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ORIGINAL ARTICLE

# LncRNA LSINCT5 drives proliferation and migration of oral squamous cell carcinoma through the miRNA-185-5p/ZNF703 axis

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## Summary

***Purpose:*** *To analyze the role of lncRNA LSINCT5 in oral squamous cell carcinoma (OSCC) progression and the molecular mechanism.*

***Methods:*** *QRT-PCR was conducted to detect LSINCT5 levels in OSCC tissues and cell lines. Survival analysis in OSCC patients based on LSINCT5 levels was performed using Kaplan-Meier method. Influence of LSINCT5 on functions of OSCC cells were assessed by CCK-8, EdU and Transwell assay. Bioinformatic analysis and dual-luciferase reporter assay were carried out to identify the interaction between LSINCT5 and the miRNA (miR)-185-5p/zNF703 axis. Rescue experiments were conducted to uncover the molecular mechanism of LSINCT5 in regulating OSCC cell functions.*

***Results:*** *LSINCT5 was upregulated in OSCC specimens and correlated to poor prognosis of OSCC. Knockdown of LSINCT5 inhibited proliferative and migratory capacities of OSCC. LSINCT5 could target and negatively regulate miR-185-5p. Moreover, miR-185-5p was the key downstream gene that was responsible for the carcinogenic role of LSINCT5 in regulating OSCC cell functions. The oncogenic gene ZNF703 was proven to be the target binding miR-185-5p.*

***Conclusions:*** *LSINCT5 is abnormally upregulated in OSCC specimens and drives its malignant progression through the miR-185-5p/ZNF703 axis.*

***Key words:*** *OSCC, LSINCT5, miRNA-185-5p, ZNF703*

## Introduction

Oral squamous cell carcinoma (OSCC) is a major global health problem. The increased incidence of OSCC has a close relation to the widely recognized risk factors, that is, smoking and drinking [1]. In addition to tobacco-related carcinogens, certain types of human papillomavirus (HPV) are also relevant to the carcinogenesis of oral tumors [2]. The 5-year survival of OSCC is lower than 50%, which is far away from satisfactory [3]. Therefore, a better understanding of the molecular mechanism of OSCC is very necessary, which is beneficial to improve the therapeutic effect.

Genetic studies have shown that variations of tumor-related genes are often observed during the development of OSCC [4]. In fact, gene diagnosis and treatment theory have shown great potentials in the treatment of OSCC [5]. Through genome-wide analyses, a large number of abnormally expressed long non-coding RNAs (lncRNAs) (> 200 nt) during OSCC progression have been detected [6]. Previous studies have demonstrated that lncRNAs are a key determinant of cancer biology [7], and they participate in various biological processes of tumor cells [8]. It is reported that lncRNA LSIN-

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CT5 is abnormally expressed in different types of tumors, which is a potential prognostic biomarker [9,10]. However, the role of LSINCT5 in OSCC is unclear.

We conducted a series of functional experiments to deeply explore the expression level of LSINCT5 in OSCC, and the *in vitro* regulations. Its carcinogenic role in OSCC was further analyzed by detecting the downstream targets.

## Methods

*Clinical specimens of OSCC*

Twenty-three OSCC and paracancerous tissues were surgically resected. Specimens were washed, pathologically confirmed and stored. None of recruited OSCC patients had preoperative treatment. This study was approved by the research ethics committee of our hospital and complied with the Helsinki Declaration. Informed consent was obtained from all patients.

*Quantitative real-time polymerase chain reaction (qRT-PCR)*

Cells or tissues were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT kit (Invitrogen, Carlsbad, CA, USA), followed by qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Tokyo, Japan). Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) and U6 served as the internal references. Each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$.

U6: Forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH: Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; miRNA-185-5p: Forward: 5'-GAAGGATCCGCATGAGAGGGTGTTGGAATGC-3', reverse: 5'- GGAGAATTCGTGCAGGGGCAGCAGACC-3'; LSINCT5: Forward: 5'-TTCGGCAAGCTCCTTTTCTA-3', reverse: 5'-GCCCAAGTCCCAAAAAGTTCT-3'; ZNF703: Forward: 5'-GATCAGGGTCCTGAAGATGC-3', reverse: 5'-CCGAGTTGAGTTTGGAGGAG-3'.

