

# Down-regulating lncRNA LINC00485 inhibits the development of osteosarcoma by regulating miR-361-5p/Twist1 axis

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## Primary research

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# Abstract

**Background:** Mounting researches have established that long-chain non-coding RNA (lncRNA) and microRNA (miRNA) occupy an essential position in osteosarcoma development. The present study attempted to explicate the functional role and mechanism of LINC00485 and miR-361-5p in regulating the osteosarcoma progression.

**Methods:** RT-PCR was employed to measure LINC00485 expression in osteosarcoma tissues and adjacent healthy tissues. Moreover, the correlation between LINC00485 expression and the clinicopathological indexes was analyzed. Further, the LINC00485 low-expressed cell model was constructed, and the CCK8 assay was employed to measure cell proliferation, followed by the flow cytometry in apoptosis testing. Besides, Transwell assay was utilized to detect cell migration and invasion ability, and Western blot examined the alternations of epithelial mesenchymal transition (EMT) marker molecules (E-cadherin and Vimentin). Furthermore, bioinformatics analysis was taken advantage of predicting the downstream molecular targets of LINC00485. RT-PCR and Western blot were carried out respectively to estimate the expression variations of miR-361-5p and Twist1. Dual luciferase activity assay and RNA co-precipitation (RIP) assay were conducted to verify the targeting relationship between LINC00485 and miR-361-5p, miR-361-5p and Twist1.

**Results:** In comparison with normal adjacent tissues, the LINC00485 expression in cancer tissues was profoundly up-regulated. LINC00485 low expression greatly attenuated proliferation, migration, invasion, and EMT in osteosarcoma cells, while enhancing apoptosis. Bioinformatics analysis claimed that miR-361-5p was a sponge miRNA for LINC00485, targeting the Twist1 expression. Further gain- and loss-experiments declared that miR-361-5p limited the osteosarcoma development by inhibiting osteosarcoma cell proliferation, metastasis and promoting apoptosis.

**Conclusions:** LINC00485 acted as an oncogene in the osteosarcoma tumorigenesis by modulating the miR-361-5p/Twist1 axis.

## Introduction

Osteosarcoma, the most prevailing cancer that starts in the bones, mainly affects children and young adults under the age of 25 [1]. High-grade osteosarcomas are the most common form and have the greatest tendency to metastasize, most often the lungs. Even with clinical improvements, patients with metastatic or recurrent tumors do not respond well to conventional therapies [2]. Moreover, although chemotherapy, combined with surgical tumor excision, partly ameliorates the clinical treatment of osteosarcoma, the poor response to chemotherapy remains a major hurdle in current antitumor research [3]. In this context, it is imperative to further make clear the molecular mechanism behind osteosarcoma, so as to identify more comprehensive and targeted therapies for osteosarcoma treatment.

Non-coding RNAs (ncRNAs) are transcribed from the mammalian genome but unable to encode proteins. A majority of the human genome constitutes non-coding elements. These non-coding RNAs may affect

gene expression and tumor progression, significantly contributing to cellular processes regulation [4]. Long non-coding RNA (lncRNA) is a kind of non-coding RNA that can be transcribed and processed like mRNAs, but does not encode proteins [5]. LncRNAs are underscored in a good number of biological processes, like cell cycle progression, apoptosis, development, stem cell pluripotency and canceration, etc. [7-10]. Similarly, lncRNAs also regulate the osteosarcoma progression from different dimensions. For example, FOXC2-AS1 level is elevated in osteosarcoma cell lines and tissues, promoting cell resistance to doxorubicin *in vivo* and *in vivo*, a phenomenon closely associated with the poor prognosis [11]; LncRNA SOX2-OT, functioning as a new biomarker or independent prognostic indicator for osteosarcoma, can regulate the proliferation and mobility of cells through regulating SOX2 [12]. Previous studies have substantiated LINC00485' key role in human lung adenocarcinoma carcinogenesis [13], but overlooked the molecular mechanism and biological functions of LINC00485 in osteosarcoma.

MiRNAs are non-coding RNA molecules measuring 18-22 nucleotides long, which function in post-transcriptional regulation through base-pairing with complementary sequences to promote or inhibit mRNA degradation and translation. MiRNAs are highly conserved throughout evolution [14, 15]. Many papers have reported that miRNAs abnormally expressed are closely entwined with tumorigenesis and progression, and are highly involved in cell development, differentiation, and cycle regulation [16, 17]. MiR-361-5p, for example, has been confirmed its effectiveness in suppressing cancers, such as gastric cancer [18], breast cancer [19], and lung cancer [20]. Nevertheless, in osteosarcoma, the upstream mechanism regulating miR-361-5p has remained uncharted.

Twist family bHLH transcription factor 1 (Twist1) is a basic transcription factor encoded by the gene located at 7p21.1. During skull development, Twist1 forms both homodimers and heterodimers binding to DNA E box sequences and regulate the transcription of genes implicated in cranial suture closure [21]. Twist1 also regulates neural tube closure, limb development, together with brown fat metabolism [22 -24]. Moreover, Twist1 has been proved its essential role in carcinogenesis malignant tumors, including sarcoma and hematological malignancies, tumor stem cells, angiogenesis, invasion, metastasis and chemotherapy resistance [25-27]. In addition, Twist1 has been recognized as one of the main regulators of the epithelial-mesenchymal transition (EMT) process. For example, Twist1 increases vimentin expression via Cul2 circular RNA, thereby promoting EMT of hepatocellular carcinoma (HCC) [28]. However, Twist1's regulation of the EMT pathway in osteosarcoma and its in-depth mechanism still awaits further validation.

In this research, LINC00485 was reported to be up-regulated in osteosarcoma samples. LINC00485 over-expression substantially enhanced osteosarcoma cell proliferation, migration, and invasion and promoted the EMT process. Further experiments clarified that LINC00485 was a driver as the disease progresses by regulating the miR-361-5p/Twist1 axis. Taken together, this research illustrated that the LINC00485/miR-361-5p/Twist signaling pathway was a new mechanism associated with the osteosarcoma progression, and was expected to provide new molecular stratification options with respect to osteosarcoma therapy.

# Materials And Methods

## Clinical samples

The study has garnered approval from the Research Ethics Committee of the People's Hospital of Longhua, Affiliated Hospital of Southern Medical University and strictly abides by the Declaration of Helsinki. Between January 2014 and June 2015, 40 osteosarcoma samples and the corresponding healthy tissues in total were harvested from People's Hospital of Longhua, Affiliated Hospital of Southern Medical University. Before the condition was diagnosed, the patients included in the study had not received anti-tumor treatment. All eligible patients had provided an informed consent in written forms.

## Cell Culture and cell transfection

We purchased human osteoblast cell lines (hFOB 1.19) and human osteosarcoma cell lines (Saos-2, MG-63, U-2 OS, SOSP-9607 and MNNG/HOS) from the Cell Culture Bank of Type Culture Collection Committee in the Chinese Academy of Sciences (Shanghai, China). In RPMI1640 medium (Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, MA, USA) and 1% penicillin/streptomycin (Invitrogen, CA, USA), the cells were cultured. They were placed in an incubator at designated conditions (37 °C, 5% CO<sub>2</sub>). Cells in the logarithmic growth phase subjected to trypsinization and subculture with 0.25% trypsin (Thermo Fisher HyClone, Utah, USA). Short hairpin RNA (sh-LINC00485), miR-361-5p mimics, and miR-361-5p inhibitors targeting LINC00485 were constructed by GenePharma (Shanghai, China). Moreover, the Lipofectamine® 3000 (Invitrogen; ThermoFisher Scientific, Inc., USA) was employed to transfect sh-LINC00485, miR-361-5p mimics, and miR-361-5p inhibitor into Saos-2 or MG-63 cells following the supplier's instructions. For further experiments, the cells were harvested when stable transfection was observed.

