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Long Noncoding RNA NEAT1 Promotes Proliferation and Invasion via Targeting miR-181a-5p in Non-Small Cell Lung Cancer

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Long noncoding RNAs (lncRNAs) have been implicated in various biological processes and pathological conditions, including tumorigenesis. However, the exact roles of NEAT1 and its underlying mechanisms in non-small cell lung cancer (NSCLC) remain largely unclear. In the present study, lncRNA NEAT1 was detected to be significantly upregulated in NSCLC tissues and closely associated with advanced TNM stages, lymph node metastasis, distant metastasis, and poor prognosis. Further experiments revealed that lncRNA NEAT1 silencing inhibited cell proliferation and invasion in vitro. In addition, mechanistic analysis showed that lncRNA NEAT1 upregulated the miR-181a-5p-targeted gene HMGB2 through acting as a competitive "sponge" of miR-181-a-5p. In conclusion, our study suggested that lncRNA NEAT1 plays an oncogenic role in NSCLC progression and provides potential mechanisms by which lncRNA NEAT1 contributes to this disease.

Key words: Non-small cell lung cancer (NSCLC); Long noncoding RNAs (lncRNAs); Nuclear-enriched abundant transcript 1 (NEAT1); miR-181a-5p; HMGB2

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide[1]. According to the pathological features, lung cancers are categorized into two subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for more than 80% of all lung cancer diagnoses[2]. Although the treatments for NSCLC, including surgery, radiotherapy, and platinum-based combination chemotherapy, have improved significantly in recent years, the prognosis of NSCLC remains poor, and the 5-year survival rate is less than 15%[3,4]. Therefore, it is urgent to enhance our understanding of the molecular mechanisms involved in NSCLC progression.

Long noncoding RNAs (lncRNAs) are the noncoding transcripts that are more than 200 nucleotides (nt) in length[5]. Growing evidence has shown that lncRNAs could influence various cellular processes such as cell growth, cell apoptosis, cell cycle progression, cell invasion, and metastasis[6,7]. Recent studies showed that aberrant lncRNA expression contributed to tumor progression, including NSCLC. For example, Li et al. found that overexpressed lncRNA ZEB1-AS1 promoted cell invasion and angiogenesis through the Wnt/β-catenin signaling in NSCLC[8]. Li et al. found that lncRNA BCAR4 promoted proliferation, invasion, and metastasis of NSCLC cells by affecting epithelial–mesenchymal transition[9]. Cui et al. showed that upregulated lncRNA SNHG1 contributed to the progression of NSCLC through the inhibition of miR-101-3p and the activation of the Wnt/β-catenin signaling pathway[10]. However, the functions and underlying molecular mechanisms of lncRNAs in NSCLC progression remain largely unknown.

In the present study, our data showed that nuclear-enriched abundant transcript 1 (NEAT1) harbors one conserved miR-181a-5p cognate site and functions as a competing endogenous RNA (ceRNA) for miR-181a-5p. We then searched TargetScan for underlying targets of miR-181a-5p that owned oncogenic characteristics and found that the oncogene HMGB2 was an underlying target of miR-181a-5p. In conclusion, we indicate that NEAT1 plays important roles in NSCLC progression by regulating the miR-181-5p/HMGB2 pathway.

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MATERIALS AND METHODS

Patients and Samples

A total of 73 NSCLC tissue samples and matched nontumor adjacent tissue specimens were obtained from patients who had pathologically confirmed NSCLC in the Department of Oncology and Thoracic Surgery, The Second Affiliated Hospital of Zhengzhou. None of the patients had undergone adjuvant treatments including radiotherapy, chemotherapy, or immunotherapy before surgical resection. All patients were classified according to the criteria of American Joint Committee on Cancer (AJCC) tumor node metastasis (TNM) staging system for lung cancer[11]. Written informed consent was obtained from all the participants, and the study was approved by the Board and Ethics Committee of The Second Affiliated Hospital of Zhengzhou. After resection, all tissues were immediately snap frozen in liquid nitrogen and then stored at −80°C until RNA extraction. The clinicopathologic characteristics of patients are summarized in Table 1.

