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Circular RNA CircMTO1 suppressed proliferation and metastasis of osteosarcoma through miR-630/KLF6 axis

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Abstract. – OBJECTIVE: Circular RNAs (circRNAs) could regulate gene expression which may induce tumor occurrence and progression. In the current study, we first investigated the expression of circMTO1 in osteosarcoma, and the underlying mechanism was further elucidated.

PATIENTS AND METHODS: Circular RNA microarrays were used to identify the differential expression of circRNAs in osteosarcoma tissues and the corresponding normal tissues. qRT-PCR was used to examine the level of circMTO1 in osteosarcoma tissues and cell lines. In addition, circMTO1 overexpression was constructed using lentiviral transfection in cell lines. Subsequently, the Cell Counting Kit-8 (CCK8), cell migration and invasion, and flow cytometry were used to investigate the effect of circMTO1 on the biological functions of cells. The Western Blot and the recovery experiments were used to explore the potential mechanism.

RESULTS: Here, we measured 20 circRNAs which were downregulated in osteosarcoma tissues using circRNA microarray. CircMTO1 expression was decreased in osteosarcoma cell lines. Besides, circMTO1 could inhibit cell proliferation, migration and invasion, and induced apoptosis in osteosarcoma cells. Bioinformatics analysis showed that circMTO1 serves as a sponge for miR-630 and KLF6 is a direct target of miR-630. Furthermore, circMTO1 functions through regulation of miR-630/KLF6 axis.

CONCLUSIONS: Our study suggests circMTO1 could suppress osteosarcoma progression by regulating miR-630/KLF6 axis, which may highlight the diagnostic and therapeutic potential of these molecules in osteosarcoma treatment.

Key Words:
CircMTO1, Osteosarcoma, MiR-630, KLF6.

Introduction

Osteosarcoma, arises from osteogenic mesenchymal cells, is the most prevalent primary bone malignancy[1]. Osteosarcoma occurs usually in infants and adolescents with an incidence of about one to three in a million annually worldwide[2]. Despite extensive advancements in osteosarcoma diagnostic and therapeutic strategies, the prognosis of patients still remains unfavorable[3]. Hence, it is of great clinical significance to investigate the molecular mechanisms underlying osteosarcoma progression.

CircRNAs (circRNAs), characterized by covalently closed loop structures with neither 5' caps nor 3' polyadenylated tails, has been documented to participate in diverse biological processes, such as transcriptional regulation, cell proliferation and differentiation[4,5]. Some studies[6,7] indicate deregulation of circRNAs serve as tumor suppressors or oncogenes in cancer occurrence and progression. CircMTO1 (mitochondrial translation optimization 1 homologue) has been demonstrated to play an inhibitory role in hepatocellular carcinoma, bladder cancer, breast cancer, colorectal cancer and cervical cancer[8-12]. However, the molecular function of circ_0007142 in osteosarcoma pathogenesis has yet to be fully explored.

MicroRNAs (miRNAs), which are a class of non-coding RNAs with 19-23 nucleotides in length, control gene expression level after transcription[13]. Competing endogenous RNA (ceRNA) hypothesis demonstrated that miRNA sponge function of circRNA has been most confirmed in human cancers[6]. In the current study, we first investigated the expression of circMTO1 in osteosarcoma, and the underlying mechanism was further elucidated.

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Patients and Methods

Clinical Specimens

Seventy clinical human osteosarcoma tissues and the corresponding normal tissues were obtained from January 2017 and December 2018 in Hong Hui Hospital. All patients did not receive any therapies before operation. Clinical and pathological information were acquired from patients' medical records and pathological reports which were shown in Table I. All the specimens were immediately snap-frozen after isolation and stored in liquid nitrogen for further use. Written informed consent was obtained from all patients, which was approved by the Ethics Committee of Hong Hui Hospital.

Circular RNA Microarrays

The total RNA was digested with RNAse R (Epicentre, Madison, WI, USA) to remove linear RNAs and enrich circular RNAs. Then, the circular RNAs were amplified, transcribed into fluorescent cRNA, and then, hybridized onto the Arraystar circRNA Array (8 × 15 K, Arraystar, Rockville, MD, USA). After washing, the arrays were scanned by the Agilent Scanner G2505C (Agilent Technologies, Santa Clara, CA, USA). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. A heat map of differentially expressed circRNAs/genes in cancer tissues and matched normal tissues was then constructed.

