Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2136-2147

DOI: 10.1159/000495831

Published online: 6 December 2018

Accepted: 28 November 2018

Original Paper

Gastric Adenocarcinoma Predictive Long Intergenic Non-Coding RNA Promotes Tumor Occurrence and Progression in Non-Small Cell Lung Cancer via Regulation of the miR-661/eEF2K Signaling Pathway

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Key Words

Long non-coding RNAs (lncRNAs) • Gastric adenocarcinoma predictive long intergenic non-coding RNA (GAPLINC) • miR-661 • Eukaryotic elongation factor-2 kinase (eEF2K) • Pathogenesis

Abstract

Background/Aims: Long non-coding RNAs (lncRNAs) play vital roles in carcinogenesis as oncogenes or tumor suppressor genes. This study explored the biological function of lncRNA gastric adenocarcinoma predictive long intergenic non-coding RNA (GAPLINC) in human non-small cell lung cancer (NSCLC). ***Methods:*** GAPLINC expression in NSCLC specimens and cell lines was detected by qRT-PCR and Western blot. The effect of GAPLINC on cell proliferation was investigated using CCK8-assay, colony formation assay, and xenograft model. The effects of GAPLINC on apoptosis and cell cycle were determined using flow cytometry. The mechanism of GAPLINC involved in NSCLC was explored using Western blot, luciferase reporter assay, and RNA fluorescence *in situ* hybridization. ***Results:*** We found that GAPLINC expression was up-regulated in NSCLC tissues and cell lines. Overexpression of GAPLINC was associated with poor prognosis in patients with NSCLC. Silencing of GAPLINC significantly inhibited cell proliferation, promoted apoptosis, and induced cell cycle arrest in the G0/G1 phase. Results from xenograft transplantation showed that GAPLINC silencing inhibited the tumor growth *in vivo*. Interestingly, GAPLINC silencing decreased the expression of eukaryotic elongation factor-2 kinase (eEF2K) protein both *in vivo* and *in vitro*. Bioinformatic analysis and luciferase reporter confirmed that miR-661 targeted GAPLINC and eEF2K 3'-UTR and was negatively correlated with the expression of GAPLINC and eEF2K. ***Conclusion:*** Our findings indicate that GAPLINC promotes NSCLC tumorigenesis by regulating miR-661/eEF2K cascade and provide new insights for the pathogenesis underlying NSCLC and potential targets for therapeutic strategy.

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Introduction

Lung cancer has become a major clinical problem worldwide. Approximately 2 million patients die from lung cancer each year, and lung cancer is one of the main causes of cancer death [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancers, and the 5-year survival rate is < 20% [2]. Early diagnosis is one of the most effective strategies to improve the survival and prognosis of patients with lung cancer [3-5]. The pathogenesis underlying NSCLC is complex, making clinical treatment difficult and prognosis poor. Therefore, a thorough understanding of the molecular mechanism and effective biomarkers and new targets for diagnosis and therapy are warranted.

The roles of non-coding RNA (ncRNA), including small ncRNAs and lncRNAs (> 200 nt) have been known for decades [6, 7]. It has been shown that lncRNA plays an important role in multiple molecule regulation, such as transcriptional regulation, post-transcriptional regulation, and modification [8-10]. For example, lncRNA GAS5 is down-regulated in NSCLC tissues and cells, and negatively correlated with miR-23a, which inhibits the occurrence of NSCLC [11]. LncRNA MIAT is up-regulated in NSCLC, overexpression of which is associated with the advanced tumor stage. LncRNA MIAT promotes NSCLC proliferation and metastasis by activating MMP9 [12]. It has been reported that lncRNA GAPLINC, as an oncogene, aggravates carcinogenesis through epigenetic regulation. A previous study reported that GAPLINC promotes the proliferation, migration, and invasion of gastric cancer cells by regulating miR-378 [13]; however, the function of GAPLINC in NSCLC remains unclear.

In this study, we explored the potential role of lncRNA GAPLINC in the occurrence and progression of NSCLC. The results showed that GAPLINC expression is up-regulated in NSCLC tissues and cells and GAPLINC promotes eEF2K expression through the regulation of miR-661, thereby accelerating the proliferation and progression of NSCLC. Our findings elucidate a new molecular mechanism and provide potential therapeutic targets for NSCLC treatment.

Materials and Methods

Tissue samples

A total of 72 NSCLC specimens were obtained from the Department of the First People's Hospital of Wenling from January 2013 to December 2016. All of the participants signed informed consent. The study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals Monitoring Committee of Zhejiang Province, China. The protocol was approved by the Committee on the Ethics of Animal Experiments at the First People's Hospital of Wenling (approval number: FHW201406).

Cell culture

NSCLC cell lines (LTEP-A2, NCI-H1299, SK-MES-1, and Calu-3) were purchased from the American Type Culture Collection [ATCC] (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium [DMEM] (Invitrogen, Carlsbad, CA, USA). The normal human bronchial epithelial cell line (NHBE) and HEK-293T were purchased from the Preservation Center for Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum [FBS] (Gibco), 100 U/ml of penicillin (Gibco), and 100 μg/ml of streptomycin (Gibco). All of the cells were incubated in a humidified air atmosphere with 5% CO_2 at 37°C.

