Original Article

Upregulation of long noncoding RNA *RAB11B-AS1* promotes tumor metastasis and predicts poor prognosis in lung cancer

Tiegang Li[1,2#], Di Wu[1,3#], Qun Liu[1,3#], Dedong Wang[1#], Jinbin Chen[1], Hongjun Zhao[1], Lan Zhang[4], Chenli Xie[5], Wei Zhu[3], Zhixu Chen[6], Yifeng Zhou[7], Soham Datta[1], Fuman Qiu[1], Lei Yang[1], Jiachun Lu[1]

[1]The State Key Lab of Respiratory Disease, The First Affiliated Hospital, The Institute for Chemical Carcinogenesis, School of Public Health, Guangzhou Medical University, Guangzhou 511436, China; [2]Institute of Lung Disease, Guangzhou Chest Hospital, Guangzhou 510095, China; [3]Guangzhou Center for Disease Control and Prevention, Guangzhou 510440, China; [4]Department of Medical Genetics and Cell Biology, GMU-GIBH Joint School of Life Sciences, Guangzhou Medical University, Guangzhou 510440, China; [5]Fifth People's Hospital of Dongguan, Dongguan 523900, China; [6]Puning People's Hospital, Puning 515300, China; [7]Department of Genetics, Medical College of Soochow University, Suzhou 215006, China

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[#]These authors contributed equally to this work.

Correspondence to: Jiachun Lu, MD, PhD. The State Key Lab of Respiratory Disease, The First Affiliated Hospital, The Institute for Chemical Carcinogenesis, School of Public health, Guangzhou Medical University, Xinzao Town, Panyu District, Guangzhou 511436, China. Email: jclu@gzhmu.edu.cn.

Background: Lung cancer (LC) is one of the leading causes of cancer-related mortality in China and worldwide. Despite the progress in diagnosis and treatment of LC, the prognosis of LC remains poor. Studies have demonstrated that long non-coding RNAs (lncRNAs) play a critical role in carcinogenesis and cancer development.

Methods: Here we examined the expression and potential function of *lnc-RAB11B-AS1* in LC both *in vitro* and *in vivo*. All experiments in this study were conducted using A549 and PC-9 cell lines according to protocols described in this paper. The clinic characteristics were analyzed using logistic regression, cox model, log rank test, biochemical analysis using qRT-PCR, transfections, nude mice model, and cell biological analysis using Transwell assay, CCK-8 assay, flow cytometry, and rescue experiments, and immunohistochemistry.

Results: The results showed that *lnc-RAB11B-AS1* was significantly overexpressed in LC tissues compared to the corresponding non-tumor tissues. Patients with a higher level of *lnc-RAB11B-AS1* expression showed a poorer overall survival rate. Functionally, overexpression of *lnc-RAB11B-AS1* promotes cell proliferation, migration and invasion abilities of LC cell lines, which suggests *lnc-RAB11B-AS1* may play an oncogenic role in LC. *lnc-RAB11B-AS1* was located in physical contiguity with *RAB11B* gene and found positively regulates the *RAB11B* expression, and the protein levels of RAB11B in LC tissues also found to positively correlated with the level of *lnc-RAB11B-AS1* expression. *RAB11B* silencing partially abrogated *lnc-RAB11B-AS1*-induced proliferation of the LC cell lines used in this study.

Conclusions: This study provided a novel evidence into the function of lncRNA-driven carcinogenesis. Our findings highlighted the importance of *lnc-RAB11B-AS1* and *RAB11B* in LC progression and indicated that *lnc-RAB11B-AS1* may serve as a novel and valuable prognostic biomarker for LC.

Keywords: lncRNA; lung cancer (LC); *lnc-RAB11B-AS1; RAB11B*; survival

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Introduction

Lung cancer (LC) is one of the most common malignant respiratory tumors and remains a serious health threat (1). The morbidity and mortality rates of LC rank first in the spectrum of cancers and have kept increasing in the past few decades in China (2-4). The 5-year overall survival rate of LC patients is 16.1% in China (5), while in rural areas is only 11.2% (6). The main reason for the poor survival rate of LC patients is that most LC patients were diagnosed at an advanced stage, and a large number of the LC patients were sustained malignant proliferation and extensive lymphatic metastasis. Although studies have identified tons of genes that are involved in LC tumorigenesis and tumor metastasis, the molecular mechanisms underlying the tumorigenesis and metastasis are not yet well understood. Therefore, a detailed understanding of the relevant mechanisms and molecular pathways of activated lncRNA in LC is crucial to discovery of new anti-cancer therapeutic targets.

Long non-coding RNAs (lncRNAs) are a sub-class of non-coding RNAs over 200 nucleotides in length and lack of protein coding capacity. LncRNAs played crucial roles in multiple biological processes through regulating genes expression of proteins involved in various processes, such as carcinogenesis, cell proliferation, migration and invasion (7). LncRNAs are divided into five types: (I) bidirectional; (II) intergenic; (III) intronic; (IV) sense; and (V) anti-sense (anti-sense lncRNAs, as-lncRNAs) (8). Previous studies have demonstrated that as-lncRNAs are functional because the special location with its natural antis-sense transcripts. In addition, recent studies have demonstrated a link between as-lncRNAs and cancer progression. For example, *ZEB1-AS1* (7) was reported to promote tumor metastasis and its overexpression predicted poor prognosis in hepatocellular carcinoma (HCC). *KRT7-AS* (9) was shown to promote cancer cell progression in gastric cancer (GC), and another study showed that *HNF1A-AS1* (10) functions as a competing endogenous RNA in colon cancer. These reports have demonstrated the involvement of as-lncRNAs in different cancers and their potential as biomarkers for the early detection, diagnosis and treatment of cancer. Recent studies showed that upstream anti-sense transcripts of as-lncRNAs played a critical rule in transcriptional regulation of corresponding gene expression (11). Sequence analysis showed that most as-lncRNAs originate from the promoters of the corresponding mRNAs in a head-to-head conformation. Thus, there seems to be an obvious potential to investigate these as-lncRNAs as an approach to study the well-known tumor-suppressors or oncogenes with a natural anti-sense transcript.

The RAS superfamily was first reported as oncogenes in mice by Jenifer Harvey in 1960s (12) and to date, over 150 genes of the RAS super-family have been identified. The RAS superfamily proteins are divided into five sub-classes: Ras, Rho, Ran, Arf and Rab (13). Approximately 60 of Rab proteins have been identified in the human genome (14). We previously found that *RAB11B* upregulated in osteosarcoma and negatively correlated with the expression level of the corresponding natural anti-sense transcript *lnc-RAB11B-AS1* (15). We found that *lnc-RAB11B-AS1* functioned as a tumor suppressor in osteosarcoma. However, another study conducted by Feng *et al.* (16) found that *lnc-RAB11B-AS1* was upregulated in GC and the overexpression was correlated with clinical stage, metastasis and overall survival of the GC patients. A recent research reported that upregulation of *lnc-RAB11B-AS1* could enhance the ability of cell migration and invasion in breast cancer cell lines both *in vitro* and *in vivo via* hypoxia-inducible factor 2 (HIF-2) (17) *Lnc-RAB11B-AS1* is a 1022-bp transcript with 3 exons and located on human chromosome 19q13.2 (chr19: 8,439,260-8,455,575, *hg19*) on the reverse strand, a region that has been associated with high risk for several cancers, such as ovarian carcinoma (18), prostate cancer (19), pancreatic cancer (20), neuroblastoma (21) and chronic obstructive pulmonary disease (22,23). Although these studies have indicated a link between *lnc-RAB11B-AS1* and cancer, the biological functions of *lnc-RAB11B-AS1* in LC remained to be clarified. Furthermore, recent studies showed that the expression level of an mRNA correlated with the level of the corresponding anti-sense transcript (11). We therefore speculated whether *lnc-RAB11B-AS1* regulates *RAB11B* expression, promotes LC progress and worsening LC prognosis.

In this study, we investigated the expression pattern and clinical significance of *lnc-RAB11B-AS1* in LC patients and examined the functions of *lnc-RAB11B-AS1* in LC cell lines. We also examined the potential function of *lnc-RAB11B-AS1* in regulating *RAB11B* expression in LC.

Methods

Study subjects

All the LC patients involved in the present study were

Han Chinese people from Southern and Eastern China. A total of 276 paired samples of LC tissues and paired normal tissues were used in the present study, 182 of which were collected from the Affiliated Hospitals of Guangzhou Medical University, the First Affiliated Hospital affiliated with Kunming University and Cancer Hospital affiliated with Kunming University between 2008 and 2015, and the rest of the samples were collected from the First Affiliate Hospital of Soochow University between 2007 and 2016. The LC patients in the study had no genetic connections with one another. The present study was approved by the Ethics Committee of Guangzhou Medical University (No. GMU201481473040) and we strictly followed the related clinical research guidelines. All study participants involved in the present study were provided written informed consent.

Cell culture

Human lung adenocarcinoma cell lines A549 and PC-9 and human embryonic kidney cell line 293 (HEK-293) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai Institute of Cell Biology, China). A549 and PC-9 cell lines were cultured in RPMI-1640 medium (Gibco, Life Technologies, USA) and HEK-293 cell line was cultured in DMEM medium (Gibco). All cell lines were grown in 10% (volume ratio) fetal bovine serum (FBS)-containing culture medium and all cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37 ℃.

qRT-PCR analysis

Total RNA was extracted from LC tissues and cell line samples using Trizol Reagent (Life Technologies) according to the manufacturer's instructions. RIN (RNA integrity number) was determined to detect RNA integrity using an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). *Lnc-RAB11B-AS1* and *RAB11B* expression levels were detected in all tissue cDNA (complementary DNA) samples using quantitative real-time polymerase chain reaction (qRT-PCR). *ACTB* expression level was used as an internal control of the qRT-PCR assay. All primers used are listed in *Table S1*. All qRT-PCR analyses were performed using ABI 7900HT system (Applied Biosystems, CA, USA), and the master mix of the qRT-PCR assay was also purchased from Applied Biosystems (ABI Power SYBR Green PCR Master Mix). The relative expression levels of the genes involved in this study were calculated using the comparative threshold cycle (Ct) ($2^{-\Delta\Delta Ct}$) method.

