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Long noncoding RNA CASC15 is upregulated in non-small cell lung cancer and facilitates cell proliferation and metastasis *via* targeting miR-130b-3p

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Abstract. – OBJECTIVE: Recent researches have discovered a class of long noncoding RNAs (lncRNAs), which are dysregulated in various tumors and linked to carcinogenesis. This study aims to uncover the molecular functions of lncRNA CASC15 in non-small cell lung cancer (NSCLC) tumorigenesis.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed to detect CASC15 expression in 55 NSCLC samples and four NSCLC cell lines. Besides, the function of CASC15 was d[illegible]ed through proliferation assay, transwell [illegible] and wound healing assay in NSCLC cells. Furthermore, the interaction between CASC15 [illegible] miR-130b-3p in NSCLC was studied by perfo[illegible]ing dual-luciferase reporter as[illegible] additio[illegible] tumor formation and metasta[illegible]ere pe[illegible] formed *in vivo*.

RESULTS: CASC15 ex[illegible]sion w[illegible]remarkably upregulated in NSC[illegible]mpl[illegible] with that in adjacent [illegible]ple[illegible]roliferat[illegible]n, invasion, and mig[illegible]n in NS[illegible]were inhibited *via* knockdow[illegible]ASC15 *in* [illegible]Moreover, RT-qPCR resu[illegible]d that miR[illegible]-3p was upregulated [illegible]knoc[illegible]n of CASC15 *in vitro*. In additio[illegible]iR-130b-3p[illegible] a direct target of CASC15 [illegible]NSCLC. Tumor[illegible]ation and metastasis [illegible]e inhibited after C[illegible]C15 was knockdow[illegible] *vivo*.

C[illegible]US[illegible]S: Our study indicates that CASC[illegible] prom[illegible]metastasis and proliferat[illegible]of [illegible]C th[illegible]gh sponging miR-130b-3p *in vitro* and *in vivo*, which may offer a new therapeutic interve[illegible]n for NSCLC patients.

Key Words: Long [illegible]ding RNA, CASC15, NSCLC, MiR-130b-3p.

Introduction

Lung cancer is one of the most common and life-threatening cancers in the world, which is the leading cause of cancer-related deaths among men and wo[illegible]6% and 2[illegible]ctively)[1,2]. It has bee[illegible]o[illegible]hat 234,00[illegible]w cases are diagnosed [illegible]ith lun[illegible]cer in America in 2018, which [illegible]contribute[illegible]54,000 deaths in the sa[illegible]ear[illegible]. As the most[illegible]mmon type of lung c[illegible]er, non-small cell lung cancer (NSCLC) ac[illegible]ts for appro[illegible]ately 85% of all lung cancer ca[illegible]. The prin[illegible] feature of NSCLC is the mig[illegible]n and i[illegible]sion of neoplasms which lead to the [illegible]gnosis of lung cancer patients[4]. Moreover, most of NSCLC cases are diagnosed at [illegible]ed stages without the opportunity to take [illegible]surgery. Thus, it's important to understand the molecular mechanism of metastasis and progression of NSCLC.

Long noncoding RNAs (lncRNAs), as one subgroup of non-coding RNAs, have been reported to participate in many cellular processes of tumorigenesis. For instance, the lncRNA-CCHE1 expression is positively related to the malignancy of colorectal carcinoma *via* regulation of ERK/COX-2 pathway[5]. LncRNA HCCL5 activated by ZEB1 accelerates cell viability, cell migration, epithelial-mesenchymal transition, and malignancy of hepatocellular carcinoma[6]. LncRNA linc-PINT functions as a tumor suppressor in gastric cancer *via* the interaction with miR-21 and is associated with a poor survival of GC patients[7]. LncRNA SNHG1 could inhibit the differentiation of Treg cells and suppress the immune escape of breast cancer[8].

In this study, we firstly found out that the expression level of CASC15 was remarkably upregulated in NSCLC samples. Moreover, further experiments revealed that CASC15 promoted cell proliferation, invasion, and migration of NSCLC *in vitro* and *in vivo*. Furthermore, we discovered that CASC15 played its function in NSCLC by sponging miR-130b-3p.

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Patients and Methods

Clinical Samples

Human tissues were obtained from 55 NSCLC patients who underwent surgery during China-Japan Union Hospital of Jilin University. All tissues were kept at –80°C. The written informed consent was obtained by all the participants in this study before the operation. This investigation was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University.

