Am J Transl Res 2020;12(8):4302-4313
www.ajtr.org /ISSN:1943-8141/AJTR0110038

Original Article

Long non-coding RNA LINC01116 is overexpressed in lung adenocarcinoma and promotes tumor proliferation and metastasis

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Received February 28, 2020; Accepted July 4, 2020; Epub August 15, 2020; Published August 30, 2020

Abstract: Long non-coding RNA LINC01116 is involved in the occurrence and progression of a variety of cancers. However, the specific role of LINC01116 in lung adenocarcinoma (LUAD) remains unclear. In this work, we found that LINC01116 was overexpressed in LUAD tissues and cell lines and that increased expression was significantly associated with worse prognoses in patients with LUAD. Univariate and multivariate Cox regression analyses indicated that LINC01116 was an independent risk factor for the prognosis of patients with LUAD. Downregulation of LINC01116 significantly inhibited cell proliferation and migration, promoted cell apoptosis, and prevented cell progression from G1 to S phase. In addition, downregulation of LINC01116 significantly inhibited the epithelial-mesenchymal transition, leading to an increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal markers N-cadherin and vimentin. In summary, our results suggest that LINC01116 may act as an oncogene in LUAD and may be a valuable prognostic biomarker for patients with LUAD.

Keywords: LINC01116, lung adenocarcinoma, prognosis, proliferation, EMT

Introduction

Lung cancer is the primary cause of tumor-related deaths worldwide, making it a public health issue of global concern [1, 2]. Non-small cell lung cancer (NSCLC) is the most common subtype of lung cancer, accounting for more than 80% of primary cases [3, 4]. Lung adenocarcinoma (LUAD), the predominant subtype of NSCLC, causes over 500,000 deaths per year around the world [5]. Although patients with LUAD can sometimes be successfully treated with targeted therapy or immunotherapy, survival rates remain low, especially in patients with metastatic disease [6]. Moving forward, it will be crucial to explore the molecular mechanisms of LUAD development and metastasis to develop better therapeutic approaches and improve survival rates.

Long non-coding RNA (LncRNA) is a type of non-coding RNA that is longer than 200 nucleotides and does not encode proteins [7]. With the development of gene sequencing and chip detection technologies, an increasing number of LncRNAs have recently been identified [8]. Crucial roles for LncRNA have been identified in the development of various cancers [9, 10]. Aberrantly expressed LncRNAs are involved in many biological processes of tumors, contributing to tumorigenesis, metastasis, and worse prognosis [11, 12]. Studies have shown that LncRNAs act as epigenetic regulators in several cancers [13, 14]. Further evidence has suggested that LncRNAs can regulate tumor occurrence and development by acting both as oncogenes and tumor suppressor genes [15, 16]. As a result, it has been suggested that LncRNAs may serve well as diagnostic or prognostic biomarkers, or even as therapeutic targets [17-19]. LINC01116 is a novel LncRNA located in 2q31.1. Previous studies have demonstrated that it is overexpressed in different types of cancers, including osteosarcoma [20], oral sq-

Table 1. Clinical information of LUAD patients in TCGA and GEO datasets

Clinical factors	TCGA dataset (n = 448)	GEO datasets (n = 381)
Vital status		
Alive	299 (66.7)	286 (75.1)
Dead	149 (33.3)	95 (24.9)
Age (years)		
≤65	213 (47.5)	233 (61.2)
>65	235 (52.5)	148 (38.8)
Gender		
Female	240 (53.6)	190 (49.9)
Male	208 (46.4)	191 (50.1)
Stage		
I	241 (53.8)	275 (72.2)
II	111 (24.8)	95 (24.9)
III/IV	96 (21.4)	8 (2.1)
T stage		-
1	151 (33.7)	-
2	241 (53.8)	-
3/4	56 (12.5)	-
N stage		-
0	294 (65.6)	-
1	87 (19.4)	-
2	67 (20.0)	-

LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; TNM, tumor-node-metastasis. Data were shown as n (%).

uamous cell carcinoma [21], nasopharyngeal carcinoma [22], and others. Studies have illustrated that LINC01116 regulates tumorigenesis [23] and promotes cell proliferation, migration, and invasion [24, 25]. Other evidence has suggested that LINC01116 can act as competing endogenous RNA (ceRNA) to regulate the expression of target genes to ultimately promote tumorigenesis [26-28]. One study indicated that LINC01116 promoted gefitinib resistance by regulating IFI44 in NSCLC [29]. However, the role of LINC01116 in LUAD remains poorly understood. A better understanding of the function and mechanism of LINC01116 in LUAD may pave the way for novel approaches in diagnosis or therapeutic management.

In this study, we investigated the role of LINC01116 in LUAD by downregulating its expression *in vitro* and by analyzing its association with prognosis in human patients.

Materials and methods

Acquisition of RNA expression data and clinical information

A dataset including RNA sequencing data from 535 LUAD tumor and 59 non-tumor tissues, along with other relevant clinical information, was downloaded from The Cancer Genome Atlas website (TCGA, https://gdc-portal.nci.nih.gov/). We performed differential expression analysis on the RNA sequencing data using the Wilcox test with R software. In subsequent analyses, a total of 448 LUAD patients were analyzed after excluding patients with incomplete clinical information or with an overall survival (OS) of less than 60 days. The clinical factors included OS, survival status, age, sex, tumor (T) stage, node (N) stage, and tumor node metastasis (TNM) stage. Three Gene Expression Omnibus (GEO) datasets were acquired from the official website (http://www.ncbi.nlm.nih.gov/geo) of GEO and included gene expression profiles, relevant clinical data, and the corresponding probe information. The R package "sva" was used to normalize the expression of different batches after merging the three GEO datasets together. A total of 381 LUAD patients were selected for subsequent analyses after excluding patients whose pathological type was not adenocarcinoma, whose overall survival was less than 60 days, or whose clinical information was incomplete. Of the 381 LUAD patients, 226 were from GSE31210, 30 were from GSE29013, and the other 125 patients were from GSE50081. The clinical factors in the GEO datasets included OS, age, sex, and TNM stage. Detailed information on the TCGA and GEO datasets is listed in **Table 1**.

Tissue collection

We collected tumor and matched adjacent non-tumor tissues from 21 patients with LUAD treated at the Second Affiliated Hospital of Xian Jiaotong University (Xian, China) from January 2016 to January 2019. Among them, 8 were women and 13 were men, and the median age was 62 years (range, 40-76 years). Our study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Xian Jiaotong University (Xian, China) and all 21 patients provided written informed consent.

