Long noncoding RNA CASC2 inhibits metastasis and epithelial to mesenchymal transition of lung adenocarcinoma via suppressing SOX4

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Abstract. OBJECTIVE: Recently, long non-coding RNAs (lncRNAs) have caught more attention for their role in tumor progression. Lung adenocarcinoma (LAC) is one of these ordinary malignant tumors. This study aims to identify whether IncRNA CASC2 (cancer susceptibility candidate 2) can regulate the metastasis of LAC, and find out its potential mechanism.

PATIENTS AND METHODS: RT-qPCR was conducted to detect CASC2 expression level in 63 LAC tissues and 4 LAC cells. Besides, statistical methods were applied to analyze clinical data and prognosis in the 63 patients. Furthermore, function experiments were performed to determine the effect of CASC2 on LAC metastasis in vitro. The potential mechanism was further explored by RT-qPCR and Western blot assay.

RESULTS: In our study, CASC2 expression level was lower in LAC tissues than that in corresponding tissues. CASC2 expression was associated with lymph node metastasis, clinical stage and survival time of these patients. Moreover, overexpression of CASC2 inhibited migrated and invaded ability of LAC cells. Then, epithelial to mesenchymal transition (EMT) process of LAC cells and SOX4 expression was suppressed by upregulating CASC2.

CONCLUSIONS: These results indicate that CASC2 could inhibit metastasis and EMT of LAC via suppressing SOX4, which may offer a new vision for interpreting the mechanism of LAC metastasis.

Key Words: lncRNA, CASC2, Lung adenocarcinoma, SOX4, EMT.

Introduction

Lung cancer (LC) ranks the second common cancer among human malignant tumors. 80% of LC is non-small-cell lung cancer (NSCLC) worldwide, of which lung adenocarcinoma (LAC) is the main subtype. The incidence of liver cancer in China accounts for more than half of the whole world. Although molecular targeted therapy is available for LAC patients, only a small part of patients benefits from those discovered driver mutations. Therefore, it is urgent to find more potential regulators and targets for treatment of LAC. Recently, aberrant long non-coding RNAs (lncRNAs) expression is identified in most solid cancers and closely associated with patients’ prognosis. Moreover, mounting evidence indicates that lncRNAs contribute to regulating oncogene expression, which further acts on progression of malignant cancers, including LAC. LncRNA CASC2, as a novel tumor suppressor, has been discovered in many human cancers, such as glioma, hepatocellular carcinoma, colorectal cancer, and thyroid carcinoma. Accumulating evidence demonstrates that CASC2 participates in tumor carcinogenesis. For instance, CASC2 knockdown promotes cell metastasis via activating Wnt signaling pathway in bladder cancer. By targeting MAPK signaling pathway, CASC2 functions as a tumor suppressor and inhibits cell growth in gastric cancer. Moreover, miR-21 can downregulate CASC2 and further promote cell growth and migration in renal cell carcinoma. CASC2 contributes to enhancing sensitivity of cells to cisplatin in cervical carcinoma. He et al detected aberrant CASC2 expression in NSCLC, and revealed the association between CASC2 expression level and postoperative prognosis.

Epithelial to mesenchymal transition (EMT) has been identified as a key step of metastasis in epithelial cancers. More evidence suggests that lncRNAs could affect the migrated and
invaded ability of cancer cells through regulating EMT. However, the role of CASC2 in the metastasis of LAC remains unknown. Our present study firstly revealed that CASC2 was downregulated in clinical LAC samples, and its expression was correlated with clinicopathological features and prognosis. Besides, overexpressed CASC2 inhibited cell migration, and invasion of LAC, as well as EMT in vitro. Further study showed that SOX4 might serve as a potential target of CASC2.

**Patients and Methods**

**Clinical Samples and Cell Lines**

Cancer tissue specimens and corresponding tissues were acquired from 63 LAC patients who received surgery at the Affiliated Hospital of Qingdao University. No patients received radiotherapy or chemotherapy before operation. All LAC tissues were stored at -80°C. An experienced pathologist assessed the clinical data. The written informed consent was available for this study. The study conformed to requirements of the Ethics Committee of the Affiliated Hospital of Qingdao University. The Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China) provided us 4 LAC cell lines including A549, SPCA1, H1975 and PC-9, and 16 HBE (human bronchial epithelial cell). Culture medium was consisted of penicillin, 10% fetal bovine serum (FBS), which was purchased by Invitrogen Life Technologies (Carlsbad, CA, USA), and Dulbecco's modified Eagle's medium (DMEM), which was obtained by Thermo Fisher Scientific (Waltham, MA, USA). Besides, cells were cultured in humidified incubator containing 5% CO₂ at 37°C.

