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MiR-654-5p regulated cell progression and tumor growth through targeting SIRT6 in osteosarcoma

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Abstract. – OBJECTIVE: MiRNAs are important regulators in cell progression, tumor formation, and development. The poor prognosis and high incidence of osteosarcoma are difficult to treat. Therefore, studying the mechanism of OS progression is conducive to the diagnosis and treatment of OS. However, the role of miRNA in OS progression has not been fully explored.

MATERIALS AND METHODS: The expression of miR-654-5p and SIRT6 was detected using qRT-PCR. Western blot was applied to measure the protein expression of SIRT6. Transfected cells proliferation was measured using MTT assay. Transwell was performed to assess cell migrated and invasive capacity. Animals experiment was used to verify the regulatory mechanism of miR-654-5p in OS.

RESULTS: In this study, we found that miR-654-3p was downregulated while SIRT6 expression was upregulated in OS tissues and cells. Meanwhile, the overexpression of miR-654-5p suppressed cell proliferation, invasion, and migration in OS cells. Otherwise, Luciferase reporter assay determined that SIRT6 was a target gene of miR-654-5p. Notably, the promotion effect of anti-miR-654-5p on cell proliferation, migration, and invasion was reversed by inhibition of SIRT6 in OS. Moreover, the promotion of miR-654-5p inhibited OS tumor growth *in vivo*.

CONCLUSIONS: MiR-654-5p regulated cell progression and tumor growth by targeting SIRT6 in OS, providing a new therapeutic target for OS.

Key Words:
MiR-654-5p, SIRT6, Osteosarcoma, Migration, Invasion, Proliferation.

Introduction

Osteosarcoma (OS) is one of the most common primary tumors, characterized by a high degree of malignancy, which can rapidly destroy surrounding tissues and metastasize, seriously affecting the prognosis of patients[1,2]. Traditional treatments such as surgery, radiotherapy and chemotherapy have certain limitations, resulting in a lower 5-year survival rate[3,4]. Therefore, the identification of new molecules abnormally expressed in OS cells provides a possibility for further interpretation of the mechanism of tumor progression and could serve as a prognostic marker for OS.

MicroRNAs are a class of endogenous, non-coding RNAs that are 19-25 nucleotides in length and silence the expression of a target gene by completely or incompletely binding to its 3' UTR, thereby regulating their target mRNA degradation or translational inhibition[5,6]. MiRNA were closely associated with many cancers, such as colorectal cancer, pancreatic cancer and OS[7-9]. MiRNAs not only affect tumor formation and cell growth, but also participate in cancer resistance and immune responses[10-12]. Moreover, accumulating studies have shown that microRNAs are abnormally expressed during the development of OS, including miR-184, miR-320, and miR-124[13-15]. Moreover, miR-654-5p is abnormally expressed during the pathological progression of OS, suggesting that it may be involved in the development of OS[16].

In this study, we found that miR-654-5p was downregulated in OS cells and tissues. However,

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the role of miR-654-5p in OS has not been fully known and clarified. To further explore the specific role of miR-654-5p and its potential molecular mechanisms, this study was conducted to investigate the effect of miR-654-5p on the biological characteristics of OS cells by *in vitro* and *in vivo* experiments, and to analyze its regulatory mechanism.

Materials and Methods

Patients and Tissues

Sixty-three pairs of OS tissues and adjacent tissues were obtained from patients who were diagnosed at Tai'an Central Hospital. All patients had not undergone chemotherapy and other therapies. All samples were stored in liquid nitrogen at -80°C for following experiments. This study was approved by the Research Ethics Committee of Tai'an Central Hospital. Written informed consent was signed by all patients.

Cells Cultured and Transfection

Human OS cell lines (U2-OS and MG-63) and normal cell lines (hFOB 1.19) were purchased from 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) in humidified atmosphere with 5% CO_2 at 37°C.

