LINC00476 suppresses the progression of non-small cell lung cancer via inducing the ubiquitination of SETDB1

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Research

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Abstract

Background: Long non-coding RNAs are involved in the tumorigenesis of non-small cell lung cancer (NSCLC). This study aims to investigate whether LINC00476 affects the proliferation, invasion, and migration of NSCLC cells via SETDB1-activated Wnt/β-catenin pathway.

Method: The expression of LINC00476, SETDB1, Wnt1, and β-catenin were determined in NSCLC tumor tissues and the paired adjacent tissues, as well as in NSCLC cell lines and bronchial epithelioid cell lines. Cell proliferation, invasion, and migration were determined using cell counting kit-8 assay and Transwell assay. The relationship between LINC00476 and SETDB1 was elucidated using RNA pull-down, RNA immunoprecipitation, and ubiquitination assays.

Result: LINC00476 was significantly downregulated, while SETDB1, Wnt1, and β-catenin were upregulated in NSCLC tumor tissues and cell lines compared to the normal ones. Overexpression of LINC00476 promoted the proliferation, invasion, and migration of NSCLC cells, as well as suppressed tumor growth in the mouse xenograft. Meanwhile, overexpression of LINC00476 induced the degradation of SETDB1 via promoting its ubiquitination. The simultaneous overexpression of LINC00476 and SETDB1 negated the inhibition of LINC00476 overexpression on the proliferation, invasion, and migration of NSCLC cells.

Conclusion: LINC00476 acts as a tumor suppressor in NSCLC via downregulating SETDB1, which provides a novel target in treating NSCLC.

Introduction

In recent years, the high incidence of lung cancer has not only made it become one of the most common malignant tumors worldwide, but also been a major killer that threatens human health. Clinically, according to the pathological characteristics, lung cancers are divided into two types, the non-small cell lung cancer (NSCLC) which is about 80–85% of the total incidence of lung cancers, and the small cell lung cancer (SCLC) [1]. Although the diagnosis and treatment of NSCLC have made great progress from the tumor resection, chemotherapy, radiotherapy, targeted therapy, and even immune checkpoint blockade therapy [2], the non-specific symptoms in the early stage of NSCLC patients often result in a late stage and poor prognosis. Therefore, it is particularly important to clarify the molecular biological mechanisms of the development of NSCLC in order to elucidate novel therapeutic targets.

SET domain bifurcated 1 (SETDB1, also known as ESET or KMT1E) is a histone H3 lysine 9 methyltransferase [3], and it has been reported to be involved in the progression and aggressiveness of several kinds of cancers, including hepatocellular cancer [4], breast cancer [5], and colorectal cancer [6]. In NSCLC clinical samples and cell lines, SETDB1 was highly expressed, and the administration of SETDB1-interfering drug mithramycin suppressed lung tumor growth in the nude mouse xenograft [7]. Meanwhile, the oncogenic effect of SETDB1 on NSCLC progression was demonstrated to be mediated via the Wnt/β-catenin pathway [8, 9]. However, the upstream molecular regulation of SETDB1 in NSCLS has not been fully elucidated.

More than 90% of the genes in human genome do not encode proteins, and these genes are described as the non-coding RNAs (ncRNAs). Among them, the long non-coding RNAs (lncRNAs) are a subtype of ncRNAs, whose length is more than 200 nucleotides [10]. Initially, lncRNAs were considered as the “rubbish” of gene transcription. However, the increasing evidence has revealed the multiple functions of lncRNAs on cell fate regulation, especially on tumorigenesis [11]. For example, lncRNA-MALAT1 [12, 13] and lncRNA-XIST [14, 15] have been elucidated to regulate the proliferation, invasion, and migration of NSCLC. Still, there have been a large amount of lncRNAs whose effects on NSCLC tumorigenesis have not been clearly identified.

In the current study, we searched the GEO database and performed the statistical analysis to identify dysregulated lncRNAs in NSCLC tumorigenesis. We found that LINC00476 was markedly downregulated in NSCLC clinical tumor tissues and cell lines. Furthermore, our study demonstrated that LINC00476 modulated the proliferation, invasion, and migration of NSCLC cells and the tumor growth via affecting the ubiquitination of SETDB1, aiming to provide a novel therapeutic target in treating NSCLC.

Materials And Methods

Clinical samples and cell lines

NSCLC patients (50 cases) who were admitted to The First Affiliated Hospital of Zhengzhou University from January 2017 to January 2019 were included in this study. During the pulmonary tumor resection, fresh tumor tissues and the paired normal adjacent tissues were collected and were frozen at -80 ℃.

Human bronchial epithelioid cell line (16HBE), human lung adenocarcinoma cell lines (SPC-A-1 and A549), human NSCLC cell line from a metastatic lymph node (H1299), human bronchioalveolar carcinoma cell line (H358), and human large cell lung cancer cell line (H460) were bought from Procell Life Technology (Wuhan, Hubei, China). They were cultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 ℃ (95% air and 5% CO_2).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to determine the relative expressions of LINC00476 and the mRNA expression of SETDB1 in tissues and cells. Total RNAs were used for the reverse transcription reactions, and qRT-PCR was performed on a Step One Plus real-time system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. The relative expression was calculated using $2^{-\triangle\triangle Ct}$.

