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LncRNA CASC11 promotes the development of lung cancer through targeting microRNA-302/CDK1 axis

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Abstract. – OBJECTIVE: To elucidate whether long non-coding RNA cancer susceptibility candidate 11 (lncRNA CASC11) could participate in the development of lung cancer through targeting microRNA-302/CDK1 axis.

PATIENTS AND METHODS: Expression levels of CASC11, microRNA-302 and CDK1 in lung cancer tissues and paracancerous tissues were determined by quantitative real-time polymerase chain reaction (qRT-PCR). CASC11 expression in lung cancer cell lines was also determined. The regulatory effect of CASC11 on proliferative potential of lung cancer cells was accessed by cell counting kit-8 (CCK-8) and colony formation assay. The binding condition between microRNA-302 to CASC11 and CDK1 was evaluated by dual-luciferase reporter gene assay. CDK1 expression in lung cancer cells with CASC11 or microRNA-302 knockdown was detected by Western blot. The proliferation of lung cancer cells was determined after transfection of microRNA-302 inhibitor or co-transfection of microRNA-302 inhibitor and si-CASC11.

RESULTS: CASC11 and CDK1 were highly expressed, whereas microRNA-302 was lowly expressed in lung cancer tissues. Identically, CASC11 was highly expressed in lung cancer cell lines (A547, H157 and SPC-A-1) than controls as well. CASC11 knockdown attenuated proliferative capacity of lung cancer cells. The opposite trend was observed by microRNA-302 knockdown. Dual-luciferase reporter gene assay verified that CASC11 could bind to microRNA-302 and microRNA-302 could bind to CDK1. CDK1 expression in lung cancer cells was negatively regulated by CASC11. MicroRNA-302 knockdown reversed the inhibitory effect of CASC11 on CDK1 expression. The proliferation of lung cancer cells co-transfected with microRNA-302 inhibitor and si-CASC11 decreased compared with those transfected with microRNA-302 inhibitor.

CONCLUSIONS: High expression of CASC11 promotes the development of lung cancer through upregulating CDK1 expression by binding to microRNA-302.

Key Words:
Lung cancer, CASC11, MicroRNA-302, CDK1, Proliferation.

Introduction

Lung cancer is the leading cause of cancer deaths, accounting for 19.4% of all cancer deaths. Globally, there were 1.82 million new cases of lung cancer in 2012[1]. Based on the pathological classification, lung cancer can be classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Among them, SCLC is derived from Kulchitsky cells with endocrine function that originate from the respiratory epithelium, accounting for about 20%[2]. SCLC is highly malignant. NSCLC is subdivided into squamous cell carcinoma, adenocarcinoma and large cell carcinoma, accounting for about 80% of lung cancer. Great progress has been made in improving the early diagnostic and therapeutic methods of lung cancer in recent years; however, the clinical outcome of lung cancer is still not optimistic[3].

Long non-coding RNA (lncRNA) is a type of cell-derived, non-coding RNAs with a length greater than 200 nucleotides. It is structurally similar to mRNA, but lacks the protein-encoding ability. Studies[4,5] have shown that lncRNA is involved in the regulation of crucial biological processes, such as epigenetics, cell cycle progression and cell differentiation. In addition, lncRNA is associated with the progression and survival of cancer patients[6-8]. LncRNA is expected to be a diagnostic and prognostic biomarker[9]. LncRNA MALAT1 expression is positively correlated with tumor size and lymphatic metastasis, but negatively correlated with survival time and survival rate of patients with lung adenocar-

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cinoma[10]. LncRNA CASC2 inhibits proliferation of gastric cancer cells through mitogen activated protein kinase (MAPK) pathway[11]. Abnormally expressed lncRNA HOTAIR is associated with tumor staging, peritoneal metastasis, lymphatic metastasis, vascular invasion, and prognosis of gastric cancer[12]. The potential function of HOTAIR in chemotherapy resistance of lung cancer has been identified[13]. CASC11 (cancer susceptibility candidate 11) is a novel lncRNA located in the gene deserts of 8q24, and about 2.1 kb upstream of c-Myc. CASC11 is involved in the processes of a variety of diseases. For example[14,15] CASC11 contains lymphoma-associated SNP rs16902359. Through targeting Wnt/β-catenin pathway, CASC11 promotes the development of colorectal cancer[16]. Cytoplasmic level of lncRNA is elevated by enhancing the stability of target genes. CASC11 attenuates the progression of colon cancer by upregulating and stabilizing hnRNP-K expression[17,18]. So far, the specific mechanism of CASC11 in lung cancer is still unclear and will be specifically explored in this study.

