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LINC00657 promotes malignant progression of oral squamous cell carcinoma *via* regulating microRNA-150

F.-Y. XU[1], X. XU[2], X.-D. HU[1]

[1]Department of Stomatology, Shaoxing Shangyu People's Hospital, Shaoxing, China
[2]Department of Oral and Maxillofacial Surgery, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China

Abstract. – OBJECTIVE: Previous studies have shown that LINC00657 is a cancer-promoting gene. However, the role of LINC00657 in oral squamous cell carcinoma (OSCC) has not been reported. This study was designed to investigate the role of LINC00657 in OSCC and its regulatory mechanism.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qPCR) was used to detect the levels of LINC00657 and microRNA-150 in 32 pairs of OSCC tissues and normal ones, and the correlation between LINC00657 and clinical indicators and OSCC patient's prognosis was analyzed. qRT-PCR further verified the levels of LINC00657 and microRNA-150 in OSCC cells. In addition, LINC00657 overexpression and knockdown models were constructed using lentivirus in OSCC cell lines Fadu and Tca8113, and Cell Counting Kit-8 (CCK-8), plate clone experiment, and 5-Ethynyl-2'-deoxyuridine (EdU) assay were carried out to evaluate the influence of LINC00657 on the biological functions of OSCC cells. Further, Luciferase reporter gene and recovery experiments were used to explore its potential mechanism.

RESULTS: qRT-PCR showed that LINC00657 expression in OSCC tissue specimens was increased in comparison to normal ones. Patients with high LINC00657 expression had higher pathological staging and lower overall survival. Besides, the cell proliferation ability of the LINC00657 silencing group was remarkably decreased, while the opposite result was observed in LINC00657 overexpression group. Subsequently, qRT-PCR demonstrated a significant decrease in microRNA-150 expression in OSCC cell lines and tissues and a negative correlation with LINC00657. Luciferase assay demonstrated that LINC00657 could be targeted by microRNA-150 in certain binding sites. In addition, cell reverse experiment also confirmed that LINC00657 and microRNA-150 can be mutually regulated, thereby jointly modulating the malignant progression of OSCC.

CONCLUSIONS: LINC00657, remarkably upregulated in OSCC tissues, showed a close association with the poor prognosis of OSCC patients. Additionally, it may accelerate the malignant progression of OSCC via regulating microRNA-150.

Key Words:
LINC00657, MicroRNA-150, Oral squamous cell carcinoma, Proliferation.

Introduction

Oral squamous cell carcinoma (OSCC) is a highly invasive tumor of head and neck malignancies, often involving adjacent bone, muscle, skin tissue, and local lymphoid tissue[1-3]. OSCC accounts for about 3% of all new malignant tumor cases, including lip cancer, soft, and hard palate cancer, bottom of mouth cancer, oropharyngeal cancer[4,5]. Although surgical operation and chemoradiotherapy techniques have made continuous progress in OSCC treatment in recent years, their invasive, metastatic and recurrent characteristics still limited the therapeutic effect. The overall five-year survival rate of OSCC patients is still only 50%-60%, seriously threatening people's health[6,7]. The aggressive and metastatic nature of the tumor is believed to be the main reason for poor prognosis of oral squamous cell carcinoma patients[8,9]. However, the underlying mechanism of OSCC remains elusive; therefore, it is of great importance to study and clarify the pathogenesis of OSCC, explore new targeted molecules to inhibit the occurrence and metastasis of OSCC, reduce the incidence, and improve the cure rate and survival rate of patients[10,11].

Slaby et al[12] have indicated that oncogenes and tumor suppressor genes need to be expanded to include not only protein-coding genes but also non-coding RNA (ncRNAs) tumor suppressor genes and lncRNAs oncogenes. According to

Corresponding Author: Feiyan Xu, BM; e-mail: 187212124@qq.com

some studies[13,14] of genome spectral analysis, only 1.5% of the entire genome DNA has the ability of encoding proteins, and the remaining of the DNA is transcribed into RNA and then not encode proteins; as a result, these RNAs are called ncRNAs. Some studies[15,16] have shown that there are about 450,000 ncRNAs in eukaryotes, including long non-coding RNA (lncRNA) and short non-coding RNA. There are about 7000-23,000 lncRNAs in the human genome, which are mRNA-like transcripts and exist as highly conserved RNA molecules with a length of 200 nt-100 kb but lack of open reading frame. They exist in the nucleus or cytoplasm like other transcription factors and do not encode proteins[16,17].