Cell Culture and Transfection

The NSCLC cell lines A549, H1299, H446, H460, and NCI-H1650 and one cultured human lung epithelial cell (BEAS-2B) were obtained from the Chinese Academy of Science Cell Bank (Shanghai, P.R. China) and maintained in an incubator with 5% CO_2 at 37°C. The cell lines were cultured in high-glucose DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). siRNAs specifically targeting NEAT1 or HMGB2 were synthesized by GenePharma (Shanghai, P.R. China). Cells were transfected with si-NEAT1, si-HMGB2, or miR-181a-5p mimics using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. The efficiency was verified by real-time quantitative (qRT)-PCR 48 h after transfection.

Real-Time Quantitative PCR

Total RNA was isolated from the frozen tissues using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and then the first cDNA was synthesized using a Reverse Transcription Kit (Takara, Dalian, P.R. China). qRT-PCR was performed with the Applied Biosystems 7900 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification conditions were set as follows: 95°C for 30 s, followed by 95°C for 10 s, and 60°C 30 s for 40 cycles. The relative expression level was calculated using the $2^{-\Delta\Delta}$Ct method, and all the experiments were presented in triplicate.

Western Blot

Cells were lysed and proteins were separated by 10% SDS polyacrylamide gel. After being electrotransferred to polyvinylidene fluoride membranes (Millipore, Billerica,

Table 1. The Relationship Between lncRNA NEAT1 Expression and the Clinicopathological Features in NSCLC Patients

		lncRNA NEAT1 Expression		
Variables	*n*	High	Low	*p* Value
Age				0.555
≥55	38	18	20	
<55	35	19	16	
Gender				0.534
Male	44	21	23	
Female	29	16	13	
Tumor size				0.412
≥3cm	37	17	20	
<3cm	36	20	16	
TNM				0.025
I, II	39	15	24	
III, IV	34	22	12	
Lymph node metastasis				0.006
Absent	27	8	19	
Present	46	29	17	
Differentiation				0.071
Well/moderately	58	19	11	
Poorly	43	18	25	
Distant metastasis				0.003
Present	40	14	26	
Absent	33	23	10	

MA, USA), the membranes were probed with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h at 37°C. GAPDH was used as an internal control. The bands were obtained with an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA).

Dual-Luciferase Reporter Analysis

Dual-luciferase report assay was performed with a luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The lncRNA NEAT1 promoter region was synthesized and cloned into a pGL3-basic vector, and the luciferase activities were detected.

CCK-8 Assay

The cell proliferation rate was detected using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Then 100 µl of cell suspension (1×10^3 cells/well) was seeded in a 96-well plate, and various concentrations of substances were added. The cells were incubated for an appropriate length of time (0, 24, 48, and 72 h) and obtained with 10 µl of CCK-8 solution added. The absorbance was measured at 450 nm using a microplate reader. All the samples were detected in triplicate.

Cell Invasion Assays

Transfected cells were harvested and plated (1×10^5 cells/well) in the top chamber of a Transwell system (Millipore). The Transwell membrane, containing 8-µm pores, was coated with Matrigel and supplied with 200 ml of serum-free DMEM. DMEM with 10% FBS as a chemoattractant was added to the bottom of the wells. After 24 h, the remaining cells on the top layer were removed. The cells that migrated to the bottom surface were stained with 0.1% crystal violet, counted, and photographed with microscopy.

Statistical Analysis

All statistical analyses were conducted using the SPSS version 18.0 software (Chicago, IL, USA). One-way analysis of variance, chi-square test, two-tailed Student's *t*-test, and rank correlation were performed as appropriate. Patients' survival was assessed by Kaplan–Meier method and log-rank tests. All experiments were performed in triplicate. The difference was significant with a value of $p<0.05$.