## Real-time polymerase chain reaction (RT-PCR)

To extract the total cellular, TRIzol (Invitrogen, Carlsbad, CA, USA) was applied. Then, the total RNA extracted was treated with deoxyribonuclease I for the genomic DNA removal. The reverse transcription reaction was operated according to guidance of the reverse transcription kit (Takara, Dalian, China). The reaction conditions were: 70 °C (10 min), on ice (5 min), 42 °C (60 min), 95 °C (5 min), and 0 °C (5 min). The fluorescence quantitative PCR reaction system is 25 µL, containing 500 ng cDNA template, 250 nmol/L upstream and downstream primers, and 12.5 µL of 2×SYBR Green (Takara, Dalian, China) PCR Master mixture.

The primer sequences of each molecule are as follows:

LINC00485, (F) 5'-CTCCAAGCAGGGGCTACAAA-3',

(R) 5'-CCAGGAGCTCAGAAAGCCAA-3';

miR-361-5p, (F) 5'-AGCCAGCGTTATCAGAATCTCCA-3',

(R) 5'-CAGTGCAGGGTCCG -3 ';

Twist1, (F) 5'-GGAGTCCGCAGTCTTACGAG-3',

(R) 5'-CCAGCTTGAGGGTCTGAATC-3 ';

GAPDH, (F) 5'- CCCACTCCTCCACCTTTGAC-3',

(R) 5'- ATGAGGTCCACCACCCTGTT-3 ';

U6, (F) 5'- ATTGGAACGATACAGAGAAGATT-3',

(R) 5'- GGAACGCTTCACGAATTTG-3 '.

The reaction tube was put into the MX3000P Real time PCR reactor and the conditions were: 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 40 s through 45 cycles, a process monitored by fluorescence signal. Here, GAPDH served as the endogenous control for LINC00485 and Twist1, U6 for miR-361-5p. The  $2^{-\Delta\Delta Ct}$  value represents the gene relative expression, and the ct value represents the PCR cycle number that emitted via a fluorescence signal that reached the set threshold during amplification.

### **CCK-8 assay**

CCK-8 assay was operated in measuring the cell viability. The Saos-2 and MG-63 cells were inoculated in 96-well plates ( $1 \times 10^3$  cells/well), followed by a 24 h incubation, during which stable cell growth was observed. Then, the incubation continued in set periods: 12 hours, 24 hours, 48 hours, and 72 hours. Following the supplier's instructions, CCK8 reagent (Beyotime Biotechnology, Shanghai, China) was added to each well (10  $\mu$ L/well). The spectrophotometer was used to measure the OD450 value (Bio-Rad, CA, USA) after one-hour cell incubation (37 °C).

### **Flow cytometry**

When the stably transfected cells were produced, osteosarcoma-derived cell lines (Saos-2, and MG-63) were harvested. Washed three times by PBS, 4 °C PBS was used to suspend the cells, adjusting the cell density to  $5 \times 10^5$  / mL. According to the instructions of the Annexin V-FITC apoptosis detection kit (Sigma-Aldrich; St. Louis, MO, USA), the apoptosis level was evaluated. Then, the cell suspension (190  $\mu$ L) prepared in step with the addition of Annexin V: FITC (5  $\mu$ L). When mixed fully, the mixture was added with 5  $\mu$ L of propidium iodide (PI) solution. Next, the sample was incubated shielded from light (20 minutes, room temperature), and then the flow cytometry analyzer was employed (BD Biosciences, Franklin Lakes, NJ, USA) to examine the apoptosis level.

### **Western Blot**

Washed 3 times with cold PBS, the cells collected were then added with RIPA lysis solution (100-200  $\mu$ L) (Beyotime Biotcchnology, Shanghai, China) to lyse the cells in ice water. The Bradford protein assay was

performed to detect the concentration of total protein. Proteins at a concentration (equal amounts) were prepared from each group and were placed in the separation gels (10%) for the SDS-PAGE electrophoresis. Next, the proteins on the gels was transferred to PVDF membranes (Millipore, Bedford, MA, USA). Further, the membranes were blocked at 4 °C for 1 h, intervened with the following primary antibodies: E-cadherin Rabbit Polyclonal (Proteintech, Cat.No. 20874-1-AP, 1: 1000), Vimentin Rabbit Polyclonal (Proteintech, Cat.No. 10366-1-AP ), and TWIST1-specific Rabbit Polyclonal (Proteintech, Cat.No. 25465-1-AP), overnight at 4 °C. Following that, HRP Conjugated Goat Anti-Rabbit IgG (H + L) Secondary Antibody (BOSTER, 1: 2000, BA1055) for hybridization (room temperature, 1 h), succeeded by TBST washing (3 times, 5 min each). Then, equal volumes of ECL chemiluminescent substrates A and B were mixed up and added evenly on the PVDF membranes. Five minutes were granted for color development. When the X-ray film had been exposed for 30 seconds to 5 minutes, the exposed film was processed in the developing solution and the fixing solution in order. Next, the film was scanned or photographed, and the gel image processing system was used to measure the gray level of the protein bands. The ratio of the gray value of the target protein bands to that of  $\beta$ -actin, the endogenous control was employed to correct the total amount of protein added. The corrected protein expression of the control group was taken as 100%. The percentage of protein content in the control group was calculated. On this basis, the protein expression level of each group was compared and analyzed. All experiments were repeated 3 times.

### **Luciferase Reporter Assay**

It was carried out with the Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA). Target fragments of wild-type LINC00485 (LINC00485-WT), wild-type Twist1 (Twist1-WT) and mutant LINC00485 (LINC00485-MT), mutant Twist1 (Twist1-MT) were amplified and integrated into pmirGLO reporter vector (Promega , Madison, WI, USA), where LINC00485-WT and Twist1-WT contain sites that bind to miR-361-5p, and LINC00485-MT and Twist1-MT do not contain sites that bind to miR-361-5p. Saos-2 cells were co-transfected with miR-361-3p mimics or negative control, respectively. In line with the manufacturer's instructions, the luciferase activity was measured forty-eight hours after transfection. All experimental procedures were done in triplicate.

### **RNA immunoprecipitation assay**

Using a Magna RIPTM RNA kit (Millipore, USA), RIP analysis was carried out. Cultured chondrocytes were suspended in RIP lysis buffer and were further incubated overnight in RIP buffer that contained human anti-Ago2 antibody beads (Millipore) (Input and normal IgG served as controls). Afterward, RNA extraction was achieved using the TRIzol reagent and the relative enrichment of [LINC00485](#)/Twist1 and miR-361-5p was tested via the RT-PCR analysis.

### **Transewell assay**

After the Saos-2 and MG-63 cells were detached from each other with 0.25% trypsin, the centrifugation followed. Then the cells were resuspended in a single well of a 24-well culture plate. Matrigel Chambers

(8  $\mu\text{m}$  poresize; Corning, Beijing, China) were put into use in the invasion assay alone, not applied to the migration assay. Transfected cells ( $5 \times 10^4$ ) were inoculated into the upper chamber, and then the Matrigel Matrigel was added. Next, the lower chamber was placed with culture medium supplemented with 10% FBS and filled with 400  $\mu\text{L}$  of RPMI-1640. After incubation (24 h, 37  $^{\circ}\text{C}$ ), the cells failed to migrate in the upper chamber were discarded. The cells were secured for 10 min using 4% paraformaldehyde and stained with 0.5% crystal violet. Following the running water washing, the number was counted under an inverted microscope. All experimental procedures were repeated in triplicate.