Patients and Methods

Patients and Samples

Thirty cases of lung cancer tissues and paracancerous tissues were collected in Cancer Hospital of China Medical University from July 2016 to December 2017. The collected tissues were pathologically confirmed. Enrolled patients signed the written informed consent prior to the study. The Ethic Committee of Cancer Hospital of China Medical University approved this experiment.

Cell Culture and Transfection

Lung mucosal cell lines (16HBE) and lung cancer cell lines (A549, H157 and SPC-A-1) were purchased from Shanghai Cell Research Institute (Shanghai, China). Cells were cultured in low-glucose Roswell Park Memorial Institute-1640 (RPMI-1640) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin at 37°C under 5% CO_2. Cells were washed with phosphate-buffered saline (PBS) at the confluence of 50-60% and incubated with the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in the serum-free medium. Complete medium was replaced 6 hours later. Transfection efficacy was verified by quantitative real-time polymerase chain reaction (qRT-PCR) at 48 h. The transfection plasmids, including si-CASC11 and microRNA-302 inhibitor, were constructed by GenePharma (Shanghai, China).

RNA Extraction

Tissues or cells were lysed in TRIzol (Invitrogen, Carlsbad, CA, USA). Subsequently, lysis was incubated with 250 μL of chloroform, mixed for 30 s and centrifuged at 4°C. The aqueous phase was aspirated and an equal volume of pre-cooled isopropanol was added. After centrifugation, the precipitate was washed with 75% ethanol. The precipitate was collected by centrifugation, air dried and finally dissolved in 20 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). RNA concentration was quantified using a spectrophotometer and RNA samples were preserved at -80°C.

Reverse Transcription and qRT-PCR

The reverse transcription system was prepared on ice using the PrimeScript RT reagent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan) to obtain the complementary Deoxyribose Nucleic Acid (cDNA). The reverse transcript template was diluted in RNase-depleted water to a final concentration of 3 ng/μL. QRT-PCR was carried out in accordance with the instruction of SYBR Green PCR Kit (TaKaRa, Otsu, Shiga, Japan) with a total reaction system of 10 μL. QRT-PCR was performed: pre-denaturation at 95°C for 15 min, and 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Primer sequences were: CASC11, F: 5'-GGAAAGCCTGTGCTCTTGGA-3', R: 5'-TCACAGTGCTGCTCAATCGT-3'; MicroRNA-302, F: 5'-GGGUCUCCCAACCCUUG-3', R: 5'-CAGTGCGTGTCGTGGAGT-3'; CDK1, F: 5'-ACGCACCCCAACTACAACTC-3', R: 5'-TCTCCTTAATGTCACGCACGA-3'; GAPDH, F: 5'-GGAATCCACTGGCGTCTTCA-3', R: 5'-GGTTCACGCCCATCACAAAC-3'; U6, F: 5'-TCCGATCGTGAAGCGTTC-3', R: 5'-GTGCAGGGTCCGAGGT-3'.

Cell Proliferation Assay

Transfected cells for 24 h were inoculated into 96-well plates at 2×10^3 per well, and 6 replicate wells were set in each group. Cells were incubated for 6 h, 24 h, 48 h and 72 h, respectively. Two hours before determination of cell viability, 10 μL of cell counting kit-8 (CCK-8) solution (Chengdu

Ruifeng Biotechnology Company, Chengdu, China) was added to each well. The absorbance of each well at 450 nm wavelengths was recorded by a microplate reader.

Colony Formation Assay

A549 and H157 cells in the logarithmic growth phase were collected, and the concentration of cell suspension was adjusted to 1×10^4 cells/mL. A total of 2000 cells per well were seeded in a 6-well plate, and cultured in a 37°C incubator for 3-4 days. Subsequently, paraformaldehyde fixation and crystal violet staining were conducted for 30 min each. The amount of colonies in each well was counted and photographed.