Transfection

All siRNAs and sequences were chemically synthesized by GenePharma (Shanghai, Chin), including siRNA for GAPLINC (si-GAPLINC), short-hairpin RNA directly targeting GAPLINC (sh-GAPLINC), miR-661 inhibitor, and miRNA negative control (miR-NC). According to the instructions, Lipofectamine 2000 (Invitrogen) was used to transfect oligonucleotides into NSCLC cells. After 48 h of transfection, cells were collected for further experiments.

Cell proliferation assay

Colony formation assay and CCK-8 test were used to determine the proliferation ability of NSCLC cells. For the colony formation assay, transfected LTEP-A2 and NCI-H1299 cells were cultured in 6-well plates (1000 cells/ well) and incubated at 37°C in 5% CO_2 for 10 days. Then, cells were stained with crystal violet after fixation with 4% paraformaldehyde and the clones were counted. CCK-8 test was performed using a CCK-8 kit (Dojindo, Tokyo, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm and the test was repeated 3 times.

Apoptosis and cell cycle analysis

Apoptosis was analyzed by flow cytometry with an Annexin V-FITC apoptosis detection kit (Invitrogen). LTEP-A2 and NCI-H1299 cells were suspended with 400 µl binding buffer and incubated with 5 µl Annexin V-FITC and 5 µl of propidium iodide (PI). The apoptosis rate was analyzed using a Beckman-Coulter CyAN ADP (Beckman Coulter, Inc., Brea, CA, USA). For cell cycle analysis, LTEP-A2 and NCI-H1299 cells were fixed with 70% ethanol. Then cells were incubated with RNA enzyme A (0.5 mg/ml) and PI (0.1 mg/ml), and measured by flow cytometry.

qRT-PCR

Total RNA was extracted from NSCLC tissues and cells using TRIzol reagent (TaKaRa, Dalian, China). The reverse transcription (RT) reaction was performed using a Prime Script™ RT Reagent Kit (TaKaRa). qRT-PCR was performed on an Applied Bio Systems 1900 using a SYBR Green Real Time PCR Kit (Bio-Rad, city, state, USA). The reaction was as follows: 95°C for 30 sec; 95°C for 8 sec; and 45 loops at 60°Cfor 45 sec. The specific primers used for RT-PCR were as follows: GAPLINC, 5'-TTGTGCAAGTCCAGAAGTGAACTGC-3' (forward) and 5'-TGTGGTTCACAGTCGTACTGTGGT-3' (reverse); miR-661, 5'-AATGGTGGGTGCAAATGTGG-3' (forward) and 5'-GAAACGCATGCCAAAAAGAC-3' (reverse); and glyceraldehyde phosphate dehydrogenase (GAPDH), 5'-CACTTCAGCAGCCAGACA-3' (forward) and 5'-GATCCACTAGGACTCGCT-3' (reverse). The relative expression of each target gene was calculated using the $2^{-\Delta\Delta CT}$ method standardized to GAPDH.

Western blot

Protein was extracted using RIPA lysate buffer (1% NP40, 0.1% SDS, 5mM EDTA, 0.5% deoxycholate sodium, and 1mM provanadate) containing protease and phosphatase inhibitors. Equal amounts of protein were separated by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies at 4°C overnight after blocking with 5% skim milk, then incubated with horseradish peroxidase-conjugated secondary antibodies. The ECL system was used to detect the signals.

Luciferase reporter assays

GAPLINC fragments containing the wild type (WT) binding site of miR-661 (GAPLINC-WT) or mutant binding sites of miR-661 (GAPLINC-Mut) were synthesized by PCR, and cloned into the pmirGLO-alkaline luciferase reporter vector (Promega, Madison, WI, USA). Briefly, 100 ng of luciferase reporter vector, 20 ng of Renilla luciferase vector (Promega), and miR-661 mimics (100 nM) or miR-NC were co-transfected into NSCLC cells by Lipofectamine 2000 (Invitrogen). After transfection for 48 h, luciferase activity was detected using the Dual Luciferase Reporter system (Promega), which was normalized by Renilla luciferase activity.

RNA fluorescence in situ hybridization

Cy3-labeled GAPLINC probes were purchased from RiboBio (Shanghai, China). RNA FISH was performed as previously described [14]. U6 snRNA and 18S rRNA probes (RiboBio) were used as positive controls.

Tumor xenograft transplantation in mice

BALB/C nude mice (n=7; weight, 8-10 g; 4 weeks old) were fed under SPF conditions. sh-GAPLINC or sh-NC stablely-transfected LTEP-A2 cells (1.0×10^7) mediated by lentivirus, were subcutaneously injected into the right posterior of nude mice. After injection, the tumor volume was measure by a digital caliper every 3 days. The tumors were removed and weighed after the mice were sacrificed. All animal procedures were approved by the Animal Experimental Ethics Committee of the First People's Hospital of Wenling.

Statistical analysis

Data are presented as the mean ± S.D. The results were analyzed by Student's t-test or one-way ANOVA using GraphPad Prism 6 software. A $P<0.05$ was considered statistically significant.