A large proportion of lncRNAs interact with adjacent protein-coding genes and function in the form of "lncRNA-miRNA" pairs[18,19]. These lncRNAs are related to each other and adjacent miRNAs in expression or function regulation[19]. Therefore, in this study, we analyzed the expressions of LINC00657 and microRNA-150 in OSCC tissue specimens, and explored the impact of this two ncRNAs on the biological functions of OSCC cells.

Patients and Methods

Patients and OSCC Samples

32 pairs of OSCC tissue specimens and adjacent ones were collected from OSCC patients diagnosed in the Department of Urology and Oncology of our hospital. All patients did not receive any treatment, such as radiotherapy and chemotherapy before the surgery and all collected tumor samples were confirmed as oral squamous cell carcinoma by postoperative pathology. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). This study complies with the Declaration of Helsinki, it was approved by the Ethics Committee of Shaoxing Shangyu People's Hospital, and all patients had signed informed consent. All patients were followed up after discharge. The follow-up included general conditions, clinical symptoms, and imaging examination.

Cell Lines and Reagents

Four human OSCC cell lines (Fadu, SCC-25, CAL-27, Tca8113) and a normal human oral cell line (Hs 680.Tg) provided by American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin in a 37°C cell incubator with 5% CO_2.

Transfection

Lentivirus transfection was performed with LINC00657 lentiviral sequence or microRNA-150 mimics or microRNA-150 inhibitor (GenePharma, Shanghai, China) when cell density reached to 70%. Cells were collected 48 hours later for cell experiments.

Cell Counting Kit-8 (CCK-8) Assay

Cells were plated in 96-well plates ($2*10^3$ cells/well) in 100 ul culture medium. Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Kumamoto, Japan) was performed based on the manufacturer's protocol.

Colony Formation Test

After 48 h of transfection, cells were collected, and 200 cells were seeded in each well of a 6-well plate and cultured with complete medium for 2 weeks. The medium was changed after one week and then twice a week. After 2 weeks, the cells were cloned, and then, fixed in 2 mL of methanol for 20 minutes. Cells were counted under a light-selective environment.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

To demonstrate the ability of LINC00657 and microRNA-150 to proliferate OSCC cells, the EDU proliferation assay (RiboBio, Nanjing, China) was performed according to the manufacturer's requirements. After transfection for 24 h, the cells were incubated with 50 μm EDU for 2 h, then, stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA), and the number of EDU-positive cells was detected by fluorescence microscopy. The display rate of EDU positive is shown as the ratio of the number of EDU positive cells to the total DAPI chromogenic cells (blue cells).

qRT-PCR

Total RNA was extracted from tissue samples using the TRIzol (Invitrogen, Grand Island, NY, USA) method. The reverse transcription reaction was performed with reference to the AMV reverse transcription kit, and 2 μg of total RNA was added to the 20 μL system for complementary deoxyribose nucleic acid (cDNA) synthesis. Real Time-PCR was performed using 2×SYBR Green PCR Master Mix (TaKaRa, Otsu, Shiga,

Japan). Primers were subjected to PCR amplification with β-actin as an internal reference. The PCR reaction was carried out on a quantitative PCR reactor. Data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA) and the relative levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used for qRT-PCR: LINC00657: forward: 5'-TGAAGGCAGAGAAGGAAGGG-3', reverse: 5'- TCCACCACATACACAGCACT-3'; microRNA-150: forward: 5'-TGTCGTGGAGTCGGCAATTCAGTTGAGCACTGG-3', reverse: 5'-ACACTCCAGCTGGGTCTCCCAACCCTT GTA-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'.

Luciferase Assay

The transcription factor expression plasmid to be tested was co-transfected with the reporter plasmid into the ccRCC cell line. The Luciferase activity was measured using a Luciferase reporter kit (Promega, Madison, WI, USA).

Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA). X^2-test and the exact probability Fisher test were used for Univariate analysis; COX regression analysis was performed for multivariate analysis. Data were expressed as mean ± standard deviation and were statistically significant when p less than 0.05.

Results

LINC00657 Was Upregulated in Human OSCC Tissues and Cell Lines

QRT-PCR analysis revealed a significant increased expression of LINC00657 both in OSCC tissue specimens and cell lines as compared to corresponding normal tissue samples (Figure 1A) and normal cell lines (Figure 1B).