Table 2. Small interfering RNA sequences

	Sequence (5'-3')
siRNA1	GCAGTGTATTAGAAGACAATT
siRNA2	GCCGCATAGTGTAACTTTATT
siRNA3	CCCATTCATTGTTGTCACTTT
siNC	TTCTCCGAACGTGTCACGTTT

Table 3. Primer sequences

	Sequence (5'-3')
LINC01116	F: TTGCCTCCCCTTACCCCGAAAG
	R: TGGGTGATGGCAGAGTGAGACC
ACTB	F: CCTGGCACCCAGCACAAT
	R: GGGCCGGACTCGTCATAC

Cell culture and transfection

Three human LUAD cell lines A549, pc-9, and H1299 and one normal bronchial epithelial cell line BEAS-2B were used in this study. All four cell lines were obtained from the Cellular Institute of Chinese Academy of Science (Shanghai, China) and were grown in RPMI-1640 (Hyclone, USA) medium supplemented with 10% fetal bovine serum (FBS; Biological Industries). All cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Three short interfering RNAs (siRNAs) against LINC01116 and one negative control were produced by GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect siRNAs when the cell density reached around 70-80%. The sequences of those siRNAs are listed in **Table 2**.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Fast1000 (Xfyangbio) and Fast200 (Xfyangbio) were used to extract total RNA from lung tissues and cell lines, respectively. The total RNA was reverse transcribed to cDNA using the PrimerScript™ RT kit (TAKARA BIO INC). TB Green® Premix Ex Taq™ II (TAKARA BIO INC) kit was used for real-time PCR and all reactions were performed in triplicate. In this study, *ACTB* was used as a control. The primers used in real-time PCR were designed and produced by Sangon Biotech Co. Ltd. (Shanghai, China) and the sequences of primers are shown in **Table 3**. All operations were performed according to the manufacturer's instructions. The $2^{-\Delta\Delta Cq}$ method [30] was used to evaluate relative gene expression.

Cell counting assay

The cell counting kit-8 (CCK-8) assay was used to assess cell proliferation. First, 2×10^3 transfected cells were seeded per well of a 96-well plate. After incubation for 24, 48, or 72 h, 100 μl medium containing 10 μl CCK-8 solution (Keygen Biotech, china) were added to each well and incubated for 2 hours. The absorbance at 450 nm was measured on a spectrophotometer (Bio Tek, USA).

EdU assay

Cell growth was measured using the EdU (5-ethynyl-2'-deoxyuridine) assay kit (RiboBio Co., Ltd, China). First, 4×10^3 transfected cells were seeded in each well of 96-well plates and incubated for 24 h. Then, the medium was replaced with one containing 50 μM EdU and cultured for another 2 h. Next, we fixed the cells with 4% paraformaldehyde, stained the EdU-positive cells with Apollo solution and stained the DNA using Hoechst reagents. All assay steps were carried out in sequence according to the kit's instructions. Finally, a fluorescence microscope (Nikon, Tokyo, Japan) was used to acquire images. The extent of cell proliferation was calculated as the EdU positivity rate = (the number of EdU-positive cells/the number of DNA-stained cells) × 100%.

Cell apoptosis assay

Cells in 6-well plates were incubated for another 24 h after transfection with siRNA. Next, the transfected cells were digested and incubated at room temperature for 15 minutes in the dark after the addition of a solution containing 500 μl of binding buffer, 5 μl of Annexin V-APC and 5 μl of 7-amino-actinomycin D (7-AAD). Finally, cell apoptosis was detected using a flow cytometer (Becton-Dickinson; BD Biosciences) within 1 h.

Cell cycle assay

A549 cells were incubated in 6-well plates for another 24 h after transfection with siRNA. Next, the transfected cells were digested and fixed in pre-cooled 70% ethanol at -20°C overnight. On the second day, fixed cells were incu-

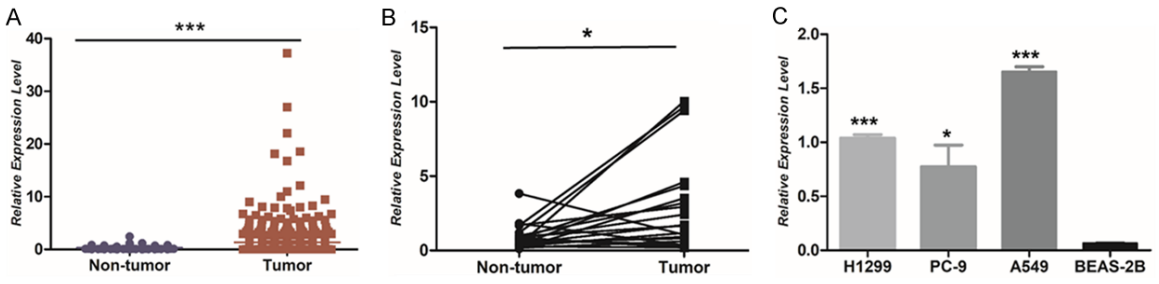

Figure 1. LINC01116 was significantly overexpressed in LUAD tissues and cell lines. A. Differential expression analysis showed that LINC01116 was overexpressed in 535 lung adenocarcinoma tumor tissues compared with 59 non-tumor lung tissues. ***P < 0.001. B. qRT-PCR showed that the expression level of LINC01116 in 21 lung adenocarcinoma tissues was higher than that in corresponding adjacent non-tumor lung tissues. *P < 0.05. C. qRT-PCR found that the expression level of LINC01116 were higher in H1299, PC-9, and A549 than that in BEAS-2B. *P < 0.05, **P < 0.01, ***P < 0.001.

bated with 500 μl working solution (RNase A: Propidium Iodide = 9:1) for 30 min at room temperature in the dark. Finally, cell cycle status was detected using a flow cytometer (Becton-Dickinson; BD Biosciences).

Transwell migration assay

Cells were grown on 24-well Transwell plates (Corning, USA) for the Transwell migration assay. First, 4 × 10^4 transfected cells were seeded in the upper chamber in 200 μl FBS-free medium, and 600 μl of medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the cells were fixed with paraformaldehyde and stained with crystal violet. The cells in the upper chamber were wiped off, and the cells in the lower chamber were counted under the microscope.

Wound healing assay

Transfected cells were grown to 100% confluence in 6-well plates, and then scratches were made using 10-μl pipette tips. Then, the complete medium was replaced with low serum medium (1% FBS). The cells migrated into the scraped areas and images were captured at 0, 24, and 48 h. The wound healing area was calculated using ImageJ software (version 1.52t).

Western blotting

Total cellular proteins were extracted using the radio immunoprecipitation assay lysate (Xi'an HEART Biotech). Proteins were separated using 10% sodium deodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% (w/v) nonfat dry milk powder in 0.1% Tris buffered saline/Tween 20 (TBST) for 2 h at room temperature and incubated with various primary antibodies overnight at 4°C. The primary antibodies against β-actin, E-cadherin, N-cadherin, and vimentin were acquired from Cell Signaling Technology (Danvers, MA, USA). Next, membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies, acquired from Cell Signaling Technology, for 1 h at room temperature. Enhanced chemiluminescence (Thermo Company, USA) was used to quantify protein bands. ImageJ software (version 1.52t) was used to analyze relative protein expression.

