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Long non-coding RNA CASC15 promotes nasopharyngeal carcinoma cell proliferation and metastasis by downregulating miR-101-3p

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Abstract. – OBJECTIVE: Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors worldwide. Recent studies have revealed that long non-coding RNAs (lncRNAs) play important roles in the progression of tumorigenesis. The aim of this study was to identify the exact role of lncRNA CASC15 in the progression of NPC.

PATIENTS AND METHODS: CASC15 expression in both 54 paired NPC patients' tissue samples and cell lines was detected by Real-Time-quantitative Polymerase Chain Reaction (RT-qPCR). Moreover, the function of CASC15 was identified by performing cell proliferation assay, transwell assay and wound healing assay in vitro. The underlying mechanism was explored through Luciferase assay and qPCR. In addition, tumor formation and metastasis assays were conducted *in vivo*.

RESULTS: CASC15 expression in tissues was markedly higher than that of adjacent non-tumor tissues. The proliferation, migration and invasion of NPC cells were significantly inhibited after knockdown of CASC15 *in vitro*. Our further experiments revealed that miR-101-3p was remarkably up-regulated by knockdown of CASC15. Meanwhile, miR-101-3p was a direct target of CASC15 in NPC. Furthermore, tumor formation and metastasis of NPC were significantly inhibited via knockdown of CASC15 in nude mice.

CONCLUSIONS: CASC15 enhances NPC cell proliferation and metastasis via sponging miR-101-3p *in vitro* and *in vivo*.

Keywords:
long non-coding RNA, CASC15, Nasopharyngeal carcinoma (NPC), MiR-101-3p.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck epithelial cancers worldwide, especially in Southern China and Southeast Asia. NPC is characterized by poor or undifferentiated cancer. Due to the advances in modulated radiotherapy and combined chemotherapy, the prognosis of patients with local and regional NPC has been significantly improved. However, most NPC patients are already in advanced stages when initially diagnosed, with an overall survival of 12 months[2,3]. Therefore, it is urgent to stimulate a better understanding of the molecular mechanisms of NPC progression.

Genome sequencing technology indicates that almost genome sequence transcripts are non-coding RNAs (ncRNAs). Long non-coding RNA (lncRNA) is a subtype of ncRNA with longer than 200 nucleotides in length. Recently, research has revealed that lncRNAs function as key regulators in numerous biological processes, including the development of diverse cancers. For example, knockdown of lncRNA MNX1-AS1 inhibits the proliferation and migration of ovarian cancer cells. Meanwhile, it may be a potential therapeutic target for ovarian cancer[4]. By depressing tumor suppressor miR-143, lncRNA UCA1 functions as an oncogene in breast cancer and participates in the modulation of cell growth and apoptosis[5]. LncRNA RUNX1-IT1 acts as a tumor suppressor in colorectal cancer by inhibiting cell migration and cell proliferation[6]. Furthermore, overexpression of lncRNA NNT-AS1 facilitates the proliferation and invasion of cervical cancer cells through the Wnt/beta-catenin signaling pathway[7].

Our study demonstrated that CASC15 was remarkably up-regulated in NPC tissues and cell lines. Moreover, knockdown of CASC15 significantly inhibited the proliferation and invasion of NPC *in vitro* and *in vivo*. In addition, our findings indicated that the function of CASC15 in NPC was associated with miR-101-3p.

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Patients and Methods

Tissue Specimens

54 paired tumor tissues and adjacent non-tumor tissues were sequentially gathered from NPC patients who underwent surgery in the Affiliated Hospital of Jiangnan University. All patients were diagnosed with NPC by two independent pathologists without any controversial. This study was approved by the Ethics Committee of the Affiliated Hospital of Jiangnan University. Informed consents were obtained from all participants before the study.

Cell Culture

NPC cancer cell lines (CNE2, CNE1, 5-8F and 6-18B) and immortalized normal nasopharyngeal epithelial cell line (NP69) were purchased from the Chinese Academy of Science (Shanghai, China). All cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Besides, the cells were cultured in an incubator containing 5% at 37°C.

Cell Transfection

Lentivirus expressing short-h RNA (shRNA) directly against CASC wa rovided by GenePharma (Shanghai hina). gative control shRNA was synthes as y ly, CASC15 shRNA or n ive shRNA was transfected into NP cells acc g to the manufacturer's proto Lipofectan 3000 reagent (Invitrogen lsb CA, USA).

