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Circ-0003998 promotes cell proliferative ability and invasiveness by binding to miR-197-3p in osteosarcoma

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Abstract. – OBJECTIVE: The aim of this study was to investigate the level of circ-0003998 in osteosarcoma tissues and cell lines, and to analyze its relation with prognosis of patients, as well as its effect on biological behaviors of osteosarcoma cells. In addition, the potential mechanism of circ-0003998 in promoting osteosarcoma cell proliferation and invasion was explored.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to examine circ-0003998 expression in 60 clinical osteosarcoma tissues and cell lines. The association between circ-0003998 expression and the overall survival rate of patients was explored. After shRNA-circ-0003998 was constructed to down-regulate circ-0003998 expression in osteosarcoma cell lines, the proliferation of osteosarcoma cells was observed through the Cell Counting Kit-8 (CCK-8) and colony formation assay. Meanwhile, cell invasiveness was detected by the transwell invasion assay. Bioinformatics was used to search for microRNAs (miRNAs) that contained the direct effect on circ-0003998. Subsequently, the luciferase reporter vector of circ-0003998 or Krüppel-like factor 10 (KLF10) containing miR-197-3p binding site was constructed. Then, the binding of circ-0003998 or KLF10 to miR-197 was detected using the Dual-Luciferase assay. Furthermore, the function recovery experiment was designed to validate the biological function of circ-0003998 and miR-197 in osteosarcoma.

RESULTS: Compared to normal control tissues and cells, the expression of circ-0003998 was significantly up-regulated in both osteosarcoma tissue samples and cell lines. Highly-expressed circ-0003998 was significantly associated with poor overall survival of patients with osteosarcoma. *In vitro* experiments revealed that the down-regulation of circ-0003998 significantly inhibited the proliferative ability and invasiveness of osteosarcoma cells. Bioinformatics analysis and Dual-Luciferase reporter gene assay indicated that circ-0003998 might bind to miR-197-3p in MG-63, and Saos-2 cell lines. Meanwhile, the functional recovery experiment demonstrated that inhibiting miR-197-3p expression could partially restore the changes in cellular biological behaviors induced by circ-0003998 down-regulation in MG-63 and Saos-2 cells. In addition, miR-197-3p was remarkably down-regulated in osteosarcoma tissues, while KLF10 was up-regulated. However, KLF10 was significantly up-regulated after the knockdown of miR-197-3p in osteosarcoma cells.

CONCLUSIONS: Circ-0003998 plays a vital role in promoting the development of osteosarcoma, whose high expression can predict poor clinical prognosis. Circ-0003998 is highly expressed in osteosarcoma tissues and cell lines. The down-regulation of its level can significantly inhibit the proliferative ability and invasiveness of osteosarcoma cells. Meanwhile, circ-0003998 up-regulates the expression of KLF10 by binding to miR-197-3p, thereby promoting osteosarcoma cell growth and invasion, and accelerating the progression of osteosarcoma.

Key Words:
Osteosarcoma, Circ-0003998, Cell proliferation, Cell invasion.

Introduction

Osteosarcoma is the most common malignant bone tumor in children and adolescents, accounting for approximately 60% of all malignant bone tumors. Osteosarcoma usually involves the end of long bones, especially fast-growing bones such as the distal femur, proximal tibia, and proximal humerus[1]. Statistics have indicated that osteosarcoma is characterized by low 5-year survival rate, high amputation, and poor postoperative functional recovery[2]. Currently, limb salvage combined with neoadjuvant chemotherapy has significantly increased the five-year disease-free survival rate to 70%. However, the above factors are still the main obstacles for clinicians due to side effects and resistance to chemotherapy drugs, as well as the spread

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of tumors[3]. Therefore, to develop new targeted therapeutic strategies for osteosarcoma, it is necessary to further elucidate the underlying molecular mechanism and to discover new biomarkers[4].