MiR-654-5p mimics (miR-654-5p), miR-654-5p inhibitor (anti-miR-65-5p), si-SIRT6, and their negative control (miR-NC, anti-NC and si-NC) were obtained from GenePHarma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were used to transfect vector and oligos into the OS cells (U2-OS and MG63) following the manufacturer's protocol.

qRT-PCR

Total RNA in tissues and cells was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). For the detection of miR-654-5p expression, TaKaRa RNA PCR kit (TaKaRa, Otsu, Shiga, Japan) and TaqMan microRNA assay (Applied Biosystems, Foster City, CA, USA) were used. For detection of SIRT6 expression, TaKaRa RNA PCR kit (TaKaRa, Otsu, Shiga, Japan) and TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA) were used. Real Time-PCR was performed by using a SYBR Green Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instruction. α-tubulin was used as an internal control. The primer sequences were used as following: miR-654-5p Forward: 5'-UGGUGGGCCGCAGAACAUGUGC-3'; miR-654-5p reverse: 5'-ACAUGUUCUGCGGCCCACGAAU-3'; α-tubulin Forward: 5'-TATCGAGCGCCCAACCTACACT-3'; α-tubulin Reverse: 5'-CACCAGGTTGGTCTGGAATTCTGTC-3'; SIRT6 Forward: 5'-GCG TGTGGAGTATTTGGATGAC-3'; SIRT6 Reverse: 5'-AGTGTGATGATGGTGAGGATGG-3'.

Western Blot

Proteins were extracted form cells and tissues and separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Then, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane and blocked by non-fat milk for 1 h at 37°C. The PVDF membrane was incubated with primary antibodies, including SIRT6 (Abcam, Cambridge, MA, USA) and α-tubulin (Abcam, Cambridge, MA, USA). Then, the horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Cell Signaling Technology (CST, Danvers, MA, USA) and was incubated with membrane. Finally, the bolt was detected using enhanced chemiluminescence (ECL) Advance Western Detection Reagents (GE Healthcare, Little Chalfont, UK).

Luciferase Reporter Assay

The wild-type or mutant miR-654-3p binding sites in 3'-UTR of SIRT6 was synthesized and inserted into pMIR-REPORT™ (Thermo-Fisher Scientific, Waltham, MA, USA) to construct SIRT6 wild-type reporter vector (SIRT6-Wild) or SIRT6 mutation reporter vector (SIRT6-Mutant). OS cells (U2-OS and MG63) were cotransfected with SIRT6-Wild or SIRT6-Mutant and miR-NC or miR-654-5p using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. After transfection, Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Cell Proliferation

Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, 2×10^3 OS cells (U2-OS and MG63) were seeded into a 96-well plate. After incubation overnight, 10 ul MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added into each plate and then cultured for 4

h at room temperature. Then, 150 mL of dimethyl sulfoxide (DMSO) were added into each well. Cell proliferation was detected at a wavelength of 450 nm by using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Invasion and Migration

Cell invasion and migration were detected using transwell migrated and invasive assay. For the invasion assay, the cells were added into the upper chamber with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). For the migration assay, the cells were seeded into the upper chamber without Matrigel. Next, DMEM with FBS were added into the below chamber served as chemoattractant. After 24 h incubation, the cells in the upper chamber were removed and the cells in the below chamber were fixed with 90% alcohol and stain with crystal violet. Finally, the cells were counted with an inverted microscope (Olympus, Tokyo, Japan).

In Vivo

To ensure the effect of miR-654-5p on tumor growth, female BALB/c nude mice at 5-6 weeks were purchased from Vitalriver (Beijing, China) and were injected into the U2-OS cells transfected with Leti-NC or leit-miR-654-5p. All animal experiments were approved by Tai'an Central Hospital Animal Care and Use Committee. Tumor volume was measured by measuring the length (L) and width (W) with Vernier caliper every day. After 30 days, the mice were sacrificed to measure the tumor weight.

Statistical Analysis

All data were presented as mean±standard deviation (SD). The data were analyzed and displayed using GraphPad Prism 7.0 (San Diego, CA, USA). Statistical evaluation was assessed using Student's *t*-test, to compare the difference between two groups. One-way analysis followed by Tukey's test was used to perform the difference analysis among multiple groups. $*p < 0.05$ was considered as statistically significant.

Results

miR-654-5p Was Downregulated in Tumor Tissues and Cells of OS

qRT-PCR analysis showed that miR-654-5p has much lower levels of OS cells (MG-63 and U2-OS) or OS tissues than normal cells or tissues, implying that miR-654-5p was implicated in the formation of OS (Figure 1A and 1B). In addition, correlation analysis between the expression of miR-654-5p and its clinicopathological parameters in OS showed that the high level of miR-654-5p was closely associated with TNM stage ($p<0.01$), lymph node ($p<0.01$, **Supplementary Table I**), and high survival rate of OS (**Supplementary Figure 1**). Thus, these results suggested that miR-654-5p played an important role in OS progression.

