Original Paper

A Novel Long Non-Coding RNA, SOX21-AS1, Indicates a Poor Prognosis and Promotes Lung Adenocarcinoma Proliferation

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Key Words

Long non-coding RNA • SOX21-AS1 • Cell proliferation • p57 • Lung adenocarcinoma

Abstract

Background: In recent years, long non-coding RNAs (lncRNAs) have been shown to be a novel class of regulators of cancer biological processes. Although lncRNAs are dysregulated in numerous cancer types, limited data are available on the expression profiles and potential functions of lncRNAs in lung adenocarcinoma (LUAD). This study evaluated the expression and biological roles of lncRNA SOX21 antisense RNA 1 (SOX21-AS1) in LUAD. **Methods:** Quantitative reverse transcription PCR (qRT-PCR) was performed to detect the expression levels of SOX21-AS1 in 68 pairs of LUAD tissues and corresponding non-tumor tissues. The effect of SOX21-AS1 on proliferation was evaluated by MTT, colony formation, EdU assays, flow-cytometric analysis and *in vivo* tumor formation assays. Real-time PCR, western-blot and immunohistochemistry were used to evaluate the mRNA and protein expression of p57. **Results:** Higher expression levels of SOX21-AS1 positively correlated with tumor size and advanced tumor-node-metastasis (TNM) stage. Multivariate analyses indicated that SOX21-AS1 expression could serve as an independent prognostic factor for overall survival of LUAD. Furthermore, knockdown of SOX21-AS1 significantly inhibited LUAD cell proliferation both *in vitro* and *in vivo* and induced cell cycle phase arrest and cell apoptosis. Importantly, through qRT-PCR and western blot analysis, we found that inhibition of SOX21-AS1 remarkably induced p57 expression. **Conclusions:** Collectively, our study demonstrates that SOX21-AS1 is involved in the development and progression of LUAD and that SOX21-AS1 may be a potential diagnostic factor as well as a target for new therapies for patients with LUAD.

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Introduction

Despite the current advances in cancer surgery, chemotherapy and molecular targeting therapy techniques for lung cancer, it remains the predominant cause of cancer-related death worldwide [1, 2]. According to the National Center for Health Statistics (NCHS), lung cancer is expected to account for 27% of all male cancer deaths and 26% of all female cancer deaths in 2016 [1, 3]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases, and lung adenocarcinoma (LUAD) is its most common histological type [4]. Currently, the 5-year overall survival rate for NSCLC patients is still approximately 15% because patients are typically at advanced stages when diagnosed [5]. Therefore, a better understanding of the molecular mechanisms underlying LUAD development and progression could improve the early diagnosis, treatment and overall prognosis of this disease.

With the development and innovation of whole genome and transcriptome sequencing technologies in the past decade, we have realized that more than 90% of the total mammalian genome can be transcribed into non-coding RNAs [6-8]. Long noncoding RNAs (lncRNAs), which are longer than 200 nt in length [9, 10], are a class of non-protein-coding RNA that have been demonstrated to play essential roles in cellular development and differentiation, in the modulation of apoptosis and in multiple biological processes [11-15]. More importantly, numerous studies have revealed that aberrant lncRNA expression is involved in a wide range of human diseases, particularly cancers [16-24]. Mounting evidence in recent years has demonstrated that lncRNAs, such as MALAT1 [25], LINC00473 [26], AGAP2-AS1 [27] play crucial roles in NSCLC development and progression. LncRNA MALAT1 is a regulator of gene expression that governs the hallmarks of lung cancer metastasis. LINC00473 is a potential biomarker for the detection of lung cancers with inactivated LKB1. In our previous study, we found that p53-regulated TUG1 is downregulated in NSCLC and acts in part through the control of HOXB7 [28]. Upregulated LUADT1 could regulate LUAD proliferation via suppression of p27 expression [29]. In this regard, identifying cancer-associated lncRNAs is essential for understanding the progression of LUAD and for establishing better treatment methods.