Circular RNA (circRNA) is an RNA molecule that is ubiquitous in animal and plant cells. It can regulate gene expression through various pathways[5]. As early as the 1970s, it was discovered that cyclic RNA molecules existed in RNA viruses. Since then, they have not received sufficient attention, and the research on circRNA is not deep enough. With the rapid development of RNA sequencing technology and bioinformatics, it has been found that compared to the previous linear splicing, circular RNAs are reverse spliced or formed by gene rearrangement. Meanwhile, they account for a large proportion of all spliced transcripts[6]. At present, increasing evidence has found that circRNA is not only closely related to the progression of many diseases but also exhibits potential value in cancer diagnosis and adjuvant therapy[7]. For example, in non-small cell lung cancer, circ-100146 acts as an oncogene by directly binding to microRNA-361-3p (miR-361-3p) and miR-615-5p[8]. Circ-ZNF609 promotes the migration of colorectal cancer cells by inhibiting Gli1 expression *via* microRNA-150[9]. In addition, circ_0009910 regulates the growth and metastasis of gastric cancer and is also associated with poor prognosis[10]. Meanwhile, the up-regulated circ-0030235 is able to predict poor prognosis of pancreatic ductal adenocarcinoma and promote cancer progression through sponge-bound of miR-1253 and miR-1294[11].

Circ-0003998 is a cyclic RNA with a splicing sequence length of 304 nt[12]. Previously, circ-0003998 has been shown to be highly expressed in non-small cell lung cancer. Up-regulated circ-0003998 promotes cell proliferative ability and invasiveness by alleviating the degradation of Notch1 *via* miR-326[12]. However, whether it exerts the same cancer-promoting effect on osteosarcoma remains unclear. Therefore, the aim of this study was to investigate the level of circ-0003998 in osteosarcoma tissues and cells, and to explore the possible mechanism through *in vitro* experiments.

Patients and Methods

Research Objects and Sample Collection

This investigation was approved by the Ethics Committee of Liaoning Provincial People's Hospital. Informed consent was obtained from each subject before the study. Osteosarcoma tissues and normal control tissues were collected from 60 patients diagnosed with osteosarcoma from Liaoning Provincial People's Hospital from December 2016 to October 2018. All patients received no treatment before surgery and were diagnosed as osteosarcoma by pathological diagnosis. Collected specimens were stored in liquid nitrogen for subsequent use.

Cell Culture

Human osteoblast cell line (hFOB) and human osteosarcoma cell lines (143B, U-2OS, MG-63, and Saos-2) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Rockville, MD, USA) medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and placed in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

For circ-0003998 silencing, shRNA against circ-0003998 was constructed into the pSicoR lentiviral vector and 293T cells were used for the virus production. Osteosarcoma cells were transfected with lentivirus and cultured for two weeks with puromycin to generate circ-0003998 silenced cell lines. An appropriate number of cells were plated into six-well plates. Cell transfection was performed according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) when cell density reached 70%-80%. MiR-197-3p mimics and inhibitor were designed and synthesized by GenePharma (Shanghai, China). The above mixture was added to a cell culture plate or a culture flask containing culture medium, and the cells were continuously cultured for 24 h in a CO_2 incubator at 37°C. Subsequent *in vitro* experiments were carried out.

RNA Extraction

Osteosarcoma tissues and cells required for the experiment were first collected. Cells were lysed in an Eppendorf (EP) tube by pipetting with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). After 250 μL of chloroform was added, the tube was shaken for 30 s, mixed and centrifuged at 4°C. The aqueous phase was aspirated, and an equal volume of pre-chilled isopropanol was added. After centrifugation, the precipitate was gently rinsed with 75% ethanol and dissolved in 30 μL of diethyl pyrocarbonate (DEPC) water (Beyotime,

Shanghai, China). Total RNA was quantified by NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA) and stored in a −80°C refrigerator for use.

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

Extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). MiRNA quantitative PCR procedures were performed using the miScript SYBR Green PCR Kit. QRT-PCR assay was performed on the ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal parameters for miRNAs and mRNAs, respectively. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study were as follows: circ-0003998, F: 5'-TCCACAGGATCCTTATTCTGC-3', R: 5'-GGCTAGGGATAAATCCCCAGGA-3'; miR-197-3p, F: 5'-GCCGGGTTGTAAACGATCCTACGG-3', R: 5'-GTGATTCCGTGTCGTTAGTGG-3'; KLF10, F: 5'-ATTGCACCGGCGTTCGGACAC-3', R: 5'-CTGGAAGTGCGTAGCCCAGATCGT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R:5'-CGCTTCAGAATTTGCGTGTCAT-3';GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Proliferation Assay

Cell Counting Kit-8 (CCK-8) assay was performed as follows. Briefly, osteosarcoma cells with different treatments were first seeded into 96-well plates at a density of 4×10^5/mL per well. After culture for 0, 24, 48, and 72 h, respectively, 10 μL of CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, followed by incubation for 1 h at 37°C in the dark. Optical density (OD) value at 450 nm was measured by a microplate reader. Five duplicate wells were set in each group.

