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Long noncoding RNA NEAT1 functions as an oncogene in human laryngocarcinoma by targeting miR-29a-3p

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Abstract. – OBJECTIVE: Long noncoding RNAs (lncRNAs) have been reported to participate in the progression and development of many human diseases. In this study, we are committed to uncover the potential function of lncRNA Nuclear Enriched Abundant Transcript 1 (NEAT1) in the development of laryngocarcinoma.

PATIENTS AND METHODS: LncRNA NEAT1 expression in laryngocarcinoma cells and 54 paired laryngocarcinoma samples was detected by Real-time quantitative polymerase chain reaction (RT-qPCR). Furthermore, the regulatory effects of NEAT1 on the proliferation and metastasis of laryngocarcinoma cells were evaluated. Biological role of NEAT1/miR-29a-3p axis was finally explored in regulating the progression of laryngocarcinoma.

RESULTS: NEAT1 was upregulated in laryngocarcinoma tissues and cell lines. NEAT1 knockdown suppressed growth and invasive abilities in laryngocarcinoma cells, while overexpression of NEAT1 enhanced such abilities. Further experiments showed that miR-29a-3p was directly targeted by NEAT1, and participated in NEAT-mediated progression of laryngocarcinoma.

CONCLUSIONS: NEAT1 is a novel oncogene in laryngocarcinoma and could enhance growth and invasion of laryngocarcinoma cells by targeting miR-29a-3p.

Key Words:
Long noncoding RNA, NEAT1, Laryngocarcinoma, MiR-29a-3p.

Introduction

Laryngocarcinoma is one of the most ordinary head and neck cancers worldwide. The major subtype of laryngocarcinoma is squamous cell carcinomas. Laryngocarcinoma brings a huge health burden and affects the life quality of affected patients. There were approximately 12,260 new cases of laryngocarcinoma diagnosed in the United States in 2013, and 3,630 cases died of laryngocarcinoma[1]. Despite advances have been made in diagnostic and therapeutic strategies, improvement on overall survival of laryngocarcinoma patients was unsatisfactory for the past decades. Thus, it is urgent to uncover the tumorigenesis of laryngocarcinoma, so as to improve the survival of the patients. Recent studies indicated that the majority of transcribed sequences have no ability of protein coding. Long noncoding RNAs (lncRNAs) are those transcripts with over 200 base pairs long. A great number of lncRNAs have been identified to be related to tumor diseases. For instance, lncRNA ATB participates in tumor progression and is correlated with prognosis of colorectal cancer[2]. SP1 induces upregulation of lncRNA TINCR, which in turn mediates cell proliferation and apoptosis of gastric cancer[3]. LncRNA H19 acts as an oncogene in thyroid cancer through regulating YES1[4]. LncRNA SOCS2-AS1 induces androgen to enhance proliferation and suppresses cell apoptosis in prostate cancer[5]. LncRNA Nuclear Enriched Abundant Transcript 1 (NEAT1) is a newly discovered oncogene in multiple types of cancers. However, the role of NEAT1 in laryngocarcinoma remains unknown. In this research, NEAT1 was highly expressed in laryngocarcinoma tissues, and enhanced cell growth and invasion of laryngocarcinoma cells. MicroRNA-29a-3p (miR-29a-3p) was the predicted target of NEAT1 and served as a tumor suppressor in laryngocarcinoma. We further analyzed the interaction between NEAT1 and miR-29a-3p in the progression of laryngocarcinoma.

Patients and Methods

Tissue Specimens

A total of 54 paired laryngocarcinoma samples and adjacent normal samples were surgical-

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ly resected from patients undergoing surgeries at Xiaogan Hospital Affiliated to Wuhan University of Science and Technology. None of patients received treatments of chemotherapy or radiotherapy before surgery. All tissue samples collected in the surgeries were frozen in liquid nitrogen immediately, and then preserved under -80°C. Signed written informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of Xiaogan Hospital Affiliated to Wuhan University of Science and Technology.