### **Xenograft assay in nude mice**

Xenograft assay was conducted to test the growth of Saos-2 and MG-63 cells with low level of LINC00485 on BALB/c nude mice (aged 4–6 weeks) that were provided by the Experimental animal center of Southern Medical University. Saos-2 and MG-63 cells transfected with the sh-LINC00485 or sh-NC were subcutaneously injected into the backs of nude mice under sterile conditions. The tumor growth was observed and photographed every 7 d for a total of 35 d. The tumor growth curve was drawn. The tumor volume was calculated as  $(a \times b^2)/2$ , in which “a” refers to the longest diameter while “b” refers to the shortest. The mice were euthanized on the 35<sup>th</sup> d via intraperitoneal injection of 150 mg/kg pentobarbital to collect and weigh the tumors. The experiment was permitted by the Animal Research Committee of the People's Hospital of Longhua, Affiliated Hospital of Southern Medical University and performed in accordance with the guidelines of the National Animal Care and Ethics Institution.

### **Data analysis**

All statistical calculations were operated via the SPSS statistical software (version 22.0, Chicago, IL, US). The measurements were represented as the mean  $\pm$  SD, and student's *t* or one-way ANOVA was used for statistical analysis; the count data was represented as frequencies. The Chi-square test was valid for analyzing the relationship between LINC00485 and the of patients' clinicopathological indicators. The relevance between the LINC00485 expression and the osteosarcoma prognosis was determined by the KM plotter assay.  $P < 0.05$  is statistically meaningful.

## **Results**

### **LINC00485 was up-regulated in osteosarcoma tissues and cell lines**

RT-PCR was conducted here to quantify the LINC00485 level, and higher expression of LINC00485 was reported in osteosarcoma samples (Figure 1A). Additionally, compared to the osteoblast cell line (hFOB 1.19), the LINC00485 level in osteosarcoma cells (Saos-2, MG-63, U-2OS, SOSP-9607 and MNNG/HOS) was also increased (Figure 1B). These results demonstrated that LINC00485 may exert a carcinogenic effect in osteosarcoma.

### **LINC00485 expression was related to osteosarcoma patients' worse survival**

Analyzing the cohort of osteosarcoma patients who provided with the prognosis information, **LINC00485** expression was found related to the overall survival of osteosarcoma patients. Meanwhile, patients with low **LINC00485** expression had better survival than those with high **LINC00485** expression ( $P < 0.001$ , Figure 1C). Besides, the correlations of **LINC00485** level and pathological indexes in osteosarcoma patients were also analyzed. The results showed that osteosarcoma patients with higher level of **LINC00485** had higher Enneking stage ( $P=0.025$ ), larger tumor size ( $P=0.0267$ ) and earlier distant metastasis ( $P=0.038$ ) (Table 1). Therefore, these results manifested that **LINC00485** was an unfavorable prognostic factor for patients with osteosarcoma.

**Table 1. The correlations of **LINC00485** level and pathological indexes in osteosarcoma patients**

Parameter		<b>LINC00485</b> level		p value
		High (n=20)	Low (n=20)	
Age	<18 years	13	10	0.3773
	≥18 years	7	10	
Gender	Male	12	11	0.749
	Female	8	9	
Enneking stage	I-IIA	5	12	0.025
	IIB-III	15	8	
Tumor size	≤8 cm	6	13	0.027
	>8 cm or discontinuous	14	7	
Distant metastashes	Yes	9	3	0.038
	No	11	17	

### **LINC00485 regulated osteosarcoma cell proliferation, apoptosis, migration and invasion**

Studying the biological function of **LINC00485** in gliomas, a knockdown **LINC00485** cell model in Saos-2 and MG-63 cells (Figure 2A) was constructed. Using CCK-8 assay to measure cell proliferation, we claimed that low **LINC00485** level can inhibit the cell proliferation (Figure 2 B-C). Flow cytometry results suggested that knocking down **LINC00485** remarkably promoted the level of apoptosis of Saos-2 and MG-63 cells (Figure 2D). Subsequently, the Transwell assay was operated to determine the abilities of cells in terms of migration and invasion, confirming that knocking down **LINC00485** significantly inhibited the migration and invasion of Saos-2 and MG-63 cells (Figure 2 E, F). In addition, Western blot was also utilized to estimate the EMT of osteosarcoma cells. It was found that knocking down **LINC00485** promoted the level of the epithelial cell markers E-cadherin, while inhibiting that of the interstitial cell marker Vimentin (Figure 2 G-H). Moreover, the *in vivo* experiments revealed that downregulation of **LINC00485** prominently inhibited the growth of Saos-2 and MG-63 cells (Figure 3A-E). Taken together, those results clarified that **LINC00485** was an oncogene of osteosarcoma progression.

### **LINC00485 can target miR-361-5p**

To further ascertain the downstream molecular mechanism of LINC00485, IncBase v.2 ([http://carolina.imis.athena-innovation.gr/diana\\_tools/web/index.php?r=Incbasev2%2Findex](http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=Incbasev2%2Findex)), a bioinformatics database, was put into use to retrieve potential target genes of LINC00485, showing that LINC00485 contained potential binding sites for miR-361-5p (Figure 4A). Next, the RIP assay was employed to verify the potential endogenous interaction between LINC00485 and miR-361-5p, elucidating that after miR-361-5p mimics were transfected, the amount of LINC00485 was enriched by the anti-Ago2 antibody (Figure 4B). In addition, luciferase reporter assay illustrated that miR-361-5p mimics greatly inhibited the luciferase activity of LINC00485-WT, but had no effect on LINC00485-MUT (Figure 4C). Next, the miR-361-5p level in transfected sh-LINC00485 cells was detected by RT-PCR, and the results reported that in comparison with the controls, the miR-361-5p expression in the LINC00485 knockdown group was remarkably increased (Figure 4D). Moreover, RT-PCR employed to measure the miR-361-5p level in osteosarcoma tissues and normal adjacent tissues showed that the level of miR-361-5p in osteosarcoma tissues was significantly reduced (Figure 4E). Pearson analysis demonstrated that LINC00485 and miR-361-5p expression was negatively correlated in osteosarcoma tissues (Figure 4F). To sum up, the data presented here supported that LINC00485 can bind to miR-361-5p in osteosarcoma cells directly, while inhibiting its expression.

### **MiR-361-5p suppressed the osteosarcoma progression**

Here, the effect of miR-361-5p on osteosarcoma cells was explored. First, we constructed a miR-361-5p over-expression model using miR-361-5p mimics (Figure 5A). Functionally, the CCK-8 assay results illustrated that miR-361-5p markedly suppressed the Saos-2 and MG-63 cells (Figure 5 B-C) from proliferating but promoted apoptosis (Figure 5D). Transwell results suggested that over-expressed miR-361-5p dramatically promoted the migration and invasion capability of osteosarcoma cells (Figure 5 E-F). Further, we tested the expression of EMT-related proteins. In contrast to the control group, miR-361-5p over-expression notably suppressed the EMT progression of osteosarcoma (Figure 5 G-H). These findings substantiated that miR-361-5p inhibited osteosarcoma development.

### **MiR-361-5p can target Twist1**

On the basis of the mirPath (<http://snf-515788.vm.okeanos.grnet.gr/index.php?r=mirpath>), we analyzed the KEGG pathway related to miR-361-5p. The relationship between miR-361-5p and Adherens junction pathway is reported to be the most significant. (Figure 6A). In exploring the downstream targets of miR-361-5p, the bioinformatics databases, micorT, miRanda, Targetscan, and PicTar, were combined in use to find potential target genes of miR-361-5p. Building on the Wayne diagram analysis, we reported that miR-361-5p contained the potential binding sites of Twist1 (Figure 6 B-C). Moreover, RIP analysis was utilized to verify the potential endogenous interaction between miR-361-5p and Twist1, demonstrating that miR-361-5p and Twist1 were preferentially enriched in the anti-Ago2 group in contrast to the IgG control group (Figure 6D). Besides, luciferase reporter assay elaborated that miR-361-5p dramatically inhibited Twist1-WT's luciferase activity, but exerted no effect on Twist1-MT (Figure 6E). Next, after miR-361-5p mimics were transfected, the expression of Twist1 in osteosarcoma cells was measured via RT-PCR and Western

blot. These findings proved that the Twist1 level in the miR-361-5p mimic group was substantially lower than the control group (Figure 6 F-G). Further, RT-PCR was taken to estimate Twist1 expression level in osteosarcoma tissues, suggesting that the Twist1 expression in osteosarcoma tissues was profoundly up-regulated compared with normal tissues (Figure 6H). Pearson correlation analysis exhibited that Twist1 was negatively correlated with miR-361-5p in osteosarcoma tissues (Figure 6I). Taken together, these data demonstrated that miR-361-5p can directly bind to Twist1 in osteosarcoma cells, while inhibiting its expression.