Subcellular fractionation

Nuclear and cytosolic fractions used in this study were extracted from LC cell lines using a nuclear/cytoplasmic isolation kit (Biovision, San Francisco, CA, USA) according to the manufacturer's protocol.

Plasmids, cell transfection and stable cell lines

The full-length complimentary DNA of human *lnc-RAB11B-AS1* and small hairpin RNA (shRNA) targeting *lnc-RAB11B-AS1* were both synthesized by GeneCopoeia (MD, USA). The sequence of the full-length *lnc-RAB11B-AS1* complimentary DNA was cloned into lentivirus expression vector pEZ-Lv206, and the shRNA targeting *lnc-RAB11B-AS1* was cloned in to the lentivirus expression vector psi-LVRH1MP. Both vectors contained a gene encoding red fluorescence protein, which could be detected by an inverted fluorescence microscope to determine transfection efficiency. The resulting constructs were verified by sequencing. Stably transfected LC cell lines were established according to the protocol of GeneCopoeia, and the transfection reagents (Lenti-Pac HIV Expression Packaging Kit) were also purchased from GeneCopoeia. All stable cell lines were verified by both fluorescence and qRT-PCR.

Cell proliferation assay

The A549 and PC-9 cell lines with stably overexpressed and down-expressed *lnc-RAB11B-AS1* were seeded in 96-well plates (200 cells per well), and the proliferation of the seeded cells were examined using the Cell Counting Kit-8 (CCK-8) (Engreen Biosystem Co. Ltd., China) according to the manufacturer's protocol. The absorbance of the cell lines was measured at 450 nm at a series of time points (0, 24, 48, 72 and 96 hours after the cells were seeded).

Flow cytometry assays

For cell cytometry assays, A549 and PC-9 cell lines with stably overexpressed and down-expressed *lnc-RAB11B-AS1* were labeled with propidium iodide (PI) (7seaharmtech, Shanghai, China) and analyzed using flow cytometry. For apoptosis assays, Annexin V-fluorescein isothiocyanate

(FITC)/PI (Multisciences, Hangzhou, China) staining was performed and the apoptosis of the cells were examined by flow cytometry according to the manufacturer's protocol.

Clonogenic assay

Stably transfected cell lines were plated into 6-well plates in triplicate at a density of 100 cells/plate and regularly cultured in routine conditions for 2 weeks. The colonies were stained with Giemsa (Beijing Solarbio Science & Technology Co. Ltd., China) and then counted (the colonies contain more than 50 cells) and imaged. The clonogenic assay was conducted in triplicate.

Cell migration and invasion assays

Transwell assays were conducted in an 8 μm Trans-well chamber (Costar, Corning Incorporated, NY, USA). A549 and PC-9 cell lines with stably overexpressed and down-expressed *lnc-RAB11B-AS1* were trypsinized and re-suspended by FBS-free culture medium with 0.1% bovine serum albumin (BSA). Cells (2×10^4) were seeded into each chamber, which was placed into a 24-well plate containing culture medium with 20% FBS (Gibco). After 48 hours culture, non-migrated cells in the upper section of the chamber were removed lightly. Migrated cells number were manually counted in 10 random fields by a microscope, and the migrated cells numbers were averaged.

Cell invasion assays were conducted using chambers with Matrigel (Corning), and assays were performed as described for migration assays.

All experiments were conducted in triplicate.

Xenograft model in nude mice

BALB/c-nu nude mice (6-week-old) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Stably transfected A549 and PC-9 cell lines were re-suspended at a concentration of 1×10^7 cells/mL in phosphate buffer (PBS). A total of 54 mice were randomly divided into 9 groups (three male mice and three female mice in each group) as follows: A549 *lnc-RAB11B-AS1* overexpression & Control, A549 *lnc-RAB11B-AS1* silenced & Control, PC-9 *lnc-RAB11B-AS1* overexpression & Control, PC-9 *lnc-RAB11B-AS1* silenced & Control, and a group of mice were injected with PBS only as a control for the whole experiments (data of this group was not shown). Each mouse was subcutaneously injected in the back flank with 0.2 mL of the indicated cell suspension (a total of 2×10^6 cells). Tumor growth was monitored over 21 days, and we measured the tumor length and width and obtained imaging every 3 days. The volume of the tumor was calculated as follows: V_{tumor} = length × width × width ×0.5. This study was approved by the Ethics Committee of Guangzhou Medical University (No. GZYDW201503512). All animal experiments were conducted according to the relevant regulations.

Immunohistochemistry (IHC)

Immunohistochemistry assay was used to detect the RAB11B protein level in the cancer patients' samples, we selected 2 pairs of tissues with a high expression level of *lnc-RAB11B-AS1*, which were confirmed by qRT-PCR experiment. The experiment was conducted at a routine procedure. The tissue samples used was formalin-fixed, were embedded in paraffin and sliced. The sections were then incubated with monoclonal antibodies against RAB11B (1:500, ab228954, abcam, USA) overnight at 4 ℃, and then incubated with a secondary antibody (1:500, ab150117, abcam), the 3,3'-diaminobenzidine (DAB) was used to be the chromogen. Hematoxylin was used to be the dying material. Finally, the RAB11B protein level was evaluated.

Luciferase assays

pGL3-*RAB11B* vector and pGL3-Control vector were co-transfected with *lnc-RAB11B-AS1* overexpression and *lnc-RAB11B-AS1* downregulation using Lipofectamine 3000 (Invitrogen) into A549 and PC-9 cell lines. The relative luciferase activity of each group was normalized by Renilla luciferase activity 48 h after transfection.

Statistical analysis

Statistical analyses were performed using SPSS 19.0. The differences between groups was calculated by χ^2 test. One-way analysis of variance test was used to examine the influence of *lnc-RAB11B-AS1* expression on *RAB11B*. Overall survival rates of different groups were calculated by the Kaplan-Meier survival analysis and the log-rank test was applied for comparison. P values less than 0.05 were considered significant.

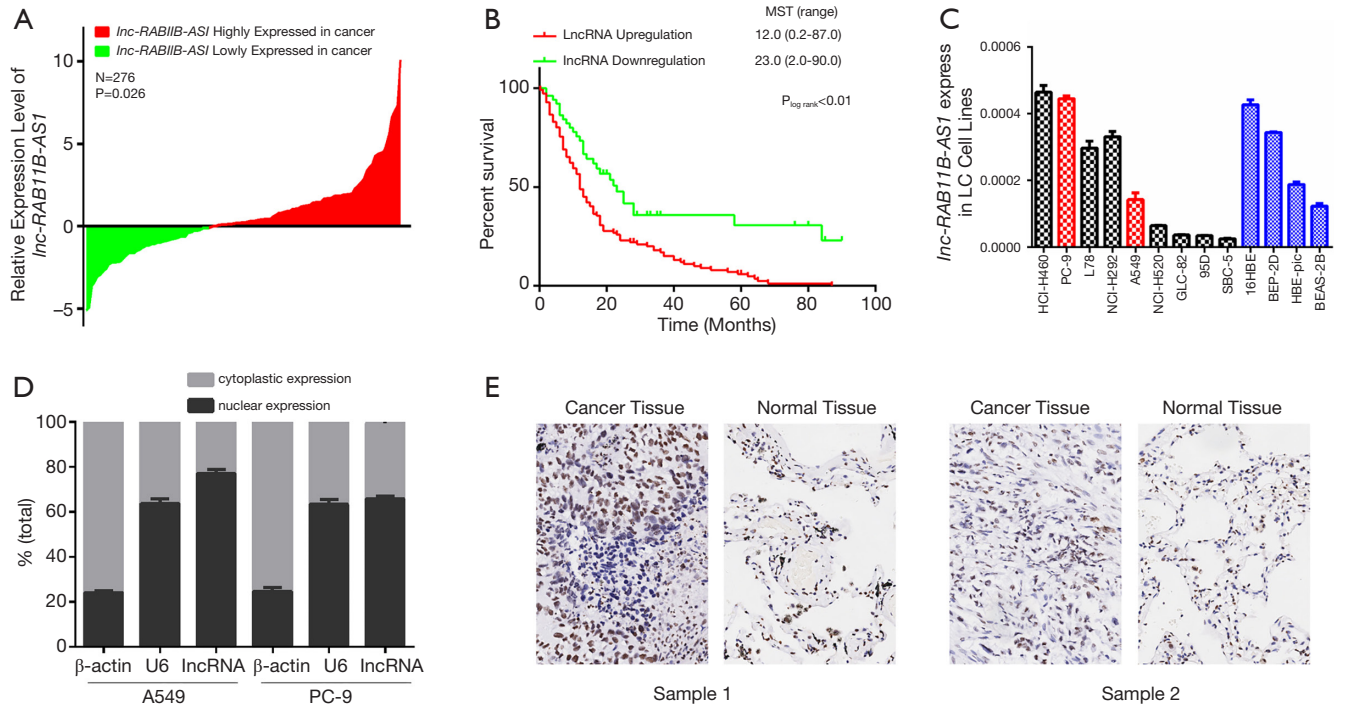


Figure 1 Expression pattern of lnc-RAB11B-AS1 and its association with lung cancer survival. (A) Expression pattern of lnc-RAB11B-AS1 in lung cancer tissues. The expression level of lnc-RAB11B-AS1 was significantly higher in lung cancerous tissues compared with adjacent normal lung tissues. Results are shown as Log (tumor/normal). P was calculated by the paired *t*-test. (B) Effect of lnc-RAB11B-AS1 expression on lung cancer survival. Patients with high expression of lnc-RAB11B-AS1 showed a significantly poorer survival than those with low expression of lnc-RAB11B-AS1. P was calculated by the log-rank test. (C) Relative expression of lnc-RAB11B-AS1 in nine lung cancer cell lines and three normal pulmonary epithelial cell lines. (D) Expression of lnc-RAB11B-AS1 in nuclear and cytoplasmic fractions of LC cells. (E) Expression of RAB11B protein in lung cancer tissues.

