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LncRNA CASC11 promotes the development of esophageal carcinoma by regulating KLF6

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression level of long non-coding RNA (lncRNA) CASC11 in esophageal carcinoma (ECa), and to further explore its relationship with clinical progression, pathological parameters, and prognosis of ECa patients.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to examine the level of lncRNA CASC11 in 45 pairs of ECa tissues and adjacent normal tissues. The relationship between the lncRNA CASC11 level and clinical progression, pathological parameters, and prognosis of ECa patients was analyzed. Meanwhile, the level of lncRNA CASC11 in the ECa cell lines was verified by qPCR as well. In addition, lncRNA CASC11 knockdown model was constructed using lentiviral transfection in ECa cell lines. Subsequently, the cell counting kit-8 (CCK8), colony formation assay, and flow cytometry were used to explore the effect of lncRNA CASC11 on the biological functions of the ECa cells. Finally, the Western Blot and the recovery experiments were used to explore the potential mechanism.

RESULTS: In this work, the qPCR results showed that the expression level of lncRNA CASC11 in the ECa tissues was remarkably higher than that of the adjacent normal tissues, and the difference was statistically significant ($p<0.05$). Compared with patients with a low level of lncRNA CASC11, the pathological stage of patients with high expression was significantly higher, while the overall survival rate was lower ($p<0.05$). Compared with negative control (NC) group, the proliferation ability of the cells in the lncRNA CASC11 knockdown group CASC11 significantly decreased, whereas cell apoptosis remarkably increased ($p<0.05$). The Western Blot results revealed that protein expression of KLF6 was remarkably up-regulated after lncRNA CASC11 knockdown. In addition, the recovery experiments found that lncRNA CASC11 and KLF6 had mutual regulation, thereby promoting the malignant progression of ECa.

CONCLUSIONS: LncRNA CASC11 expression was remarkably up-regulated in ECa, which was associated with the pathological stage and poor prognosis of ECa. In addition, lncRNA CASC11 could promote the malignant progression of ECa by mutual regulation of KLF6.

Key Words:
LncRNA CASC11, KLF6, Esophageal carcinoma (ECa), Proliferation.

Introduction

Esophageal carcinoma (ECa) is a highly malignant gastrointestinal tumor, which can be divided into two types, including: esophageal adenocarcinoma (ECA) and esophageal squamous cell carcinoma (ESCC). ECA is more common in Western countries, while ESCC is more prevalent in developing countries[1-3]. At present, surgical treatment is the preferred method for early ECa. However, many patients in the advanced stage have lost the opportunity to undergo surgery[4,5]. Therefore, the overall five-year survival rate of patients with ECa is extremely low[6]. Although multiple genetic and epigenetic changes have been found in ECa, the exact pathogenesis of ECa remains to be further elucidated[7-9]. Therefore, further exploration of the occurrence and development of ECa at the molecular level has important practical and theoretical value.

Long non-coding RNAs (lncRNAs) are a type of non-coding RNAs with over than 200 nucleotides in length[9-11]. In humans, lncRNA is widely distributed within genes. Although lncRNA does not encode proteins, it participates in a complex and important gene regulatory network, which subtly regulates gene expression[12,13]. Researchers[13-15] have shown that lncRNAs play important roles in normal tissue development. Meanwhile, they are of great importance in regulating cellular pluripotency and cell differentiation. In addition,

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lncRNAs are involved in the control of multiple molecular pathways. This can cause changes in the gene levels, ultimately regulating cell proliferation, apoptosis, and migration[15,16]. Therefore, the disorder of lncRNAs is closely related to many diseases in humans, such as malignant tumors[16].

LncRNA CASC11 is located at chromosome 8p12. Few reports[17,18] have focused on lncRNA CASC11 in the literature at home and abroad. A number of studies[17-20] have shown that lncRNA CASC11 plays an important role in the development of malignancies, such as non-small cell lung cancer, gastric cancer, bladder cancer, hepatocellular carcinoma, etc.. However, no reports have elucidated the role of lncRNA CASC11 in ECa. Therefore, for the first time, this study comprehensively analyzed the expression level and biological effects of lncRNA CASC11 in ECa. Furthermore, the molecular mechanism of its regulation was explored as well.

In this work, the quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was used to detect lncRNA CASC11 expression in ECa tissues and adjacent normal tissues. The correlation between the expression level of lncRNA CASC11 and the clinico-pathological features of ECa patients was analyzed as well. Subsequently, the effect of lncRNA CASC11 on the proliferation and apoptosis of ECa cell lines EC-109 and KYSE30 was detected. Our findings provided clues to explain the molecular mechanisms of ECa development and provided potential biomarkers for early diagnosis and population screening of ECa.

