



hsa_circ_0000006 induces tumorigenesis through miR-361-3p targeting immunoglobulin-like domains protein 1 (LRIG1) in osteosarcoma

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Background: Osteosarcoma (OS) is considered to be the most highly prevalent bone tumor. In the progression of different human cancers, the role of circular RNAs (circRNAs) has been extensively studied. Microarray analysis has indicated that hsa_circ_0000006 expression was lower in OS, but the mechanism of hsa_circ_0000006 in regulating the progression of OS remains elusive.

Methods: The expression of cancer-related genes at the transcriptional and translational levels was assessed by RT-qPCR and western blotting (WB). Colony formation and Cell Counting Kit-8 (CCK-8) assays were used to evaluate the proliferative potential of cells. The transwell assay was used to examine the invasive and migratory potential of cells. Furthermore, dual-luciferase reporter (DLR) and RNA pull-down assays were performed for the validation of the targeting sites of hsa_circ_0000006, miR-361-3p, and the 3'-untranslated region (3'-UTR) of immunoglobulin-like domains protein 1 (LRIG1) mRNA. Moreover, the protein levels of epithelial-to-mesenchymal transition (EMT) markers were analyzed by WB.

Results: The expression of hsa_circ_0000006 and LRIG1 were found to be down-regulated in OS tissues and cells, while miR-361-3p was up-regulated. Knockdown of hsa_circ_0000006 promoted the progression and development of OS, as well as EMT. Furthermore, hsa_circ_0000006 was revealed as a sponge of miR-361-3p, which negatively regulates miR-361-3p expression. LRIG1 was found to be an miR-361-3p target. In OS cells, the LRIG1 expression level was decreased, with elevated expression of miR-361-3p. Advanced studies demonstrated that hsa_circ_0000006 regulates LRIG1 expression through sponging miR-361-3p, then promotes the tumorigenesis of OS.

Conclusions: hsa_circ_0000006 is associated with the progression and development of OS through miR-361-3p by target LRIG1, which is a significant biomarker and effective therapeutic target for patients with OS.

Keywords: hsa_circ_0000006; miR-361-3p; immunoglobulin-like domains protein 1 (LRIG1); osteosarcoma (OS)

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Introduction

Osteosarcoma (OS) is considered to be the most highly prevalent bone tumor, and is particularly diagnosed in childhood and in adult patients (1). Traditional treatments of OS involve neoadjuvant chemotherapy and surgical removal of the tumor followed by adjuvant chemotherapy (1). Five-year overall survival rates in patients with OS have risen to 60–75% since the advent of chemotherapy (2). Nevertheless, 30–40% of OS patients are metastatic and recurrent, and the survival rate is less than 30%, indicating that the survival rate has not been substantially improved (3,4). Therefore, finding new approaches to treat OS patients with poor prognosis is extremely urgent. It has been reported that genetic variants are associated with OS (5,6). A type of endogenous noncoding RNA known as circular RNAs (circRNAs) contain no 5'-end cap and 3'-end poly-A tail. They exist in a circular structure established by covalent bonding. CircRNAs are widely expressed in a variety of cells (7,8). Several studies have revealed that circRNAs have a considerable role in numerous processes associated with pathology, including those in cancer (9–12). More and more evidence suggests that circRNAs regulate the progression of OS and act as candidate biomarkers for predicting the prognosis of cancer (13–15). However, there are still many circRNAs that need to be discovered in OS. According to the microarray GSE140256 analysis, *hsa_circ_0000006* was found to have abnormal expression in OS.

The function of circRNAs in miRNA regulation has recently gained considerable attention (16). MiRNAs are small non-coding RNAs and contain a single-strand with 19–25 nucleotides. They regulate gene expression in both plants and animals (16). In cancers, several biological processes, such as proliferation, invasion, differentiation, and apoptosis, have been linked with miRNAs (17,18). Recently, studies have revealed that circRNAs contain miRNA response elements (MRE), which can serve as competing endogenous RNAs (ceRNAs) and bind with miRNAs to work as miRNA sponges in cells. Furthermore, the inhibitory activity of miRNA against its target genes was eliminated, and target gene expression was increased (19,20). Then, miR-361-3p was found to be abnormally expressed in OS by the microarray GSE65071. Numerous reports have also revealed that miR-361-3p affects the progression and developmental process of tumors, such as non-small cell lung carcinoma and thyroid cancer, among others (21,22). However, the *hsa_circ_0000006*-miR-361-3p regulatory network in the progression of OS is poorly understood.

Furthermore, the microarray GSE87624 was

reviewed and it was found that leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1) is abnormally expressed in OS. LRIG1 was identified more than 20 years prior. LRIG1 was found to be down-regulated or absent in many carcinomas and acts as a tumor suppressor (23). For example, in prostate cancer, malignant glioma, and other cancers, LRIG1 acts as a tumor suppressor (24,25). However, there are still few studies on the mechanism of LRIG1 in OS.

In the present research, based on the results of GSE140256, GSE65071, and GSE87624, we identified a new OS-related circRNA, namely *hsa_circ_0000006*. To further understand the role of *hsa_circ_0000006* in the progression of OS, the mechanism of *hsa_circ_0000006* was investigated in OS cells and tissues. The expression of *hsa_circ_0000006* was significantly suppressed in OS tissues and cells, and acted as an miR-361-3p sponge, affecting LRIG1 expression. Finally, *hsa_circ_0000006* was also shown to regulate the progression and development of OS in cells. Overall, our data provides insights into diagnostic biomarkers of OS. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-3076>).

Methods

Clinical samples

We collected 30 OS tissue samples and para-carcinoma tissue samples from surgical specimens, which were from the archives of PLA General Hospital, Beijing, China. In the present study, all the experimental procedures were carried out according to the Declaration of Helsinki (as revised in 2013) and approval was obtained from the Institutional Review Board of General Hospital of Chinese People's Liberation Army (No. C2020-011-01). Before the beginning of the study, each patient signed written informed consent.