Western blot analysis

The protein expressions of SETDB1, Wnt1, β-catenin in tissues and cells were determined using western blot analysis. The primary antibodies used in this study were anti-SETDB1 (ab12317, 1:1000, Abcam,

Cambridge, UK), anti-Wnt1 (ab15251, Abcam), anti-β-catenin (1:1000, #8480, Cell Signaling Technology, Danvers, MA, USA), and anti-β-actin (ab8226, 1:500, Abcam, Cambridge, UK).

Cell transfection

The LINC00476-overexpressing plasmid (pcDNA-LINC00476) and its control plasmid (pcDNA), the small interfering RNA against LINC00476 (si-LINC00476) and its control RNA (si-control), the LINC00476-overexpression lentivirus vector (Lenti-LINC00476) and its control lentivirus (Lenti-GFP), and the SETDB1-overexpression lentivirus vector (Lenti-SETDB1) and its control lentivirus (Lenti-GFP) were obtained from RiboBio Technology (Guangzhou, Guangdong, China). Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) was used to transfect the vectors into tumor cells in accordance with manufacture's constructions.

Cell proliferation, invasion, and migration

Cell proliferation of tumor cells was determined using Cell counting kit-8 (CCK-8) assay [16]. Tumor cells were seeded into 96-well plates at a density of 5×10^4/μL. The CCK-8 solution (10 μL, Dojindo, Kumamoto, Japan) was added to each well for 0, 24, 48, and 72 h, respectively. The absorbance at 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell invasion and migration were determined using Transwell assay [17]. The pore size in the Transwell assay was 8.0 μm (Costar, Shanghai, China). For cell migration assay, the upper chamber was coated with 200 μL serum-free DMEM, while the lower chamber was coated with 600 μL DMEM supplemented with 10% FBS. Tumor cells at a density of 1×10^5 per well were seeded into the upper chamber, followed by the culture for 24 h. The cells in the lower chamber were fixed with methanol for 10 min and stained with crystal violet for 10 min. After being washed with PBS, cells were counted from five randomly selected fields at 200 × magnification. For cell invasion assay, the membrane of the upper chamber was pre-coated with 30 mg/cm^2 Matrigel (BD Biosciences, San Jose, CA, USA) for 1h. Then the performance was the same as the cell migration assay.

In vivo experiments

Male BALB/c nude mice (n=10) were bought from the Shanghai Lab Animal Research Center (Shanghai, China). The xenograft model of lung cancer was established as previously described [18]. A549 cells were transiently transfected with Lenti-GFP/Lenti-LINC00476 for 48 h. Then the cells were collected and were added to the mixture of FBS/Matrigel (1:1) before being injected into the posterior flank of the nude mouse (n=5 in each group). The tumor volumes were measured every 3 days using a Vernier caliper (tumor volume= length × width^2 × 0.5). Mice were sacrificed 21 days after the injection. The expressions of LINC00476 and SETDB1 in tumor tissues were determined using qRT-PCR and western blot analysis, respectively.

RNA immunoprecipitation

The interaction between LINC00479 and SETDB1 was determined in A549 cells using RNA immunoprecipitation (RIP). A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Temecula, CA, USA) was used according to the manufacturer's instructions. SETDB1 antibody (ab12317, Abcam, Cambridge, UK) was used during the immunoprecipitation, and IgG was used as the control. The fold change of LINC00476 was determined using qRT-PCR.

RNA pull-down assay

The interaction between LINC00479 and SETDB1 was determined in A549 cells using RNA pull-down assay. Biotin-labeled LINC00479 and control probe (NC, negative control) were purchased from RiboBio Technology (Guangzhou, Guangdong, China). The probes were co-incubated with Streptavidin-labeled magnetic beads and A549 cells overnight at 4°C. Then the cells were lysed using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA). After being incubated with magnetic beads for 1 h, the cell lysates were collected and the protein expression of SETDB1 was detected using western blot analysis.

Ubiquitination assay and Cycloheximide treatment

A549 and H460 cells were transfected with HA-tagged ubiquitin (HA-Ub), Flag-tagged SETDB1 (Flag-SETDB1), and pcDNA/pcDNA-LINC00476 for 48 h, followed by the treatment of MG132 (a proteasome inhibitor). After MG132 treatment for 4 h, cells were collected. The cell lysates were immunoprecipitated with SETDB1 antibody (ab12317, Abcam, Cambridge, UK) before being immunoblotted with HA antibody (ab9110, Abcam, Cambridge, UK). The protein expression of SETDB1 was determined using western blot analysis.

To investigate the effect of LINC00476 on the degradation of SETDB1, we transfected A549 and H460 cells with pcDNA/pcDNA-LINC00476, followed by the treatment of 125 μg/mL cycloheximide (CHX, a protein synthesis inhibitor). The protein expression of SETDB1 was determined at 0, 1, 2, and 3 h after the CHX treatment using western blot analysis.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Data analysis imaging used SPSS 18.0 and Graphpad Prism 5.0. Statistical analyses between two groups were done by using the Student's t-test. One-way analysis of variance (ANOVA) followed by the LSD post hoc test was used to compare among multiple groups. Differences were considered statistically significant if $p<0.05$.