In this study, we identified a new lncRNA, SOX21 antisense RNA 1 (SOX21-AS1), which is located at chromosome 13q32.1 and is transcribed into a 2986 nt transcript. SOX21-AS1 was remarkably overexpressed in LUAD tissues compared with corresponding non-tumor tissues. We then discovered that elevated expression of SOX21-AS1 was associated with poor patient prognosis. Knockdown of SOX21-AS1 was found to suppress the proliferation of LUAD cells both *in vitro* and *in vivo*. Furthermore, our results showed that SOX21-AS1 may act as an oncogene partly through the repression of p57 in lung cancer. Our results demonstrate that lncRNA SOX21-AS1 may represent a novel indicator of poor prognosis and is a potential biomarker for the diagnosis and treatment of LUAD.

Materials and Methods

Patients and tissue samples

A total of 68 primary NSCLC patients who had undergone surgeries at the First Affiliated Hospital of Nanjing Medical University (China) between 2012 and 2014 were enrolled in this study. None of these patients had received chemotherapy or radiotherapy prior to surgery. Histological classifications and tumor-node-metastasis (TNM) stages were determined according to the National Comprehensive Cancer Network (NCCN) criteria. This study was approved by the Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, People's Republic of China), and written informed consent was obtained from all participants. The clinical characteristics of the NSCLC patients are summarized in Table 1.

Cell cultures

Six human NSCLC adenocarcinoma cell lines (A549, SPC-A1, NCI-H1299, NCI-H1650, PC9, and H1975), three NSCLC squamous carcinomas cell lines (H1703, SK-MES-1 and H226), and a normal human bronchial

epithelial cell line (16HBE) were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 or DMEM (GIBCO-BRL) supplemented with 10% fetal bovine serum (10% FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in incubators at 37°C with 5% CO₂.

RNA extraction and qRT-PCR analyses

Total RNA was extracted from frozen tissues or cultured cells using the TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. For qRT-PCR, the isolated RNA was reverse transcribed to cDNA using a PrimeScript RT reagent Kit (Takara, Dalian China). Real-time PCR analyses were performed with SYBR Green (Takara, Dalian China). The results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The gene-specific primers were as follows: SOX21-AS1 forward, 5'-GGCTCAGGTTTAGGCGAGTG-3'; and reverse, 5'-AGAGGCTCTCCTACTCGTGC-3'; GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3'; and reverse, 5'-GCCAATACGACCAATCC-3'; p15 forward, 5'-GGACTAGTGGAGAAGGTGCG-3'; and reverse, 5'-GGGCGTGCCCATCATCATG-3'; p16 forward,

5'-CACCGAATAGTTACGGTCCG-3'; and reverse, 5'-GCACGGGTCCGGTGAGAGTG-3'; p21 forward, 5'-GTCCAC-TGGGCCGAAGAG-3'; and reverse, 5'-TGCGTTCACAGGTGTTTCTG-3'; p27 forward, 5'-TGCAACCGACGATT-CTTCTACTCAA-3'; and reverse, 5'-CAAGCAGTGATGTATCTGATAAACAAGG-3'; and p57 forward, 5'-CTAGC-CAGCAGGCATCGAG-3'; and reverse, 5'-GTGGTGGACTCTTCTGCGTC-3'. qRT-PCR and data collection were carried out on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expression of SOX21-AS1 was calculated and normalized using the 2^{-ΔΔCt} method relative to GAPDH.

siRNA and transfection of LUAD cells

Cells cultured on six-well plates were transfected with siRNA or non-specific control siRNA (si-NC) using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Non-specific siRNA (si-NC) was purchased from Invitrogen. Typically, cells were seeded on six-well plates and then transfected the next day with specific siRNA (100 nM) and control siRNA (100 nM) using Lipofectamine. Cells were harvested after 48 h for qRT-PCR and other experiments. The siRNA sequences were as follows: 5'AACAGAAACAGAGCUUCUCGCAUU3' (sense) and 5'AAUGCGAGAAGCCUCUGUUUCUGUU3' (antisense) for siRNA 1# and 5'CAGUUAACUACAGUGUCUCACUUA3' (sense) and 5'UAAGUGAGACACUGUAAGUUAACUG3' (antisense) for siRNA 2#.