*Cell culture*

The oral cancer cell lines Fadu, SCC-25, CAL-27 and Tca8113, and human oral mucosal epithelial cell line (OMEC) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cell passage was limited in 30 generations. Cell transfection was routinely conducted using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells were used at 24 h.

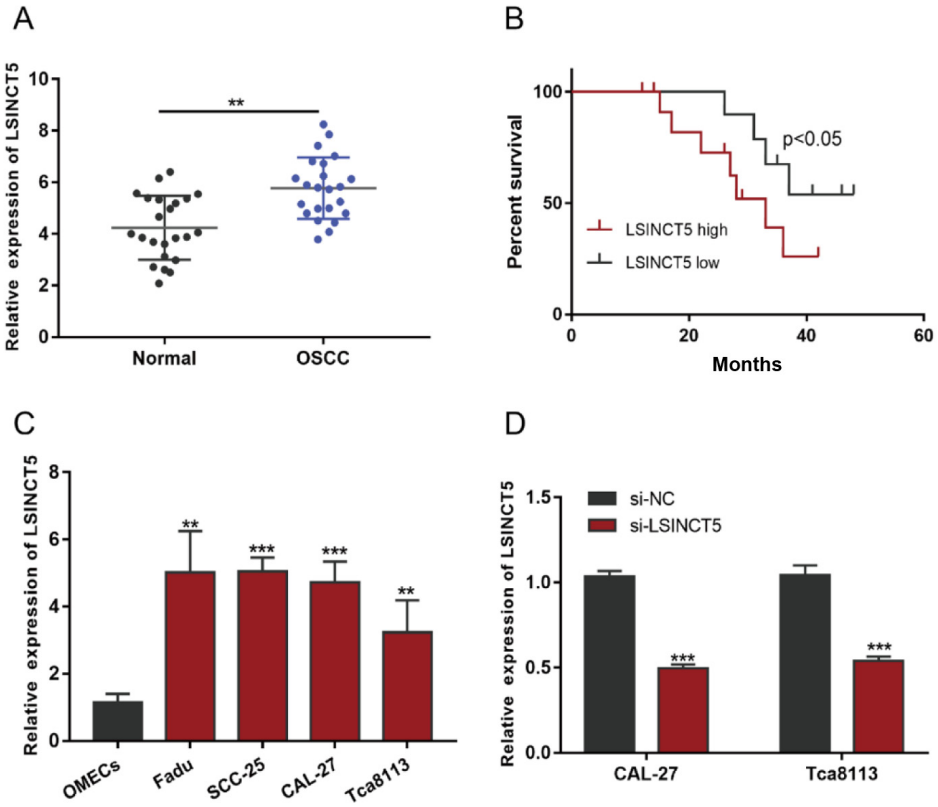


**Figure 1.** LSINCT5 was upregulated in OSCC and predicted poor prognosis. **A:** LSINCT5 was upregulated in OSCC tissues; **B:** High level of LSINCT5 predicted poor prognosis of OSCC; **C:** LSINCT5 was upregulated in OSCC cell lines; **D:** Transfection of si-LSINCT5 significantly downregulated LSINCT5 in OSCC cells. **p<0.01, ***p<0.001.

*Cell counting kit-8 (CCK-8) assay*

Cells were inoculated in 96-well plates with $1\times10^3$ cells/well. At 0, 24, 48 and 72 h, optical density (OD) at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

*5-Ethynyl-2'- deoxyuridine (EdU) assay*

Cells were pre-inoculated in 96-well plates with $1\times10^5$ cells/well. They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100, and 30-min reaction in 400 μL of 1×ApolloR. Afterwards, cells were dyed in 4',6-diamidino-2-phenylindole (DAPI) for another 30 min in the dark. Positive EdU-stained cells were calculated.