RNA Extracti and Real Time-Quan tive olymerase Chain Re on (

Total RN tracte om NPC cells or tissue s ples ing zol reagent (TaKaRa, ga, Extracted RNA was the evers ranscri d into complementary d yribo acids (cDNAs) through the Transcription Kit (TaKaRa, Otsu, Shiga, n). Primer sequences used for Real Time-Qu tive Polymerase Chain Reaction (RT-qPCR) ere as follows: CASC15 forward: 5′-CACACGCATGGAAAACCCAG-3′ and reverse: 5′-GAGGACCTGAGCTGTAAGCC-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse: 5′-TGGTGAAGACGCCAGTGGA -3′. The specific procedure for PCR was as follows: pre-denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Relative expression was calculated by t method. This experiment was repeat three

Cell Proliferation A say

Cell viability was onitored a to the instructions of Ce ounti Kit-8 (K-8; Dojindo Molecular chno es, K mamoto, Japan) assay. Bri CNE1 s were seeded into 96-w plates nigh fter incubation with CAS shRNA , the cells were incubat 10 µL of K-8 for 3 h in the dark. A orban 450 nm was measured by an enzyme-linked i sorbent assay (ELISA) r ystem (Multi an Ascent, LabSystems, sinki, Finland).

S h W nd Assay

Ce e first transferred into 6-well plates and cultured in RPMI-1640 medium overnight. ratched with a plastic tip, the cells were ultur d in serum-free RPMI-1640. Wound closure was viewed at 48 h. Each assay was independently repeated three times.

Transwell Assay

Transwell chambers with 8 µm pores were provided by Corning (Lowell, MA, USA). First, the membrane was pre-coated with 50 µL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Subsequently, the cells were seeded into the upper chamber of a 24-well plate. Meanwhile, 20% FBS-RPMI-1640 was added to the lower chamber of culture inserts. After incubation for 24 h, these inserts were fixed with methanol for 30 min and stained with hematoxylin for 20 min. The number of migrated cells was counted under a light microscope (Olympus, Tokyo, Japan).

Luciferase Reporter Gene Assay

DIANA LncBASE Predicted v.2 was used to predict the potential target gene and fragment sequences containing CASC15 reaction sites. The Luciferase reporter gene assay (Promega, Madison, WI, USA) was used to detect the Luciferase activity of NPC cells. The Luciferase reporter gene vector was constructed and transfected into

CNE1 cells. CASC15 3′-untranslated region (3′-UTR) wild-type (WT) sequence named CASC15-WT was 5′-GGUGAGGAUCUUAUUACU-GUA-3′, and mutant sequence of CASC15 3′-UTR missing the binding site with miR-101-3p named CASC15-MUT was 5′-GGUGAGGAUCUUAU-AUGACAU-3′.

Xenograft Model

For tumor formation assay, transfected CNE1 cells were subcutaneously injected into NOD/SCID mice (6 weeks old). Tumor diameters were detected every 5 days after inoculation. Tumor volume was calculated as the formula: volume= length × width^2 × 1/2. The mice were sacrificed, and tumors were extracted after 4 weeks. For tumor metastasis assay, transfected CNE1 cells were injected into the tail vein of NOD/SCID mice (6 weeks old). The mice were sacrificed, and lung tissues were extracted after 4 weeks. The number of metastatic nodules in lung tissues was counted. Animal experiments were approved by the Animal Ethics Committee of the Jiangnan University.

Statistical Analysis

Statistical Product and Service Soluti (SPSS) 21.0 (IBM Corp., Armonk, NY, USA) w used for all statistical analysis. Ind nt-sam-ple *t*-test was used to compar erence between the two groups. $p<0.$ was c dered statistically significant.

Results

Expression Level of CASC15 in NPC Tissues and Cell Lines

RT-qPCR was first conducted to detect CASC15 expression in 54 patients' tissu PC cell lines. The results demonstrat at CA 5 was significantly up-regulated umor tissu han that of the adjacent norm ues (Figu A). Compared with NP69 ells, C 15 ex ssion was markedly highe NPC cel 1B).