Cell culture and transfection

The non-cancer cell line hFOB1.19, and human OS cell lines U2OS, Saos-2, HOS, and MG-63 were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). The hFOB1.19 cell line was grown in DMEM, while the HOS and MG-63 cell lines were grown in EMEM. The U2OS and Saos-2 cell lines were grown in α -MEM, enriched with fetal bovine serum (FBS, 10%), along with penicillin and streptomycin (100 U/mL each).

Table 1 qRT-PCR primer sequences

Gene	Sequence (5'-3')
hsa_circ_0000006	F: GCCAATATGAGCCAG
	R: CTTTCTTGGGAATCCAG
hsa-miR-361-3p	F: CACTCCAGCTGGGTCCCCCAGGTGTGATTC
	R: CTCAACTGGTGTTCGTGGAGTCGGCAATTCAGTTGAGAAATCAGA
LRIG1	F: CACGCAGTTGGGCACTTTTG
	R: TTGGGACAGCTTGGATCAC

qRT-PCR, quantitative real-time PCR; LRIG1, immunoglobulin-like domains protein 1.

Furthermore, 37 °C temperature and 5% CO₂ flow were adjusted in the cell culture incubator. The overexpressed hsa_circ_0000006 plasmids, si-hsa_circ_0000006, miR-361-3p mimics/inhibitors, si-LRIG1, and negative control were provided by RiboBio (Guangzhou, China). The transfection was conducted via Lipofectamine 2000 (Invitrogen, USA), following the manufacturer's protocol.

qRT-PCR analysis

TRIzol[®] reagent was provided by Invitrogen and was used for total RNA extraction from OS cells (1×10⁵), following the manufacturer's protocol. The isopropanol precipitates in the upper aqueous phase were collected by centrifugation at 4 °C then washed, followed by drying at 22 to 25 °C. Next, to determine the concentration of RNA, 20 to 30 mL of water (DEPC-treated) was added, followed by storing RNA at -80 °C in a freezer. Subsequently, qRT-PCR detection was carried out according to the instructions for the TAKARA OneStep PrimeScript[®] miRNA cDNA Synthesis Kit, followed by the SYBR Green I fluorescence assay. The relative expression (RE) of RNA was determined by the 2^{-ΔΔCt} method. All experiments were conducted in triplicate. *Table 1* comprises the sequences of primers used in qRT-PCR experiments.

Cell Counting Kit-8 (CCK-8) assays

The proliferative potential of cells was evaluated via CCK8 assays. Based on the manufacturer's instructions, the seeding of 1×10⁵ cells was carried out into 96-well plates with 100 μL per well. At 24, 48, 72, and 96 hrs, 10 μL of CCK-8 reagent (Beyotime Institute of Biotechnology) was added into the culture medium, followed by incubating the cells at 37 °C for 4 hrs. The optical density (OD) was recorded via

a microplate reader (BioTek Instruments) at 450 nm. All experiments were conducted in triplicate.

Colony formation assay

In 6-well plates, 500 transfected cells in each well were grown for 14 days, followed by washing the cultured cells in PBS, then the cells were fixed with 4% paraformaldehyde. Cells were then stained with a 0.1% crystal violet solution, followed by counting the colonies individually. All experiments were repeated in triplicate.

Transwell assays

Cell migration and invasion were examined via transwell assays. In the upper chambers, 1×10⁵ OS cells were grown with 150 μL of medium (without serum). For invasion, the transwell chamber (Corning) was paved with Matrigel mix (BD Biosciences), while migration assays were paved in the absence of Matrigel mix. Medium with FBS (10%) was added into the lower chamber, followed by culturing for 24 hrs at 37 °C with 5% CO₂ exposure. The upper chambers were then set and stained for 20 min with 0.1% crystal violet (Kaigen, China). Then, images were taken under a microscope, followed by counting the cells. The experiments were repeated in triplicate.

Western blotting (WB) analysis

Cells were treated with lysis buffer for the radioimmunoprecipitation assay (RIPA, Beyotime, China). Bicinchoninic acid (BCA) analysis was used to determine the protein concentration (Beyotime, China), and 10% SDS-PAGE was used to isolate the same amount of protein, which was then transferred to PVDF membranes (Millipore,

USA). Anti-N-cadherin, anti-LRIG, anti-E-cadherin, and anti-Vimentin were obtained from Cell Signaling Technology, USA, and were used at dilutions 1:1,000, 1:2,000, 1:1,000, and 1:1,000, respectively. Anti-Slug (1:2,000) and anti-Snail (1:2,000) were provided by Abcam, USA. The membranes were then exposed to secondary antibodies (1:5,000) for 40 min. Finally, Millipore's ECL chemiluminescent reagent was utilized for the visualization of blots, followed by analyzing the obtained data using Image Lab Software.

Dual-luciferase reporter (DLR) assays

The pmir-reporter was provided by Promega, USA to transfect the *hsa_circ_0000006* WT, *hsa_circ_0000006* MUT, LRIG1 WT, or LRIG1 MUT sequences. After 48 h, the DLR assay was performed after the OS cells were treated with miR-361-3p mimics or miR-NC.

RNA pull-down assay

The biotinylated RNA pull-down assay was conducted to evaluate the link between *hsa_circ_0000006*/LRIG1 and miR-361-3p. The biotin-labeled miR-361-3p probe and NC probe were provided by GenePharma. Briefly, 1×10^5 HOS cells were obtained, followed by their lysis and sonication. C-1 magnetic beads (Life Technologies) were used to incubate the probes for 3 h at 25 °C. Cell lysates with the miR-361-3p probe or oligo probe were incubated for 24 hrs at 4 °C, followed by washing with PBS buffer. Trizol reagent was used to purify the bound RNA, and then qRT-PCR was conducted.