Results

Expression pattern of lnc-RAB11B-AS1 in LC

The clinical characteristics of the 276 patients with LC included in this study are shown in *Table S2* [this data was published in one of our previous studies which was a study based on the same study subjects (24)]. As shown in *Figure 1A*, *lnc-RAB11B-AS1* was significantly upregulated in cancerous tissues from the LC patients compared with corresponding normal tissues, with an average 1.47-fold increased expression (P=0.026).

Associations between lnc-RAB11B-AS1 expression and LC progression

We next categorized LC patients based on *lnc-RAB11B-AS1* expression using the medium level in LC tissues. Patients with expression levels equal to or greater than the cut-off value were categorized in the high *lnc-RAB11B-AS1* expression group, and those with expression levels lower than the cut-off value were defined as the low *lnc-RAB11B-AS1* expression group. The expression status of *lnc-RAB11B-AS1* was statistically different in patients at different clinic stages (P<0.001), with lymph node metastasis (N stage) (P<0.001) and with distant metastasis (M stage) (P<0.001) (*Table 1*). These results showed that the LC patients with a higher level of *lnc-RAB11B-AS1* expression had a higher risk for advanced clinic stage, N stage and M stage.

Associations between the lnc-RAB11B-AS1 expression and LC prognosis

We next analyzed the effect of *lnc-RAB11B-AS1* expression on LC survival. We obtained complete follow-up data from 163 patients (110, 60.4% in southern population, 53,

Table 1 Association between the expression level of *lnc-RAB11B-AS1* and the clinic characteristics of the patients

Clinic characteristics	Southern samples, N (%)		P[a]	Eastern samples, N (%)		P[a]	Merged samples, N (%)		P[a]
	Low	High		Low	High		Low	High	
Age									
<60	38 (36.2)	67 (63.8)	0.462	21 (41.2)	30 (58.8)	0.233	59 (37.8)	97 (62.2)	0.180
≥60	32 (41.6)	45 (58.4)		23 (53.5)	20 (46.5)		55 (45.8)	65 (54.2)	
Sex									
Female	23 (44.2)	29 (55.8)	0.312	15 (53.6)	13 (46.4)	0.392	38 (47.5)	42 (52.5)	0.182
Male	47 (36.2)	83 (63.8)		29 (43.9)	37 (56.1)		76 (38.8)	120 (61.2)	
Family history of cancer									
No	92 (38.5)	99 (61.5)	0.971	40 (47.6)	44 (52.4)	0.648	102 (41.6)	143 (58.4)	0.755
Yes	8 (38.1)	13 (61.9)		4 (40.0)	6 (60.0)		12 (38.7)	19 (61.3)	
Family history of lung cancer									
No	64 (37.9)	105 (62.1)	0.554	44 (48.4)	47 (51.6)	0.099	108 (41.5)	152 (58.5)	0.750
Yes	6 (46.2)	7 (53.8)		0 (0)	3 (100.0)		6 (37.5)	10 (62.5)	
Smoking									
No	32 (47.8)	35 (52.2)	0.049	15 (50.0)	15 (50.0)	0.671	47 (48.5)	50 (51.5)	0.076
Yes	38 (33.0)	77 (67.0)		29 (45.3)	35 (54.7)		67 (37.4)	112 (62.6)	
Drinking									
No	53 (38.7)	84 (61.3)	0.913	38 (52.1)	35 (47.9)	0.057	91 (43.3)	119 (56.7)	0.222
Yes	17 (37.8)	28 (62.2)		6 (28.6)	15 (71.4)		23 (34.8)	43 (65.2)	
Surgery									
No	46 (38.0)	75 (62.0)	0.862	28 (48.3)	30 (51.7)	0.717	74 (41.3)	105 (58.7)	0.987
Yes	24 (39.3)	37 (60.7)		16 (44.4)	20 (55.6)		40 (41.2)	57 (58.8)	
Chemotherapy									
No	21 (33.3)	42 (66.7)	0.301	10 (34.5)	19 (65.5)	0.110	31 (33.7)	61 (66.3)	0.069
Yes	49 (41.2)	70 (58.8)		34 (52.3)	31 (47.7)		83 (45.1)	101 (54.9)	
Radiotherapy									
No	43 (41.3)	61 (58.7)	0.356	18 (37.5)	30 (62.5)	0.065	61 (40.1)	91 (59.9)	0.661
Yes	27 (34.6)	51 (65.4)		26 (56.5)	20 (43.5)		53 (42.7)	71 (57.3)	
Clinical stages									
I + II	44 (62.0)	27 (38.0)	<0.001	20 (69.0)	9 (31.0)	0.004	64 (64.0)	36 (36.0)	<0.001
III + IV	26 (23.4)	85 (61.5)		24 (36.9)	41 (63.1)		60 (34.1)	116 (65.9)	
T									
1+2	43 (41.4)	61 (58.6)	0.356	23 (51.1)	22 (48.9)	0.423	66 (44.3)	83 (55.7)	0.274
3+4	27 (34.6)	51 (65.4)		21 (42.9)	28 (57.1)		48 (37.8)	79 (62.2)	

Table 1 (*continued*)

Table 1 (*continued*)

Clinic characteristics	Southern samples, N (%)		P[a]	Eastern samples, N (%)		P[a]	Merged samples, N (%)		P[a]
	Low	High		Low	High		Low	High	
N									
0	34 (51.5)	32 (48.5)	0.006	27 (62.8)	16 (37.2)	0.004	61 (56.0)	48 (44.0)	<0.001
1+2+3	36 (31.0)	80 (69.0)		17 (33.3)	34 (66.7)		53 (31.7)	114 (68.3)	
M									
0	62 (48.1)	67 (51.9)	<0.001	33 (55.0)	27 (45.0)	0.034	95 (50.3)	94 (49.7)	<0.001
1	8 (15.1)	45 (84.9)		11 (32.4)	23 (67.6)		19 (21.8)	68 (78.2)	
Histological types									
Adenocarcinoma	37 (43.0)	49 (57.0)	0.325	20 (48.8)	21 (51.2)	0.471	57 (44.9)	70 (55.1)	0.747
Squamous cell carcinoma	21 (41.2)	30 (58.8)		12 (38.7)	19 (61.3)		33 (40.2)	49 (59.8)	
Large cell carcinoma	1 (16.7)	5 (83.3)		2 (40.0)	3 (60.0)		3 (27.3)	8 (72.7)	
Small cell lung cancer	4 (21.1)	15 (78.9)		6 (75.0)	2 (25.0)		10 (37.0)	17 (63.0)	
Other carcinomas[b]	7 (35.0)	13 (65.0)		4 (44.4)	5 (55.6)		11 (37.9)	18 (62.1)	

[a], Pearson χ^2 test P value; [b], mixed-cell or undifferentiated carcinoma.

56.4% in eastern population) [listed in *Table S3*, and this data was published in one of our previous studies which was a study based on the same study subjects (24)]. We conducted Kaplan-Meier survival analysis and log-rank test, and found that the LC patients with a higher level of *lnc-RAB11B-AS1* expression experienced a significantly shorter medium survival time than the ones with a lower *lnc-RAB11B-AS1* expression level (12.0 *vs.* 23.0 months, $P_{log\text{-}rank}$ <0.01; *Figure 1B*).

We conducted a COX regression for multivariate analysis and found that the chemotherapy (HR =0.67; 95% CI, 0.45–0.99, P=0.048) and high *lnc-RAB11B-AS1* expression (HR =1.76; 95% CI, 1.09–2.85, P=0.020) were statistically associated with the overall survival of the patients (*Table 2*). We further analyzed the merged samples in a stratification way; the results are shown in *Table 3*.