LINC00657 Was Correlated With Metastasis Occurrence as Well as Survival of OSCC Patients

We divided the collected OSCC tissue specimens into high LINC00657 expression and low expression group, and further explored the association of LINC00657 with gender, pathological stage, age, and metastasis of OSCC patients. Table I indicates that LINC00657 expression showed a positive correlation with metastasis incidence, but

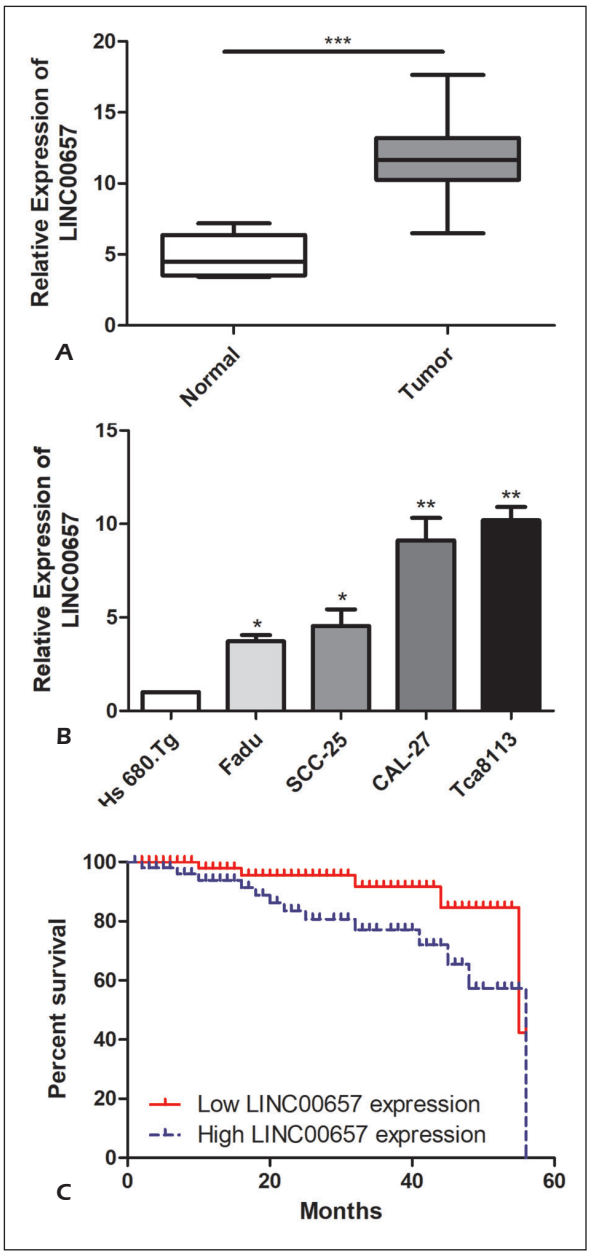

Figure 1. LINC00657 is highly expressed in oral squamous cell carcinoma tissues and cell lines. **A**, qPCR was used to detect the difference in expression of LINC00657 in tumor tissues and adjacent tissues of oral squamous cell carcinoma. **B**, qPCR was used to detect the expression level of LINC00657 in oral squamous cell carcinoma cell lines. **C**, Kaplan-Meier survival curve of patients with oral squamous cell carcinoma based on LINC00657 expression; the prognosis of patients with high expression was significantly worse than that of low expression group. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Table I. Association of LINC00657 and miR-150 expression with clinicopathologic characteristics of oral squamous cell carcinoma.

Parameters	No. of cases	LINC00657 expression		*p*-value	miR-150 expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.264			0.540
<60	11	7	4		3	8	
≥60	21	9	12		8	13	
Gender				0.480			0.710
Male	16	9	7		5	11	
Female	16	7	9		6	10	
T stage				0.009			0.012
T1-T2	21	14	7		4	17	
T3-T4	11	2	9		7	4	
Lymph node metastasis				0.127			0.210
No	22	13	9		6	16	
Yes	10	3	7		5	5	
Distance metastasis				0.264			0.082
No	21	12	9		5	16	
Yes	11	4	7		6	5	

not with the other three indexes (Figure 1C). In addition, Kaplan-Meier survival curve showed that highly expressed LINC00657 was relevant to the poor prognosis of OSCC patients ($p<0.05$; Figure 1C), suggesting that LINC00657 may be a new biological indicator for predicting the prognosis of OSCC.

Upregulation/Downregulation of LINC00657 Promoted/Inhibited Cell Growth

To specify the impact of LINC00657 on OSCC cell functions, we constructed LINC00657 overexpression (Fadu cells) and knockdown (Tca8113 cells) models and verified the transfection efficiency by qRT-PCR (Figure 2A). Further, the results of CCK-8, EDU, and cell cloning experiments revealed that LINC00657 silencing remarkably inhibited the proliferation ability of OSCC cells while LINC00657 overexpression promoted that (Figure 2B-2D).

