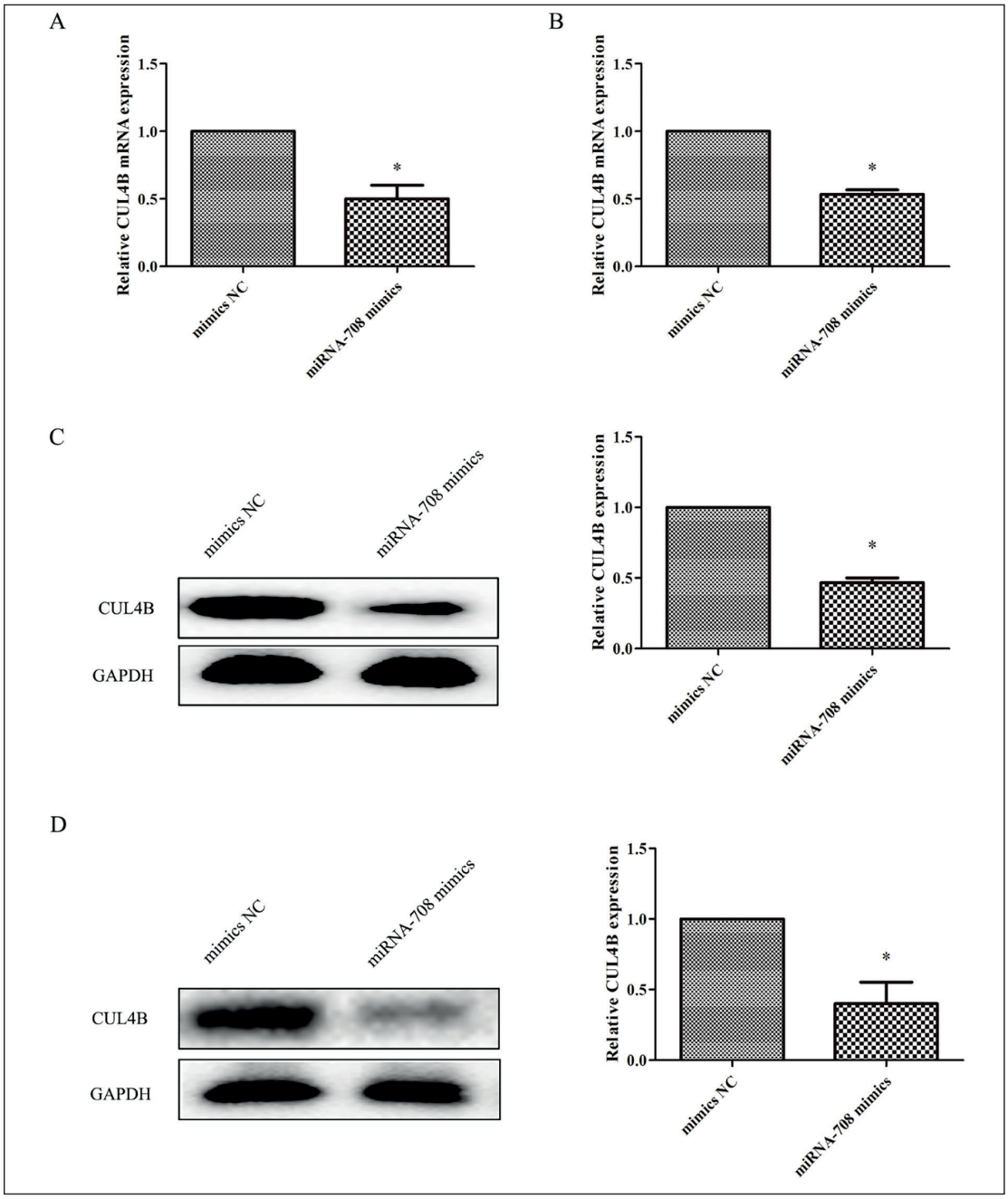

Figure 4. CUL4B expression was negatively regulated by miRNA-708. ***A,*** and ***B,*** qRT-PCR showed the mRNA level of CUL4B were down-regulated in miRNA-708 over-expression group compared with the NC group. ***C,*** and ***D,*** The Western blot showed that the protein expression levels of CUL4B were down-regulated in miRNA-708 over-expression group compared with the NC group. $p < 0.05$.