*Transwell assay*

Transwell chambers (8 μm; Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate, where $1\times10^4$ cells were applied in the upper layer of the chamber, and 600 μL of medium containing 10% FBS was applied in the bottom. After cell culture for 48 h, migratory cells to the bottom were captured following fixation and staining, which were counted in 5 randomly selected fields per sample.

*Dual-luciferase reporter assay*

Binding sites between miR-185-5p and LSINCT5 or ZNF703 were predicted using online tools, which were used for generating luciferase vectors. Cells were co-transfected with luciferase vectors (wild-type and mutant-type ones) and miR-185-5p inhibitor or negative control (NC). At 48 h, 35 μL of fresh medium and 35 μL of luciferase substrate were replaced per well for 10-min mixture, followed by measurement of luciferase activity.

*Statistics*

Data processing was conducted using GraphPad7.0 (LaJolla, CA, USA). Two-paired independent *t*-test was performed for comparing differences between groups.

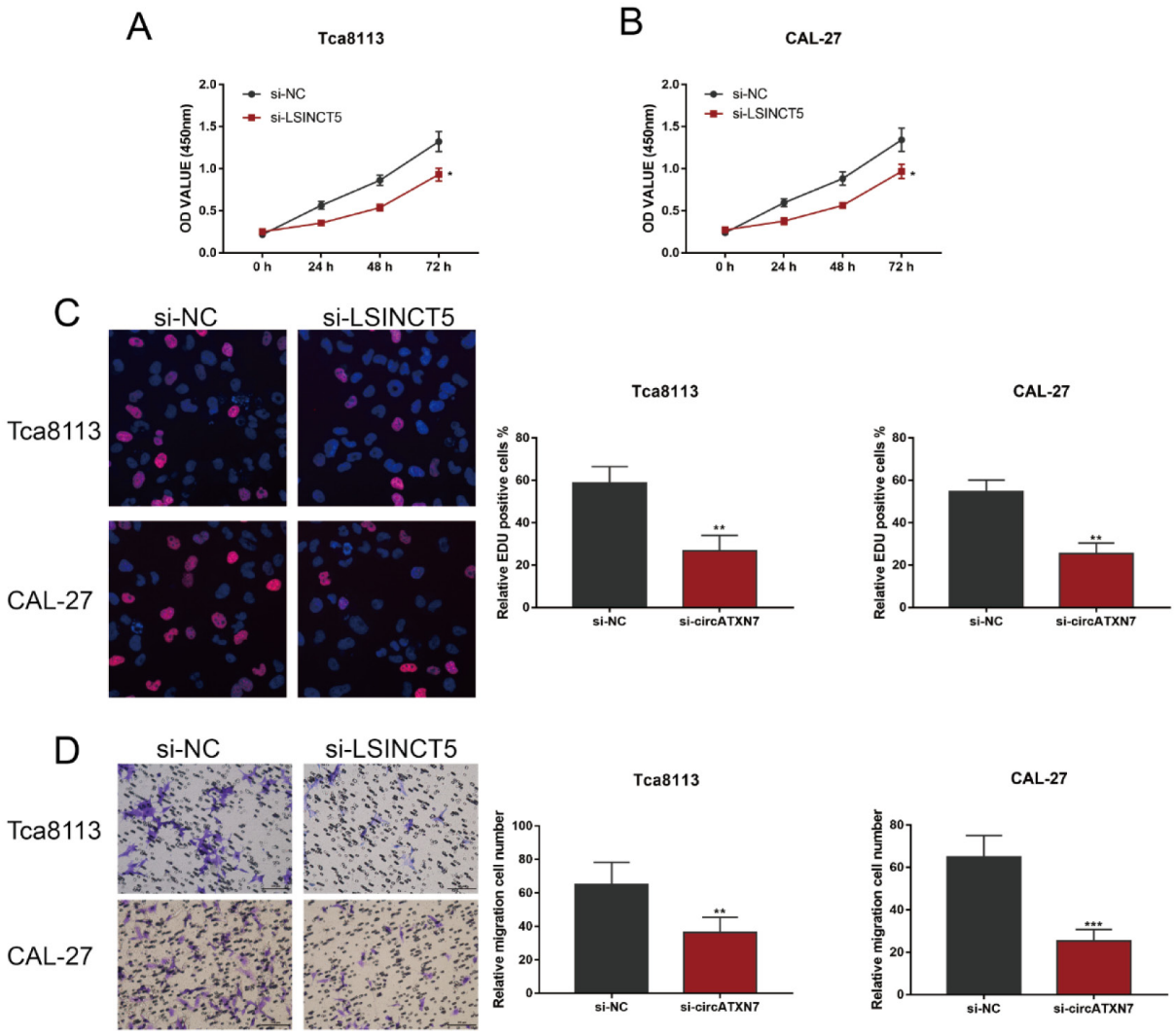


**Figure 2.** Knockdown of LSINCT5 inhibited proliferative and migratory capacities of OSCC. **A, B:** Knockdown of LSINCT5 reduced viability of OSCC cells; **C:** Knockdown of LSINCT5 reduced EdU-positive rate of OSCC cells; **D:** Knockdown of LSINCT5 reduced the migratory cell number of OSCC cells (magnification 200×). *p<0.05, **p<0.01, ***p<0.001.

Kaplan-Meier method was used for survival analysis along with log-rank test. Correlation between LSINCT5 and miR-185-5p levels was assessed by Pearson's correlation test. Significant difference was set at $p<0.05$.