Patients and Methods

Patients and ECa Samples

45 pairs of ECa tissues and corresponding adjacent normal tissues were obtained from patients who underwent surgical resection. All collected tissues were frozen and stored in -80°C refrigerator for subsequent RNA extraction. The study was approved by the Ethics Oversight Committee of our hospital. No patient received chemotherapy before surgery. Informed consent was obtained from patients and their families. The pathological classification and staging criteria of ECa were performed according to the international association of cancer (UICC) ECa staging criteria.

Cell Lines and Reagents

Four human ECa cell lines (OE19, OE33, TE-1, KYSE30, and EC-109) and one human normal esophageal epithelial cell line (HEEC) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) and fetal bovine serum (FBS) in an incubator at 37°C with 5% CO_2.

Cell Transfection

LncRNA CASC11 knockdown model was constructed by transfection of sh-lncRNA CASC11 (100 nmol/L) in ECa cells KYSE30 and EC-109. All cells were divided into three groups, including: lncRNA CASC11 lentivirus transfection group 1 (sh-lncRNA CASC11-1), lncRNA CASC11 lentivirus transfection group 2 (sh-lncRNA CASC11-2), as well as negative control group (sh-NC). KLF6 knockout model was constructed by the transfection of KLF6 knockout vector in KYSE30 or EC-109 cells. The cells were first plated into 6-well plates and grown to a density of 70%. Cell transfection was performed according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Subsequently, the cells were collected for qPCR analysis and other functional experiments.

Cell Counting Kit-8 (CCK8) Assay

48 h after transfection, the cells were harvested and plated into 96-well plates at a density of 2000 cells per well. Subsequently, the cells were cultured for 6 h, 24 h, 48 h, and 72 h respectively. The cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was then added to each well, followed by incubation for 2 hours in the dark. Optical density (OD) of each well at the absorption wavelength of 490 nm was measured by a microplate reader.

Colony Formation Assay

After the proportion of cells reached 90%, the cells were scratched using a 100 uL tip, with the back line of the plate as the reference line. The position and thickness of the scratches in each hole were kept the same. After scratching, the cells were washed with prepared Phosphate-Buffered Saline (PBS) solution to remove the necrotic cells. Then, the cells were cultured in medium containing 10% serum. At 6 h and 24 h after the test, each group of the cell plates was placed under a microscope for observation and photographing.

Flow Cytometry

Cell apoptosis was detected using the Annexin V FITC (fluorescein isothiocyanate) Apoptosis

Kit. ECa cells (KYSE30 and EC-109) were first transfected with lncRNA CASC11 small interference RNA and negative control NC, respectively. After 72 h of incubation, the cells were harvested and operated according to the apoptosis kit instructions. Experimental results were analyzed by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA). The cells were divided into living cells, necrotic cells, and apoptotic cells. Finally, the proportion of the apoptotic cells in each group was calculated. The experiment was repeated for 3 times.

Quantitative Real-Time Polymerase Chain Reaction (QPCR)

The total RNA was extracted from cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA). The concentration of the extracted RNA was measured by an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). Subsequently, the RNA samples were reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). The QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The primers used for qPCR reaction were as follows: lncRNA CASC11: forward: 5'-GGACACCAACTATTGCTTCA-3', Reverse: 5'-TCCAGGCTCCAAATGTAGG-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', Reverse: 5'-AACGCTTCACGAATTTGCGT-3'; KLF6: forward: 5'-CCAGAGAGTTCCAGCACAGA-3', Reverse: 5'-CCGACTCCATCTGTTCCTCA-3'; β-actin: forward: 5'-CAGAGCTCCTCGTCTTGCC-3', Reverse: 5'-GTCGCCACCATGAGAGAC-3'.

The data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA). The relative expression levels of mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method. This experiment was repeated for 3 times.

Western Blot

72 h after transfection, the cells were collected and the total proteins were extracted. The concentration of the extracted protein was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electroporated onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in skimmed milk, the membranes were incubated with KLF6 mAb (1:500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (1:2000) at 4°C overnight. On the next day, the membranes were washed with Tris-Buffered Saline and Tween 20 (TBST) for 3 times and incubated with the corresponding secondary antibody (1:1000) at room temperature for 1 h. Then, the membranes were washed again with TBST for 3 times. The immuno-reactive bands were exposed by the enhanced chemiluminescence (ECL) assay.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. The measurement data were expressed as mean±standard deviation (x±s). The mean difference of lncRNA CASC11 level folds between the groups was analyzed by the paired sample test. The relationship between lncRNA CASC11 and the clinicopathological features was tested by the χ^2-test. The survival analysis was performed using the Kaplan-Meier method, and the survival curves were plotted. $p<0.05$ was considered statistically significant.