Dual-Luciferase Reporter Gene Assay

Potential target sites between microRNA-302 with CASC11 and CDK1 were predicted by bioinformatics. Wild type or mutant-type sequences of CASC11 3'UTR and CDK1 3'UTR were inserted into the pGL3 promoter vector, respectively. A549 and H157 cells were seeded into 24-well plates with 5×10^5 cells/well one day prior to transfection. Luciferase reporter vector (0.12 μg) and 40 nM microRNA-302 mimic or negative control was co-transfected into cells. At 48 hours, luciferase activity was determined using the dual-luciferase reporter assay kit (Promega, Madison, WI, USA). The experiment was repeated for three times.

Western Blot

Total protein was extracted using the cell lysate for determining protein expression of CDK1 by Western blot. After quantification of the total protein using bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA), the sample was loaded at 80 μg/well and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. The transferred polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) was blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by chemiluminescence.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) and Graphpad statistical software (Version X; La Jolla, CA, USA). The chi-square test was used for analyzing the classification data, and the Student's *t*-test was used for the measurement data. All measurement data were expressed as mean±SD (Standard Deviation). The difference was statistically significant at $p<0.05$.

Results

CASC11 was Highly Expressed in Lung Cancer Tissues and Promoted Proliferative Potential of Lung Cancer Cells

Expression level of CASC11 in 30 pairs of lung cancer tissues and paracancerous tissues was determined by qRT-PCR. CASC11 was highly expressed in lung cancer tissues compared with that of paracancerous tissues (Figure 1A). Identically, qRT-PCR data also showed higher expression level of CASC11 in lung cancer cell lines (A549, H157 and SPC-A-1) relative to normal lung mucosal cells (16HBE) (Figure 1B). We thereafter selected A549 and H157 cells, showing a large difference in CASC11 expression. By transfection of si-CASC11 in A549 and H157 cells, relative expression of CASC11 was markedly inhibited as qRT-PCR data indicated (Figure 1C). Colony formation assay revealed that CASC11 knockdown could inhibit the proliferative potential of lung cancer cells (Figure 1D). Similar results were obtained in the CCK-8 assay as well (Figure 1E).

CASC11 Bound to MicroRNA-302

We predicted potential binding targets for CASC11 and microRNA-302 through bioinformatics (Figure 2A). Based on the binding sites, wt CASC11 3'UTR and mut CASC11 3'UTR were first constructed. As dual-luciferase reporter gene data showed, luciferase activity markedly decreased in lung cancer cells co-transfected with microRNA-302 mimic and wt CASC11 3'UTR. We did not observe a significant change of luciferase activity in mut CASC11 3'UTR group, demonstrating the binding between microRNA-302 and CASC11 (Figure 2B). Furthermore, microRNA-302 was upregulated by transfection of si-CASC11 in A549 and H157 cells (Figure 2C). On the contrary, microRNA-302 expression was downregulated, whereas CASC11 expression was upregulated by transfection of microRNA-302 inhibitor (Figure 2D and 2E).

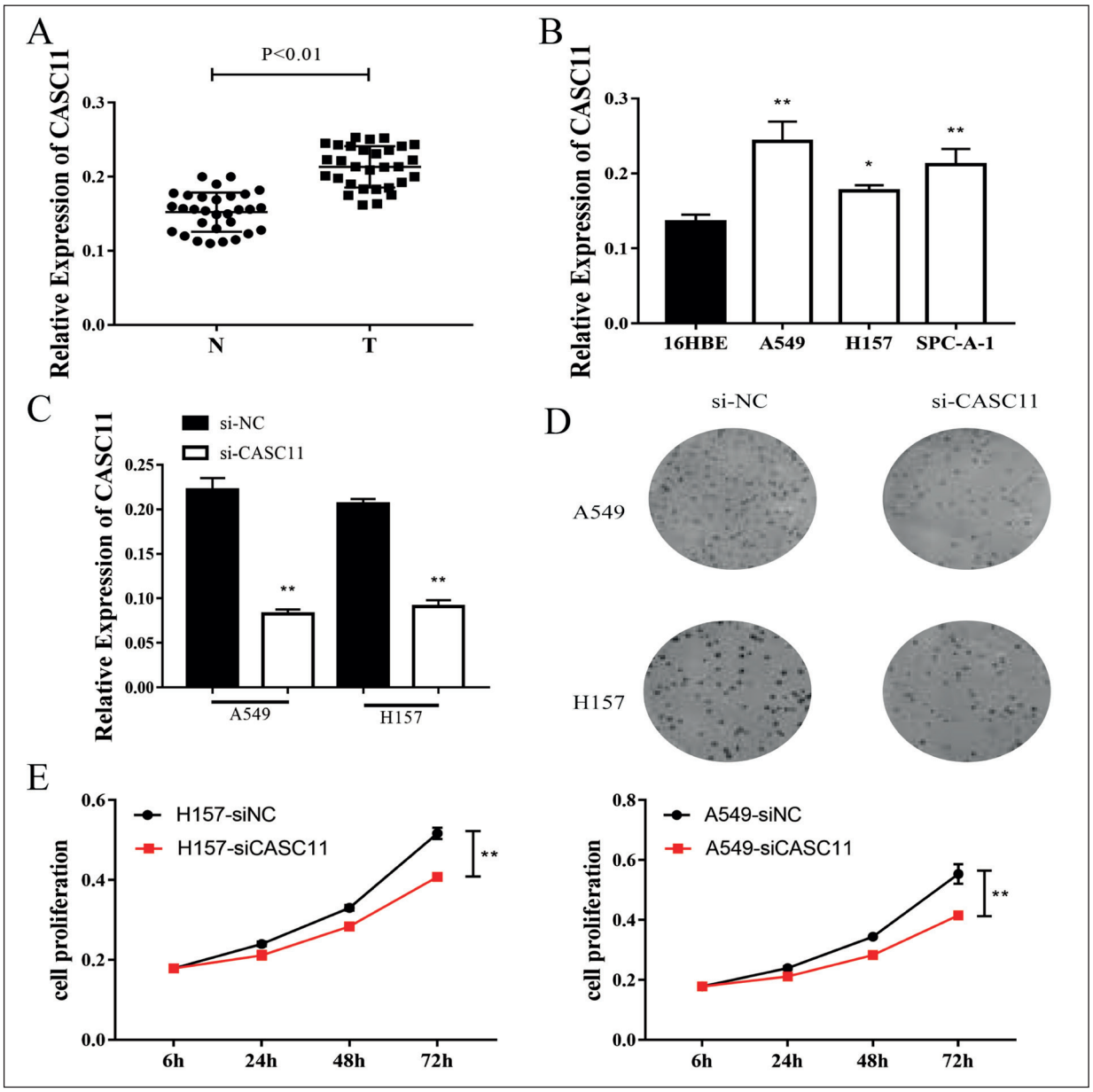

Figure 1. CASC11 was highly expressed in lung cancer. ***A,*** CASC11 was highly expressed in lung cancer tissues compared with paracancerous tissues. ***B,*** CASC11 was highly expressed in lung cancer cell lines (A549, H157 and SPC-A-1) relative to normal lung mucosal cells (16HBE). ***C,*** Transfection efficacy of si-CASC11 in A549 and H157 cells. ***D,*** Colony formation assay showed that transfection of si-CASC11 inhibited proliferation of A549 and H157 cells. ***E,*** CCK-8 assay showed that transfection of si-CASC11 inhibited proliferation of A549 and H157 cells. **p*<0.05, ***p*<0.01.

MicroRNA-302 was Lowly Expressed in Lung Cancer Tissues and Promoted Proliferative Potential of Lung Cancer Cells

QRT-PCR was conducted to determine microRNA-302 expression in 30 pairs of lung cancer and paracancerous tissues. MicroRNA-302 expression was lower in lung cancer tissues compared to that of paracancerous tissues (Figure 3A). We found that microRNA-302 was negatively correlated with CASC11 (Figure 3B). MicroRNA-302 inhibitor was then transfected into A549 and H157 cells. Colony formation assay revealed that microRNA-302 knockdown could markedly enhance the proliferative potential, but was further reversed by CASC11 knockdown (Figure 3C). CCK-8 assay yielded the identical results (Figure 3D).