Cell Culture and Transfection

Human normal osteoblast cells (hFOB 1.19) and four osteosarcoma cell lines (HOS, U2OS, Saos-2 and MG63) were attained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Solarbio, Beijing, China) and 1% penicillin/streptomycin (Solarbio, Beijing, China) at 37°C in a humidified atmosphere containing 5% CO_2. CircMTO1 overexpression, miR-630 mimics as well as negative controls, were all designed and synthesized by GenePharma (Shanghai, China). As for cell transfection, circMTO1 expression vector (Invitrogen, Carlsbad, CA, USA), miR-630 mimics and circ_0007142 overexpression (Gene-Pharma, Shanghai, China) were, respectively, alone or in combination transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's protocol. At 48 h after transfection, the cells were harvested for RT-PCR analyses or Western blotting.

Cell Proliferation

HOS and U2OS cells (2×10^3 cells/well) with different transfections were seeded in 96-well plates and cultured for 0, 24, 48, 72, 96 h. Then, the cells were added with 10 µl of CCK-8 solution (Beyotime, Shanghai, China) for 3 hours incubation. Subsequently, the OD value at the wavelength of 450 nm was measured using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Cell Apoptosis

After being cultured in DMEM for 24 h, the treated cells were harvested and washed twice with PBS. Then, the cells were suspended in the binding buffer, fixed in ice-cold 70% ethanol,

Table I. The correlation of circMTO1 expression with clinical parameters in patients with osteosarcoma.

		CircMTO1 expression		
Variables	Clinical parameters	High (n = 38)	Low (n = 32)	*p*-value
Age (years)				0.536
Gender	Male	20	11	0.126
	Female	18	21	
Age (years)	< 18	17	14	0.934
	≥ 18	21	18	
Size (cm)	< 5	21	17	0.858
	≥ 5	17	15	
Differentiation	Well/Moderate	21	14	0.337
	Poor	17	18	
Enneking stage	I	18	11	0.041*
	II	12	14	
	III	8	7	

*$p < 0.05$.

stained with Annexin V-FITC and propidium iodide (PI). The quantitative detection was immediately performed using a FACScan (Beckman Coulter, Brea, CA, USA) and the data were analyzed using FlowJo software.

Cell Migration and Invasion

In the migration assay, cells suspended in serum-free medium were added to the upper chamber. The medium supplemented with 10% FBS was used as an attractant in the lower chamber. In the invasion assay, the cells were inoculated in the upper chamber preprocessing with Matrigel. After 24 hours of incubation, cells on the lower surface of the transwell membrane were fixed in 4% paraformaldehyde for 30 min and stained with 1% crystal violet for 30 min. The number of migrative and invasive cells was counted in five different areas under a light microscope.

Real-Time Quantitative PCR Analysis

Total RNA was isolated from osteosarcoma tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. cDNA synthesis was conducted using the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA) for miR-630 and using One Step PrimeScript cDNA kit (Qiagen, Hilden, Germany) for circMTO1 and KLF6. RT-PCR was performed using the SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) and the Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression was analysis by using $2^{-\Delta\Delta CT}$ method and normalized to GAPDH or U6. The primers were as follows: circMTO1 forward 5'-TTACCAGCCGAGTAGAGTTCC-3' and reverse 5'-ATCCATTCCTTCAGGTTCCAAC-3'; miR-630 forward 5'-GTCAGCGCAGTATTCTGTAC-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'; lnc-NC 5'-UGGACAACAUGGGCUCU-3'; KLF6 forward 5'-TCAAATGCTATCCCCTTTCC-3' and reverse 5'-CCAGGGCTAGGAAGTAGGAG-3'; U6 forward 5'-GCTCGCTTCGGCAGCACA-3' and reverse 5'-GAGGTATTCGCACCAGAG GA-3'; and GAPDH forward 5'-ACCACAGTCCATGCCATCCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3'.