MicroRNA-150 Is a Direct Target of LINC00657

Luciferase assay verified that LINC00657 can specifically combine with microRNA-150 (Figure 3A, 3B). In addition, qRT-PCR results showed that microRNA-150 expression was negatively regulated by LINC00657 (Figure 3C). Meanwhile, it was found that microRNA-150 was remarkably downregulated in 32 pairs of OSCC tumor tissues (Figure 3D, E), which suggests a negative correlation between LINC00657 and microRNA-150 in OSCC tissues (Figure 3F). These results showed that microRNA-150 was a direct target of LINC00657. LINC00657 could repress the expression of miR-150 by functioning as sponge.

Upregulation/Downregulation of MicroRNA-150 Inhibited/Promoted Cell Growth

To investigate the influence of microRNA-150 on OSCC cell growth, we overexpressed and knocked out microRNA-150 in the Fadu and Tca8113 cell lines (Figure 4A) and examined cell proliferation by CCK-8, EDU, and cell cloning experiments. The results revealed that microRNA-150 mimics significantly attenuated the proliferation ability of OSCC cells while microRNA-150 inhibitor oppositely enhanced that (Figure 4B-4D).

LINC00657 Modulated MicroRNA-150Expression in Human OSCC Cells

To confirm the interaction between LINC00657 and microRNA-150 in OSCC cells, we overexpressed microRNA-150 in a cell line that silenced LINC00657, or overexpressed microRNA-150 in a cell line overexpressing LINC00657 (Figure 5A). The results of CCK-8, EDU, and cell cloning experiments indicated that microRNA-150 could offset the effect of LINC00657 on OSCC cell proliferation (Figure 5B-5D). In sum, we concluded that LINC00657 could modulate microRNA-150 expression in human OSCC cells.