Statistical analysis

SPSS 22.0 (IBM, Chicago, USA) and GraphPad Prism 7 were used for statistical analysis. Statistical significance was analyzed by ANOVA. The Pearson correlation coefficient was used to evaluate the correlations. Data were expressed as the mean \pm SD. Group variations were regarded as statistically significant when $P < 0.05$ between groups.

Results

***hsa_circ_0000006* is down-regulated in OS tissues and cells**

In the analysis of microarray GSE140256 from platform

GPL27741, circRNAs with different expression levels were filtered according to the criteria of $\log_2|\text{fold change}| > 3$ and $P < 0.05$, and the expression of *hsa_circ_0000006* was found to be reduced in OS tissues, as depicted in *Figure 1A*. Furthermore, *hsa_circ_0000006* level was determined in OS tissues as well as cell lines to explore the expression profile of *hsa_circ_0000006* in OS. The qRT-PCR results demonstrated that the expression of *hsa_circ_0000006* was considerably reduced in OS tissues relative to that in neighboring tissues, as depicted in *Figure 1B* ($P < 0.001$). Likewise, the expression of *hsa_circ_0000006* was significantly decreased in U2OS, Saos2, HOS, and MG63 cells compared to hFOB1.19 cells (*Figure 1C*, $P < 0.001$ and 0.01, respectively), as depicted in *Figure 1B*. HOS cells showed the lowest level of *hsa_circ_0000006* expression. Then, we carried out subsequent experiments on this cell line.

***hsa_circ_0000006* knockdown facilitates OS cell migration, invasion, and proliferation**

To study the cell transfection efficacy of *hsa_circ_0000006*, the level of *hsa_circ_0000006* was silenced or overexpressed in HOS cells. In comparison to the si-NC group, *hsa_circ_0000006* expression was considerably inhibited after transfection with si-circ_0000006. Meanwhile, *hsa_circ_0000006* expression was strikingly increased compared to the vector group (*Figure 2A*), which suggested that the transfection was successful.

Then, the proliferative potential of cells was further evaluated. The CCK-8 and colony formation assays showed that the down-regulation and up-regulation of *hsa_circ_0000006* significantly stimulated and attenuated cell proliferation (*Figure 2B, 2C*, $P < 0.001$), respectively, which revealed that *hsa_circ_0000006* enhanced the proliferative potential of OS cells.

Moreover, transwell assays were conducted to identify the impact of *hsa_circ_0000006* on the invasive and migratory potential of OS cells. The data demonstrated that the invasive and migratory abilities of HOS cells were markedly promoted by down-regulation of *hsa_circ_0000006* but significantly suppressed by up-regulation of *hsa_circ_0000006* (*Figure 2D, 2E*, $P < 0.001$). These findings indicated that *hsa_circ_0000006* assisted OS cell migration and invasion. Furthermore, the expression levels of markers [linked with epithelial-to-mesenchymal transition (EMT)] were determined by WB to explore the possible mechanism of *hsa_circ_0000006* in anti-tumor abilities. After *hsa_circ_0000006*

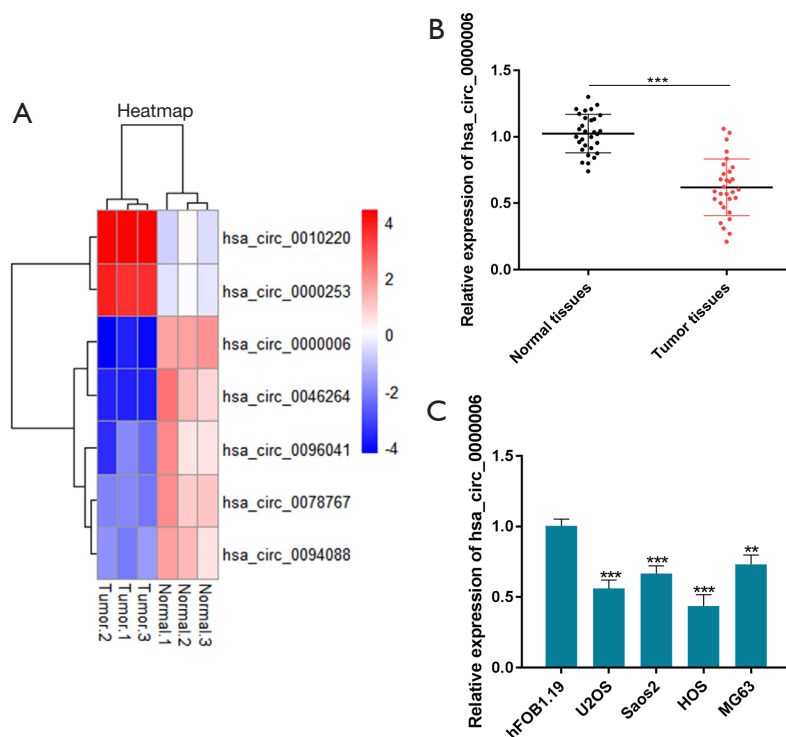


Figure 1 RE of hsa_circ_0000006 in OS tissues and cell lines. (A) Analysis of microarray GSE140256 from platform GPL27741 containing OS tissues and the adjacent tissues. (B) RE of hsa_circ_0000006 in OS as well as neighboring tissue samples determined by qRT-PCR. (C) RE of hsa_circ_0000006 in OS cell lines and hFOB1.19 determined by qRT-PCR. ** $P < 0.01$, *** $P < 0.001$. RE, relative expression; OS, osteosarcoma; qRT-PCR, quantitative real-time PCR.