Colony formation assay was conducted as well. Transfected osteosarcoma cells were trypsinized, re-suspended and counted. After 14 days of culture, formed visible colonies were stained with crystal violet. Finally, stained colonies were photographed, counted, and compared between different groups.

Cell Invasion Assay

Cell invasion assay was performed using Matrigel invasion chambers. First, 1×10^5 of osteosarcoma cells suspended in serum-free medium were seeded into the upper chamber. Meanwhile, the culture medium containing 10% fetal calf serum (FCS) was added to the lower chamber. After 24 h of incubation, the cells remaining on the upper membrane were discarded, while cells adhering to the membrane were fixed and stained with 0.5% crystal violet. Finally, the number of invaded cells was counted under a microscope. Five fields of view were randomly selected for each sample.

Luciferase Reporter Gene Assay

Wild type and mutant circ-0003998 or KLF10 were cloned into pGL3-control vector. 5×10^4 of osteosarcoma cells were seeded into 24-well plates. After 48 h of co-transfection with corresponding plasmid and miR-197-3p mimics or negative control, the luciferase activity of each group was measured using the Dual-Luciferase reporter assay system.

Western Blot Experiment

Transfected osteosarcoma cells were collected and protein lysate (Beyotime, Shanghai, China) was added to cells (Beyotime, Shanghai, China) to extract total proteins. After centrifugation, the supernatant was collected, and the concentration of extracted protein was determined according to the instructions of bicinchoninic acid (BCA) protein quantification kit (Beyotime, Shanghai, China). Then, the protein samples were mixed with protein loading buffer and heated at 100°C to be denatured. Subsequently, they were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% of skimmed milk powder blocking solution, the membranes were incubated with primary antibodies and corresponding secondary antibody. Finally, immune-reactive bands were exposed and observed.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA). GraphPad Prism 6 software (La Jolla, CA, USA) was used for statistical analysis mapping. Measurement data were expressed as mean ± standard deviation. A two-sample *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

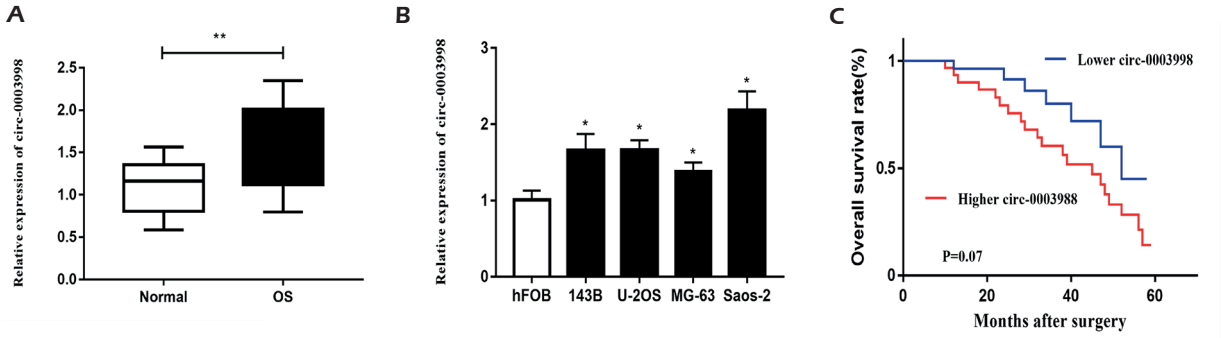

Figure 1. Circ-0003998 was highly expressed in osteosarcoma tissues. **A,** Circ-0003998 was highly expressed in osteosarcoma tissues. **B,** Circ-0003998 was highly expressed in osteosarcoma cell lines. **C,** High expression of circ-0003998 indicated poor prognosis of patients. *p<0.05; **p<0.01