Results

LncRNA CASC11 Was Highly Expressed in ECa Tissues and Cell Lines

The expression level of lncRNA CASC11 in 45 pairs of ECa tissues and adjacent normal tissues, as well as ECa cell lines was detected by qPCR. The results showed that the level of lncRNA CASC11 in ECa tissues significantly increased when compared with that in adjacent normal tissues, and the difference was statistically significant ($p<0.05$) (Figure 1A). *In vitro* experiments showed that lncRNA CASC11 was remarkably highly expressed in ECa cells compared with normal esophageal epithelial cells (HEEC) ($p<0.05$). CASC11 expression was the highest in KYSE30 and EC-109 ECa cell lines (Figure 1B), which were then selected for subsequent experiments. All these results indicated that lncRNA CASC11 was highly expressed in ECa tissues and cell lines.

LncRNA CASC11 Level Was Correlated With Pathological Stage and Overall Survival of ECa Patients

According to the expression levels of lncRNA CASC11, the ECa patients were divided into two groups, including: high expression group and

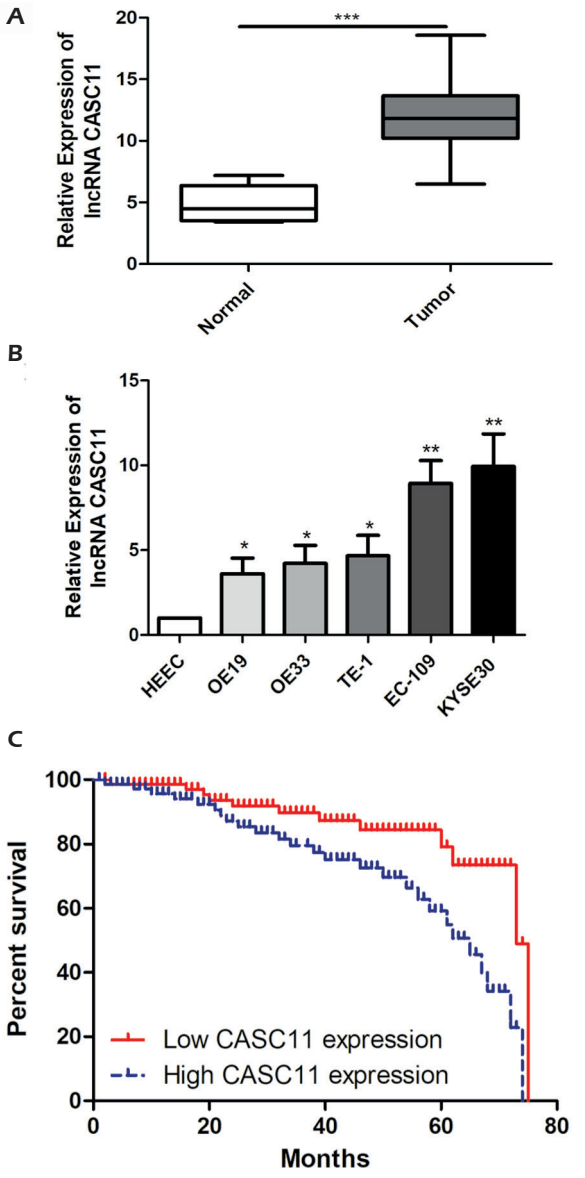

Figure 1. LncRNA CASC11 was highly expressed in ECa tissues and cell lines. **A,** QRT-PCR was used to detect the expression of lncRNA CASC11 in ECa tissues and adjacent non-tumor tissues. **B,** QRT-PCR was used to detect the expression level of lncRNA CASC11 in ECa cell lines. **C,** Kaplan-Meier survival curve of ECa patients based on lncRNA CASC11 expression. The prognosis of patients with high expression was significantly worse than that of those with low expression. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

low expression group. The number of patients in each group was counted. The Chi-square test was used to analyze the relationship between lncRNA CASC11 expression with age, gender, pathological stage, lymph node metastasis, and distant metastasis of ECa patients. As shown in Table I, the high level of lncRNA CASC11 was positively correlated with the pathological stage of ECa, whereas it was not associated with age, gender, lymph node metastasis, and distant metastasis. To further explore the relationship between the expression level of lncRNA CASC11 and the prognosis of ECa patients, we collected the relevant follow-up data. The Kaplan-Meier survival curves demonstrated that the high level of lncRNA CASC11 was remarkably associated with the poor prognosis of ECa. A higher level of lncRNA CASC11 indicated the significantly worse prognosis of patients (p<0.05; Figure 1C). Therefore, lncRNA CASC11 level was correlated with the pathological stage and overall survival of ECa patients.

Knockdown of LncRNA CASC11 Inhibited Cell Proliferation and Promoted Cell Apoptosis

To explore the role of lncRNA CASC11 in the proliferation and apoptosis of ECa cells, a silencing model of lncRNA CASC11 was first constructed (Figure 2A). The CCK-8 and colony formation assays were used to detect the effect of lncRNA CASC11 on cell proliferation. As shown in Figures 2B and 2C, the proliferation rate of the cells in sh-lncRNA CASC11 group significantly decreased when compared with the sh-NC group (p<0.05). Subsequently, cell apoptosis was detected by flow cytometry. Annexin V-FITC/PI double staining results showed that the apoptosis of sh-lncRNA CASC11 group was remarkably higher than that of the sh-NC group (p<0.05, Figure 2D). We concluded that the knockdown of lncRNA CASC11 inhibited cell proliferation and promoted cell apoptosis.