circ_0000006 silencing, E-cadherin (epithelial marker) was down-regulated, while N-cadherin, Vimentin, Slug, Snail, and Twist (mesenchymal markers) were up-regulated in HOS cells. However, the up-regulation of hsa_circ_0000006 exerted opposite effects (Figure 2F, $P < 0.05$). Hence, we inferred that hsa_circ_0000006 stimulated proliferation, migration, invasion, and EMT signaling pathways in OS.

hsa_circ_0000006 as a molecular sponge antagonized miR-361-3p availability

Bioinformatics analysis (starBase, circInteractome, and GSE65071) revealed that hsa_circ_0000006 was the potential target of miR-361-3p, and miR-361-3p significantly attenuated the DLR activity of hsa_circ_0000006, as indicated in Figure 3A ($P < 0.001$). In addition, an RNA pull-down assay was conducted and the data indicated that enrichment was obtained in the mutant hsa_circ_0000006 group relative to the bio-NC group (Figure 3B, $P < 0.001$). These data proved the interaction of miR-361-3p with hsa_circ_0000006. In Figure 3C, the

silencing of hsa_circ_0000006 up-regulated the miR-361-3p level in HOS cells ($P < 0.001$), whereas overexpression of hsa_circ_0000006 showed the opposite result. Furthermore, the expression of miR-361-3p was significantly elevated in OS tissues (Figure 3D, $P < 0.001$). The expression of hsa_circ_0000006 and miR-361-3p were inversely correlated, as indicated by the Pearson analysis (Figure 3E). In short, hsa_circ_0000006 can act as a sponge molecule of miR-361-3p to regulate the expression of miR-361-3p.

LRIG1 was the potential target of miR-361-3p

StarBase, TargetScan, miRDB, miRTarBase, and GSE87624 were used for predicting the target of miR-361-3p, and the results revealed that LRIG1 had binding sequences with miR-361-3p, as depicted in Figure 4A. The results of the DLR assay indicated that the activities of HOS cells were considerably lowered by transfection with miR-361-3p relative to the control in the LRIG1 3'-untranslated region (3'-UTR)-WT group, and the variations were not observed in the LRIG1 3'-UTR-MUT group, as depicted in