Results

Circ-0003998 Was Highly Expressed in Osteosarcoma Tissues

The mRNA level of circ-0003998 in 60 osteosarcoma tissues and normal control tissues or in osteosarcoma cell lines was first detected by Real Time quantitative PCR. The results showed that circ-0003998 was significantly highly expressed in osteosarcoma tissues and cell lines (Figure 1A and 1B). Among all cell lines, MG-63 and Saos-2 cells expressed the highest level of circ-0003998. Therefore, these two cell lines were selected for subsequent experiments. According to circ-0003998 expression in osteosarcoma, the patients were divided into two groups, namely, circ-0003998 high expression group and circ-0003998 low expression group. Combining the clinical information, we found that the overall survival rate of patients in circ-0003998 high expression group was significantly reduced when compared to those in circ-0003998 low expression group (Figure 1C). Results suggested that a high level of circ-0003998 might predict the poor prognosis for osteosarcoma.

Knockdown of Circ-0003998 Inhibited Osteosarcoma Cell Proliferative Ability and Invasiveness

Circ-0003998 shRNA was then transfected in MG-63 and Saos-2 cells. QRT-PCR revealed that circ-0003998 shRNA transfection significantly decreased the expression of circ-0003998 (Figure 2A). The proliferation of MG-63 and Saos-2 cells was explored after circ-0003998 was down-regulated. The results of the CCK-8 assay showed that knockdown of circ-0003998 significantly inhibited the proliferation of osteosarcoma cells (Figure 2B). Similarly, the colony formation assay showed a significant reduction in the number of formed colonies in sh-circ-0003998 group (Figure 2C). Next, we analyzed the effect of circ-0003998 on the invasion ability of osteosarcoma cells by transwell invasion assay. Results indicated that the inhibition of circ-0003998 markedly attenuated the invasive ability of MG-63 and Saos-2 cells (Figure 1D). The above results suggested that the growth and metastasis abilities of osteosarcoma cells were significantly restricted after circ-0003998 down-regulation.

Circ-0003998 Could Directly Bind to MiR-197-3p

The miRNAs that could bind to circ-0003998 were predicted by bioinformatics, with higher binding scores for miR-136, miR-145, miR-326, and miR-197-3p. Subsequently, we functionally analyzed the levels of these miRNAs in osteosarcoma. Finally, miR-197-3p was selected for subsequent experiments (Figure 3A). The binding relation between miR-197-3p and circ-0003998 was determined by the luciferase reporter gene assay. Results showed that after miR-197-3p mimics transfection in MG-63 and Saos-2 cells, the luciferase activity of circ-0003998-WT group was significantly reduced. However, no significant difference was observed in circ-0003998-MUT 3'UTR group (Figure 3B). Results indicated that miR-197-3p could directly bind to circ-0003998. Subsequently, we detected microRNA-197-3p expression in osteosarcoma tissues and normal control tissues by qRT-PCR. Results showed that miR-197-3p was highly expressed in osteosarcoma tissues (Figure 3C). In addition, a negative correlation was observed between the expression levels of circ-0003998 and miR-197-3p in osteo-

sarcoma tissues (R=-0.7873, $p<0.001$) (Figure 3D). After knocking down circ-0003998 in MG-63 and Saos-2 cell lines, the miR-197-3p level increased significantly (Figure 3E). The above findings demonstrated that circ-0003998 was able to bind to miR-197-3p and inhibit its expression in osteosarcoma.

Circ-0003998 Promoted Proliferative Ability and Invasiveness of Osteosarcoma Cells by Binding to MiR-197-3p

The effect of miR-197-3p on cell proliferative ability and invasiveness was explored by *in vitro* experiments. Results showed that knocking down alone weakened the proliferation ability of MG-63 cells. However, simultaneous knockdown of miR-197-3p and circ-0003998 could reverse this effect (Figure 4A-4B). Meanwhile, the invasion of MG-63 showed a similar changing trend (Figure 4C). The above results demonstrated that circ-0003998 might promote the progression of osteosarcoma by inhibiting miR-197-3p expression.