**Plasmid Construction**

Ribobio (Guangzhou, China) helped us synthesizing CASC2 sequence. Then, sequence was sub-cloned into pcDNA 3.1. For transfection in LAC cells, pcDNA-CASC2 accompanied with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used. Besides, empty pcDNA vector (EV) was used as control. 48 h later, detection of CASC2 expression level in these cells was conducted using qRT-PCR.

**Quantitative RT-PCR**

Firstly, total RNA was separated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer’s instructions. Next, RNA was reverse-transcribed to cDNAs via reverse Transcription Kit (TaKaRa Biotechnology Co., Dalian, China). qRT-PCR was performed on ABI 7500 RT-PCR detection system (Applied Biosystems, Foster City, CA, USA). Following are the primers using for qRT-PCR: CASC2, forwards 5'-GCACATTGGACGGTGTTTCC-3' and reverse 5'-CCCA-GTCCCTTCAGGGTCAC-3'; SOX4, forward 5'-GGTCTCTTCTTCTGTCCAGGCTC-3' and reverse 5'-CGGAATCGGCACCTAAGGAG-3'; GAPDH, forward 5'-AGCCACATCGGCACTAACGAG-3'; and reverse 5'-GCCAACATCGGCACTAACGAG-3'. The thermal cycle was as follows: 30 s at 95°C, 5 s at 95°C for 40 cycles, 35 s at 60°C.

**Western Blot**

Reagent radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) was utilized to extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, they were replaced to the polyvinylidene fluoride (PVDF) membrane, which was incubated with antibodies. Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-E-cadherin, rabbit anti-N-cadherin, rabbit anti-SOX4 and rabbit anti-GAPDH, as well as goat anti-rabbit secondary antibody. Chemiluminescent film was applied for assessment of protein expression with ImageJ software.

**Wound Healing Assay**

Cells were transferred into 6-well plates, were cultured in DMEM overnight. After they were scratched with a plastic tip, cells were cultured in serum-free DMEM. Wound closure was viewed at different time points. Each assay was independently repeated in triplicate.

**Matrigel Assay**

To detect the invasion of LAC cells, 5 ×10⁴ cells in 200 µL serum-free DMEM were transformed to top chamber of an insert (8 µm pore size; Millipore, Billerica, MA, USA) coated with 50 µg Matrigel (BD Biosciences, San Jose, CA, USA). The bottom chamber was added with DMEM containing 10% FBS. After the top surface of chambers was cultured for
48 h, it was wiped by cotton swab and immersed for 10 min with precooling methanol. The followings were stained in crystal violet for 30 min. The data for invasion was counted from three fields per membrane.

**Statistical Analysis**

Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). $\chi^2$ test, Student t-test and Kaplan-Meier method were selected when appropriate. Results were presented as mean ± SD. $p<0.05$ was considered statistically significant.

**Results**

**Downregulated CASC2 is Detected in Human LAC Tissues**

First, RT-qPCR was conducted for detecting CASC2 expression in 63 pairs of LAC tissues and corresponding tissues. As the result, CASC2 was significantly downregulated in LAC tissue samples (Figure 1A). Clinicopathological features in those LAC patients were presented in Table I. Results revealed that downregulated CASC2 was related to lymph node metastasis and clinical stage (Figure 1B-C).

**Low Expression of CASC2 is Correlated with Postoperative Prognosis in LAC Patients**

Patients’ survival after surgery was analyzed through Kaplan-Meier method. We divided 63 LAC patients into two groups, high-CASC2 and low-CASC2, via median expression. Results showed that patients in high-CASC2 group had better survival than those in low-CASC2 group (Figure 1D).

Figure 1. Expression levels of CASC2 were decreased in LAC tissues. (A) CASC2 expression was significantly decreased in the LAC tissues compared with adjacent tissues. (B) CASC2 expression was significantly lower in patients with advanced clinical stage. (C) CASC2 expression was significantly lower in patients with lymph nodes metastasis. (D) Kaplan–Meier overall survival curves showed overall survival of low-CASC2 group (n=34) was significantly shorter than that of high-CASC2 group (n=29). ($p<0.001$, log-rank test). *$p<0.05$; **$p<0.01$. 