Expression levels of lnc-RAB11B-AS1 in LC cell lines

We next examined *lnc-RAB11B-AS1* expression patterns in 9 LC cell lines and 4 normal lung epithelial cell lines and found varying levels of expression (*Figure 1C*). PC-9 and A549 cells showed relatively high *lnc-RAB11B-AS1* expression and thus we selected these cell lines for further evaluation. In both cell lines. *lnc-RAB11B-AS1* was mostly localized in the nucleus (*Figure 1D*), which supports the possibility of its role as a transcriptional regulator in LC development.

lnc-RAB11B-AS1 overexpression promotes proliferation ability and decreases apoptosis of LC cells in vitro

We next conducted CCK-8 assays to assess the influence of *lnc-RAB11B-AS1* on LC cell proliferation. We generated PC-9 and A549 cell lines stably expressing a *lnc-RAB11B-AS1* overexpression construct or shRNA targeting *lnc-RAB-AS1* as described in Methods. CCK-8 results showed that *lnc-RAB11B-AS1* upregulation significantly increased cell proliferation rates *in vitro*, while *lnc-RAB11B-AS1* silencing resulted in reduced cell proliferation rates in both cell lines (P<0.05; *Figure 2A*). We further examined the effect of *lnc-RAB11B-AS1* expression on the cell cycle and found that overexpression of *lnc-RAB11B-AS1* resulted in fewer cells in G1 and more cells in M phase, while cells with stably down-expressed *lnc-RAB11B-AS1* showed increased cells in G1 phase and fewer cells in M phase (P<0.05; *Figure 2B*). Similar results were found in both PC-9 and A549 cell lines. In addition, overexpression of *lnc-RAB11B-AS1* caused an increase in apoptosis, while stably down-expressed *lnc-RAB11B-AS1* led to a decrease in apoptosis in both PC-9

Table 2 COX model of the significant variables

Variables	HR (95% CI)	Cox model P value
Age	1.43 (1.00–2.04)	0.053
Sex	1.28 (0.80–2.04)	0.311
Smoking	1.58 (1.00–2.50)	0.051
surgery	0.71 (0.47–1.08)	0.108
Chemotherapy	0.67 (0.45–0.99)	0.048
Radiotherapy	0.70 (0.49–1.02)	0.060
Stage	1.17 (0.95–1.44)	0.136
lnc-RAB11B-AS1 over-expression	1.76 (1.09–2.85)	0.020

Table 3 Association between the expression level of *lnc-RAB11B-AS1* and the prognosis of the lung cancer patients

Variables	*lnc-RAB11B-AS1* lowly expressed		*lnc-RAB11B-AS1* highly expressed		Adjusted HR (95% CI)[a]	P_{homo}[b]	P_{inter}[c]
	Case N	Death, N (%)	Case, N	Death, N (%)			
Age							
<60	28	13 (46.4)	70	64 (91.4)	2.58 (1.42–4.71)	0.361	0.362
≥60	23	16 (69.6)	42	40 (95.2)	1.74 (0.96–3.16)		
Sex							
Female	25	16 (64.0)	29	26 (89.7)	2.02 (1.12–3.65)	0.742	0.712
Male	26	13 (50.0)	83	78 (94.0)	2.34 (1.23–4.46)		
Family history of cancer							
No	45	24 (53.3)	100	93 (93.0)	2.40 (1.52–3.78)	0.227	0.112
Yes	6	5 (83.3)	12	11 (91.7)	1.15 (0.38–3.46)		
Family history of lung cancer							
No	49	27 (55.1)	108	100 (92.3)	2.20 (1.43–3.38)	0.417	0.760
Yes	2	2 (100.0)	4	4 (100.0)	65.32 (0.02-251377)		
Smoking							
No	30	17 (56.7)	34	30 (88.2)	2.45 (1.32–4.54)	0.373	0.381
Yes	21	12 (59.8)	78	74 (94.9)	1.63 (0.82–3.01)		
Drinking							
No	43	28 (65.1)	80	73 (91.3)	1.80 (1.16–2.80)	0.092	0.083
Yes	8	1 (12.5)	32	31 (96.9)	10.44 (1.42–76.80)		
Stage							
I + II	32	18 (56.3)	23	23 (100.0)	1.91 (0.86–4.27)	0.805	0.776
III + IV	19	11 (57.9)	89	81 (91.0)	2.15 (1.31–3.53)		

Table 3 (*continued*)

Table 3 (*continued*)

Variables	*lnc-RAB11B-AS1* lowly expressed		*lnc-RAB11B-AS1* highly expressed		Adjusted HR (95% CI)[a]	P_{homo}[b]	P_{inter}[c]
	Case N	Death, N (%)	Case, N	Death, N (%)			
Surgery							
No	35	21 (60.0)	75	70 (93.3)	1.88 (1.15–3.08)	0.327	0.416
Yes	16	8 (50.0)	37	34 (91.9)	3.05 (1.33–7.02)		
Chemotherapy							
No	12	10 (83.3)	40	36 (90.0)	1.20 (0.59–2.47)	0.094	0.100
Yes	39	19 (48.7)	72	68 (94.4)	2.55 (1.52–4.27)		
Radiotherapy							
No	28	18 (64.3)	64	62 (96.9)	2.10 (1.23–3.57)	0.797	0.826
Yes	23	11 (47.8)	48	42 (87.5)	2.35 (1.20–4.61)		
Histological types							
Adenocarcinoma	26	14 (53.8)	50	46 (92.0)	1.98 (1.08–3.62)	0.967	0.411
Squamous cell carcinoma	16	10 (62.5)	34	33 (97.1)	2.20 (1.08–4.50)		
Large cell carcinoma	1	1 (100.0)	4	4 (100.0)	–		
Small cell lung cancer	4	2 (50.0)	11	9 (81.2)	2.80 (0.59–13.31)		
Other carcinomas[d]	4	2 (50.0)	13	12 (92.3)	2.66 (0.58–12.15)		

[a], HRs were adjusted for age, sex, stage and metastasis in a Cox regression model; [b], P value of homogeneity test between strata for the related HRs of the lowly expressed and highly expressed *lnc-RAB11B-AS1*; [c], P value of test for the multiplicative interaction between the expression level of *lnc-RAB11B-AS1* and selected variables on cancer death in Cox regression models; [d], mixed-cell or undifferentiated carcinoma.

and A549 cell lines (P<0.05; *Figure 2C*).

lnc-RAB11B-AS1 overexpression increases colony formation ability of LC cells

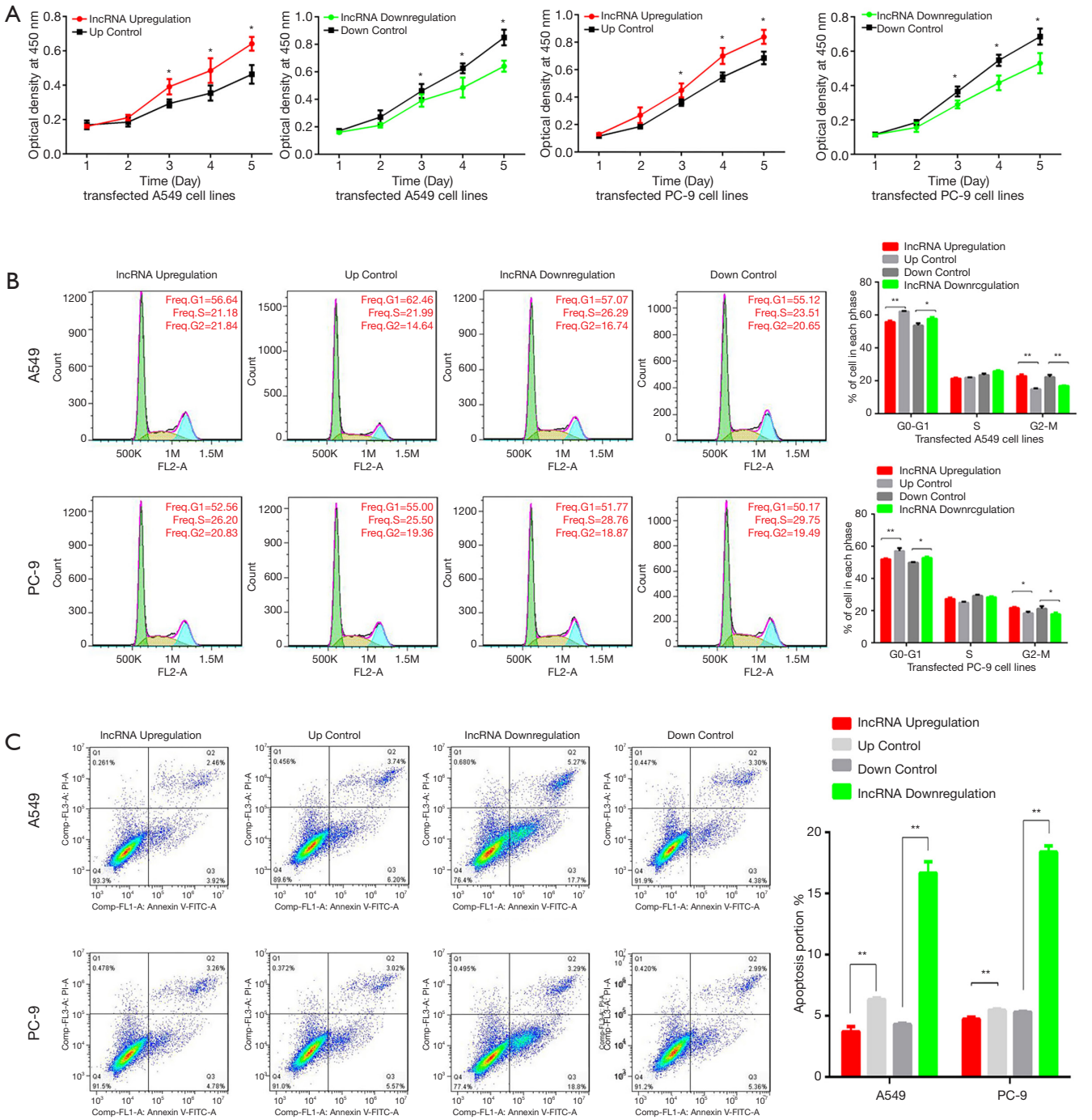
We next evaluated whether *lnc-RAB11B-AS1* expression impacted the clonogenic ability of LC cell lines. The results showed that the colony formation counts in LC cell lines were statistically increased when *lnc-RAB11B-AS1* was upregulated compared with control groups, while silencing *lnc-RAB11B-AS1* significantly reduced the colony numbers both in PC-9 and A549 cell lines (P<0.05; *Figure 2D*).

lnc-RAB11B-AS1 overexpression promotes LC cell migration and invasion abilities in vitro

As epithelial-mesenchymal transition (EMT) is an important property of tumor metastasis, we next performed TRANSWELL assays to explore the influence of *lnc-RAB11B-AS1* on cell migration and invasion abilities. Cell migration assay results showed that upregulation of *lnc-RAB11B-AS1* statistically increased the migratory ability of the LC cell lines, while *lnc-RAB11B-AS1* knockdown significantly reduced this ability (P<0.05; *Figure 3A*). Similar effects were also observed in invasion assays (P<0.05; *Figure 3B*).

lnc-RAB11B-AS1 promotes tumor growth in vivo

To study the influence of *lnc-RAB11B-AS1* on the proliferation abilities of LC cells *in vivo*, we generated a BALB/c-nu nude mice xenograft model using stably transfected PC-9 and A549 cell lines. A statistically increase in tumor volume and weight was observed in the *lnc-RAB11B-AS1* upregulation groups (both PC-9 and A549 cell lines), while the *lnc-RAB11B-AS1* downregulation groups showed a statistically decrease in tumor volume and weight (P<0.05; *Figure 3C,D*).