Knockdown o ASC Repressed Proliferation a stasi CNE1 NPC Cells

In our y, CNE1 was chosen for knockd CASC15 *in vitro*. RT-qPCR was utilize o de CASC15 expression (Figure 2A). CCK-8 assay d that the growth ability o cells was s nificantly repressed after SC15 knock down (Figure 2B). Scratch wound say showe at the migrated length of CNE1 decreas markedly after CASC15 was k d d (Figure 2C). Furthermore, transwell showed that the number of invaded cells was significantly reduced after knock-down SC15 (Figure 2D).

The Interaction Between MiR-101-3p and CASC15 in NPC

DIANA LncBASE Predicted v.2 was used to search for microRNAs that contained complementary base with CASC15. MiR-101-3p

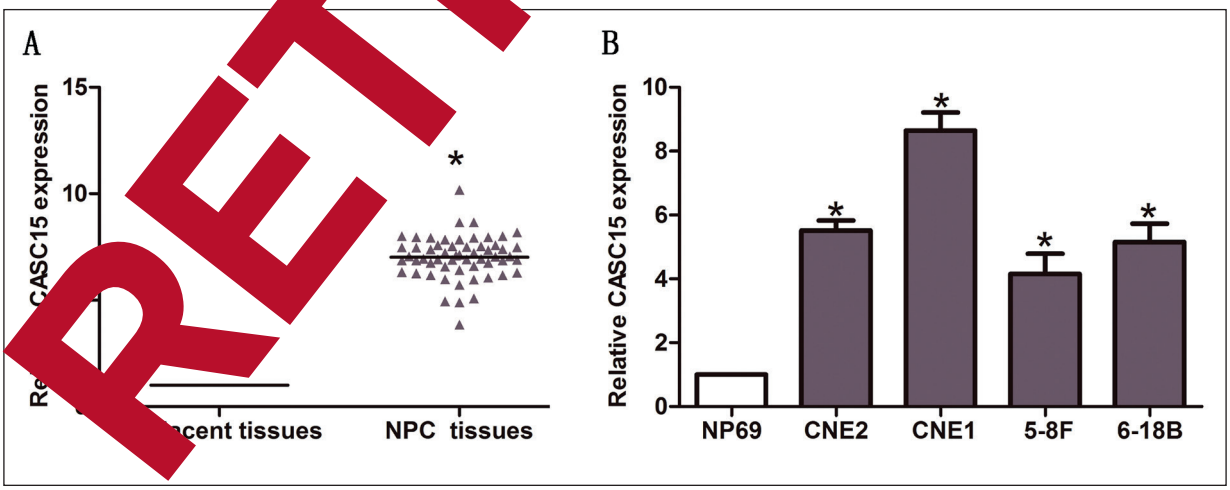

Figure 1. Expression level of CASC15 increased significantly in NPC tissues and cell lines. **A,** CASC15 expression increased markedly in NPC tissues when compared with adjacent normal tissues. **B,** Expression levels of CASC15 relative to GAPDH in human NPC cell lines and immortalized normal nasopharyngeal epithelial cell line (NP69) were determined by RT-qPCR. Data were presented as mean ± standard error of the mean. *$p<0.05$.