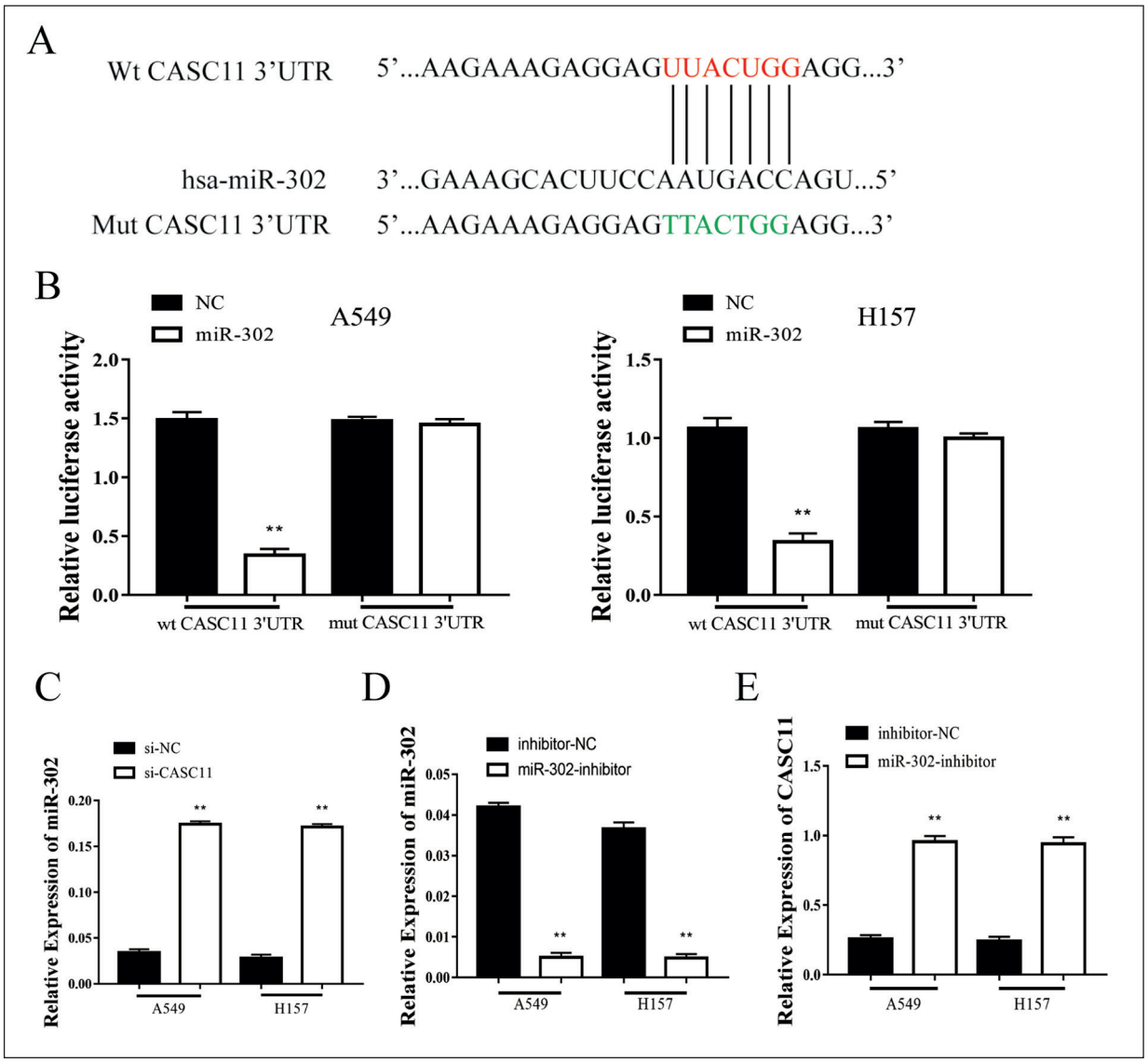

Figure 2. CASC11 bound to microRNA-302. ***A,*** Potential binding sites between microRNA-302 and CASC11 by bioinformatics. ***B,*** Dual-luciferase reporter gene assay confirmed that CASC11 could bind to microRNA-302. ***C,*** Transfection of si-CASC11 upregulated microRNA-302 expression in A549 and H157 cells. ***D,*** Transfection efficacy of microRNA-302 inhibitor in A549 and H157 cells. ***E,*** Transfection of microRNA-302 inhibitor upregulated CASC11 expression in A549 and H157 cells. $^{**}p<0.01$.