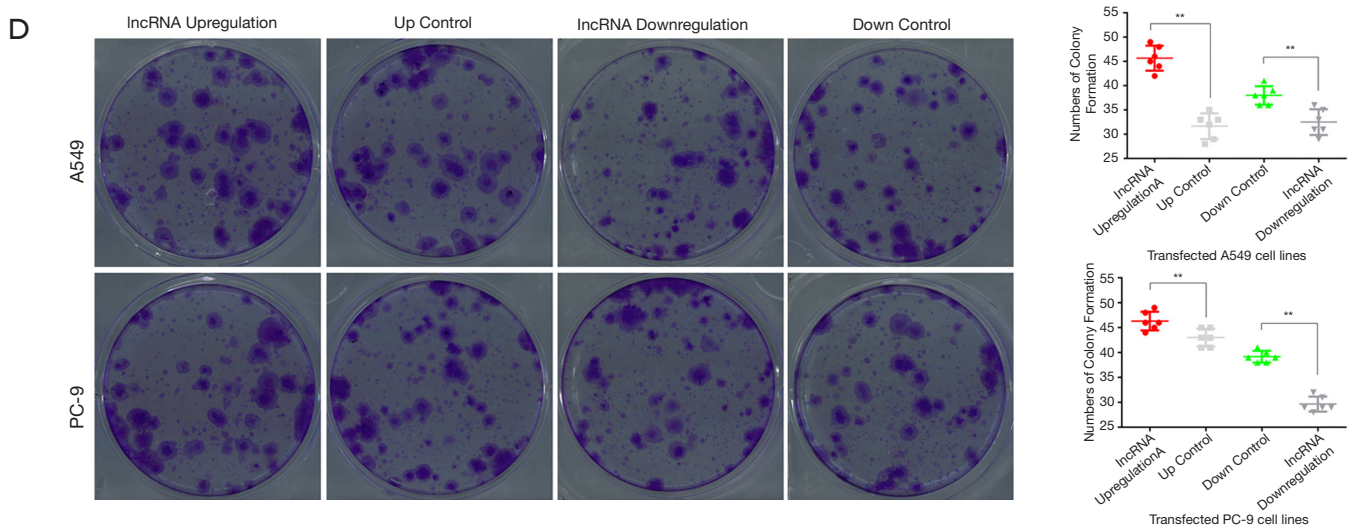
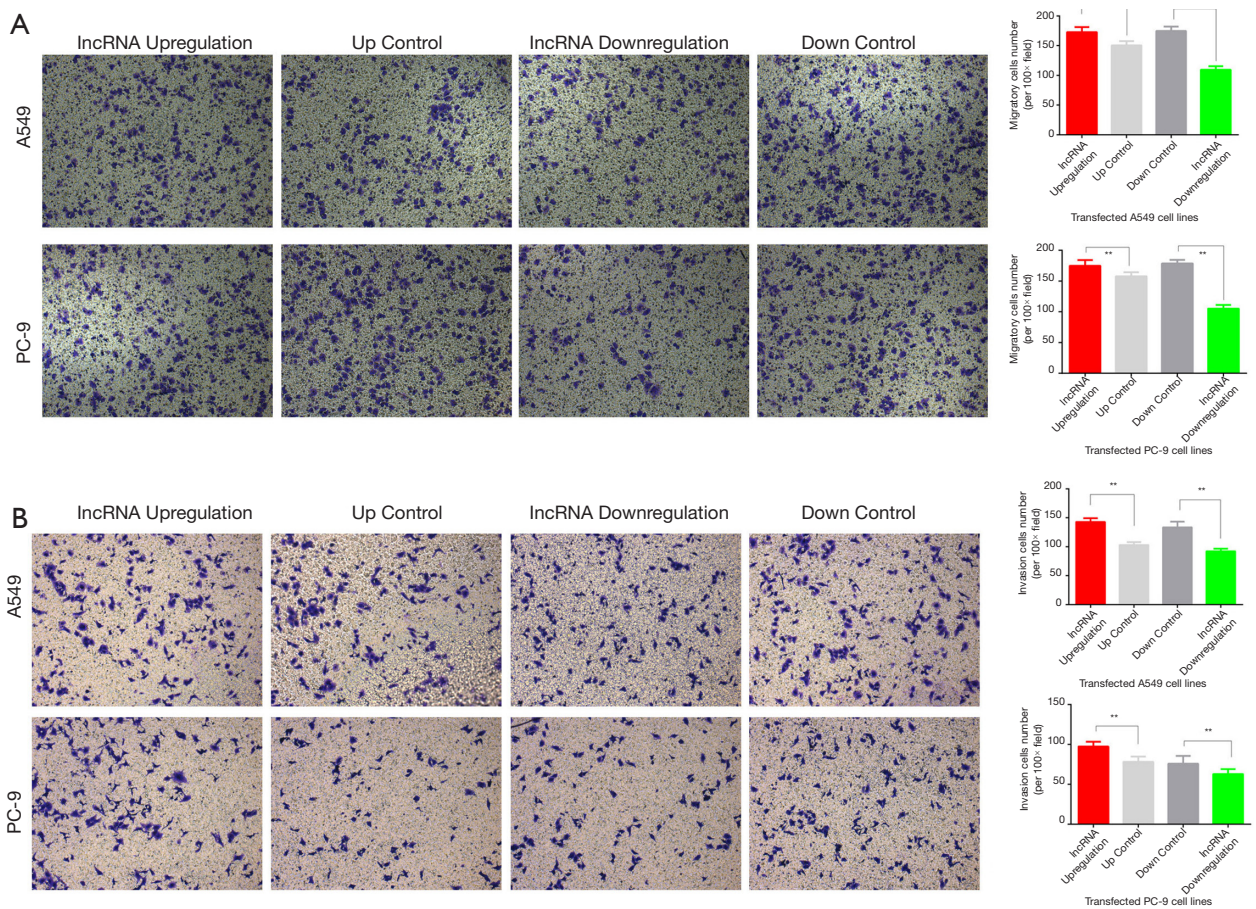


Figure 2 Lnc-RAB11B-AS1 promotes cancer cell proliferation, apoptosis, colony formation and cell cycle progression in LC cells *in vitro*. (A) Lnc-RAB11B-AS1 over-expression significantly enhanced lung cancer cell viability, while silencing of lnc-RAB11B-AS1 reduced cell viability of both A549 and PC-9 cell lines; (B) over-expression of lnc-RAB11B-AS1 decreased the numbers of G1 phase cells and increased the numbers of cells in M phase, while silencing of lnc-RAB11B-AS1 had the opposite effects; (C) Lnc-RAB11B-AS1 over-expression caused a decrease in apoptosis and silencing increased the apoptosis rate in both A549 and PC-9 cell lines; (D) the tablet clone formation efficiency of the transformed cell lines showed significant increases in lnc-RAB11B-AS1 overexpressed cell lines and decreases in lnc-RAB11B-AS1 silenced cell lines. *, P value <0.05; **, P value <0.01.