MiR-197-3p Could Combine With KLF10

The target genes that could bind to miR-197-3p were predicted through bioinformatics, and KLF10 was selected (Figure 4D). Subsequently, we performed a functional analysis to confirm the binding relation between miR-197-3p and KLF10. The luciferase reporter gene assay revealed that after transfection of miR-197-3p mimics in Saos-2 cells, the luciferase activity decreased markedly in KLF10-WT 3'UTR group. However, no significant changes were observed in KLF10-MUT 3'UTR group (Figure 4E). This indicated that miR-197-3p could indeed bind to KLF10. Subsequently, we examined the level of KLF10 in osteosarcoma and normal control tissues by qRT-PCR. Results showed that KLF10 was significantly highly expressed in osteosarcoma tissues (Figure 4F). After the overexpression of miR-197-3p in Saos-2 cells, the mRNA and protein expression of KLF10 were both markedly reduced. However, miR-197-3p knockdown resulted in the opposite result (Figure 4G-4H). All these findings suggested that miR-197-3p might exert its biological function by degrading KLF10.

Discussion

Current standardized treatments for OS include complete surgical resection of the primary tumor and all metastases, as well as control of micro-metastatic lesions with neoadjuvant chemotherapy and postoperative adjuvant chemother-

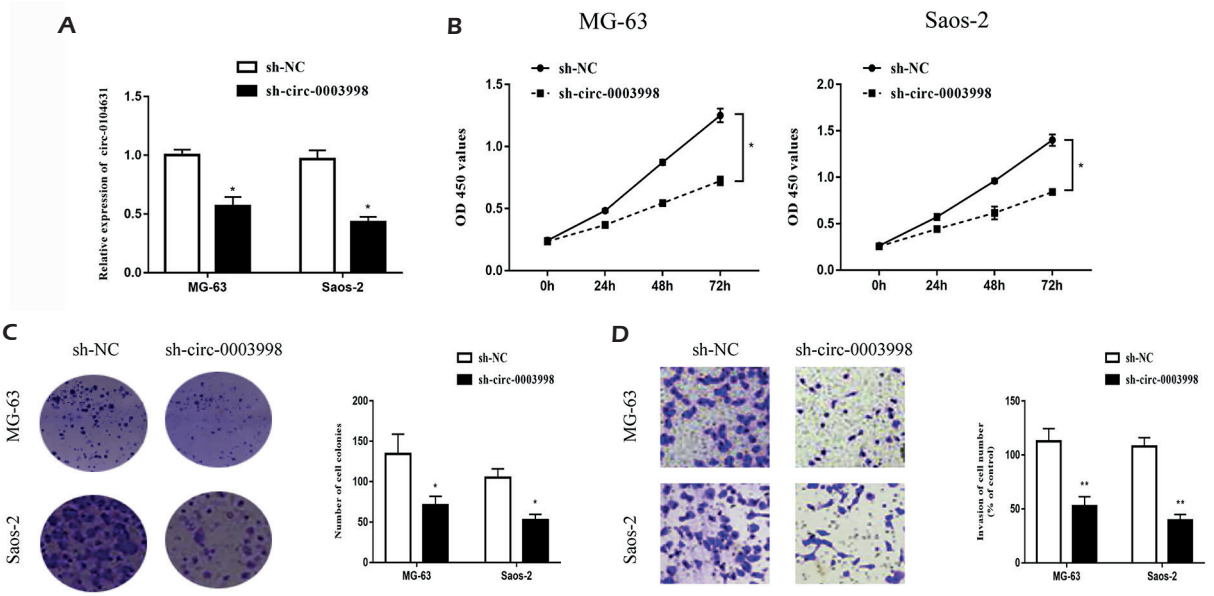

Figure 2. Knockdown of circ-0003998 inhibited proliferation and invasion of osteosarcoma cells. **A,** Expression of circ-0003998 after transfection of circ-0003998 shRNA in MG-63 and Saos-2 cell lines was detected. **B-C,** CCK-8 assay and colony formation assay examined changes in cell proliferation ability after knockdown of circ-0003998 in MG-63 and Saos-2 cell lines. **D,** Transwell invasion assay detected changes in cell invasive ability after knockdown of circ-0003998 in MG-63 and Saos-2 cell lines. (Magnification: 20×). *$p<0.05$; **$p<0.01$