Statistical analysis

Kaplan-Meier survival analyses were conducted using the R package "survival", and the two-sided log-rank test was used to compare differences. GraphPad Prism 6.0 Software (GraphPad Inc) was used to analyze experimental data. Student's t-test and one-way ANOVA were used to analyze differences in means across groups and P < 0.05 was considered statistically significant.

Results

LINC01116 was overexpressed in LUAD tissues and cell lines

We analyzed the differential expression of RNA in the 535 LUAD tissues and 59 non-tumor tissues from RNA-sequencing data obtained from the TCGA database and found that LINC01116 was significantly overexpressed in tumor tissues relative to non-tumor tissues (log FC = 2.06, P = 9.78 × 10^{-06}, **Figure 1A**). In order to

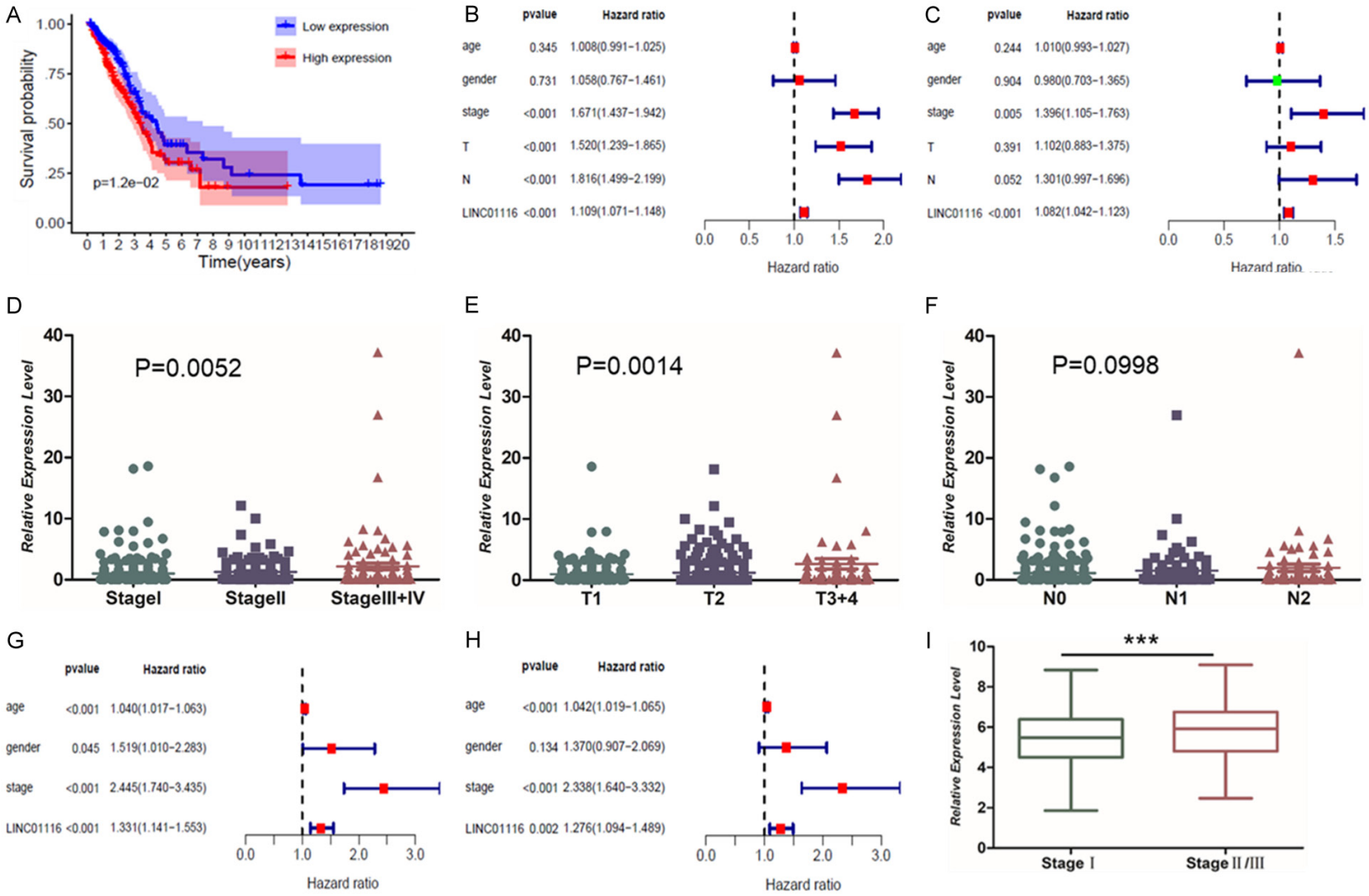

Figure 2. LINC01116 was significantly associated with the prognosis of LUAD patients. A. Kaplan-Meier survival curves of overall survival in 448 LUAD patients from the TCGA dataset, based on LINC01116 expression. The LUAD patients with high expression of LINC01116 tended to have worse prognosis. B. Univariate Cox regression analysis of LINC01116 and other clinical factors in 448 LUAD patients in the TCGA dataset. T stage, N stage, TNM stage, and LINC01116 expression were closely related to overall survival and acted as risk factors of prognosis. C. Multivariate Cox regression analysis of LINC01116 and other clinical factors in 448 LUAD patients in TCGA dataset. LINC01116 was still significantly associated with prognosis after adjusting for clinical factors. D. The expression of LINC01116 among different TNM stages in the TCGA dataset. Patients in the advanced TNM stages were more likely to have higher expression of LINC01116. E. The expression of

LINC01116 among different T stages in the TCGA dataset. Patients with advanced T stage were more likely to have higher expression of LINC01116. F. The expression of LINC01116 among different N stages in the TCGA dataset. Patients in advanced N stage were more likely to highly express LINC01116. G. Univariate Cox regression analysis of LINC01116 and other clinical factors in 381 LUAD patients in the GEO datasets. LINC01116 and TNM stage were highly related to overall survival and acted as risk factors for prognosis. H. Multivariate Cox regression analysis of LINC01116 and other clinical factors in 381 LUAD patients in the GEO datasets. LINC01116 still was significantly associated with overall survival after adjusting for other clinical factors. I. The expression level of LINC01116 between TNM stage I and stage II/III in GEO datasets. The expression of LINC01116 in TNM stage I was significantly lower than that in stage II/III. ***P < 0.001.

