

LINC00961 suppresses cell proliferation and induces cell apoptosis in oral squamous cell carcinoma

L.-N. PAN¹, Y.-R. SUN²

¹Department of Stomatology, Shungeng Branch, Jinan Stomatological Hospital, Jinan, China

²Department of Pediatric Dentistry, Jinan Stomatological Hospital, Jinan, China

Abstract. – OBJECTIVE: Oral squamous cell carcinoma (OSCC) is still one of the most frequent neck and head malignancies and is one of the most common cancers in the world. The main purpose of this research was to illustrate the functional role of LINC00961 in OSCC and provide novel insight of biomarkers and therapeutic strategies in OSCC.

PATIENTS AND METHODS: The relative expression level of LINC00961 was evaluated by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell counting kit-8 (CCK-8) assay was involved for determining the ability of cell proliferation. Flow cytometric analysis was performed to detect the cell cycle and cell apoptosis. Expressions of AKT, p-AKT, BCL2, Bax protein levels were detected in Western blotting. Transfected cells were used to perform tumor xenograft formation assay.

RESULTS: Low-expression of LINC00961 was detected in both OSCC tissues and cell lines. Through CCK-8 assay and flow cytometric analysis, we validated that up-regulated LINC00961 suppressed cell proliferation and promoted cell apoptosis in OSCC. Besides, over-expressed LINC00961 suppressed PI3K/AKT signaling pathways. In tumor xenograft formation assay, over-expressed LINC00961 inhibited tumor formation.

CONCLUSIONS: Our research verified that LINC00961 functioned as a tumor suppressor in OSCC. Regulation of PI3K/AKT might be the underlying mechanism of the tumor suppressor role of LINC00961. The current study might bring a novel insight of biomarkers and therapeutic strategies in OSCC.

Key Words

LINC00961, OSCC, Proliferation, Apoptosis, PI3K/AKT.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most frequent tumor types among the neck and head malignancies¹. Oral cancer now becomes

the third most common tumor type in developing areas and is one of the most common cancers in the world². Despite years of work in studying OSCC, the overall survival rate of OSCC is still not favorable due to many factors³. Though all the work has been done, the underlying molecular mechanism of OSCC progression remains unknown. Hence, we hope our findings may provide some novel insights of finding therapeutic targets and biomarkers of OSCC.

Long non-coding RNAs (LncRNAs) were a type of transcript with more than 200 nucleotides (nt) in length and without the ability of coding proteins⁴. LncRNAs have been reported⁵⁻⁷ to take part in many tumor progressions and are involved in many biological behaviors including cell proliferation, cell metastasis, cell apoptosis, and cell cycle. LncRNAs have been reported⁸⁻¹⁰ to exert their functions in diverse cancers such as breast cancer, pancreatic cancer, and prostate cancer. However, the physiological role of LINC00961 in OSCC has never been reported. Therefore, in the current investigation, we mainly focused on the biological function and its underlying mechanism in the development of OSCC.

In this research, we examined the relative expression level of LINC00961 in OSCC, and it turned out that LINC00961 was down-regulated in OSCC tissues and cell lines. Through CCK8 assay and flow cytometric analysis, we considered that LINC00961 can suppress cell proliferation and promote cell apoptosis in OSCC *in vitro*. Subsequently, we recruited Western blotting assay. The protein expression levels of p-AKT, BCL2, and Bax are consistent with the results of experiments *in vitro*. Besides, in the tumorigenicity assay, the data indicated that LINC00961 can inhibit the tumor formation *in vivo*. In summary, our study validated that LINC00961 can suppress cell proliferation and induce cell apoptosis in oral squamous cell carcinoma.

Patients and Methods

Tissue Specimens

35 pairs of OSCC tissues and normal tissues were involved in this work. All tissue specimens were obtained from patients got surgery treatment in Jinan Stomatological Hospital from 2016-2017. Tissue specimens were immediately put into liquid nitrogen. All tumor tissues were approved by pathological examination. This research was approved by the Ethics Committee of Jinan Stomatological Hospital. The written informed consents were signed from all participants before the study.