### **Effect of LINC00485 / miR-361-5p axis on osteosarcoma cell proliferation, apoptosis, migration, invasion and EMT process**

To further investigate what effects LINC00485 and miR-361-5p exerted on proliferation, migration, EMT and invasion of osteosarcoma cells, we divided MG-63 cells into three groups: sh-NC, sh-LINC00485, and sh-LINC00485+miR-361-5p inhibitor. Subsequently, the expression of LINC00485, miR-361-5p and Twist1 were measured respectively. The results clarified that after knocking down LINC00485, the miR-361-5p level was elevated, while the LINC00485 and Twist1 expressions were down-regulated (Figure 7 A-C). Compared with the sh-LINC00485 group, further inhibiting miR-361-5p significantly promoted Twist1 expression (Figure 7 C). Following that, we examined the proliferation, apoptosis, migration, invasion, and EMT of MG-63 cells, respectively. The results elucidated that compared to the sh-LINC00485 group, miR-361-5p inhibition enhanced the proliferation of MG-63 cells (Figure 7D), attenuated apoptosis (Figure 7E), elevated cell migration, invasion, and EMT. (Figure 7 E-G). These results are a proof that LINC00485 inhibition can exert antitumor effects by up-regulating miR-361-5p.

## **Discussion**

Osteosarcoma is the most prevailing cancer that arises in bones. Through surgical resection and multi-drug chemotherapy, 70% of patients with high-grade osteosarcoma and localized limb tumors have sustained long-term survival. However, treatments limited to chemotherapy associated with resistance development do not reflect well on patients who defy surgical resection, carrying with primary migration, or diseases with high recurrence [29]. Accumulating researches have shown that lncRNA is an essential cell regulator in tumor progression, including osteosarcoma, which brings new ideas for the treatment of osteosarcoma [30-32]. This study first explored a new lncRNA, namely LINC00485. Our findings suggested that LINC00485 promoted osteosarcoma carcinogenesis and progression, and regulated osteosarcoma cell proliferation, migration, and invasion by targeting the miR-361-5p/Twist1 axis.

As the latest generation of gene sequencing technology develops and bioinformatics advances, more and more non-coding RNAs (ncRNAs) are considered to be related to various human tumor diseases [33]. For example, NCK1-AS1 participates in the occurrence and development of tumors by affecting the level of related proteins. In cervical cancer, NCK1-AS1 serves as a molecular sponge for miR-134 which targets matrix metalloproteinase (MMP) -2 and MMP-9, thereby promoting the proliferation, migration, and invasion of bone cervical cancer cells [34]. Previous studies have shown that LINC00485 is up-regulated

in lung adenocarcinoma (LAC) cells. Moreover, inhibiting LINC00485 attenuates the expression of various oncogenes, CHEK1, Bcl-2, VEGF and HIF-1 $\alpha$ , in LAC cells, and also enhances the sensitivity of LAC cells to cisplatin [13]. Nevertheless, LINC00485's role in osteosarcoma has yet been fully elucidated. Here, we found a significant upregulation of LINC00485 in osteosarcoma samples compared to normal controls. In addition, by analyzing the biological function of LINC00485 in osteosarcoma through loss-of-functions, we reported that LINC00485 knockdown effectively suppressed cell proliferation and migration in osteosarcoma, and enhanced cell apoptosis. All these results proved the carcinogenic effect that LINC00485 exerted on osteosarcoma.

In recent years, multiple lines of evidence have clarified that various miRNAs are implicated in regulating the osteosarcoma occurrence and development, and are abnormally expressed in cancer, functioning as oncogenes to exert effects [35]. For example, Circ\_0001658 propels the proliferation and metastasis of osteosarcoma cells in way of regulating the miR-382-5p / YB-1 axis [36]. Knocking down miR-9 in human osteosarcoma cell lines inhibits their metastatic potential [37]. Taking miR-361-5p as an example, it is known as a tumor-suppressor miRNA and suppresses glycolysis, proliferation, and invasion of breast cancer by targeting FGFR1 and MMP-1 [38]. Moreover, it restrains the migration and invasion of glioma by targeting SND1 [39]. Consistent with the reports mentioned above, our research clarified that the miR-361-5p level in osteosarcoma samples was notably down-regulated in comparison with adjacent healthy tissues and cells. Further, miR-361-5p over-expression suppressed the proliferation and metastasis of osteosarcoma cells, while knocking down miR-361-5p reversed the effects. The statistics confirmed that miR-361-5p suppressed osteosarcoma progression.

Additionally, lncRNA acts as a sponge for miRNA, reducing its regulatory effects on mRNA. For example, SNHG12 is up-regulated in colorectal cancer (CRC) and promotes CRC cell proliferation and invasion as a sponge for miR-16 [40]. Notably, lncRNA-miRNA interaction was essential in the progression of osteosarcoma. For example, the highly expressed lncRNA DLEU2 in osteosarcoma cells suppresses the osteosarcoma proliferation and metastasis by sponging miR-337-3p [41]. Furthermore, lncRNA MSC-AS1 was reported to be up-regulated in osteosarcoma tissues that was associated with the poor prognosis reported by osteosarcoma patients. At the same time, lncRNA MSC-AS1 inhibition reduces the malignant phenotype of osteosarcoma cells and increases their sensitivity to cisplatin by up-regulating miR-142 [42]. Thus, exploring the interaction of lncRNA-miRNA in the progression of osteosarcoma is of great significance. In light of the relevance between the expression and functions of LINC00485 and miR-361-5p in osteosarcoma, we speculated that LINC00485 may be a ceRNA in osteosarcoma. Using the bioinformatics database, we identified that miRNAs may interact with LINC00485, namely miR-361-5p. And further studies showed that the miR-361-5p expression increased profoundly after the LINC00485 knockdown. Moreover, the luciferase activity assay confirmed that LINC00485 directly bound to miR-361-5p. Therefore, we speculated that LINC00485, after up-regulated in osteosarcoma, further inhibits the miR-361-5p expression, a tumor suppressor molecule, thereby exerting a carcinogenic effect.

EMT refers to a physiological process where epithelial cells acquire the motile and invasive properties of mesenchymal cells by losing their cell polarity and cell-cell adhesion [43]. The process where epithelial-

like cells are converted into mesenchymal-like cells is characteristic of the weakened expression of epithelial marker E-cadherin and the increased expression of mesenchymal markers, Vimentin and N-cadherin [44]. As a tumor prone to spread from the initially affected bone to more sites, it is notable that osteosarcoma is also accompanied by significant EMT changes. Inhibiting the EMT of osteosarcoma cells can not only suppress the development of metastasis, but also decrease the resistance to osteosarcoma chemotherapy [45-46]. Twist1, belonging to the bHLH (basic helix loop helix) transcription factor family, has been confirmed by mounting studies that it can regulate the EMT of tumor cells. For example, it enhances tumor metastasis by inhibiting the E-cadherin transcription [47]. Interestingly, recent studies have described that non-coding RNA affects tumor EMT by regulating Twist1. In osteosarcoma, for instance, LncRNA AFAP1-AS1 propels tumor progression and EMT by regulating RhoC/ROCK1/p38MAPK / Twist1 Signaling Pathway [48]. MicroRNA-300, by reducing Twist1, suppresses cell viability, inhibits cell migration, and promotes osteosarcoma cell apoptosis [49]. In the present research, we explained that Twist1 was remarkably up-regulated in osteosarcoma samples. Moreover, based on bioinformatics analysis and expression regulation experiments, we determined that miR-361-5p can interfere in the 3'UTR end of Twist1 and negatively regulated its expression. This result led us to further verify whether LINC00485 can indirectly regulate Twist1 expression through miR-361-5p to exert its carcinogenic effect. These findings exhibited that LINC00485 knockdown reduces the level of mRNA and protein expression levels of Twist1. The experimental data revealed the biological functions of LINC00485 which partly regulated the Twist1 expression through miR-361-5p.