CDK1 Was Highly Expressed in Lung Cancer Tissues and Bound to MicroRNA-302

Expression level of CDK1 in 30 pairs of lung cancer tissues and paracancerous tissues was determined by qRT-PCR. Higher level of CDK1 was observed in lung cancer tissues than that of paracancerous tissues (Figure 4A). Besides, CDK1 expression was positively correlated to CASC11 expression (Figure 4B). The potential binding sites between CDK1 and microRNA-302 were predicted by bioinformatics, followed by construction of wt CDK1 3'UTR and mut CDK1 3'UTR. Lower luciferase activity was seen in cells co-transfected with microRNA-302 mimic and wt CDK1 3'UTR, whereas no significant difference was found in mut CDK1 3'UTR group (Figure 4D). We therefore confirmed the binding between microRNA-302 and CDK1. CDK1 expression was downregulated in A549 and H157 cells transfected with si-CASC11, but were markedly upregulated after transfection of microRNA-302 inhibitor as qRT-PCR revealed. Interestingly, CDK1 expression was reduced by co-transfection of si-CASC11

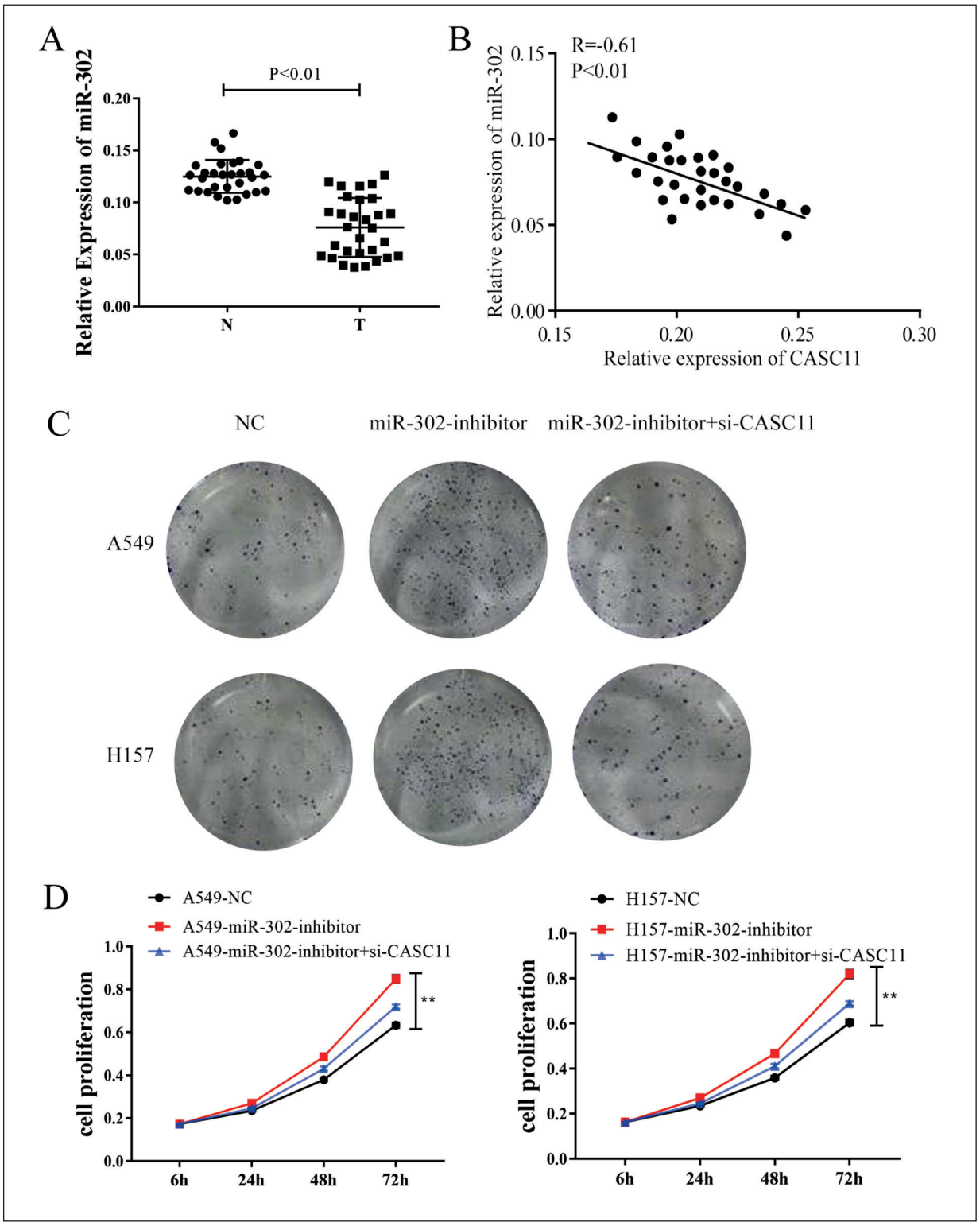

Figure 3. MicroRNA-302 was lowly expressed in lung cancer. ***A,*** MicroRNA-302 was lowly expressed in lung cancer tissues compared with paracancerous tissues. ***B,*** MicroRNA-302 was negatively correlated to CASC11. ***C,*** Colony formation assay showed that transfection of microRNA-302 inhibitor enhanced proliferation, which was reversed by co-transfection of si-CASC11. ***D,*** CCK-8 assay showed that transfection of microRNA-302 inhibitor enhanced proliferation, which was reversed by co-transfection of si-CASC11. $^{**}p<0.01$.

and microRNA-302 inhibitor (Figure 4E). Western blot analyses demonstrated the same results at the protein level of CDK1 (Figure 4F). The above results altogether revealed that CASC11 participated in the development of lung cancer through promoting CDK1 by targeting microRNA-302.

Discussion

Lung cancer shows the highest morbidity and mortality in China[19]. Risk factors for lung cancer include genetics, smoking, air pollution, occupational exposure, etc. Due to the insufficient early