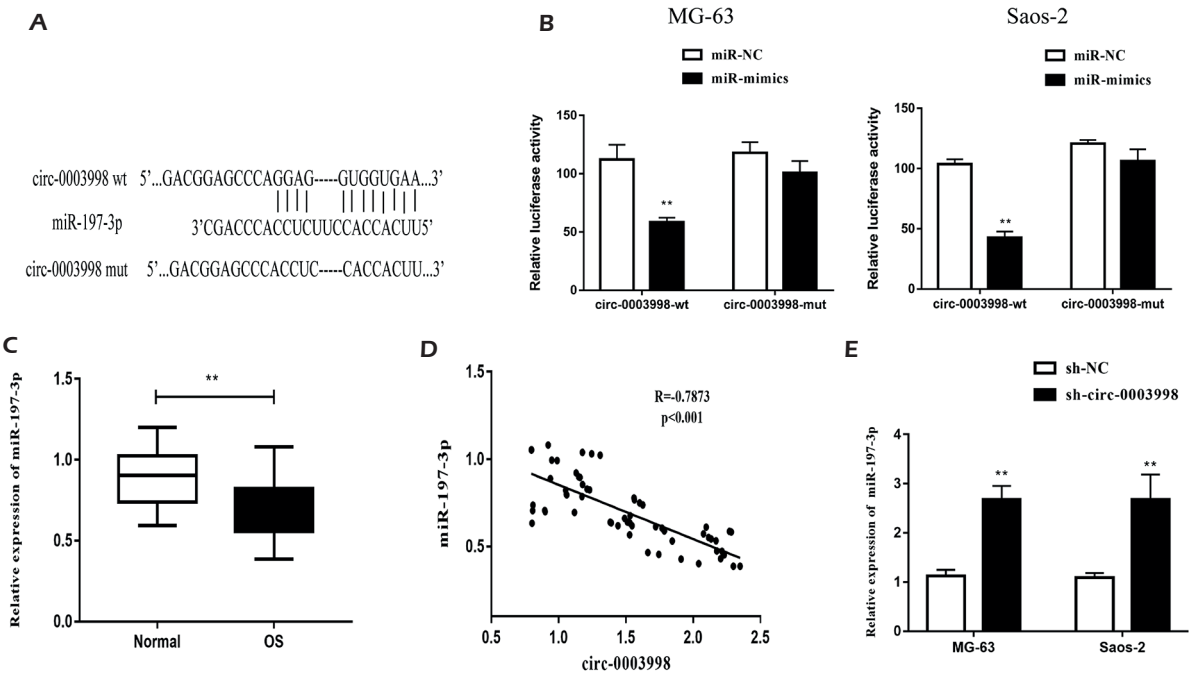

Figure 3. Circ-0003998 was capable of binding to miR-197-3p. **A,** Bioinformatics was used to predict the binding sites of circ-0003998 to miR-197-3p. **B,** Dual-Luciferase reporter assay detected the binding relation between miR-197-3p and circ-0003998. **C,** QRT-PCR was used to detect the expression of miR-197-3p in osteosarcoma tissues. **D,** Expression of circ-00039980 in osteosarcoma tissues was significantly negatively correlated with the expression of miR-197-3p. **E,** Expression of miR-197-3p increased significantly after interference with circ-0003998 in MG-63 and Saos-2 cell lines. $^{**}p<0.01$

apy. With chemotherapy and complete surgical resection, up to 70% of patients with local limb tumors can achieve long-term survival[13]. However, current treatment therapy may lead to long-term morbidity, easily induced secondary cancer, and even fatal complications[14]. For patients with unresectable or recurrent tumors, the prognosis is relatively poor with extremely low 5-year survival rate[15,16].

More than 10% of the genes in all tested cells or tissues are capable of producing circular RNA. A large number of circular RNAs are highly abundant and have cellular or tissue specificity[17]. In addition, during the epithelial-mesenchymal transition of human cells, the expression levels of hundreds of circular RNAs have changed. Recent studies[18] have shown that circular RNA can adsorb microRNAs or bind proteins. Changes in the level of circular RNA can lead to changes in the level of tumor-related genes, thereby affecting the occurrence and development of tumors. So far, few studies[19,20] have explored the role of circular RNA in osteosarcoma. For example, the expression of circ_001569 is up-regulated in osteosarcoma, which promotes cell proliferation and cisplatin resistance by activating the Wnt/beta-catenin signaling pathway. Meanwhile, over-expressed circ-0007534 is able to predict poor prognosis of osteosarcoma. Furthermore, it regulates cell growth and apoptosis by targeting the AKT/GSK-3β signaling pathway[21]. In addition, circ-NT5C2 acts as an oncogene in the proliferation and metastasis of osteosarcoma cells by targeting miR-448[22].