## Conclusion

In summary, this study demonstrated that LINC00485 was up-regulated in OS and promoted OS cell proliferation and invasion as a sponge for miR-361-5p. Therefore, the new LINC00485-miR-361-5p-Twist1 axis might be considered as a promising therapy in metastatic OS patients. In future research, more attention should be focused on the detailed understanding of this network at the animal level or in more cases outside the scope of this report.

## Declarations

### Ethics approval and consent to participate

Our study was approved by the Ethics Review Board of The People's Hospital of Longhua, Shenzhen, Southern Medical University.

### Consent for publication

Not applicable.

### Availability of data and materials

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

Conceived and designed the experiments: Hao Zhang, Yawei Hu, Xinle Luo;

Performed the experiments: Yawei Hu, Xinle Luo, Hao Zhang;

Statistical analysis: Jianhua Zhou, Shaochu Chen;

Investigation and data record: Ming Gong, Yue Deng;

Wrote the paper: Yawei Hu, Xinle Luo.

All authors read and approved the final manuscript.

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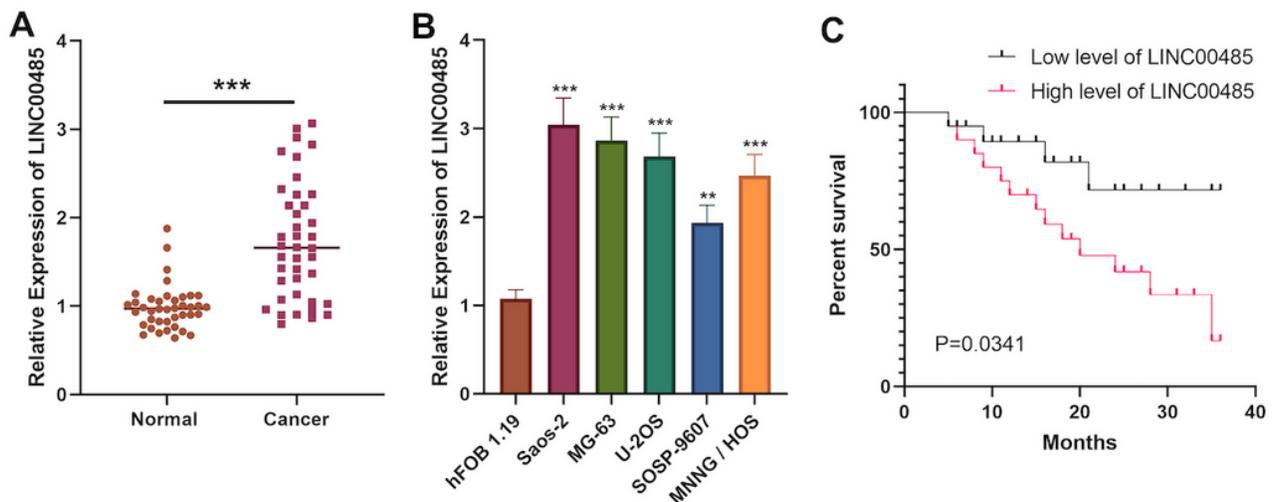
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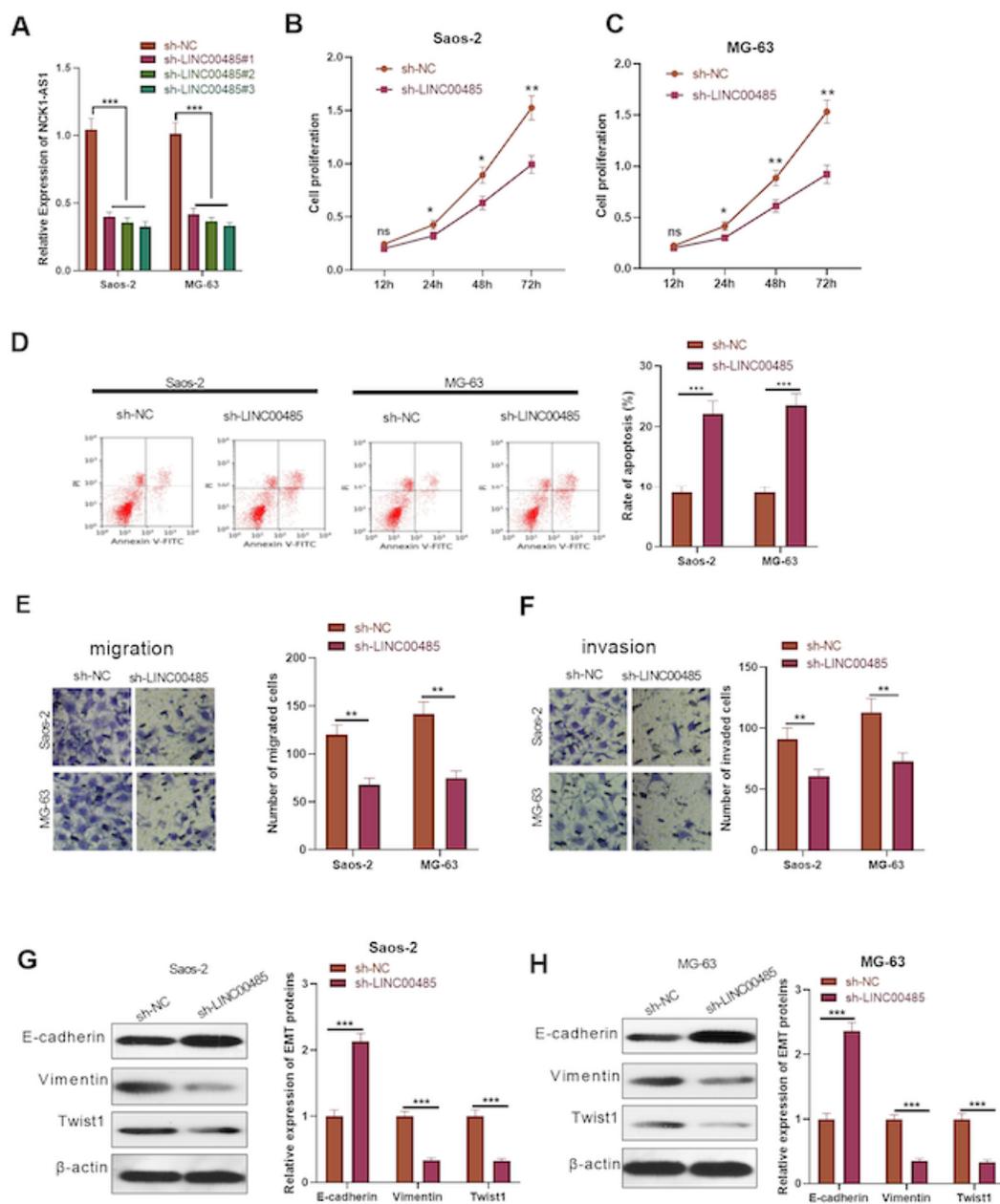
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## Figures