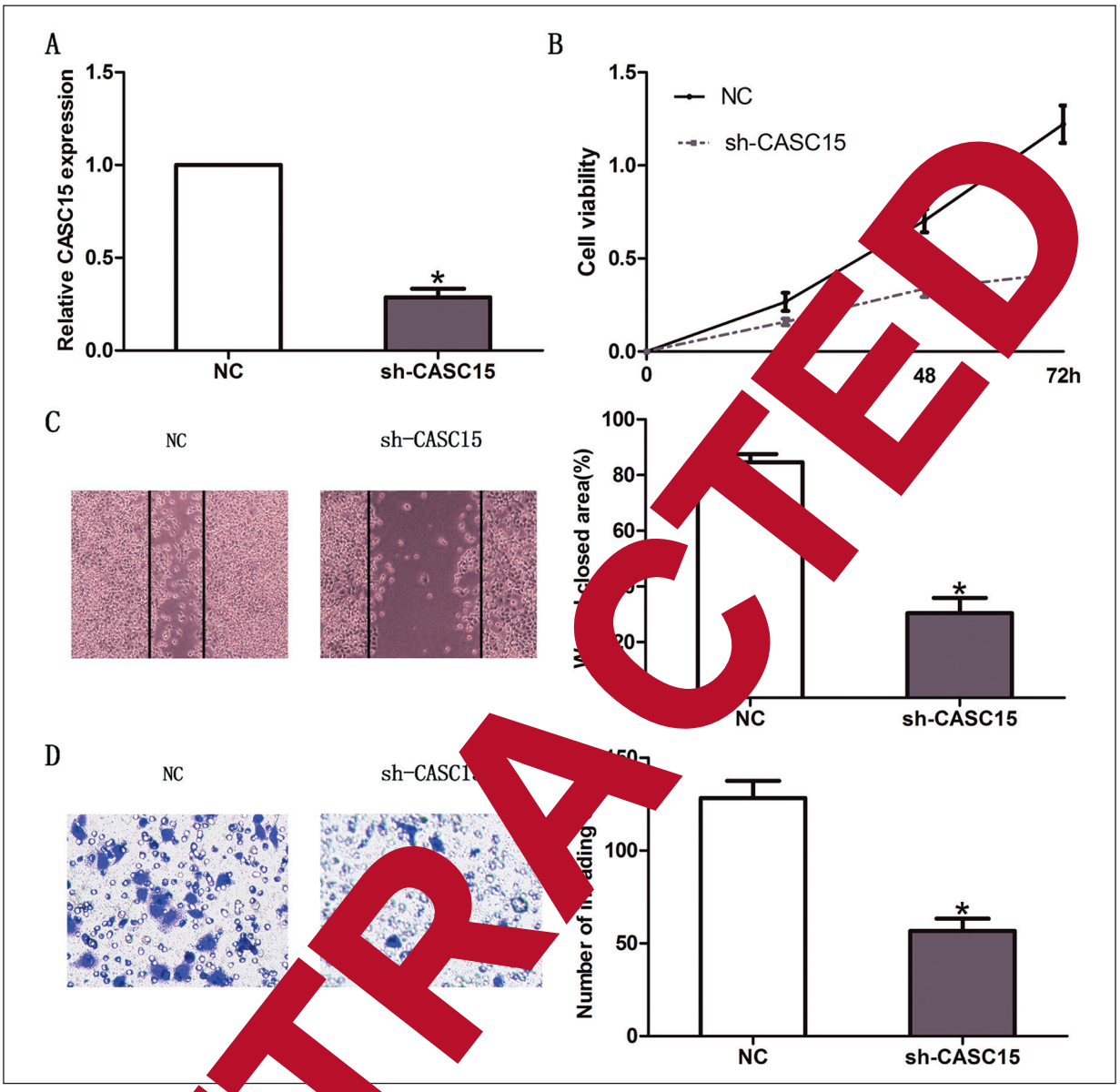

Figure 2. Knock[illegible]n of CASC15 inhi[illegible]d CNE1 NPC cell proliferation, migration and invasion. **A,** CASC15 expression in NPC cells tr[illegible]cted w[illegible] negative control shRNA (NC) or CASC15 shRNA (sh-CASC15) was detected by RT-qPCR. GAPDH was [illegible] as an [illegible]rnal control. **B,** CCK-8 assay showed that knockdown of CASC15 significantly repressed the proliferation [illegible] **C,** W[illegible] healing assay showed that the migrated length of NPC cells in sh-CASC15 group decreased marke[illegible] comp[illegible] with the NC group (magnification: 40×). **D,** Transwell assay showed that knockdown of CASC15 [illegible]cantly [illegible]s[illegible] invasion of NPC cells (magnification: 40×). The results represented the average of three inde[illegible]rimen[illegible] ± standard error of the mean). *p<0.05, compared with control cells.

wa[illegible]d from these miRNAs that were intera[illegible]with CASC15 (Figure 3A). RT-qPCR assay sho[illegible]that the expression of miR-101-3p was signific[illegible]ntly higher in sh-CASC15 cells than that of negative control shRNA cells (Figure 3B). The Luciferase reporter gene assay revealed that co-transfection of CASC15-WT and miR-101-3p remarkably decreased Luciferase activity. However, no significant differences were observed in the Luciferase activity after co-transfection of CASC15-MUT and miR-101-3p (Figure 3C). In addition, the results of the linear correlation analysis showed that the expression of miR-101-3p was negatively correlated with CASC15 expression in NPC tissues (Figure 3D).