Results

LncRNA GAPLINC is highly expressed in NSCLC tissues and cells and indicates a poor prognosis

qRT-PCR was used to detect the expression of lncRNA GAPLINC in NSCLC tissue samples. The clinicopathologic data of the NSCLC patients are shown in Table 1. The results showed that the expression of lncRNA GAPLINC was significantly up-regulated in 35 NSCLC tissues compared with adjacent non-cancerous tissues (Fig. 1A). According to TNM staging, GAPLINC in stage III was significantly up-regulated compared to stage I-II (Fig. 1B). Compared with NHBE cells, GAPLINC was significantly overexpressed in the NSCLC cell lines (LTEP-A2, NCI-H1299, SK-MES-1, and LTEP-A2; Fig. 1C). NSCLC patients were divided into high- and low-expression groups on the basis of the median value (0.83) of GAPLINC expression (Fig. 1D). Survival rates were assessed by Kaplan-Meier survival curves and the logarithmic rank test. The prognosis in the high GAPLINC-expression group was poorer than the low-expression group (Fig. 1E). Overall, the data concluded that lncRNA GAPLINC was up-regulated in NSCLC tissues and cells, and GAPLINC expression is associated with poor prognosis in patients with NSCLC. In addition, we performed a RNA fluorescence *in situ* hybridization (FISH) assay to detect the subcellular localization of GAPLINC. The data showed that GAPLINC was located in the nucleus and cytoplasm (mainly located in the cytoplasm; Fig. 1F).

LncRNA GAPLINC silencing inhibits NSCLC cell proliferation in vitro

The up-regulation of GAPLINC in NSCLC tissues and cells promotes us to consider that GAPLINC might have an oncogneic activity in NSCLC. To confirm this hypothesis, we investigated the effect of GAPLINC on cell proliferation of NSCLC *in vitro* using a cell dysfunction assay. GAPLINC expression was significantly down-regulated in LTEP-A2 and NCI-H1299 cell lines after siRNA-mediated knockdown (Fig. 2A). Results of the CCK-8 assay showed that GAPLINC silencing significantly inhibited the proliferation NSCLC cells *in*

Table 1. The relationship between GAPLINC expression and clinicopathological parameters. *P < 0.05 represents statistical difference

Parameters	n	Low expression n (%)	High expression n (%)	P value
Age (years)				
≤60	26	10 (33.3)	16 (38.1)	0.4877
>60	46	20 (66.7)	26 (61.9)	
Gender				
Male	51	18 (60.0)	33 (78.6)	0.6143
Female	21	12 (40.0)	9 (21.4)	
Smoking status				
Smoking	52	19 (63.3)	33 (78.6)	0.0265
Non-smoking	20	11 (37.7)	9 (21.4)	
Lymphonode metastasis				
Positive	45	16 (53.3)	29 (69.0)	0.0061*
Negative	27	14 (46.7)	13 (31.0)	
Differentiation				
Well	35	14 (46.7)	21 (50.0)	0.3329
Moderate/poor	37	16 (53.3)	21 (50.0)	
TNM				
I-II	43	17 (60.0)	26 (61.9)	0.0134*