Luciferase Reporter Assay

CcircMTO1 and smad5 3'-UTR containing wild type (WT) or Mutant (Mut) miR337 binding sites were synthesized and inserted into Luciferase reporter vectors pGL3 vector. Cells were co-transfected with the Luciferase reporter constructs, miR-630 mimics, and Renilla Luciferase construct (Promega, Madison, WI, USA) and incubated for 24 h. The relative Luciferase activities were measured using the Dual-Luciferase Reporter System (Promega, Madison, WI, USA).

Western Blotting Analysis

Proteins were extracted from cultured cells by RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing a mixture of protease inhibitors (100X; Beijing ComWin Biotech Co, Ltd, Beijing, China). Equal quantities of protein (30 μg protein/lane) were separated *via* SDS-PAGE (Bio-Rad, Hercules, CA, USA) on a 10% gel and transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk at room temperature for 1.5 h, followed by incubation with the following primary antibodies: KLF6 (1:1000, sc-36563, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and GAPDH (1:5000; cat. No. ab 9484; Abcam, Cambridge, UK) overnight, the goat horseradish peroxidase-conjugated anti-rabbit IgG secondary Ab (1:5,000; cat. No. sc-2054; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were employed to incubate the PVDF membranes for 1 h at room temperature. Quantity One software version 4.62 (Bio-Rad, Hercules, CA, USA) was utilized for densitometry.

Statistical Analysis

All statistical analyses were performed using the SPSS 17.0 software (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 6 (La Jolla, CA, USA). All data were expressed as the mean ± standard deviation. Differences were calculated with Student's *t*-test or one-way ANOVA followed by Tukey post-hoc test. Chi-squared and Fisher's exact tests were applied in the analysis of categorical variables. Pearson's correlation analysis was used to analyze the expression correlation. Kaplan-Meier method and log-rank test were utilized to analyze the overall survival rate of patients. The value of p less than 0.05 indicated a statistically significant difference.

Results

CircMTO1 Was Decreased in Osteosarcoma Tissues and Correlated With Poor Prognosis

To evaluate the expression of circRNAs in osteosarcoma, we initially analyzed altered ex-

pression of circRNAs between osteosarcoma tissues and adjacent normal tissues using circRNA microarray. The results demonstrated that 20 circRNAs were downregulated in osteosarcoma tissues (Figure 1A). The biggest decrease was the level of circMTO1 expression among these 20 circRNAs (Figure 1B). Meanwhile, we calculated the expression of circMTO1 in 70 pairs of osteosarcoma tissues and adjacent normal tissues. The results verified that circMTO1 expression was remarkably declined in osteosarcoma tissues compared with adjacent normal tissues (Figure 1C). Besides, we analyzed the relationship between circMTO1 expression and clinicopathological features of osteosarcoma patients. The results demonstrated that circMTO1 had no relationship with age, gender and tumor size but correlated with Enneking stage (Figure 1D; Table I). Kaplan-Meier survival curves indicated that patients with low circMTO1 expression had significantly shorter overall survival time than those with high circMTO1 expression (Figure 1E).

CircMTO1 Inhibits Cell Proliferation, Migration and Invasion, Induced Apoptosis in Osteosarcoma Cells

Next, we determined the circMTO1 expression level in osteosarcoma cell lines by qRT-PCR assay. Compared with hFOB 1.19 cells, circMTO1 expression was significantly decreased in four osteosarcoma cell lines (Figure 2A). CircMTO1 expression was the lowest in HOS and U2OS cells (Figure 2A). Thus, we chose HOS and U2OS cells for follow-up experiments and established stable circMTO1 overexpression model by transfecting these cells with circMTO1 (Figure 2B). CCK-8 assay showed circMTO1 overexpression reduced the proliferation of HOS and U2OS cells significantly (Figure 2C). In addition, overexpression of circMTO1 inhibited the migration and invasion capacities of cells (Figure 2D). Furthermore, flow cytometry analysis manifested circMTO1 overexpression promoted a higher cell apoptosis percentage than the control group (Figure 2E).