In the present study, we found that the expression of circ-0003998 was significantly up-regulated in osteosarcoma tissues and cell lines. High level of circ-0003998 predicted poor prognosis of patients with osteosarcoma. Subsequently, *in vitro* experiments demonstrated that the proliferation and invasion abilities were significantly attenuated after circ-0003998 down-regulation in osteosarcoma cells. Previous studies have also verified that circ-RNAs can exert their biological functions by regulating the expression of miRNAs. Therefore, we speculated that circ-0003998 might promote the osteosarcoma progression by regulating the expressions of certain miRNAs. Using bioinformatics prediction tool, several miRNAs (such as miR-136, miR-145, miR-326,

miR-197-3p, etc.) were found to have potential binding sites with circ-0003998. MiR-197-3p was selected as a possible binding target because it has been observed to be significantly down-expressed in osteosarcoma[23]. Subsequently, circ-0003998 was verified to be able to bind to miR-197-3p. Knocking down circ-0003998 in cells could enhance the expression of miR-197-3p. Meanwhile, cellular function experiments demonstrated that circ-0003998 significantly promoted the proliferation and invasiveness of osteosarcoma cells, while miR-197-3p inhibited cell growth and metastasis.

Bioinformatics website predicted that circ-0003998 was able to competitively bind to miR-197-3p with KLF10. Krupple-like factor 10 (KLF10) plays a vital role in transforming growth factor-β (TGF-β)-mediated cell growth and differentiation[24]. TGF-β binds to its transmembrane receptor kinase to initiate a unique intracellular signaling cascade, including Smads signaling, transcription factors and signaling pathways