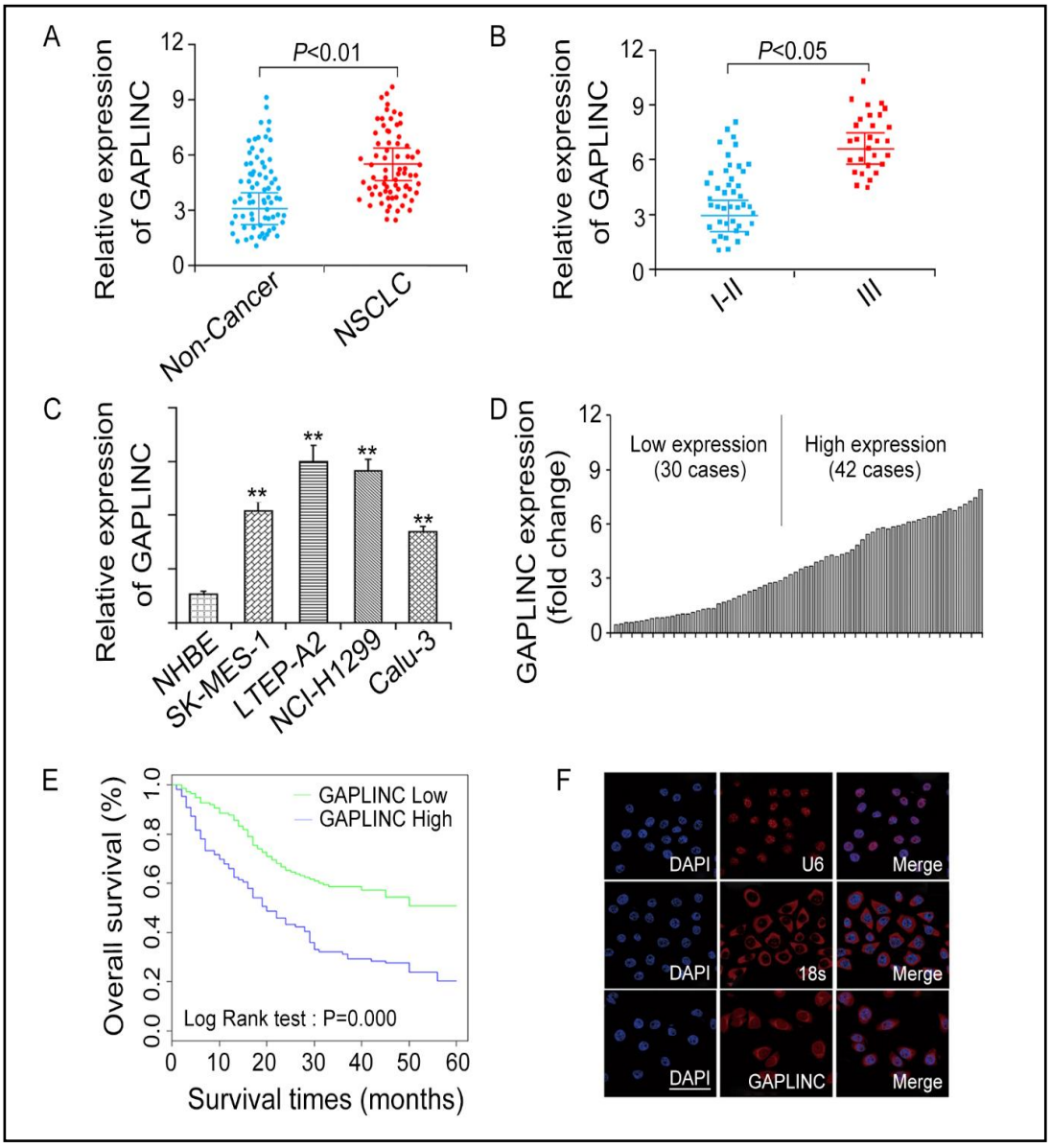

Fig. 1. LncRNA GAPLINC was highly expressed in NSCLC tissues and cells and indicates a poor prognosis. (A) qRT-PCR results showed the expression of GAPLINC in 72 NSCLC tissues and adjacent non-cancerous tissues. (B) The expression of GAPLINC in TNM stages I-II and III. (C) The expression of GAPLINC in NSCLC cell lines and NHBE cells. (D) NSCLC patients were grouped according to GAPLINC expression (high or low). (E) Survival rate of NSCLC patients was assessed by Kaplan-Meier survival curves. (F) GAPLINC localization examined by RNA-FISH (scale bar= 50 μm). The U6 and 18S were used as positive controls. **p<0.01 indicates a significant difference.

vitro (Fig. 2B). In line with these results, the colony formation assay showed that GAPLINC silencing reduced the number of clones of LTEP-A2 and NCI-H1299 cells (Fig. 2C). Taken together, our results indicated that lncRNA GAPLINC silencing inhibits the proliferation of NSCLC cells *in vitro*.