Cell Culture

Human laryngocarcinoma cell lines M4E, TU212, Hep-2 and M2E and the normal nasopharyngeal epithelial cell line NP69 (Shanghai Model Cell Bank, Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin. Cells were maintained in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

GenePharma (Shanghai, China) provided us lentivirus expressing short-hairpin RNA (shRNA) against NEAT1 (NEAT1/shRNA) and lentivirus against NEAT1 (NEAT1). NEAT1/shRNA was cloned into the pGPH1/Neo vector, which was then used for transfection of M2E cells. NEAT1 lentivirus was cloned into the pGPH1/Neo vector (GenePharma, Shanghai, China), which was then used for transfection of Hep-2 cells. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Reverse Transcription System Kit (TaKaRa, Dalian, China) was utilized to generate first strand complementary deoxyribose nucleic acid (cDNA). RT-qPCR analyses were performed using SYBR Green I (TaKaRa, Dalian, China) in triplicate. Primers were as follows: NEAT1 primers forward 5′-GCTCTGGGACCTTCGTGACTCT-3′, reverse 5′-CTGCCTTGGCTTGGAAATGTAA-3′; β-actin primers forward 5'-GATGGAAATCGTCAGAGGCT-3' and reverse 5'-TGGCACTTAGTTGGAAATGC-3'. The relative fold change in expression was calculated by $2^{-\Delta\Delta Ct}$ method. Thermal cycle was as follows: 30 s at 95°C, 5 s at 95°C and 35 s at 60°C, for a total of 40 cycles.

Cell Proliferation Assay

2×10^3 transfected cells were seeded in 96-well plates and cell proliferation was assessed by Cell Proliferation Reagent Kit I (MTT; Roche, Basel, Switzerland) at 0 h, 24 h, 48 h, 72 h. Absorbance at 490 nm was assessed using an enzyme-linked immunosorbent assay (ELISA) reader system (Multiskan Ascent, LabSystems, Helsinki, Finland).

Colony Formation Assay

Hep-2 cells in logarithmic growth phase were washed with PBS, digested with trypsin and centrifuged at 100 rpm/min for 3 min. After cell density was adjusted to 1×10^4/L, cells were seeded in the 6-well plates with 2000 cells per well. Cell culture was continued for 1-2 weeks until visible colonies were formed. Subsequently, cells were fixed with 4% methanol for 30 min and stained with 0.1% crystal violet (0.1%; Sigma-Aldrich, St. Louis, MO, USA) for another 30 min, followed by the detection of colony formation.

Transwell Assay

24 h after transfection, 2×10^5 cells suspended in 100 μL of serum-free DMEM were applied on the top chamber of an 8-μm culture inserts (Corning, Corning, NY, USA) pre-coated with 50 μg Matrigel Matrix dilution (BD, Bedford, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal bovine serum (FBS) was added to the bottom chamber. 24 h later, these inserts were treated by methanol for 30 min and stained by hematoxylin for 20 min. Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany) was utilized for counting penetrating cells in three random fields (magnification ×20).

Dual-Luciferase Reporter Gene Assay

3′-Untranslated Region (3′-UTR) of NEAT1 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-29a-3p binding site in NEAT1 3′-UTR, which

was named as mutant (MUT) 3′-UTR. Cells were co-transfected with WT-3′-UTR/MUT-3′-UTR and miR-ctrl/miR-29a-3p mimics for 48 h. Luciferase activity was determined by the dual-luciferase reporter gene assay system (Promega, Madison, WI, USA).

RNA Immunoprecipitation Assay (RIP)

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was used for RIP assay. Co-precipitated RNAs were detected by RT-qPCR.

Statistical Analysis

In this study, Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) was utilized for statistical analysis. Data analysis was conducted by two-tailed Student's *t*-test. It was considered of statistically significance when $p<0.05$.

Results

Expression Level of NEAT1 in Laryngocarcinoma Tissues and Cells

Firstly, RT-qPCR was conducted for detecting NEAT1 expression in 44 paired cases of laryngocarcinoma tissues and 4 laryngocarcinoma cell lines. As a result, NEAT1 was remarkably upregulated in laryngocarcinoma tumor samples compared with that of adjacent non-tumor samples (Figure 1A). Expression level of NEAT1 in laryngocarcinoma cells was also significantly higher than that of nasopharyngeal epithelial cell line NP69 (Figure 1B).