Cell Lines

Three OSCC cell lines CAL-27, UM-1, OSC-4, and one human normal oral epithelial keratinocytes were obtained from Shanghai Cell Bank (Shanghai, China). All cell lines were cultured by Roswell Park Memorial Institute-1640 (RPIM-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C with 5% CO₂.

Cell Transfection

To over-express LINC00961, we selected OSC-4 cells to transfect with the lentiviral vector following the standard instructions. For knock-down of LINC00961, the selected CAL-27 cells were transfected with shRNA following the standard protocol. All the plasmids were obtained from GenePharma (Shanghai, China).

Isolation of Total RNA and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Tissue specimens stored in liquid nitrogen and cell lines were used to extract total RNA *via* TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the standard protocol. Complementary deoxyribose nucleic acids (cDNAs) were synthesized *via* Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) according to the standard protocol. LINC00961 expression level was assessed through SYBR Green real-time PCR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as a normalization. GAPDH forward 5'-3': TATCGGACGCCTGGTTAC, reverse 5'-3': TATCGGACGCCTGGTTAC; LINC00961 forward 5'-3': CTGTTCTGGATGGAGCGAA, reverse 5'-3': ACAGTCACCACGAACAGCAC.

Cell Counting Kit-8 Assay (CCK-8)

The cell counting kit-8 assay was involved in examining the cell proliferation in OSCC. The transfected cells were plated into 96-well plates (6 × 10³/well), and then, the CCK-8 solution (Beyotime, Shanghai, China) (10 µL/well) was used to stain cells for 2 hours at 37°C. The optical density (OD) value (450 nm) was then evaluated.

Flow Cytometric Analysis

For detecting the apoptotic cells, the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (Vazyme, Nanjing, China) was performed according to the instruction. To examine the cell cycle, the transfected cells were immersed in 70% ethanol at -20°C overnight before stained with PI (Vazyme, Nanjing, China). The flow cytometric analysis was taken place by BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometry.

Western Blot

The total protein was isolated by radioimmunoprecipitation assay (RIPA) buffer (Thermo, Waltham, MA, USA) and phenylmethanesulfonyl fluoride (PMSF). The protein lysates isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Then, the membrane was immunostained at 4°C by primary antibodies overnight. The primary rabbit antibodies used in the current study were including anti-AKT (CST, Danvers, MA, USA), anti-p-AKT (CST, Danvers, MA, USA), anti-BCL2 (CST, Danvers, MA, USA), anti-Bax (CST, Danvers, MA, USA). The rabbit anti-GAPDH (CST, Danvers, MA, USA) was taken as a loading control. The protein relative expression level was determined by Image Lab software.

Xenograft Model

The current study was approved by the Animal Ethics Committee of Jinan Stomatological Hospital Animal Center. Transfected OSC-4 or CAL-27 cells (6 × 10⁵/mL) were injected into two flanks of nude mice (6 weeks old) subcutaneously. The tumor's growth was monitored and recorded every week. The formula (volume = length × width² × 1/2) was used to calculate the tumor volume. Tumors were extracted after 4 weeks.

Statistics Analysis

All experiments in this study were performed three times independently at least. All data re-

corded were exhibited as mean \pm standard deviation (SD). The Student's unpaired *t*-test was used to undergo statistics analysis. In this study, $p < 0.05$ was considered to be significant.

Results

LINC00961 Had Low-Expression Level in OSCC Tissues and Cell Lines

For the determination of the relative expression level of LINC00961 in OSCC, we used the qRT-PCR assay. As shown in Figure 1A, LINC00961 was down-regulated in OSCC tissues when compared with para-tumor tissues. Consistently, the expression level of LINC00961 was down-regulated in OSCC cell lines. Thus, all the results indicated that LINC00961 had a low-expression level in OSCC.

LINC00961 Suppressed Cell Proliferation in OSCC

To validate the ability of LINC00961 in OSCC, we transfected the selected cell lines with lentivirus for over-expressing LINC00961 or down-expressing LINC00961. The transfection efficiency was examined by the qRT-PCR (Figure 2A). Subsequently, we recruited CCK8 assay for examining the ability of LINC00961 on cell proliferation. As shown in Figure 2B, the OD value of the LINC00961 up-regulated group was significantly increased in comparison with control group.