Results

LINC00476 is lowly expressed in NSCLC tumor tissues and cell lines

The GSE118370 dataset which refers to the invasive NSCLC samples in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) showed that a large amount of lncRNAs and mRNAs were dysregulated in NSCLC as shown in the volcano plot (**Fig. 1A**). We screened top 15 downregulated

lncRNAs and top 4 upregulated lncRNAs by adjusting p value < 0.05 and logFC < -1 or > 1 (**Table. 1**). Among them, LINC00476 was significantly downregulated in NSCLC tissues (adjust p = 0.027395) by using GSE118370 dataset (**Fig. 1B**). Meanwhile, the overall survival (171 in high-level group and 430 in low-level group, p = 0.0056, HR =0.61, 95% CI = 0.42-0.87) in NSCLC patients with low-level LINC00476 was shorter than those with high-level LINC00476 (**Fig. 1C**). These data suggested that the lower level of LINC00476 may be associated with poor prognosis of NSCLC patients. In the clinical tumor samples, which were collected during surgery by us, the relative LINC00476 expression was significantly lower than that in adjacent tissues (0.362 ± 0.144 vs 0.998 ± 0.157, p < 0.01, **Fig. 1D**). In addition, the low LINC00476 expression was related to the high grade of tumor stage and lymph node metastasis (both p < 0.01, **Table. 2**). The protein expression of SETDB1 was upregulated in tumor tissues compared with that in adjacent tissues (**Fig. 1E**). The lowly expressed LINC00476 and the highly expressed SETDB1 were also determined in NSCLC cell lines, including SPC-A-1, A549, H1299, H460, and H358, in comparison with normal bronchial epithelioid cell line (16HBE) (**Fig. 1F**). Moreover, the downregulation of LINC00476 in A549 and H460 cells were more obvious than other NSCLC cell lines (p < 0.01 vs p < 0.05). The protein expressions of Wnt1 and β-catenin were upregulated in NSCLC cell lines compared to the normal bronchial epithelioid cell line (**Fig. 1F**).

Overexpression of LINC00476 inhibits NSCLC cell proliferation, migration, and invasion in vitro and suppresses NSCLC tumor growth in vivo

Given that LINC00476 expression was dramatically reduced in A549 and H460 cells, we used these cells in the next experiments. After overexpressing LINC00476 via pcDNA-LINC00476 transfection, the relative expression of LINC00476 was markedly induced (p < 0.01, **Fig. 2A**). The overexpression of LINC00476 significantly suppressed cell proliferation of A549 cells at 24 h (0.240 ± 0.036 vs 0.467 ± 0.033, p = 0.040), 48 h (0.400 ± 0.062 vs 0.660 ± 0.029, p = 0.037), and 72 h (0.517 ± 0.057 vs 1.080 ± 0.086, p = 0.009) and H460 cells at 24 h (0.297 ± 0.034 vs 0.597 ± 0.068, p = 0.007), 48 h (0.383 ± 0.040 vs 0.677 ± 0.050, p = 0.044), and 72 h (0.503 ± 0.040 vs 1.220 ± 0.106, p = 0.019) (**Fig. 2B**). The overexpression of LINC00476 also inhibited cell invasion of H460 cells (91.000 ± 18.493 vs 263. 000 ± 18.019, p = 0.022) and A549 cells (192.333 ± 16.214 vs 408.667 ± 20.039, p = 0.008) (**Fig. 2C**), as well as cell migration of H460 cells (397.333 ± 20.270 vs 616.667 ± 28.674, p = 0.021) and A549 cells (366.000 ± 22.760 vs 669.333 ± 25.091, p = 0.000) (**Fig. 2D**). For the in vivo experiments, the overexpression of LINC00476 markedly suppressed the tumor growth during the 21 days (**Fig. 2E**). Then the upregulated expression of LINC00476 (**Fig. 2F**) and the downregulated expressions of SETDB1, Wnt1, and β-catenin (**Fig. 2G**) were confirmed in tumor tissues of Lenti-LINC00476 injected mice compared with the Lenti-GFP injected mice.

LINC00476 induces the degradation of SETDB1 via promoting its ubiquitination

SETDB1 was expressed in the LINC00476 pull-down compound (**Fig. 3A**), and the fold change of LINC00476 was increased in SETDB1-immunoprecipitated complex compared with the IgG-immunoprecipitated complex (p < 0.05, **Fig. 3B**), suggesting the interaction between LINC00476 and SETDB1. In A549 and H460 cells, the overexpression of LINC00476 reduced SETDB1 protein expression,

while the knockdown of LINC00476 enhanced SETDB1 protein expression (**Fig. 3C**), indicating the negative regulatory relationship between LINC00476 and SETDB1. However, either overexpressing or knocking down LINC00476 did not affect the mRNA level of SETDB1 ($p > 0.05$, **Fig. 3D**). Therefore, we supposed that LINC00476 modulated the protein stability of SETDB1. After overexpressing LINC00476 in A549 and H460 cells, the degradation of SETDB1 protein was accelerated at 1, 2, and 3 h after the treatment of the protein synthesis inhibitor CHX (**Fig. 3E**). In addition, the overexpression of LINC00476 increased the ubiquitin-bound SETDB1 (**Fig. 3F**), suggesting that LNC00476 induced the degradation of SETDB1 protein via increasing its ubiquitination.