Decreased MiRNA-708 and Increased CUL4B Was Expressed in Osteosarcoma Tissues

The above studies illustrated that miRNA-708 functioned as a tumor-suppressor in osteosarcoma progression by depending on CUL4B. Finally, we detected the expression of miRNA-708 in osteosarcoma tissues. The results of qRT-PCR showed that decreased miRNA-708 was expressed in osteosarcoma tissues (Figure 5A). Furthermore, miRNA-708 expression was lower in tissues with IIB-III stage than that in IA-IIA stage (Figure 5B). We also detected the mRNA level of CUL4B in osteosarcoma tissues. The results of qRT-PCR

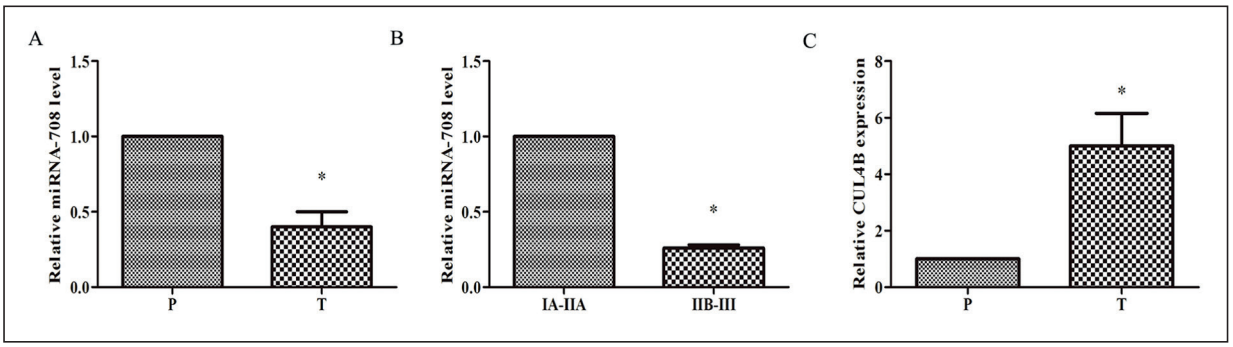

Figure 5. Decreased miRNA-708 and increased CUL4B was expressed in osteosarcoma tissues. ***A,*** The detection of qRT-PCR showed that decreased miRNA-708 was expressed in osteosarcoma tissues. ***B,*** miRNA-708 expression was lower in IIB-III than that in IA-IIA. ***C,*** qRT-PCR assay showed increased CUL4B expression in osteosarcoma tissues. $p < 0.05$.

assay showed increased CUL4B expression in osteosarcoma tissues (Figure 5C). Those results showed that the expression of CUL4B is negatively regulated by miRNA-708, which is consistent with the above findings *in vitro.*

Discussion

Osteosarcoma, as the most common primary malignant bone tumor, occurs in the metaphysis of long tubular bones. Osteosarcoma is characterized by a high degree of malignancy and poor prognosis, early occurrence of local and distant metastases[24]. Since the 1970s, with the advancement of chemotherapy, the 5-year survival rate of osteosarcoma patients has increased to 50%-80%[25]. However, the survival rate of patients has not changed in the past 20 years. 20%-30% of the newly diagnosed patients still have undergone tumor metastasis[26]. Therefore, the gene therapy for human diseases has become a hot spot in recent years.

MiRNA-708 is located on chromosome 11 (11q14.1)[27]. MiRNA-708 is also found within intron 1 of the ODZ4 gene, which encodes the Teneurin Transmembrane Protein 4 (Tenm4). In our study, we discovered that the expression of miRNA-708 was reduced in osteosarcoma cells. Furthermore, through CCK8 and apoptosis assays, we found that up-regulated miRNA-708 inhibited cell proliferation and induced cell apoptosis. MiRNA-708 could directly bind to CUL4B 3'UTR area. CUL4B expression was negatively regulated by miRNA-708. Decreased miRNA-708 was expressed in osteosarcoma tissues, and furthermore, miRNA-708 expression was lower in tissues with IIB-III stage than that in IA-IIA stage. We uncovered that increased CUL4B expression in osteosarcoma tissues, which was negatively regulated by miRNA-708 and was consistent with the findings *in vitro.*

CUL4B is one of the two members in cullin4 (CUL4) family. CUL4B plays an important role in central nervous system[28]. CUL4B is involved in DNA damage and repair[29], cell cycle progression, apoptosis and signaling pathways[30]. Dysregulation of CUL4B has been confirmed in the different human diseases. In pancreatic cancer, CUL4B could enhance cells metastasis and proliferation *via* epithelial-mesenchymal transition through Wnt/β-catenin signaling pathway[31]. Another work[32] reported that CUL4B could promote cells invasion and metastasis in gastric cancer. In bladder cancer, CUL4B also could enhance cells metastasis and induce epithelial-to-mesenchymal transition *via* the Wnt/β-catenin signaling pathway[33].

In this research, we discovered that miRNA-708 expression was reduced in osteosarcoma cells. Up-regulated miRNA-708 inhibited cell proliferation and induced cell apoptosis. MiRNA-708 could negatively regulate CUL4B by directly bind to CUL4B 3'UTR. Decreased miRNA-708 was expressed in osteosarcoma tissues, and miRNA-708 expression was lower in tissues with IIB-III stage than that in IA-IIA stage. CUL4B expression was increased in osteosarcoma tissues.

Conclusions

We showed that miRNA-708/CUL4B axis contributes to cell proliferation and apoptosis of osteosarcoma cells, which could provide a potential therapeutic target in treatment for osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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