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LncRNA CASC19 promotes the proliferation, migration and invasion of non-small cell lung carcinoma *via* regulating miRNA-130b-3p

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Abstract. – OBJECTIVE: To uncover the biological role of long non-coding RNA (lncRNA) CASC19 in the pathogenesis of non-small cell lung carcinoma (NSCLC) and the potential mechanism.

PATIENTS AND METHODS: Expression pattern of lncRNA CASC19 in NSCLC tissues and cell lines was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Survival analysis on the correlation between CASC19 level and prognosis of NSCLC patients was conducted by introducing for the Kaplan-Meier estimator. After the transfection of si-CASC19 in A549 and PC9 cells, changes in viability, migratory, and invasive capacities were evaluated. Dual-luciferase reporter gene assay was performed to explore the interaction between microRNA-130b-3p (miRNA-130b-3p) and CASC19/ZEB2. Their interactive effects on the progression of NSCLC were finally investigated through rescue experiments.

RESULTS: LncRNA CASC19 was upregulated in NSCLC tissues and cell lines. NSCLC patients with high expression of CASC19 presented a worse survival. Knockdown of CASC19 attenuated proliferative, migratory, and invasive capacities of A549 and PC9 cells. CASC19 sponged miRNA-130b-3p and negatively regulated its level. ZEB2 was the direct target of miRNA-130b-3p. The knockdown of miRNA-130b-3p reversed the regulatory effects of CASC19 on A549 and PC9 cells.

CONCLUSIONS: CASC19 sponges miRNA-130b-3p to regulate ZBR2 as a ceRNA, thus accelerating the progression of NSCLC by regulating proliferative, migratory, and invasive capacities of tumor cells.

Key Words:
CASC19, MiRNA-130b-3p, CeRNA, Non-small cell lung carcinoma (NSCLC).

Introduction

Lung carcinoma is one of the main lethal malignancy in the world. Its incidence and malignancy are very high and an effective treatment is lacking. Non-small cell lung carcinoma (NSCLC) accounts for 80-85% of all lung cancer cases[1]. Diagnostic rate and therapeutic strategy of NSCLC have been improved in recent years. However, strong invasiveness and high metastatic rate of NSCLC result in the poor prognosis of affected patients. It is reported that the median survival of NSCLC is less than 1 year and the 5-year survival is less than 5%[2]. Hence, it is urgent to develop effective early-stage diagnostic methods and therapeutic targets of NSCLC, thus improving the clinical outcome of NSCLC patients.

Long non-coding RNA (lncRNA) is a type of non-coding RNA with a transcript length of more than 200 nt that could regulate gene expressions. LncRNA could not encode proteins due to the lack of the open reading frame[3]. Some reports[4] have shown the vital functions of lncRNA in diverse aspects of tumor biology. Relevant lncRNAs are identified to influence the occurrence and metastasis of NSCLC[5,6]. Cui et al[7] illustrated that PVT1 is upregulated in lung carcinoma tissues and cell lines, which is closely related to lymphatic metastasis, disease-free survival, and overall survival of lung carcinoma. Ono et al[8] pointed out that silence of HOTAIR attenuates NSCLC cells to proliferate and invade. CASC19 is a newly discovered lncRNA located on 8q24 region of the chromosome[9]. Recently, Sinovieva et al[10] illustrated the upregulated CASC19 in colorectal cancer. Also, the potential mechanism requires further explorations. The biological role of CASC19 in NSCLC, however, is rarely reported.

Serving as a ceRNA, lncRNA interacts with miRNAs to mediate target gene expressions, thereafter affecting the progression of NSCLC[11]. For instance, SNHG20 sponges miR-154 to upregulate ZEB2 and RUNX2, further accelerating the proliferative, migratory, and invasive ca-

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pacities of NSCLC cells[12]. UCA1 competitively binds to miR-193a-3p to further inhibit ERBB4 level, thus stimulating NSCLC cell growth[13]. In-depth researches on lncRNAs involving in NSCLC may provide promising targets for NSCLC treatment.