**Figure 1**

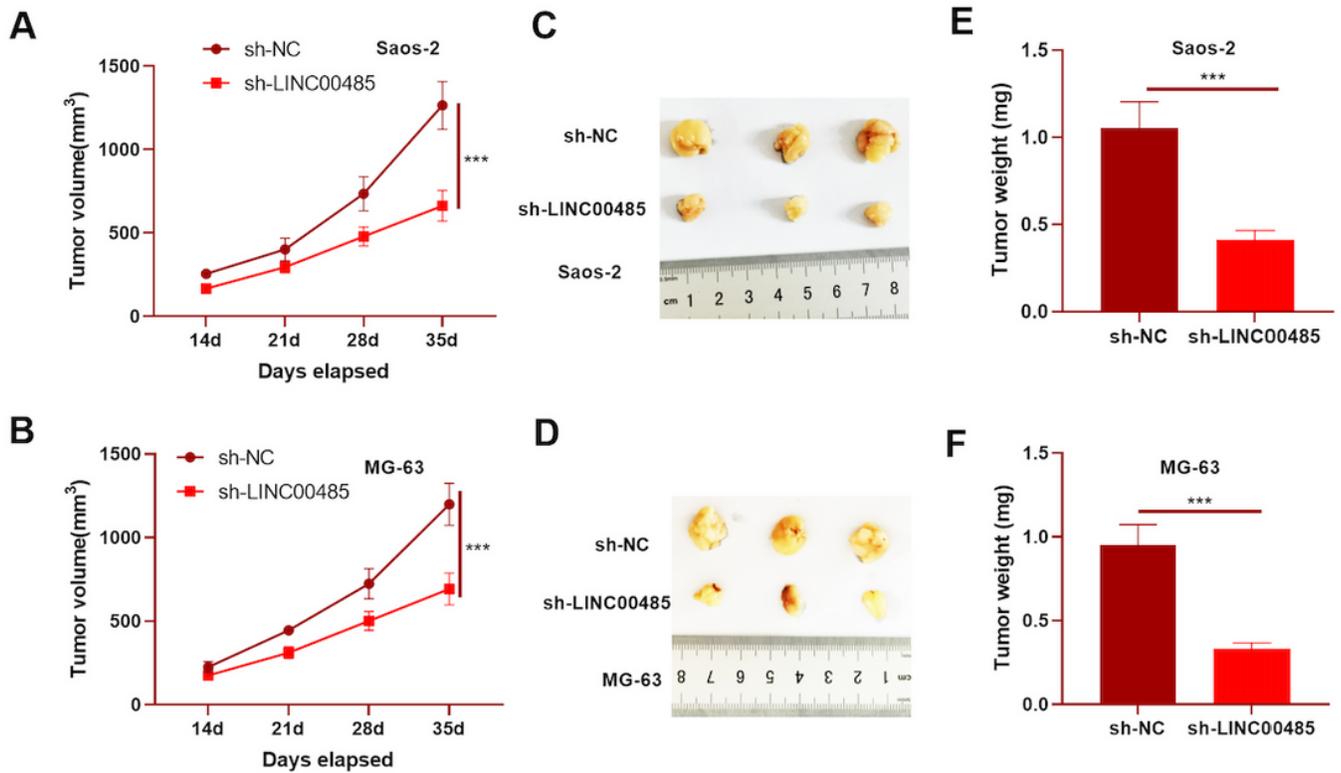
LINC00485 was increased in osteosarcoma tissues and cell lines. A. qRT-PCR measured LINC00485 level in LINC00485 osteosarcoma tissue samples, \*\*\*  $P < 0.001$ . B. RT-PCR detected the LINC00485 level in osteoblast cell lines (hFOB 1.19) and osteosarcoma cell lines (Saos-2, MG-63, U-2OS, SOSP-9607 and MNNG / HOS), \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , vs. hFOB 1.19 group. C. KM was employed to analyze the relevance between the LINC00485 level and the osteosarcoma prognosis.



**Figure 2**

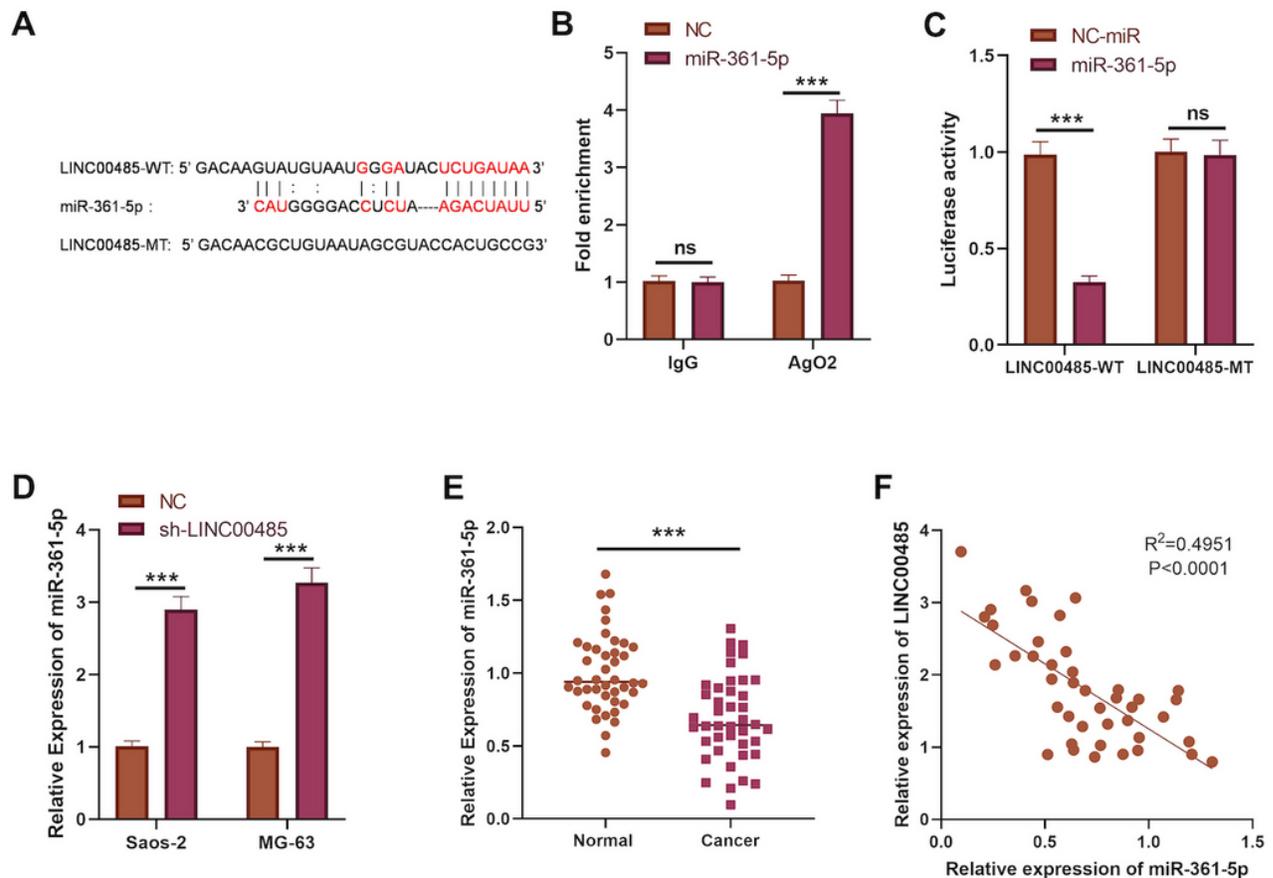
LINC00485 exerted a carcinogenic effect on regulating osteosarcoma cell proliferation, apoptosis, migration and invasion. A knockdown LINC00485 cell model was constructed in Saos-2 and MG-63 cells, \*\*\*  $p < 0.001$ . B-C. CCK-8 assay was employed to measure cell proliferation, NS  $p > 0.05$ , \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , vs. sh-NC group. D. Flow cytometry measured the apoptosis level of Saos-2 and MG-63 cells, \*\*\*  $p < 0.001$ , vs. sh-NC group.