Cell Culture

The NSCLC cell lines (SPCA1, H1299, PC-9, and H358) were obtained from the Chinese Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). The normal human bronchial epithelial cell line (16HBE) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) and 10% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA) were used to incubate the cells in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

For transfection, lentivirus expres[illegible] short-hairpin RNA (shRNA) targeting CASC[illegible] was compounded and then [illegible] pLent[illegible] EF1a-EGFP-F2A-Puro vecto[illegible]oset[illegible]nc., San Diego, CA, USA). CASC[illegible] shRNA [illegible]ASC15/shRNA) and scramble ve[illegible]were [illegible] 293T cells and then [illegible] tra[illegible]n in H1299 NSCLC cells. The [illegible]re transfe[illegible] process was performed by u[illegible]fectamine [illegible] (Invitrogen, Carlsbad, [illegible]A, U[illegible]

RNA E[illegible]ction and Re[illegible] Tim[illegible]uantit[illegible]ive Polym[illegible]rase Ch[illegible] Reac[illegible] (RT-qPCR)

To[illegible] was ex[illegible]ted from cultured NS[illegible]C ce[illegible] pati[illegible] tumor tissues by using T[illegible]l rea[illegible]KaRa Bio, Inc., Otsu, Shiga, [illegible]pan) an[illegible]en reverse-transcribed to compl[illegible] deoxyribose nucleic acids (cDNAs) [illegible]ough [illegible]erse Transcription Kit (TaKaRa, Ot[illegible], Shiga, Japan). Real-time PCR was performed [illegible] a FastStart Universal SYBR Green Master ki[illegible]oche, Basel, Switzerland). Expression data were normalized by β-actin levels. The relative expression was calculated by performing the $2^{-\Delta\Delta CT}$ method. The primers used were: CASC15, forward 5′-CACACGCATGGAAAACCCAG-3′ and reverse 5′-GAGGACCTGAGCTGTAAGCC-3′; β-actin, forward 5′-GATGGAAATCGTCAGAGGCT-3′ and reverse 5′-TGGCACTTAGTTGGAAATGC-3′. The thermal cycle was as follows: 30 sec at 95°C, 5 sec for 40 cycles at 95°C, 35 sec at 60°C.

Cell Proliferation Assay

Following the protocol (Dojind[illegible]olecular Technologies, Inc., Kumamo[illegible]Japan), [illegible] proliferation of these treated [illegible] in 96-we[illegible] was monitored by cell [illegible]nting [illegible]-8 (CC[illegible] assay every 24 h. S[illegible]ophot[illegible]ter (Thermo Scientific, Waltha[illegible] MA[illegible] was uti[illegible]d to measure the abs[illegible]nce at [illegible]m.

Wound [illegible]ssay

1.0 × 10[illegible] cells w[illegible]eed into a 6-well plate. Three [illegible]lel lines w[illegible]made on the back of ea[illegible]. After reache[illegible]out 90% confluenc[illegible]ells were scratched with a pipette tip and c[illegible]red in a m[illegible]m. Cells were photographed u[illegible] a light mi[illegible]scope after 48 h. Each assay wa[illegible]pende[illegible] repeated in triplicate.

Transwell Assay

[illegible]m pore size insert was provided by Corn[illegible]g ([illegible]ning, NY, USA). 4 ×10^4 cells in 150 µL serum-free DMEM were transformed to the top chamber of the insert coated with 50 µg Matrigel (BD, Bedford, MA, USA). The bottom chamber was filled with DMEM and FBS. 48 h later, the top surface of chambers was immersed for 10 min with precooling methanol and was stained in crystal violet for 30 min.

Dual-Luciferase Reporter Assay

DIANA LncBASE Predicted v.2 was used to predict the potential target of microRNAs and fragment sequences containing CASC15 reaction sites. For the luciferase assay, the 3′-UTR of CASC15 was cloned into the pGL3 vector (Promega, Madison, WI, USA), which was identified as wild-type (WT) 3′-UTR. Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used for site-directed mutagenesis of the miR-130b-3p binding site in CASC15 3′-UTR, which was named as mutant (MUT) 3′-UTR. Cells were transfected with WT-3′-UTR or MUT-3′-UTR and negative control or miR-130b-3p for 48 h. Then, the luciferase assay was conducted on the dual luciferase reporter assay system (Promega, Madison, WI, USA).