This research mainly explored the biological function of CASC19 in the progression of NSCLC and the potential molecular mechanism. Our conclusions may provide new targets for the clinical treatment of NSCLC.

Patients and Methods

Patients and Samples

Tumor tissues and matched normal tissues were surgically harvested from 30 patients diagnosed with NSCLC in the Thoracic Surgery Department, Tumor Hospital, Peking University. Tissue samples were immediately preserved in liquid nitrogen. This investigation was approved by the Medical Ethics Committee and informed consent was obtained from each subject.

Cell Culture and Transfection

Bronchial cell line HBE and lung carcinoma cell lines (A549, H322, PC9, and GLC-B2) were provided by Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μg/ml penicillin and 0.1 mg/ml streptomycin in a 37°C, 5% CO_2 incubator. After cell growth to 70-80% of confluence, cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in the 96-well plate with 2×10^3 cells per well. Absorbance (A) at 450 nm was recorded at the established time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) to depict the viability curve.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular RNA extraction was conducted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription. The extracted complementary deoxyribose nucleic acid (cDNA) was applied for PCR using the SYBR Green method. Primer sequences were as follows: CASC19: F: 5'-CTCAGCATTTGCCATACTACAT-3', R: 5'-TTCTAACCCAGGCACTCCAA-3'; MiRNA-130b-3p: F: 5'-GCCGCCAGTGCAATGATGAA-3', R: 5'-GTGCAGGGTCCGAGG-3'; ZEB2: F: 5'-AGGAGCAGGTAATCG-3', R: 5'-TGGGCACTCGTAAGG-3'.

Transwell

Matrigel was used to pre-coat the transwell chamber overnight at 4°C. The cell density was adjusted to 2×10^5/mL in serum-free medium. 500 μL of medium containing 10% FBS and 200 μL of cell suspension were added in the basolateral and apical chamber of the 24-well plate, respectively. 24 h later, cells were fixed in methanol for 30 min and stained with 0.1% crystal violet for another 30 min. Invasive cells were observed and photographed using an inverted microscope. The migration assay was conducted in the same procedures except for Matrigel pre-coating (BD Biosciences, San Diego, CA, USA).

Dual-Luciferase Reporter Gene Assay

Based on the predicted binding sequences between miRNA-130b-3p and CASC19/ZEB2, wild-type and mutant-type luciferase plasmids of CASC19/ZEB2 were constructed. Cells seeded in the 24-well plate with 2×10^5 cells per well were co-transfected with CASC19/ZEB2-WT or CASC19/ZEB2-MUT and miRNA-130b-3p mimics or NC. 48 h later, cells were lysed for determining relative luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. The Spearman correlation test was performed for evaluating the correlation between the two genes. Survival analysis was carried out using the Kaplan-Meier estimator. $p<0.05$ was considered as statistically significant.

Results

Upregulation of CASC19 in NSCLC

Compared with matched normal tissues, CASC19 was upregulated in 30 cases of NSCLC tissues (Figure 1A). Identically, CASC19 was highly expressed in lung carcinoma cell lines relative to the bronchial epithelial cell line (Figure