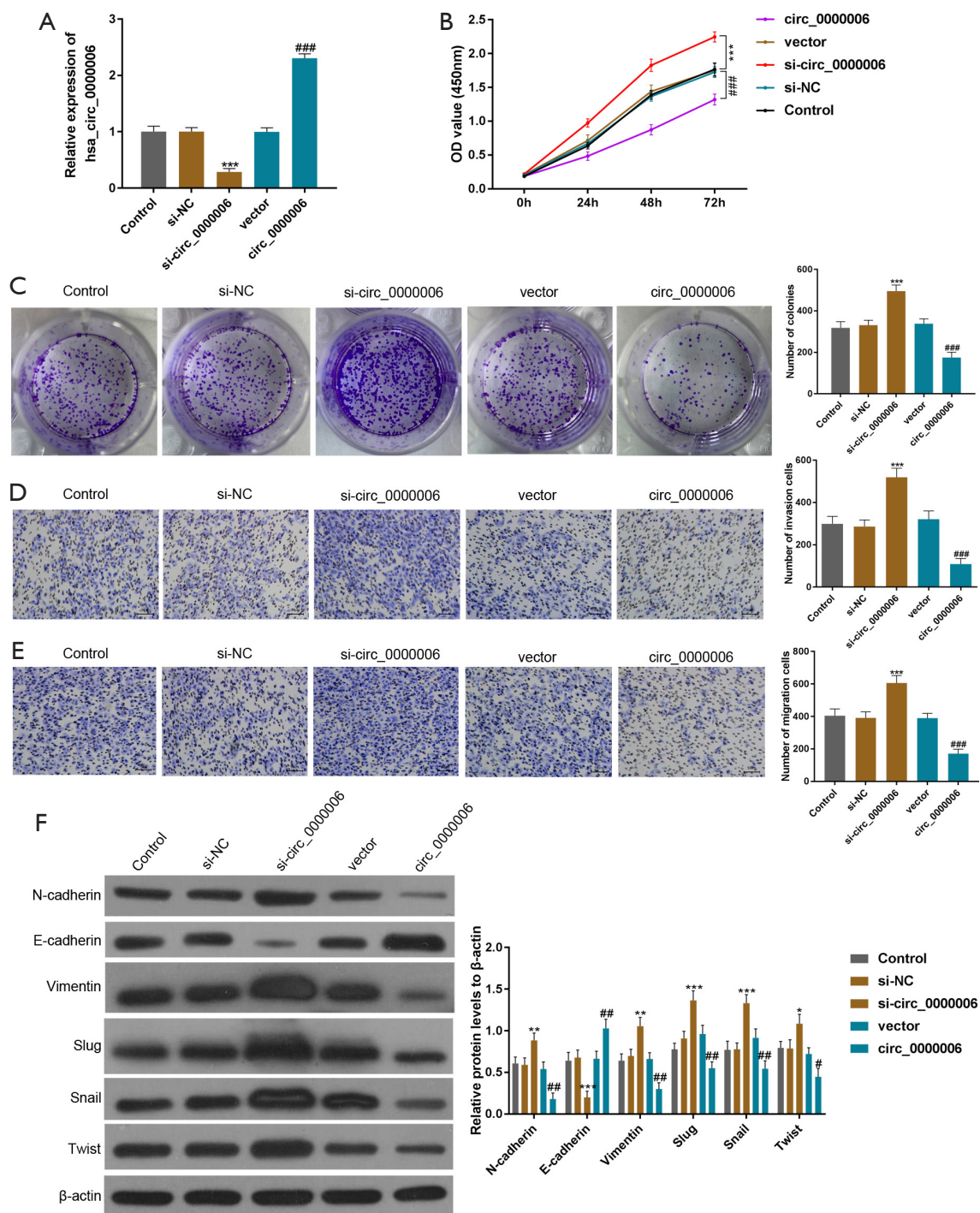


Figure 2 The cell activity and EMT mechanism of hsa_circ_0000006 in OS cells. (A) An overexpressed or silenced elevated or decreased expression of hsa_circ_0000006 by a method based on lentivirus in HOS cells. (B,C) The capacity for proliferation was determined in HOS cells (transfected with si-circ/oe-circ or NC/vector) by using CCK-8 and colony formation assays (dyed with crystal violet, >50 cells). (D,E) Cell invasion and migration were assessed by transwell assays in HOS cells transfected with si-circ/oe-circ or NC/vector (dyed with crystal violet, 50 μ m). (F) The expression levels of epithelial-to-mesenchymal transition markers were detected in HOS cells by WB. The obtained data were expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$. EMT, epithelial-to-mesenchymal transition; OS, osteosarcoma; HOS, human osteosarcoma; NC, normal control; CCK-8, Cell Counting Kit-8; WB, western blotting.

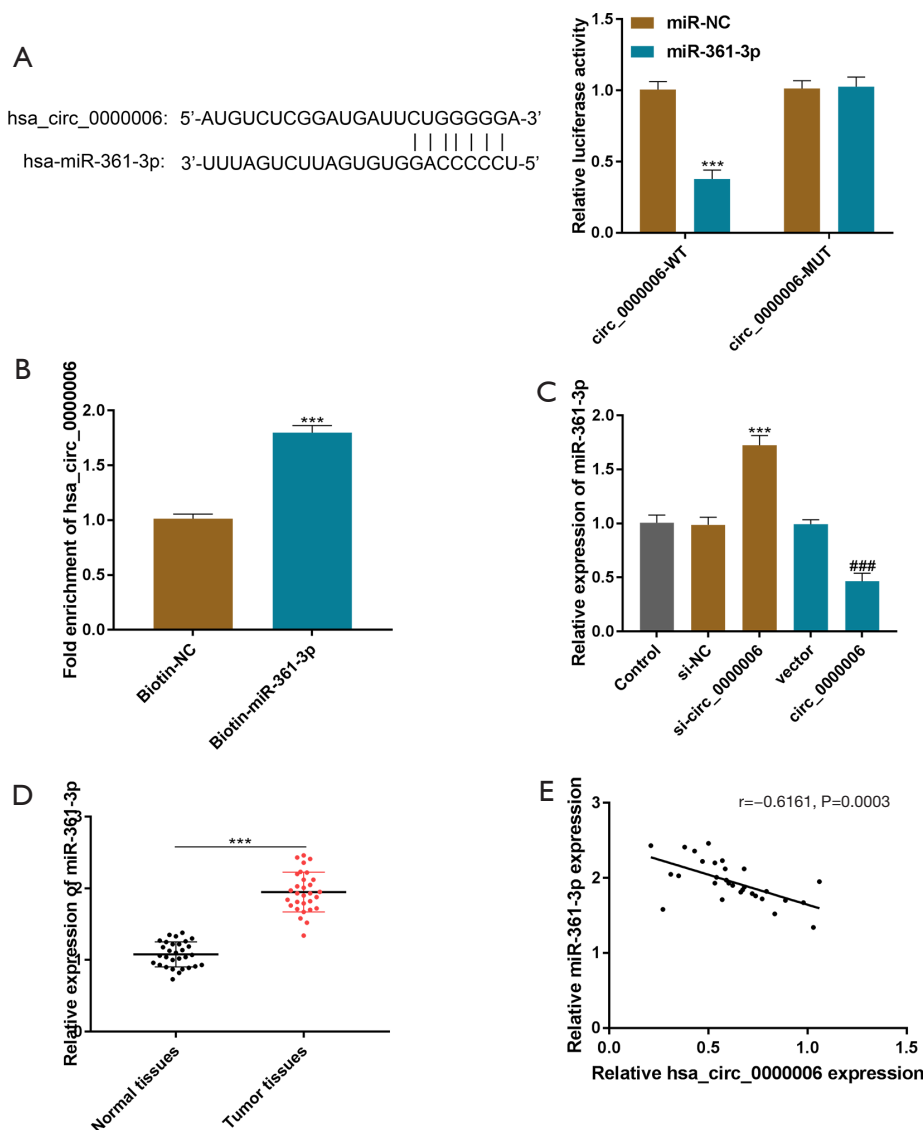


Figure 3 hsa_circ_0000006 was found to bind with miR-361-3p and negatively regulated its expression. (A) The DLR assay was employed to evaluate the association between hsa_circ_0000006 3'-UTR and miR-361-3p. (B) The levels of hsa_circ_0000006 and miR-361-3p enrichment in HOS cells were evaluated by the RIP assay. (C) The RE of miR-361-3p was determined by qRT-PCR in HOS cells (transfected with si-hsa_circ_0000006 or oe-hsa_circ_0000006). (D) MiR-361-3p expression in OS and nearby normal tissues was evaluated by qRT-PCR. (E) The link between the expression of hsa_circ_0000006 and miR-361-3p was evaluated by Pearson analysis. *** $P < 0.001$; ### $P < 0.001$. NC, normal control; DLR, dual-luciferase reporter; 3'-UTR, 3'-untranslated region; HOS, human osteosarcoma; RE, relative expression; OS, osteosarcoma.