Table 4. Average expression level of LINC01116 in different clinical stages

	Clinical stages	Average expression level
TCGA dataset	T1	0.987 ± 0.1583
	T2	1.222 ± 0.1408
	T3+4	2.657 ± 0.8565
	stage I	1.003 ± 0.1389
	Stage II	1.274 ± 0.1851
	Satge III/IV	2.181 ± 0.5197
	N0	1.126 ± 0.1375
	N1	1.510 ± 0.3524
	N2	1.979 ± 0.5988
GEO datasets	Stage I	5.334 ± 0.0843
	Stage II/III	5.914 ± 0.1327

TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus Data were shown as mean ± SD.

verify this result, we used qRT-PCR to measure the expression of LINC01116 in 21 paired LUAD tumor tissues and adjacent non-tumor tissues and found a consistent result: LINC-01116 expression was significantly higher in LUAD tissues (P = 0.0102, **Figure 1B**). Next, we detected the expression of LINC01116 in LUAD cell lines (A549, H1299, and PC-9) and normal bronchial epithelial cells (BEAS-2B) by qRT-PCR. Again, we found that the expression of LINC01116 in the three LUAD cell lines was significantly higher than in BEAS-2B (P < 0.0001 for H1299, P = 0.023 for PC-9, and P < 0.0001 for A549, **Figure 1C**). A549 cells, which showed the highest expression of LINC01116 compared with the others, were chosen for the subsequent experiments.

LINC01116 was a significant independent prognostic biomarker in LUAD

Kaplan-Meier survival analysis was performed on the TCGA dataset to evaluate the relationship between overall survival (OS) and the expression of LINC01116. High expression of LINC01116 was significantly associated with worse outcomes (P = 0.012, **Figure 2A**). Using the median expression value as a cut-off point, the low-expression group had a higher median survival (4.38 years) compared with that in high-expression group (3.37 years). The 3- and 5-year survival rates were 63.9% and 39.1% in the low-expression group, and 53.9% and 30.2% in high-expression group, respectively. To further explore the prognostic value of LINC01116 in LUAD, univariate Cox regression analysis was performed. Here, LINC01116 was a prognostic risk factor of LUAD patients (hazard ratio (HR) = 1.109, P < 0.0001, **Figure 2B**). Next, we performed multivariate Cox regression analysis to evaluate whether LINC01116 expression remained an independent prognostic risk factor in the presence of other clinical factors such as age, sex, T stage, N stage, and TNM stage. Indeed, LINC01116 was still an independent prognostic risk factor after adjusting for the other clinical factors (HR = 1.082, P < 0.0001, **Figure 2C**). Further, we analyzed the correlation between LINC0116 and T stage, N stage, and TNM stage among samples in the TCGA dataset. The expression of LINC01116 was higher in patients with advanced TNM stage (P < 0.0052; **Figure 2D**) and T stage (P = 0.0014, **Figure 2E**). However, although no significant difference was found between the expression of LINC01116 and N stage (P = 0.0998, **Figure 2F**), LINC01116 tended to be more highly expressed in patients with advanced N stage.

To further verify the prognostic value of LINC-01116, we analyzed LINC01116 in a dataset consisting of three GEO datasets (GSE31210, GSE50081, and GSE29013). Using the median expression of LINC01116 as the cut-off, the 3- and 5-year survival rates were 90.2% and 77.3% in the low-expression group and 79.2% and 69.2% in the high-expression gro-

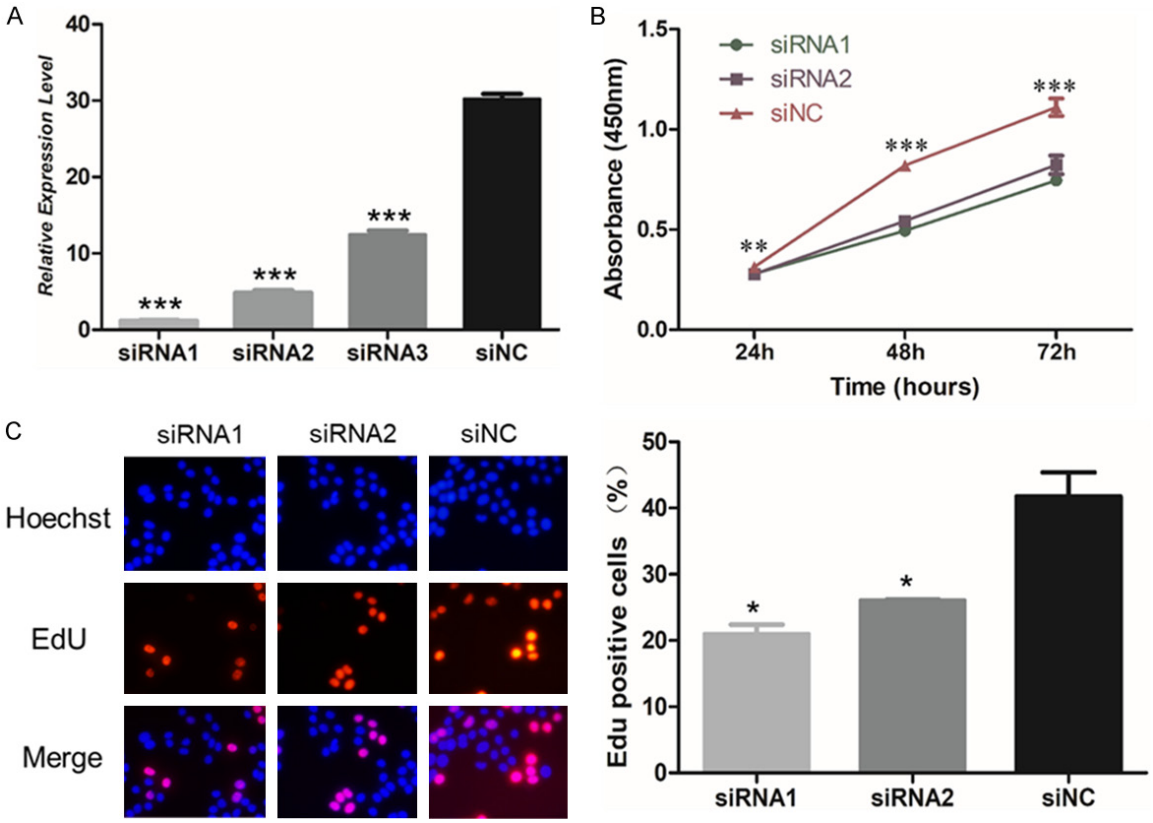

Figure 3. Downregulation of LINC01116 suppressed cell proliferation. A. qRT-PCR showed that the expression of LINC01116 in A549 cells was significantly reduced after transfection with siRNAs. ***P < 0.001. B. Transfected cells were seeded in 96-well plates. After incubation for 24, 48, and 72 h, cells were treated with 100 μl medium containing 10 μl CCK-8 solution for another 2 h. Absorbance at 450 nm was measured. The absorbance in LINC01116-downregulated groups was lower than the negative control group at 24 h, 48 h, and 72 h. **P < 0.01, ***P < 0.001. C. Transfected cells were seeded in 96-well plates and incubated for 24 h, then cultured for another 2 h after replacing the medium with 50 μM EdU-containing medium. Next, cells were fixed with 4% paraformaldehyde, and EdU-positive cells were stained with Apollo solution. DNA was stained with Hoechst reagents. Finally, we acquired the images and calculated the EdU positivity rates. The EdU positivity rate in LINC01116 downregulated groups was significantly lower than that of the negative control group. *P < 0.05.