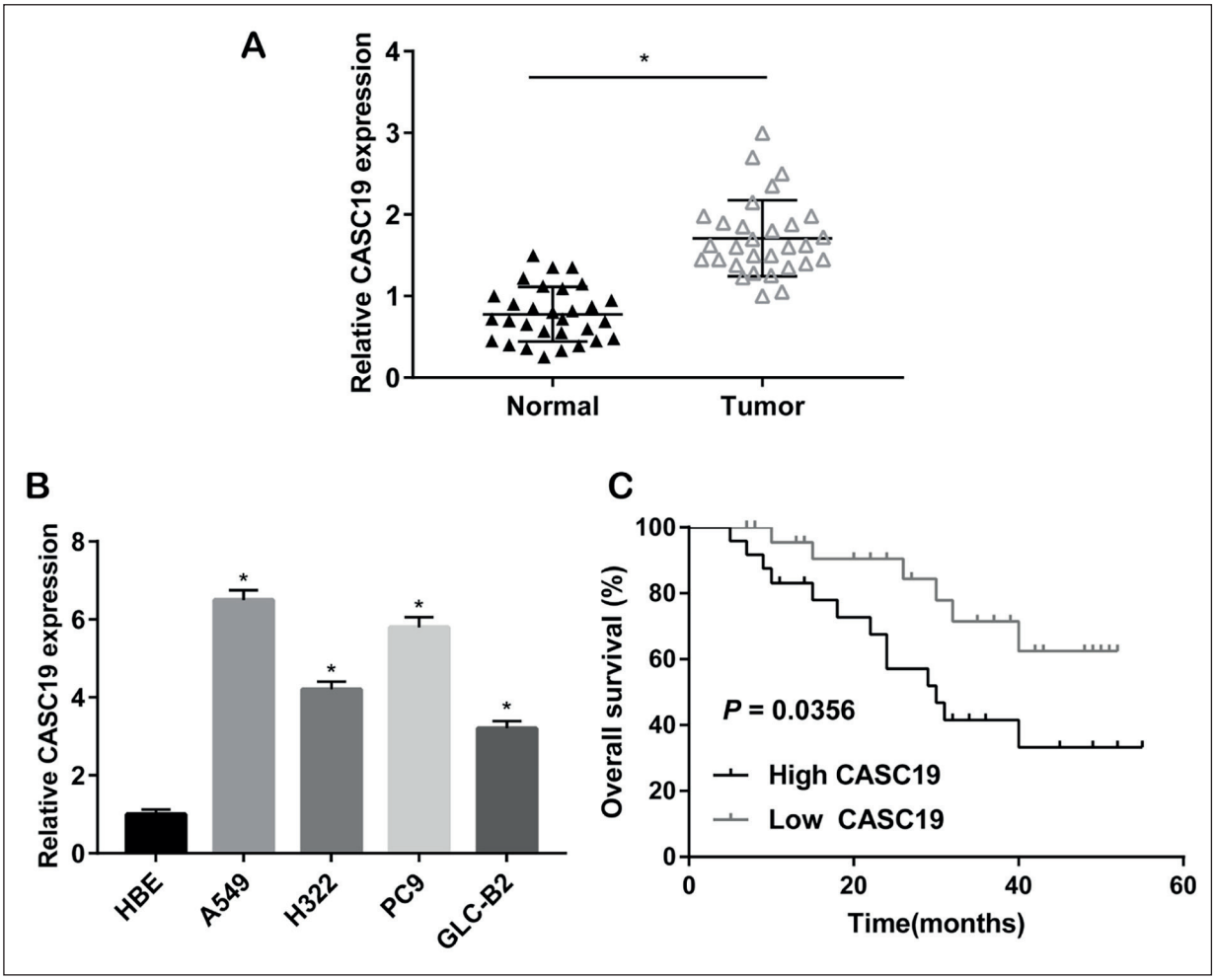

Figure 1. Upregulation of CASC19 in NSCLC. ***A,*** Relative level of CASC19 in NSCLC tissues and matched normal tissues. ***B,*** Relative level of CASC19 in bronchial cell line HBE and lung carcinoma cell lines (A549, H322, PC9, and GLC-B2). ***C,*** Kaplan-Meier survival analysis in NSCLC patients with high and low expression of CASC19.

1B). Among the four detected lung carcinoma cell lines, A549 and PC9 cells expressed the highest level of CASC19 and were selected for the following experiments. Kaplan-Meier curves revealed worse survival in NSCLC patients with high expression of CASC19 than those with low expression (Figure 1C). It is suggested that the upregulated CASC19 in NSCLC may predict poor prognosis.