## Results

*LSINCT5 was upregulated in OSCC and predicted poor prognosis*

A total of 23 pairs of OSCC and paracancerous tissues were collected. LSINCT5 was upregulated in OSCC tissues than that of controls (Figure 1A). Survival analysis showed that OSCC patients expressing high level of LSINCT5 had shorter overall survival in comparison to those expressing low level of LSINCT5 (Figure 1B). It is suggested that LSINCT5 could be utilized as a prognostic factor. Subsequently, LSINCT5 was detected to be identically upregulated in OSCC cell lines (Figure 1C). Transfection of si-LSINCT5 markedly downregulated LSINCT5 in CAL-27 and Tca8113 cells (Figure 1D).

*Knockdown of LSINCT5 inhibited proliferative and migratory capacities of OSCC*

CCK-8 results uncovered that knockdown of LSINCT5 reduced the viability in Tca8113 and CAL-27 cells (Figure 2A and 2B). EdU assay consistently showed lower EdU-positive cell rate after knockdown of LSINCT5 in OSCC cells, indicating the inhibited proliferative capacity (Figure 2C). Transwell assay was conducted to explore the regulatory effect of LSINCT5 on OSCC migration and it was shown that transfection of si-LSINCT5 declined the migratory cell number, suggesting that LSINCT5 could stimulate migratory capacity of OSCC (Figure 2D).