Tumor Formation and Metastasis Assay

Transfected H1299 cells (6×10^5/mL) were replaced into NOD/SCID mice (6 weeks old) subcutaneously. Tumor diameters were detected every 5 days after inoculation. Tumor volume was calculated as the formula (volume = length × width^2 × 1/2). Tumors were extracted after 4 weeks. Transfected H1299 cells (6×10^5/mL) were injected into the tail vein of NOD/SCID mice (6 weeks old). The mice were sacrificed, and the lungs were extracted after 4 weeks. Then, the number of metastatic nodules in the lung was counted.

Statistical Analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was utilized to conduct the statistical analysis. The two-tailed Student's *t*-test was performed to analyze the significance. When $p<0.05$, the data were considered statistically significant.

Results

Expression Level of CASC15 in Tissu and Cells of NSCLC

RT-qPCR was conducted for detecting CAS expression in 55 patients' tissues and four NSC cell lines. CASC15 was signifi regulate in tumor tissue samples tha t in a cent tissues (Figure 1A). In addi CASC1 evel was significantly higher in NS cell 16HBE (Figure 1B).

Knockdown of CASC15 Repressed Cell Growth, Migration and Invasion in NSCLC Cells

According to CASC15 expression in NSCLC cells, we chose H1299 NSCLC cells for knockdown of CASC15. The CASC15 (CASC15/shRNA) and the scramble or (NC) were synthetized and transduced in 299 cells. Then, the CASC15 expression was d ined by RT-qPCR (Figure 2A). Res of Cc ssay suggested that the cell gro ability of cells was inhibited afte ASC15 as kno down (Figure 2B). T the ts of would healing assay sho d t migrate ngth of NSCLC cell inhibi y kno own of CASC15 (Fig C). In addi esults of the transw s showed tha number of invaded NS LC ce s reduced after CASC15 was k down (F 2D).

Knockdown of CASC15 Inhibited NSCLC Tumorigenesis Via Sponging MiR-130b-3p

ed DI A LncBASE Predicted v.2 to predic target microRNAs of CASC15. MiR-130b-3p was one of those predicted microRNAs which was reported to suppress tumorigenesis in many tumors. The binding area of CASC15 by miR-130b-3p was shown in Figure 3A. Moreover, miR-130b-3p was upregulated in CASC15/shRNA group compared with NC group (Figure 3B). Furthermore, the results of luciferase assay showed that luciferase activity was significantly reduced through co-transfection of CASC15-WT

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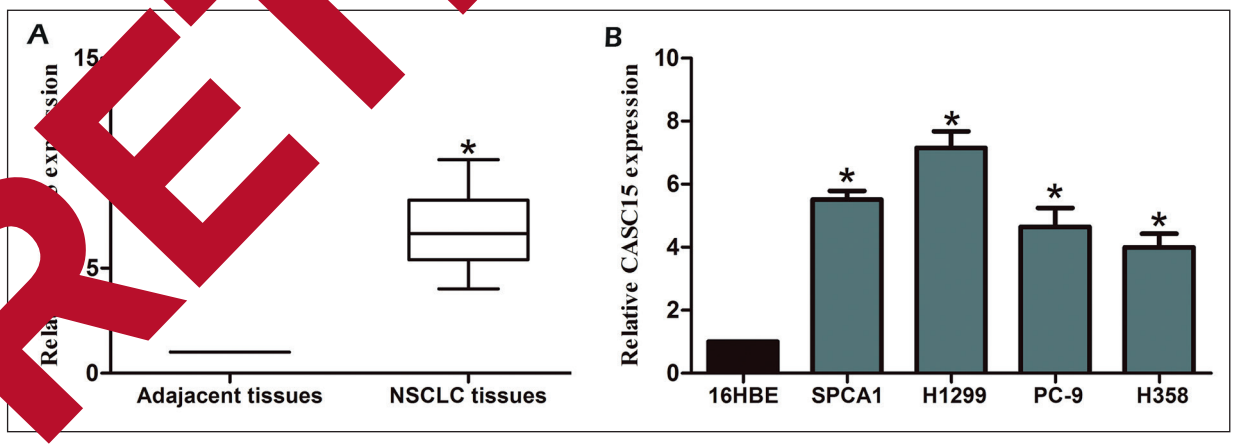