Hence, all the data suggested that LINC00961 can inhibit cell proliferation in OSCC.

Up-Regulated LINC00961 Can Inhibit Cell Cycle Progression and Promote Cell Apoptosis In Vitro

To figure out the effect of LINC00961 on cell cycle and cell apoptosis, flow cytometric analysis was involved in this study. As Figure 3A showed, the ratio of G1/0 fraction in LINC00961 up-regulated group was increased when compared with control group. While in LINC00961 down-regulated group, the ratio of S fraction was increased. It suggested that LINC00961 can suppress cell cycle progression in OSCC cell lines. As Figure 3B showed, apoptotic cells were significantly increased in LINC00961 over-expression group while decreased in LINC00961 down-expression group. Taken together, the results showed that LINC00961 can inhibit the cell cycle progression and induce the cell apoptosis in OSCC cell lines.

The Influence of LINC00961 on OSCC Cell Lines Might Be Relied on the PI3K/AKT Signaling Pathway

For exploring the underlying mechanism of LINC00961 on the progression of OSCC, we used Western blotting assay to determine if there were alternation on some markers in the PI3K/AKT pathway. As shown in Figure 4, in LINC00961 up-regulated group, the expression level of phosphorylation-AKT was significantly

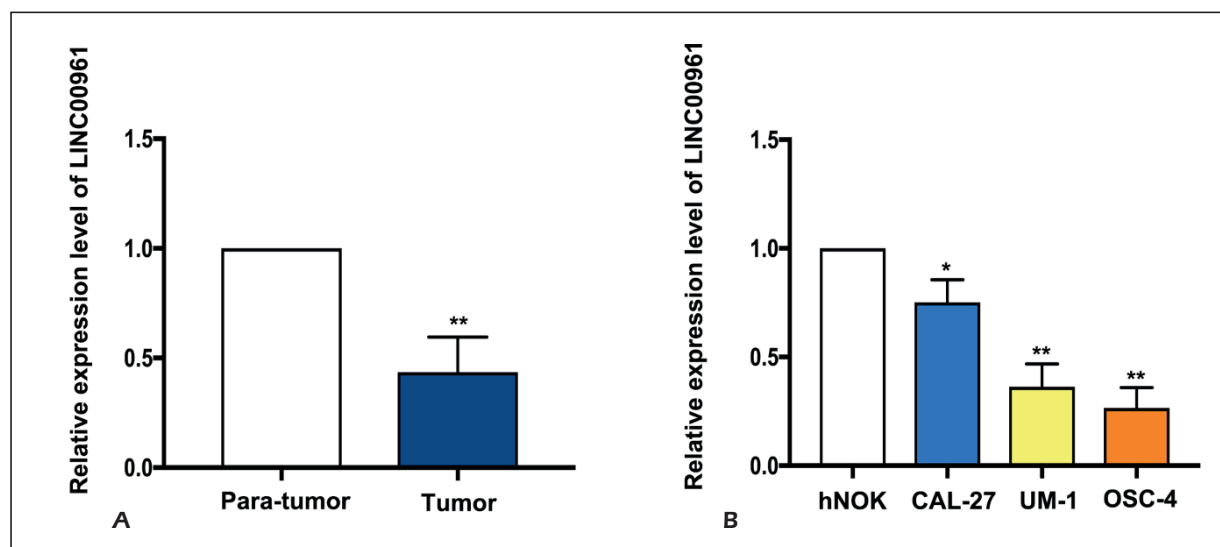


Figure 1. LINC00961 had low-expression level in OSCC tissues and cell lines. **A**, The expression level of LINC00961 in OSCC tissues and para-tumor tissues. **B**, Analysis of LINC00961 expression level in OSCC cell lines. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