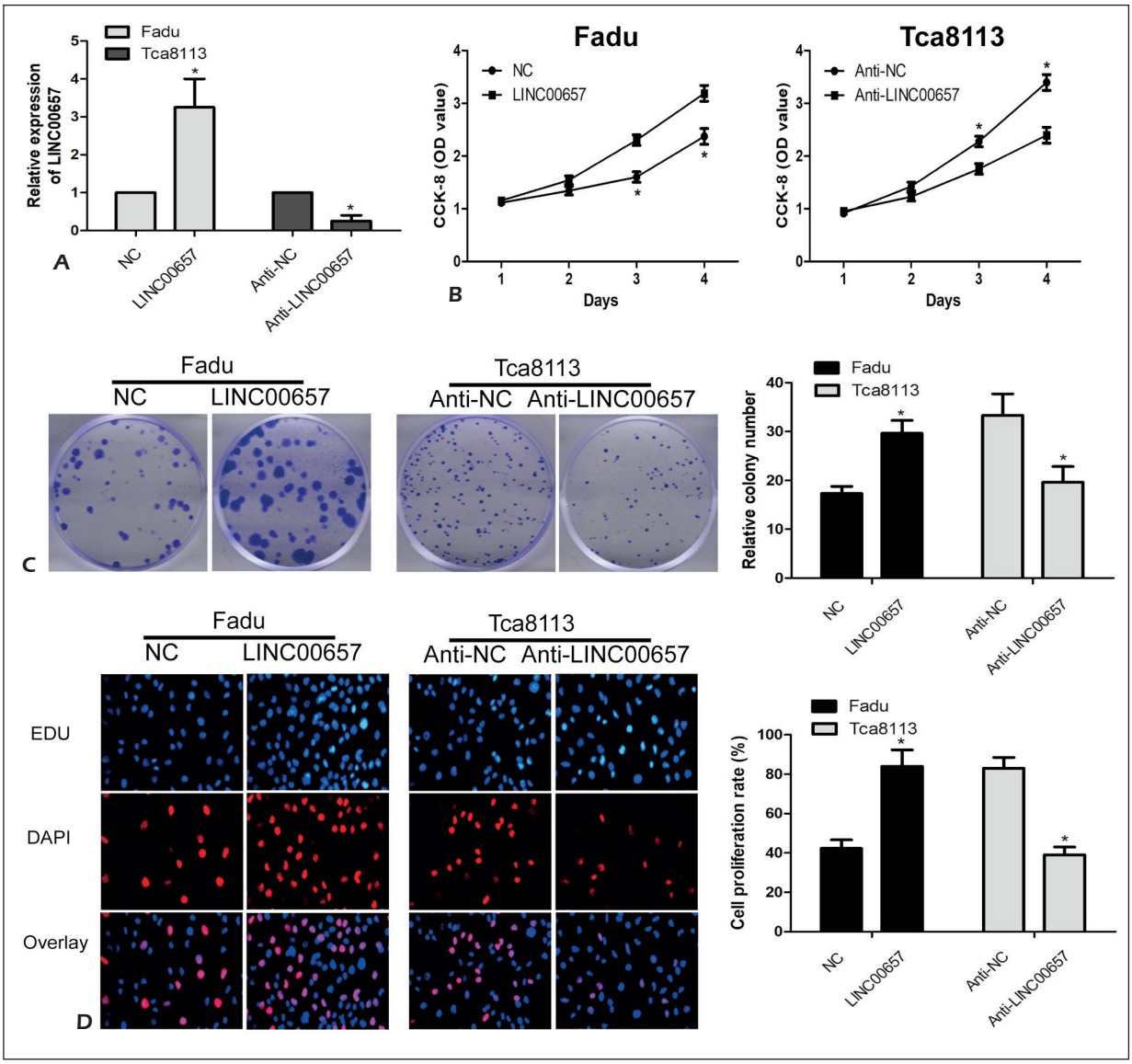

Figure 2. Overexpression/silencing LINC00657 promotes/suppresses the proliferation of oral squamous cell carcinoma cells. **A**, qPCR verified the transfection efficiency of LINC00657 after transfection of the LINC00657 overexpression/knockout vector in the Fadu and Tca8113 cell lines. **B**, CCK-8 assay detects the effect of transfection of LINC00657 on the proliferation of oral squamous carcinoma cells in Fadu and Tca8113 cell lines. **C**, Cell cloning experiments examined the ability of the Fadu and Tca8113 cell lines to transfect LINC00657 for the proliferation of oral squamous cell carcinoma (magnification: 10×). **D**, The EDU assay detects the ability of LINC00657 transfected with Fadu and Tca8113 cell lines to proliferate oral squamous cell carcinoma. (magnification: 400×). Data are mean ± SD, *$p<0.05$.

Discussion

OSCC is one of the most malignant head and neck tumors, accounting for about 75% of head and neck tumors, which seriously threatens human health[1-3]. Development of oral squamous carcinoma involves a multi-step process. It is often caused by mutations, inactivation of oncogenes, and tumor suppressor-related genes, or accumulation of multiple genes. Squamous cell carcinoma can gradually acquire anti-apoptosis, malignant proliferation, and immune escape during gene mutation inactivation[4-6]. The complex biological process of tissue carcinogenesis is mainly manifested in the overexpression of oncogenes and the downregulation of tumor suppressor genes[7,8].