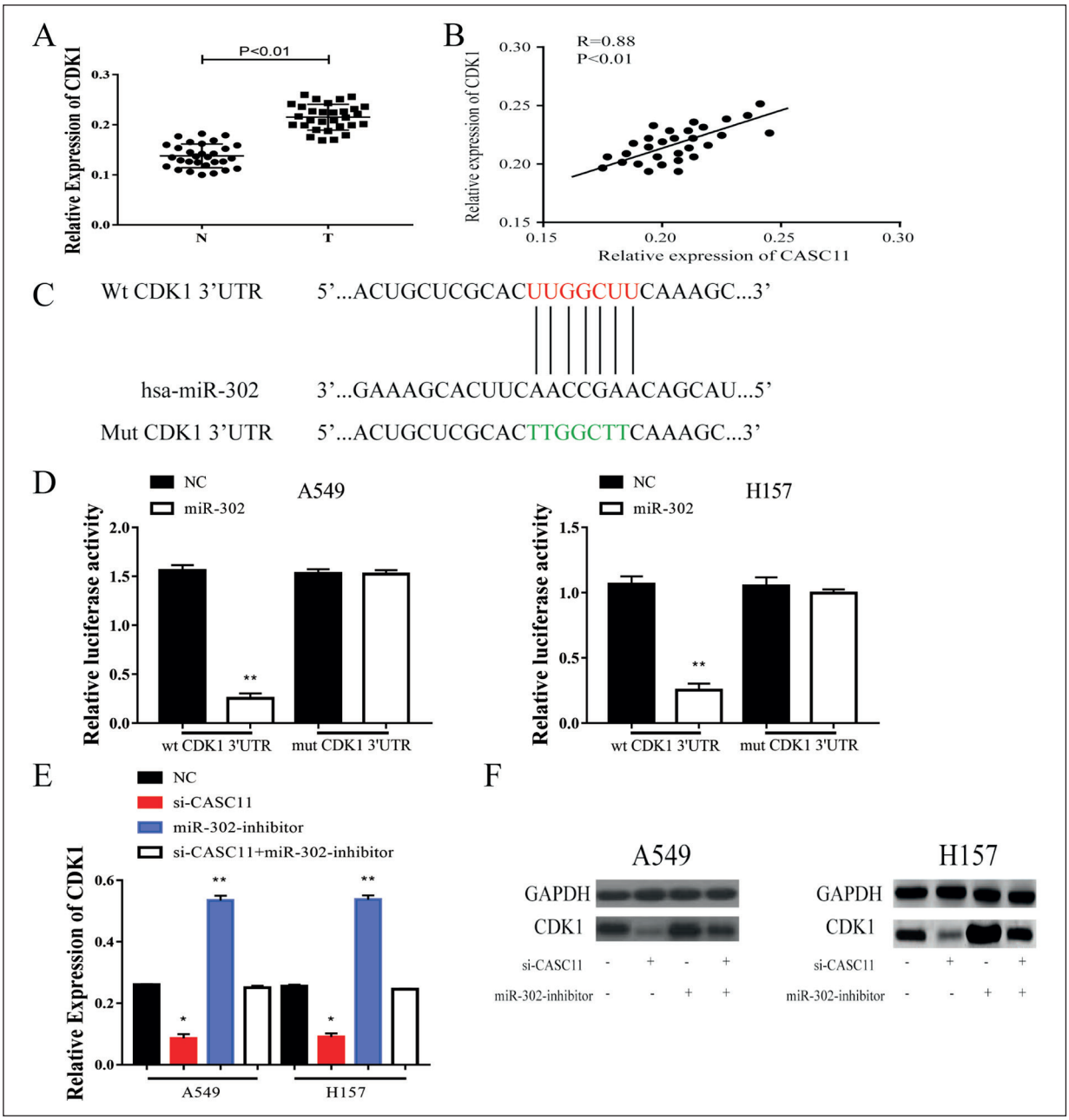

Figure 4. CDK1 was highly expressed in lung cancer tissues and bound to microRNA-302. ***A,*** CDK1 was highly expressed in lung cancer tissues compared with paracancerous tissues. ***B,*** CDK1 was positively correlated to CASC11. ***C,*** Potential binding sites between microRNA-302 and CDK1 by bioinformatics. ***D,*** Dual-luciferase reporter gene assay detected that CDK1 could bind to microRNA-302. ***E, F,*** The mRNA and protein levels of CDK1 were inhibited in A549 and H157 cells transfected with si-CASC11, which were upregulated after transfection of microRNA-302 inhibitor. Co-transfection of si-CASC11 and microRNA-302 inhibitor decreased the mRNA and protein levels of CDK1 than those transfected with microRNA-302 inhibitor alone. *$p<0.05$, **$p<0.01$.

diagnosis of lung cancer, most affected patients have been progressed in the advanced stage or accompanied with serious complications. Currently, the therapeutic efficacy of lung cancer is still unsatisfactory. Relative lncRNAs in lung cancer have been discovered in recent years. The regulatory role of lncRNA has been identified in the occurrence and development of lung cancer. It regulates gene expressions mainly at epigenetics, transcription and post-transcriptional levels, thereafter mediating chromosomal modification, transcriptional activation or interference[20]. LncRNA UCA1, H19 and HOTAIR are crucial lncRNAs in the development of lung cancer[21-23]. Cyclin-dependent kinase 1 (CDK1) is a member of the serine/tyrosine kinase family[24]. To date, 13 different cyclin-dependent kinases (CDKs) and more than 25 different cyclins have been discovered in human genome. However, only CDK1, CDK2, CDK4 and CDK6 are directly involved in the cell cycle progression[25]. CDK1 exerts an important role in the cell cycle progression from