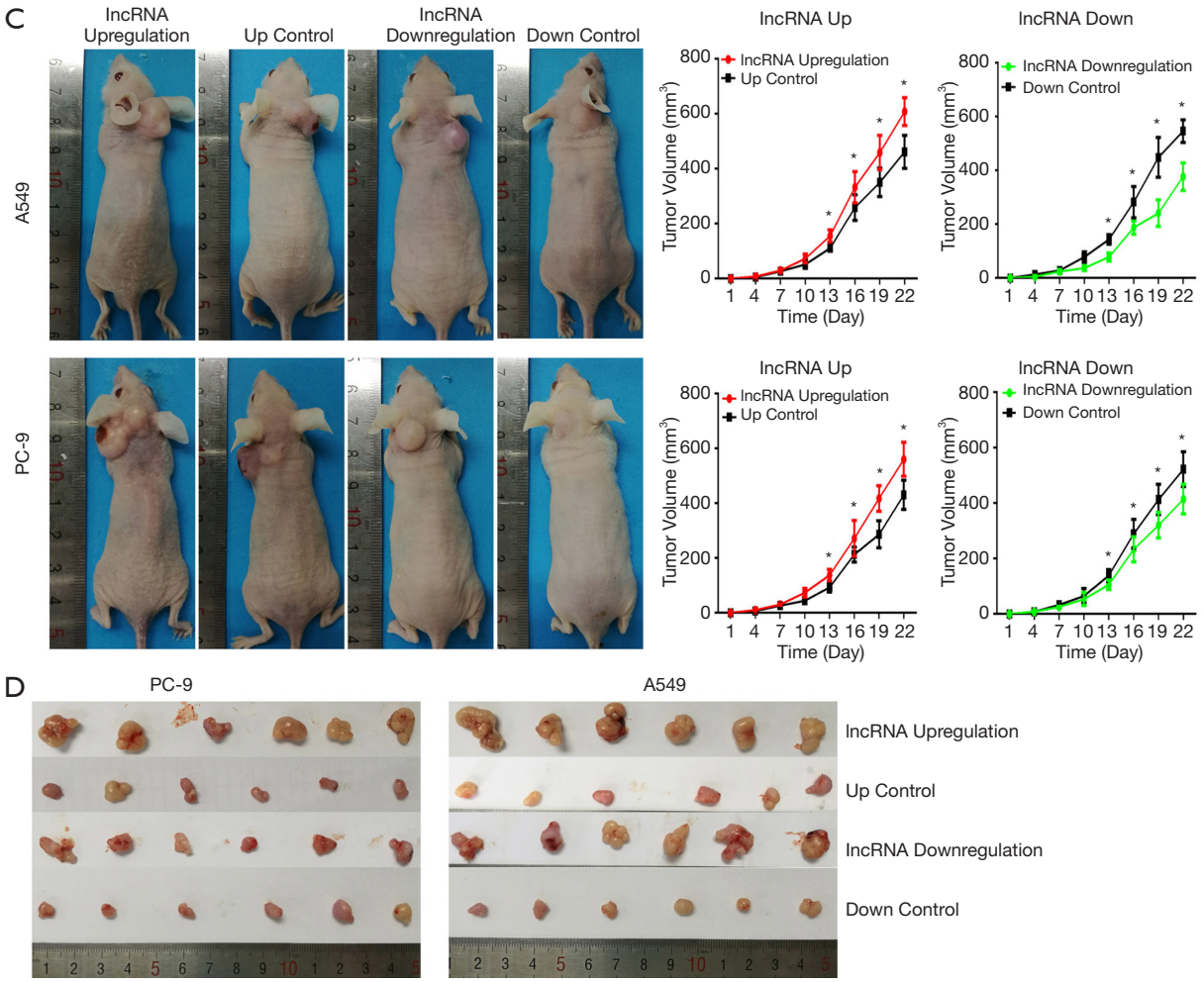


Figure 3 Lnc-RAB11B-AS1 promotes cancer cell migration and invasion activities in LC cells *in vitro* and enhances tumor growth *in vivo*. (A,B) Cell lines with overexpressed lnc-RAB11B-AS1 showed significantly higher migration and invasion activities compared with controls, while cell lines silenced for lnc-RAB11B-AS1 exerted significantly lower abilities; (C,D) tumor growth was increased in xenograft mice implanted with stable transfected in lnc-RAB11B-AS1 overexpressed cells and attenuated in xenograft mice implanted with lnc-RAB11B-AS1 silenced cells compared with controls. All results are shown as mean ± SD. P<0.05, calculated by the one-way ANOVA test. *, P value <0.05; **, P value <0.01.

The expression of lnc-RAB11B-AS1 was positively correlated with its sense-cognate gene RAB11B in LC

Sequence analysis showed that *lnc-RAB11B-AS1* is located in physical contiguity with *RAB11B* (*Figure 4A*) and forms a 'head-to-tail' pairing pattern with 448 bp of full complementarity sequence. We next detected the expression levels of *RAB11B* by qRT-PCR using the same groups of LC tissue samples used to detect *lnc-RAB11B-AS1* levels and conducted a correlation analysis. A statistical increase of *RAB11B* expression level was observed in LC tissues when compared with the paired normal tissues (P=0.023; *Figure 4B*). The correlation analysis demonstrated a positive correlation between the expression levels of *lnc-RAB11B-AS1* and *RAB11B* (R =0.5044, P<0.001; *Figure 4C*).

To confirm these findings, we also examined the levels of *RAB11B* expression in the *lnc-RAB11B-AS1* stable transfected cell lines. Our results showed that *RAB11B* mRNA level was statistically increased when *lnc-RAB11B-AS1* was upregulated, and decreased in those cells with *lnc-RAB11B-AS1* silencing (*Figure 4D*).

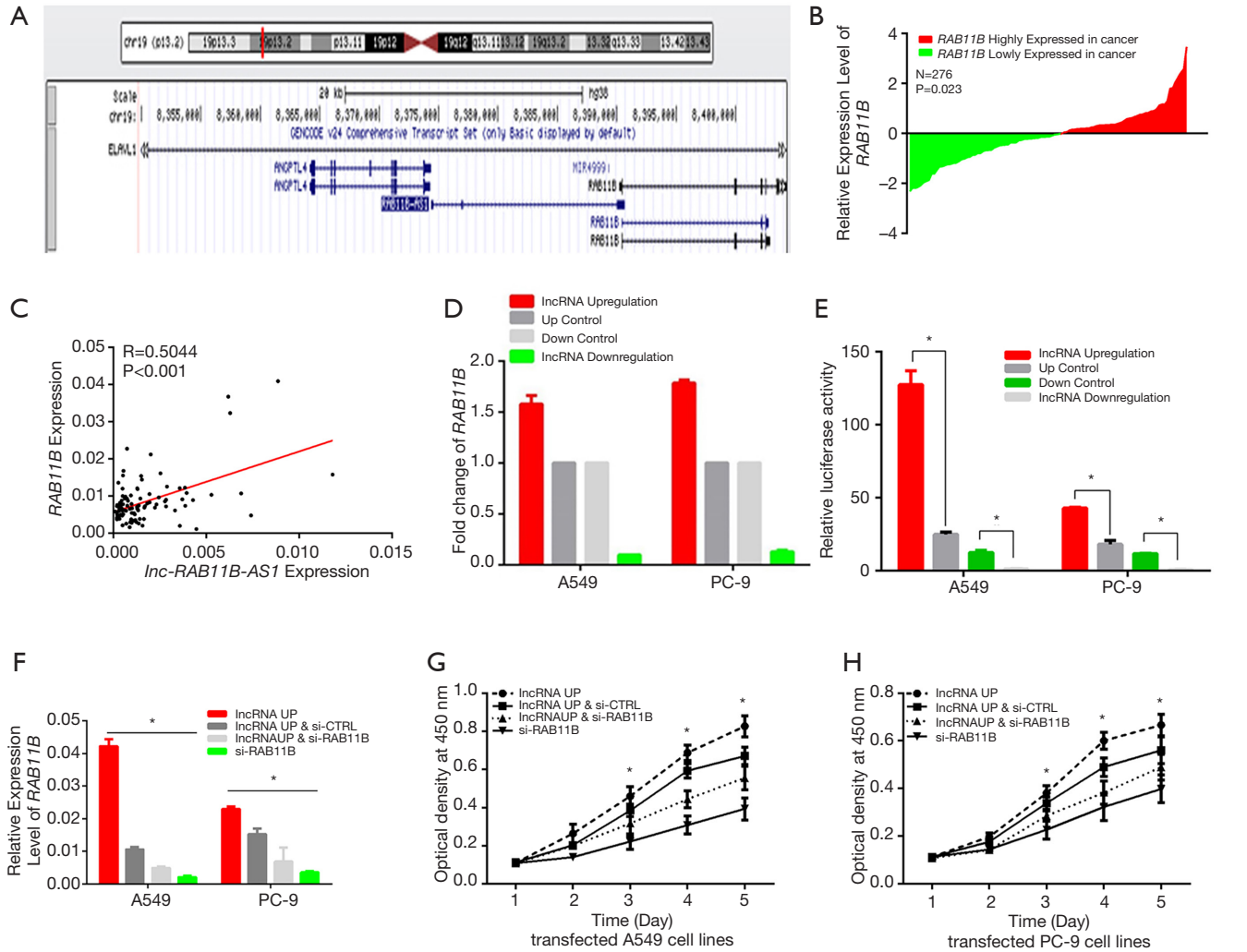


Figure 4 Correlation between lnc-RAB11B-AS1 and RAB11B expression. (A) The relative position of lnc-RAB11B-AS1 and RAB11B in human genome; (B) relative expression level of RAB11B in LC patients (N=276). P<0.01; (C) correlation between lnc-RAB11B-AS1 and RAB11B; (D) high expression of lnc-RAB11B-AS1 resulted in high RAB11B expression, while low expression of lnc-RAB11B-AS1 caused low RAB11B expression in stably transfected A549 and PC-9 cell lines; (E) upregulation of lnc-RAB11B-AS1 induced a significant increase in the luciferase activity of pGL3-Promoter-RAB11B in LC cell lines; (F) relative expression of RAB11B was measured by qPCR in A549 and PC-9 cells stably silenced for lnc-RAB11B-AS1 and transfected with si-RAB11B or si-CTRL; (G,H) CCK-8 assays were conducted to detect the effect of si-RAB11B on stably overexpression lnc-RAB11B-AS1 cell lines. *, P value <0.05; **, P value <0.01.

lnc-RAB11B-AS1 promotes LC progress via correlated with RAB11B

We next performed luciferase reporter assay to more closely examine the correlation between *lnc-RAB11B-AS1* and *RAB11B* gene. Reporter assays were performed in LC cell lines using a pGL3-*RAB11B*. Transfection of the reporter in cells with *lnc-RAB11B-AS1* upregulation resulted in a significant increase in the luciferase activity in both LC cell lines, while *lnc-RAB11B-AS1* silenced cell lines showed decreased luciferase activity (*Figure 4E*). The above results showed a positive relationship between *lnc-RAB11B-AS1* and the promoter of *RAB11B* in LC cell lines.