Cell proliferation analyses and colony formation assays

Cell proliferation was measured using a Cell Proliferation Reagent Kit I (MTT) (Roche Applied Science). The A549 and NCI-H1299 cells were transfected with si-SOX21-AS1 or si-NC (3000 cells/well) and were plated in each well of 96-well plates. Cell viability was documented every 24 h according to the manufacturer's recommendations. For the cells to form colonies, a total of 700 si-SOX21-AS1 or si-NC A549 and NCI-H1299 cells were placed onto fresh six-well plates and maintained in media containing 10% FBS, replacing the medium every 4 d. After 2 weeks, the colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma, St. Louis, MO). The visible colonies were manually counted. Each experiment was repeated at least three times independently.

Table1. The clinic-pathological factors of 68 NSCLC patients

Clinical factors	Number of cases	(%) of patients
Sex		
Male	29	42.6
Female	39	57.4
Age		
≤60	36	52.9
>60	32	47.1
Histological grade		
High	13	19.1
Middle	19	27.9
Middle to low	12	17.6
Low	23	33.8
Other	1	1.5
Histological classification		
SCC (Squamous cell carcinoma)	39	57.4
AD (adenocarcinoma)	28	41.2
Other	1	1.5
Tumor stage		
I	21	30.9
II	27	39.7
III	18	26.5
IV	2	2.9
Tumor (T)		
T1	21	30.9
T2	28	41.2
T3	14	20.6
T4	5	7.4
Lymph node metastasis (N)		
N0	30	44.1
N1	31	45.6
N2	6	8.8
N3	1	1.5
History of smoking		
Ever	36	52.9
Never	32	47.1

Flow-cytometric analyses

Transfected cells were harvested 48 h after transfection by trypsinization. Subsequent double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) was performed using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. The cells were analyzed using a flow cytometer (FACScan; BD Biosciences) equipped with Cell Quest software (BD Biosciences). The cells were categorized as viable cells, dead cells, early apoptotic cells, or apoptotic cells, and then the relative ratios of apoptotic cells were compared with control transfection from each experiment. For the cell-cycle analysis, the cells were stained with PI using the CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences) following the manufacturer's protocol and were then analyzed by FACScan. The percentages of cells in the G0–G1, S, and G2–M phases were counted and compared. All of the samples were assayed in triplicate.

Ethynyldeoxyuridine (EdU) analysis

A 5-ethynyl-2-deoxyuridine (EdU) labeling/detection kit (Ribobio, Guangzhou, China) was used to assess cell proliferation. The cells were cultured in 96-well plates at 5×10^3 cells/well. Forty-eight hours after transfection, 50 μ M EdU labeling media was added to the 96-well plates, and they were incubated for 2 h at 37°C under 5% CO₂. After treatment with 4% paraformaldehyde and 0.5% Triton X-100, the cells were stained with the anti-EdU working solution. DAPI was used to label the cell nuclei. The percentage of EdU-positive cells was calculated after fluorescence microscopy analysis. Five fields of view were randomly assessed for each treatment group.

Tumor formation in nude mice

Five-week-old male athymic BALB/c nude mice were maintained under specific pathogen-free conditions and were manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. A549 cells were stably transfected with shSOX21-AS1 or empty vector using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were collected and harvested at a concentration of 2×10^7 cells/ml. One hundred microliters of the cell suspension was subcutaneously injected into a single side of the posterior flank of each mouse. Tumor volumes and weights were examined every 2 days in mice from the control (four mice) or shSOX21-AS1 (four mice) groups; the tumor volumes were measured ($\text{length} \times \text{width}^2 \times 0.5$). Sixteen days after injection, the tumors were removed from all of the animals and, after the tumor weights were measured, were used for further analysis. The primary tumors were excised, and the tumor tissues were used to perform qRT-PCR analysis of SOX21-AS1 expression levels and immunostaining analysis of Ki-67 and p57 protein expression.