Overexpression of MiR-654-5p Suppressed Cell Proliferation, Invasion and Migration in OS

To investigate the effect of miR-654-5p on cell progression in OS, miR-654-5p was transfected into MG-63 and U2-OS cells, which stably expressed high miR-654-5p (Figure 2A). MTT assay performed that miR-654-5p transfection inhibited cell proliferation in MG-63 and U2-OS cells (Figure 2B). Furthermore, cell migrated capacity of MG-63 and U2-OS cells

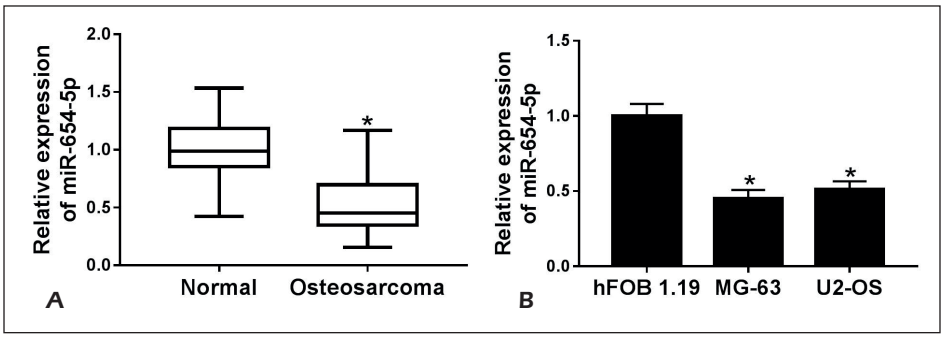

Figure 1. MiR-654-5p was downregulated in tumor tissues and cells of OS. **A**, The expression of miR-654-5p was detected by qRT-PCR in normal tissues and OS tissues. **B**, The expression of miR-654-5p was measured in OS cell lines (U2-OS and MG-63) and normal cell lines (hFOB 1.19) via qRT-PCR. $*p<0.05$.