RESULTS

lncRNA NEAT1 Was Upregulated in NSCLC and Indicated a Poor Prognosis

To explore whether NEAT1 was dysregulated in NSCLC, we analyzed NEAT1 expression in NSCLC tissues and adjacent nontumor tissues using qRT-PCR. Our results showed that NEAT1 was increased in NSCLC tissues compared to the adjacent nontumor tissues ($p<0.05$) (Fig. 1A). Furthermore, we explore the expression of NEAT1 in NSCLC cell lines (A549, H1299, H446, H460, and NCI-H1650) and a normal human lung epithelial cell (BEAS-2B). The qRT-PCR results showed that the expression of NEAT1 was significantly higher in NSCLC cells than in BEAS-2B cells ($p<0.05$) (Fig. 1B). We then analyzed the association between NEAT1 expression level and clinicopathological characteristics. These results indicated that the high expression of lncRNA NEAT1 was not only associated with advanced TNM stages, lymph node metastasis, and distant metastasis ($p<0.05$) (Table 1) but also an independent factor for prognosis of NSCLC ($p<0.05$) (Fig. 1C). These data suggested that high lncRNA NEAT1 expression was related to poor prognosis and might be crucial in NSCLC initiation, progression, and development.

lncRNA NEAT1 Promoted the Proliferation and Invasion of NSCLC Cells In Vitro

To investigate the biological effects of lncRNA NEAT1 on NSCLC, we established loss-of-function models in NSCLC cell lines A549 and H460. The qRT-PCR analysis showed that the expression of lncRNA NEAT1 was strikingly downregulated in the si-NEAT1 group compared with the si-NC group ($p<0.05$) (Fig. 2A). The CCK-8 assay showed that lncRNA NEAT1 inhibition significantly decelerated the tumor cell proliferation in A549 and H460 cell lines ($p<0.05$) (Fig. 2B). Furthermore, we found that decreased expression of lncRNA NEAT1 could suppress tumor cell invasion ability through a Transwell chamber assay ($p<0.05$) (Fig. 2C). These data indicated that lncRNA NEAT1 depletion could inhibit cell proliferation and invasion in NSCLC cells in vitro.

lncRNA NEAT1 Directly Targeted miR-181a-5p in NSCLC Cells

Recently, lots of studies demonstrated that lncRNAs could function as a ceRNA by competitively binding miRNAs in tumor progression[12,13]. Thus, we examined whether NEAT1 has a similar mechanism in NSCLC; we predicted miRNA target sites using the online program starBase 2.0. Our data showed that miR-181a-5p had putative binding sites with lncRNA NEAT1 ($p<0.05$) (Fig. 3A). To further verify if NEAT1 was a direct target of miR-181-5p, dual-luciferase reporter assay was performed. Our findings showed that miR-181a-5p mimics reduced luciferase activity of pmirGLO-NEAT1-Wt but not of pmirGLO-NEAT1-Mut ($p<0.05$) (Fig. 3B). Moreover, we found that NEAT1 suppression increased miR-181a-5p expression both in A549 and H460 cell lines ($p<0.05$) (Fig. 3C). In addition, we applied a