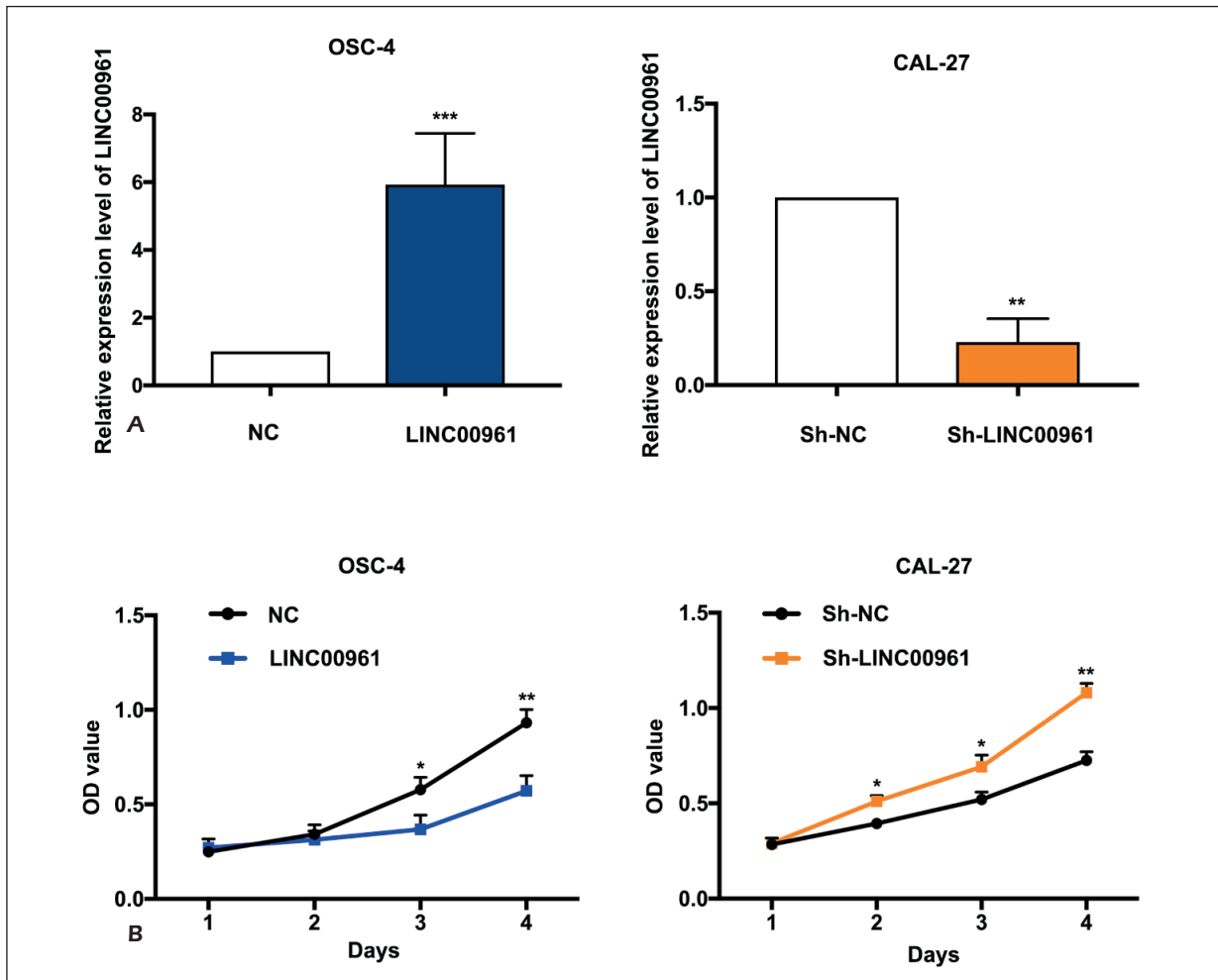


Figure 2. LINC00961 suppressed cell proliferation in OSCC. **A**, Transfection efficiency was evaluated by qRT-PCR. **B**, The cell proliferation ability was determined by CCK-8 assay. Data are presented as the mean \pm SD of three independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

decreased. Besides, the alternation of BCL2 and Bax indicated that LINC00961 over-expression can induce cell apoptosis. In sum, LINC00961 functioned as a tumor suppressor in OSCC partially through regulating the PI3K/AKT signaling pathway.