Figure 1. Expression levels of CASC15 were upregulated in NSCLC tissues and cell lines. **A,** CASC15 expression was significantly upregulated in the NSCLC tissues compared with adjacent tissues. **B,** Expression levels of CASC15 relative to β-actin were determined in the human NSCLC cell lines and normal human bronchial epithelial cell (16HBE) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

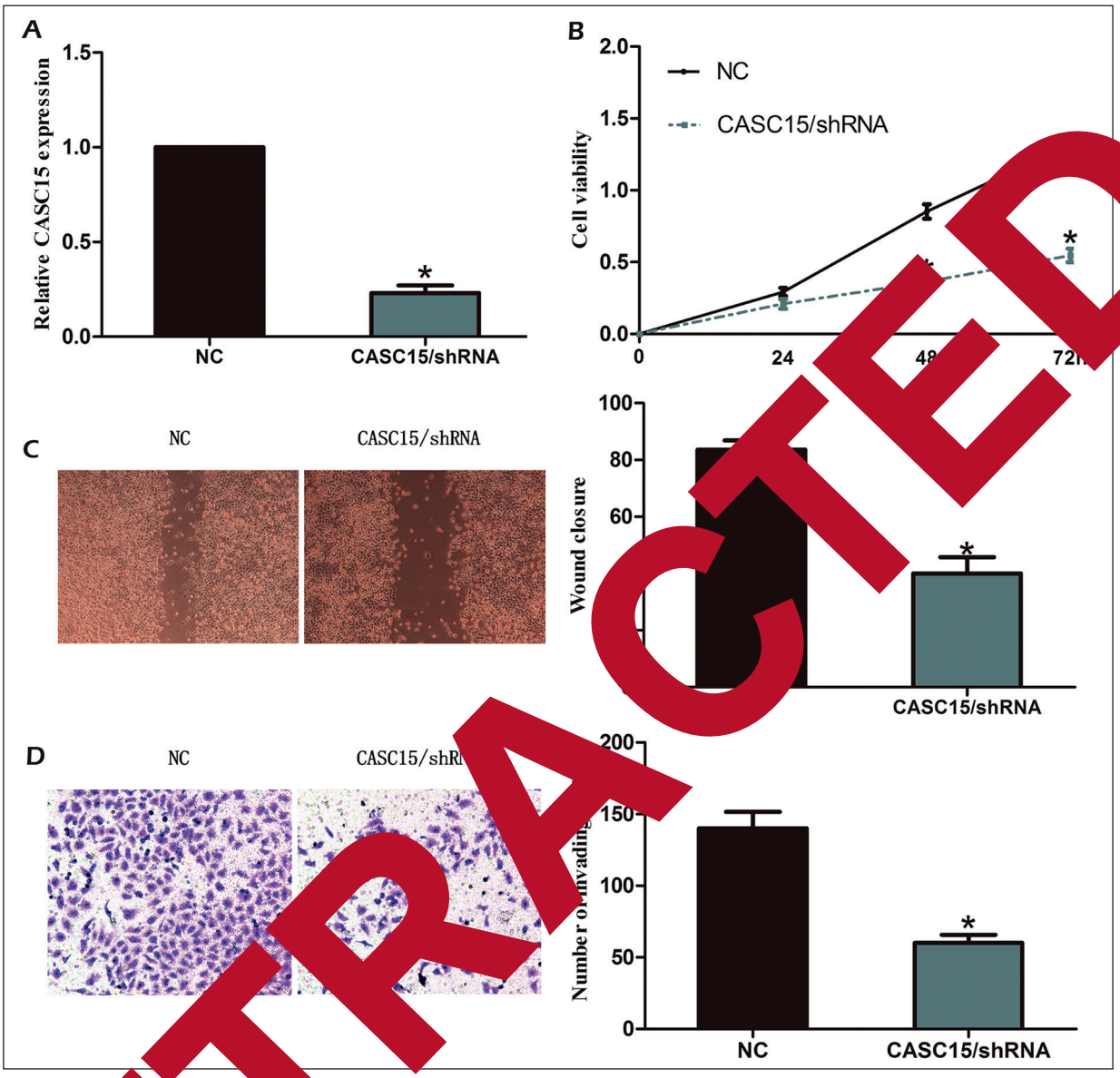

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Figure 2. ...kdown of CA... ...hibited H1299 NSCLC cell proliferation, migration, and invasion. **A,** CASC15 expression in NSCL... ...s transduced with ...le vector (NC) or CASC15 shRNA (CASC15/shRNA) was detected by RT-qPCR. β-actin was us... ...an inter...l control. **B,** C...8 assay showed that knockdown of CASC15 significantly repressed cell proliferation in H12... ...SCLC ce..., Wound healing assay showed that the migrated length of cells in CASC15/shRNA group was significantly decre... ...on... with N... group in H1299 NSCLC cells (magnification: 10×). **D,** Transwell assay showed that number of invaded ...SCLC ce... ...s decreased in CASC15/shRNA group compared with NC group (magnification: 40×). The results ...sent t... ...age o... ...e independent experiments (mean ± standard error of the mean). *$p<0.05$.