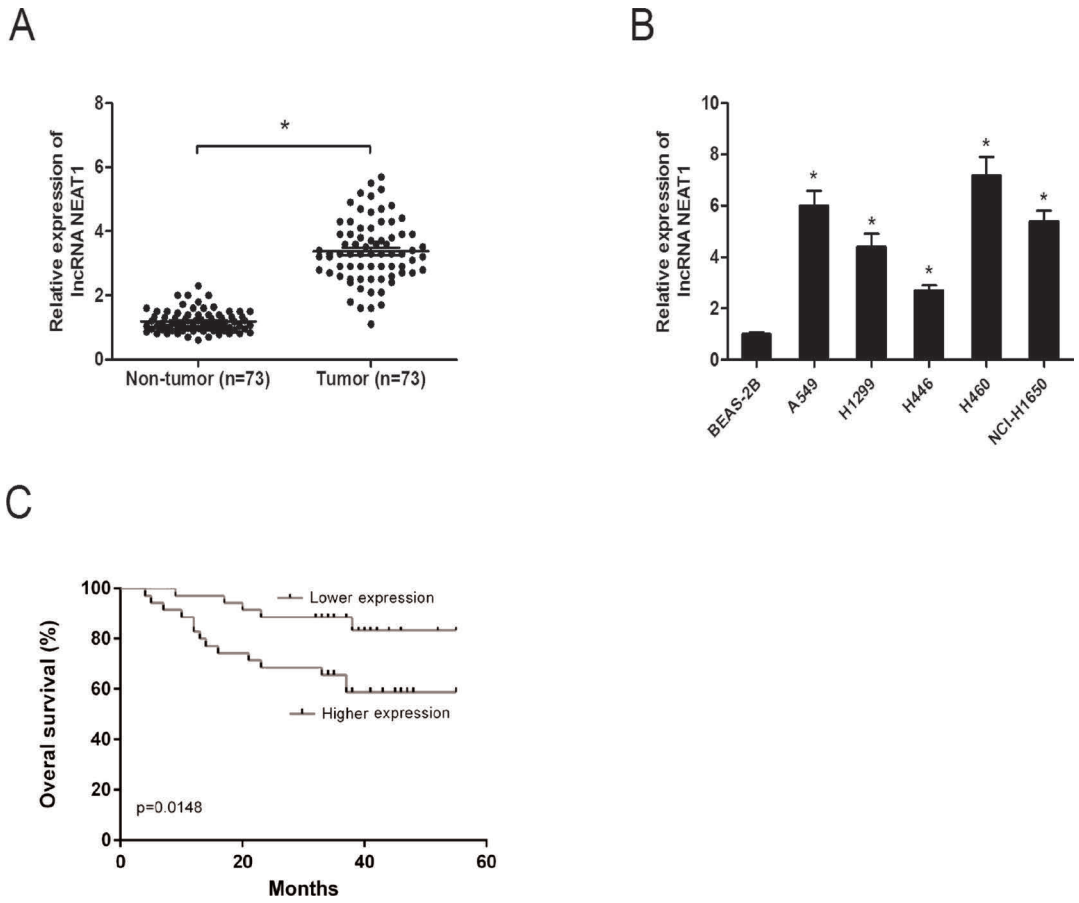

Figure 1. The expression of long noncoding RNA (lncRNA) nuclear-enriched abundant transcript 1 (NEAT1) in non-small cell lung cancer (NSCLC) tissues and cell lines. (A) lncRNA NEAT1 mRNA expression in NSCLC tissues and adjacent nontumor tissues. (B) lncRNA NEAT1 mRNA expression in NSCLC cell lines (A549, H1299, H446, H460, and NCI-H1650) and a normal human lung epithelial cell line (BEAS-2B). (C) Overall survival of 73 NSCLC patients who underwent surgery was compared between the high lncRNA NEAT1 expression group and the low lncRNA NEAT1 expression group. *$p<0.05$.

pull-down assay by a biotin-labeled specific NEAT1 probe. miR-181a-5p was precipitated as revealed by qRT-PCR ($p<0.05$) (Fig. 3D). Furthermore, we explored miR-181a-5p expression in 40 pairs of NSCLC tissues. The qRT-PCR results showed that the expression of miR-181a-5p was significantly decreased and had an inverse correlation with lncRNA NEAT1 expression in NSCLC tissues ($r^2=0.2484$, $p<0.05$) (Fig. 3E and F). These data revealed that NEAT1 might directly bind to miR-181a-5p in NSCLC.

HMGB2 Was a Target Gene of miR-181a-5p and Was Regulated by NEAT1

We used TargetScan to predict target genes for miR-181a-5p, and HMGB2 was determined to be one of the best candidates (Fig. 4A). We used luciferase reporter assays to verify whether HMGB2 expression was really regulated by miR-181a-5p, and the results demonstrated that miR-181a-5p mimics reduced the luciferase activity of the wild-type (Wt) NEAT1 3′-UTR, but not the mutant (Mut) HMGB2 3′-UTR ($p<0.05$) (Fig. 4B). These data indicated that HMGB2 was a target gene of miR-181a-5p. To further explore whether HMGB2 was regulated by lncRNA NEAT1, we transfected si-NEAT1-2 into A549 and H460 cells. Western blot showed that lncRNA NEAT1 suppression decreased the protein levels of HMGB2, and the miR-181a-5p inhibitors restored the reduction of HMGB2 expression in lncRNA NEAT1 suppression in NSCLC cells ($p<0.05$) (Fig. 4C). Taken together, these results suggested that lncRNA NEAT1 might exert oncogenic functions by modulating the miR-181a-5p/HMGB2 axis.