Western blot assay and antibodies

Cell protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to 0.22- μ m nitrocellulose (NC) membranes (Sigma, USA), and incubated with specific antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). GAPDH antibody was used as control. Anti-p57 was purchased from Cell Signaling Technology (Boston, MA, USA).

Statistical analysis

All statistical analyses were performed using SPSS 22.0 software (IBM, SPSS, USA). The significance of differences between groups was estimated by Student's t-test and the χ^2 test as appropriate. The overall survival (OS) rates were calculated using the Kaplan–Meier method with the log-rank test applied for comparison. Survival data were evaluated using univariate and multivariate Cox proportional hazards models. Variables with a value of $P < 0.05$ in univariate analyses were used in subsequent multivariate analyses on the basis of Cox regression analyses. Two-sided P values were calculated, and a probability level of 0.05 was considered to be statistically significant.

Results

SOX21-AS1 expression is upregulated in human LUAD tissues and correlates with poor prognosis

We first analyzed RNA-Seq data (from TCGA: The Cancer Genome Atlas) for lncRNAs from lung adenocarcinoma tissues (n = 291) using the bioinformatics tool lncRNator (<http://lncrnator.ewha.ac.kr/index.htm>). The results revealed that SOX21-AS1 expression levels were upregulated approximately 5-fold in lung adenocarcinoma tissues (Fig. 1A). Then, we analyzed the expression of SOX21-AS1 in 40 LUAD tissue samples and adjacent

Fig. 1. Relative SOX21-AS1 expression in LUAD tissues and its clinical significance. A. RNA-Seq data from TCGA for lncRNAs in lung adenocarcinoma were analyzed using the bioinformatics tool lncRNator (<http://lncrnator.ewha.ac.kr/index.htm>). Lung adenocarcinoma: Normal (n = 46) and Tumor (n = 245). B. SOX21-AS1 expression in LUAD tissues (n=40) compared with non-cancerous tissues (n=40) analyzed using TCGA database. C. Relative expression levels of SOX21-AS1 in NSCLC tissues (n=68) compared with corresponding non-tumor tissues (n=68). SOX21-AS1 expression was examined using qPCR and was normalized to GAPDH expression. The Δ Ct value was determined by subtracting the GAPDH Ct value from the SOX21-AS1 Ct value (relative to a single reference value). Smaller Δ Ct values indicate higher expression. D. SOX21-AS1 expression was classified into two groups according to the median SOX21-AS1 expression level value in NSCLC tissue samples. E. SOX21-AS1 expression was significantly higher in patients with higher pathological stages (III/IV) than in those with lower pathological stages (I/II). F. SOX21-AS1 expression was significantly higher in patients with larger tumor sizes (>3 cm) than in those with smaller tumor sizes ($\leq$ 3 cm). G. Kaplan-Meier overall survival (OS) curves according to SOX21-AS1 expression levels. H. Kaplan-Meier survival plots demonstrating that high SOX21-AS1 expression levels correlated with worse OS in lung cancer patients (n = 1145). *, $P < 0.05$, and **, $P < 0.01$.