NEAT1 Promoted Proliferation in Laryngocarcinoma Cells

Laryngocarcinoma cell line M2E was selected for establishing NEAT1 knockdown model. Transfection efficiency of shNEAT1 was verified by RT-qPCR (Figure 2A). CCK-8 assay revealed that NEAT1 knockdown decreased the viability of M2E cells (Figure 2B). Colony formation assay revealed that knockdown of NEAT1 decreased the number of colonies in M2E cells (Figure 2C). Transfection of NEAT1 lentivirus in Hep-2 cells markedly upregulated NEAT1 level (Figure 2D). As CCK-8 assay revealed, overexpression of NEAT1 enhanced the viability of Hep-2 cells (Figure 2E). Moreover, the number of colonies was elevated in Hep-2 cells overexpressing NEAT1 (Figure 2F).

NEAT1 Promoted Invasion in Laryngocarcinoma Cells

To identify the function role of NEAT1 in the metastasis of laryngocarcinoma, transwell assay was conducted and found that knockdown of NEAT1 in M2E cells attenuated invasive ability, manifesting as fewer invasive cells (Figure

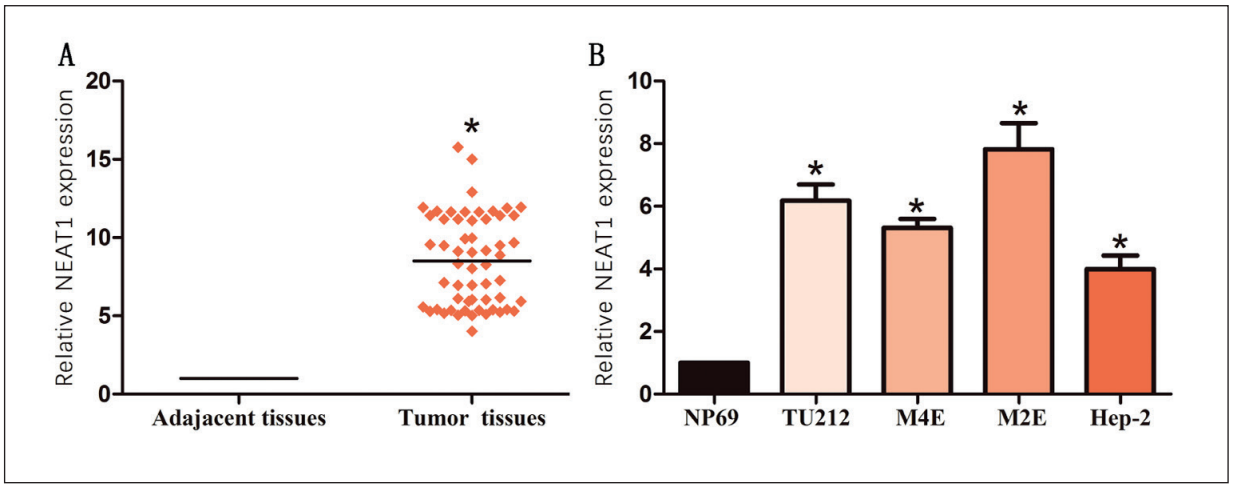

Figure 1. Expression levels of NEAT1 were upregulated in laryngocarcinoma tissues and cell lines. ***A***, NEAT1 expression was significantly upregulated in the laryngocarcinoma tissues compared with adjacent tissues. ***B***, Expression levels of NEAT1 were detected in the human laryngocarcinoma cell lines and NP69 (normal nasopharyngeal epithelial cell line) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