Up-Regulated LINC00961 Can Inhibit Tumor Formation In Vivo

To examine the ability of LINC00961 on tumor formation *in vivo*, we generate tumorigenicity assay. As Figure 5A, 5B showed, in LINC00961 over-expression group, tumor volume and tumor weight were significantly lower than negative control group. Besides, we examined the LINC00961 expression level in generated tumors of nude mice (Figure 5C). The results showed that the tumors of LINC00961 over-expression group had signifi-

cantly higher LINC00961 expression level. All data indicated that LINC00961 can inhibit tumor formation *in vivo*.

Discussion

OSCC is still one of the most common neck and head malignancies in the world due to the lack of efficient approaches for early-stage diagnosis and treatment strategies¹¹. Hence, it is vital for seeking for novel biomarkers and therapeutic targets of OSCC¹². Hence, in this study, we wonder if LINC00961 play an important role in OSCC progression and figure out the underlying molecular mechanism. We hope our findings can provide some novel insight of OSCC development and progression.

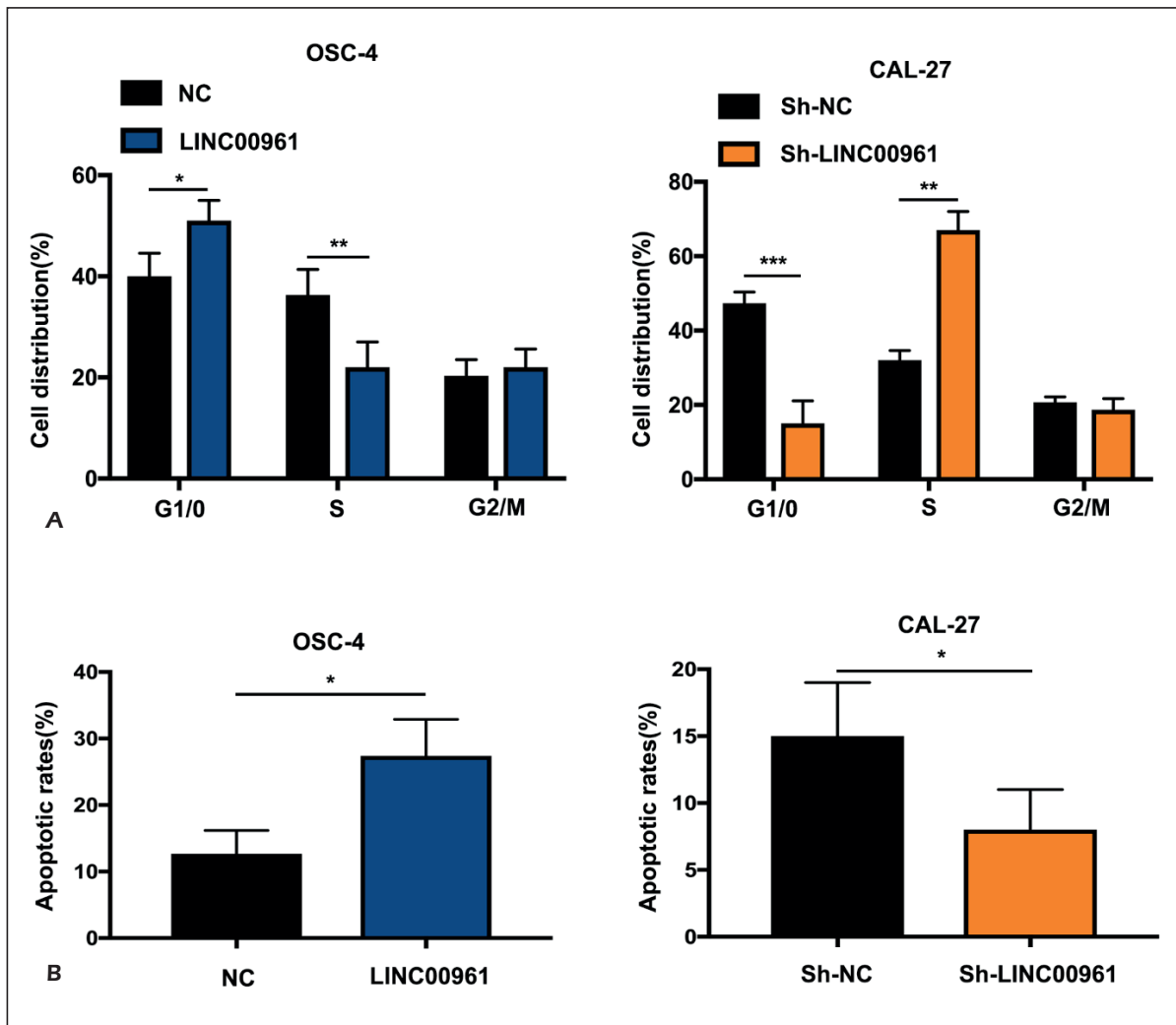


Figure 3. Up-regulated LINC00961 can inhibit cell cycle progression and promote cell apoptosis *in vitro*. **A**, Cell cycle progression was detected by flow cytometric analysis in transfected groups. **B**, Flow cytometric analysis was performed to detect the apoptotic rates in transfected cells. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