lnc-RAB11B-AS1 promotes LC progression via upregulating RAB11B

We next analyzed the associations between the expression

Table 4 Association between the expression level of *RAB11B* and the clinic characteristics of the patients

Clinic characteristics	Southern samples, N (%)		P[a]	Eastern samples, N (%)		P[a]	Merged samples, N (%)		P[a]
	Low	High		Low	High		Low	High	
Age									
<60	46 (43.8)	59 (56.2)	0.762	22 (43.1)	29 (56.9)	0.919	68 (43.6)	88 (56.4)	0.856
≥60	32 (41.6)	45 (58.4)		19 (44.2)	24 (55.8)		51 (42.5)	69 (57.5)	
Sex									
Female	24 (46.2)	28 (53.8)	0.570	12 (42.9)	16 (57.1)	0.923	36 (45.0)	44 (55.0)	0.686
Male	54 (41.5)	76 (58.5)		29 (43.9)	37 (56.1)		83 (42.3)	113 (57.7)	
Family history of cancer									
No	67 (41.6)	94 (58.4)	0.348	37 (44.0)	47 (56.0)	0.807	104 (42.4)	141 (57.6)	0.529
Yes	11 (52.4)	10 (47.6)		4 (40.0)	6 (60.0)		15 (45.2)	16 (54.8)	
Family history of lung cancer									
No	73 (43.2)	96 (56.8)	0.740	40 (44.0)	51 (56.0)	0.715	113 (43.5)	147 (56.5)	0.640
Yes	5 (38.5)	8 (61.5)		1 (33.3)	2 (66.7)		6 (37.5)	10 (62.5)	
Smoking									
No	31 (46.3)	36 (53.7)	0.478	14 (46.7)	16 (53.3)	0.683	45 (46.4)	52 (53.6)	0.419
Yes	47 (40.9)	68 (59.1)		27 (42.2)	37 (57.8)		74 (41.9)	105 (58.1)	
Drinking									
No	60 (43.8)	77 (56.2)	0.655	31 (42.5)	42 (57.5)	0.675	91 (43.3)	119 (56.7)	0.897
Yes	18 (40.0)	27 (60.0)		10 (47.6)	11 (52.4)		28 (42.4)	38 (57.6)	
Surgery									
No	54 (44.6)	67 (55.4)	0.497	25 (43.1)	33 (56.9)	0.899	79 (44.1)	100 (55.9)	0.643
Yes	24 (39.3)	37 (60.7)		16 (44.4)	20 (55.6)		40 (41.2)	57 (58.8)	
Chemotherapy									
No	24 (38.1)	39 (61.9)	0.345	12 (41.4)	17 (58.6)	0.770	36 (39.1)	56 (60.9)	0.344
Yes	54 (45.4)	65 (54.6)		29 (44.6)	36 (55.4)		83 (45.1)	101 (54.9)	
Radiotherapy									
No	47 (45.2)	57 (54.8)	0.462	22 (45.8)	26 (54.2)	0.658	69 (45.4)	83 (54.6)	0.397
Yes	31 (39.7)	47 (60.3)		19 (41.3)	27 (58.7)		50 (40.3)	74 (59.7)	
Clinical stages									
I + II	40 (56.3)	31 (43.7)	0.003	18 (62.1)	11 (37.9)	0.016	58 (58.0)	42 (42.0)	<0.001
III + IV	38 (34.2)	73 (65.8)		23 (35.4)	42 (64.6)		61 (34.7)	115 (65.3)	
T									
1+2	50 (48.1)	54 (51.9)	0.100	19 (42.2)	26 (57.8)	0.794	69 (46.3)	80 (53.7)	0.246
3+4	28 (35.9)	50 (64.1)		22 (44.9)	27 (55.1)		50 (39.4)	77 (60.6)	

Table 4 (*continued*)

Table 4 (*continued*)

Clinic characteristics	Southern samples, N (%)		P[a]	Eastern samples, N (%)		P[a]	Merged samples, N (%)		P[a]
	Low	High		Low	High		Low	High	
N									
0	35 (53.0)	31 (37.0)	0.036	26 (60.5)	17 (39.5)	0.002	61 (56.0)	48 (44.0)	<0.001
1+2+3	43 (37.1)	73 (62.9)		15 (29.4)	36 (70.6)		58 (34.7)	109 (65.3)	
M									
0	63 (48.8)	66 (51.2)	0.011	32 (53.3)	28 (46.7)	0.012	95 (50.3)	94 (49.7)	<0.001
1	15 (28.3)	38 (71.7)		9 (26.5)	25 (73.5)		24 (27.6)	63 (72.4)	
Histological types									
Adenocarcinoma	39 (45.3)	47 (54.7)	0.693	20 (48.8)	21 (51.2)	0.600	59 (46.5)	68 (53.4)	0.612
Squamous cell carcinoma	20 (39.2)	31 (60.8)		10 (32.3)	21 (67.7)		30 (36.6)	52 (63.4)	
Large cell carcinoma	4 (66.7)	2 (33.3)		2 (40.0)	3 (60.0)		6 (54.5)	5 (45.5)	
Small cell lung cancer	7 (36.8)	12 (63.2)		4 (50.0)	4 (50.0)		11 (40.7)	16 (59.3)	
Other carcinomas[b]	8 (40.0)	12 (60.0)		5 (55.6)	4 (44.4)		13 (44.8)	16 (55.2)	

[a], Pearson χ^2 test P value; [b], mixed-cell or undifferentiated carcinoma.

levels of *RAB11B* and LC progression. We stratified the LC patients into high and low expression groups using the medium level in LC tissues. Patients with expression levels equal to or greater than the cut-off value were categorized into the high *RAB11B* expression group, and those with expression levels lower than the cut-off value were defined as the low *RAB11B* expression group. The status of *RAB11B* expression was statistically different in patients at different clinic stages (P<0.001), N stage (P<0.001) and M stage (P<0.001) (*Table 4*). These results indicate that patients with a higher level of *RAB11B* expression would have a higher risk for advanced clinic stages, N stage and M stage.

We also examined the effect of *RAB11B* on the proliferation ability of LC cell lines using CCK-8 assays. We generated PC-9 and A549 cell lines expressing a siRNA targeting *RAB11B* as described in Methods. The above results showed that LC cell lines with silenced *RAB11B* showed reduced proliferation compared with control cells (*Figure 4F,G,H*). These results indicate that *RAB11B* functions in regulating LC cell proliferation and imply a role for *RAB11B* in promoting LC progression.

Discussion

Our previous studies found that *lnc-RAB11B-AS1* was involved in cell cycle and metastasis regulation in osteosarcoma (15), and was reported to correlate with the advanced clinic stage, metastasis, and overall survival rate in gastric cancer patients (16) and promote hypoxia-mediated angiogenesis and metastasis in breast cancer patients (17). However, its role in LC development remains to be clarified. In this study, we identified a novel mechanism by which *lnc-RAB11B-AS1* functions to promote LC development through regulating *RAB11B* expression. *lnc-RAB11B-AS1* was overexpressed in LC tissues compared with the paired normal tissues. High expression levels of *lnc-RAB11B-AS1* correlated with an advanced cancer stage, N, M status and poor overall survival rate in LC patients. The above data suggest that *lnc-RAB11B-AS1* could act as a potential prognostic biomarker for the survival of LC patients and may be a novel potential therapeutic target in treating LC patients.

Our previous studies found that *lnc-RAB11B-AS1* was down-regulated in osteosarcoma (15). However, Feng

et al. (16) and Niu *et al.* (17) reported that *lnc-RAB11B-AS1* was over-expressed in gastric cancer and breast cancer respectively. This is the first study to describe the expression pattern and function of *lnc-RAB11B-AS1* in LC. We observed significantly higher expression levels of *lnc-RAB11B-AS1* in LC tissues compared with corresponding non-cancerous tissues and had a positive correlation between the expression levels of *lnc-RAB11B-AS1* and *RAB11B*. The expression level of *lnc-RAB11B-AS1* was statistically correlated with clinic stage, N stage and M stage in LC patients. LC patients with upregulated *lnc-RAB11B-AS1* expression showed a poorer prognosis and worse overall survival rate compared with those with downregulated *lnc-RAB11B-AS1* expression, indicating that *lnc-RAB11B-AS1* may function as a prognostic biomarker for LC. Gain-of-function and loss-of-function experiments conducted in this study showed that *lnc-RAB11B-AS1* promoted cell cycle changes and induced apoptosis, proliferation, migration and invasion abilities of LC cells *in vitro*. Furthermore, xenograft experiments showed that overexpression of *lnc-RAB11B-AS1* increased tumor growth *in vivo*. These results indicate that *lnc-RAB11B-AS1* may play an oncogenic role in LC development and progression.

Increasing studies have shown that as-lncRNAs play a crucial role in cancer development and some may function as cancer biomarkers. For example, *lnc-MUC5B-AS1* promotes metastasis through regulating its natural antisense transcript *MUC5B* in lung adenocarcinoma (25). *Lnc-ZEB1-AS1* was reported to promote tumor metastasis and predicts poor prognosis in liver cancer (7). *Lnc-ZEB2-AS1* was reported to promote proliferation and inhibit apoptosis in human lung cancer cells (26). Thus, in addition to these lncRNAs, *lnc-RAB11B-AS1* may be another novel preventive and therapeutic biomarker for LC. The xenograft model showed that *lnc-RAB11B-AS1* plays a crucial role in controlling LC cell proliferation *in vivo*, which indicates that *lnc-RAB11B-AS1* might be a therapeutic target of LC.