LINC00476 suppresses NSCLC cell proliferation, invasion, and migration via the SETDB1/Wnt/β-catenin axis

A549 and H460 cells were transfected with LINC00476 overexpression plasmid (pcDNA-LINC00476), or co-transfected with SETDB1 overexpression lentivirus (Lenti-SETDB1) for 48 h. The overexpression of LINC00476 significantly inhibited the cell proliferation, while such response was negated by the simultaneous overexpression of LINC00476 and SETDB1 (**Fig. 4A**). Similarly, the co-transfection restored the inhibition of cell invasion and migration which were caused by LINC00746 overexpression (**Fig. 4B** & **4C**). These data indicated that LINC00476 suppressed the cell proliferation, invasion, and migration of NSCLC cells via modulating SETDB1. In addition, the co-transfection restored the reduction of SETDB1, Wnt1, and β-catenin protein levels which were downregulated by LINC00476 overexpression (**Fig. 4D**). Taken together, our findings indicated that LINC00476 suppressed NSCLC cell proliferation, invasion, and migration via the SETDB1/Wnt/β-catenin axis.

Discussion

The critical regulatory functions of lncRNAs have been elucidated recently, especially in cancers [19]. In the current study, a novel functional lncRNA, LINC00476, was identified in NSCLC, and it acted as a tumor suppressor via inducing the ubiquitination and degradation of the oncogenic protein SETDB1. Our findings provide a new insight into the mechanism of LINC00476/SETDB1 axis in NSCLC, suggesting that LINC00476 may be a potential therapeutic target of NSCLC.

LncRNAs have been shown to be implicated in multiple biological functions of cells, including cell cycle progression, cell differentiation, gene expression regulation, and chromatin modification [20]. In NSCLC, these functions of lncRNAs make them to be valuable in cancer diagnosis, treatment, and predicting and improving prognosis. A study conducted by Weber et al showed that the expression of serum lncRNA-MALAT1 was dysregulated in 45 NSCLC patients in comparison with healthy controls, indicating lncRNA-MALAT1 as a promising diagnostic biomarker due to its high stability, specificity, and minimal invasiveness [21]. LncRNA-HOTAIR was demonstrated to contribute to cisplatin resistance in lung adenocarcinoma cells via the downregulation of p21 expression [22], thus the knockdown of lncRNA-HOTAIR by RNA interference technologies has been shown to reduce viability and invasiveness of lung

cancer [23]. The elevation of lncRNA-H19 predicted a poor prognosis of NSCLC patients and was an independent prognostic factor for overall survival [24]. Inspired by these studies, we searched the GEO database for the potential dysregulated lncRNAs in NSCLC samples, and we found that LINC00476 was significantly downregulated in NSCLC tumor tissues in comparison with the paired normal tissues. Consistent with the dataset, the downregulated LINC00476 was also determined in clinical tumor samples compared with the adjacent tissues. In addition, the low expression of LINC00476 was relative to the high clinical stage and lymph node metastasis, indicating LINC00476 may be a protective factor against NSCLC.

LINC00476 is a newly found lncRNA whose functions have not been fully understood. LINC00476 is located in chromosome 9 at q22.32, and is expressed in many types of tissues, including in the lungs. In our study, the expression of LINC00476 was relatively lower in NSCLC tumor tissues than that in the paired adjacent tissues. Similarly, the expression of LINC00476 was relatively lower in five NSCLC cell lines (SPC-A-1, A549, H1299, H460, and H358) than that in the bronchial epithelioid cell line (16HBE). Moreover, the downregulation of LINC00476 in A549 and H460 cell lines seemed more obvious than in SPC-A-1, H1299, and H358 cell lines ($p < 0.01$ vs $p < 0.05$). Since these cell lines were originated from different types of NSCLC, such as adenocarcinoma and large cell carcinoma, whether the expression of LINC00476 is associated with lung cancer cell types deserves further investigations.

The regulatory functions of lncRNAs can be exerted by several ways. The lncRNA-protein interaction plays an important role in the posttranslational modification of proteins via affecting the protein stability [25]. For instance, lncRNA-XLOC_006390 was reported to promote pancreatic carcinogenesis via blocking c-Myc ubiquitination and stabilizing c-Myc protein [26]. In the current study, the binding between LINC00476 and SETDB1 was confirmed using RNA pull-down and RIP assays. The unchanged mRNA level while the negatively changed protein level of SETDB1 by overexpressing or knocking down LINC00476 suggested that the SETDB1 expression may be regulated by LINC00476 at posttranslational levels. The further CHX experiment and ubiquitination assay indicated that the LINC00476-SETDB1 interaction was modulated by LINC00476-induced ubiquitination and degradation of SETDB1. Our findings provide more evidence in lncRNA-protein interactions in NSCLC.

In conclusion, this study indicated that LINC00476 acted as a tumor suppressor in NSCLC. The overexpression of LINC00476 may be a potential therapeutic strategy in treating NSCLC.

Declarations

Ethics approval and consent to participate

This study was approved by the Institute Research Medical Ethics Committee of The First Affiliated Hospital of Zhengzhou University. Written informed consents were obtained from all the patients involved.

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None.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare that they have no competing interests.

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Tables

Table 1. Downregulated and upregulated lncRNAs in tumor tissues of NSCLC patients

Gene symbol	ID	Adjust P value	P value	logFC
Downregulated lncRNAs				
LOC101930541	1561211_at	0.002532	9.99e-07	-2.6812646
PCAT19	230132_at	0.002997	2.14e-06	-2.2812843
LOC101927699	244308_at	0.005738	1.27e-05	-2.5506702
LINC00312	220244_at	0.007418	2.63e-05	-2.3581367
LOC101060604	1555216_a_at	0.009194	4.21e-05	-1.7056772
LOC101928612	232227_at	0.009813	4.91e-05	-2.0472014
LOC101928551	221337_s_at	0.01742	1.52e-04	-1.8459938
LOC101927069	1560169_at	0.017712	1.59e-04	-1.9597048
LINC00312	240306_at	0.018904	1.83e-04	-2.7696985
LINC01359	1562103_at	0.020012	2.07e-04	-1.9505817
LINC00845	238298_at	0.025347	3.44e-04	-2.6691229
CARMN	1558828_s_at	0.026325	3.71e-04	-1.775856
LINC00476	**239799_at**	**0.027395**	**4.06e-04**	**-1.5113358**
LOC101929398	1560826_at	0.02797	4.33e-04	-2.997071
PTCSC1	233111_at	0.030061	4.88e-04	-1.1507019
Upregulated lncRNAs				
BLACAT1	232105_at	0.003996	5.85e-06	2.224308
LINC01123/LINC01106	242222_at	0.009194	3.97e-05	2.2867043
LOC105369635	240284_x_at	0.025186	3.34e-04	1.2656658
LOC100505938	230641_at	0.030278	5.06e-04	1.5238666

NSCLC, non-small cell lung cancer. FC, fold change.

Table 2. The association of LINC00476 expression with clinicopathological features of NSCLC patients

Clinicopathological features	Sample (n=50)	LINC00476 expression		P value (High vs Low)
		Low (n=28)	High (n=22)	
Gender				0.826
Male	23	13	10	
Female	27	15	12	
Age (years)				0.613
<65	29	16	13	
≥65	21	12	9	
Smoking history				0.545
Yes	26	14	12	
No	24	14	10	
Histological subtype				0.437
Squamous cell carcinoma	22	15	7	
Adenocarcinoma	28	13	15	
Tumor size (cm)				0.329
≤5	24	15	9	
>5	26	11	15	
TNM stage				**0.003**
I-II	24	6	18	
III-IV	26	22	4	
Lymph node metastasis				**0.001**
Yes	30	21	9	
No	20	7	13	

NSCLC, non-small cell lung cancer.

Figures

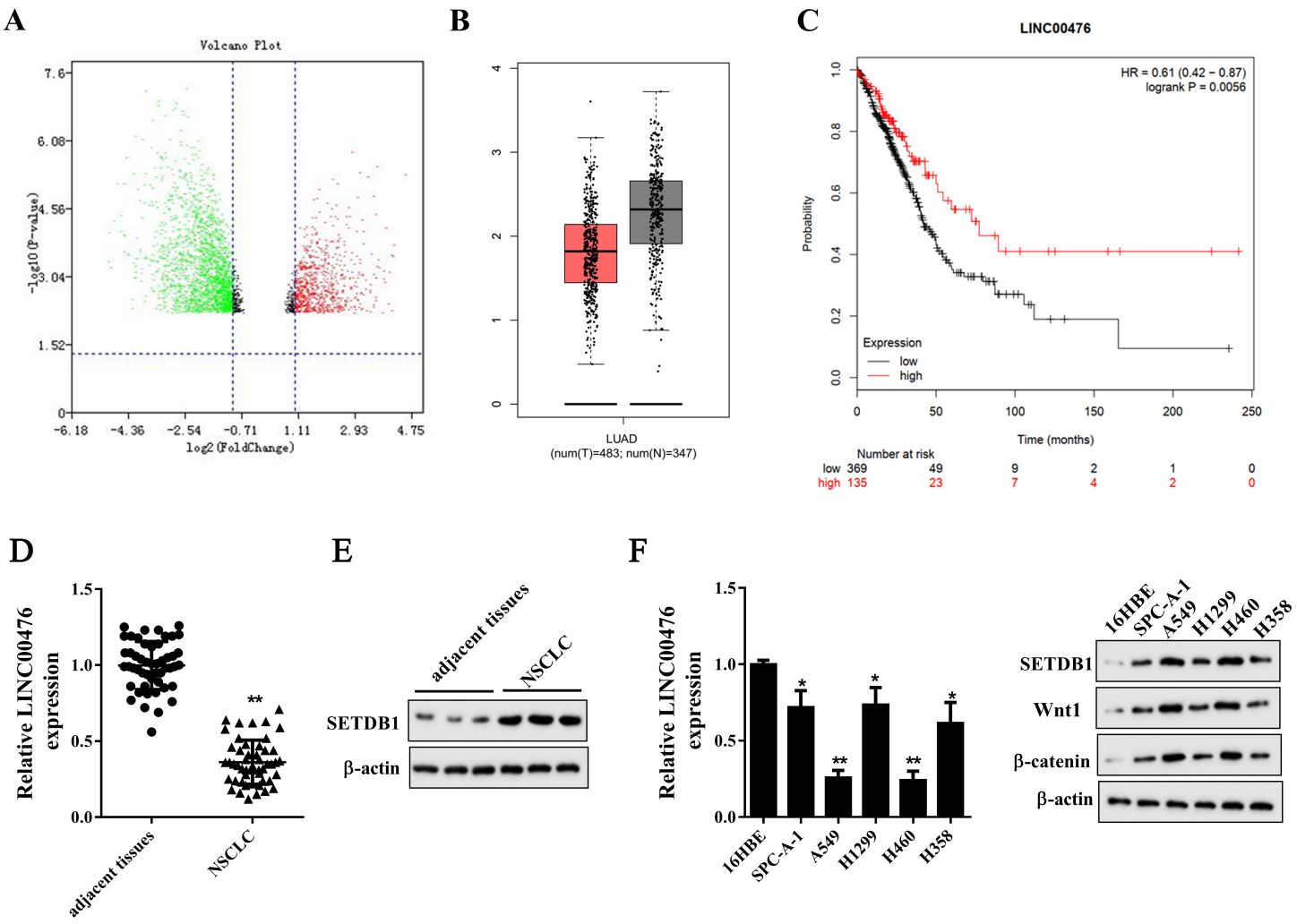

Figure 1

LINC00476 is lowly expressed in NSCLC tumor tissues and cell lines A. The volcano plot of dysregulated lncRNAs and mRNAs in NSCLC samples (GSE118370 dataset) by searching the GEO database. B. The logFC of LINC00476 was analyzed by using GSE118370 dataset. C. The overall survival in NSCLC patients with different levels of LINC00476 expression. D. The relative expression of LINC00476 in NSCLC tumor tissues and adjacent tissues was determined using qRT-PCR. E. The protein expression of SETDB1 in NSCLC tumor tissues and adjacent tissues was determined using western blot analysis. F. The relative expression of LINC00476 and the protein expressions of SETDB1, Wnt1, and β-catenin in normal bronchial epithelioid cell line (16HBE) and in NSCLC cell lines (SPC-A-1, A549, H1299, H460, and H358) were determined using qRT-PCR and western blot analysis, respectively. *p < 0.05, **p < 0.01 vs adjacent tissues or 16HBE.