LncRNAs have been reported^{13,14} to be potential biomarkers in diverse cancers and have essential roles in tumor progression. As reported¹⁵, lncRNA ROR can promote cell proliferation in renal cancer and is negatively associated with a favorable prognosis. LncRNA MEG3 was proved⁶ to inhibit the cell proliferation and metastasis in gastric cancer through regulating the p53 signaling pathway. It is validated¹⁶ that lncRNA FEZF1-AS1 may enhance the epithelial-mesenchymal transition (EMT) *via* inhibiting E-cadherin and regulating the WNT pathway in non-small cell lung cancer. It is also reported¹⁷ that lncRNA CPS1-IT1 could suppress cell proliferation, invasion, and metastasis in colorectal

cancer. Nevertheless, the physiological role of LINC00961 in OSCC has rarely been studied. Herein, we carried out this research to figure out the role of LINC00961 in OSCC. Through qRT-PCR assay, we validated that lnc00961 was down-regulated in OSCC. Subsequently, we used the CCK8 assay and flow cytometric analysis to verify that LINC00961 can inhibit cell proliferation, cell cycle progression, and induce cell apoptosis in OSCC cell lines. Besides, the tumorigenicity assay was involved in our study. The results indicated that LINC00961 can suppress the tumor formation *in vivo*. Taken together, our study validated that LINC00961 functioned as a tumor suppressor in OSCC.

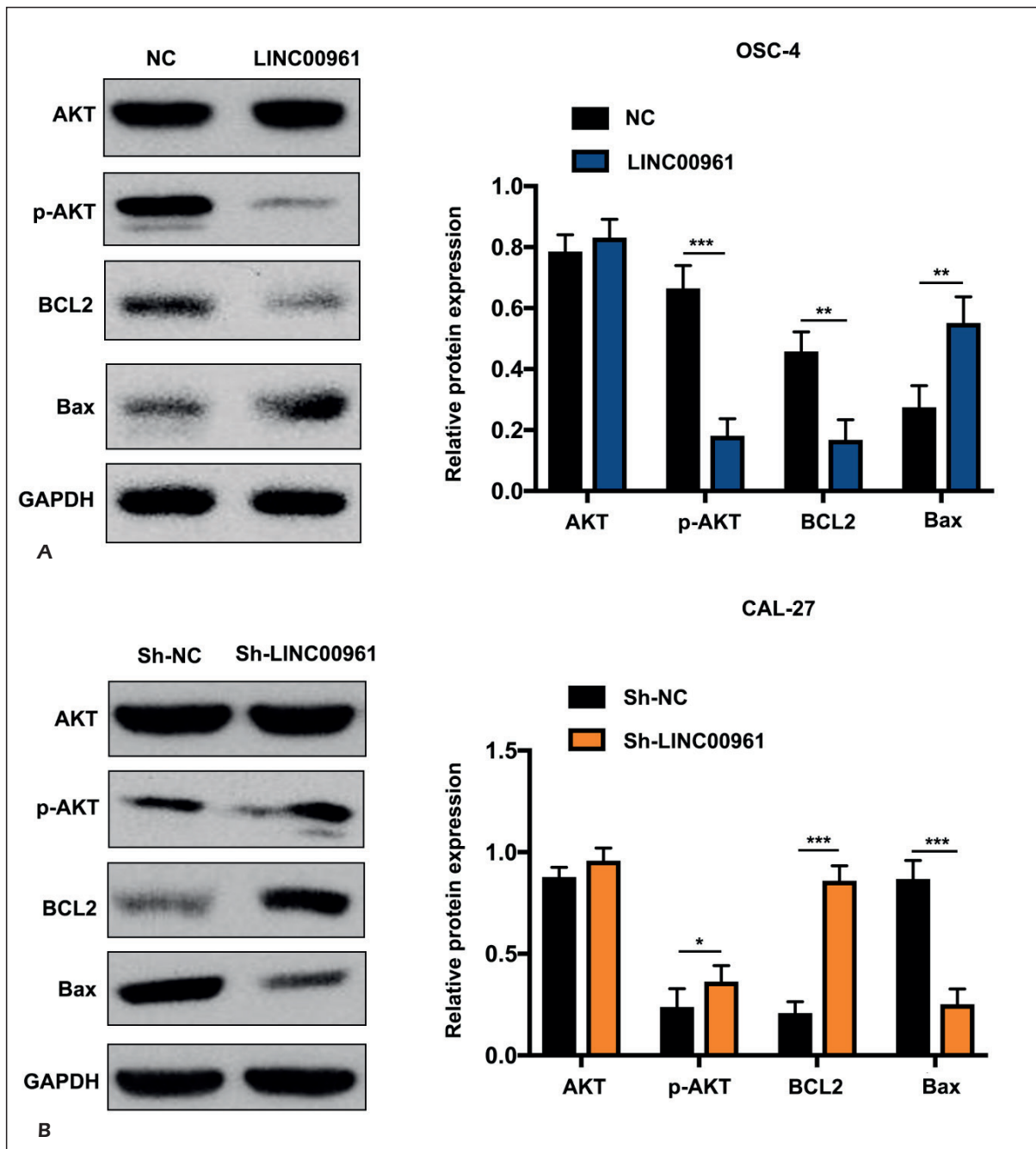


Figure 4. The influence of LINC00961 on OSCC cell lines might be relied on the PI3K/AKT signaling pathway. **A**, AKT, p-AKT, BCL2, Bax protein expression levels were examined in over-expression LINC00961 group. **B**, AKT, p-AKT, BCL2, Bax protein expression levels were examined in down-expression LINC00961 group. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To explore the underlying mechanism of the role of LINC00961 in OSCC, we examined the alternation of the protein expression level of p-AKT, BCL2, and Bax. The results indicated that the influence of LINC00961 may be through regulating the PI3K/AKT signaling pathway. The PI3K/AKT pathway was reported^{18,19} to be essential in

various cancer progression and development. As reported^{20,21}, the PI3K/AKT signaling pathway was related to numerous cell physiological behaviors including cell proliferation and cell apoptosis. In sum, all the results indicated that LINC00961 may exert its physiological functions in OSCC by regulating PI3K/AKT signaling pathway.

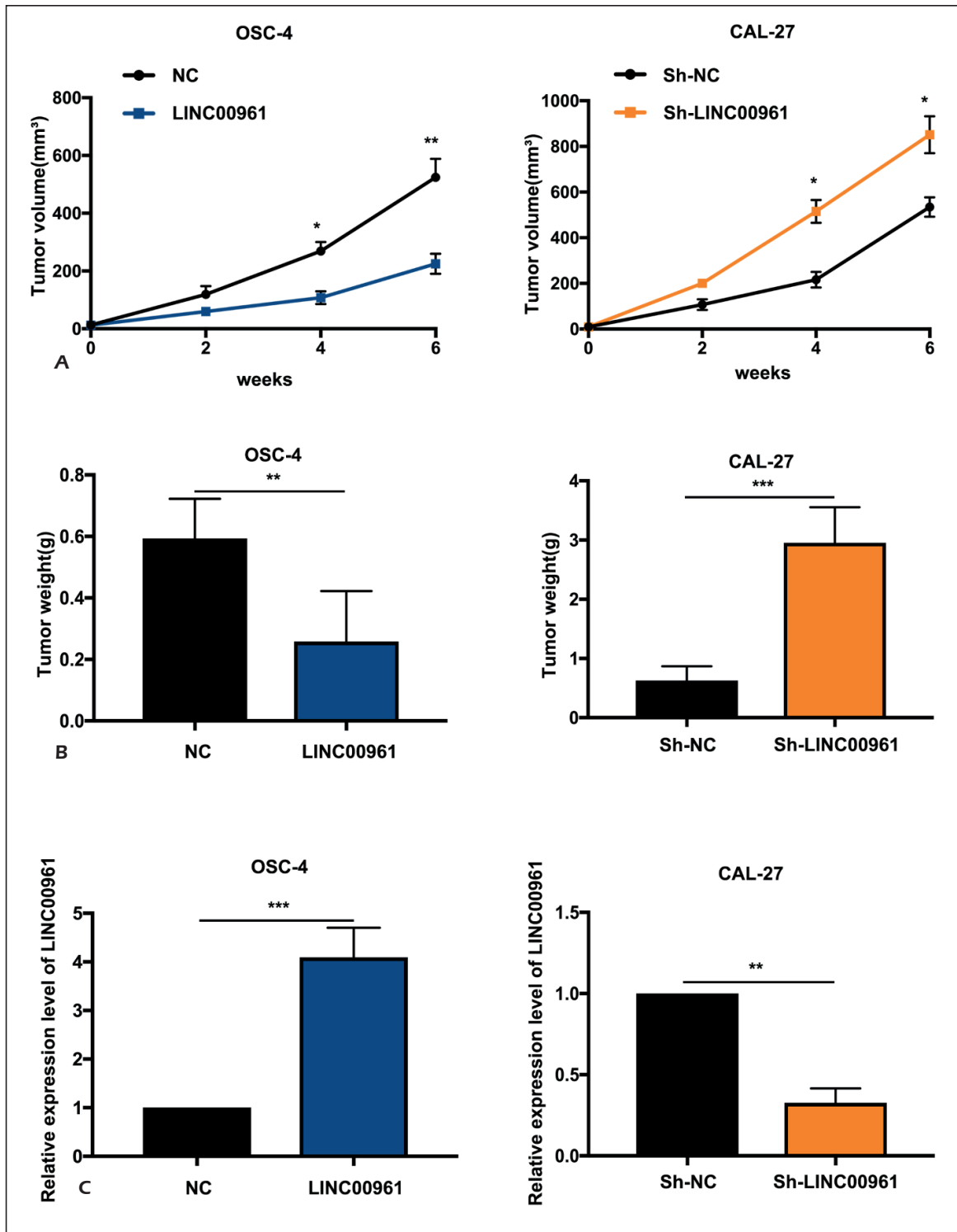


Figure 5. Up-regulated LINC00961 can inhibit tumor formation *in vivo*. **A**, After tumor extraction, tumor volume was calculated respectively and made into a graph; **B**, Tumor weight was recorded; **C**, The relative expression of LINC00961 in tumors was examined by qRT-PCR. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Conclusions

In the current study, we showed that LINC00961 was down-regulated in OSCC tissues and cell lines. Through CCK8 assay and flow cytometric analysis, we validated that LINC00961 can inhibit cell proliferation, cell cycle progression, and induce cell apoptosis. In tumorigenicity assay, the results exhibited that LINC00961 could suppress tumor formation *in vivo*. Through Western blotting assay, we verified that LINC00961 may play a vital role in the OSCC progression *via* regulating PI3K/AKT signaling pathway. We provide some findings of the role of LINC00961 in OSCC and we hope our findings could lead to some novel insight of seeking for therapeutic targets and biomarkers in OSCC.

Conflict of Interests

The authors declare that they have no conflict of interest.

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