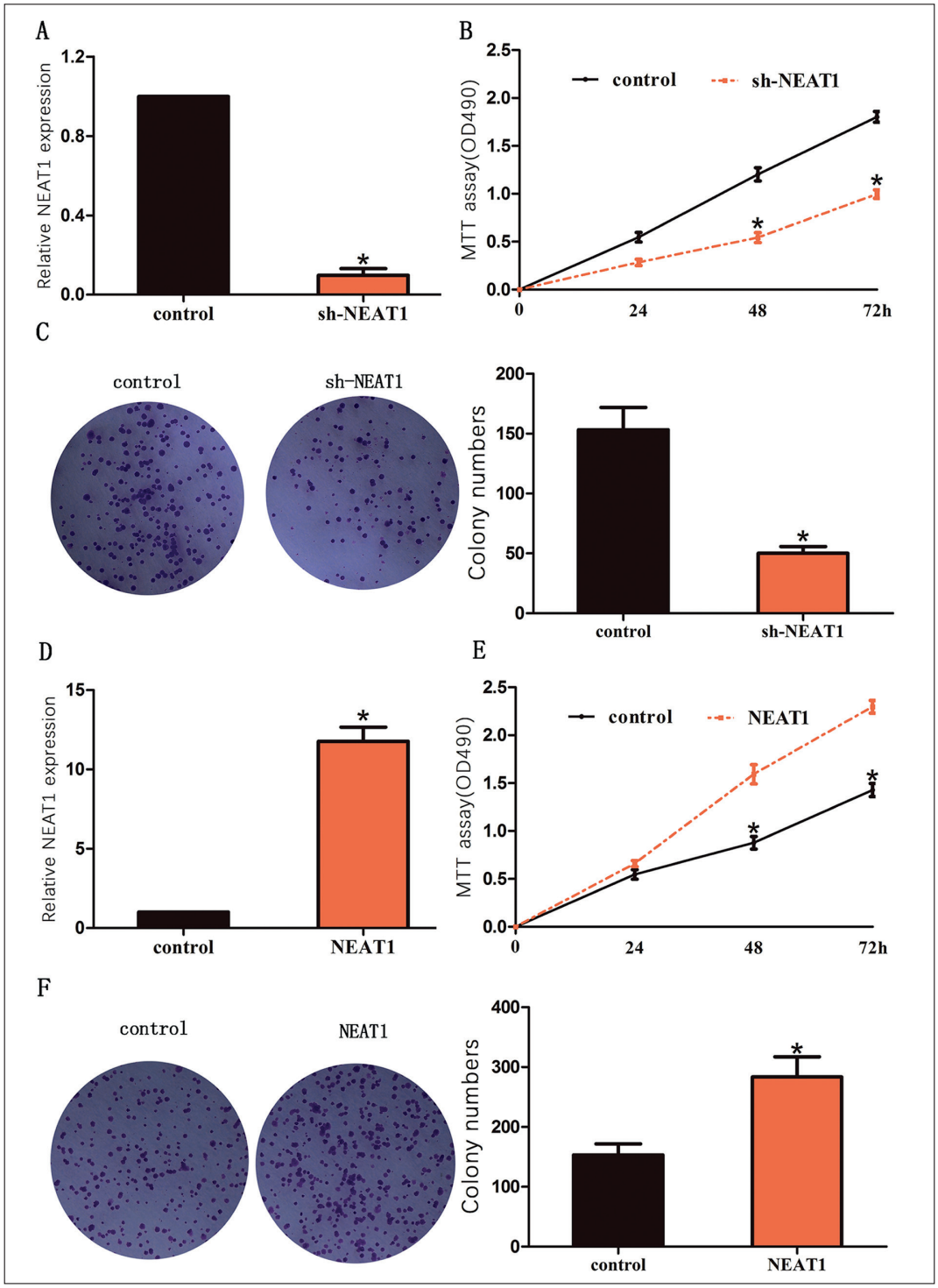

Figure 2. NEAT1 promoted proliferation of laryngocarcinoma cells. ***A,*** NEAT1 expression in M2E laryngocarcinoma cells transfected with NEAT1 shRNA (NEAT1/shRNA) and the control vector was detected by RT-qPCR. β-actin was used as an internal control. ***B,*** Cell proliferation assay showed that cell viability was significantly inhibited in M2E cells after knockdown of NEAT1. ***C,*** Colony formation assay showed that number of colonies was significantly reduced via knockdown of NEAT1 in M2E cells. ***D,*** NEAT1 expression in Hep-2 cells transfected with NEAT1 lentivirus (NEAT1) and the control vector was detected by RT-qPCR. β-actin was used as an internal control. ***E,*** Cell proliferation assay showed that cell viability was significantly promoted in Hep-2 cells after overexpression of NEAT1. ***F,*** Colony formation assay showed that number of colonies was obviously increased *via* overexpression of NEAT1 in Hep-2 cells. The results represent the average of three independent experiments (mean ± standard error of the mean). **p*<0.05.