a... ...-130b-3p, while no significant chang- ...of lu... ...ase activity were observed through ...o-transfection of CASC15-MUT and miR-130b- ...Figure 3C).

Knockdown of CASC15 Inhibited Tumor Formation and Metastasis In Vivo

The ability of CASC15 in tumor formation and metastasis was detected *in vivo*. The tumor size in CASC15/shRNA group was smaller compared with NC group (Figure 4A). The number of metastatic nodules in the lung of the CASC15/shRNA group was significantly reduced compared with NC group (Figure 4B). Moreover, the expression level of CASC15 and miR-130b-3p in dissected tumor tissues was detected by RT-qPCR. The results showed that CASC15 was lower-expressed in CASC15/shRNA group compared with NC

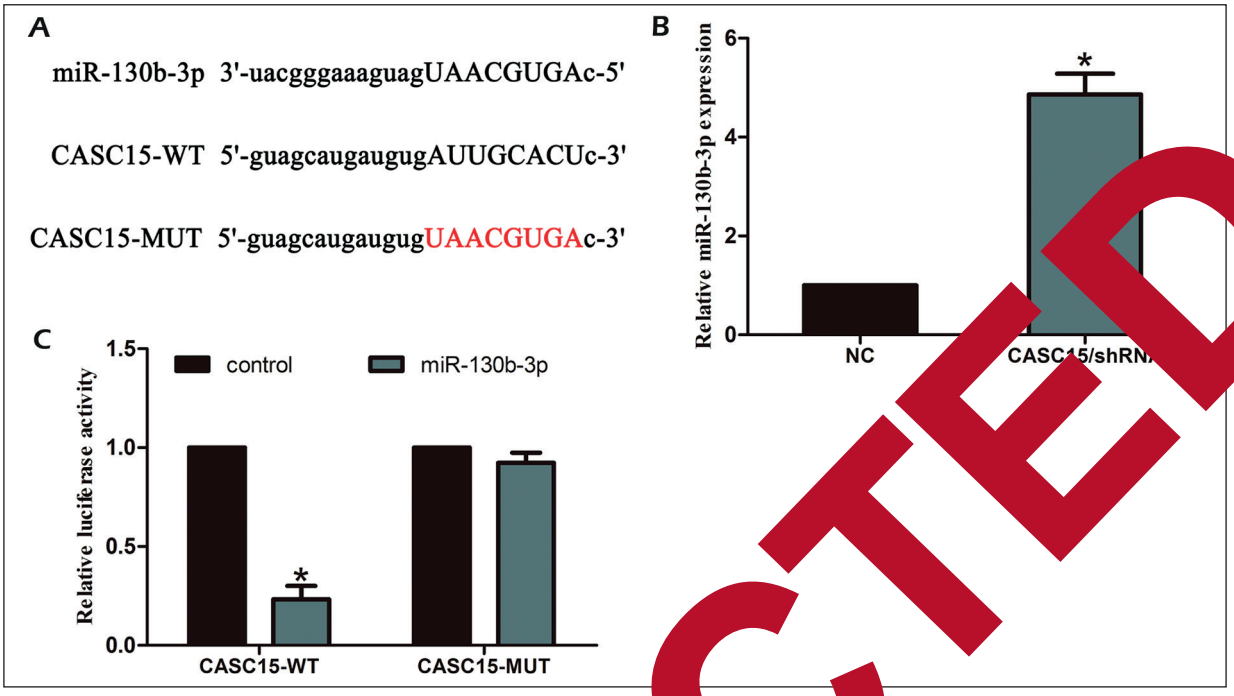

Figure 3. Interaction between miR-130b-3p and CASC15 in NSC[illegible]A. The bindi[illegible]es of miR-130b-3p on CASC15. **B,** The miR-130b-3p expression was increased in CASC15/shRNA grou[illegible]pared wi[illegible]C group. **C,** Co-transfection of miR-130b-3p and CASC15-WT strongly decreased the lucif[illegible]se activity, wh[illegible]on of miR-130b-3p and CASC15-MUT did not change the luciferase activity either. The r[illegible]esent the aver[illegible] three independent experiments. Data are presented as the mean ± standard error of the mean[illegible]