KLF6 Was Lowly Expressed in ECa Tissues and Cell Lines

To further explore the way in which lncRNA CASC11 affected the malignant progression of ECa, the Western Blot was performed. The results demonstrated that sh-lncRNA CASC11 remarkably reduced the level of KLF6 (Figure 3A). Subsequent qPCR results verified the above results as well (Figure 3B). Compared with adjacent normal tissues, the expression level of KLF6 in ECa tissues was remarkably lower, and the difference was statistically significant (p<0.05, Figure 3C). In addition, compared with HEEC, KLF6 was remarkably lower in ECa cells, and the difference was statistically significant (p<0.05, Figure 3D). These results suggested that KLF6 might

play a role as a tumor suppressor gene in ECa. Furthermore, the relationship between lncRNA CASC11 and KLF6 was detected by qPCR. The results showed that CASC11 expression was negatively correlated with KLF6 expression in the ECa tissues (Figure 3E).

KLF6 Modulated LncRNA CASC11 Expression in Human ECa Cells

To further explore the interaction between lncRNA CASC11 and KLF6 in ECa cells, we knocked out KLF6 in ECa cells of lncRNA CASC11 silencing group. This confirmed that there was some mutual interaction between lncRNA CASC11 and KLF6. At the cellular level, we constructed a KLF6 knockout vector model by transfection of small interference RNA. The transfection efficiency of KLF6 was examined by Western Blot and qPCR (Figures 4A and 4B). Subsequently, CCK-8, colony formation assay and flow cytometry showed that the knock-out of KLF6 in KNF6 and EC-109 cells with the silence of lncRNA CASC11 could remarkably enhance the proliferation of ECa cells and decrease cell apoptosis ($p<0.05$, Figures 4C and 4E). These results suggested that KLF6 modulated lncRNA CASC11 expression in human ECa cells.

Discussion

Due to the occultation of ECa, most patients have already been in an advanced stage when diagnosed. Currently, comprehensive treatment with radiotherapy and chemotherapy is not effective. Therefore, searching for new treatments is the key to improve the overall survival of patients with ECa[2,4-6]. Previous studies have indicated that proliferation, apoptosis, invasion, and migration are the most evident biological characteristics of malignant tumors. They are the root causes of malignant tumors as well. Therefore, how to inhibit the proliferation, invasion, and migration of malignant tumors is of great significance in controlling their progression and improving the survival rate of patients[7-9]. The malignant progression of tumors has been confirmed as a complex process involving multiple factors. In recent years, the regulation of lncRNA on tumor cell proliferation and apoptosis-related gene expression is a hot topic.

With the completion of the Human Genome Project, it has been found that in addition to approximately 20,000 protein-coding genes, the genome contains a large number of non-coding RNAs (ncRNAs)[10-12]. LncRNAs can regulate gene expression and function differently from that of miRNAs. It can not only affect the translational regulation of proteins, but also function through various pathways such as transcriptional active protein degradation of genes[13-15]. CASC11 is one of the newly discovered lncRNAs. It acts as a tumor-promoting gene by targeting the downstream gene level in several malignancies[17-20]. A large number of researches have confirmed that high level of CASC11 exists in a variety of malignant

Table I. Association of lncRNA CASC11 expression with clinicopathologic characteristics of esophageal cancer.

Parameters	Number of cases	lncRNA CASC11 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.259
<60	17	12	5	
≥60	28	15	13	
Gender				0.286
Male	22	14	8	
Female	23	11	12	
T stage				0.008
T1-T2	27	21	6	
T3-T4	18	7	11	
Lymph node metastasis				0.128
No	30	22	8	
Yes	15	7	7	
Distance metastasis				0.067
No	35	25	10	
Yes	10	4	6	