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**CASC2 Suppresses Cell Migration and Invasion of LAC Cells**

According to CASC2 expression in LAC cells (Figure 2A), we chose A549 cells for lentivirus transfection. The CASC2 expression in cells transfected with pcDNA-CASC2 and empty pcDNA vector (EV) was determined by qRT-PCR (Figure 2B). The migration in pcDNA-CASC2 cells was decreased compared with EV cells (Figure 2C). Besides, Matrigel assay demonstrated that numbers of invading cells were reduced in pcDNA-CASC2 cells compared with EV cells (Figure 2D).

**Overexpression of CASC2 Inhibits EMT in LAC cells**

To determine the function of CASC2 on EMT process, Western blot was conducted and results showed that N-cadherin was downregulated and E-cadherin was upregulated at protein level after CASC2 was overexpressed in A549 cells (Figure 3).

**Correlation Between Expression of SOX4 and CASC2**

Firstly, SOX4 expression was detected in LAC cells and tissue samples. SOX4 was remarkably highly expressed in LAC tissue specimens compared with corresponding samples (Figure 4A). SOX4 was upregulated in LAC cells (Figure 4B). Furthermore, expression of CASC2 was negatively associated with SOX4 expression in LAC tissues (Figure 4C). Western blot was conducted and results showed that SOX4 was downregulated at protein level after CASC2 was overexpressed in A549 cells (Figure 4D).

**Discussion**

In our investigation, CASC2 was found downregulated in tissue samples and cells of LAC, and significant correlation was seen between clinical stage, lymph node metastasis and patients’ prognosis. Furthermore, the migrated and invaded ability was inhibited in LAC cells after CASC2 was overexpressed. Data above suggests that CASC2 serves as a tumor suppressor and inhibits the aggressiveness of LAC.

Then, we further explored whether CASC2 could regulate EMT of LAC cells. Moreover, results of Western blot assay demonstrated that level of N-cadherin was decreased and that of E-cadherin was increased once CASC2 was overexpressed. Indeed, N-cadherin is one of the mesenchymal phenotype cell biomarkers, while E-cadherin is one of the epithelial phenotype cell biomarkers. These data indicated that CASC2 made effect in LAC metastasis via re-

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*p<0.05 is considered as statistically significant.

Table I. Correlation between lncRNA CASC2 expression and clinicopathological characteristics in LAC patients.
gulating EMT. However, it remained unknown through what mechanism CASC2 influenced the process of EMT.

Accumulating evidence reveals that SOX4 acts as an oncogene and induces EMT process of cancers. For instance, the expression level of SOX4 is correlated with poor prognosis of prostate cancer16. Upregulated SOX4 promotes cell growth, and invasion of colorectal cancer17. Moreover, SOX4 acts as a critical regulator of EMT and is associated with epigenetic reprogramming18. In breast cancer, EMT induced by SOX4 promotes tumor progression in vivo19. Several studies reveal that SOX4 emerges as a potential target, which can be regulated by non-coding RNA. SOX4 is the direct target of miR-338-3p.
which suppresses metastasis of breast cancer in vivo\textsuperscript{20}. Besides, IncRNA UCA1, as a ceRNA of SOX4, promotes cell growth in esophageal cancer\textsuperscript{21}. Wang et al\textsuperscript{22} show that SOX4 is highly expressed in NSCLC tissues and is associated with overall survival of NSCLC patients\textsuperscript{22}. In our study, overexpression of CASC2 inhibited the expression of SOX4. Moreover, CASC2 expression was negatively correlated with SOX4 expression in LAC tissues.

**Conclusions**

CASC2 was downregulated in LAC tissue samples and cells, and is associated with clinicopathological characteristics and postoperative prognosis. Besides, overexpression of CASC2 could inhibit migration, invasion, and EMT process of LAC through regulating SOX4. These findings implied that CASC2 could act as a prospective therapeutic target for LAC.

**Conflict of interest**

The authors declare no conflicts of interest.

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**Figure 3. Overexpression of CASC2 suppressed EMT process of LAC cells.** The level of EMT-related protein was significantly changed by upregulating CASC2 in A549 cells. *p*<0.05.

**Figure 4. Correlation between SOX4 and CASC2 in LAC tissues and LAC cell lines.** (A) SOX4 was significantly higher expressed in LAC tissues compared with corresponding normal tissues. (B) SOX4 was higher expressed in LAC cell lines compared with 16HBE cells. (C) Correlation analysis revealed that CASC2 expression was negatively correlated with SOX4 expression in LAC tissues. (D) Western blot assay showed that SOX4 was downregulated at protein level after CASC2 was overexpressed in A549 cells. *p*<0.05.
References