<0.001. E-F. Transwell evaluated cell migration and invasion ability, \*\* p <0.01. G-H. Western blot tested the level of EMT-related proteins E-cadherin and Vimentin in osteosarcoma cells, \*\*\* p <0.001.



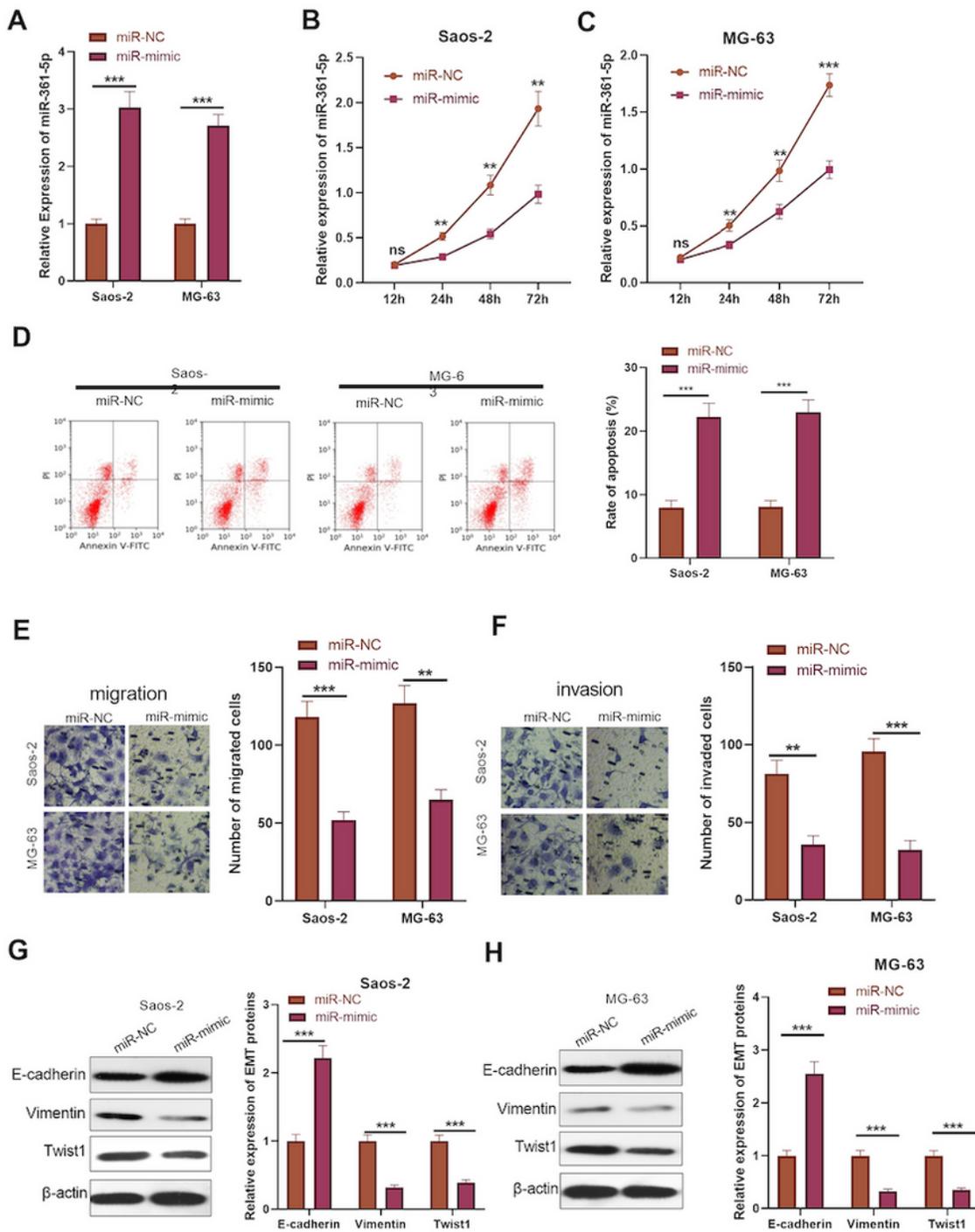
**Figure 3**

Downregulation of LINC00485 attenuate the growth of osteosarcoma cells. Saos-2 and MG-63 cells transfected with the sh-LINC00485 or sh-NC were subcutaneously injected into the backs of nude mice to conduct xenograft assay. A and B. Tumor volumes were counted. C and D. The tumor images were shown. E and F. Tumor weights were measured in different groups. \*\*\* p <0.001.