Knockdown of CASC19 Attenuated Proliferative, Migratory, and Invasive Capacities of NSCLC

Transfection of si-CASC19 markedly downregulated CASC19 level in A549 and PC9 cells, demonstrating an effective transfection efficacy (Figure 2A). The CCK-8 assay showed that transfection of si-CASC19 suppressed viability in A549 and PC9 cells at 48, 72, and 96 h (Figure 2B). The transwell assay revealed the inhibited migratory and invasive capacities in lung carcinoma cells with CASC19 knockdown (Figure 2C).

CASC19 Sponged MiRNA-130b-3p

TargetScan and miRanda predicted binding sequences between CASC19 and miRNA-130b-3p were depicted (Figure 3A). The luciferase activity markedly decreased in A549 cells co-transfected with miRNA-130b-3p mimics and CASC19-WT, verifying the binding relation between CASC19 and miRNA-130b-3p (Figure 3B). Furthermore, si-CASC19 and pcDNA-CASC19 were constructed. Transfection of si-CASC19 upregulated miRNA-130b-3p level. Conversely, the transfection of pcDNA-CASC19

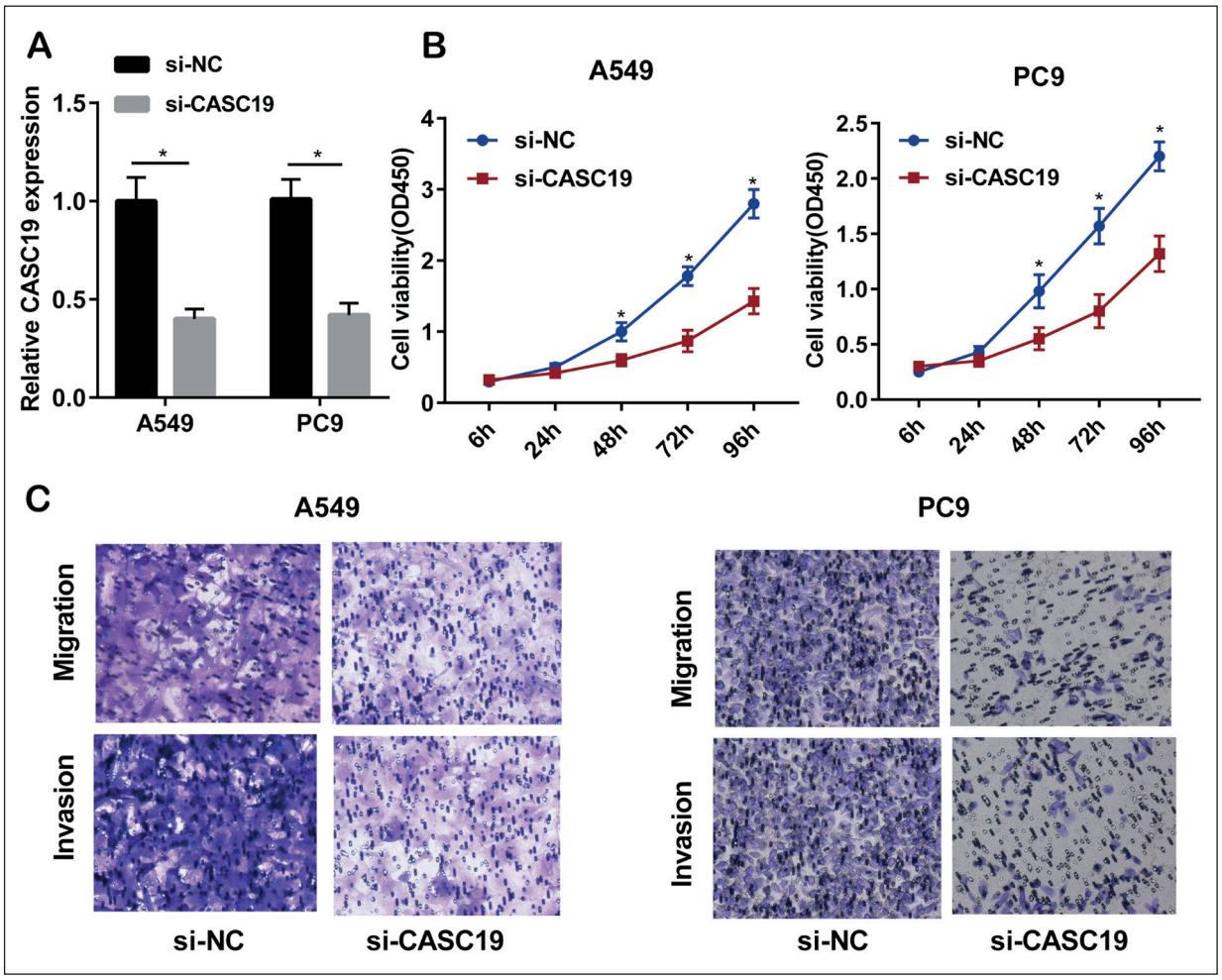

Figure 2. Knockdown of CASC19 attenuated proliferative, migratory and invasive capacities of NSCLC. ***A,*** Transfection efficacy of si-CASC19 in A549 and PC9 cells. ***B,*** CCK-8 assay showed the viability in A549 and PC9 cells transfected with si-NC or si-CASC19 at 6, 24, 48, 72, and 96 h. ***C,*** Transwell assay showed the migration and invasion in A549 and PC9 cells transfected with si-NC or si-CASC19 (magnification: 40×).