up, respectively. Univariate Cox regression analysis showed that LINC01116 was closely related to prognosis (HR = 1.331, P < 0.001, **Figure 2G**). Further, multivariate Cox regression analysis demonstrated that LINC01116 was an independent prognostic biomarker after adjusting for age, sex, and TNM stage (HR = 1.276, P = 0.002, **Figure 2H**). The relationship between LINC01116 and TNM stage was further investigated. We found that LINC01116 was significantly correlated with the TNM stage: the expression of LINC01116 in LUAD patients with TNM stage I was significantly lower than in LUAD patients with TNM stage II/III (P = 0.0003, **Figure 2I**). The average expression of LINC01116 in each clinical subgroup is listed in **Table 4**.

Downregulation of LINC01116 suppressed cell proliferation

To better understand the role of LINC01116 in the development of LUAD, we transfected A549 cells with siRNAs to downregulate the expression of LINC01116. As expected, qRT-PCR showed that siRNA1 and siRNA2 significantly reduced the expression of LINC01116 compared with the negative control siRNA (**Figure 3A**). The knockdown rates were 96.1% for siRNA1, 84.0% for siRNA2, and 58.7% for siRNA3. Thus, siRNA1 and siRNA2 were chosen for further experiments and named the group transfected negative control siRNA as siNC group. The results of the CCK-8 assay indicated that absorbance at 450 nm (indicating cell proliferation)

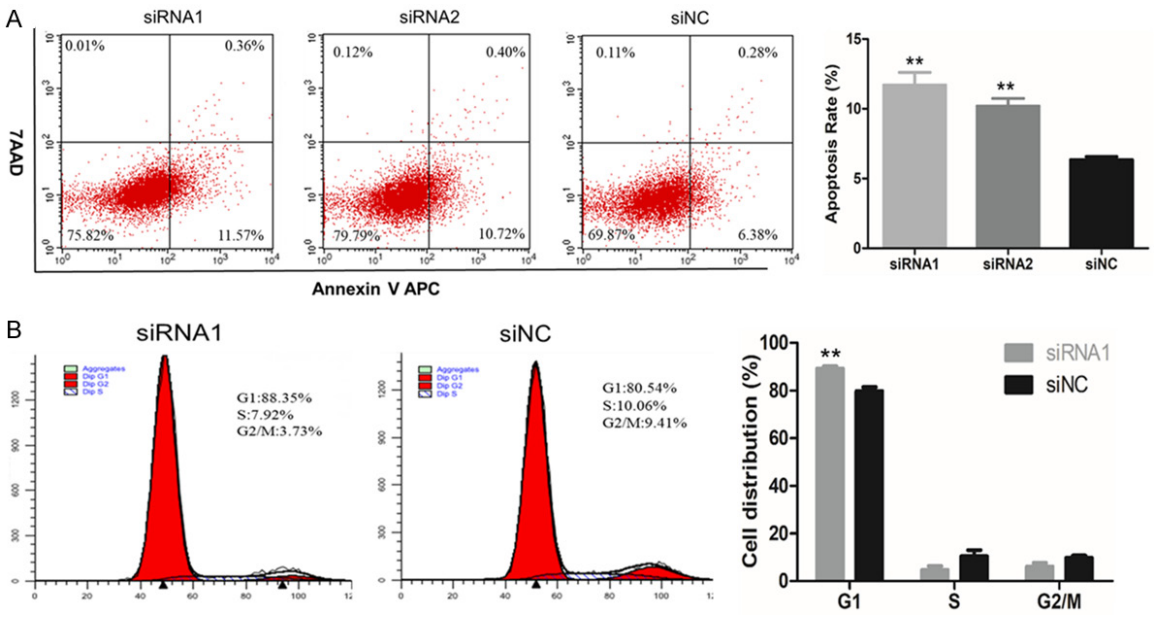

Figure 4. Downregulation of LINC01116 inhibited cell apoptosis and prevented G1 to S phase transition. A. After transfecting for 24 h, cells were digested, treated with 500 μl of binding buffer, 5 μl Annexin V-APC, and 5 μl 7-AAD at room temperature for 15 minutes in the dark. Finally, cell apoptosis was analyzed using a flow cytometer. Cell apoptosis in the siRNA1 and siRNA2 groups was significantly higher than the siNC group. **P < 0.01. B. After transfection for 24 h, cells were digested and fixed in pre-cooled 70% ethanol at -20°C overnight. On the second day, fixed cells were incubated with 500 μl working solution (RNase A: propidium iodide = 9:1) for 30 min at room temperature in the dark. Finally, cell cycle status was detected using a flow cytometer. The proportion of cells in G1 phase in the siRNA1 group was significantly higher than that in the siNC group. **P < 0.01.

was significantly reduced in the siRNA1 and siRNA2 groups at 24, 48, and 72 h compared with the siNC group (**Figure 3B**). The absorbance at 450 nm was 0.28 ± 0.01 for the siRNA1 group, 0.28 ± 0.01 for the siRNA2 group, and 0.31 ± 0.02 for the siNC group at 24 h. At 48 h, it was 0.49 ± 0.04 for the siRNA1 group, 0.54 ± 0.06 for the siRNA2 group, and 0.82 ± 0.04 for the siNC group. At 72 h, it was 0.75 ± 0.03 for the siRNA1 group, 0.82 ± 0.10 for the siRNA2 group, and 1.15 ± 0.08 for the siNC group. An EdU assay showed that the EdU-positive rates in LINC01116-knockdown groups were markedly lower than in the negative control group (**Figure 3C**). The EdU-positive rates were 20.95 ± 1.45% for the siRNA1 group, 26.00 ± 0.20% for the siRNA2 group, and 41.75 ± 3.65% for the siNC group. These results suggested that downregulation LINC01116 significantly suppressed cell proliferation.