Although our results indicate that *lnc-RAB11B-AS1* might function as an oncogene in LC, the underlying molecular mechanisms remain to be clarified. Recent studies showed that methylation of the promoter of as-lncRNAs could be a potential strategy to dysregulate the expression of as-lncRNA, and thus affect the corresponding mRNA expression. For example, a previous report revealed a CpG island spanning the transcription start site of *lnc-ZEB1-AS1*, and *lnc-ZEB1-AS1* methylation was negatively correlated with the *lnc-ZEB1-AS1* expression (7). We observed a similar mechanism in *lnc-RAB11B-AS1* in osteosarcoma in our previous study (15). We found that DNA hypomethylation of the promoter could result in an increase in the expression level of *lnc-RAB11B-AS1*. Our data showed that *lnc-RAB11B-AS1* distributes mainly in the nucleus and partly in the cytoplasm. Literatures showed that nuclear lncRNAs involved in transcriptional regulation both *in cis* and *in trans*, and played a crucial role in the modulation of chromosomal interactions, chromatin looping, transcription factor trapping, gene methylation, recruitment of transcription factors, and chromatin modification (27,28). Thus, we speculate that hypomethylation in the promoter region may cause the upregulation of *lnc-RAB11B-AS1* in LC. Canzio *et al.* (29) reported a new mechanism of the antisense lncRNAs functioned in distance-independent enhancer and promotor demethylation, thus could be a potential mechanism of how *lnc-RAB11B-AS1* regulate *RAB11B*.

Sequence analysis showed that *lnc-RAB11B-AS1* is located in physical contiguity with *RAB11B*, and formed a 'head-to-tail' pairing pattern with 448 bp full complementarity sequence. Therefore, we can suggest that *lnc-RAB11B-AS1* may function as a super-enhancer or co-factor in the transcriptional regulation way (30) to regulate the target gene *RAB11B*. Luciferase reporter assays showed that *lnc-RAB11B-AS1* overexpression promotes *RAB11B* expression, which supports the super-enhancer hypothesis. Furthermore, the qPCR results showed a positive correlation between the expression of *lnc-RAB11B-AS1* and the expression of *RAB11B*, which demonstrated the positive regulation of *lnc-RAB1B-AS1* on *RAB11B* in LC.

RAB11B is a member of the *RAB11* family, which includes *RAB11A*, *RAB11B* and *RAB25* (or *RAB11C*) (31). Multiple diseases have been correlated with RAB11 family proteins, such as Alzheimer's disease, Huntington's disease and type 2 diabetes, and RAB11 proteins have been associated with carcinogenesis and cancer development (32). Gebhardt *et al.* (33) found RAB11 proteins were functional in human skin cancer. Yoon *et al.* (34) demonstrated that *RAB11* enhances the invasiveness and migration capabilities of breast cancer cells. *RAB11B* was first reported by Lai *et al.* (35) in mice in 1994 and has been associated with various diseases (35), such as breast cancer (36) and non-small cell lung cancer (37). Dysregulated 19p13.2 genomes were also correlated with pancreatic cancer, smoking associated chronic obstructive pulmonary disease (22,23,38), colon cancer (39) and ovarian mucinous carcinoma (18).

In this study, we found that overexpression of *lnc-*

RAB11B-AS1 positively correlated with *RAB11B* expression level in LC tissues as well as stably transfected LC cell lines. We also examined whether *lnc-RAB11B-AS1* regulates the *RAB11B* promoter activity, and indeed, *lnc-RAB11B-AS1* exhibited a significant effect on promoting *RAB11B* promoter activity. Furthermore, *RAB11B* inhibition partially abrogated *lnc-RAB11B-AS1*-induced LC cell lines invasion and cancer metastasis, while silencing of *lnc-RAB11B-AS1* suppressed *RAB11B* expression and decreased cell invasion and cancer metastasis. Although we found that *lnc-RAB11B-AS1* had a positive regulation on *RAB11B* in LC on RNA level, we did not detect if the RAB11B protein was influenced by *lnc-RAB11B-AS1*, the mechanism of how *lnc-RAB11B-AS1* was upregulated in LC remains to be clarified, and that's what we plan to explore next.

In conclusion, our results provide the first evidence that *lnc-RAB11B-AS1* may function as an oncogene to facilitate cell proliferation, invasion and migration ability, which possibly through the regulation on *RAB11B* in LC. We found that upregulation of *lnc-RAB11B-AS1* in LC cell lines statistically promoted tumor growth, metastasis, cell proliferation and inhibit apoptosis both *in vivo* and *in vitro*, which increased the possibility that *lnc-RAB11B-AS1* might be a promising new biomarker and therapeutic target for LC.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm.2020.04.52). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was approved by the Ethics Committee of Guangzhou Medical University (No. GMU201481473040) and we strictly followed the related clinical research guidelines. All study participants involved in the present study were provided written informed consent.

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Supplementary

Table S1 Primers used in this study

Gene	Forward primer (5'->3')	Reverse primer (5'->3')
lnc-RAB11B-AS1	GGAACATGTTTACATGGACTTTGT	TCTTTGTTCTTGTTTGTTTTCTTTCT
RAB11B	CGTACTACCGTGGTGCAGTG	ATGACGATGTTGCTGTCTGC
RAB11A	TCTCAGGGCAGTTCCTACAGA	ACCACATTGTTGCTTGGAGAC
RAB25	GACTGCTCTTCCTGGAGACCT	TCTGTCTCTGCTTGGACACCT
ACTB	GGCGGCACCACCATGTACCCT	AGGGGCCGGACTCGTCATACT

Table S2 Demographics and clinical characteristics of studied samples

Characteristics	Southern samples, N (%)	Eastern samples, N (%)	Pearson χ^2	P
Total	182 (65.9)	94 (34.1)		
Age(years)				
<60	105 (57.7)	51 (54.3)	0.298	0.585
≥60	77 (42.3)	43 (45.7)		
Sex				
Female	52 (28.6)	28 (29.8)	0.045	0.8329
Male	130 (71.4)	66 (70.2)		
Family history of cancer				
No	161 (88.5)	84 (89.4)	0.050	0.822
Yes	21 (11.5)	10 (10.6)		
Smoking				
No	67 (36.8)	30 (31.9)	0.653	0.419
Yes	115 (63.2)	64 (68.1)		
Drinking				
No	137 (75.3)	73 (77.7)	0.194	0.660
Yes	45 (24.7)	21 (23.3)		
Gold stage				
I + II	71 (39.0)	29 (30.1)	2.287	0.515
III + IV	111 (61.0)	65 (69.9)		
Histological types				
Adenocarcinoma	86 (47.3)	41 (43.6)	1.681	0.794
Squamous cell carcinoma	51 (28.0)	31 (33.0)		
Large cell carcinoma	6 (3.3)	5 (5.3)		
Small cell lung cancer	19 (10.4)	8 (8.5)		
Other carcinomas*	20 (11.0)	9 (9.6)		

*, mixed-cell or undifferentiated carcinoma.

Table S3 Association between the clinic characteristics and the prognosis of the lung cancer patients

Variables	Southern samples		Eastern samples		Merged samples		MST (month)	log-rank P value	Crude HR (95% CI)
	Case, N	Death, N (%)	Case, N	Death, N (%)	Case, N	Death, N (%)			
Total	110	88 (80%)	53	45 (84.9)	163	133 (81.6)			
Age									
<60	64	50 (78.1)	34	27 (79.4)	98	77 (78.6)	24.5		1.00 (Ref.)
≥60	46	38 (82.6)	19	18 (94.7)	65	56 (86.2)	22.5	0.575	1.10 (0.78–1.56)
Sex									
Female	38	29 (76.3)	16	13 (81.3)	54	42 (77.8)	26.8		1.00 (Ref.)
Male	72	59 (81.9)	37	32 (86.5)	109	91 (83.5)	22.2	0.345	0.84 (0.58–1.21)
Family history of cancer									
No	100	79 (79.0)	45	38 (84.4)	145	117 (80.7)	24.4		1.00 (Ref.)
Yes	10	9 (90.0)	8	7 (87.5)	18	16 (88.9)	19.0	0.353	1.28 (0.76–2.17)
Smoking									
No	44	32 (72.7)	20	15 (75.0)	64	47 (73.5)	29.4		1.00 (Ref.)
Yes	66	56 (84.9)	33	30 (90.9)	99	86 (86.9)	20.0	0.015	1.56 (1.09–2.22)
Drinking									
No	83	67 (80.7)	40	34 (85.0)	123	101 (82.1)	24.2		1.00 (Ref.)
Yes	27	21 (77.8)	13	11 (84.6)	40	32 (80.0)	21.3	0.600	1.11 (0.75–1.66)
Stage									
I + II	42	31 (73.8)	13	10 (76.9)	55	41 (74.5)	32.2		1.00 (Ref.)
III + IV	68	57 (83.8)	40	35 (87.5)	108	92 (85.2)	19.1	0.004	1.73 (1.19–2.52)
Histological types									
Adenocarcinoma	56	44 (78.6)	20	16 (80.0)	76	60 (78.9)	29.0		1.00 (Ref.)
Squamous cell carcinoma	30	25 (83.3)	18	20 (90.0)	48	45 (93.8)	19.7	0.114	1.38 (0.93–2.04)
Large cell carcinoma	2	2 (100.0)	3	3 (100.0)	5	5 (100.0)	12.6	0.070	2.34 (0.93–5.86)
Small cell lung cancer	11	9 (81.8)	4	2 (50.0)	15	11 (73.3)	12.9	0.095	1.76 (0.91–3.40)
Other carcinomas[a]	11	8 (72.7)	6	6 (100.0)	17	14 (93.3)	15.6	0.083	1.68 (0.93–3.00)

[a], mixed-cell or undifferentiated carcinoma. MST, median survival time; HR, hazard ratio; ref, reference.