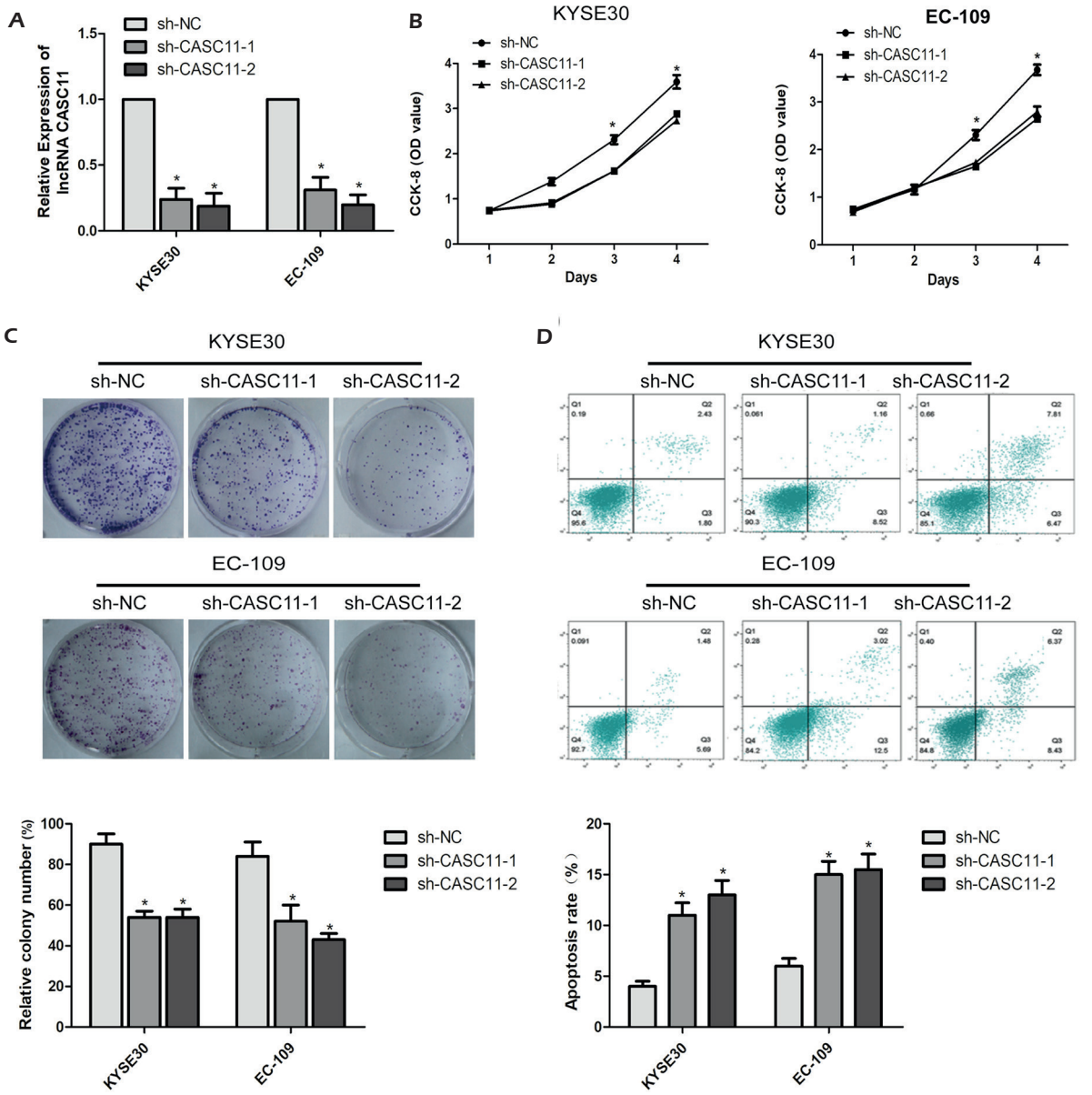

Figure 2. Silencing lncRNA CASC11 inhibited the proliferation of ECa cells and promoted apoptosis. **A,** QRT-PCR verified the interference efficiency of lncRNA CASC11 in KYSE30 and EC-109 cell lines. **B,** CCK-8 assay detected the effects of interference with lncRNA CASC11 on the proliferation of KYSE30 and EC-109 cell lines. **C,** Colony formation assay was performed to detect the effect of lncRNA CASC11 on the proliferation of KYSE30 and EC-109 cell lines (magnification '40). **D,** Flow cytometry assay was performed to detect the apoptosis of ECa cells after interference with lncRNA CASC11. Data were expressed as mean ± SD, *$p<0.05$.

tumors. Meanwhile, highly expressed CASC11 provides favorable conditions for the proliferation and differentiation of malignant tumor cells to some extent. However, the exact role of CASC11 in ECa has rarely been reported[17-20]. Based on previous findings, our study first verified the expression level of CASC11 in 45 pairs of ECa tumor tissues and adjacent tissues. The results showed that CASC11 expression was remarkably up-regulated, which was also positively correlated with the pathological stage and poor prognosis of ECa. All these findings suggested that CASC11 might play an oncogenic role in ECa development.