CircMTO1 Serves As a Sponge for MiR-630

Given that circRNA has been shown to act as miRNA sponge[14,15], we further predicted the potential circRNA-miRNA interactions using circinteractome (https://circinteractome.nia.nih.gov/). As shown in Figure 3A, miR-630 was observed to have a potential binding site with circMTO1. Luciferase reporter assay revealed that miR-630 overexpression repressed the Luciferase activity of only circMTO1 wild type in HOS and U2OS cells (Figure 3B). Besides, miR-630 level was significantly upregulated in osteosarcoma

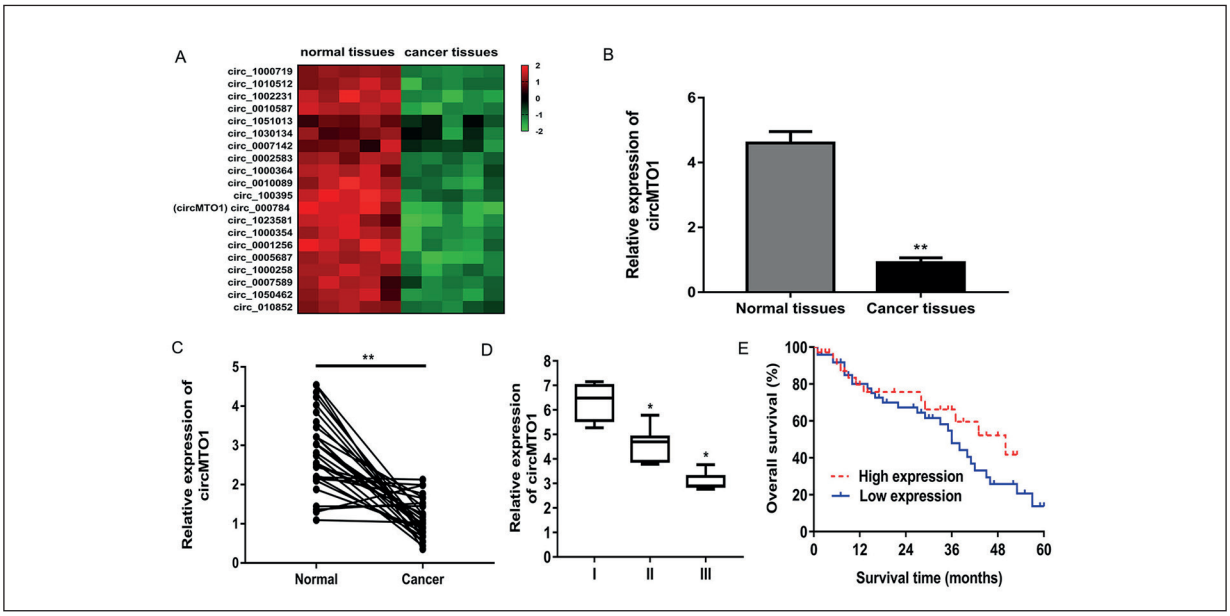

Figure 1. CircMTO1 was decreased in osteosarcoma tissues and correlated with poor prognosis. **A,** 20 circRNAs were downregulated in osteosarcoma tissues. **B,** The expression of circMTO1 was most down-regulated in osteosarcoma tissues. **C,** The expression of circMTO1 in 70 pairs of osteosarcoma tissues and adjacent normal tissues. **D,** CircMTO1 expression was significantly decreased in patients with advanced clinical stage. **E,** Kaplan-Meier curves of overall survivals and log-rank test showed that patients with low circMTO1 expression had poor overall survivals. $^{*}p<0.05$, $^{**}p<0.01$ compared to control group.