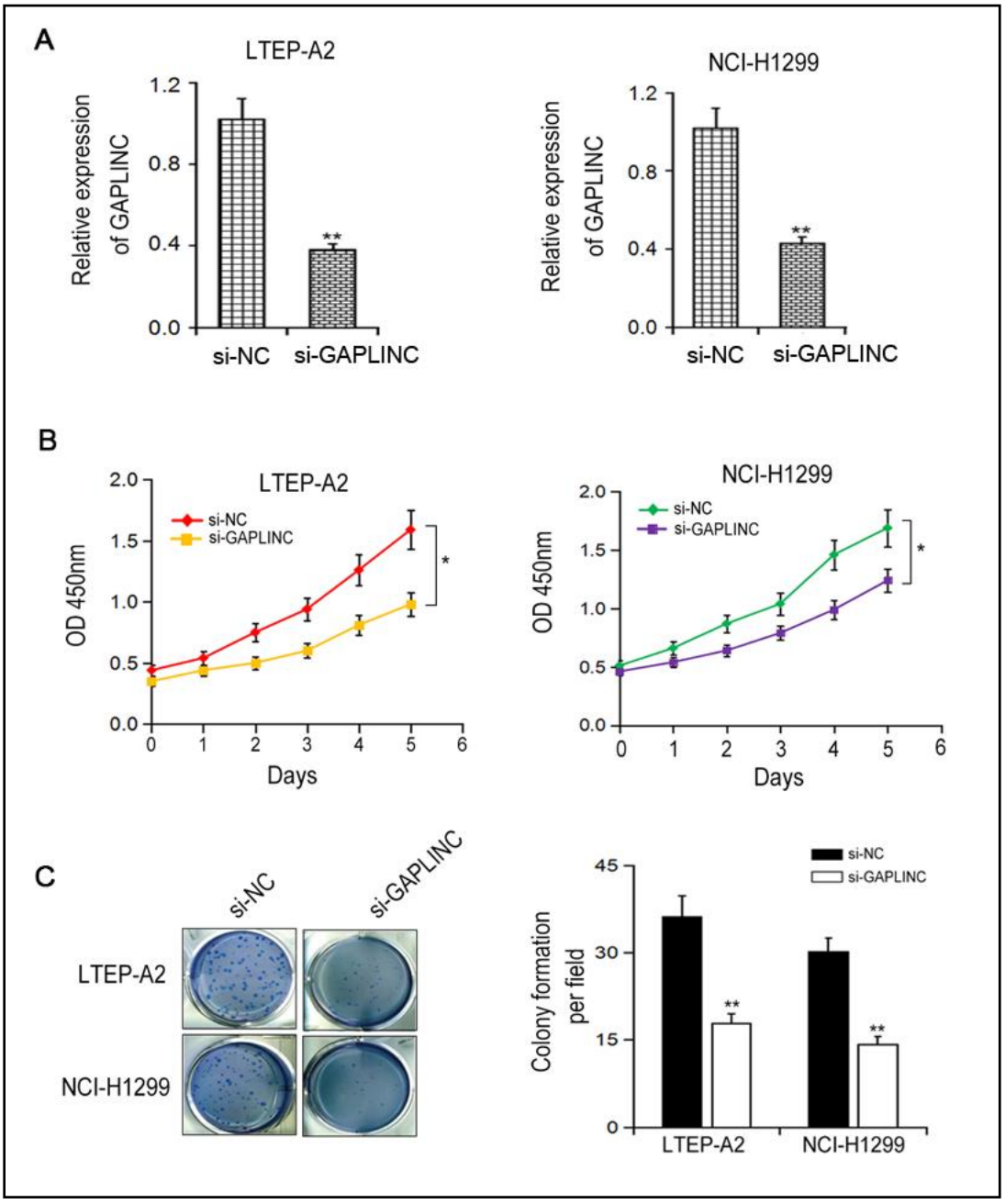

Fig. 2. LncRNA GAPLINC silencing inhibits cell proliferation of NSCLC in vitro. (A) qRT-PCR results showed the expression of lncRNA GAPLINC in LTEP-A2 and NCI-H1299 cell transfected with GAPLINC-siRNA. (B) CCK-8 results of OD values in LTEP-A2 and NCI-H1299 cells. (C) Results of the colony formation assay showed the number of LTEP-A2 and NCI-H1299 colonies. *p<0.05, **p<0.01 indicates a significant difference.

LncRNA GAPLINC silencing induces cell cycle arrest and promotes NSCLC cell apoptosis

To understand the mechanism underlying GAPLINC-induce cell proliferation, we analyzed the effects of GAPLINC silencing on apoptosis and cell cycle distribution by flow cytometry. Flow cytometry showed that GAPLINC silencing accelerated apoptosis of LTEP-A2 and NCI-H1299 cells compared with the control transfected cells (Fig. 3A, B). In addition, flow cytometry analysis revealed that GAPLINC silencing induced a cell cycle arrest in G0/G1 phase (Fig. 3C, D). Collectively, these results suggest that GAPLINC silencing induces G0/G1 phase arrest and enhances apoptosis in NSCLC cells.

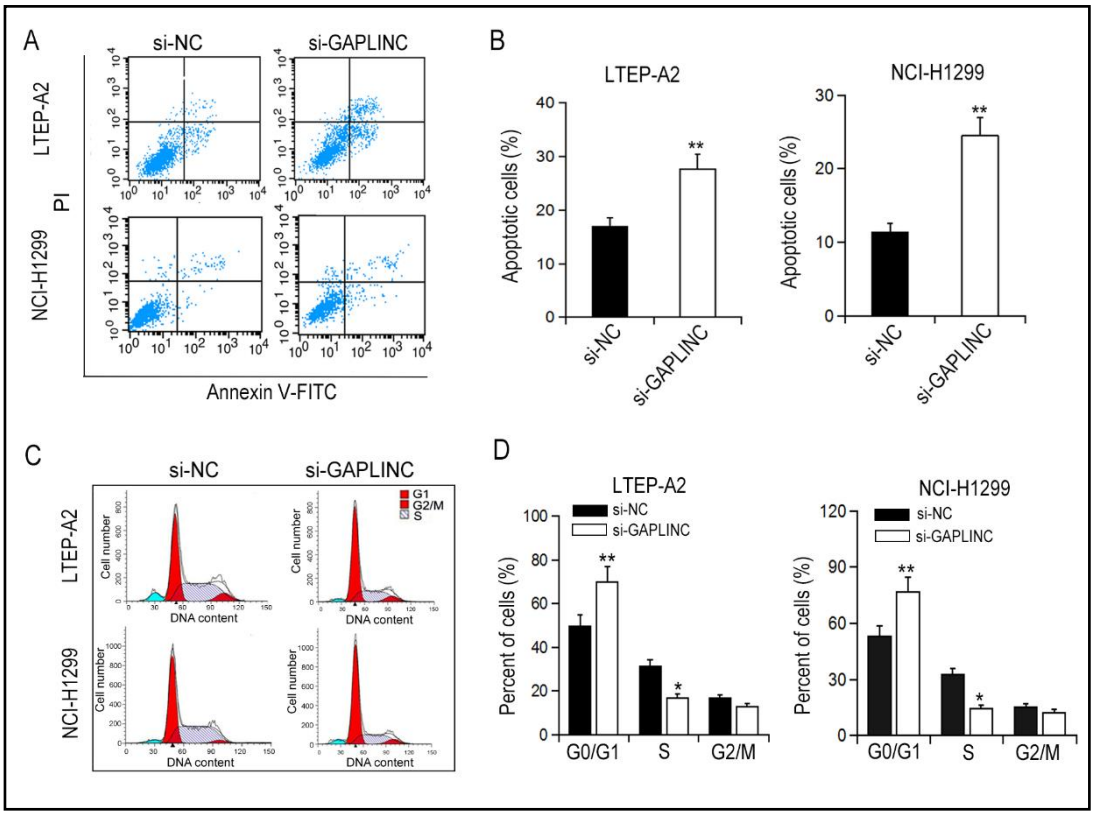

Fig. 3. LncRNA GAPLINC silencing induced cell cycle arrest in the G0/G1 phase and promoted NSCLC cell apoptosis. (A-B) Flow cytometry showed apoptosis of si-NC and si-GAPLINC-transfected LTEP-A2 and NCI-H1299 cells. (C-D) Flow cytometry showed the cell cycle distribution of si-NC and si-GAPLINC-transfected LTEP-A2 and NCI-H1299 cells. *p<0.05, **p<0.01 indicates a significant difference.