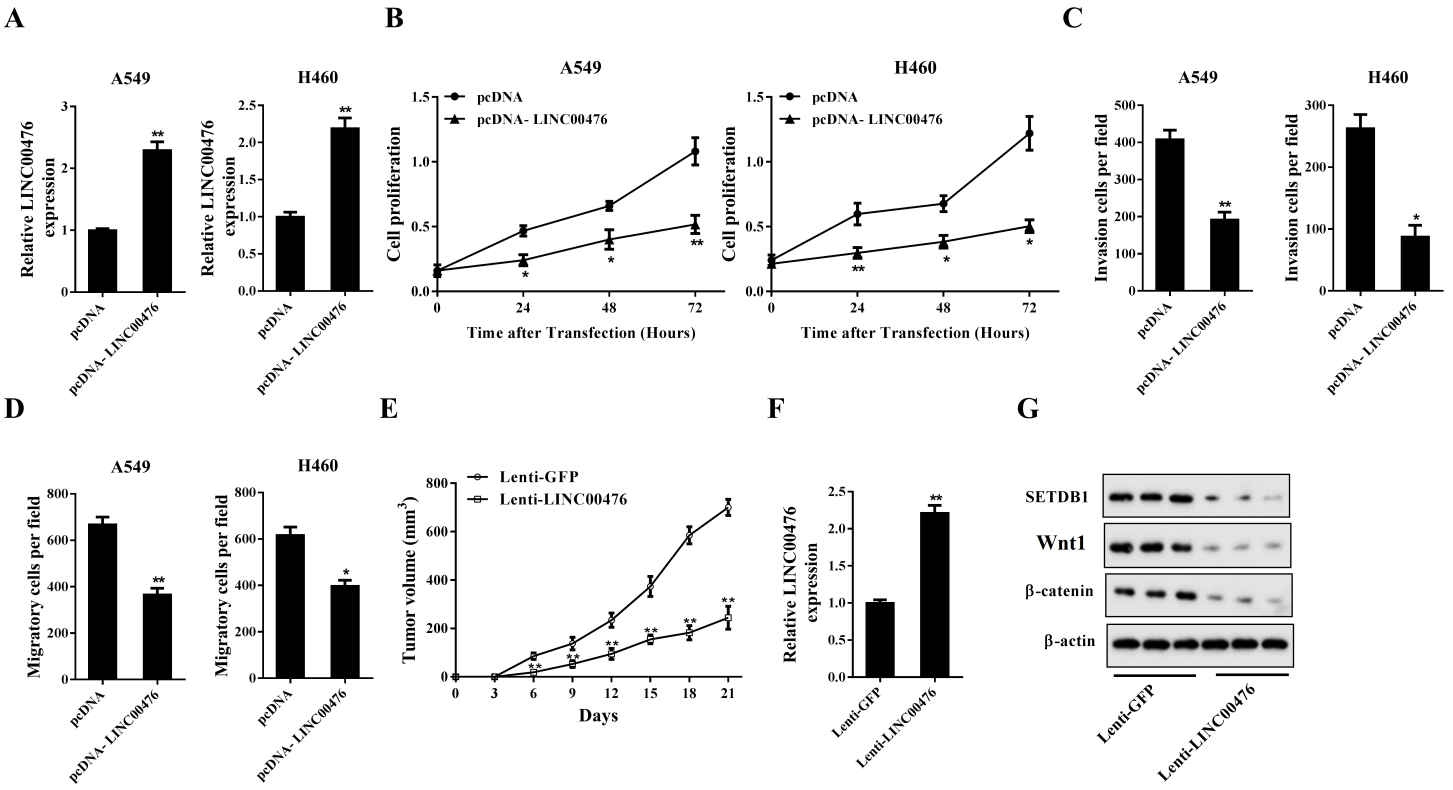

Figure 2

Overexpression of LINC00476 inhibits NSCLC cell proliferation, migration, and invasion in vitro and suppresses NSCLC tumor growth in vivo NSCLC cell lines A549 and H460 cells were transfected with LINC00476 overexpression plasmid (pcDNA-LINC00476) or the control plasmid (pcDNA) for 48 h. The relative expression of LINC00476 (A), the cell proliferation (B), the cell invasion (C) and the cell migration (D) were determined using qRT-PCR, cell counting kit-8 (CCK-8) assay, and Transwell assay. A549 cells were transfected with LINC00476 overexpression lentivirus (Lenti-LINC00476) or the control lentivirus (Lenti-GFP) for 48 h. BALB/c nude mice were injected with the transfected A549 cells which were mixed with FBS/Matrigel (1:1) solution in the posterior flank (n=5 in each group). E. The tumor volumes were measured every 3 days using vernier caliper (tumor volume= length × width2 × 0.5). The relative expression of LINC00476 (F) and the protein expressions of SETDB1, Wnt1, and β-catenin (G) were determined in tumor tissues using qRT-PCR and western blot analysis. *p < 0.05, **p < 0.01 vs pcDNA or Lenti-GFP.

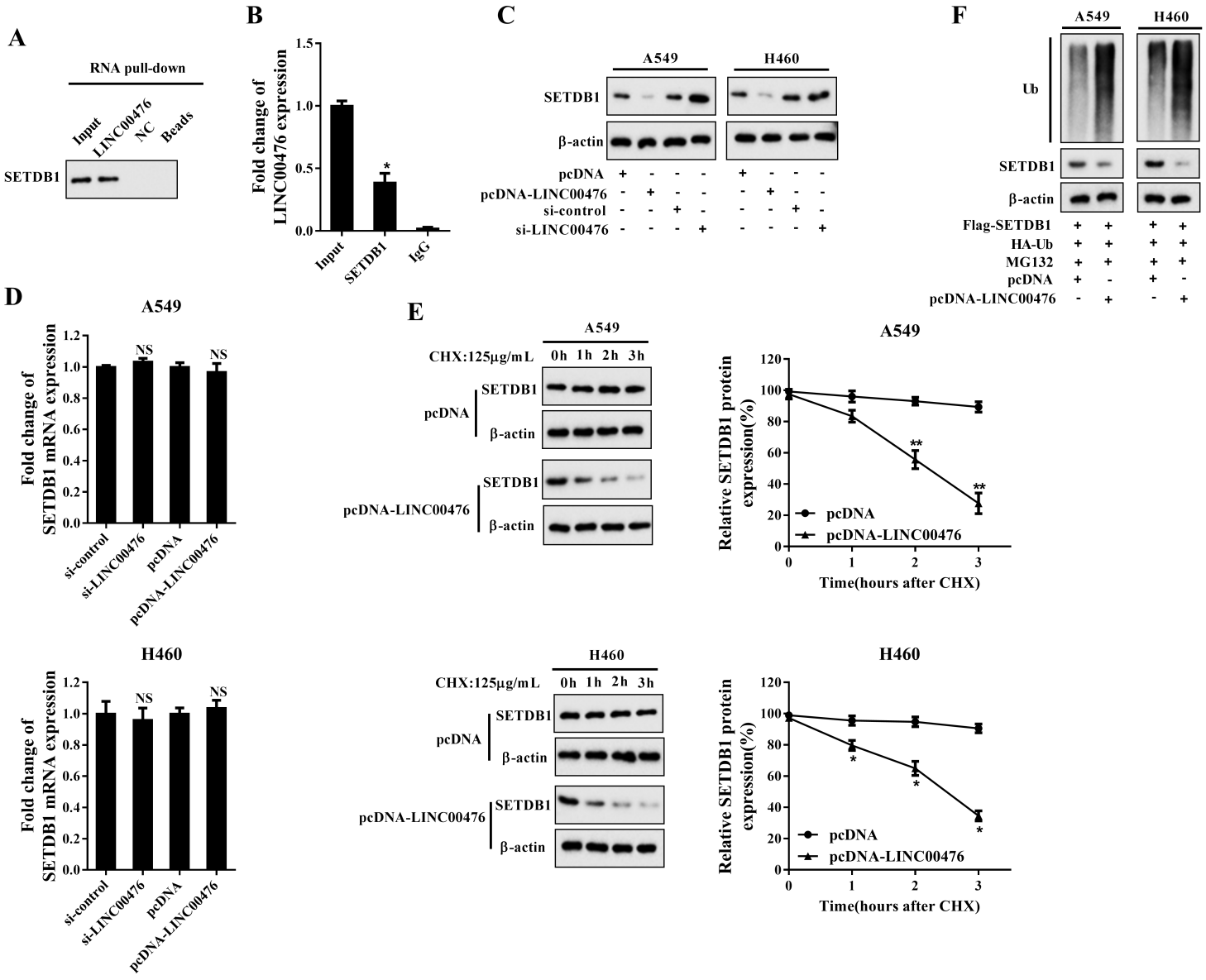

Figure 3

LINC00476 induces the degradation of SETDB1 via promoting its ubiquitination A. RNA pull-down assay showed that SETDB1 was expressed in the LINC00476 pull-down compound. B. RNA immunoprecipitation (RIP) assay showed that the fold change of LINC00476 was increased in SETDB1-immunoprecipitated complex compared with the IgG-immunoprecipitated complex. A549 and H460 cells were transfected with the LINC00476 overexpression plasmid (pcDNA-LINC00476) or the control plasmid (pcDNA), or transfected with the LINC00476 small interfering RNA (si-LINC00476) or the control siRNA (si-control) for 48 h. The protein expression (C) and the mRNA expression (D) of SETDB1 were determined using western blot analysis and qRT-PCR, respectively. E. The protein level of SETDB1 was determined at 1, 2, and 3 h after the treatment of the protein synthesis inhibitor CHX (125 μg/mL). F. A549 and H460 cells were transfected with HA-Ub, Flag-SETDB1, and pcDNA/pcDNA-LINC00476 for 48 h. The proteasome inhibitor MG132 was added for 4 h before cell collection. Cell lysates were