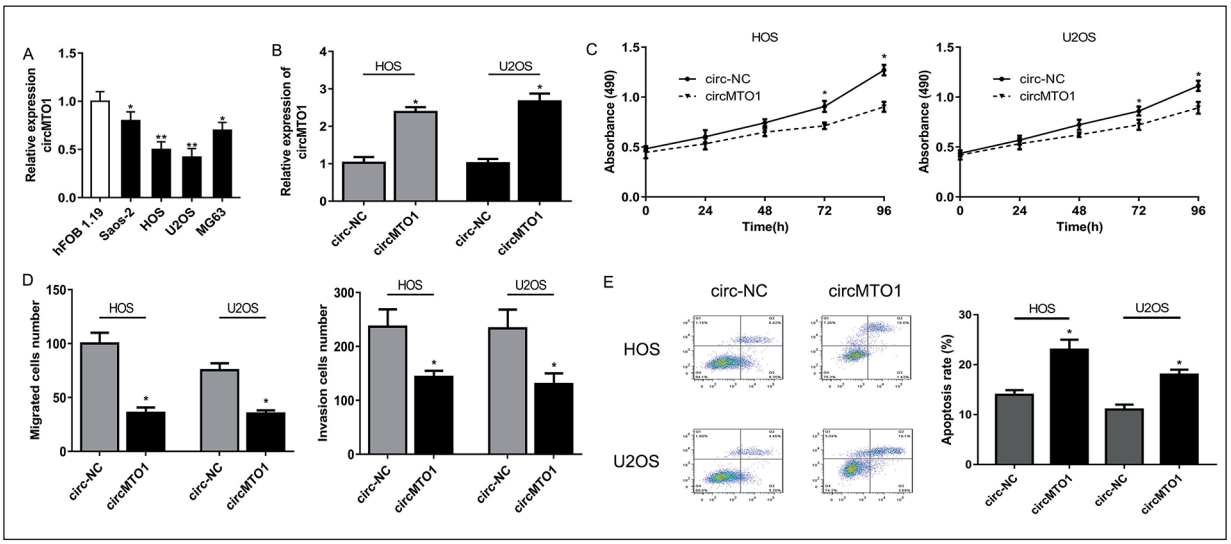

Figure 2. CircMTO1 inhibits cell proliferation, migration and invasion, induced apoptosis in osteosarcoma cells. **A,** CircMTO1 expression level was remarkably enhanced in cancer cell lines than that in normal cell line. **B,** CircMTO1 overexpression was established in HOS and U2OS cells. **C,** CircMTO1 overexpression reduced the proliferation of HOS and U2OS cells significantly. **D,** Transwell assay showed circMTO1 overexpression inhibited the migration and invasion ability of cells. **E,** Flow cytometry revealed that circMTO1 overexpression markedly augmented apoptosis in cells compared with the non-transfected cells. $^{*}p<0.05$ compared to control group.