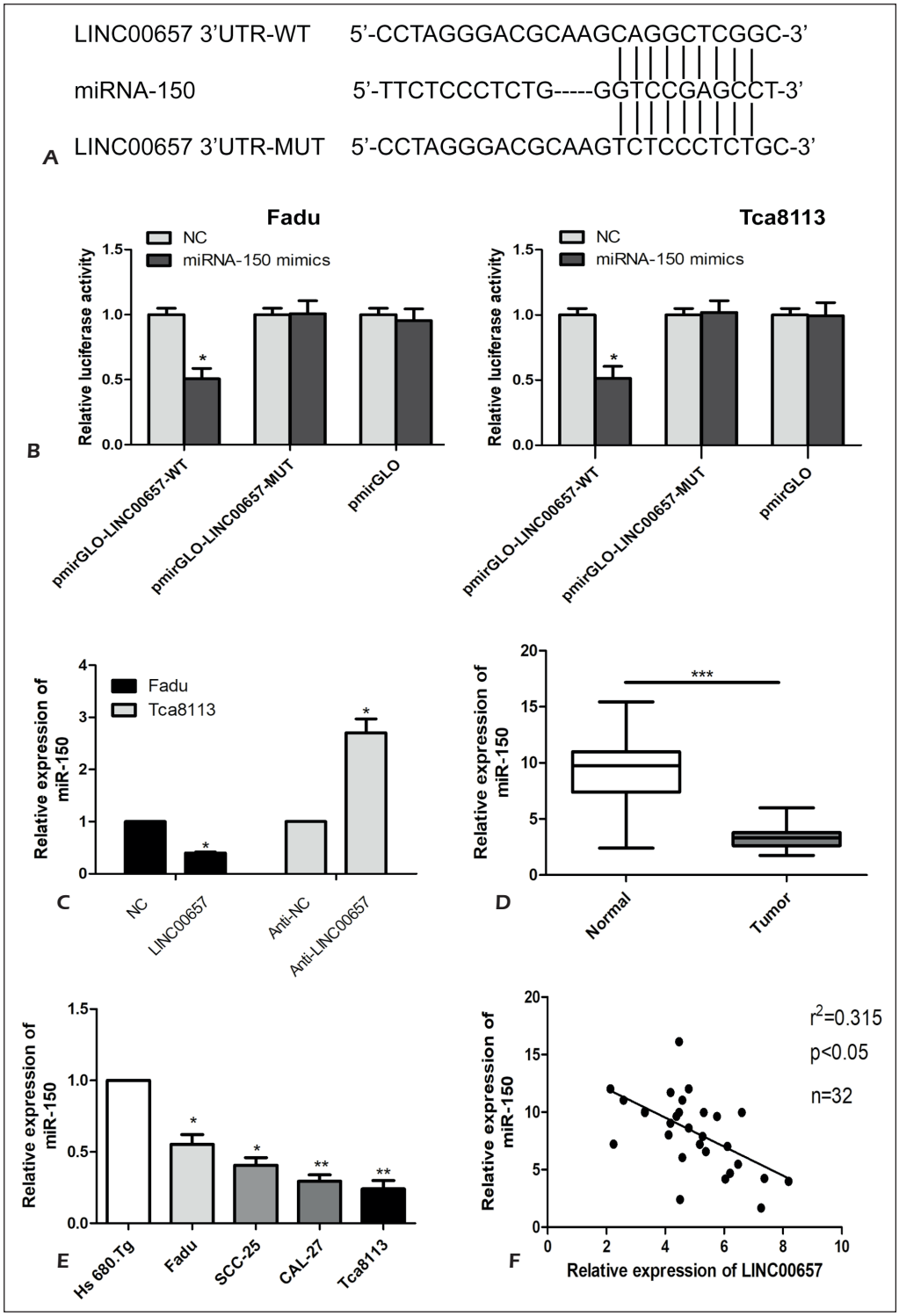

Figure 3. LINC00657 direct targeting of miR-150. **A-B**, The Dual-Luciferase reporter assay validated the direct targeting of LINC00657 and miR-150. The dual luciferase reporter gene assay in the Fadu and Tca8113 cell lines showed that overexpression of miR-150 significantly attenuated the Luciferase activity of the wild-type LINC00657 vector ($p<0.001$) without attenuating the vector containing the mutant ($p>0.05$) or Luciferase activity of the empty vector ($p>0.05$). **C**, qPCR detected differential expression of miR-150 after overexpression/silencing of LINC00657. **D**, qPCR was used to detect the differential expression of miR-150 in oral squamous cell carcinoma and adjacent tissues. **E**, qPCR was used to detect the expression level of miR-150 in oral squamous cell carcinoma cell lines. **F**, the expression level of LINC00657 and miR-150 in oral squamous cell carcinoma was significantly negatively correlated. Data are mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