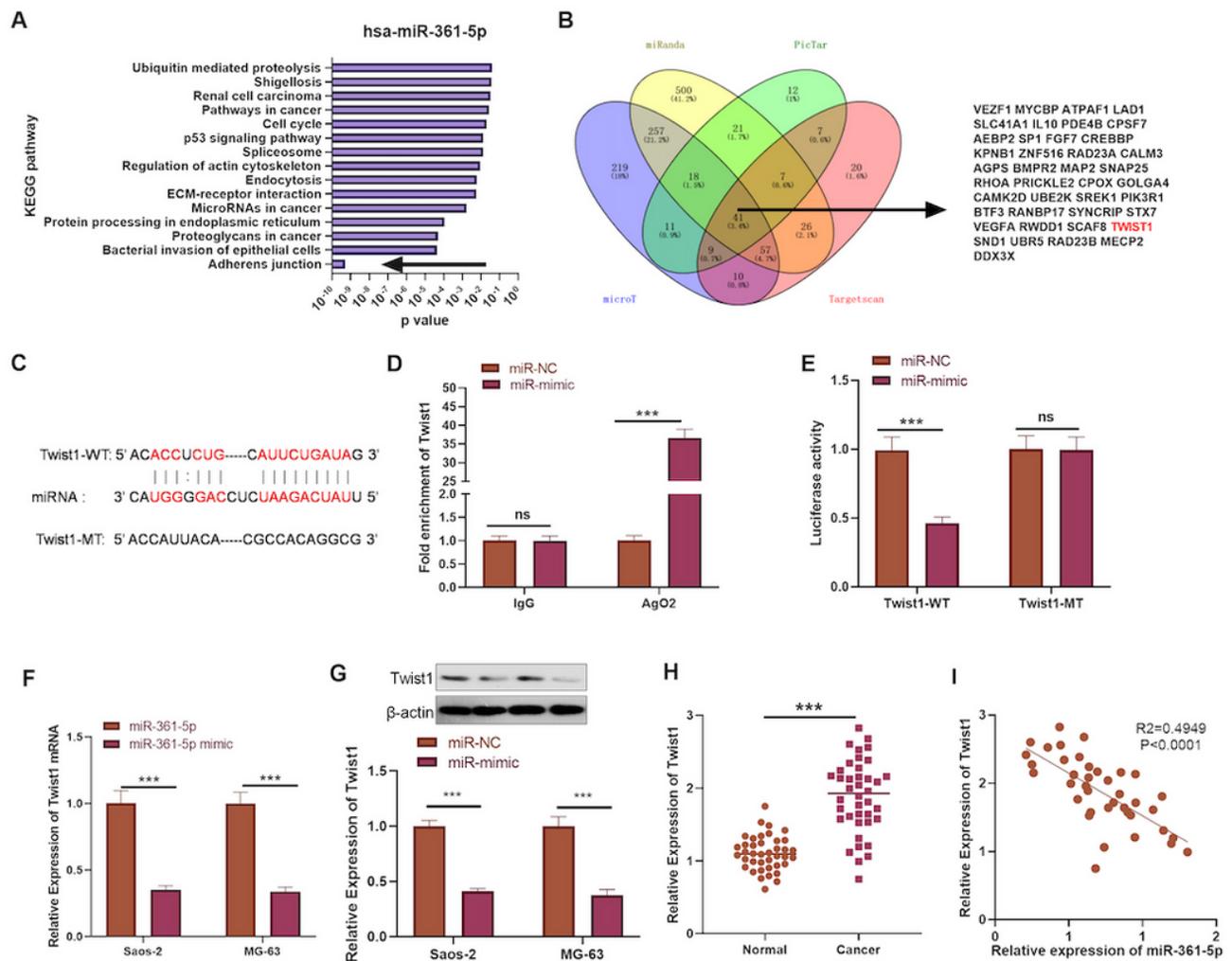
**Figure 4**

LINC00485 can target miR-361-5p. A. The bioinformatics database Starbase (<http://starbase.sysu.edu.cn/>) exhibited the potential target genes of NCK1-AS1. B. RIP verified the potential endogenous interaction between LINC00485 and miR-361-5p, NS  $p > 0.05$ , \*\*\*  $p < 0.001$ . C. luciferase reporter assay measured the luciferase activity of cells transfected with LINC00485-WT and LINC00485-MUT, NS  $p > 0.05$ , \*\*\*  $p < 0.001$ . D. RT-PCR was utilized to appraise the miR-361-5p expression in cells, \*\*\*  $p < 0.001$ . E. RT-PCR detected the miR-361-5p level in osteosarcoma tissues and normal tissues, \*\*\*  $p < 0.001$ . F. Pearson correlation analysis between LINC00485 and miR-361-5p expression.



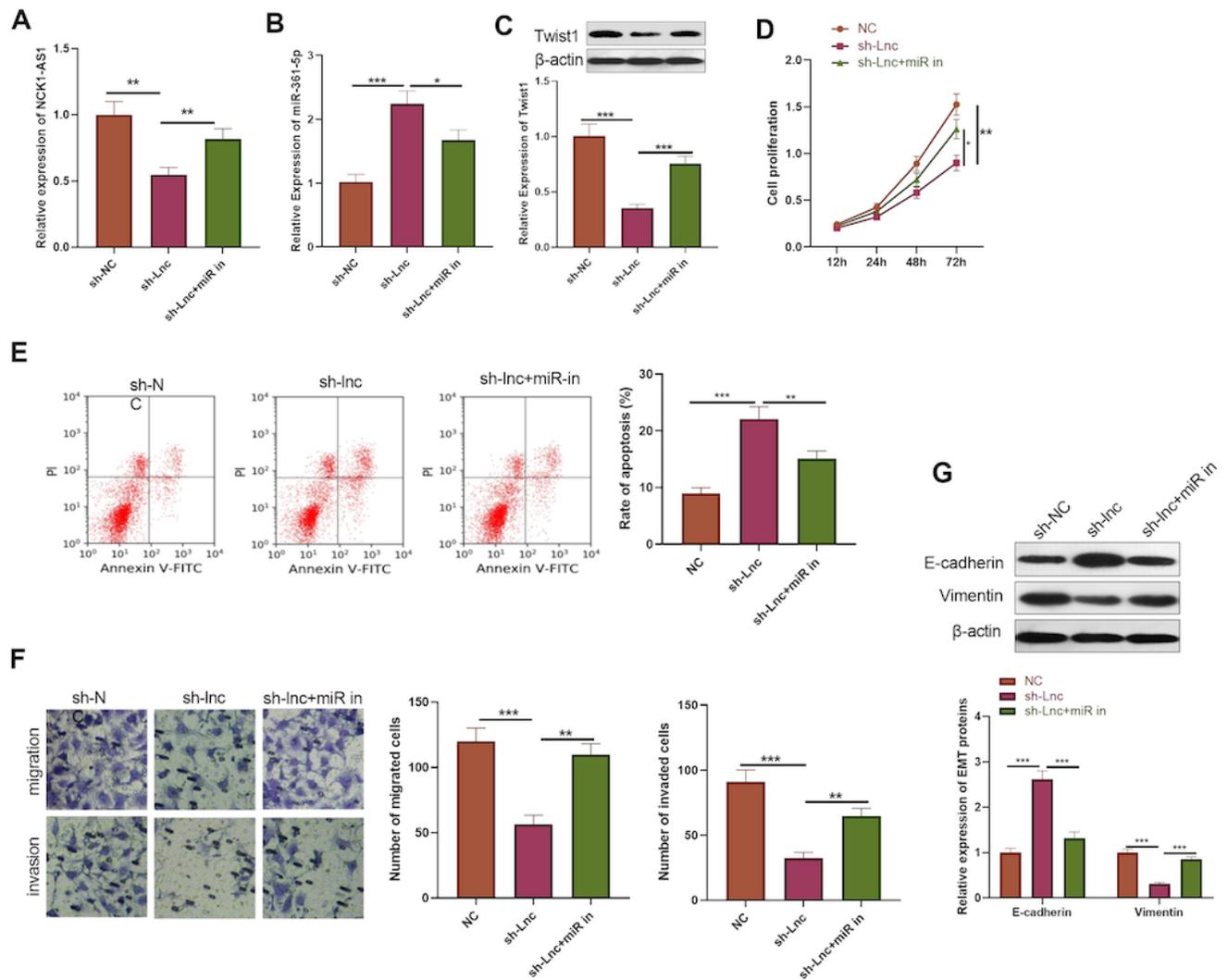
**Figure 5**

MiR-361-5p suppressed osteosarcoma progression. A. miR-361-5p mimic was utilized to construct the miR-361-5p over-expression model, \*\*\*  $p < 0.001$ . B-C. CCK-8 was applied to determine the proliferation and apoptosis of Saos-2 and MG-63 cells, NS  $p > 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , vs. miR-NC group. E-F. Transwell measured the migration and invasion ability of osteosarcoma cells, \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . G-H. estimated the level of EMT-related proteins E-cadherin and Vimentin, \*\*\*  $p < 0.001$ .



**Figure 6**

MiR-361-5p can target Twist1. A. MirPath (<http://snf-515788.vm.okeanos.grnet.gr/index.php?r=mirpath>) analyzed the KEGG pathway related to miR-361-5p. B. The bioinformatics databases micorT, miRanda, Targetscan, and PicTar were employed to pinpoint the potential target genes of miR-361-5p. C. Wayne diagram analysis of potential binding sites of miR-361-5p. D. RIP analysis verified the potential endogenous interaction between miR-361-5p and Twist1, NS  $p > 0.05$ , \*\*\*  $p < 0.001$ . E. Luciferase reporter assay was used to test the luciferase activity of Twist1-WT and Twist1-MT, NS  $p > 0.05$ , \*\*\*  $p < 0.001$ . F. Twist1 expression was measured in osteosarcoma cells after transfection with miR-361-5p mimic by RT-PCR, \*\*\*  $p < 0.001$ . G. Western blot tested the Twist1 expression in osteosarcoma cells after transfection with miR-361-5p mimic, \*\*\*  $p < 0.001$ . H. RT-PCR was used to appraise the Twist1 expression in osteosarcoma tissues, \*\*\*  $p < 0.001$ . I. Pearson correlation analysis of the relevance between Twist1 and miR-361-5p expression.



**Figure 7**

Effect of LINC00485 / miR-361-5p axis on osteosarcoma cell proliferation, apoptosis, migration, invasion and EMT process. Osteosarcoma cell line MG-63 was transfected with sh-LINC00485 and miR-361-5p inhibitors, respectively. A. qRT-PCR was used to estimate the NCK1-AS1 expression. B. qRT-PCR was applied to determine the miR-361-5p level. C. Western blot analysis of Twist1 expression. D. CCK8 detected the proliferation of MG-63 cells, \*\*  $p < 0.01$ , vs.sh-NC; \*  $p < 0.05$ , vs. Sh-Lnc + miR in group. E. Flow cytometry detected the apoptosis of MG-63 cells. F. Transwell detected the migration and invasion of MG-63 cells. G. Western blot measured the level of EMT-related proteins E-cadherin and Vimentin in MG-63 cells. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .