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LncRNA LACAT1 promotes proliferation of oral squamous cell carcinoma cells by inhibiting microRNA-4301

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Abstract. – OBJECTIVE: To investigate the expression of lncRNA LACAT1 in oral squamous cell carcinoma (OSCC) tissues, and to further investigate its potential mechanism in OSCC as well as its relationship with clinicopathology and prognosis.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was used to analyze LACAT1 level in 78 pairs of OSCC tumor tissues and adjacent tissues, and the interplay between LACAT1 level and OSCC clinical indices along with patient's prognosis was analyzed. Further, the expression level of LACAT1 in OSCC cell line was verified by qRT-PCR. In addition, after LACAT1 knockdown model was constructed using lentivirus and transfected into OSCC cell lines, cell cloning assay and flow cytometry were used to analyze the impact of LACAT1 on biological functions of OSCC cells, and finally its association with microRNA-4301 was explored.

RESULTS: In this experiment, qRT-PCR results revealed that LACAT1 level in OSCC tumor tissue specimens was significantly higher than that in paracancerous tissues. In addition, the pathology stage in patients with higher level of LACAT1 was also higher while the overall survival rate was lower. Compared with sh-NC group, the cell proliferation ability of sh-LACAT1 group was significantly decreased while cell apoptosis was oppositely increased. QRT-PCR results showed that microRNA-4301 level was significantly elevated in cells of sh-LACAT1 group, suggesting that the expression of above two molecules was negatively correlated. Meanwhile, cell reverse experiment also demonstrated that LACAT1 and microRNA-4301 can be mutually regulated, and thereby promote the malignant progression of OSCC.

CONCLUSIONS: The LACAT1 level was found significantly increased in OSCC tissues and cells, which resulted in an advanced OSCC pathological stage and a poor prognosis of patients. In addition, it was found that LACAT1 may promote the malignant progression of OSCC by modulating microRNA-4301 activity.

Key Words:
LACAT1, microRNA-4301, Oral squamous cell carcinoma, Malignant progression.

Introduction

Oral cavity squamous cell carcinoma (OSCC), accounting for about 3% of all new malignant tumors, includes lip cancer, gingival cancer, tongue cancer, soft and hard sputum cancer, jaw cancer, oral cancer, and oropharyngeal cancer, and is a highly invasive tumor in head and neck malignant tumors, often invading adjacent bone, muscle, skin tissue and local lymphoid tissue[1-4]. Although surgery, radiotherapy and chemotherapy have made continuous progress in the treatment of OSCC in recent years, its invasive, metastatic and recurrent characteristics still make the treatment efficacy limited, which seriously threaten people's health. The overall five-year survival rate of OSCC patients is still only 50-60%[5,6]. The strong proliferation of tumor cell is considered to be the main cause of poor prognosis in patients with OSCC[7]. However, its underlying mechanism remains elusive. Hence, it is crucial to clarify the pathogenesis of OSCC and explore new targeting molecules that inhibit the occurrence, growth and metastasis of OSCC, so as to reduce the incidence rate and improve the survival rate of patients[2]. Recent studies[8,9] have pointed out that oncogenes and tumor suppressor genes need to be broadly defined, not only protein-coding genes but also "non-coding RNA (ncRNAs) tumor suppressor genes" and "ncRNAs oncogenes". Recent studies on genomic profiling have shown that 90% of the human genome has been sequenced, only 1.5% of the entire genome has the ability to encode proteins, and the remaining 88.5% of DNA is transcribed

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into RNA without encoding proteins, which are called non coding RNAs (ncRNAs)[10-13]. Some studies have shown that approximately 450,000 non-coding RNAs are present in eukaryotes, including long non-coding RNAs (lncRNAs) and short non-coding RNAs (miRNAs)[14]. About 7,000-23,000 lncRNAs in the human genome are messenger RNA transcripts, which exist as highly conserved RNA molecules with a molecular length of 200 nt-100 kb, and are located in the nucleus or cytoplasm like other transcription factors[15,16]. In this work, we analyzed the expression of LACAT1 in 78 pairs of OSCC tumor tissues and adjacent tissues, and explored the effects of LACAT1 on the biological functions of OSCC cells. Previous studies have indicated that microRNA-4301 can accelerate the process of tumor cell proliferation and differentiation, thus affecting the development of tumors. This study was designed to investigate the association between LACAT1 and clinical parameters and prognosis of patients with OSCC and further explore its role in cancer promotion.

Patients and Methods

Patients and OSCC Samples

We collected OSCC tissues and paracancerous specimens from 78 pairs of surgically resected OSCC patients, which referred to the 8th edition of UICC/AJCC oral squamous cell carcinoma tumor node metastasis (TNM) staging criteria. All patients were diagnosed as OSCC by postoperative pathology analysis, and no anti-tumor treatment such as radiotherapy or chemotherapy was given before surgery. The study has been approved by the Ethics Oversight Committee, and patients and their families had been fully informed that their specimens would be used for scientific research and signed relevant informed consent.

Cell Lines and Reagents

Four human oral squamous cell carcinoma cell lines (Fadu, SCC-25, CAL-27, Tca8113) and a normal human oral cell line (Hs 680.Tg) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) medium and fetal bovine serum (FBS) were all provided by Life Technologies (Gaithersburg, MD, USA). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C incubator with 5% CO_2.

shRNA Transfection

Negative control (sh-NC) and shRNA containing the LACAT1 interference sequence (sh-LACAT1) were purchased from Shanghai Jima Company (Shanghai, China). Cells were seeded in 6-well plates and grown to a cell density of 70%, transfected with lentiviral vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h. Then quantitative Real-time polymerase chain reaction (qRT-PCR) analysis and cell function experiments were performed.

Cell Proliferation Assay

The cells after 48 h of transfection were harvested and the cells were plated into 78-well plates at 2000 cells per well. The cells were cultured for 6 h, 24 h, 48 h and 72 h respectively, and then cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added in. After incubation for 2 hours, the optical density (OD) value of each well was measured in the microplate reader at 490 nm.

Colony Formation Assay

After 48 h of transfection, cells were collected, and 200 cells were seeded in each well of a 6-well plate and cultured in complete medium for 2 weeks. The medium was changed after one week, and then changed twice a week. After 2 weeks, the cells were cloned, the medium was discarded, and the cells were washed twice with phosphate-buffered saline (PBS). Next, the cells were fixed in 2 mL of methanol for 20 minutes. After methanol was aspirated, cells were stained with 0.1% crystal violet staining solution for 20 minutes. At last, cell colonies were photographed and counted under a light-selective environment.

Flow Cytometry Analysis

In order to detect apoptotic cells, Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, Nanjing, China) was used according to the protocol. To determine the cell cycle, transfected cells in 70% ethanol were stained with propidium iodide (PI) for 30 minutes. The analysis was performed in a BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometer and FlowJo software.

QRT-PCR

Total RNA was extracted from OSCC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa,

Otsu, Shiga, Japan). QRT-PCR reactions were performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System. The primers for qRT-PCR reaction were as follows: LACAT1:F: 5'-TGTTCGTCATGGGTGTG-3',R:5'-ATGGCATGGACTGTGGTCAT-3'; β-actin:F:5›-CCTGGCACCCAGCACAAT-3›,R:5›-TGCCGTAGGTGTCCCTTTG-3›; microRNA-4301:F:5›-CAAAGGCGTCGTCAATCACC-3›,R:5›-TTCCAAACCTTTGAGGGCGA-3›;U6:F:5›-UUCUCCGAACGUGUCACGUTT-3›,R:5›-ACGUGACACGUUCGGAGAATT-3›. Data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA) and the relative expression levels of detective genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Dual Luciferase Reporter Assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected with the reporter gene plasmid into the OSCC cell line. A specific luciferase substrate was added, and luciferase reacted with the substrate to generate fluorescence. By measuring the intensity of the fluorescence, the activity of the luciferase could be determined to confirm whether the transcription factor could interact with the target promoter fragment.

Statistical Analysis

The procedure was performed using the Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Data were expressed as mean ± standard deviation ($\bar{x}\pm s$), and p-values < 0.05 were considered statistically significant. The continuous variables were analyzed using the t-test, and the categorical variables were analyzed using the χ^2-test or Fisher's exact probability method. Kaplan-Meier analysis was used to evaluate the prognosis survival time of patients, and the difference between different curves was compared by Log-rank test.

Results

LACAT1 Had a High Level in OSCC Tissues and Cell Lines

We examined the expression of LACAT1 in 78 pairs of OSCC tissue specimens as well as adjacent ones and in OSCC cell lines by qRT-PCR. The results revealed that LACAT1 level was significantly increased in OSCC tissues compared with the non-tumor tissues, and the difference was statistically significant (Figure 1A). Compared with Hs 680.Tg, a normal human oral cell line, LACAT1 was highly expressed in OSCC cells (Figure 1B).

LACAT1 Expression was Correlated with Clinical Stage and Overall Survival in OSCC Patients

Based on the qRT-PCR results of 78 pairs of OSCC tissues and paracancerous tissues, the LACAT1 expression was divided into high expression group and low expression group. Chi-square test was applied to figure out the interplay between LACAT1 and age, gender, clinical stage, lymph node or distant metastasis of OSCC patients. Table I suggested that high level of LACAT1 was positively related to OSCC pathological stage, but not with age, gender, lymph node or distant metastasis (Figure 1C). In addition, in order to explore the relationship between the expression of LACAT1 and the prognosis of OSCC patients, we collected relevant follow-up data. Kaplan-Meier survival curves indicated that high LACAT1 was significantly relevant to the poor prognosis of OSCC patients, and the higher the LACAT1 expression level, the worse the prognosis (Figure 1D).

Knockdown of LACAT1 Inhibited Cell Proliferation and Promoted Cell Apoptosis

To explore the effect of LACAT1 on the proliferation of OSCC cells, we first successfully constructed the LACAT1 interference model (Figure 2A) and performed CCK8 and colony formation assays to test cell proliferation in the sh-NC and sh-LACAT1 group and flow cytometry to detect cell apoptosis. As shown in Figure 2B and 2C, the cell proliferation ability was obviously weakened in the sh-LACAT1 group compared to the sh-NC group, while flow cytometry result revealed that cell apoptosis was significantly enhanced in the sh-LACAT1 group (Figure 2D).

Knockdown of LACAT1 Changed the Expression of microRNA-4301

To further explore the ways in which LACAT1 promotes the malignant progression of OSCC, we found a possible relationship between microRNA-4301 and LACAT1 through relevant bioinformatics analysis. Subsequently, we examined the expression changes of microRNA-4301 after

knockdown of LACAT1 by qRT-PCR, and microRNA-4301 was found significantly increased after LACAT1 knockdown (Figure 3A). In addition, microRNA-4301 level in 78 pairs of OSCC tumor tissues or OSCC cell lines was found significantly decreased compared with adjacent normal tissues or normal cell line (Figure 3B, 3C). In addition, we examined the expression of LACAT1 and microRNA-4301 in 78 pairs of OSCC tissue samples by qRT-PCR and found the expression of this two was negatively correlated (Figure 3D).

LACAT1 Bound to microRNA-4301

As shown in Figure 3E, in order to further verify the targeting of microRNA-4301 to LACAT1, a luciferase reporter assay was performed. The results indicated that overexpressed microRNA-4301 obviously attenuated the luciferase activity of the wild-type LACAT1 vector ($p<0.05$) without attenuating the luciferase activity of the mutant vector or empty vector, further demonstrating that LACAT1 can be targeted by microRNA-4301through this binding site.

MicroRNA-4301 Modulated LACAT1 Expression in Human OSCC Cells

To investigate the interplay between microRNA-4301 and LACAT1, we constructed a microRNA-4301 knockdown vector based on the sh-LACAT1 cell line and confirmed the transfection efficiency by qRT-PCR (Figure 4A). Subsequently, cell proliferation was examined by CCK8 and colony formation assays, it was found that the cell proliferation of the sh-LACAT1+microRNA-4301 inhibitor group was obviously increased comparing to the sh-LACAT1+NC group (Figure 4B and 4C). Furthermore, flow cytometry experiment revealed that cell apoptosis in the sh-LACAT1+microRNA-4301 inhibitor group was significantly lower than that in the sh-LACAT1+NC one (Figure 4D).

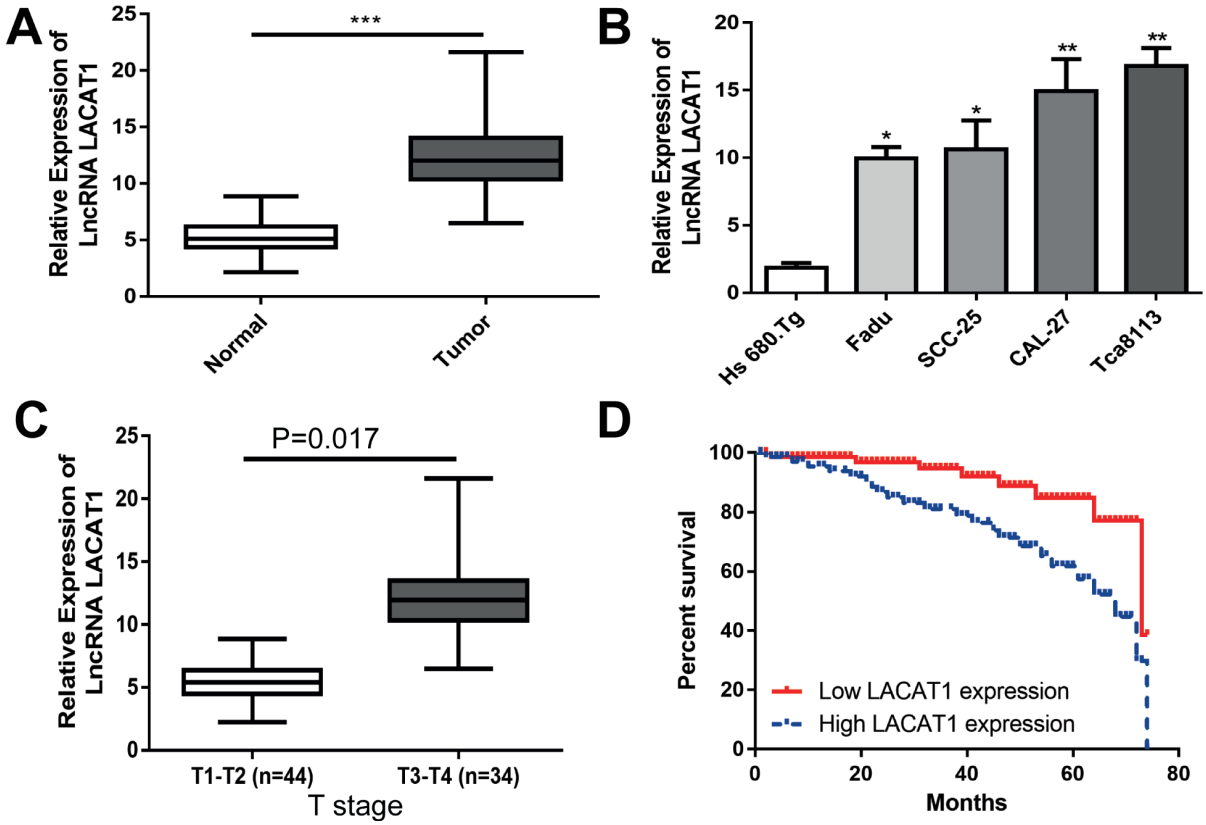

Figure 1. LACAT1 is highly expressed in oral squamous cell carcinoma tissues and cell lines. ***A,*** qRT-PCR was used to detect LACAT1 expression in tumor tissues and paracancerous tissues of oral squamous cell carcinoma; ***B,*** qRT-PCR was used to detect the expression level of LACAT1 in oral squamous cell carcinoma cell lines; ***C,*** qRT- PCR was used to detect the expression of LACAT1 in different pathological stages of oral squamous cell carcinoma; ***D,*** Kaplan Meier survival curve of patients with oral squamous cell carcinoma based on LACAT1 expression was shown; 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 LncRNA LACAT1 expression with clinicopathologic.

Parameters	Number of cases	LncRNA LACAT1 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.522
< 60	33	20	13	
≥ 60	45	24	21	
Gender				0.103
Male	38	25	13	
Female	40	19	21	
T stage				0.017
T1-T2	44	30	14	
T3-T4	34	14	20	
Lymph node metastasis				0.157
No	46	29	17	
Yes	32	15	17	
Distance metastasis				0.233
No	58	35	23	
Yes	20	9	11	

Characteristics of oral squamous cell carcinoma.

Discussion

It has been demonstrated that the underlying mechanism of OSCC pathogenesis is the broken balance in cellular self-regulated cell networks and gene expression programs, i.e., even the slightest disturbances in these signaling pathways can lead to cell transformation. For decades, studies have revealed that DNA damage responses, growth arrest, cell survival and apoptotic pathways can control these key gene mutation factors, mainly including oncogene activation and tumor suppressor gene inactivation[4-6]. In summary, the molecular biological mechanism of OSCC is related to a multi-gene epigenetic and metabolic process[2,7]. However, little is known about the potential molecules of OSCC carcinogenesis. Therefore, the identification of new molecular markers that can provide new opportunities for the prognosis and treatment of OSCC is needed[2]. Classical molecular biology theory states that genetic information is transmitted in the form of genetic coding, and RNA acts only on DNA sequences and proteins that encode proteins[5-7]. With the development of molecular biology theory and the Human Genome Project, it has been recognized that only 1.5% of genes in the human genome are protein-coding genes, and more than 90% of genes are transcribed into non-coding RNAs that do not have protein-coding functions (Non-coding RNAs)[8-10]. Long-chain non-coding RNAs are RNAs that are greater than 200 nucleotides in length and lack a meaningful open reading frame and protein-coding function, accounting for approximately 80% of all non-coding RNAs[11-13]. By regulating gene expression and other pathways, LncRNA is widely involved in many processes of cell life activities, such as cell proliferation, differentiation, and chromosome inactivation[13,14]. To date, multiple lncRNAs have been shown to regulate gene expression at transcriptional, post-transcriptional levels and by epigenetic ways, including modification of chromosomes, maintenance of mRNA stability, and inhibition of miRNA degradation of mRNA[17,18]. In the field of cancer research, LncRNA also presents pivotal modulating functions[15,19]. Studies demonstrated that LncRNA can regulate biological behaviors such as apoptosis, proliferation, cycle and metastasis of tumor cells[15]. In addition, researchers have found important regulatory functions of LncRNA in a growing number of tumors, including breast cancer, lung cancer, pancreatic cancer, osteosarcoma, hepatocellular carcinoma, leukemia, etc.[15,20,21]. LncRNA is abnormally expressed in a series of malignant tumors, which indirectly indicates that it has the dual role of tumor promotion and tumor suppression[20,21]. Studies[22] have confirmed that LncRNA is characteristically expressed in different types of tumor tissues and peripheral blood, and different types of tumors have their corresponding LncRNA expression profiles. Many LncRNAs are associated with tumor etiology, pathology, clinical staging, tumor hormone

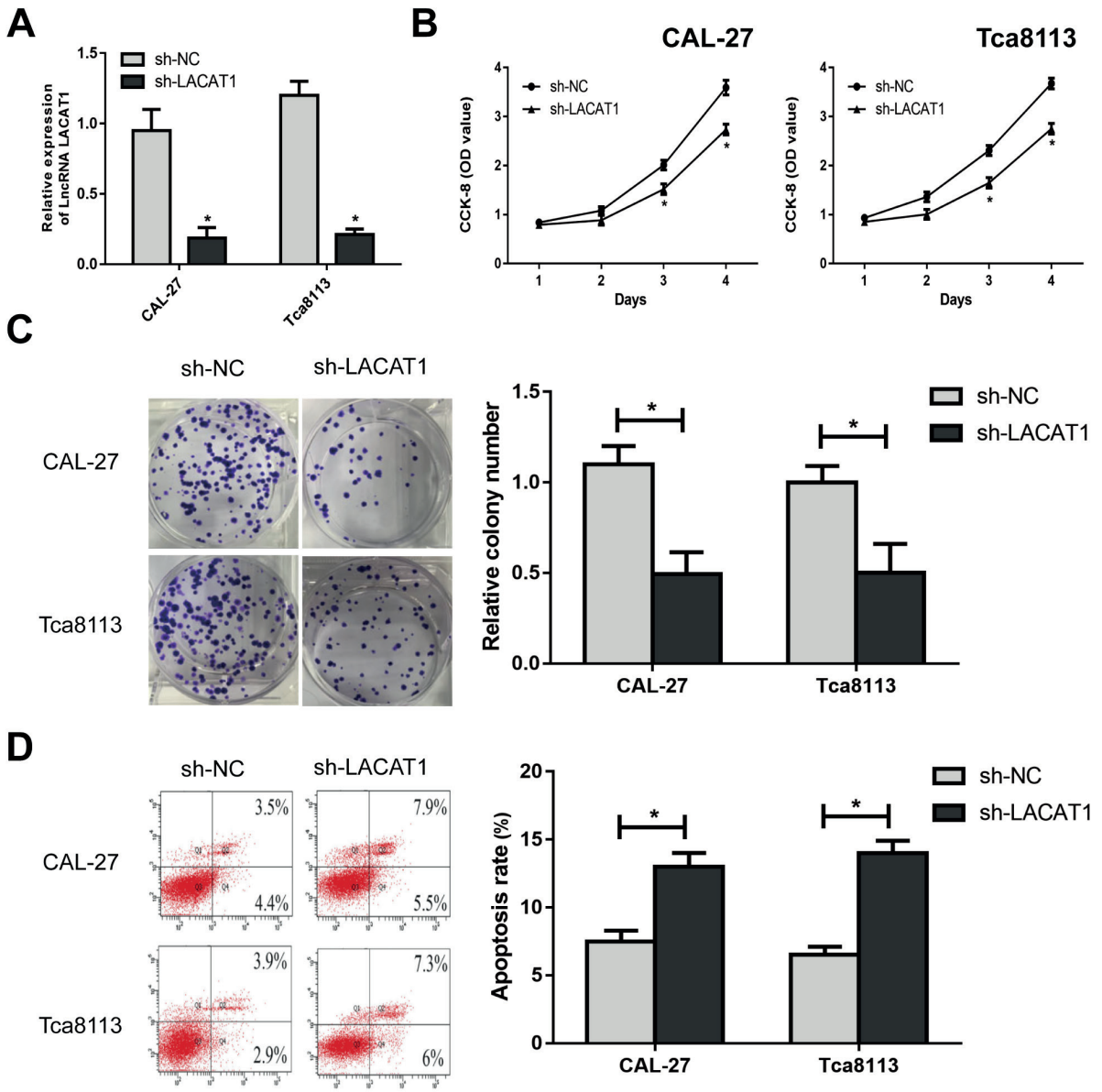

Figure 2. Silencing LACAT1 inhibits proliferation and promotes apoptosis of oral squamous cell carcinoma cells. ***A,*** qRT-PCR verified the interference efficiency of LACAT1 after transfection of LACAT1 knockout vector in CAL-27 and Tca8113 cell lines; ***B,*** CCK-8 assay was used to detect cell proliferation after LACAT1 was knockdowned in CAL-27 and Tca8113 cell lines; ***C,*** Clonal formation assay was used to detect the effect of silencing LACAT1 on proliferation of CAL-27 and Tca8113 cell lines; ***D,*** Flow cytometry was used to detect the effect of silencing LACAT1 on cell apoptosis of CAL-27 and Tca8113 cells. The data are mean± SD, $*p < 0.05$.

secretion, tumor resistance, and prognosis. This study investigated the relationship between LACAT1 and the clinical features of OSCC, as well as its role in malignant progression of OSCC. We first verified the expression of LACAT1 in 78 pairs of OSCC tissues and adjacent ones and found that LACAT1 was obviously up-regulated and positively correlated with the pathological stage and poor prognosis of OSCC. From this, we believed that LACAT1 might play a role in promoting cancer in OSCC. To further explore the effects of LACAT1 on the biological function of OSCC, we constructed a LACAT1 knockdown model using lentivirus. The results of CCK8, colony formation assay and flow cytometry showed that LACAT1 could indeed promote the development of OSCC and play a pivotal role in OSCC, but its specific molecular mechanism remains

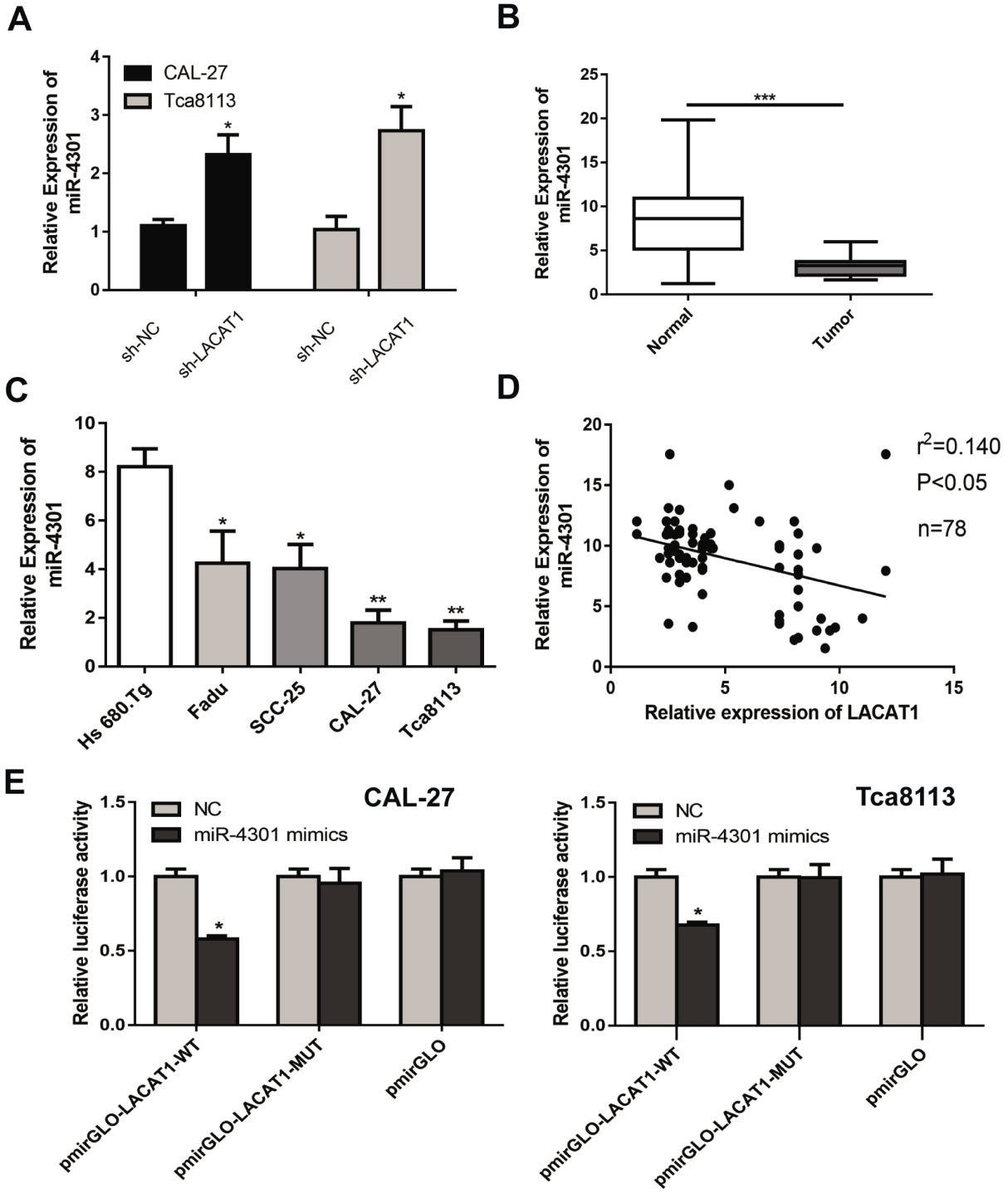

Figure 3. LACAT1 can directly target miR-4301. ***A,*** qRT-PCR verified the mRNA expression level of miR-4301 after transfection of LACAT1 in oral squamous cell carcinoma cell lines; ***B,*** qRT-PCR detection of miR-4301 in oral squamous cell carcinoma and adjacent tissues ***C,*** qRT-PCR detection of miR-4301 expression in oral squamous cell carcinoma cell lines; ***D,*** LACAT1 and miR-4301 expression level was significantly negatively correlated in oral squamous cell carcinoma tissue; E. Dual luciferase reporter assay demonstrated direct targeting of LACAT1 and miR-4301. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

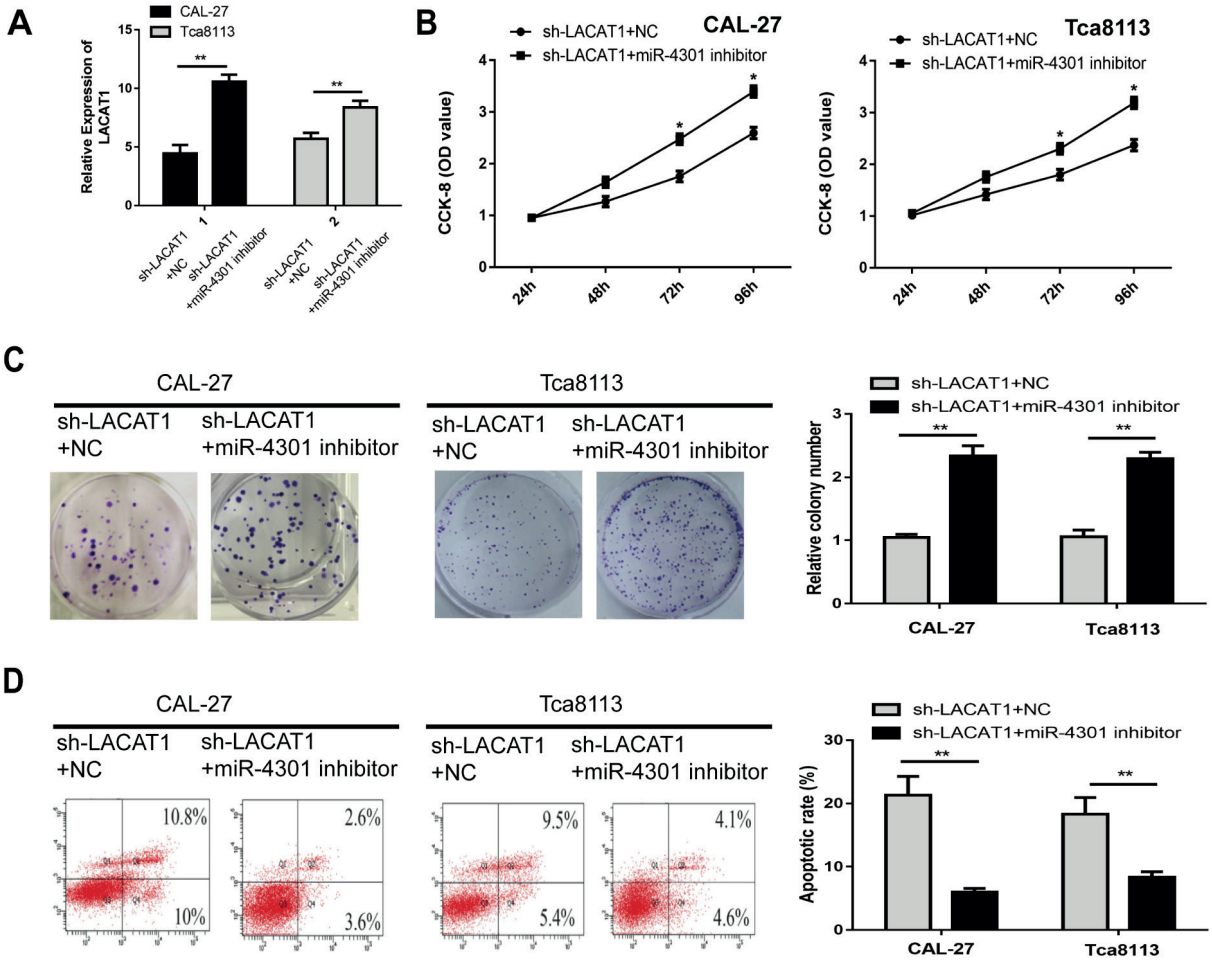

Figure 4. LACAT1 regulates the expression of miR-4301 in oral squamous cell carcinoma tissues and cell lines. ***A,*** qRT-PCR detection of LACAT1 expression levels in LACAT1 and miR-4301 co-transfected cell lines; ***B,*** CCK-8 assay was used to detect proliferation of oral squamous cell carcinoma cells after co-transfection of LACAT1 and miR-4301; ***C,*** Clonal formation assay was used to detect the proliferation of oral squamous cell carcinoma cells after co-transfection of LACAT1 and miR-4301; ***D,*** Flow cytometry assay was used to detect cell apoptosis of oral squamous cell carcinoma after co-transfection of LACAT1 and miR-4301. Data are mean ± SD, *$p<0.05$.

elusive. Numerous studies[17,23] in recent years have shown that a large proportion of lncRNAs interact with adjacent protein-coding genes and function as a "lncRNA-mRNA" pair. There is a correlation between expression or functional regulation between these lncRNAs and adjacent mRNAs[17]. LncRNAs mostly participate in protein translation processes through epigenetic, transcriptional and post-transcriptional multi-layer regulation of adjacent gene expression[24]. The "lncRNA-mRNA" pair is also a new model in epigenetics that regulates diseases[24,25]. In this investigation, we found through cell reverse experiments that there existed a mutual regulation between LACAT1 and microRNA-4301. As the research continues to deepen, further understanding of the biological function of microRNA-4301 and its role in the development of tumors will be more conducive to the diagnosis, treatment and prognosis of tumors. This undoubtedly brings good news to many cancer patients and their families, bringing new hope and dawn to human conquest of cancer. To demonstrate whether LACAT1 promotes the development of OSCC by regulating microRNA-4301, we examined the expression of microRNA-4301 after knockdown of LACAT1 by qRT-PCR. This suggested that LACAT1 promoted OSCC cell proliferation and inhibited cell apoptosis through microRNA-4301. In addition, we also showed that silencing microRNA-4301 restored OSCC cell

activities after LACAT1 knockdown, suggesting that LACAT1 might promote malignant progression of OSCC by regulating microRNA-4301.

Conclusions

We demonstrated that the expression of LACAT1 in OSCC was found significantly increased, and this increase can lead to an advanced OSCC pathological stage and a poor prognosis of patients. In addition, LACAT1 was showed to be able to promote the malignant progression of OSCC by regulating microRNA-4301.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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