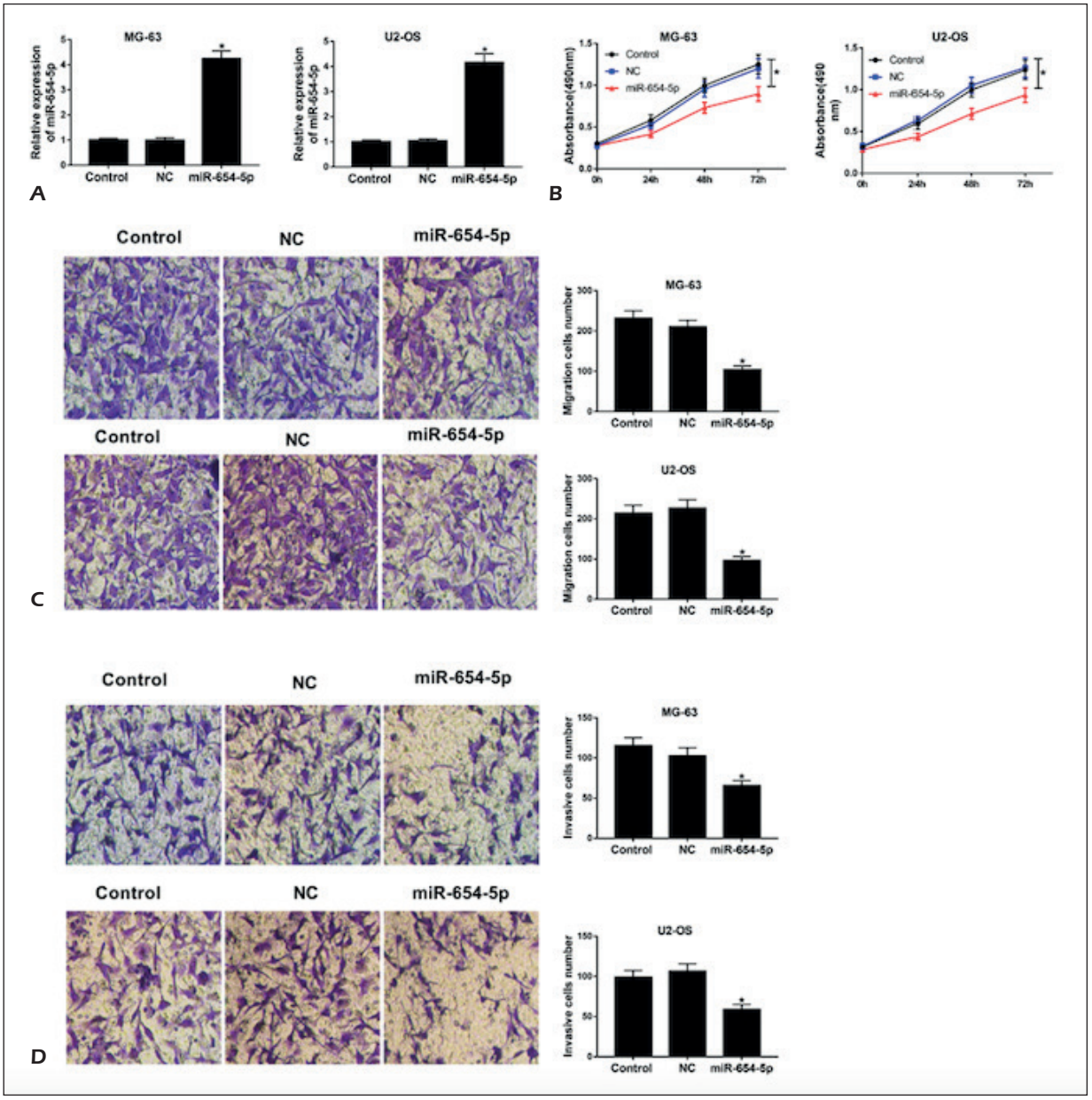

Figure 2. Overexpression of miR-654-5p suppressed cell proliferation, invasion and migration in OS. **A**, The expression of miR-654-5p was detected in control, NC, miR-654-5p groups of MG-63 and U2-OS cells with qRT-PCR assay. **B**, MTT assay was used to measure cell proliferation in control, NC, miR-654-5p groups of MG-63 and U2-OS cells. **C-D**, Transwell assay was used to assess cell migration and invasion (100×) in control, NC, miR-654-5p groups of MG-63 and U2-OS cells. *$p<0.05$.

transfected with miR-654-3p was much lower than that of MG-63 and U2-OS cells transfected with NC (Figure 2C). Similarly, as shown in Figure 2D, cell invasion in miR-654-5p groups was significantly decreased compared with that in NC and control groups. Thus, the promotion of miR-654-5p repressed cell proliferation, invasion, and migration in OS.

SIRT6 was a Target Gene of MiR-654-5p in OS

To further explore the regulatory mechanism of miR-654-5p in OS, SIRT6 was predicted to be a target gene using TargetScan (http://www.targetscan.org/vert_71/) (Figure 3A).

Then, Luciferase reporter assay was applied to ensure the relationship between SIRT6 and miR-

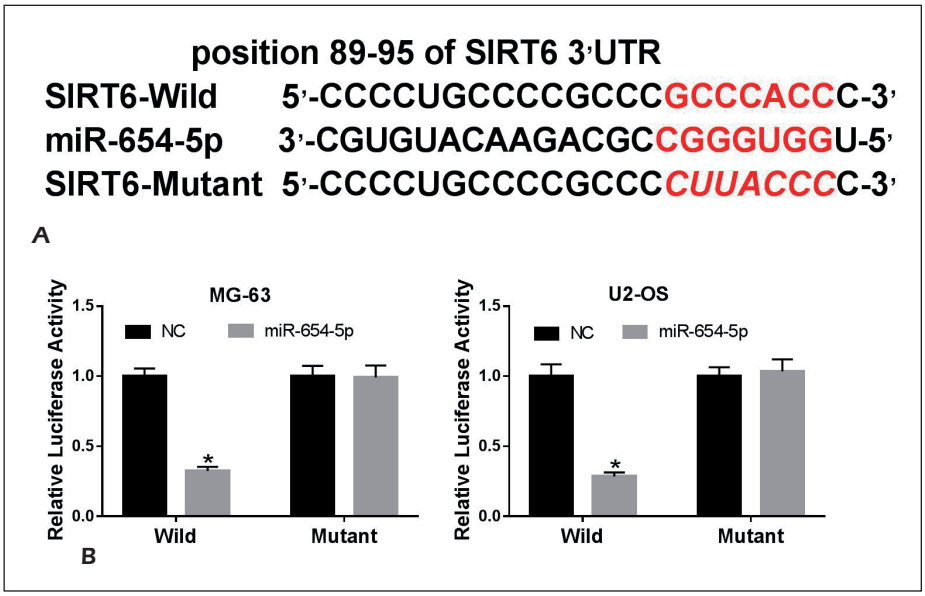

Figure 3. SIRT6 was a target gene of miR-654-5p in OS. **A**, SIRT6 has binding sites in 3'UTR with miR-654-5p using TargetScan. **B**, Luciferase activities of SIRT6-Wild and SIRT6-Mutant were measured in NC and miR-654-5p groups using Luciferase reporter assay in MG-63 and U2-OS cells. *p<0.05.

654-5p. The results showed that when miR-654-5p bound to the SIRT6-Wild, but not SIRT-Mutant, the Luciferase activities were significantly reduced in MG-63 and U2-OS cells (Figure 3B). In total, miR-654-5p directly targeted SIRT6 in OS cells.

SIRT6 was Upregulated in Tumor Tissues and Cells of OS

Then, we also found that SIRT6 expression was induced in OS tissues (Figure 4A) and the negative correlation between miR-654-5p and SIRT6 in OS tissues was verified by Pearson correlation analysis (Figure 4B). Additionally, the mRNA and protein expression of SIRT6 was increased in OS cells (MG-63 and U2-OS) (Figure 4C). Moreover, in MG-63 and U2-OS cells, the expression of SIRT6 was induced by anti-miR-654-5p transfection while it was reduced by miR-654-5p transfection (Figure 4D). Thus, the regulatory network between miR-654-5p and SIRT6 played an important role in the development of OS.

Inhibition of MiR-654-5p Promoted Cell Growth, Which Was Impaired by the Reduction of SIRT6 in OS

Rescue experiment was applied to prove the regulatory network between miR-654-5p and SIRT6. The results of Western blot demonstrated that anti-miR-654-5p promoted SIRT6 protein expression, which was inhibited by si-SIRT6 transfection in MG-63 and U2-OS cells (Figure 5A). MTT assay and transwell assay determined that the promotion effect of anti-miR-654-5p on cell proliferation, migration, and invasion were reversed by inhibition of SIRT6 in MG-63 and U2-OS cells (Figure 5B-5D). These data indicated that miR-654-5p affected cell growth by modulating SIRT6 in OS.

Overexpression of MiR-654-5p Inhibited Tumor Growth and Repressed SIRT6 Expression in OS In Vivo

Next, we demonstrated the regulatory mechanism of miR-654-5p in mice. The results showed that the tumor volume in Leti-miR-654-5p group was significantly decreased compared with that in Leti-NC groups (Figure 6A). Moreover, tumor weight in Leti-miR-654-5p group was significantly less than that in Leti-NC group (Figure 6B). In addition, Leti-miR-654-5p induced the expression of miR-654-5p and decreased SIRT6 protein expression *in vivo*. Therefore, the promotion of miR-654-5p inhibited tumor growth and repressed SIRT6 expression in OS *in vivo* (Figure 6C-6D).

Discussion

OS is the most common primary malignancy with a poor prognosis. MiRNAs have been shown to play an important role in cancer treatment and diagnosis. Moreover, miRNAs are also widely involved in cell metabolism and progression, regu-

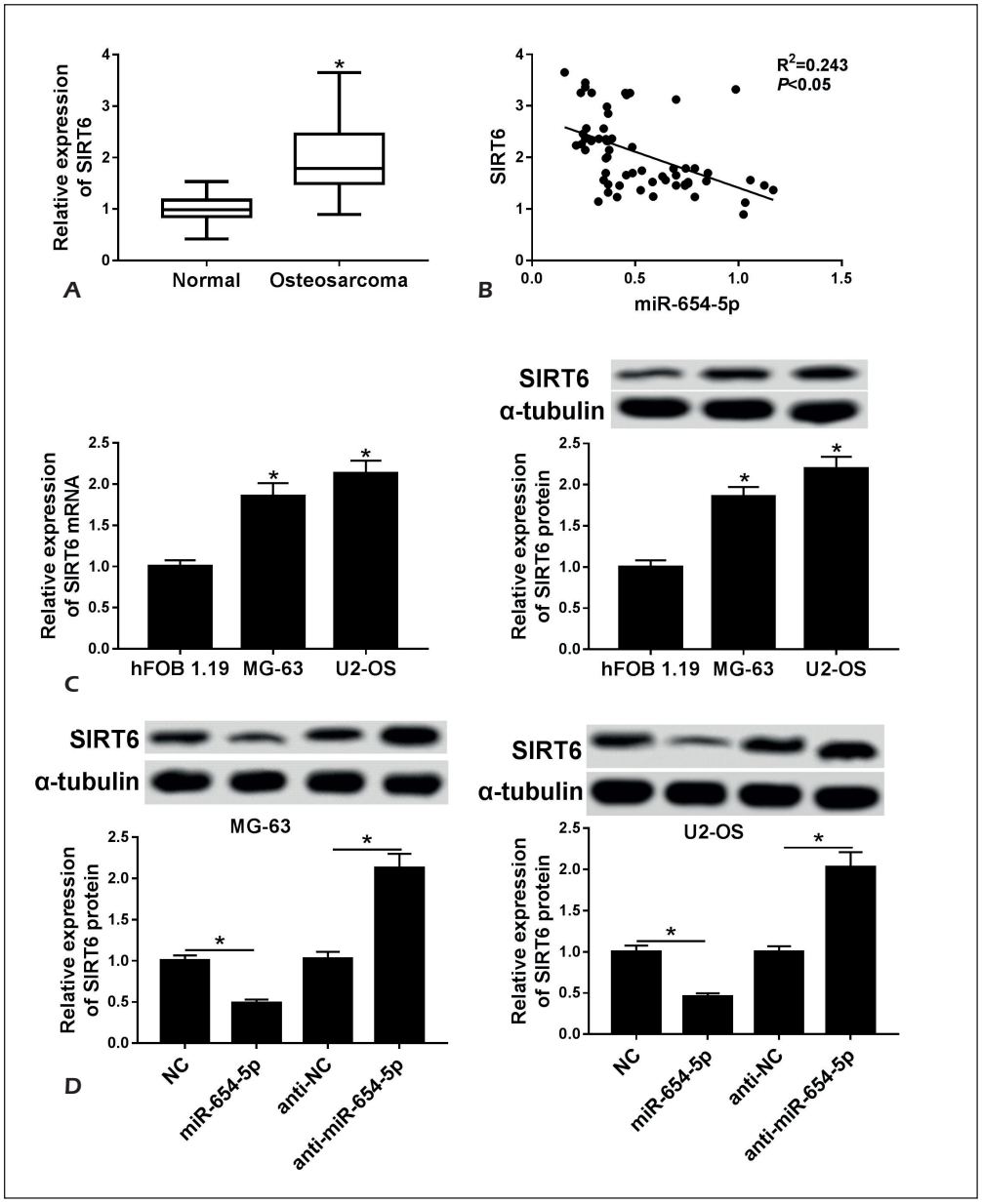

Figure 4. SIRT6 was upregulated in tumor tissues and cells of OS. **A**, The expression of SIRT6 was detected by qRT-PCR in normal tissues and OS tissues. **B**, Pearson's correlation analysis analyzed the relationship between miR-654-5p and SIRT6 in OS tissues. **C**, The mRNA and protein expression of SIRT6 was measured in OS cell lines (U2-OS and MG-63) and normal cell lines (hFOB 1.19) via qRT-PCR and Western blot. **D**, The protein expression of SIRT6 was detected in NC, miR-654-5p, anti-NC and anti-miR-654-5p groups of U2-OS and MG-63 cells via Western blot. *$p<0.05$.

lating cell proliferation, invasion, and apoptosis in a variety of cancers[17-19]. In OS, miRNA plays an indispensable role in many biological processes, from OS formation to migration and invasion[20-22]. Consistently, the expression level of miR-92b was upregulated in OS tissues, and the overexpression of miR-92b promoted tumor proliferation, migration, and invasion in OS *via* targeting RECK[23]. In addition, the inhibition of miR-183 promoted cell migration and invasion by modulating Ezrin in OS[24]. MiR-654-5p is under-expressed in OS[16], suggesting that miR-654-5p plays a role in the formation of OS. In fact, miR-654-5p has been shown to be involved in the regulation of other cancer cell metabolism. MiR-654-5p was highly expressed in tumor tissues and attenuated cell progression in breast cancer by targeting EPSTI1[25]. These results further demonstrated the importance of miR-654-