normal tissue samples from TCGA. As shown in Fig. 1B, SOX21-AS1 expression levels were significantly upregulated in cancerous tissues ($P < 0.01$). We also evaluated the expression levels of SOX21-AS1 in 68 pairs of LUAD tissues and adjacent normal tissues by qRT-PCR with normalization to GAPDH. As shown in Fig. 1C, SOX21-AS1 expression levels in tumors were significantly higher ($P < 0.05$) in 72.1% (49/68) of the samples compared to those of the corresponding normal counterparts, with a 4.29-fold upregulation. The clinical pathological features of the 68 lung adenocarcinoma cases are summarized in Table 1. To further understand the significance of SOX21-AS1 overexpression in LUAD, we used *t*-tests to examine the correlation of SOX21-AS1 expression levels and clinical-pathological factors in patients. The results demonstrated that there was an obvious positive correlation

between increased SOX21-AS1 levels and advanced tumor-node-metastasis (TNM) stage (6.3712 ± 7.88046 vs $2.48202.09170$, $P = 0.042$) and larger tumor size (4.7446 ± 5.64389 vs 1.4372 ± 1.18797 , $P < 0.001$). Patients in stages III/IV or with tumors sizes greater than 3 cm were associated with higher SOX21-AS1 expression levels, whereas patients in stages I/II or with tumors sizes less than 3 cm were associated with lower SOX21-AS1 expression levels (Fig. 1E and F). Furthermore, as shown in Fig. 1D, 68 LUAD patients were divided into two groups according to the median value of their SOX21-AS1 expression levels: a high-SOX21-AS1 group (above the median, $n = 34$) and a low-SOX21-AS1 group (below the median, $n = 34$). A Chi-squared test was performed to assess the clinical-pathological factors between the two groups. As shown in Table 2, the SOX21-AS1 expression levels also correlated with TNM stage ($P = 0.033$) and tumor size ($P < 0.001$). However, there was no remarkable relationship between SOX21-AS1 expression and other clinical factors such as gender ($P = 0.806$), age ($P = 0.627$), or smoking history ($P = 0.627$) in our study (Table 2).

To further evaluate the effects of SOX21-AS1 expression on the prognosis of patients with LUAD, we used Kaplan-Meier survival analysis and log-rank tests for overall survival (OS). As shown in Fig. 1G, the overall survival rate over 3 years for the high SOX21-AS1 group was 44.1%, while it was 23.5% for the low SOX21-AS1 group. The median survival time for the low SOX21-AS1 group was 46.061 ± 2.491 months, but it was only 31.736 ± 2.912 months for the high SOX21-AS1 group. Remarkably, overexpression of SOX21-AS1 was associated with shorter OS ($P = 0.001$). Similarly, the correlation between SOX21-AS1 expression levels and the survival of NSCLC patients was also supported by Kaplan-Meier Plotter analysis (www.kmplot.com), which indicated that higher SOX21-AS1 expression correlated with worse OS using publicly available data from 1145 lung cancer patients [30] (Fig. 1H).

Univariate analysis revealed that TNM stage (I/II, III/IV), histological grade (low, middle or high), and SOX21-AS1 expression levels could be viewed as prognostic factors (Table 3). However, there was no statistically significant correlation between SOX21-AS1 and other clinical-pathological characteristics, including sex (male or female) and age (≤ 60 or > 60). Multivariate analysis further showed that SOX21-AS1 expression was a significant independent predictor of poor survival for LUAD patients ($P = 0.005$), as was TNM stage ($P = 0.004$) and histological grade ($P = 0.010$). These results indicate that SOX21-AS1 could be a useful marker for the prognosis or progression of LUAD.

Table 2. Correlation between SOX21-AS1 expression and clinicopathological characteristics of 68 NSCLC patients. * $p < 0.05$. ** $p < 0.01$

Characteristics	Expression of SOX21-AS1		p value*
	low(n=34)	high(n=34)	
Sex			0.806
Male	14	15	
Female	20	19	
Age			0.627
≤ 60	19	17	
> 60	15	17	
Histological grade			0.145
Low or undiffer	15	21	
Middle or high	19	13	
Histological classification			0.806
SCC (Squamous cell carcinoma)	20	19	
AD (adenocarcinoma or other)	14	15	
TNM stage			0.033*
I and II	28	20	
III and IV	6	14	
Lymph node metastasis			0.066
Negative	17	13	
Positive	17	21	
Tumor size			$< 0.001^{**}$
≤ 3 cm	19	4	
> 3 cm	15	30	
History of smoking			0.627
Ever	19	17	
Never	15	17	