Downregulation of LINC01116 promoted cell apoptosis and prevented cell cycle progression

Flow cytometry was used to detect changes in cell apoptosis and cell cycle status after decreasing the expression of LINC01116. Consistent with our prior experiments, downregulation of LINC01116 significantly promoted cell apoptosis (**Figure 4A**). The cell apoptosis rates were 11.70 ± 0.92% for the siRNA1 group, 10.19 ± 0.55% for the siRNA2 group, and 6.34 ± 0.23% for the siNC group. The cell cycle stage was also affected by the downregulation of LINC01116. Downregulation of LINC01116 prevented cells from transitioning from G1 to S phase (P = 0.0077, **Figure 4B**). The proportion of cells in G1 phase was 89.24 ± 0.92% in the siRNA1 group and 79.77 ± 1.67% in the siNC group.

Downregulation of LINC01116 suppressed cell migration

A Transwell migration assay and wound healing assay were used to detect changes in cell migration ability after downregulation of LINC01116. The number of cells migrating from the upper chamber to the lower chamber in the siRNA1 and siRNA2 groups was significantly lower than that in the siNC group (**Figure 5A**). The number of migrating cells in each 10x field

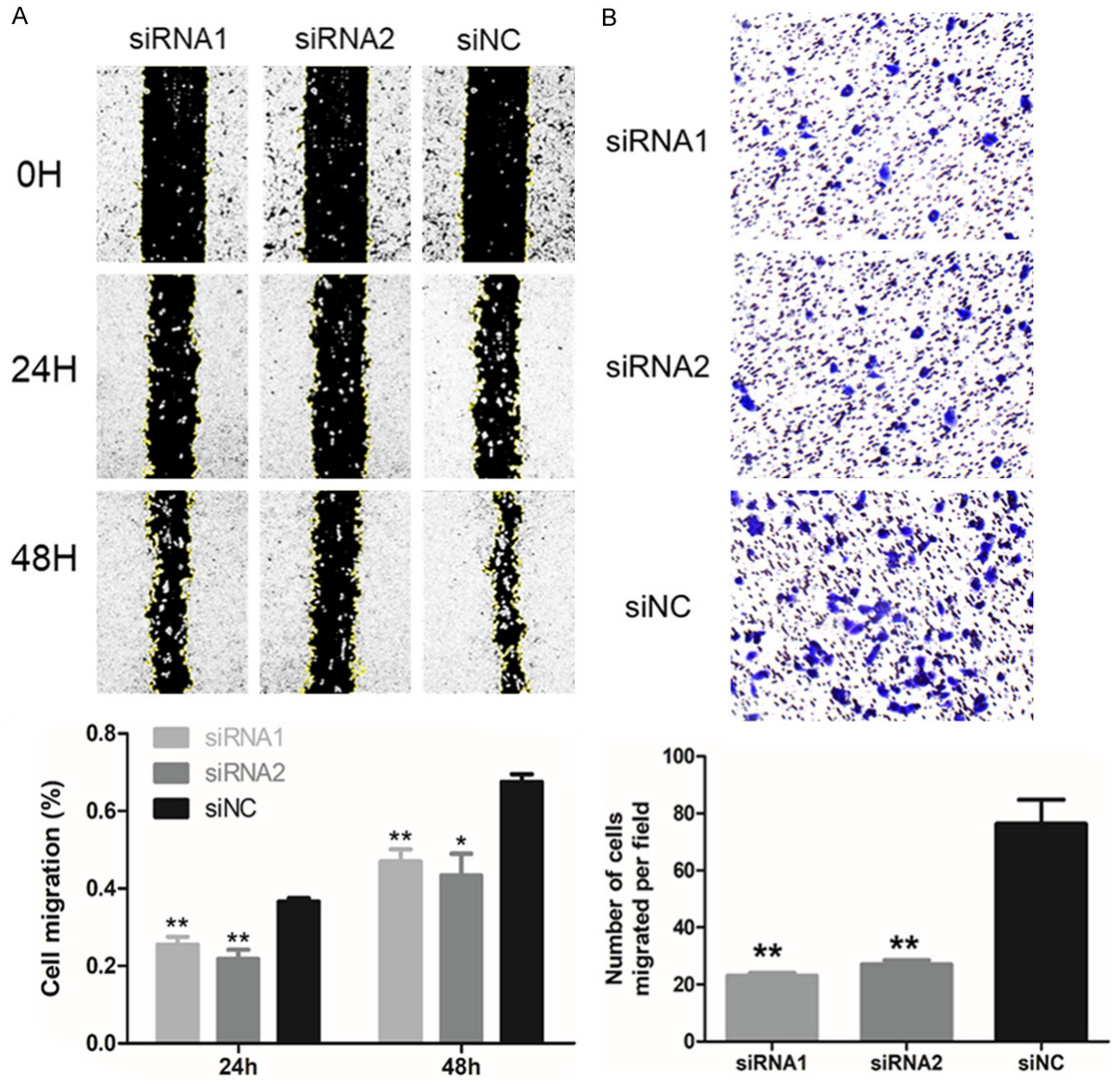

Figure 5. Downregulation of LINC01116 significantly suppressed LUAD cell migration. A. Scratches were made when transfected cells reached 100% confluence in 6-well plates and then, cells were cultured in low serum medium (1% FBS). Images were acquired at 0, 24, and 48 h after making scratches. The wound healing area in LINC01116-downregulated groups was significantly decreased after 24 h and 48 h. *P < 0.05, **P < 0.01. B. Transfected cells were seeded in upper chambers with 200 μl FBS-free medium, and 600 μl medium with 10% FBS was added to the lower chambers. After incubating for 24 h, the cells were fixed with paraformaldehyde and stained with crystal violet. We acquired images and calculated the number of cells that had migrated to the lower chamber. The number of migrated cells was significantly lower in the LINC01116-downregulated groups relative to the negative control group. **P < 0.01.

of view under the microscope was 23.00 ± 1.00 for the siRNA1 group, 27.00 ± 1.53 for the siRNA2 group, and 76.33 ± 8.45 for the siNC group. In the wound healing assay, the cell migration area in the LINC01116-knockdown groups was significantly less than in the negative control group (**Figure 5B**). The area of wound healing was 25.52 ± 1.95% for the siRNA1 group, 21.76 ± 2.41% for the siRNA2 group, and 36.54 ± 0.94% for the siNC group at 24 h. At 48 h, the area of wound healing was 46.95 ± 3.13% for the siRNA1 group, 43.31 ± 5.66% for the siRNA2 group, and 67.51 ± 1.99% for the siNC group.