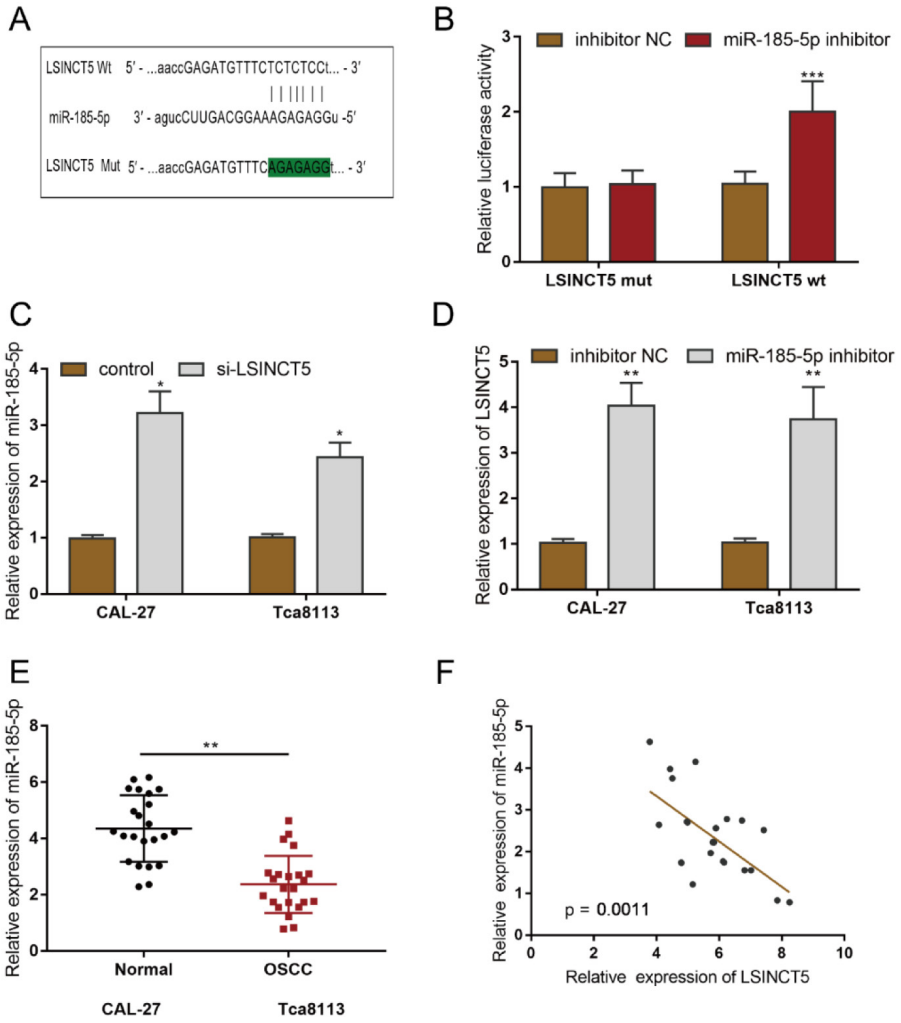


**Figure 3.** MiRNA-185-5p was the target of LSINCT5. **A:** A binding site in LSINCT5 3'UTR that was paired to that of miRNA-185-5p; **B:** Binding relationship between LSINCT5 and miRNA-185-5p; **C:** Knockdown of LSINCT5 upregulated miRNA-185-5p in OSCC cells; **D:** Knockdown of miRNA-185-5p upregulated LSINCT5 in OSCC cells; **E:** MiRNA-185-5p was downregulated in OSCC tissues; F: MiRNA-185-5p was negatively correlated to LSINCT5. *p<0.05, **p<0.01, ***p<0.001.

*MiR-185-5p was the target of LSINCT5*

To clarify the downstream molecular mechanism of LSINCT5 in regulating OSCC cell functions, its potential targets were predicted. We found that LSINCT5 3'UTR contained a binding site that paired to miR-185-5p 3'UTR (Figure 3A). Dual-luciferase reporter assay further verified the binding between miR-185-5p and LSINCT5 (Figure 3B). In CAL-27 and Tca8113 cells with LSINCT5 knockdown, miR-185-5p was markedly upregulated (Figure 3C). LSINCT5 level, identically, was enhanced by knockdown of miR-185-5p (Figure 3D). In comparison to normal tissues, miR-185-5p was downregulated in OSCC specimens (Figure 3E). As expected, a negative correlation was identified between LSINCT5 and miRNA-185-5p levels in OSCC tissues (Figure 3F). These results indicated that miRNA-185-5p was a downstream gene of LSINCT5, which may mediate its carcinogenetic role in OSCC.