Table 3. Univariate and multivariate analysis of clinic pathologic factors for overall survival in 68 patients with NSCLC. *HR hazard ratio. * $p < 0.05$. ** $p < 0.01$

Risk factors	Univariate analysis			Multivariate analysis		
	HR*	p value	95% CI	HR	p value	95% CI
SOX21 expression	1.040	0.001**	1.015~1.065	1.080	0.005**	1.023~1.140
TNM stage (I / II, III/IV)	50.180	0.001**	4.509~558.462	2.838	0.004**	1.399~5.760
Histological grade (low, middle or high)	0.459	0.012*	0.250~0.844	0.429	0.010*	0.226~0.816
Tumor size (≤ 3 cm, > 3 cm)	1.778	0.081	0.931~3.396			
Histological classification (SCC, AD or another)	1.444	0.219	0.803~2.595			
Age (≤ 60 , > 60)	0.755	0.347	0.420~1.357			
N (negative, positive)	1.493	0.189	0.821~2.715			
History of smoking (ever, never)	0.954	0.876	0.529~1.721			
Sex (male, female)	1.266	0.431	0.704~2.276

Fig. 2. SOX21-AS1 promotes LUAD cell proliferation *in vitro*. A. Analysis of SOX21-AS1 expression levels in 9 NSCLC cell lines compared with a normal bronchial epithelial cell line (16HBE) using qRT-PCR. B. The relative expression levels of SOX21-AS1 in A549 and H1299 cells transfected with si-NC or si-SOX21-AS1 (si-SOX21-AS1 1# and si-SOX21-AS1 2#) were measured using qPCR. C. MTT assays were performed 24 h after transfection to determine the cell viability of the transfected A549 and H1299 cells. D. Colony-forming assays were conducted 24 h after transfection to determine the proliferation of the transfected A549 and H1299 cells. The colonies were counted and captured. E. EdU (red)/DAPI (blue) assays were conducted 24 h after transfection to determine the viability of the transfected A549 and H1299 cells. The data represent the mean $\pm$ SD from three independent experiments. *, $P < 0.05$, and **, $P < 0.01$.

Fig. 3. The effects of SOX21-AS1 on LUAD cell cycle and apoptosis *in vitro*. A, B. Forty-eight hours after transfection, the cell cycle stages of A549 cells were analyzed by flow cytometry. The bar chart represents the percentages of cells in the G1/G0, S, or G2/M phases, as indicated. C, D. A549 and H1299 cells were stained and analyzed by flow cytometry 48 h after transfection. LR, early apoptotic cells; UR, terminal apoptotic cells. All experiments were conducted in biological triplicates with three technical replicates. Data are presented as the mean $\pm$ SD. *, $P < 0.05$, and **, $P < 0.01$.

Fig. 4. SOX21-AS1 could regulate the expression of p57. A. The expression levels of cell cycle-related genes were determined after knockdown of SOX21-AS1 using qRT-PCR. Significant upregulation of p57 was observed in A549 and H1299 cells. B. Western blot assays confirmed the expression of p57 after transfection. GAPDH protein was used as an internal control. The results shown are from three independent experiments. *, $P < 0.05$, and **, $P < 0.01$.