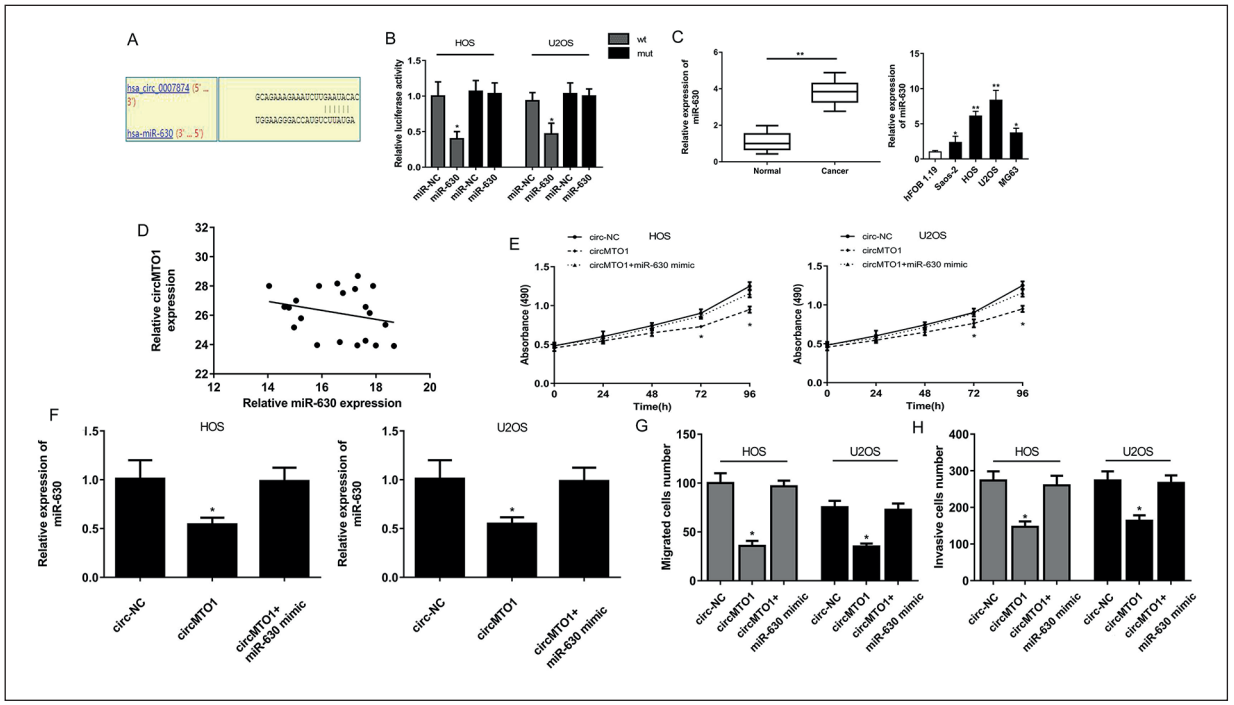

Figure 3. CircMTO1 serves as a sponge for miR-630. **A,** Bioinformatics analysis showed that miR-630 has a potential binding site with circMTO1. **B,** Luciferase reporter assay revealed miR-630 overexpression repressed the luciferase activity of only circMTO1 wild type in HOS and U2OS cells. **C,** MiR-630 level was significantly upregulated in osteosarcoma tissues and cell lines. **D,** Correlation analysis showed a negative relationship between circMTO1 and miR-630 in osteosarcoma tissues. **E,** CCK-8 assay revealed that miR-630 mimic could rescue the suppression by circMTO1 overexpression in the proliferation of two cells. **F,** After cells were transfected with circMTO1, miR-630 expression was significantly downregulated which was elevated by miR-630 mimic. **G, H,** Transwell assay showed that miR-630 mimic could rescue the suppression by circMTO1 in the migration and invasion number of two cells. $^{*}p<0.05$, $^{**}p<0.01$ compared to control group.

tissues and cell lines (Figure 3C). Correlation analysis showed a negative relationship between circMTO1 and miR-630 in osteosarcoma tissues (Figure 3D). CCK-8 assay revealed that miR-630 mimic could rescue the suppression by circMTO1 over-expression in the proliferation of two cells (Figure 3E). After cells were transfected with circMTO1, miR-630 expression was significantly down-regulated which was elevated by miR-630 mimic (Figure 3F). Transwell assay showed that miR-630 mimic could rescue the suppression by circMTO1 in the migration and invasion number of two cells (Figure 3G, 3H).

KLF6 Is a Direct Target of MiR-630

Mounting evidence suggest that miRNAs exert roles *via* binding to 3'-UTR of target mRNAs. We further analyzed the candidate genes regulated by miR-630 using TargetScan (http://www.targetscan.org/) and identified KLF6 as a potential target candidate (Figure 4A). The results indicated that miR-630 can bind to KLF6 mRNA (Figure 4A) and a binding interaction between them was shown by Luciferase reporter assays (Figure 4B). Additionally, we found that the level of KLF6 mRNA was significantly decreased in osteosarcoma tissues and cell lines (Figure 4C). Meanwhile, a reverse correlation between miR-630 and KLF6 was validated in osteosarcoma tissues (Figure 4D). Furthermore, miR-630 mimic obviously suppressed KLF6 expression at both mRNA and protein levels (Figure 4E).

CircMTO1 Functions Through Regulation of MiR-630/KLF6 Axis

Subsequently, we explored the effect of circMTO1 on the expression of KLF6 in cells using qRT-PCR and Western blot. We observed