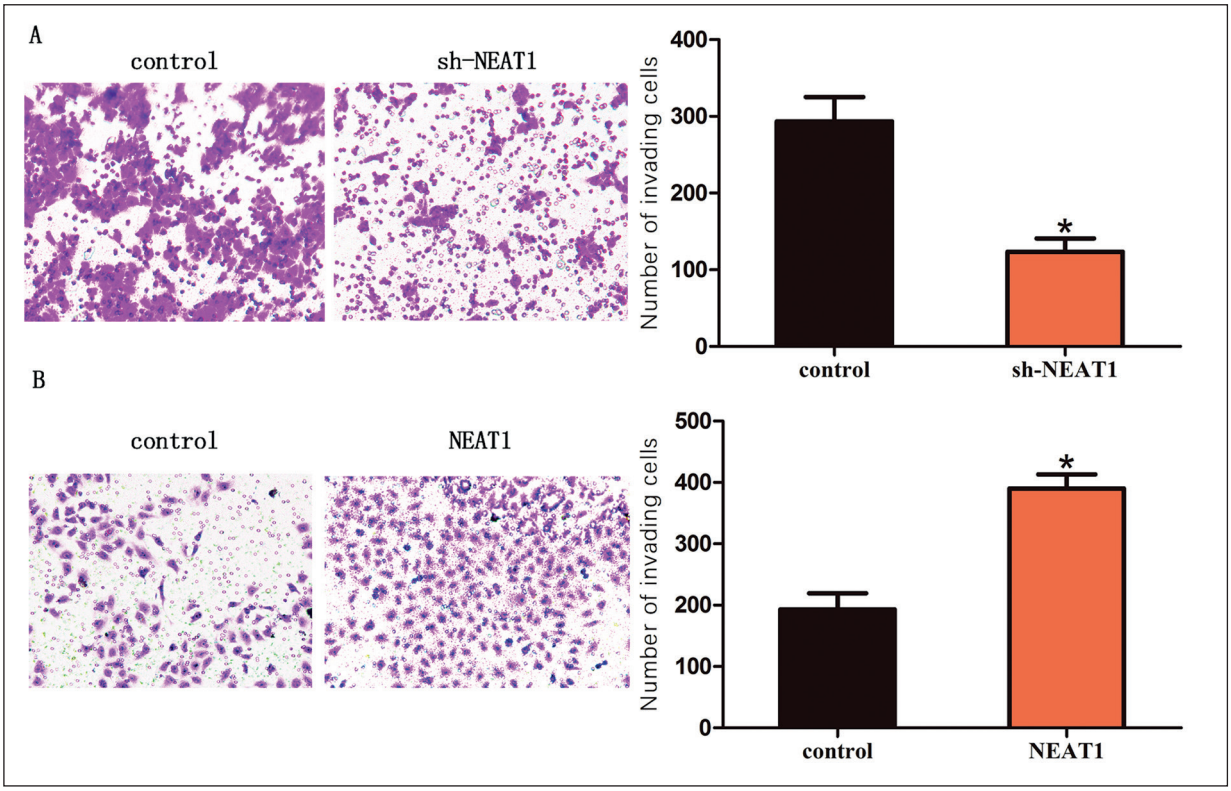

Figure 3. NEAT1 promoted invasion of laryngocarcinoma cells. ***A***, Transwell assay showed that number of invasive cells was remarkably reduced *via* downregulation of NEAT1 in M2E cells. ***B***, Transwell assay showed that number of invasive cells remarkably increased *via* upregulation of NEAT1 in Hep-2 cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$.

3A). Conversely, NEAT1 overexpression in Hep-2 cells enhanced the number of invasive cells (Figure 3B).

The Interaction Between NEAT1 and miR-29a-3p in Laryngocarcinoma

The miRNAs that contained complementary base with NEAT1 were searched by conducting Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php). As a tumor suppressor, miR-29a-3p was discovered as interacted with NEAT1 (Figure 4A). RT-qPCR assay revealed that miR-29a-3p expression was remarkably upregulated in laryngocarcinoma cells with NEAT1 knockdown (Figure 4B). In addition, miR-29a-3p was downregulated after NEAT1 overexpression (Figure 4C). Co-transfection of miR-29a-3p mimics and NEAT1-WT significantly depressed luciferase activity (Figure 4D). Furthermore, RIP assay demonstrated that miR-29a-3p could be remarkably enriched in NEAT1 group, suggesting that NEAT1 might serve as a miR-29a-3p sponge (Figure 4E). In summary, these data demonstrated that miR-29a-3p served as a direct target of NEAT1.