*MiR-185-5p abolished the role of LSINCT5 in regulating OSCC cell functions*

The involvement of miR-185-5p in LSINCT5-induced changes of OSCC cell functions was then explored. Transfection efficacy of miR-185-5p inhibitor was examined in Tca8113 and CAL-27 cells (Figure 4A). Interestingly, reduced viability and EdU-positive rate in OSCC cells with LSINCT5 knockdown were reversed by co-knockdown of miR-185-5p (Figure 4B-4D). Similarly, the inhibited migratory capacity of OSCC caused by knockdown of LSINCT5 was partially abolished by co-transfection of miR-185-5p inhibitor (Figure 4E). Taken

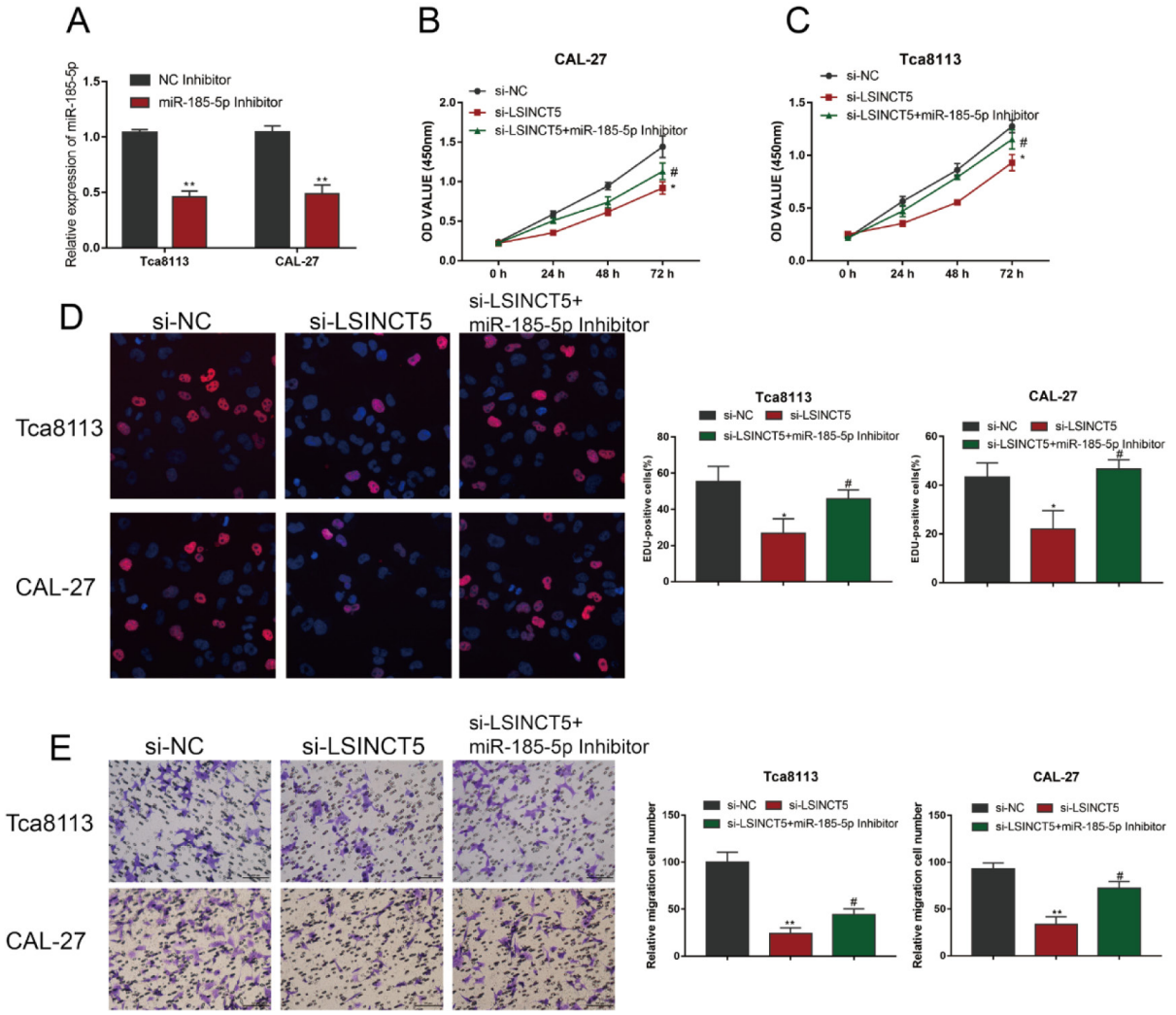


**Figure 4.** MiRNA-185-5p abolished the role of LSINCT5 in regulating OSCC cell functions. **A:** Transfection of miRNA-185-5p inhibitor significantly downregulated miRNA-185-5p in OSCC cells; **B, C:** Co-knockdown of LSINCT5 and miRNA-185-5p reversed the