Cell proliferation and apoptosis are the orderly regulation of tumor progression under the control of genes to maintain the stability of the body environment[21,22]. Unlike passive cell necrosis, apoptosis is an active process. Meanwhile, it is closely

related to the activation, expression, and regulation of a series of genes[23]. The occurrence and development of malignant tumors are closely correlated with the proliferation and apoptosis of tumor cells. Apoptosis has a negative regulatory effect, which can effectively inhibit tumor growth. Therefore, how to induce tumor cell apoptosis has become a new direction of tumor therapy[21-23]. In this study, we investigated the relationship between CASC11 and the clinical features of ECa, as well as the role of CASC11 in ECa development and progression. ECa cell lines KYSE30 and EC-

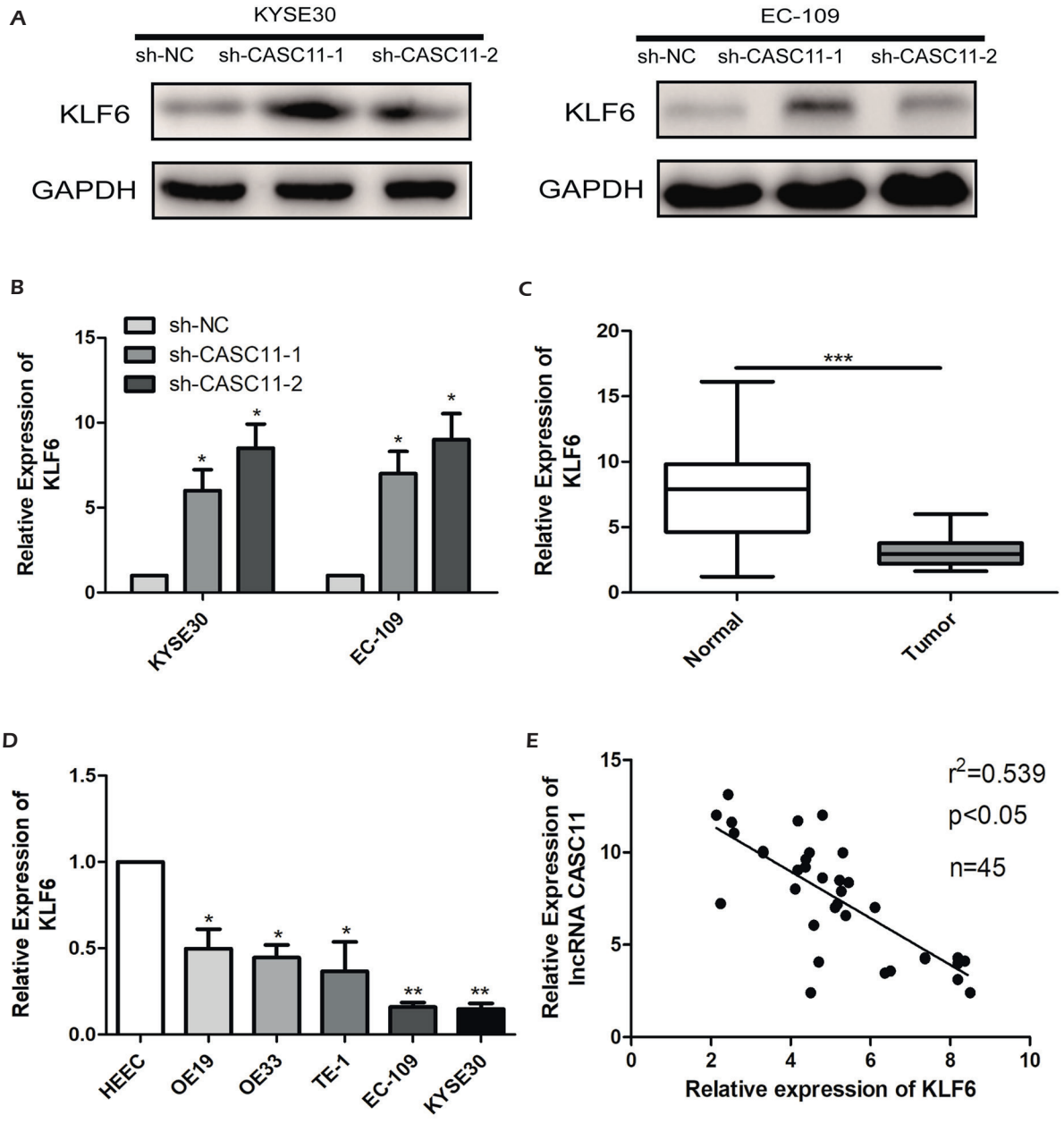

Figure 3. LncRNA CASC11 regulated the expression of KLF6 in ECa tissues and cell lines. **A,** Western Blot verified the expression level of KLF6 in KYSE30 and EC-109 cell lines after interference with lncRNA CASC11. **B,** QRT-PCR verified the expression level of KLF6 in KYSE30 and EC-109 cell lines after interference with lncRNA CASC11. **C,** QRT-PCR was used to detect the expression of KLF6 in ECa tissues and adjacent non-tumor tissues. **D,** QRT-PCR was used to detect the expression level of KLF6 in ECa cell lines. **E,** A significant negative correlation was observed between the expression levels of lncRNA CASC11 and KLF6 in ECa tissues. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

109 were selected as subjects. CASC11 was used for lentiviral transfection to investigate the effects of CASC11 silence on the biological characteristics of the ECa cells. Furthermore, its regulation on downstream related protein level was detected as well. Our results found that silencing CASC11 could remarkably inhibit the proliferation of ECa cells. The flow cytometry results demonstrated that the silence of CASC11 significantly increased the apoptosis of ECa cells. These results indicated that CASC11 played a role as a tumor-promoting gene in ECa development, suggesting that the targeted regulation of lncRNA CASC11 might be a new approach for the ECa treatment.