DISCUSSION

With the accomplishment of the Human Genome Project, there is a growing consensus that the vast majority of the human genomes do not code for proteins[14].

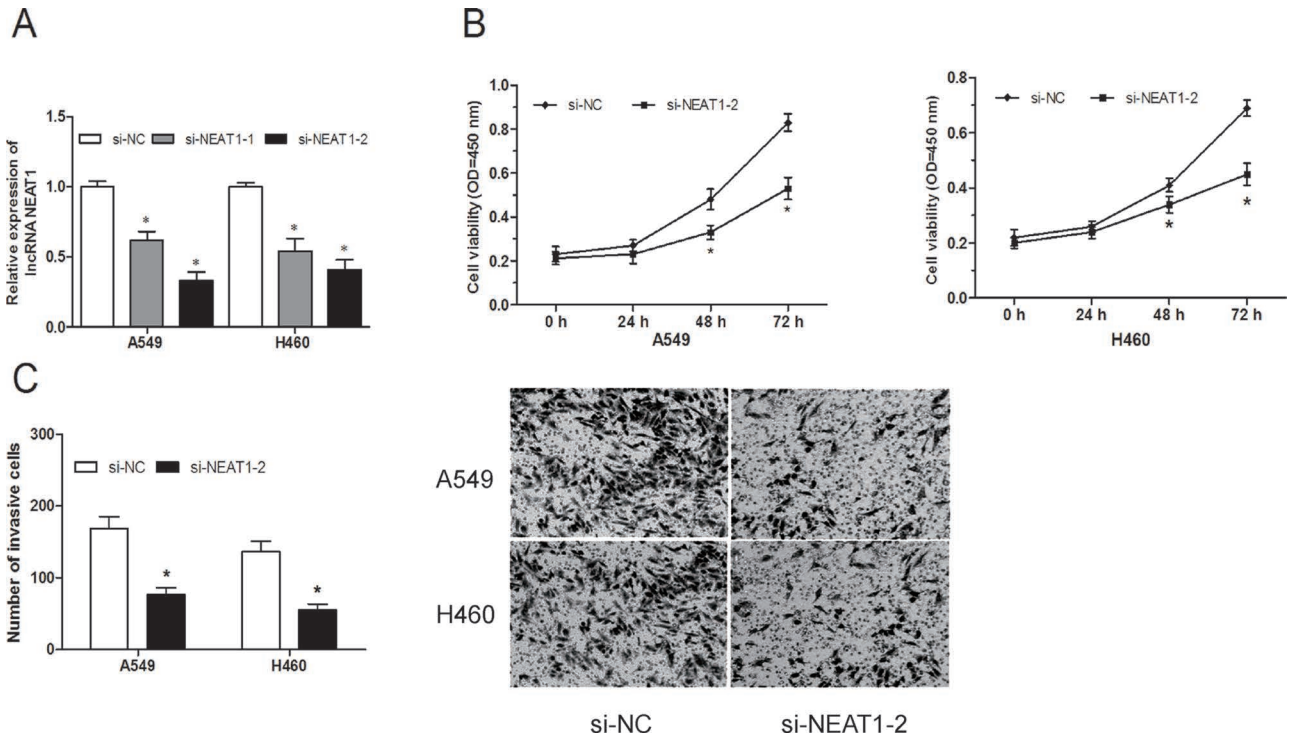

Figure 2. lncRNA NEAT1 promotes NSCLC cell proliferation and invasion in vitro. (A) real-time quantitative (qRT)-PCR analysis was performed to detect the expression of NEAT1 in A549 and H460 cells transfected with si-NEAT1 or si-NC. (B) The cell counting kit-8 (CCK-8) assay was used to determine the proliferation of A549 and H460 cells. (C) The Transwell invasion assay was performed to determine the invasion ability of A549 and H460 cells. $*p<0.05$.

The lncRNAs are referred to as RNA transcripts with no protein-coding potential and longer than 200 nt, which accounts for a large proportion of the human transcriptome[5]. Accumulating evidence suggested that lncRNAs play great therapeutic potential for human diseases, including cancer. For example, Zhang et al. showed that upregulation of lncRNA MALAT1 correlated with tumor progression and poor prognosis in clear cell renal cell carcinoma[15]. Zhang et al. revealed that lncRNA ANRIL expression was increased in cervical cancer and could promote carcinogenesis via the PI3K/Akt pathway[16].