G2 phase to M phase. It is reported that CDK1 expression is associated with postoperative distant metastasis of colon cancer[26]. Multiple studies have found that abnormal activation of CDK1 is observed in various primary tumors, such as glioma, breast cancer, colon cancer, prostate cancer, lung cancer, and esophageal cancer. High expression of CDK1 indicates a poor survival in tumors[27-29]. Our study found that the expression level of CASC11 was higher in lung cancer tissues relative to paracancerous tissues. Proliferative potential of lung cancer cells decreased after CASC11 knockdown. Further experiments revealed that CASC11 could bind to microRNA-302 and inhibit its expression. MicroRNA-302 was lowly expressed in lung cancer tissues and negatively correlated with CASC11 expression. Knockdown of microRNA-302 increased cell proliferation, and more importantly, reversed the regulatory effect of CASC11 on proliferative behaviors of lung cancer cells. Subsequently, microRNA-302 was verified to bind to CDK1. Compared with paracancerous tissues, CDK1 expression remained higher in lung cancer tissues and was positively regulated by CASC11. CDK1 expression was upregulated by transfection of microRNA-302 inhibitor. Interestingly, microRNA-302 reversed the regulatory effect of CASC11 on CDK1 expression. Hence, we confirmed that CASC11 promoted the development of lung cancer by targeting the microRNA-302/CDK1 axis, which may be a potential target for lung cancer treatment.

Conclusions

CASC11 is highly expressed in lung cancer tissues. It promotes the development of lung cancer through regulating the microRNA-302/CDK1 axis. We believe that the CASC11/microRNA-302/CDK1 axis may serve a potential therapeutic target for lung cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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