Figure 4A ($P < 0.001$). Furthermore, an RNA pull-down assay was conducted and the results revealed that enrichment was obtained in the mutant miR-361-3p group relative to the bio-NC group (Figure 4B, $P < 0.001$). These data proved that LRIG1 was the downstream molecule of miR-361-3p. Then, qRT-PCR and WB revealed that the transcriptional

and translational level of LRIG1 was considerably lowered by elevated expression of miR-361-3p, and was increased by the reduced expression of miR-361-3p (Figure 4C, 4D, $P < 0.001$). Also, the transcriptional and translational level of LRIG1 was considerably lowered in OS tissues relative to neighboring normal tissues (Figure 4E, 4F). Pearson analysis

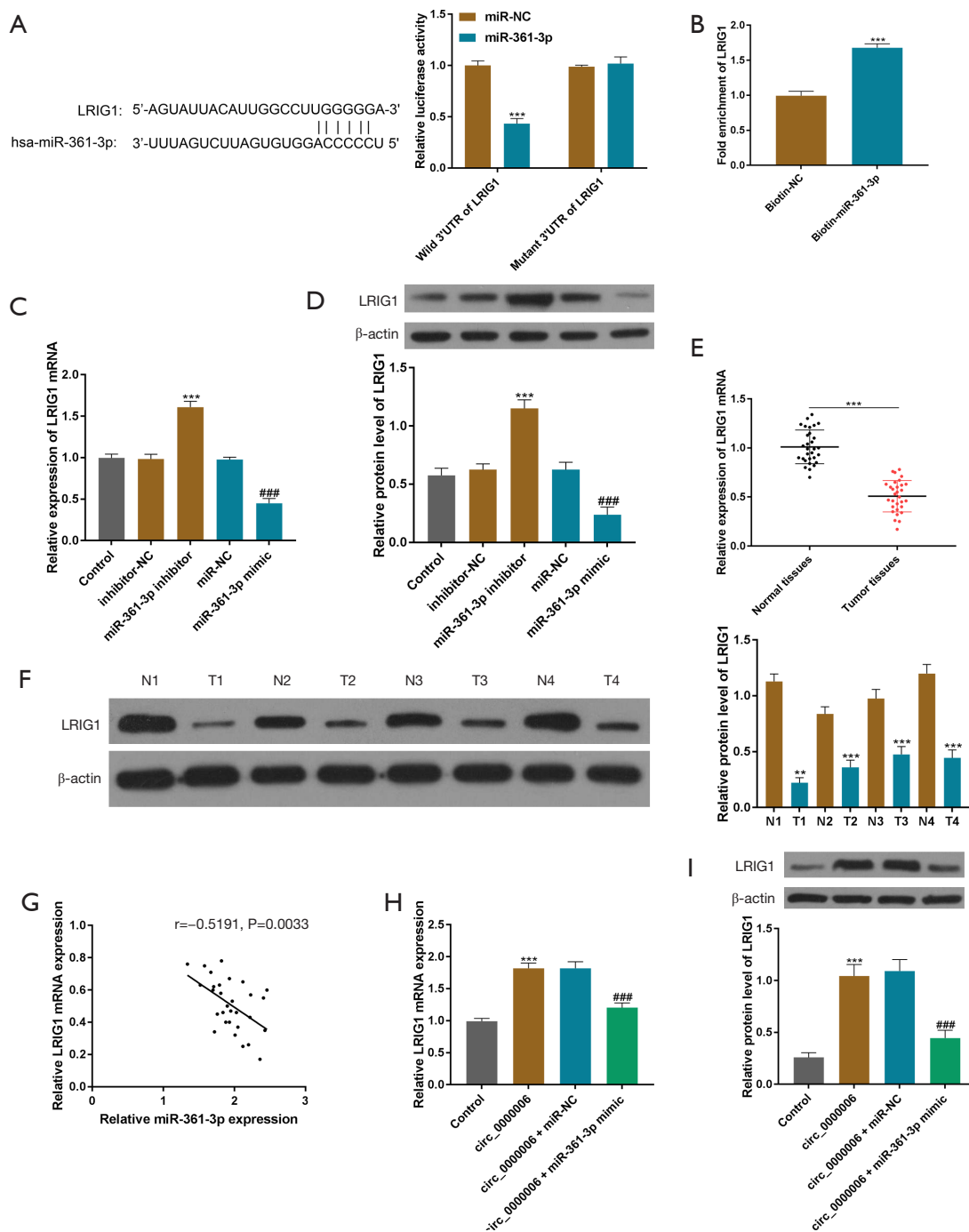


Figure 4 MiR-361-3p targeted LRIG1 and regulated its expression. (A) The interaction between LRIG1-3'-UTR and miR-361-3p was assessed by the DLR assay. (B) The RIP assay was used for measuring the enrichment of LRIG1 and miR-361-3p in HOS cells. (C,D) The RE of LRIG1 was determined by qRT-PCR/WB assays in HOS cells (transfected with miR-361-3p-mimics or miR-361-3p-inhibitor). (E,F) The expression of LRIG1 in OS and neighboring normal tissues was determined by qRT-PCR/WB assays. (G) Pearson analysis was performed to evaluate the relationship between the expression of LRIG1 and miR-361-3p. (H,I) The RE of LRIG1 was determined by qRT-PCR/WB assays in HOS cells transfected with hsa_circ_0000006 or hsa_circ_0000006 + miR-361-3p mimics. ** $P < 0.01$, *** $P < 0.05$, 0.001, ### $P < 0.001$. NC, normal control; LRIG1, immunoglobulin-like domains protein 1; 3'-UTR, 3'-untranslated region; DLR, dual-luciferase reporter; HOS, human osteosarcoma; RE, relative expression; OS, osteosarcoma; WB, western blotting.

suggested that miR-361-3p expression was negatively associated with LRIG1 expression, as depicted in *Figure 4G*. To further evaluate the association between hsa_circ_0000006 and LRIG1 in OS, qRT-PCR and WB were carried out to examine LRIG1 expression. The obtained data suggested that the transcriptional and translational level was elevated by hsa_circ_0000006 and that it recovered following transfection with an miR-361-3p mimic in HOS cells (*Figure 4H,4I*). Overall, miR-361-3p directly targeted LRIG1 and inversely regulated LRIG1 expression.

hsa_circ_0000006 promotes the proliferative, migratory, and invasive potential of OS cells through the hsa_circ_0000006/miR-361-3p/LRIG1 axis

To verify whether hsa_circ_0000006 played a tumor-promotive role through the hsa_circ_0000006/miR-361-3p/LRIG1 axis, miR-361-3p mimics or si-LRIG1 were used to construct the rescue experiments. The data obtained from the qRT-PCR and WB assays revealed that over-expression of hsa_circ_0000006 elevated the transcriptional and translational level of LRIG1 in HOS cells, while the effects caused by overexpressing hsa_circ_0000006 were reversed by miR-361-3p mimics or si-LRIG1 (*Figure 5A,5B*, $P < 0.001$).