KLF6, a member of the KLFs family, is a zinc finger protein transcription factor discovered in recent years. Many studies have shown that KLF6 is involved in cell growth, proliferation, differentiation, and apoptosis. Meanwhile, it participates in the pathophysiological processes of tissue and organ damage and repair. It has been gradually realized that KLF6 is involved

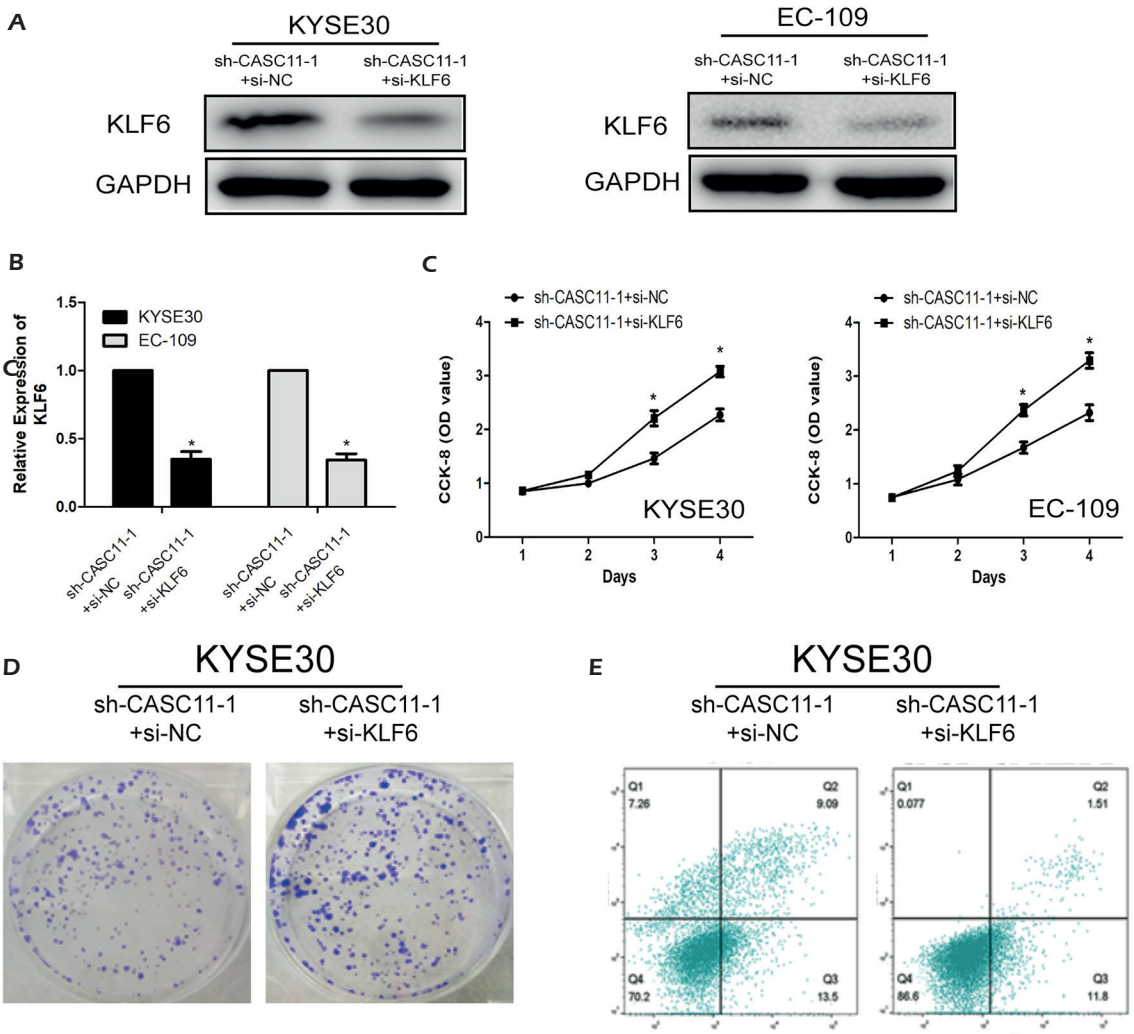

Figure 4. LncRNA CASC11 regulated the mechanism of action of KLF6 in ECa cells. **A,** Expression level of KLF6 in lncRNA CASC11 and KLF6 co-transfected cell lines was detected by Western blotting. **B,** Expression level of KLF6 in lncRNA CASC11 and KLF6 co-transfected cell lines was detected by RT-PCR. **C-E,** CCK-8, colony formation assay and flow apoptosis assay were used to detect the roles of lncRNA CASC11 and KLF6 in the regulation of ECa cell proliferation and apoptosis (magnification '40). Data were expressed as mean ± SD, *$p<0.05$.