NEAT1 is a newly identified nuclear-restricted lncRNA, which has earned the reputation as a transcriptional regulator for numerous genes[17]. Recent studies showed that NEAT1 dysregulation promoted tumorigenesis in a variety of human cancers. For example, Zhang et al. found that lncRNA NEAT1 was closely related with the progression of breast cancer via promoting proliferation and EMT[18]. Peng et al. revealed that lncRNA NEAT1 impacted cell proliferation and apoptosis of colorectal cancer via regulation of Akt signaling[19]. Wang et al. indicated that lncRNA NEAT1 promoted laryngeal squamous cell cancer through regulation of the miR-107/CDK6 pathway[20]. However, its potential biological mechanisms of NEAT1 on NSCLC are still unclear.

In the present study, our data showed that lncRNA NEAT1 expression was significantly upregulated in NSCLC tissues compared to adjacent nontumor tissues. High NEAT1 expression was associated with advanced TNM stages, lymph node metastasis, distant metastasis, and poor overall survival in NSCLC patients. In addition, we detected the biological role of NEAT1 in NSCLC progression. Our data showed that knockdown of the NEAT1 expression significantly reduced NSCLC cell proliferation and invasion in vitro. These data suggested that lncRNA NEAT1 could act as an oncogene in NSCLC progression.

Recent studies showed that lncRNAs could act as ceRNAs, abrogating the endogenous suppressive effect of these miRNAs on their targeted transcripts[13]. For example, Yang et al. showed that lncRNA XLOC_008466 functioned as an oncogene in human NSCLC by targeting miR-874[21]. Zhang et al. indicated that lncRNA UCA1 promoted cell progression by acting as a ceRNA of ATF2 in prostate cancer[22]. Tong et al. showed that lncRNA MEG3 suppressed glioma cell proliferation, migration, and invasion by acting as a ceRNA of miR-19a[23]. Therefore, we hypothesized that lncRNA NEAT1 also targeted miRNAs in NSCLC. In the present study, we identified miR-181a-5p as an inhibitory target of NEAT1 by sequence