Furthermore, we investigated whether miR-361-3p mimics or si-LRIG1 might reverse the role of hsa_circ_0000006 in OS cells. The expression of hsa_circ_0000006 was elevated which suppressed the proliferation, invasion, and migration of HOS cells, as determined by the CCK-8, colony formation, and transwell assays. The results were reversed by the miR-361-3p mimic or si-LRIG1 (*Figure 5C-5F*).

Beyond that, WB showed that the protein levels of N-cadherin, Vimentin, Slug, Snail, and Twist (mesenchymal markers) were all reduced by hsa_circ_0000006 overexpression, while their levels were all restored by co-transfection with hsa_circ_0000006 overexpression and miR-361-3p mimic or si-LRIG1. In contrast, the trend of E-cadherin expression followed the opposite pattern observed in N-cadherin, Vimentin, Slug, Snail, and Twist (*Figure 5G*). According to the results discussed earlier, we postulated that hsa_circ_0000006 worked as a ceRNA for miR-361-3p to control the expression of LRIG1.

Discussion

In children and adolescents, OS is a very invasive tumor with a high mortality rate (26). In OS patients, poor health

outcomes are major complications in clinical practice. Hence, it is necessary to identify new molecular targets and evaluate their possible mechanisms in OS. CircRNAs are non-coding RNAs that were discovered more than 40 years ago (27). Studies have revealed that circRNAs contribute significantly to the development and prognosis of many carcinomas (19,28). Up to now, few OS-linked circRNAs have been well known (13). In the present study, we determined the contribution of the hsa_circ_0000006/miR-361-3p/LRIG1 pathway in OS progression.

In this study, we showed that the expression of hsa_circ_0000006 was significantly reduced in OS tissues by microarray analysis of GSE140256. Then, we found that hsa_circ_0000006 served as an oncogene to promote the proliferation, metastasis, invasion, and EMT of OS cells based on the *in vitro* studies. Through bioinformatics comprehensive analysis, we found that hsa_circ_0000006 may be the target molecule of miR-361-3p. And the role of miR-361-3p has been revealed in many studies. For instance, Xu *et al.* revealed that miR-361-3p attenuated the development and migration of cervical cancer (29). Yang *et al.* confirmed that miR-361-3p attenuated the proliferative, migratory, and invasive potential of retinoblastoma cells as well as autophagy, and enhanced apoptosis in retinoblastoma underlined cells (30). Then, in our study, we found that the expression of miR-361-3p was significantly increased in OS cells and tissues, as predicted by bioinformatics and confirmed by DLR and RNA pull-down assays, and was negatively associated with hsa_circ_0000006 expression. Furthermore, hsa_circ_0000006 knockdown stimulated the expression of miR-361-3p.

To comprehensively evaluate the mechanism of miR-361-3p in OS, the prediction of its target genes was carried out by bioinformatics analyses. LRIG1 was validated to be a target of miR-361-3p by the DLR and RNA pull-down underlined assays. The protein/mRNA levels of LRIG1 and miR-361-3p were assessed in OS cells and the data revealed that the miR-361-3p inhibitor resulted in increased LRIG1 expression, which suggested that the expression of LRIG1 is negatively regulated by miR-361-3p. Also, we found that LRIG1 was significantly down-regulated in OS tissues, and showed a negative correlation with miR-361-3p expression. Moreover, the protein/mRNA expression of LRIG1 was enhanced by overexpression of hsa_circ_0000006 in OS cells, precisely by reversing the miR-361-3p-mimic.

To verify whether hsa_circ_0000006 served its tumor-promotive role through the hsa_circ_0000006/miR-361-3p/LRIG1 axis, the rescue experiments were designed and