Discussion

Laryngocarcinoma is one of the most general malignancies in the world. The survival is obviously worse in patients with metastasis than those with non-metastatic disease. Developing effective and targeted therapies for laryngocarcinoma are necessary to improve the clinical outcomes of affected patients. LncRNAs exert a variety of regulatory roles in the regulation of potential activities and splicing events *via* small RNA regulatory pathways. Although many therapy methods are available for laryngocarcinoma, the prognosis of laryngocarcinoma patients remains poor. A plenty of lncRNAs have been revealed to play important roles in oncogenesis and progression of laryngocarcinoma. Nuclear Enriched Abundant Transcript 1 (NEAT1) encodes two isoforms of

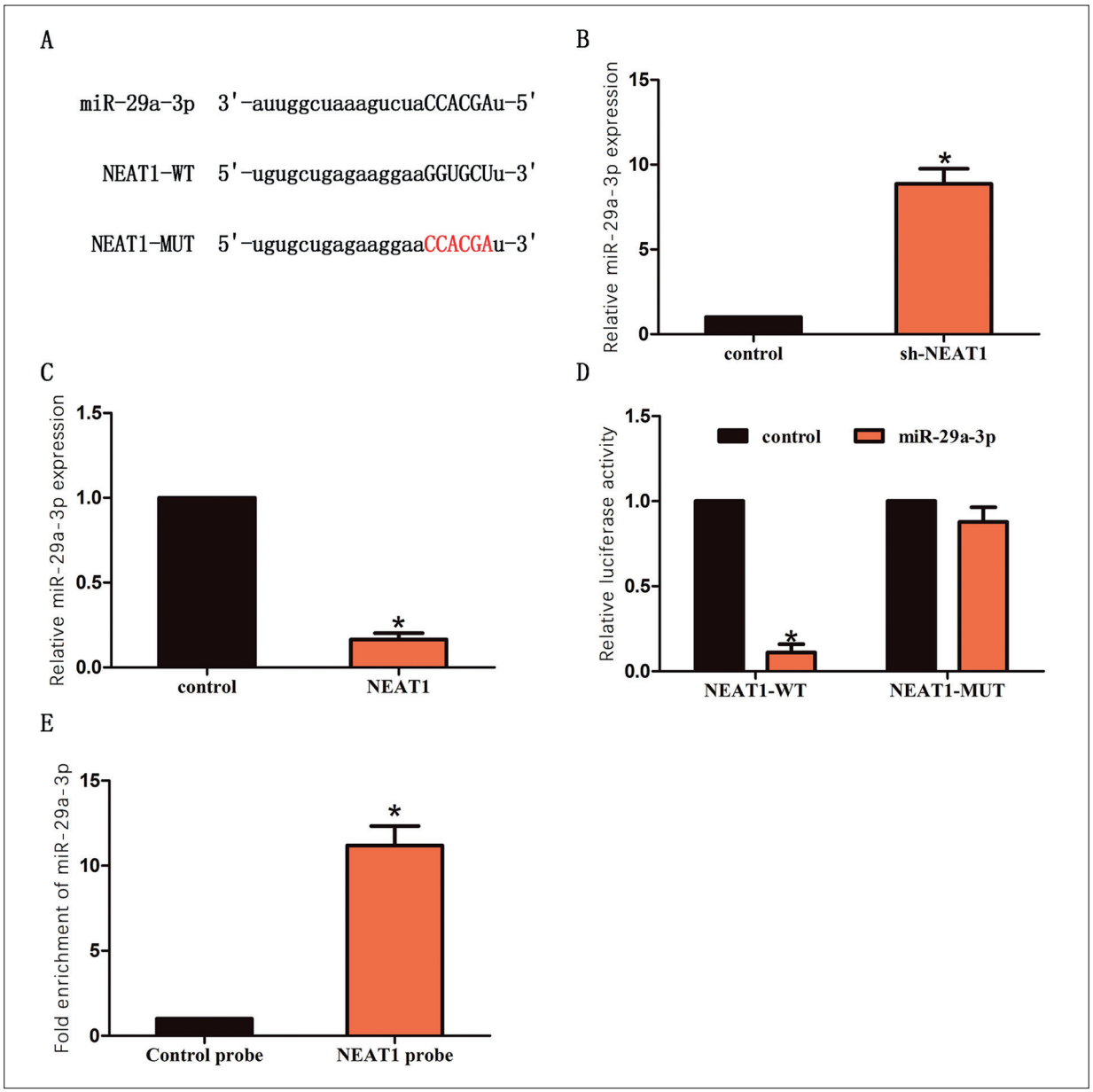

Figure 4. Reciprocal repression between NEAT1 and miR-29a-3p. ***A,*** The binding sites of miR-29a-3p on NEAT1. ***B,*** MiR-29a-3p expression was upregulated in sh-NEAT1 group compared with control group. ***C,*** MiR-29a-3p expression was downregulated in NEAT1 lentivirus group compared with control group. ***D,*** Co-transfection of miR-29a-3p and NEAT1-WT strongly decreased the luciferase activity, while co-transfection of mir-control and NEAT1-WT did not change the luciferase activity, and co-transfection of miR-29a-3p and NEAT1-MUT did not change the luciferase activity either. ***E,*** RIP assay results demonstrated that miR-29a-3p could be remarkably enriched in the NEAT1 group compared with control group. The results represent the average of three independent experiments Data are presented as the mean ± standard error of the mean. *$p<0.05$.

lncRNA, namely 3.7-kb NEAT1-1 and about 23-kb NEAT1-2, which play a crucial role in nuclear paraspeckles and serve as a regulator in RNA splicing and transcription. Recent researches have revealed that NEAT1 acts as an oncogene in multiple types of cancers. For instance, HuR upregulates NEAT1 to promote progression and tumorigenesis of ovarian cancer[6]. Through regulation of miR-211/HMGA2 axis, NEAT1 functions as an oncogene by facilitating cell proliferation and invasion in breast cancer[7]. Knockdown of NEAT1 inhibits cell proliferation and promotes cell apop-