inhibited viability in OSCC cells with LSINCT5 knockdown; **D:** Co-knockdown of LSINCT5 and miRNA-185-5p reversed the inhibited EdU-positive rate in OSCC cells with LSINCT5 knockdown; **E:** Co-knockdown of LSINCT5 and miRNA-185-5p reversed the inhibited migration in OSCC cells with LSINCT5 knockdown (magnification 200×). *p<0.05 and **p<0.01 *vs.* si-NC group; #p<0.05 *vs.* si-LSINCT5 group.

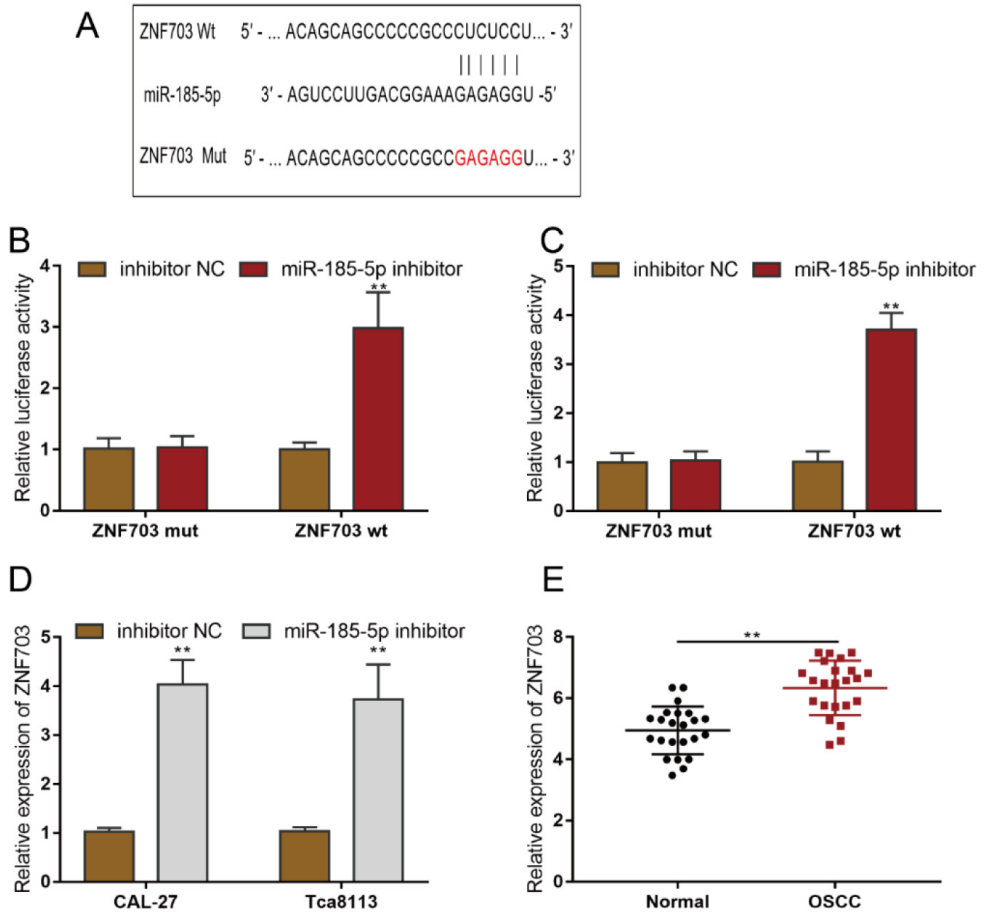


**Figure 5.** ZNF703 was the target gene of miRNA-185-5p. **A:** A binding site in ZNF703 3'UTR that was paired to that of miRNA-185-5p; **B, C:** Binding relationship between ZNF703 and miRNA-185-5p; **D:** Knockdown of miRNA-185-5p upregulated ZNF703 in OSCC cells; **E:** ZNF703 was upregulated in OSCC tissues. **p<0.01.

together, miR-185-5p displayed an anti-cancer role in OSCC, and LSINCT5 drove the malignant phenotypes of OSCC by downregulating miR-185-5p.