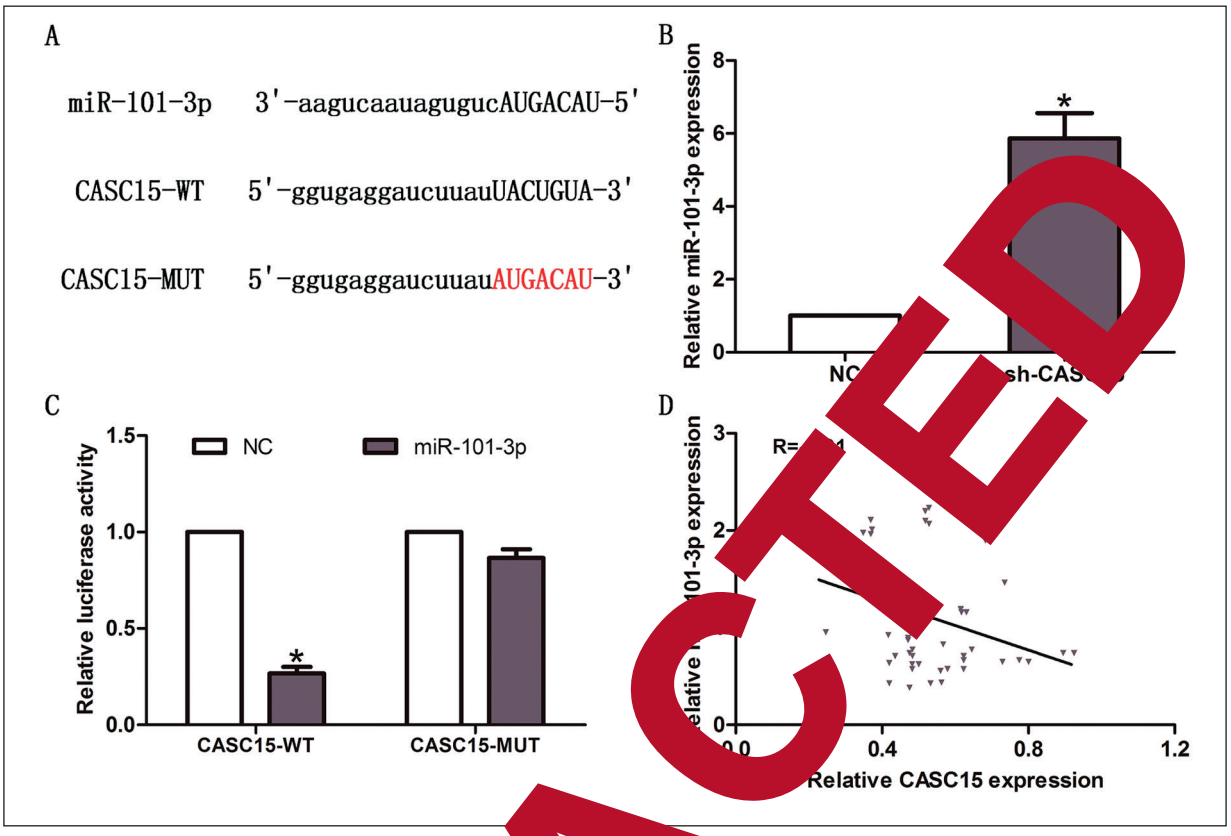

Figure 3. The association between CASC15 and miR-101-3p. **A,** The binding sites of miR-101-3p on CASC15. **B,** The expression of miR-101-3p increased remarkably in the sh-CASC15 group when compared with negative control shRNA (NC) group. **C,** Co-transfection of miR-101-3p and CASC15-WT strongly decreased Luciferase activity, while co-transfection of miR-101-3p and CASC15-MUT did not affect Luciferase activity. **D,** Linear correlation between the expression level of miR-101-3p and CASC15 in NPC tissues. The results represent the average of three independent experiments. Data were presented as mean ± standard error of the mean. $P < 0.05$.