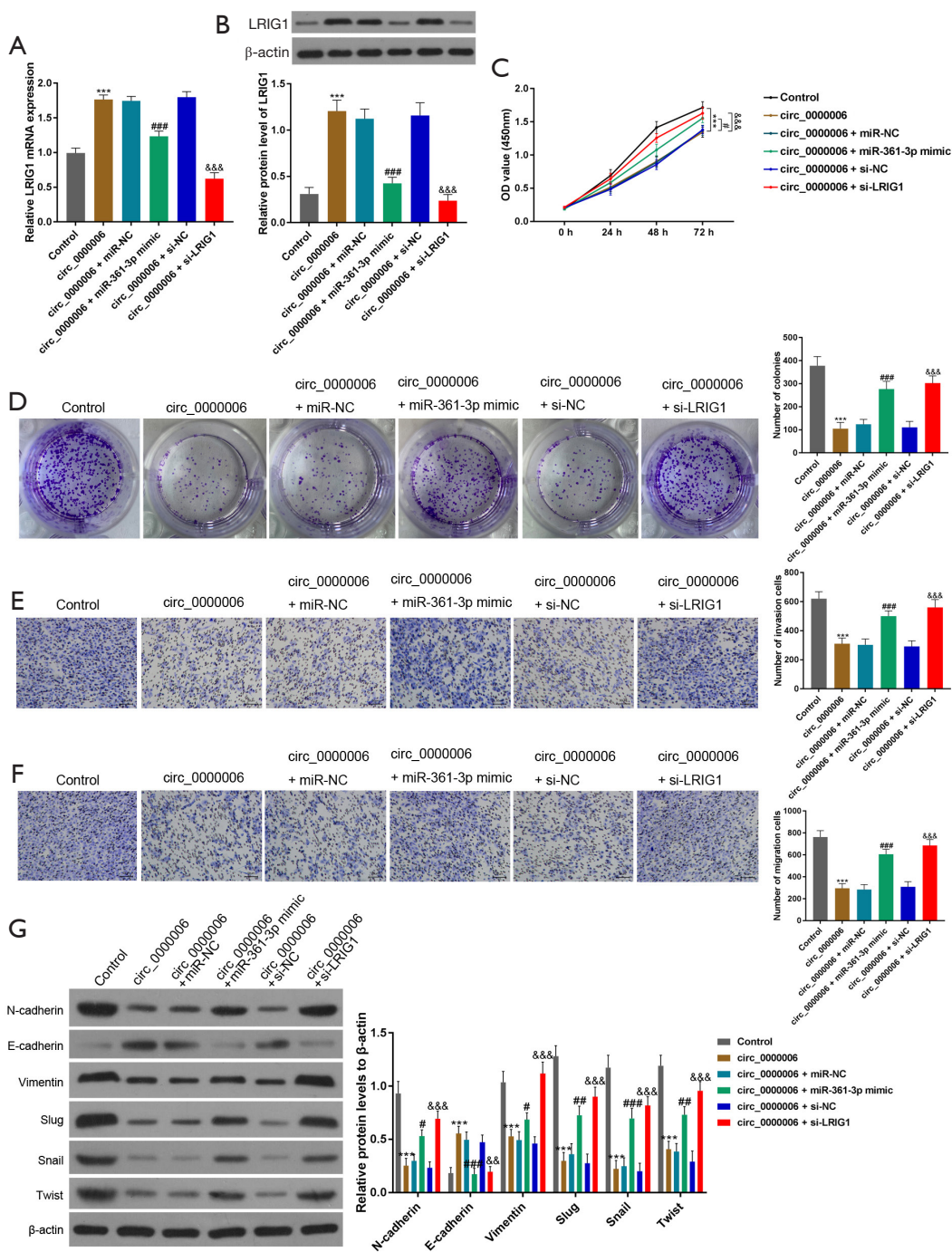


Figure 5 hsa_circ_0000006 enhanced the proliferative, migratory, and invasive potential of OS cells via the hsa_circ_0000006/miR-361-3p/LRIG1 axis. (A,B) Relative transcriptional and translational levels of LRIG1 in HOS cells transfected with the confirmed inhibitor, mimics, NC, si-circ, or oe-circ as determined by qRT-PCR and WB, respectively. (C,D) CCK-8 and colony formation assays were used to estimate the proliferative potential of HOS cells transfected with the indicated inhibitor, mimics, NC, si-circ, or oe-circ (dyed with crystal violet, >50 cells). (E,F) Cell invasive and migratory abilities were evaluated by transwell assays in HOS cells transfected with the indicated mimics, inhibitor, NC, si-circ, or oe-circ (dyed with crystal violet, 50 μ m). (G) The expression of the epithelial marker E-cadherin was detected in HOS cells by WB. The obtained data were expressed as mean \pm SD. *** P <0.001; # P <0.05; ## P <0.01; ### P <0.001; &&& P <0.01; &&& P <0.001. LRIG1, immunoglobulin-like domains protein 1; NC, normal control; OS, osteosarcoma; HOS, human osteosarcoma; WB, western blotting; CCK-8, Cell Counting Kit-8.

carried out using miR-361-3p mimics or si-LRIG1. The data obtained from qRT-PCR and WB suggested that elevated expression of hsa_circ_0000006 increased the transcriptional and translational level of LRIG1 in HOS cells. At the same time, miR-361-3p mimics or si-LRIG1 reversed the effects caused by elevated expression of hsa_circ_0000006.

Furthermore, we explored whether miR-361-3p mimics or si-LRIG1 could reverse the role of hsa_circ_0000006 in OS cells. The data obtained from the CCK-8, colony formation, and transwell assays revealed that elevated expression of hsa_circ_0000006 attenuated the proliferative, invasive, and migratory potential of HOS cells, while the results were reversed by the miR-361-3p mimic or si-LRIG1.

Subsequent rescue experiments confirmed that the oncogenic role of LRIG1 was reversed by hsa_circ_0000006. Additionally, it was revealed that increased expression of hsa_circ_0000006 attenuated the expression of LRIG1 in OS cells. However, the function of LRIG1 was weakened by adding the miR-361-3p mimic or si-LRIG1. Therefore, our results demonstrated that hsa_circ_0000006 acted as a ceRNA by harboring miR-361-3p to neutralize its carcinogenic impact on the target gene LRIG1 in OS development. These results suggest that hsa_circ_0000006 may be a novel candidate biomarker for human OS.

Conclusions

To conclude, our work demonstrated that the expression of hsa_circ_0000006 was considerably lowered in OS tissues and cells. Furthermore, hsa_circ_0000006 as a novel oncogene in OS could promote the proliferative, migratory, and invasive potential of cells, as well as EMT, through the miR-361-3p/LRIG1 axis. The underlined mechanisms may aid in exploring new therapeutic strategies for OS.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://dx.doi.org/10.21037/atm-21-3076>

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. In the present study, all the experimental procedures were carried out according to the Declaration of Helsinki (as revised in 2013) and approval was obtained from the Institutional Review Board of General Hospital of Chinese People's Liberation Army (No. C2020-011-01). Before the beginning of the study, each patient signed written informed consent.

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