Knockdown of SOX21-AS1 inhibits cell proliferation *in vitro*

To gain insight into the biological functional of SOX21-AS1 in LUAD cells, we first detected the expression of SOX21-AS1 in a normal human bronchial epithelial cell line (16HBE) and diverse human NSCLC cell lines using qRT-PCR analysis. As shown in Fig. 2A, SOX21-AS1 expression was significantly upregulated in two LUAD cell lines (A549 and H1299) compared with 16HBE. Thus, the A549 and H1299 cell lines were chosen for further study. Next, we synthesized two different siRNAs to silence endogenous SOX21-AS1 in A549 and H1299 cells. Forty-eight hours after transfection, SOX21-AS1 expression was knocked down by 79.6% in A549 cells and by 86.7% in H1299 cells relative to the control siRNA-transfected cells (Fig. 2B). Subsequent MTT assays demonstrated that knockdown of SOX21-AS1 expression significantly attenuated the cell viability rate in A549 and H1299 cells (Fig. 2C). In addition, colony-formation assays revealed that colony-forming ability was greatly reduced following downregulation of SOX21-AS1 in A549 and H1299 cells (Fig. 2D). Similarly, microscopic analysis of 5-ethynyl-2-deoxyuridine (EdU) (red)/DAPI (blue) immunostaining assays confirmed these findings (Fig. 2E). Collectively, these data indicate that SOX21-AS1 exerts an oncogenic effect to promote the proliferation of lung adenocarcinoma cells.

Fig. 5. SOX21-AS1 promotes the tumorigenesis of LUAD cells *in vivo*. A. Empty vector or sh-SOX21-AS1 was transfected into A549 cells, which were then injected individual into nude mice (n = 4). B. The tumor volumes were calculated every two days after injection. The mean tumor volumes are indicated by points, and the bars indicate SD (n = 4). C. The tumor weights are represented as the mean tumor weights $\pm$ SD. D. qRT-PCR analysis was performed to detect the average expression levels of SOX21-AS1 in the xenograft tumors (n = 4). E. The tumor sections were examined using H&E staining and IHC staining with antibodies against Ki-67 and p57. The error bars indicate the mean $\pm$ standard error. F. Correlation analysis revealed that SOX21-AS1 expression levels were inversely correlated with p57 expression levels in lung cancer from TCGA data. *, $P < 0.05$, and **, $P < 0.01$.

To further explore whether SOX21-AS1 impacts the proliferation of LUAD cells through cell cycle regulation, we tested cell cycle progression using flow cytometric analysis. The results showed that A549 cells transfected with si-SOX21-AS1 had cell-cycle arrest at the S phase compared with cells transfected with si-NC (Fig. 3A and B). Additionally, we performed flow cytometric analysis to determine whether cell proliferation was influenced by cell apoptosis. The results indicated that knockdown of SOX21-AS1 significantly induced LUAD cell apoptosis (Fig. 3C and D).

Knockdown of SOX21-AS1 induces p57 expression

To investigate the role of SOX21-AS1 in S arrest, we examined the effect of SOX21-AS1 silencing on the expression of cyclin-dependent protein kinase inhibitors via qRT-PCR. Strikingly, we discovered that the inhibition of SOX21-AS1 led to increased levels of p57 in both A549 and H1299 cells (Fig. 4A). To further determine whether the protein levels of p57 were increased with the knockdown of SOX21-AS1, we performed a Western blot assay. The repression of SOX21-AS1 increased the protein levels of p57 in both A549 and H1299 cells compared with control cells (Fig. 4B). Taken together, these results indicated that SOX21-AS1 promotes LUAD cell proliferation partially by silencing p57 expression.

Knockdown of SOX21-AS1 inhibits LUAD cell tumorigenesis in vivo

To confirm the effect of SOX21-AS1 expression on tumorigenesis *in vivo*, we used a xenograft mouse model. A549 cells stably transfected with sh-SOX21-AS1 or an empty vector were subcutaneously injected into male nude mice. We observed that all mice developed xenograft tumors at the injection site. As shown in Fig. 5A and B, SOX21-AS1 silencing substantially suppressed tumor growth compared to the control group. In addition, up to the 16th day after injection, the average tumor weight in the sh-SOX21-AS1 group was obviously lower than that in the control group (Fig. 5C). Subsequently, qRT-PCR analysis also revealed that the average expression level of SOX21-AS1 in tumors formed from sh-SOX21-

AS1 cells was lower than in tumors formed in the control group (Fig. 5D). Moreover, using immunohistochemical staining, the tumors developed from sh-SOX21-AS1 cells displayed lower Ki-67 staining and higher p57 staining than the tumors formed from empty vector transfected cells (Fig. 5E). Furthermore, correlation analysis revealed that SOX21-AS1 expression levels negatively correlated with p57 expression levels in lung cancer samples from TCGA data. These results further indicate the important role of SOX21-AS1 in lung adenocarcinoma cell proliferation.