group (Figure 4C), while miR-1[illegible]was high[illegible]er-expressed in CASC15/shR[illegible]grou[illegible]mpared with NC group (Figure 4D[illegible]

Discussion

Evidence h[illegible]rove[illegible]lncRNAs participates in the re[illegible]sion of N[illegible] development. For exampl[illegible]y sponging to [illegible]124-3p, lncRNA OGF[illegible] has b[illegible] reported[illegible] to take part in cell pr[illegible]ation [illegible]SCLC. LncRNA HEIH accelerates [illegible]tastasis [illegible]proliferation of NSCLC *vitro* [illegible]RNA [illegible]NCR1 promotes the tumor p[illegible]ssio[illegible]LC through PRNCR1-miR-48[illegible]EY2 ne[illegible]k[11]. By sponging miR-27b-3p, l[illegible]KCNQ1OT1 facilitates cell proliferation [illegible]sion in the progression of NSCLC [illegible]rough upregulating HSP90AA1[12].

[illegible]ancer susceptibility candidate 15(CASC15), l[illegible]d on chromosome 6p22.3, is firstly discovered in silico. Some researches have indicated that CASC15 plays an important role in tumorigenesis in several cancers. For example, CASC15 enhances cell metastasis and hepatocarcinogenesis in hepatocellular carcinoma, which is also correlated with poor prognosis of the patients[13]. CASC15 is also reported to promote cell proliferation in gastric cancer and is a risk factor for gastric cancer patients' prognosis[14]. In addition, by regulating the expression of SOX4, CASC15 is found to participate in RUNX1-rearranged acute leukemia[15]. Our study showed that the expression of CASC15 was upregulated in both NSCLC tissues and cells. Furthermore, after CASC15 was knocked down, the cell growth ability, migrated and invaded ability were suppressed in NSCLC. These results indicated that CASC15 functioned as an oncogene and promoted the tumorigenesis of NSCLC.

Then, bioinformatics analysis was used to predict the potential target microRNAs of CASC15. MiR-130b-3p, as one of those predicted RNAs, takes part in regulating numerous biological processes of cancers. For example, miR-130b-3p inhibits cell growth and promotes cell apoptosis *via* regulating CYLD in gastric cancer[16]. MiR-130b-3p is aberrantly expressed in prostate cancer and inhibits prostate cancer metastasis *via* targeting MMP2[17,18]. Moreover, miR-130b-3p