LncRNA GAPLINC silencing inhibits the tumor growth of NSCLC in vivo

To further study the effect of GAPLINC on tumor growth of NSCLC, we used LTEP-A2 cells for xenograft transplantation in mice (Fig. 4A). The tumor volume in the shGAPLINC-transfected LTEP-A2 group was significantly decreased compared with the sh-NC-transfected LTEP-A2 group (Fig. 4B). Moreover, silencing of GAPLINC remarkably reduced the tumor weight of NSCLC (Fig. 4C). Eukaryotic elongation factor-2 kinase (eEF2K) has been reported to contribute to carcinogenesis [15-16]. Intriguingly, western blot analysis showed that the protein expression of eEF2K in the GAPLINC silencing group decreased significantly compared with the control group (Fig. 4D, E). Taken together, these results suggest that GAPLINC silencing inhibits tumor growth and down-regulated eEF2K protein expression in NSCLC tumor.

GAPLINC regulates eEF2K expression positively through miR-661

To further confirm the regulatory effect of GAPLINC on eEF2K expression, we knocked down GAPLINC in NSCLC cells. Western blot analysis showed that GAPLINC silencing decreased the expression of eEF2K protein in both LTEP-A2 and NCI-H1299 cells (Fig. 5A, B). Next, we sought to investigate the potential mechanism of eEF2K modulation by GAPLINC. LncRNA has been reported to exert its function by acting as a miRNA sponge. Bioinformatic prediction indicated that miR-661 targeted the 3'-untranslated regions (3'-UTRs) of GAPLINC and eEF2K (Fig. 5C, F). The luciferase reporter assay confirmed that miR-661 targeted GAPLINC via molecular binding (Fig. 5D). Moreover, in contrast to the up-regulation of GAPLINC in NSCLC tissues, miR-661 expression was decreased in NSCLC tumor