downregulated miRNA-130b-3p level in A549 cells (Figure 3C). A negative correlation was subsequently identified between expressions of CASC19 and miRNA-130b-3p in NSCLC tissues (Figure 3E). Compared with adjacent normal tissues, miRNA-130b-3p was downregulated in NSCLC tissues (Figure 3D). The above data confirmed that CASC19 bound to miRNA-130b-3p and negatively regulated its level in NSCLC.

Knockdown of MiRNA-130b-3p Reversed Regulatory Effects of CASC19 on Cellular Behaviors of NSCLC

To uncover the involvement of miRNA-130b-3p in CASC19-mediated cellular behaviors of NSCLC, a series of rescue experiments were conducted. Transfection of si-CASC19 in A549 and PC9 cells upregulated miRNA-130b-3p level, which was reversed by co-transfection of anti-miRNA-130b-3p (Figure 4A). The inhibited viability in lung carcinoma cells with CASC19 knockdown was reversed after knockdown of miRNA-130b-3p (Figure 4B). Similarly, decreased migratory and invasive cell numbers were elevated after the knockdown of miRNA-130b-3p in A549 and PC9 cells (Figure 4C). We believed that CASC19 accelerated the progression of NSCLC by binding to miRNA-130b-3p and inhibiting its level.

Moreover, we predicted the target gene of miRNA-130b-3p in the same way and ZEB2 was finally selected (data not shown). Decreased luciferase activity in A549 cells co-transfected