CASC15 Knockdown Inhibited Tumor Formation and Metastasis In Vivo

The ability of CASC15 to affect tumor formation and metastasis was detected *in vivo*. The results indicated that tumor size in the sh-CASC15 group was significantly smaller when compared with negative control shRNA group (Figure 4A). The weight of dissected tumors in sh-CASC15 group was remarkably smaller than that of negative control shRNA group (Figure 4B). Meanwhile, the number of metastatic nodules in lung tissues of the sh-CASC15 group was significantly reduced when compared with the negative control shRNA group (Figure 4C). Subsequently, the expression levels of CASC15 and miR-101-3p in dissected tumor tissues were detected by RT-qPCR. The results showed that CASC15 was lowly expressed in the sh-CASC15 group compared with the negative control shRNA group (Figure 4D). However, miR-101-3p was highly expressed in the sh-CASC15 group when compared with the negative control shRNA group (Figure 4E).

Discussion

Currently, it has been reported that altered expressions of genes are associated with the progression of NPC. For example, lncRNA H19 promotes the invasion of NPC cells by regulating the expression of EZH2[8]. LncRNA-LET acts as a tumor suppressor gene in NPC by inhibiting the proliferation, adhesion and invasion of NPC cells[9]. By modulating miR-363-5p/S100A1 signaling pathway, lncRNA FOXD2-AS1 aggravates carcinogenesis of NPC[10]. Furthermore, the silence of lncRNA XIST suppresses the progression of NPC by acting as a sponge for miR-491-5p[11].