Downregulation of LINC01116 suppressed epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a crucial mechanism in tumor metastasis. The-

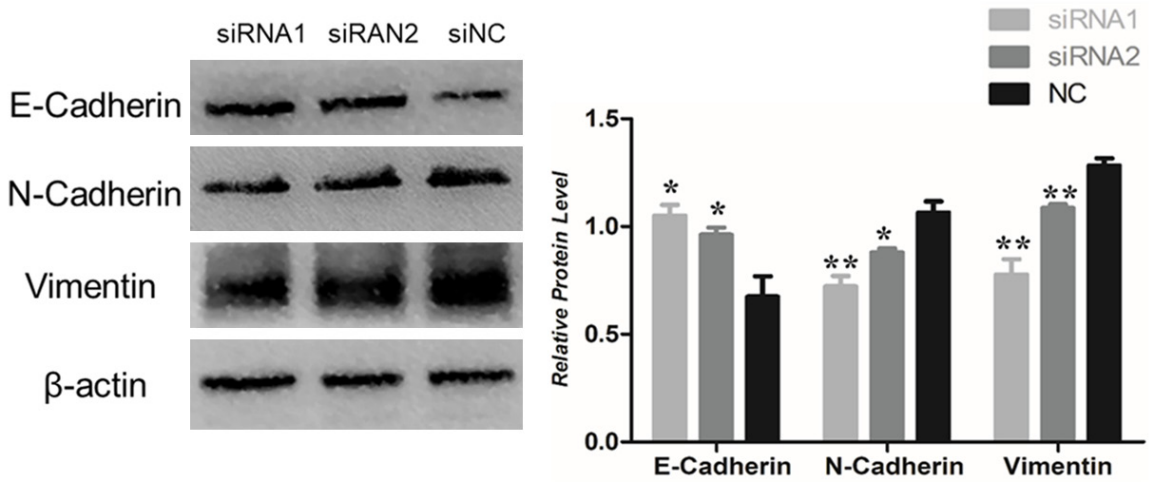

Figure 6. Downregulation of LINC01116 inhibited the EMT. Protein expression of the epithelial marker E-cadherin was upregulated, but the expression of the mesenchymal markers, N-cadherin and vimentin, were downregulated in LINC01116-downregulated groups relative to the negative control group. β-actin was used as a control. *P < 0.05, **P < 0.01.

refore, we detected the relative expression of EMT-related proteins, such as the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and vimentin. E-cadherin was significantly upregulated, but N-cadherin and vimentin were both downregulated in LINC01116-knockdown groups compared to the negative control group (**Figure 6**).

Discussion

Lung adenocarcinoma is a subtype of lung cancer with high morbidity and mortality [31]. The survival rate of patients with LUAD remains low because of a lack of effective early diagnostic markers and a high rate of metastasis [4]. In recent years, an increasing number of abnormally expressed genes have been found to play crucial roles in the occurrence and development of various cancers [11]. Thus, exploring the function and mechanisms of action of differentially expressed LncRNAs in LUAD may provide novel insights to set the foundation for new methods of diagnosis and treatment.

LncRNA LINC01116 is abnormally expressed in many kinds of human cancers [21, 23, 24]. As a result, the function of LINC01116 in various tumors has become a topic of increasing scientific interest. It has been suggested that LINC01116 is involved in biological processes such as cell growth, invasion, migration, and others. Previous studies have indicated that LINC01116 regulates the expression of target genes by acting as a ceRNA. For example, LINC01116 competes with miR-145 to regulate the expression of ESR1 in breast cancer [26]. Several reports have shown that LINC01116 affects tumor occurrence and progression via regulating known oncogenes or tumor suppressor genes such as *MYC* [22], *P53* [20], etc. One recent study suggested that a novel isoform of LINC01116 may act on sulforaphane to suppress prostate cancer [32]. Clearly, these reports confirm that LINC01116 plays an important role in the progression of several cancers. However, to date, only a few studies have assessed the relationship between LINC01116 and lung cancer, and the specific functions and mechanisms of LINC01116 in LUAD remain poorly understood.

This study provides the first analysis of the expression of LINC01116 in LUAD tissues and cell lines. LINC01116 was significantly overexpressed in LUAD tissues and cell lines relative to normal cells, and high LINC01116 expression was correlated with worse outcomes. Univariate and multivariate Cox regression analyses indicated that LINC01116 was an independent prognostic risk factor for LUAD. LUAD patients with advanced T stage, N stage, and TNM stage were more likely to have high expression of LINC01116. The expression of LINC01116 was positively correlated with other prognostic factors for LUAD, further confirming that LINC 01116 may serve well as a prognostic risk factor. These studies indicated that LINC01116 is

an independent predictor of prognosis in patients with LUAD.

Downregulation of the expression of LINC01116 in A549 cells affected many biological processes. The results of the CCK-8 and EdU assays indicated that cell proliferation was significantly suppressed after siRNA knockdown of LINC01116. To further explore the changes in apparent cell proliferation, we measured changes in cell apoptosis and cell cycle using flow cytometry. Downregulation of LINC01116 promoted cell apoptosis and prevented G1-S phase transition. These results suggested that LINC01116 may promote cell proliferation via inhibiting cell apoptosis and promoting cell cycle progression. In addition, Transwell and wound healing assays were used to measure changes in cell migration ability after knockdown of LINC01116. Cell migration was significantly suppressed in downregulated groups. Thus, we hypothesize that LUAD patients with high expression of LINC01116 are more likely to have metastases.

The EMT is a crucial stage of the progression toward metastasis, contributing to the local infiltration of cancer cells [33, 34]. Therefore, we detected the expression of EMT-related protein markers to determine whether downregulation of LINC01116 had an impact on EMT. Indeed, in LINC01116-knockdown cells, the expression of the epithelial marker E-cadherin was increased, but the expression of the mesenchymal markers, N-cadherin and vimentin, were both decreased. These results suggest that LINC01116 may promote the metastasis of LUAD by regulating EMT.

In conclusion, these experiments found that LINC01116 was overexpressed in LUAD tissues and cell lines and that increased expression was significantly associated with worse prognosis. Cox regression analysis demonstrated that LINC01116 was an independent prognostic risk factor for worse outcomes among patients with LUAD. Downregulation of LINC01116 markedly inhibited cell proliferation and migration, promoted cell apoptosis, and slowed progression through the cell cycle. Further, LINC01116 promoted the expression of EMT markers. Together, these results suggest that LINC01116 may be involved in the development and metastasis of LUAD and may have value as a prognostic indicator or therapeutic target.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (8167-2300).

Disclosure of conflict of interest

None.