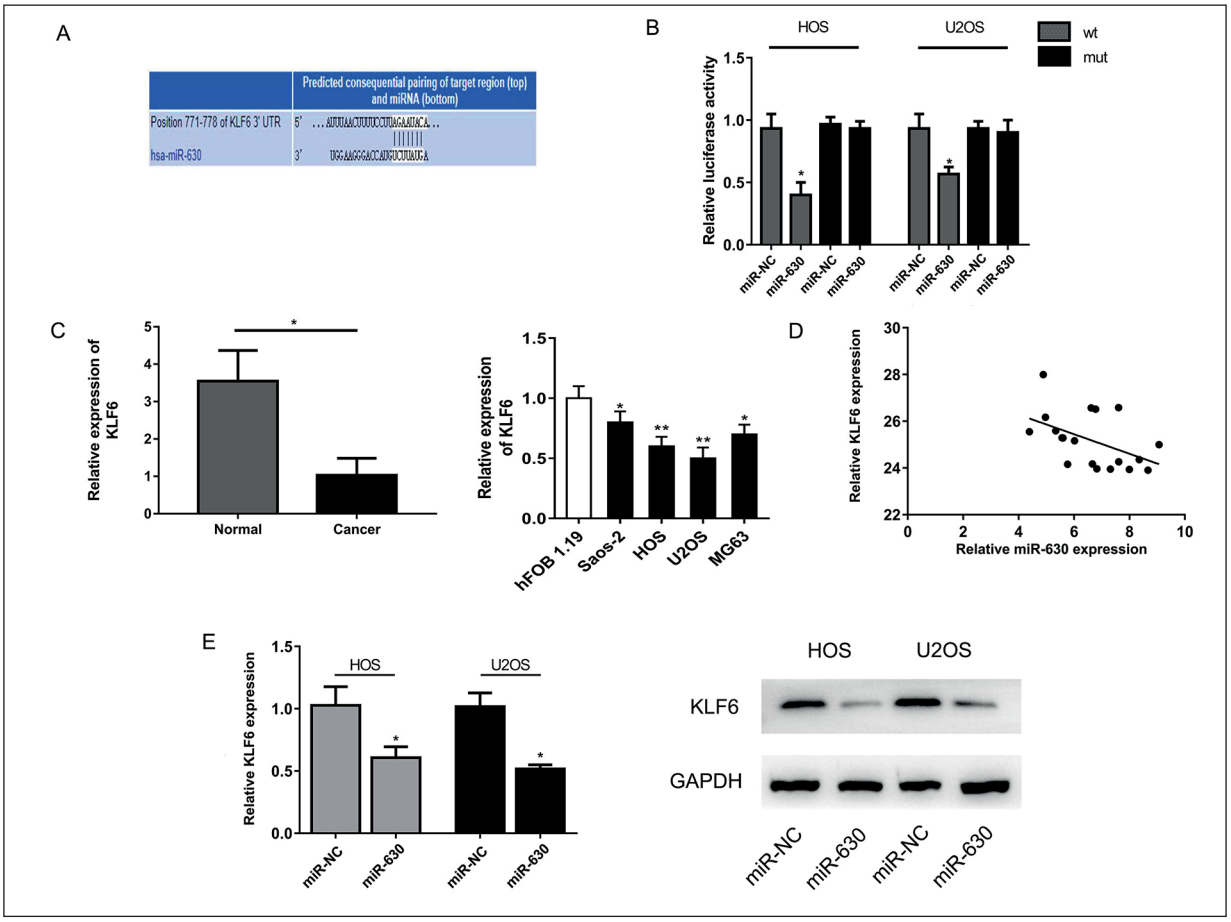

Figure 4. KLF6 is a direct target of miR-630. **A,** KLF6 is a direct target of miR-630 using TargetScan. **B,** Luciferase reporter assay validated miR-630 significantly decreased the luciferase activity of wild type (WT) but not the mutant (Mut) 3'-UTR of KLF6 in cells. **C,** KLF6 expression was significantly decreased in cancer tissues and cells. **D,** A reverse correlation was found between miR-630 and KLF6 in cancer tissues. **E,** miR-630 mimic significantly suppressed KLF6 expression at both mRNA and protein levels. $^{*}p<0.05$, $^{**}p<0.01$ compared to control group.

that circMTO1 overexpression upregulated the expression level of KLF6 in HOS and U2OS cells, which was reversed by miR-630 mimic (Figure 5).