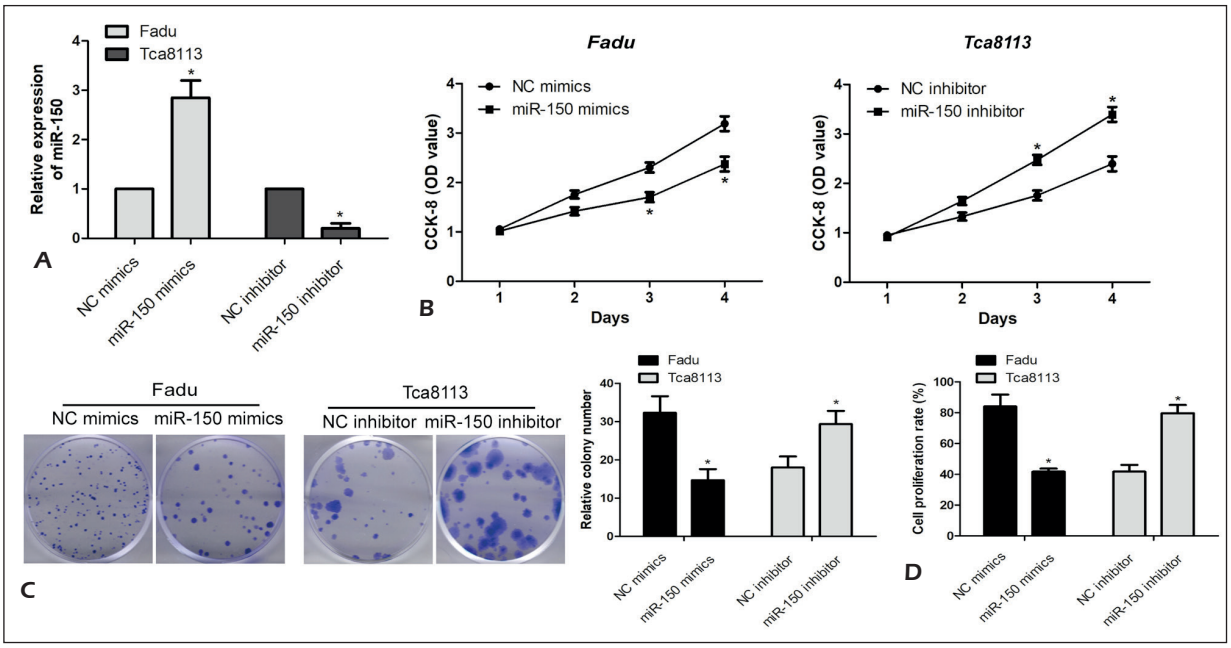

Figure 4. Overexpression/silencing of miR-150 inhibits/promotes the proliferation of oral squamous cell carcinoma cells. **A**, qPCR verified the transfection efficiency of LINC00657 after transfection of the miR-150 overexpression/silencing vector in the Fadu and Tca8113 cell lines. **B**, CCK-8 assay detects the effect of overexpression/silencing of miR-150 on proliferation of oral squamous carcinoma cells in Fadu and Tca8113 cell lines. **C**, Cell cloning assays tested the ability of overexpressing/silencing miR-150 on proliferation of oral squamous cell carcinoma cells in the Fadu and Tca8113 cell lines (magnification: 10×). **D**, The EDU assay detects the ability of overexpressing/silencing miR-150 on the proliferation of oral squamous carcinoma cells in the Fadu and Tca8113 cell lines. Data are mean ± SD, *$p<0.05$.

Many oncogenes and tumor suppressor genes, such as transcription factors, growth factors, nuclear proteins, and enzymes, have been found to be involved in the carcinogenesis process through the regulation of transcription and post-transcription levels[9-11]. Therefore, looking for abnormal expression of lncRNA and miRNA in OSCC and analyzing their correlation with clinical prognosis will help improve the level of diagnosis and treatment of OSCC and improve the clinical prognosis of patients[10,11].

Cell carcinogenesis and metastasis is a complex process involving multiple factors, genes and links, among which there are complex gene regulatory factors[9-11]. Oral squamous cell carcinoma is closely related to chronic inflammation, and anti-infection therapy is ineffective since its lymph node metastasis is related to chemokines and their receptors[7,8]. Long non-coding RNA (lncRNAs) plays an important role in cell development and metabolism, as well as the occurrence and development of tumors[12-15]. In this study, the levels of LINC00657 and microRNA-150 in 32 OSCC patients were observed to be abnormally regulated and were remarkably associated with pathological staging and poor prognosis of OSCC patients. Therefore, LINC00657 was considered to play a role in promoting cancer in OSCC while microRNA-150 was considered to play an inhibitory role in OSCC. To further clarify the impact of LINC00657 and microRNA-150 on the biological function of OSCC, overexpression and knockdown models of LINC00657 were constructed by lentivirus. CCK-8, cell cloning test, and EDU experiments revealed that LINC00657 could promote the development of OSCC, but its specific molecular mechanism was not clear.

Most lncRNAs regulate the expression of adjacent genes through transcriptional and post-transcriptional levels and participate in the protein translation process[15,16]. LncRNA-miRNA is also a new model in the epigenetics of disease regulation[17-19]. MicroRNA-150 is a key molecule in the miRNA family. In this study, we found that microRNA-150 was downregulated in OSCC tissues and inhibited the proliferation rate of OSCC cells. Bioinformatics analysis revealed that LINC00657 sequence contained a binding site of microRNA-150, and the direct binding of LINC00657 to downstream microRNA-150 was