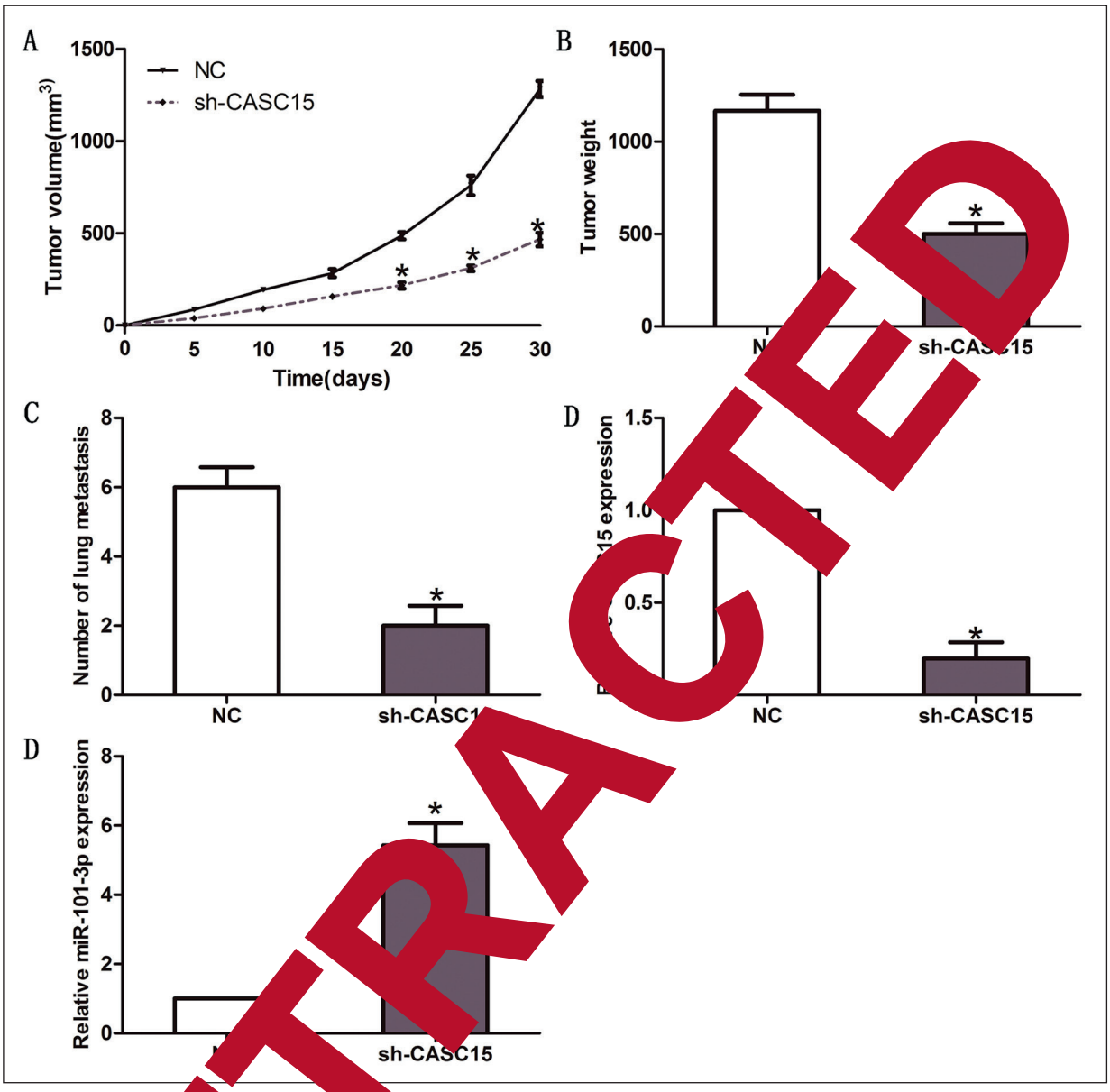

Figure 4. Knock[illegible] of CASC15 inhi[illegible] tumor formation and metastasis of NPC *in vivo*. **A,** Tumor size in sh-CASC15 group was signi[illegible]tly sm[illegible]r than that of the NC group. **B,** The weight of dissected tumors in the sh-CASC15 group was markedly sm[illegible] than t[illegible]C group. **C,** The number of metastatic nodules in lung tissues of the sh-CASC15 group was significantly l[illegible] c[illegible]ured wi[illegible]e NC group. **D,** CASC15 was lowly-expressed in dissected tumors of sh-CASC15 group when compared w[illegible]NC gr[illegible]. **E,** MiR-101-3p was highly-expressed in dissected tumors of sh-CASC15 group compared with the [illegible]up. [illegible]esented the average of three independent experiments (mean ± standard error of the mean). *p<0[illegible]d with[illegible]cells.

[illegible] cancer susceptibility candidate 15 (CASC[illegible] also known as LINC00340, was initially ide[illegible]d as an active lncRNA in silico. Lessard et a[illegible] have proved that overexpression of CASC15 promotes cell migration and phenotype switching in melanoma. CASC15 enhances the metastasis of hepatocellular carcinoma cells, which is also correlated with poor prognosis of patients[13]. Up-regulation of CASC15 promotes cell proliferation in gastric cancer and functions as a risk factor for gastric cancer prognosis[14]. Moreover, CASC15 participates in RUNX1-rearranged acute leukemia by regulating the expression of SOX4[15]. In the present study, CASC15 was found significantly up-regulated in both NPC tissues and cell lines. Fur-

thermore, after CASC15 was knocked down, the growth, migration and invasion abilities of NPC cells were markedly suppressed. These results indicated that CASC15 functioned as an oncogene and promoted the tumorigenesis of NPC.

Recently, emerging studies have explored the relationship between the two subgroups of ncRNAs, namely lncRNAs and miRNAs. Researchers have found that lncRNAs function as molecular sponges or ceRNAs in regulating the expression of miRNAs, thereby achieving their biological functions in diverse cancers. In our work, bioinformatics software predicted that miR-101-3p was a possible target gene of CASC15 in NPC. Liu et al[16] have evaluated the expression of miR-101-3p in tumors and its targeted genes. For instance, lncRNA SPRY4-IT1 promotes the progression of bladder cancer *via* upregulating EZH2. By inhibiting miR-101-3p, upregulation of lncRNA SNHG1 promotes the progression of non-small cell lung cancer *via* activating the Wnt/β-catenin signaling pathway[17]. Through silencing p27 and sponging miR-101-3p, lncRNA SNHG6 facilitates cell invasion and proliferation in gastric cancer by regu epithelial-mesenchymal transition[18]. More r, lncRNA SPRY4-IT1 enhances the prolifera and invasion of colorectal cancer cells by ser ing as a ceRNA of miR-101-3p[19]. is study, luciferases reporter gene assa dic d that miR-101-3p was directly targe by CA 15 in NPC. Meanwhile, miR-101 xpr significantly up-regulated fte down of CASC15. Knockdown ASC15 edly inhibited tumor formati d metastas *vivo*. Meanwhile, the exp sio of CASC15 was regulated by miR 01-3p in ted tumor tissues after knoc wn of CASC1 nude mice. All these res show d that CASC15 acted as a miR-101-3p nge NPC.

Co ions

SC15 NPC cell proliferation and m y sponging miR-101-3p. Our findings impli lncRNA CASC15 could serve as a promising arker for NPC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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