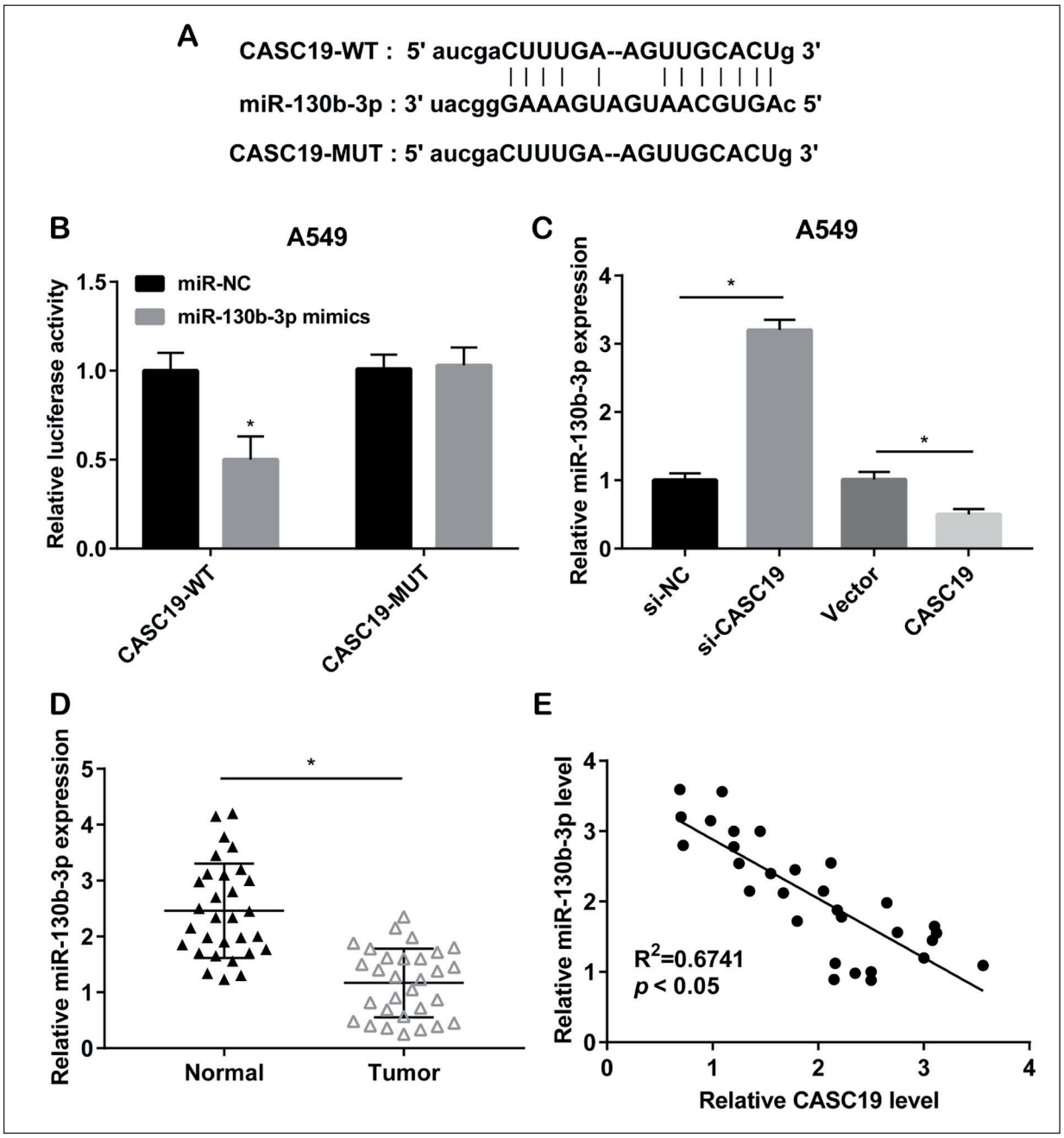

Figure 3. CASC19 sponged miR-130b-3p. ***A,*** Predicted binding sequences between CASC19 and miR-130b-3p. ***B,*** Relative luciferase activity in A549 cells co-transfected with miR-NC/miR-130b-3p mimics and CASC19-WT/CASC19-MUT. ***C,*** Relative level of miR-130b-3p in A549 cells transfected with si-NC, si-CASC19, vector or pcDNA-CASC19. ***D,*** Relative level of miR-130b-3p in NSCLC tissues and matched normal tissues. ***E,*** Negative correlation between expressions of and CASC19 and miR-130b-3p in NSCLC tissues.

with miRNA-130b-3p mimic and ZEB2-WT confirmed the binding relationship between miRNA-130b-3p and ZEB2 (Figure 4D). Transfection of miRNA-130b-3p mimics or si-CASC19 down-regulated ZEB2 level in A549 cells. The down-regulated level of ZEB2 in cells transfected with si-CASC19 was elevated after co-transfection of anti-miRNA-130b-3p (Figure 4E). Collectively, CASC19/miRNA-130b-3p/ZEB2 axis was verified to influence the progression of NSCLC.