tosis in colorectal cancer through regulating Akt Signaling[8]. NEAT1 facilitates malignancy of biological behaviors in gastric cancer and induces chemotherapy resistance[9]. In this study, NEAT1 was firstly identified as a novel oncogene in laryngocarcinoma. Our study showed that NEAT1 was upregulated in laryngocarcinoma tissues. Furthermore, NEAT1 knockdown in laryngocarcinoma cells suppressed proliferation and invasion. Overexpression of NEAT1 achieved the opposite trends. As more researchers has focused on the interaction between lncRNAs and microRNAs recently[10-13], we predicted the potential miRNAs downstream of NEAT1 on Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php). MiR-29a-3p was selected from those potential microRNAs due to its role of tumor suppressor in many tumors including laryngocarcinoma. Through competitively sponging miR-29a-3p, lncRNA H19 promotes the progression of clear cell renal cell carcinoma by regulating E2F1 expression[14]. MiR-29a-3p depresses cell proliferation and migration in hepatocellular carcinoma by downregulation of IGF1R[15]. MiR-29a-3p is upregulated in H.Pylori infected gastric tissues and it promotes cell migration *via* EMT pathway[16]. Through directly targeting OTUB2, miR-29a-3p depresses tumor growth and cell proliferation in papillary thyroid carcinoma by inhibiting NF-κB signaling[17]. In this study, miR-29a-3p was verified to directly bind to NEAT1, and miR-29a-3p was significantly enriched by NEAT1 as RIP assay showed. In addition, miR-29a-3p expression was suppressed by overexpressed NEAT1, while downregulated NEAT1 could reverse the effect of miR-29a-3p. On the other hand, knockdown of NEAT1 upregulated miR-29a-3p. All the above results suggested that NEAT1 negatively regulated miR-29a-3p in laryngocarcinoma cells.

Conclusions

We demonstrated that NEAT1 is remarkably upregulated in laryngocarcinoma, which facilitates proliferation and invasion of laryngocarcinoma cells through sponging miR-29a-3p. NEAT1 could serve as a prospective therapeutic target for laryngocarcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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