Discussion

Over the past few decades, the roles of lncRNAs in human disease, including cancer, have attracted much attention [31-33]. Mounting studies have revealed that lncRNAs have crucial roles in various cancer types by functioning as potential oncogenes or tumor suppressors [18, 34-37]. For instance, lncRNA HOTAIR, a famous lncRNA, is a strong prognostic marker for diverse human cancers [38-40]. In our previous studies, we found that SP1-induced overexpression of the lncRNA TINCR promotes cell proliferation by influencing KLF2 mRNA stability in gastric cancer [41]. We also explored whether the E2F1-mediated upregulation of linc00668 predicts poor prognosis in gastric cancer and if it regulates cell proliferation via the silencing of CKIs [42]. However, only a handful of lncRNAs are known to be involved in NSCLC tumorigenesis as the study of NSCLC lncRNAs is an emerging field.

In the present study, we identified a novel lncRNA, SOX21-AS1, using bioinformatics methods. First, we analyzed the expression profiles of SOX21-AS1 in LUAD samples in the TCGA database and validated the results in a cohort of 68 pairs of LUAD tumorous and normal tissue samples. The results showed that SOX21-AS1 was upregulated in LUAD tissues and obviously correlated with poor prognosis and shorter survival. Specifically, patients with higher SOX21-AS1 expression levels seemed to harbor larger tumor sizes and were characterized by higher TNM stages. We further determined that SOX21-AS1 expression was an independent predictor for overall survival. Moreover, silencing SOX21-AS1 impaired cell proliferation and facilitated cell apoptosis *in vitro* and inhibited tumorigenesis of LUAD cells *in vivo*, implying that SOX21-AS1 could be a biomarker and therapeutic target for LUAD.

p57Kip2 is deemed to be a candidate tumor suppressor gene that blocks cell proliferation by inhibiting cell cycle progression and by promoting apoptosis and cell differentiation [43, 44]. The functional roles of p57 have been illustrated in carcinomas of the stomach, liver, prostate, breast, bladder, and other organs [45-49]. Therefore, the expression of p57 is frequently repressed in a wide variety of cancers to facilitate cell proliferation [50-52]. In this study, we examined the effect of SOX21-AS1 inhibition on the expression of several CKI family proteins involved in the S checkpoint of the cell cycle in A549 and H1299 cells. After inhibition of SOX21-AS1, we observed a significant increase in the expression of p57 (Fig. 4A, B). These data further verified that knockdown of SOX21-AS1 can induce p57 expression and SOX21-AS1 could play an important role in the cell cycle of LUAD. A large number of long non-coding RNAs have recently been reported to have a direct role in recruiting RNA-binding proteins to specific loci and in repressing gene expression in multiple cancers including NSCLC [23, 27, 53]. Thus, we hypothesize that SOX21-AS1 may repress p57 transcription via binding to related proteins, but further work is needed for confirmation.

Collectively, in this study, we clarified that SOX21-AS1 was upregulated in LUAD tumorous tissues and cell lines and correlated with poor prognosis in LUAD patients. Our findings imply that SOX21-AS1 could be a potential diagnostic and prognostic marker in patients with LUAD. In future studies, we will increase the number of samples for further investigation and attempt to elucidate the concise molecular mechanisms underlying the altered expression of SOX21-AS1 in LUAD.

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Disclosure Statement

The authors declare no conflicts of interest.

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