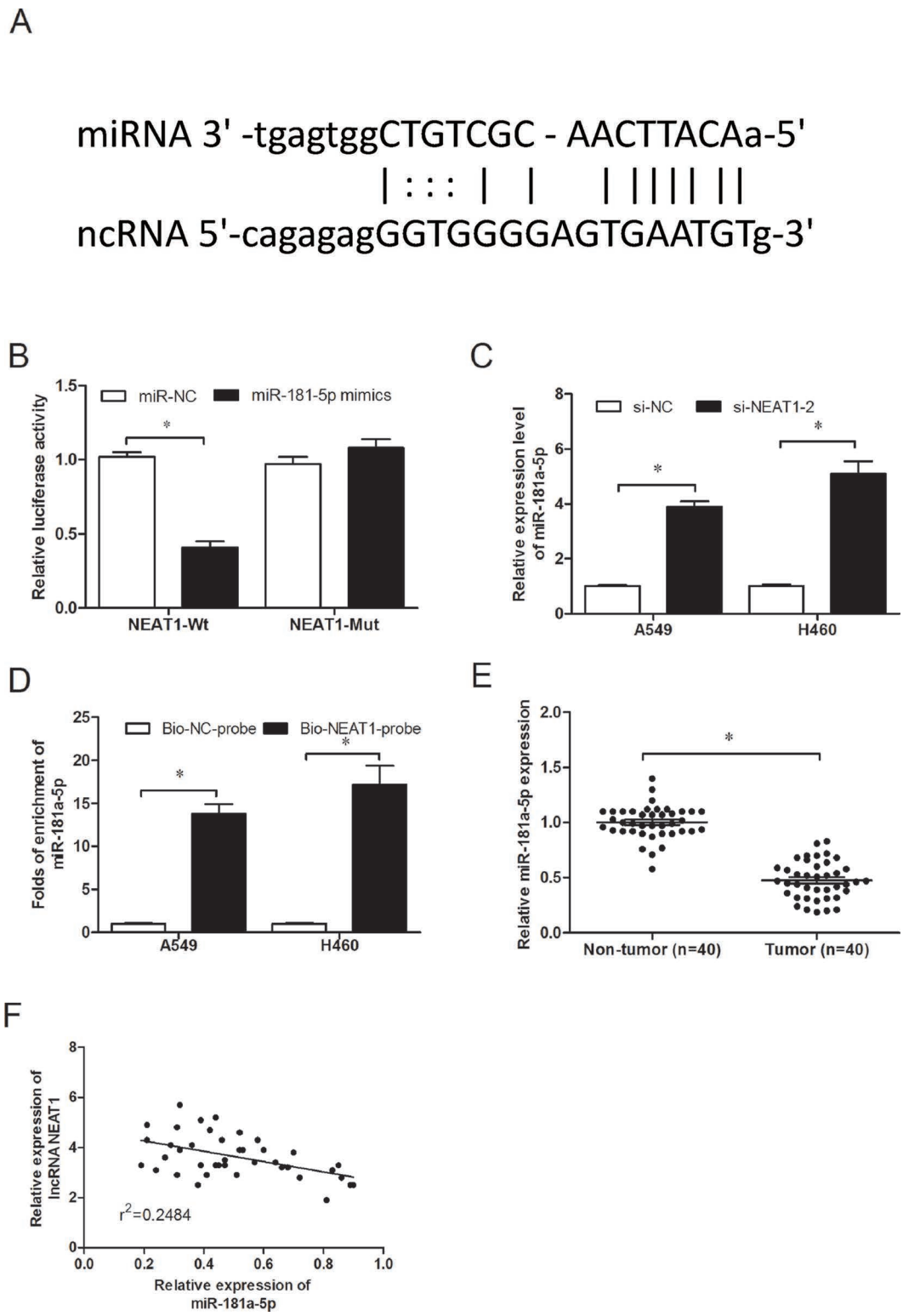

Figure 3. lncRNA NEAT1 directly targets miR-181a-5p in NSCLC. (A) The predicted miR-181a-5p binding sites on lncRNA NEAT1. (B) Luciferase activity in cells cotransfected with miR-181a-5p mimics and luciferase reporters containing the NEAT1-Wt or NEAT1-Mut transcript. (C) Detection of miR-181a-5p using qRT-PCR in the si-NEAT1-transfected A549 and H460 cells compared with the control group. (D) Detection of miR-181a-5p using qRT-PCR in the sample pulled down by biotinylated NEAT1 probe. (E) Expression of miR-181a-5p in 40 pairs of NSCLC tissues and paired adjacent nontumor tissues. (F) The correlation between lncRNA NEAT1 and miR-181a-5p levels was measured in 40 NSCLC tissues. *$p<0.05$.