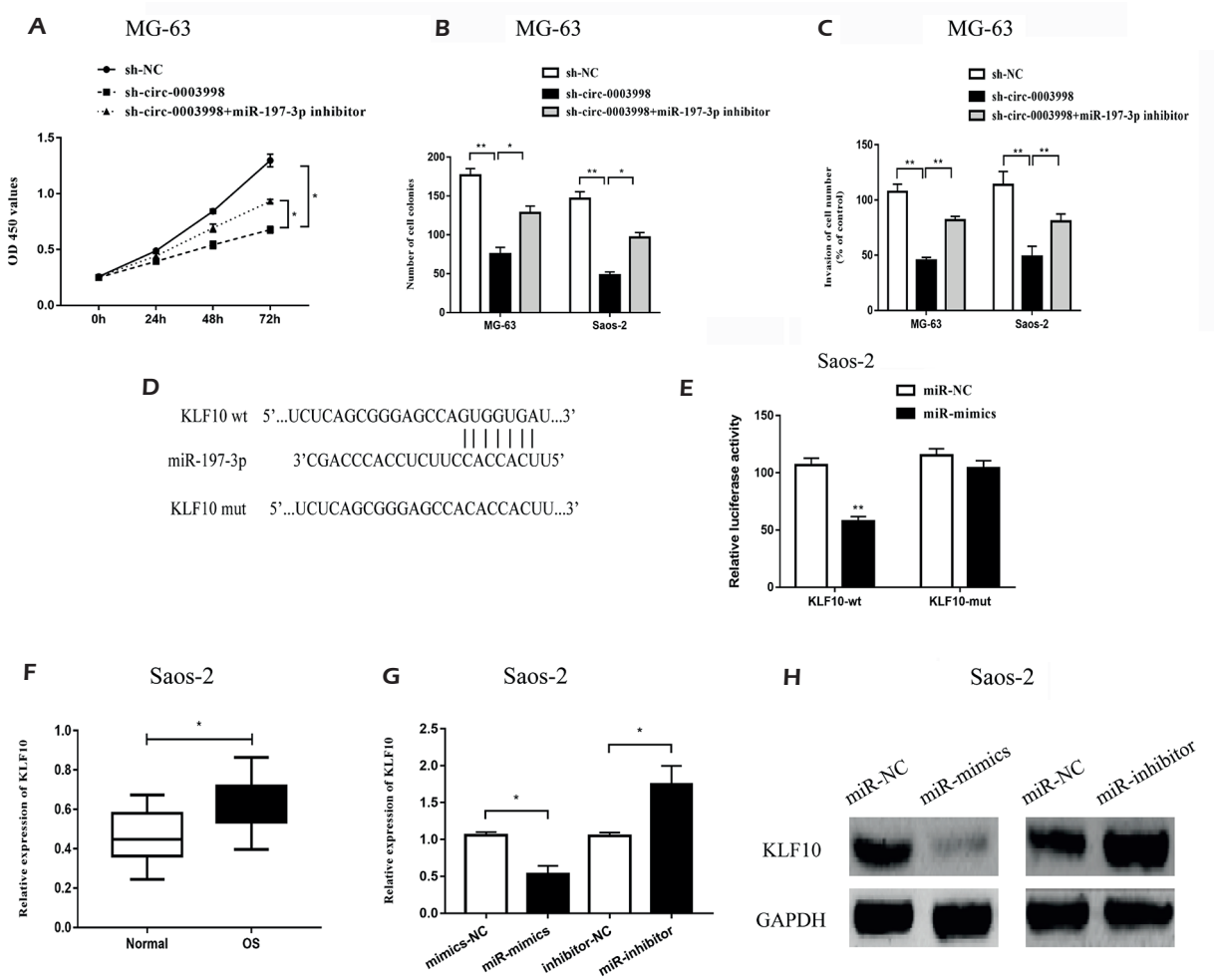

Figure 4. Circ-0003998 promoted cell proliferation and invasion by competitively binding to miR-197-3p with KLF10. **A-B,** CCK8 assay and colony formation assay showed that interference with circ-0003998 in MG-63 cells significantly inhibited cell proliferation, while simultaneous inhibiting miR-197-3p and circ-0003998 could reverse the results. CCK-8 assay: **(A)** Colony formation assay; **(B-C)** After circ-0003998 was silenced in MG-63 cells, the invasion ability of cells was weakened. However, the inhibitory effect was reversed after simultaneous interfering with miR-197-3p. **D,** Bioinformatics website was used to predict the binding sites of miR-197-3p to KLF10. **E,** Dual-Luciferase reporter gene results showed that miR-197-3p could bind to the 3'-UTR of KLF10. **F,** KLF10 was highly expressed in osteosarcoma tissues. **G,** Overexpressing miR-197-3p in Saos-2 cells significantly down-regulated the mRNA level of KLF10, while knockdown of miR-197-3p promoted KLF10 expression. **H,** Overexpressing miR-197-3p in Saos-2 cells significantly down-regulated the protein level of KLF10, while knockdown of miR-197-3p promoted KLF10 expression. *$p<0.05$; **$p<0.01$.

independent of Smads[25]. Currently, it has been found that TGF-β exerts different biological effects on different types of cells under different conditions. This can eventually induce the apoptosis of epithelial cells, inhibit immune function, regulate cell growth and differentiation, stimulate the proliferation of interstitial cells, produce extracellular matrix, and induce *in vivo* tissue fibrosis reaction[26]. In the present study, KLF10 was found highly expressed in osteosarcoma tissues. Results also confirmed that KLF10 expression was significantly reduced after the overexpression of miR-197-3p in osteosarcoma cells. However, opposite results were observed after inhibition of miR-197-3p. Circ-0003998 might protect KLF10 from degradation *via* inhibiting the level of miR-197-3p. However, the biological function of KLF10 in osteosarcoma remains the further study.

Conclusions

Circ-0003998 plays a vital role in promoting the development of osteosarcoma. High expression of circ-0003998 predicts poor clinical prognosis of patients. Meanwhile, it is highly expressed in osteosarcoma tissue specimens and cell lines. Down-regulating its level significantly inhibits the proliferative ability and invasiveness of osteosarcoma cells. Furthermore, circ-0003998 up-regulates the expression of KLF10 by binding to miR-197-3p and promotes osteosarcoma cell growth and invasion, eventually accelerating the progression of osteosarcoma.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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