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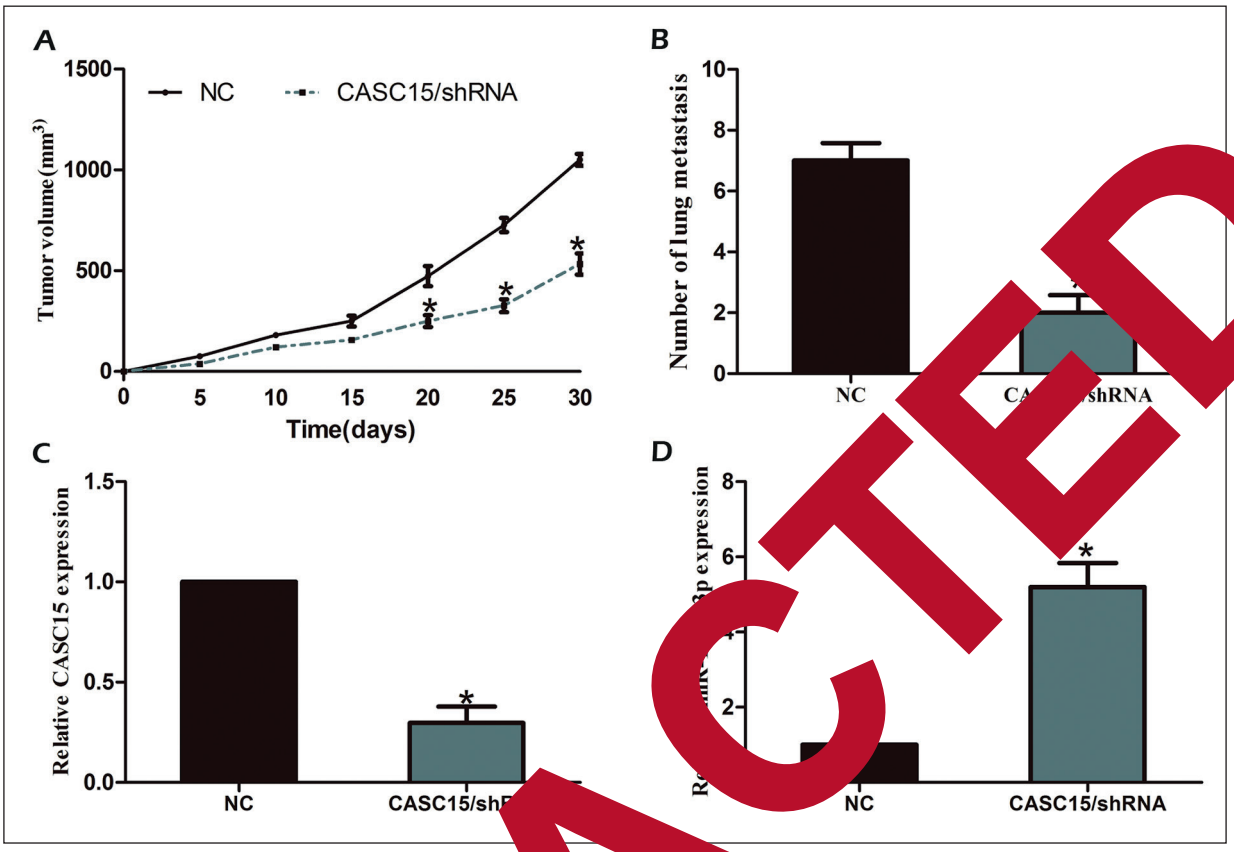

Figure 4. Knockdown of CASC15 inhibited tumor for[illegible]on a[illegible]of NSCLC *in vivo*. **A,** The tumor size in CASC15/shRNA group was smaller compared with NC group. [illegible]mber of metastatic nodules in the lung from the CASC15/shRNA group was significantly reduce[illegible]ared to NC[illegible]p. **C,** CASC15 of those dissected tumors was lower-expressed in CASC15/shRNA group compare[illegible]roup. **D,** [illegible]-130b-3p of those dissected tumors was higher-expressed in CASC15/shRNA group compared[illegible]NC gr[illegible] The resu[illegible]present the average of three independent experiments (mean ± standard error of the mean). *[illegible]05, as co[illegible]ed with the [illegible]ontrol cells.

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is downregulat[illegible]east cance[illegible]l inhibits cell invasion[illegible]d m[illegible]ion *in vitr*[illegible]. In our study, the [illegible]raction be[illegible]n miR-130b-3p and CASC1[illegible]as further ex[illegible]d. MiR-130b-3p was [illegible]egulate[illegible]after CAS[illegible]15 was knocked do[illegible] *vitr*[illegible] hat's more, the luciferase assay i[illegible]owed t[illegible]miR-130b-3p could be [illegible]ctly [illegible]ted b[illegible]ASC15. Moreover, a neg-a[illegible]corre[illegible]s discovered between miR-13[illegible]p and [illegible]SC15 expression in NSCLC ti[illegible]. The results above revealed that CASC15 [illegible]y re[illegible]ts function in NSCLC *via* sponging [illegible]iR-130b-3p.

[illegible]o further verify the function of CASC15 in N[illegible]LC *in vivo*, tumor formation and metastasis assay were conducted. Results of the tumor formation assay revealed that knockdown of CASC15 could inhibit tumor formation in nude mice. Results of tumor metastasis assay revealed that knockdown of CASC15 could significantly suppress tumor metastasis in nude mice. Through the detection of CASC15 and miR-130b-3p expression in those extracted tumors, we found that CASC15 was downregulated and miR-130b-3p was upregulated in nude mice treated with CASC15/shRNA.

Conclusions

LncRNA CASC15 is a new biomarker in the progression of NSCLC and could enhance NSCLC cell proliferation, migration, and invasion through sponging miR-130b-3p.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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