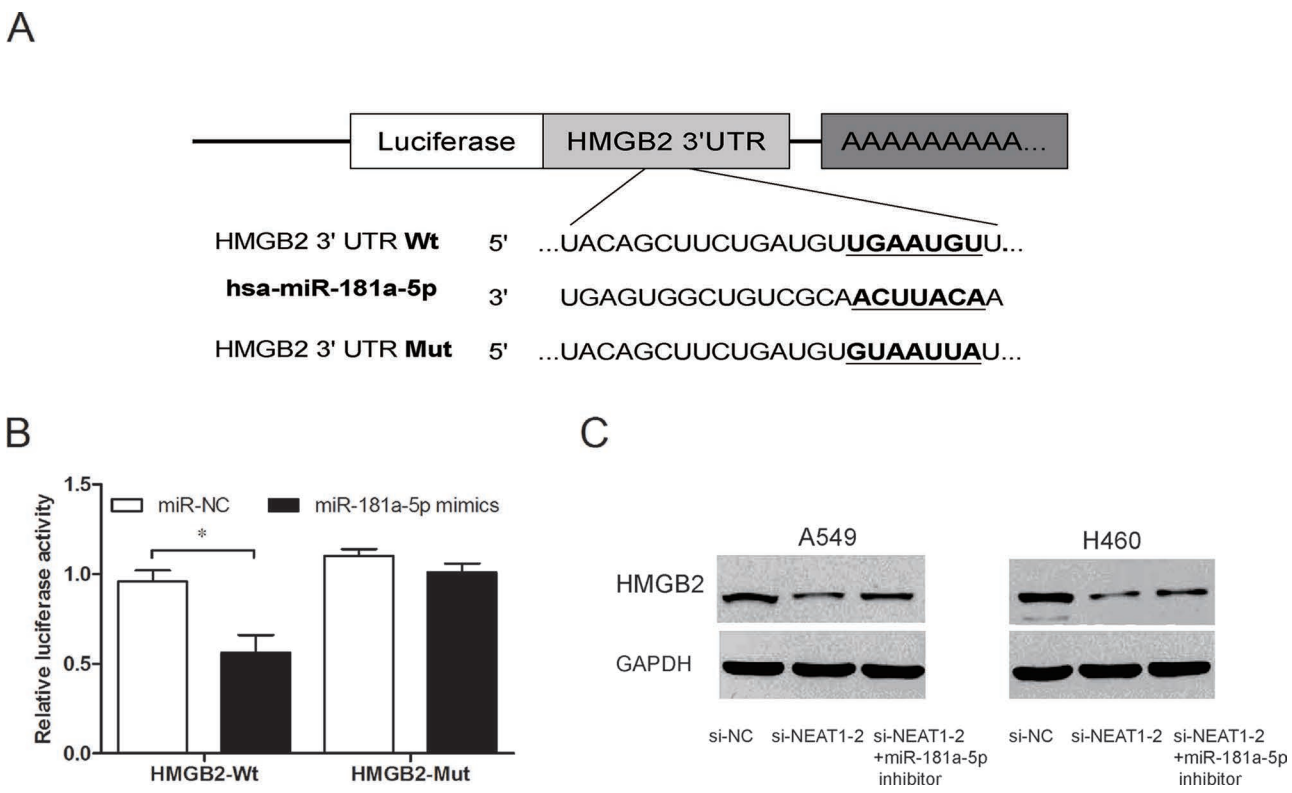

Figure 4. HMGB2 is a target gene of miR-181a-5p and is regulated by lncRNA NEAT1. (A) Predicted binding sites between HMGB2 and miR-181a-5p. (B) Luciferase activity in cells cotransfected with miR-181a-5p mimics and luciferase reporters containing the HMGB2-Wt or HMGB2-Mut transcript. (C) Protein level of HMGB2 in A549 and H460 cells transfected with si-NEAT1 or si-NC in the presence or absence of the miR-181a-5p inhibitor. $*p < 0.05$.

complementarity analysis and luciferase reporter assay. Reduced expression of NEAT1 increased miR-181a-5p expression in NSCLC cells. In addition, our data showed that the expression of miR-181a-5p was significantly decreased and had a remarkably negative correlation with NEAT1 expression in NSCLC tissues. These data indicated that miR-181a-5p is a direct target of NEAT1.

Previous studies showed the critical role of miR-181a-5p on suppressing NSCLC progression[24,25]. In the present study, we searched for the potential gene effectors involved in its function. miR-181a-5p can regulate a number of target genes. In our study, we verified HMGB2 as a direct target of miR-181a-5p, and luciferase reporter assays confirmed that miR-181a-5p targeted HMGB2 mRNA at its 3′-UTR. Moreover, Western blot showed that lncRNA NEAT1 suppression decreased the protein levels of HMGB2, and the miR-181a-5p inhibitors restored the reduction of HMGB2 expression in NEAT1-suppressed NSCLC cells. Taken together, these results suggested that NEAT1 eliminated the repression on HMGB2 induced by miR-181a-5p and exerted oncogenic functions by modulating the miR-181a-5p/HMGB2 axis.

In conclusion, our research showed that NEAT1 acts as an oncogenic lncRNA to promote the progression of NSCLC by the miR-181a-5p/HMGB2 axis. These data showed a potential mechanism underlying the tumor oncogenic role of NEAT1 in NSCLC development, suggesting NEAT1 could be a useful therapeutic target for the treatment of NSCLC.

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