immunoprecipitated with anti-SETDB1 and were immunoblotted with anti-HA. *p < 0.05, **p < 0.01 vs pcDNA.

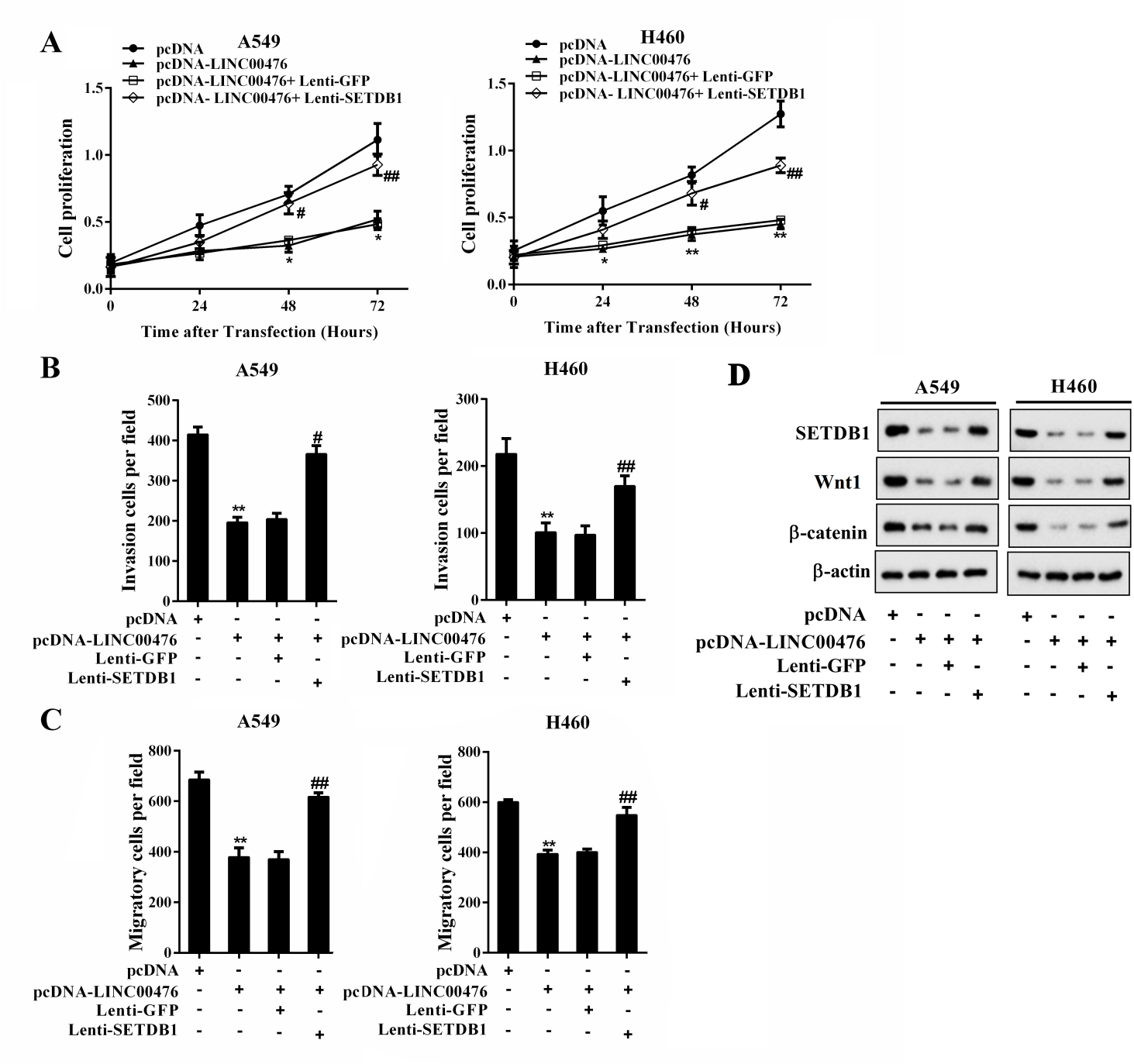

Figure 4

LINC00476 suppresses NSCLC cell proliferation, invasion, and migration via the SETDB1/Wnt/β-catenin axis A549 and H460 cells were transfected with LINC00476 overexpression plasmid (pcDNA-LINC00476), or co-transfected with SETDB1 overexpression lentivirus (Lenti-SETDB1) for 48 h. The cell proliferation (A), the cell invasion (B) and the cell migration (C) were determined using cell counting kit-8 (CCK-8) assay and Transwell assay. D. The protein expressions of SETDB1, Wnt1, and β-catenin were determined using western blot analysis.