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References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69: 7-34.
[2] Mao Y, Yang D, He J and Krasna MJ. Epidemiology of lung cancer. Surg Oncol Clin N Am 2016; 25: 439-445.
[3] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2017. CA Cancer J Clin 2017; 67: 7-30.
[4] Skřičková J, Kadlec B and Venclíček O. Non-small cell lung cancer. Vnitr Lek 2018; 63: 861-874.
[5] Imielinski M, Berger AH, Hammerman PS, Hernandez B, Pugh TJ, Hodis E, Cho J, Suh J, Capelletti M, Sivachenko A, Sougnez C, Auclair D, Lawrence MS, Stojanov P, Cibulskis K, Choi K, de Waal L, Sharifnia T, Brooks A, Greulich H, Banerji S, Zander T, Seidel D, Leenders F, Ansén S, Ludwig C, Engel-Riedel W, Stoelben E, Wolf J, Goparju C, Thompson K, Winckler W, Kwiatkowski D, Johnson BE, Jänne PA, Miller VA, Pao W, Travis WD, Pass HI, Gabriel SB, Lander ES, Thomas RK, Garraway LA, Getz G and Meyerson M. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. Cell 2012; 150: 1107-1120.
[6] Herbst RS, Morgensztern D and Boshoff C. The biology and management of non-small cell lung cancer. Nature 2018; 553: 446-454.
[7] Jarroux J, Morillon A and Pinskaya M. History, discovery, and classification of lncRNAs. Adv Exp Med Biol 2017; 1008: 1-46.
[8] Jathar S, Kumar V, Srivastava J and Tripathi V. Technological developments in lncRNA biology. Adv Exp Med Biol 2017; 1008: 283-323.
[9] Fang Y and Fullwood MJ. Roles, functions, and mechanisms of long non-coding RNAs in cancer. Genomics Proteomics Bioinformatics 2016; 14: 42-54.
[10] Sanchez Calle A, Kawamura Y, Yamamoto Y, Takeshita F and Ochiya T. Emerging roles of long non-coding RNA in cancer. Cancer Sci 2018; 109: 2093-2100.

[11] Balas MM and Johnson AM. Exploring the mechanisms behind long noncoding RNAs and cancer. Noncoding RNA Res 2018; 3: 108-117.
[12] Weidle UH, Birzele F, Kollmorgen G and Rüger R. Long non-coding RNAs and their role in metastasis. Cancer Genomics Proteomics 2017; 14: 143-160.
[13] Forrest ME and Khalil AM. Review: regulation of the cancer epigenome by long non-coding RNAs. Cancer Lett 2017; 407: 106-112.
[14] Kondo Y, Shinjo K and Katsushima K. Long non-coding RNAs as an epigenetic regulator in human cancers. Cancer Sci 2017; 108: 1927-1933.
[15] Li M, Wang Q, Xue F and Wu Y. lncRNA-CYTOR works as an oncogene through the CYTOR/miR-3679-5p/MACC1 axis in colorectal cancer. DNA Cell Biol 2019; 38: 572-582.
[16] Wei GH and Wang X. lncRNA MEG3 inhibit proliferation and metastasis of gastric cancer via p53 signaling pathway. Eur Rev Med Pharmacol Sci 2017; 21: 3850-3856.
[17] Botti G, Marra L, Malzone MG, Anniciello A, Botti C, Franco R and Cantile M. LncRNA HOTAIR as prognostic circulating marker and potential therapeutic target in patients with tumor diseases. Curr Drug Targets 2017; 18: 27-34.
[18] Jiang N, Meng X, Mi H, Chi Y, Li S, Jin Z, Tian H, He J, Shen W, Tian H, Pan J, Fang S, Jin X, Zhou C and Gong Z. Circulating lncRNA XLOC_009167 serves as a diagnostic biomarker to predict lung cancer. Clin Chim Acta 2018; 486: 26-33.
[19] Chandra Gupta S and Nandan Tripathi Y. Potential of long non-coding RNAs in cancer patients: from biomarkers to therapeutic targets. Int J Cancer 2017; 140: 1955-1967.
[20] Zhang ZF, Xu HH, Hu WH, Hu TY and Wang XB. LINC01116 promotes proliferation, invasion and migration of osteosarcoma cells by silencing p53 and EZH2. Eur Rev Med Pharmacol Sci 2019; 23: 6813-6823.
[21] Chen Z, Tao Q, Qiao B and Zhang L. Silencing of LINC01116 suppresses the development of oral squamous cell carcinoma by up-regulating microRNA-136 to inhibit FN1. Cancer Manag Res 2019; 11: 6043-6059.
[22] Xing H, Sun H and Du W. LINC01116 accelerates nasopharyngeal carcinoma progression based on its enhancement on MYC transcription activity. Cancer Med 2020; 9: 269-277.
[23] Ye J, Zhu J, Chen H, Qian J, Zhang L, Wan Z, Chen F, Sun S, Li W and Luo C. A novel lncRNA-LINC01116 regulates tumorigenesis of glioma by targeting VEGFA. Int J Cancer 2020; 146: 248-261.
[24] Su X, Zhang J, Luo X, Yang W, Liu Y, Liu Y and Shan Z. LncRNA LINC01116 promotes cancer cell proliferation, migration and invasion in gastric cancer by positively interacting with lncRNA CASC11. Onco Targets Ther 2019; 12: 8117-8123.
[25] Fang YN, Huang ZL, Li H, Tan WB, Zhang QG, Wang L and Wu JL. LINC01116 promotes the progression of epithelial ovarian cancer via regulating cell apoptosis. Eur Rev Med Pharmacol Sci 2018; 22: 5127-5133.
[26] Hu HB, Chen Q and Ding SQ. LncRNA LINC01116 competes with miR-145 for the regulation of ESR1 expression in breast cancer. Eur Rev Med Pharmacol Sci 2018; 22: 1987-1993.
[27] Zhang B, Yu L, Han N, Hu Z, Wang S, Ding L and Jiang J. LINC01116 targets miR-520a-3p and affects IL6R to promote the proliferation and migration of osteosarcoma cells through the Jak-stat signaling pathway. Biomed Pharmacother 2018; 107: 270-282.
[28] Zhang N, Shuai K, Cheng J, Yang W and Kan Z. LncRNA linc01116 prometes glioma cell migration and invasion by modulation of radixin targeted by miR-31. Int J Clin Exp Pathol 2019; 12: 1078-1086.
[29] Wang H, Lu B, Ren S, Wu F, Wang X, Yan C and Wang Z. Long noncoding RNA LINC01116 contributes to gefitinib resistance in non-small cell lung cancer through regulating IFI44. Mol Ther Nucleic Acids 2020; 19: 218-227.
[30] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.
[31] Nasim F, Sabath BF and Eapen GA. Lung cancer. Med Clin North Am 2019; 103: 463-473.
[32] Beaver LM, Kuintzle R, Buchanan A, Wiley MW, Glasser ST, Wong CP, Johnson GS, Chang JH, Löhr CV, Williams DE, Dashwood RH, Hendrix DA and Ho E. Long noncoding RNAs and sulforaphane: a target for chemoprevention and suppression of prostate cancer. J Nutr Biochem 2017; 42: 72-83.
[33] Lamouille S, Xu J and Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 2014; 15: 178-196.
[34] Micalizzi DS, Farabaugh SM and Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia 2010; 15: 117-134.