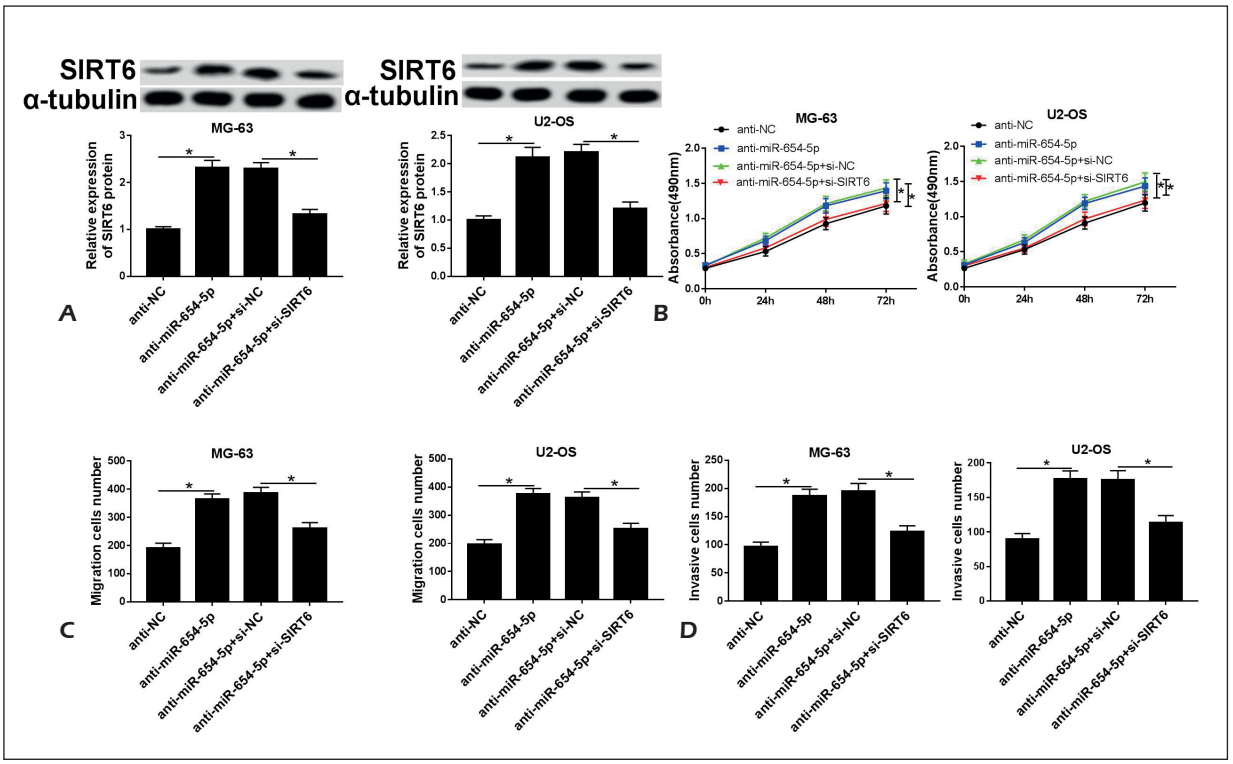

Figure 5. Inhibition of miR-654-5p promoted cell growth, which was impaired by the reduction of SIRT6 in OS. **A**, The protein expression of SIRT6 was detected in anti-NC, anti-miR-654-5p, anti-miR-654-5p+si-NC and anti-miR-654-5p+si-SIRT6 groups of MG-63 and U2-OS cells via Western blot. **B**, Cell proliferation was measured via MTT assay in anti-NC, anti-miR-654-5p, anti-miR-654-5p+si-NC and anti-miR-654-5p+si-SIRT6 groups of MG-63 and U2-OS cells. **C**-**D**, Cell migration (**C**) and invasion (**D**) were calculated via transwell assay in anti-NC, anti-miR-654-5p, anti-miR-654-5p+si-NC and anti-miR-654-5p+si-SIRT6 groups of MG-63 and U2-OS cells. *p<0.05.