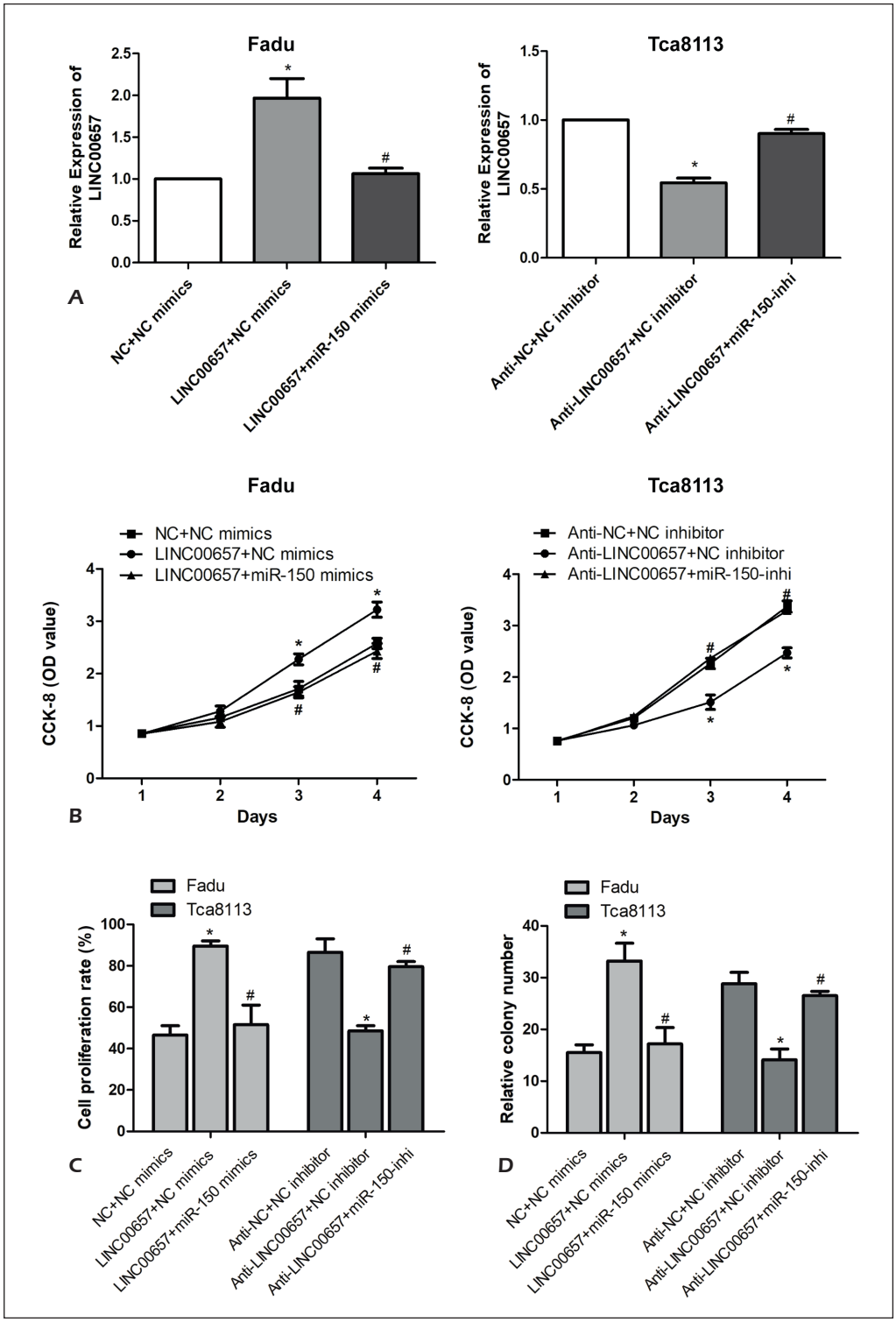

Figure 5. LINC00657 regulates the expression of miR-150 in oral squamous cell carcinoma. ***A***, The expression level of LINC00657 in LINC00657 and miR-150 co-transfected cell lines was detected by qPCR. ***B***, CCK-8 assay was used to detect the role of LINC00657 and miR-150 in the proliferation of oral squamous cell carcinoma cells in the Fadu and Tca8113 cell lines after co-transfection. ***C***, The EDU assay detects the ability of LINC00657 and miR-150 to co-transfect and regulate the proliferation of oral squamous carcinoma cells in the Fadu and Tca8113 cell lines. ***D***, The cell clone assay was used to detect the ability of LINC00657 and miR-150 to co-transfect in Fadu and Tca8113 cell lines to regulate the proliferation of oral squamous cell carcinoma cells. Data are mean ± SD, $*^{\#}p<0.05$.

verified by Dual-Luciferase gene reporter assay, and the LINC00657 vector that mutated the binding site of microRNA-150 failed to enrich microRNA-150, thus further confirming the binding site of LINC00657 and microRNA-150.

Conclusions

In summary, LINC00657, abnormally upregulated in OSCC tissues, shows a close association with the pathological stage, and poor prognosis of OSCC patients. Additionally, it may promote the malignant progression of OSCC through the modulation of microRNA-150.

Conflict of Interests

The authors declare that they have no conflict of interest.

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