Discussion

Osteosarcoma is the most common malignant bone tumors occurring among children and adolescents, with poor prognosis when metastasis is detected. CircRNAs have been discovered for more than 40 years. Li et al[16] illustrates dysregulation of circRNAs play important regulatory roles in tumorigenesis and metastasis of osteosarcoma. For instance, circ_0001721 have been revealed to participate in osteosarcoma progression. And circRNA LRP6 could promote the development of osteosarcoma[17]. Besides, circular RNA circITCH acts as a tumor suppressor in osteosarcoma[18]. Nevertheless, the function of circMTO1 on osteosarcoma remains to be uncovered. Initially, we found the level of circMTO1 expression was remarkably declined in osteosarcoma tissues and cell lines. The analysis of the relationship between clinicopathologic characteristics and circMTO1 manifested that circMTO1 was related to the progress of osteosarcoma. Meanwhile, we also determined that circMTO1 overexpression suppressed cells proliferation, migration and invasion, tumor growth *in vivo*, whereas induced apoptosis in osteosarcoma cells. Taken together, circMTO1 played a critical role in the development and progression of osteosarcoma, being an anti-oncogene in the malignancy.

It is accepted that circRNAs may execute its regulatory function in gene expression by sponging miRNAs[19]. Through miRNAs screening, we showed that circMTO1 functionally act as the sponge for miR-630. MiR-630 is located at chromosome 15q24.1 and has been identified to potentially act as oncogenes in a variety of tumors[20-23]. This study also showed that miR-630 expression level was remarkably increased in osteosarcoma tissues and cell lines. MiR-630 mimic could rescue the suppression by circMTO1 overexpression in the proliferation, migration and invasion of cancer cells. Bioinformatics analysis found that KLF6 was the downstream target of miR-630. Krüppel-like factors (KLFs) are highly conserved zinc-finger proteins that regulate cellular transcription machinery[24]. KLF6 is known as a tumor repressor gene and was reported inactivated in various malignances[25-27]. Our data showed KLF6 expression was significantly decreased in osteosarcoma tissues and cell lines. Besides, miR-630 remarkably suppressed KLF6 expression in cells. Furthermore, we observed the level of KLF6 was remarkably elevated in cells after transfection with circMTO1, which was reversed by miR-630. These data implied circMTO1 may function through regulation of miR-630/KLF6 axis in osteosarcoma progression.

Conclusions

Altogether, we first identified and confirmed that circMTO1 was downregulated in osteosarcoma. CircMTO1 overexpression could suppress osteosarcoma progression by regulating miR-630/KLF6 axis, which may highlight the diagnostic and therapeutic potential of these molecules in osteosarcoma treatment.

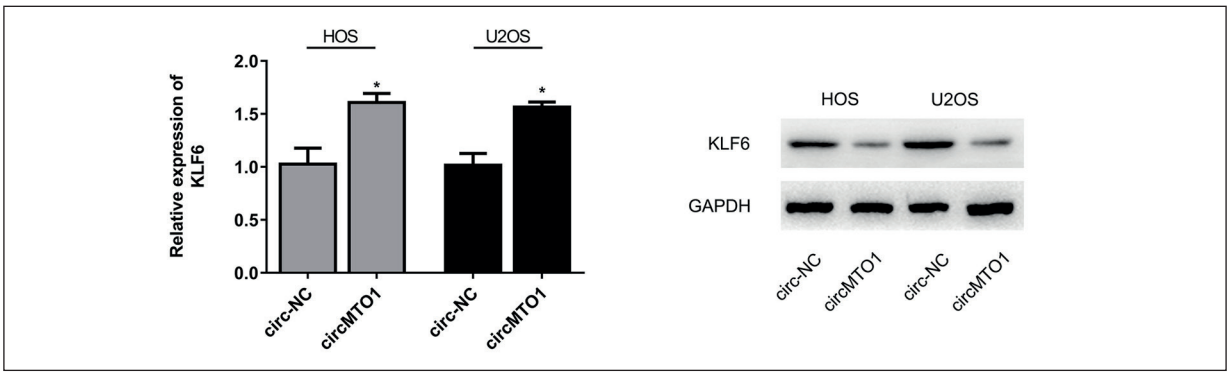

Figure 5. CircMTO1 functions through regulation of miR-337/smad5 axis. CircMTO1 overexpression up-regulated the expression level of KLF6 in HOS and U2OS cells, which was reversed by miR-630 mimic. $^{*}p<0.05$ compared to control group.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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