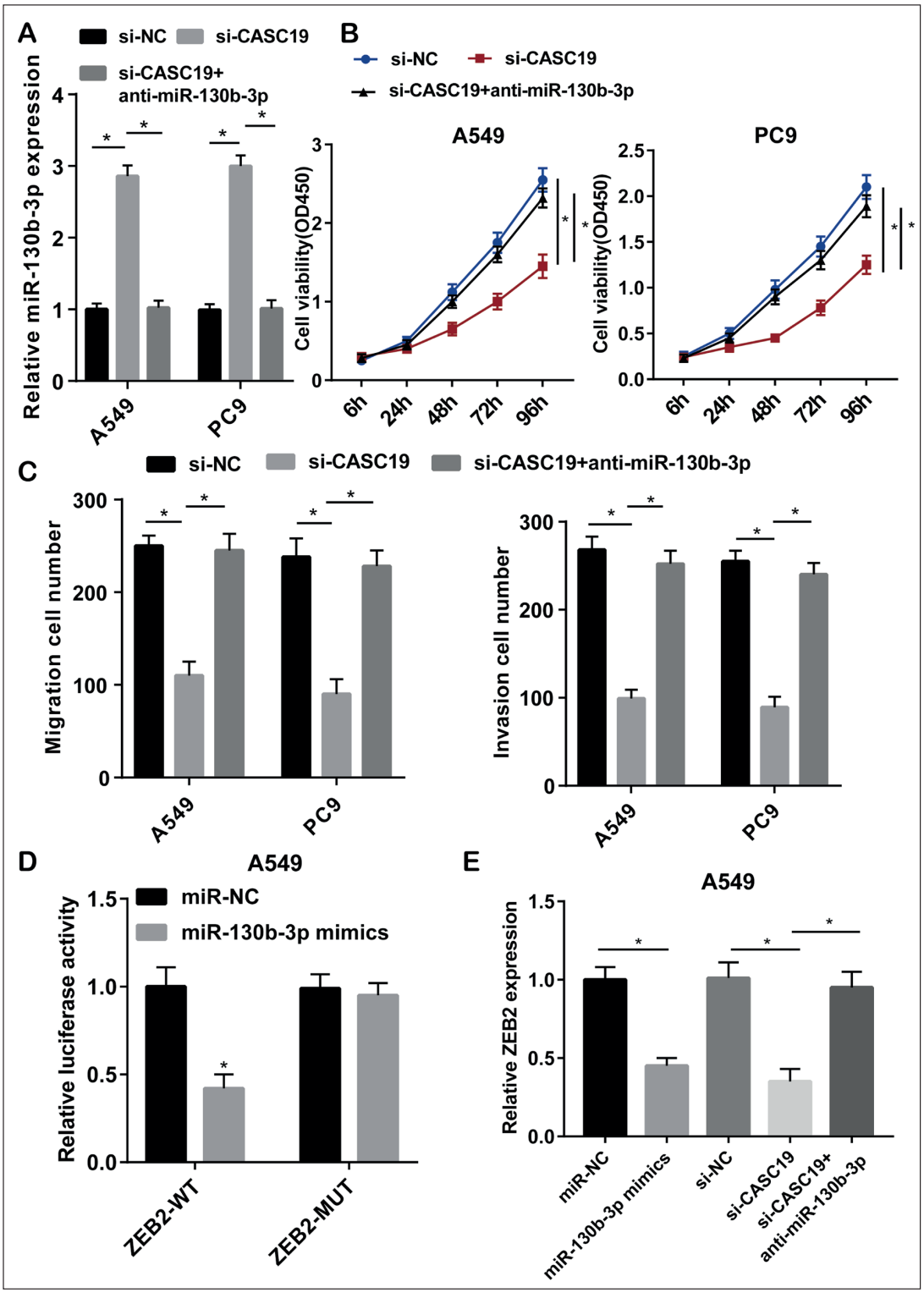

Figure 4. Knockdown of miR-130b-3p reversed regulatory effects of CASC19 on cellular behaviors of NSCLC. ***A,*** Relative level of miR-130b-3p in A549 and PC9 cells transfected with si-NC, si-CASC19 and si-CASC19+anti-miR-130b-3p. ***B,*** CCK-8 assay showed the viability in A549 and PC9 cells transfected with si-NC, si-CASC19, and si-CASC19+anti-miR-130b-3p at 6, 24, 48, 72, and 96 h. ***C,*** Migratory and invasive cell numbers in A549 and PC9 cells transfected with si-NC, si-CASC19 and si-CASC19+anti-miR-130b-3p. ***D,*** Relative luciferase activity in A549 cells co-transfected with miR-NC/miR-130b-3p mimics and ZEB2-WT/ZEB2-MUT. ***E,*** Relative level of ZEB2 in A549 cells transfected with miR-NC, miR-130b-3p mimic, si-NC, si-CASC19 and si-CASC19+anti-miR-130b-3p.

Discussion

Lung carcinoma is a malignancy with the highest morbidity and mortality, affecting 1.8 million newly onset people and 1.6 million deaths per year[14]. Comprehensive treatments, including surgical procedures, chemotherapy, and radiotherapy for NSCLC have made great strides. Nevertheless, the 5-year survival of NSCLC is still low[15]. Tumor metastasis is the major reason for the poor prognosis of NSCLC[16]. Metastatic mechanism of NSCLC is a complex and dynamic process involving various factors. It is of great significance to uncover the etiology and pathogenesis of NSCLC, which contributes to developing new strategies for preventing and treating NSCLC[17].

Some studies[18] have shown that 18% of the protein-coding genes that produce lncRNAs are associated with cancer, while only 9% of all human protein-encoding genes are associated with cancer. Owing to the vital functions in gene expression regulation, lncRNAs are believed to participate in diverse cellular behaviors both in physiological and pathological processes[19,20]. Therefore, identification of tumor-related lncRNAs helps to uncover the occurrence and progression of tumors[21]. Several lncRNAs have been reported to be abnormally expressed in lung