5p's role in cancer formation and therapy. In this paper, we found that the increase of the expression of miR-654-5p contributed to the inhibition of proliferation, migration, and invasion of OS cells, demonstrating that miR-654-5p was involved in the formation and cell growth of OS. However, miR-654-5p was upregulated in oral squamous cell carcinoma (OSCC) and promoted proliferation, which is associated with poor prognosis[26], which might be due to the difference in cancer tissue that leads to inconsistent expression of miR-654-5p in different cancers.

The regulation of miR-654-5p is inseparable from the regulation of its downstream target genes. In this experiment, we first discovered and demonstrated that SIRT6 is the target gene of miR-654-5p. SIRT6 is highly expressed in OS cells and tissues. Moreover, miR-654-5p overexpression inhibits the expression of SIRT6 in OS cells. SIRT6 is also involved in cancer cell progression, such as proliferation, apoptosis, invasion, and immune response[27-30]. Besides, the overexpression of SIRT6 promoted massive apoptosis in cancer cells[31]. In this paper, SIRT6 has been shown to be involved in the regulation of OS cell growth. Inhibition of miR-654-5p promoted cell growth, which was impaired by the reduction of SIRT6 in OS. Therefore, miR-654-5p regulated cell progression and tumor growth by targeting SIRT6 in OS. Moreover, we also found that increasing the expression of miR-654-5p inhibits OS tumor growth by inhibiting the expression of SIRT6 *in vivo*.

Conclusions

We first discovered that miR-654-5p regulates osteosarcoma cell progression by targeting SIRT6. Moreover, miR-654-5p could serve as a prognostic marker for OS and its regulatory network further elucidates the mechanism of OS formation.

Conflict of Interests

The authors declare no conflict of interest.

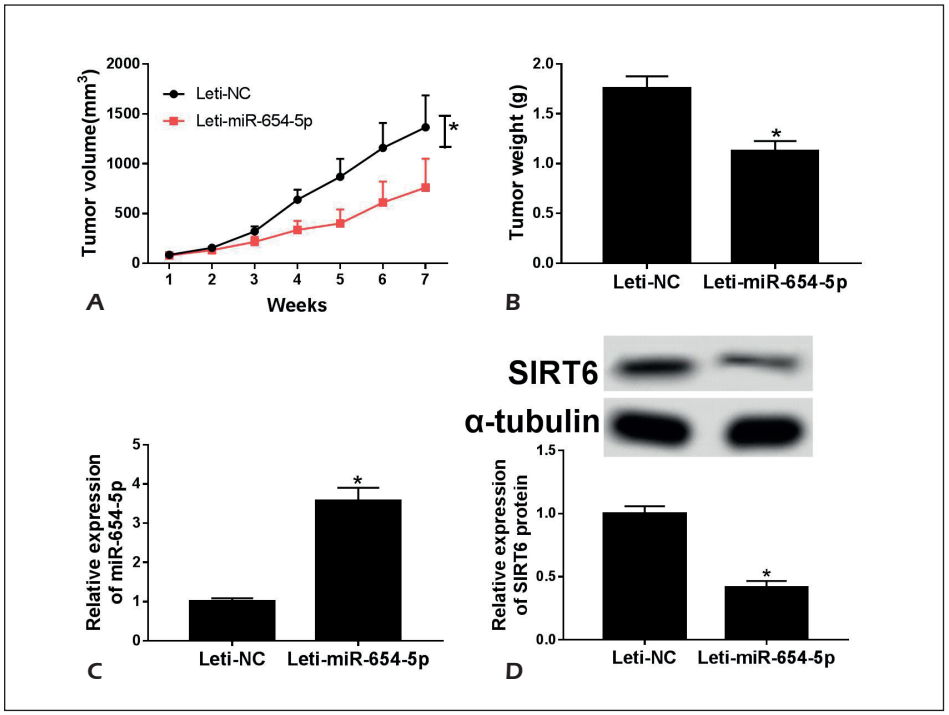

Figure 6. Overexpression of miR-654-5p inhibited tumor growth and repressed SIRT6 expression in OS in vivo. **A**, Tumor volumes were calculated between Leti-miR-654-5p and Leti-NC group. **B**, Tumor weights were evaluated in Leti-miR-654-5p and Leti-NC group. **C**, qRT-PCR assay detected miR-654-5p expression in Leti-miR-654-5p and Leti-NC group. **D**, Western blot assay was applied to measure the protein expression of SIRT6 in Leti-miR-654-5p and Leti-NC group. *$p<0.05$.

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