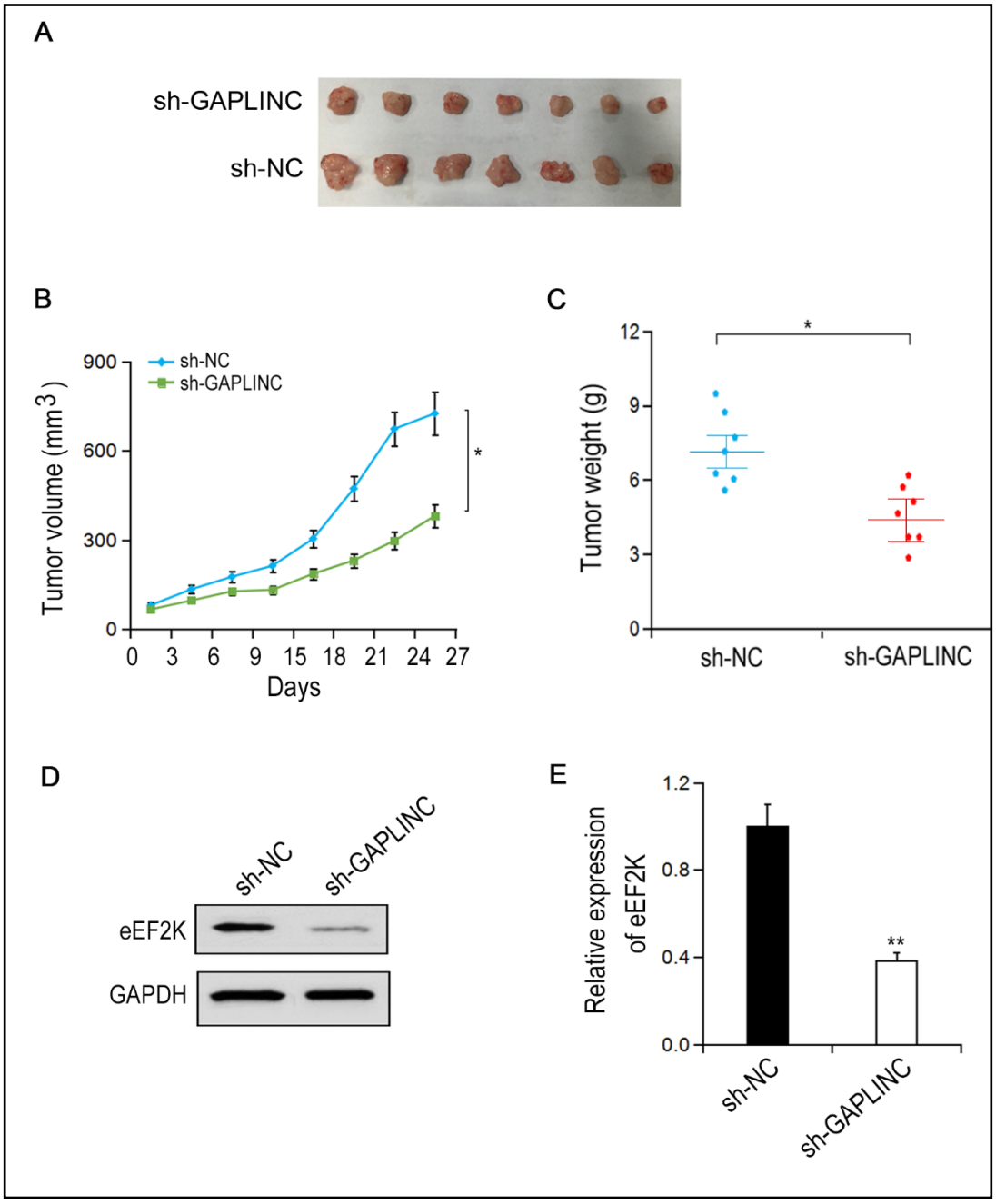

Fig. 4. LncRNA GAPLINC silencing inhibited tumor growth of NSCLC in vivo. (A) Images of xenograft injected with LTEP-A2 cells. (B) The tumor volume was measured every 3 days after subcutaneous injection. (C) The tumor weight was measured after the mice were sacrificed. (D) Western blotting analysis of eEF2K protein. (E) Quantitative data of eEF2K protein expression. *p<0.05, **p<0.01 indicated a significant difference.

tissues compared with adjacent non-cancerous tissues (Fig. 5E). In addition, luciferase reporter assay confirmed the molecular interaction between miR-661 and eEF2K (Fig. 5G). The expression of miR-661 increased significantly in si-GAPLINC-transfected LTEP-A2 cells compared with si-NC-transfected cells, while the expression of eEF2K mRNA decreased (Fig. 5H). Compared with the control groups, the expression of eEF2K mRNA and GAPLINC were increased significantly in miR-661 inhibitor-transfected LTEP-A2 cells, whereas the expression of eEF2K mRNA and GAPLINC were decreased in miR-661 mimic-transfected

LTEP-A2 cells (Fig. 5I). Western blot analysis showed that miR-661 inhibitor increased the expression of eEF2K, and this effect was abolished by co-transfection with si-GAPLINC (Fig. 5J, K). Together, GAPLINC promotes eEF2K expression through the negative regulation of miR-661, suggesting a regulatory pathway involving GAPLINC/miR-661/eEF2K.