*ZNF703 was the target gene of miRNA-185-5p*

MiRs induce gene degradation through binding miR 3'UTR that induces gene degradation [11]. Here, we proved that the oncogenic gene ZNF703 was the target gene of miR-185-5p (Figure 5A-5C). ZNF703 level was negatively regulated by miR-185-5p in OSCC cells, and it was highly expressed in clinical specimens of OSCC (Figure 5D, 5E). The anti-cancer role of miR-185-5p in OSCC may be linked to the downregulation of ZNF703.

## Discussion

Recent research on lncRNAs has greatly deepened our understanding of oncogenes and tumor-suppressor genes [12]. LncRNAs are a new class of molecules that can be used as tumor diagnostic markers owing to their advantages of extensive distribution, high abundances and stable expressions in the body fluids [13]. Clinical symptoms and signs of OSCC are not obvious. Due to the deficiency of effective diagnostic biomarkers, a large number of OSCC patients are diagnosed in the middle or advanced stage at the time of initial diagnosis [14,15]. Emerging role of lncRNAs in OSCC has been identified [16]. In the present study, we focused on the biological potentials of LSINCT5 in the malignant progression of OSCC. Our experimental data have shown that LSINCT5 was upregulated in OSCC tissues and cell lines. After analysis of long-term follow-up data of recruited OSCC patients, it is concluded that high expression of LSINCT5 indicated poor overall survival of OSCC. We believed that LSINCT5 may be a functional oncogene involved in OSCC progression. Subsequently, *in vitro* experiments suggested that knockdown of LSINCT5 inhibited the proliferative and migratory capacities of OSCC.

It is previously reported that LSINCT6 is involved in tumor progression either through targeting downstream vital miRs or regulating signaling pathways [17,18]. Multiple transcription factors

are also able to mediate LSINCT5 expression. For example, EZH2 induced the regulatory effect of LSINCT5 on APC transcription, thus driving osteosarcoma progression [19]. The deterioration of bladder cancer is triggered by LSINCT5 *via* activating the Wnt signaling [20]. Direct target on mRs is another way that LSINCT5 affects tumor progression. Through enhancing the genomic stability of HMGA2, LSINCT5 maintains tumor activity by enhancing contents of oncogenes [21]. Our study revealed for the first time that LSINCT5 drove the malignant progression of OSCC through the ceRNA theory. MiR-185-5p was the downstream target binding LSINCT5, which is generally considered as a tumor-suppressor gene. MiR-185-5p is capable of stimulating apoptosis of prostate cancer cells [22]. Through targeting ROCK2, miR-185-5p protects the aggravation of liver cancer [23]. In oral cancer, miR-185-5p prevents malignant transformation of tumor cells with the involvement of the ZNF703 and YWHAZ [24,25].

The interaction between miR-185-5p and ZNF703 has been verified in our experiments. ZNF703 is a confirmed oncogene in oral cancer. High level of ZNF703 stimulates OSCC growth and metastasis *via* the PI3K/AKT/GSK-3β signaling, which is a molecular marker for the poor prognosis of OSCC [26]. Consistently, ZNF703 was upregulated in OSCC tissues. To sum up, the oncogenic role of LSINCT5 in OSCC relied on the miR-185-5p/ZNF703 axis. LSINCT5/miR-185-5p/ZNF703 feedback loop may be promising in the clinical treatment of OSCC.

## Conclusions

LSINCT5 is abnormally upregulated in OSCC specimens and drives its malignant progression through the miR-185-5p/ZNF703 axis.

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## Conflict of interests

The authors declare no conflict of interests.

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