carcinoma, showing crucial regulatory effects on disease progression, such as MALAT1, HOTAIR, SCAL1, and MEG3[22-26]. In this paper, CASC19 was markedly upregulated in NSCLC tissues and cell lines. Knockdown of CASC19 attenuated proliferative, migratory, and invasive capacities of NSCLC cells. Notably, NSCLC patients with high expression of CASC19 presented worse prognosis, suggesting the carcinogenic role of CASC19 in NSCLC.

Increasing evidence has illustrated that lncRNAs could interact with miRNAs to influence target gene expressions, thereafter altering the biological functions of downstream genes. Xu et al[27] demonstrated that XIST sponges miR-374a as a ceRNA to upregulate LARP1 level, and thus accelerates proliferative, migratory, and invasive capacities of NSCLC cells. Chen et al[28] illustrated the role of PVT1 in stimulating NSCLC metastasis *via* regulating MMP9 by binding to miR-200a and miR-200b. This work predicted the binding sequences of CASC19 and miRNA-130b-3p using TargetScan and miRanda, and subsequently verified through the dual-luciferase reporter gene assay. Moreover, a negative correlation was identified between CASC19 and miRNA-130b-3p. The regulatory effects of CASC19 on cellular behaviors of lung carcinoma cells were reversed by the knockdown of miRNA-130b-3p. It is suggested that CASC19 was a ceRNA to sponge miRNA-130b-3p. ZEB2 is a key regulator in the progression of NSCLC[29]. Li et al[30] indicated that XIST accelerates TGF-β-induced EMT in NSCLC *via* recruiting ZBR2 as a ceRNA. Our results showed that ZBR2 was the direct target of miRNA-130b-3p and its level could be regulated by CASC19 and miRNA-130b-3p. Collectively, CASC19/miRNA-130b-3p/ZBR2 axis mediated the progression of NSCLC.

Conclusions

We showed that CASC19 sponges miRNA-130b-3p to regulate ZBR2 as a ceRNA, thus accelerating the progression of NSCLC by regulating proliferative, migratory, and invasive capacities of tumor cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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