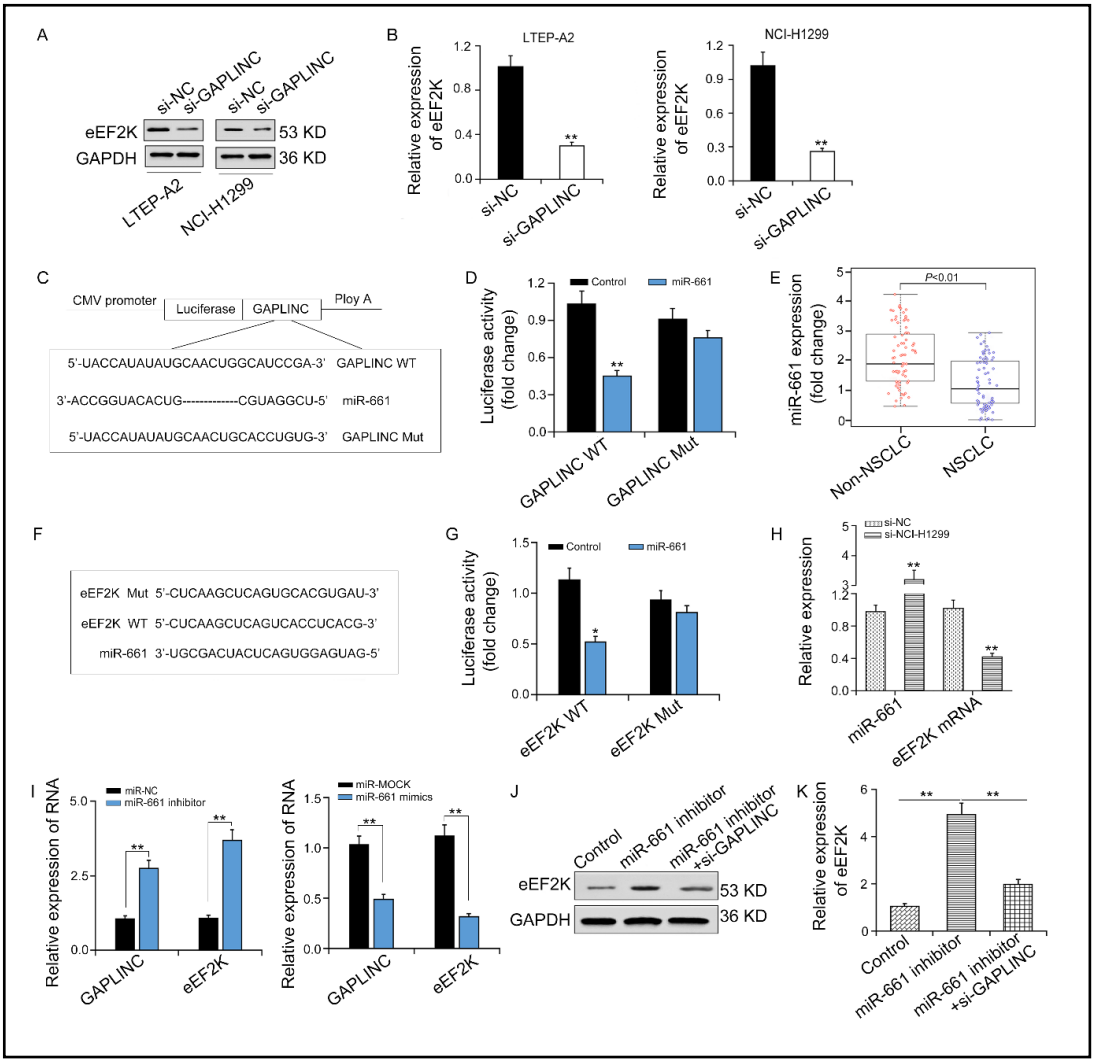

Fig. 5. GAPLINC positively regulates eEF2K expression via a miR-661 sponge. (A-B) Western blot showed the expression of eEF2K protein in LTEP-A2 and NCI-H1299 cells transfected with si- GAPLINC. (C) Predicted miR-66-binding sites in GAPLINC 3'-UTR. (D) Luciferase reporter assay showed luciferase activity of GAPLINC WT/Mut after miR-661 transfection. (E) The expression of miR-661 in NSCLC tumor tissues compared with adjacent non-cancerous tissues. (F) Predicted miR-66-binding sites in eEF2K 3'-UTR. (G) Luciferase reporter assay showed luciferase activity of eEF2K WT/Mut after miR-661 transfection. (H) The expression of miR-661 and eEF2K mRNA in si-GAPLINC-transfected LTEP-A2 cells. (I) The results of qRT-PCR showed that the expression of eEF2K mRNA and GAPLINC in miR-661 inhibitor- or miR-661 mimic-transfected LTEP-A2 cells. (J, K) Western blot showed the expression of eEF2K protein in LTEP-A2 cells co-transfected with miR-661 inhibitor and si-GAPLINC. *p<0.05, **p<0.01 indicated a significant difference.

Discussion

A large body of evidence indicates that lncRNA plays a role as an oncogene or tumor suppressor gene in various tumors through transcriptional and post-transcriptional regulation [17-19]. For example, lncRNA XIST is significantly up-regulated in NSCLC tissues compared with adjacent normal tissues, XIST inhibits proliferation, migration, and invasion of NSCLC cells, and XIST induces G0/G1 phase arrest by sponging miR-367/141 [20]. *In vivo* experiments have shown that HOTAIR knockdown reduces tumor growth and invasion and enhances apoptosis and miR-613 expression [21]. Jiang et al. [22] reported that down-regulation of LINC00961 is associated with advanced stage, lymph node metastasis, and short overall survival in NSCLC patients. Chen et al. [23] showed the carcinogenic effect of FOXD2-AS1 in clinical specimens and *in vitro* cell experiments and revealed the potential pathway of FOXD2-AS1/miR-363-5p/S100A1. Li et al. [20] found that lncRNA XIST promotes TGF-β-induced EMT, cell invasion, and metastasis by regulating the miR-367/miR-141-ZEB2 pathway in NSCLC.

In this study, we carried out a series of experiments to explore the biological function of lncRNA GAPLINC in the pathogenesis of NSCLC. Because NSCLC is a multi-gene disease, the biological mechanism is very complex. Indeed, there is a need to identify the molecular basis underlying NSCLC, which will in turn improve the diagnosis and treatment of NSCLC patients. In the current study, we found that the expression of GAPLINC in NSCLC tissues was significantly higher than adjacent non-cancerous tissues. In addition, up-regulation of GAPLINC predicted a poor prognosis for NSCLC patients. *In vitro* experiments revealed that GAPLINC knockdown inhibited cell proliferation of NSCLC.

The lncRNA/miRNA/mRNA pathway has been shown to be an important regulator of NSCLC. For example, lncRNA MALAT1 is significantly up-regulated in 5 kinds of human NSCLC cells. Bioinformatic analysis has predicted a correlation between miR-124 and MALAT1 and indicated that STAT3 is the new mRNA target of miR-124 [24]. In this study, the results of bioinformatic tools on line and luciferase experiments showed that miR-661 targeted the 3'-UTR of lncRNA GAPLINC, and GAPLINC served as a molecular sponge for miR-661. Our results also indicated that miR-661 targeted 3'-UTR of eEF2K. Our findings indicated that the GAPLINC/miR-661/eEF2K pathway plays a regulatory role in the tumorigenesis of NSCLC.

Conclusion

In conclusion, our findings suggest that lncRNA GAPLINC is overexpressed in NSCLC tissues and cell lines, which predicts a poor prognosis. In addition, GAPLINC promotes eEF2K expression by sponging miR-661 to promote NSCLC tumorigenesis. This study provides new insights into the pathogenesis and potential therapeutic strategies for NSCLC.

Abbreviations

American (Type Culture Collection, ATCC); Dulbecco's (Modified Eagle Medium, DMEM); eukaryotic (elongation factor-2 kinase, eEF2K); fetal (bovine serum, FBS); gastric (adenocarcinoma predictive long intergenic non-coding RNA, GAPLINC); Long (non-coding RNAs, lncRNAs); normal (human bronchial epithelial cell line, NHBE); non-small (cell lung cancer, NSCLC); non-coding (RNA, ncRNA).

Acknowledgements

This work was supported by the Foundation of Wenling Science and Technology Bureau, Wenling city, Zhejiang Province, China (No. 2016C31BA0032).

Disclosure Statement

The authors declare no conflicts of interest.

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