Figure continued

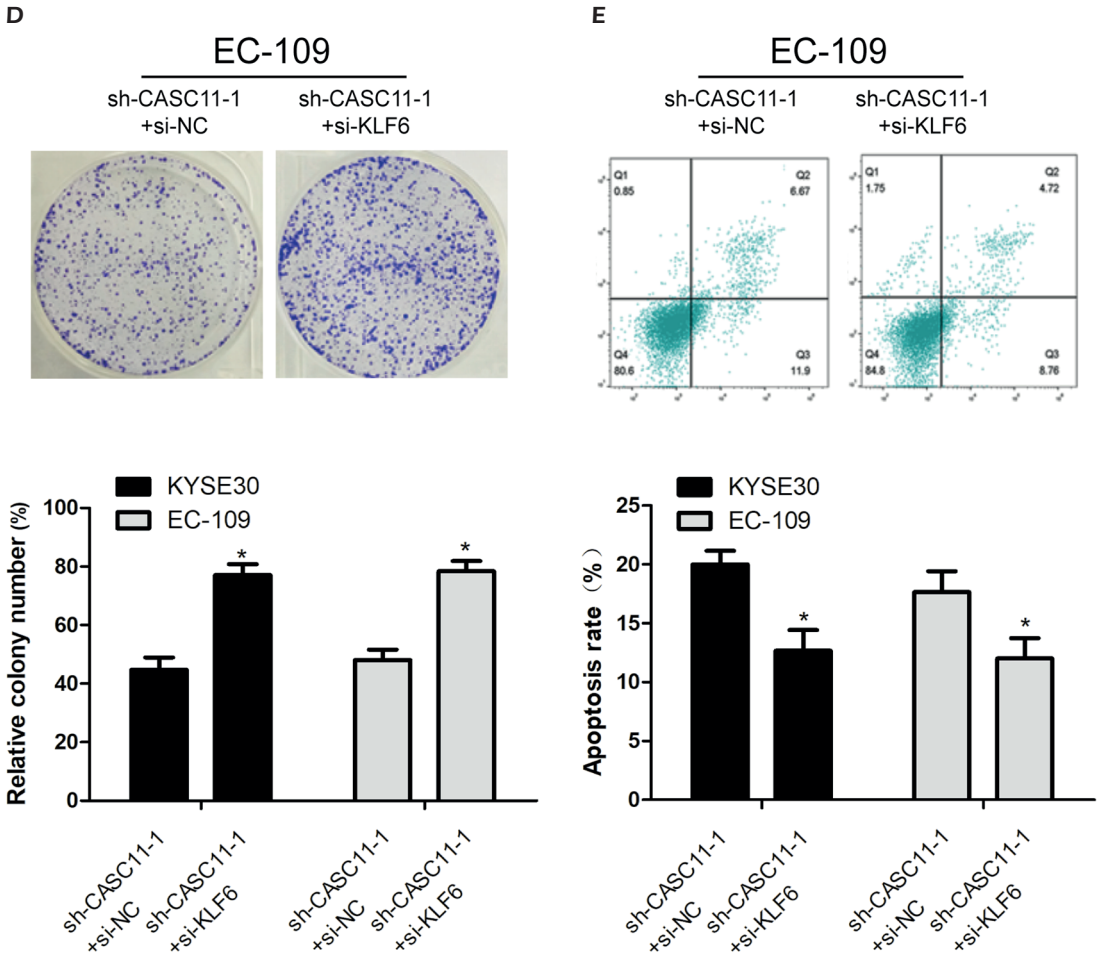

Figure 4. *(Continued).* **C-E,** CCK-8, colony formation assay and flow apoptosis assay were used to detect the roles of lncRNA CASC11 and KLF6 in the regulation of ECa cell proliferation and apoptosis (magnification '40). Data were expressed as mean ± SD, **p*<0.05.

in the development of malignant tumors[24,25]. To prove whether CASC11 promoted the development of ECa by regulating KLF6, significantly up-regulated expression level of KLF6 was observed after the silence of CASC11. Moreover, CASC11 promoted the proliferation of ECa and inhibited apoptosis by regulating KLF6. As the research continues to deepen, further understanding of the interaction between CASC11 and KLF6 will be more conducive to the diagnosis, treatment, and prognosis of malignant tumors.

Conclusions

We showed that lncRNA CASC11 expression was remarkably up-regulated in ECa, which was closely associated with pathological staging and poor prognosis of ECa. In addition, CASC11 promoted the malignant progression of ECa by regulating KLF6.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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