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Original Article

Upregulation of SPLUNC1, a novel prognostic predictor of lung adenocarcinoma, promotes cell proliferation and migration

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Abstract: Short palate, lung, nasal epithelium clone 1 (SPLUNC1) has been demonstrated to be involved in inflammatory responses to irritants in the upper airway. However, the function of SPLUNC1 in lung adenocarcinoma (ADC) and its clinical prognostic significance for this condition are unclear. Western blot and immunohistochemistry were carried out to analyze the expression of SPLUNC1 in lung ADC cell lines and tissue samples. Loss-of-function assays using SPLUNC1 targeted siRNAs were performed to determine its effects on proliferation, invasiveness, and migration of lung ADC cells *in vitro*. The expression of SPLUNC1 was significantly increased in lung ADC cell lines and tissues ($P<0.05$). High expression of SPLUNC1 was recorded in 54.7% (75 of 137) of lung ADC samples. Univariate and multivariate survival analyses showed that SPLUNC1 expression is an independent prognostic indicator of lung ADC patient survival ($P=0.002$). This was particularly true for acinar predominant ADC ($P=0.004$). Furthermore, disruption of endogenous SPLUNC1 through siRNA-mediated knockdown led to inhibition of the aggressive phenotype of lung ADC cells ($P=0.0124$ and $P=0.0084$). Our findings suggest that enhanced expression of SPLUNC1 is a predictor of poor prognosis of lung ADC, and plays an important role in disease progression.

Keywords: SPLUNC1, lung adenocarcinoma, prognosis, tumor progression

Introduction

Lung cancer is a frequent and important contributor to worldwide incidence of cancer and subsequent mortality [1]. Non-small-cell lung cancer (NSCLC) is thought to originate in epithelial cells, and is comprised of diverse histological subtypes. Lung adenocarcinoma (ADC) is the most common histological subtype of NSCLC, and in most countries accounts for almost half of all cases of lung cancer [2]. Despite improvements in diagnostic approaches and the introduction of new therapeutic agents over the last several years, the 5-year survival rate of patients diagnosed with lung cancer remains poor [3]. The aggressive and heterogeneous nature of lung cancer has thwarted efforts to reduce NSCLC-associated mortality. Hence, there is an urgent need to identify and establish reliable prognostic molecular markers that could assist in the clinical management of patients diagnosed with lung ADC.

Short palate, lung, nasal epithelium clone 1 (SPLUNC1) is one among the ten members of the PLUNC family, that are encoded by adjacent genes within a 300 kb region of chromosome 20. *SPLUNC1*, also referred to as *SPURT, LUNX,* or *NASG,* encodes a secreted protein found in abundance in the sputum and tracheobronchial secretions. Because of its structural homology with bactericidal permeability-increasing protein (BPI), SPLUNC1 was recently renamed BPI fold-containing protein, family A, member 1 (BPIFA1). SPLUNC1 plays a role in a number of physiological and pathological processes, including maintenance of liquid volume [4] and tension [5, 6] at the surface of airways, and mediating inflammatory response to irritants in

the upper airways [7-12]. In tumorigenesis, SPLUNC1 (LUNX) was identified as a marker of micrometastasis in NSCLC [13]. It was also identified as a marker to distinguish between gastric hepatoid adenocarcinoma and primary hepatocellular carcinoma [14]. However, despite its important diagnostic value in other forms of cancer, the expression and function of SPLUNC1 in lung ADC and its clinical prognostic significance for this condition is unclear.

In the present study, we examined the expression level of SPLUNC1 protein in primary ADC tissues using immunohistochemistry and western blot, and determined its correlation with clinicopathologic features, including the survival of patients diagnosed with lung ADC. Next, we performed RNA interference-mediated knockdown of SPLUNC1 expression to explore its role in regulating the properties and behavior of lung ADC cells. Our data shows that SPLUNC1 protein is higher in lung ADC tissues and cell lines, and that this upregulation is correlated with tumor progression and poor prognosis in patients. Furthermore, we demonstrate that knockdown of *SPLUNC1* expression inhibits proliferation, invasion and migration of lung ADC cells, thus playing an important role in disease progression. Our findings strongly suggest that increased SPLUNC1 expression level can be used to predict poor prognosis of lung ADC.

Materials and methods

Tumor tissue sample

Fresh and formalin-fixed, paraffin-embedded, lung tumor tissue samples were obtained from patients diagnosed with primary lung ADC in Nanfang Hospital, Southern Medical University (Guangzhou, China). The use of human tissues for the purpose of this study was approved by the ethics committee of Nanfang Hospital, Southern Medical University. Tumors were staged according to the newly revised 2009 TNM Classification for Lung Cancer. Pathological classification was based on the new edited multidisciplinary classification criteria [15]. None of the patients received pre-operative chemotherapy or radiotherapy. Because adjuvant chemotherapy was adopted as a standard therapeutic approach only since 2004, stage III lung ADC patients were subjected to systemic chemotherapy. On the other hand, no postoperative therapy was administered to patients at stages I-II. Patients diagnosed with stage III lung ADC and those displaying pathological evidence of N2 disease, received postoperative mediastinal radiotherapy. Patient follow-up was completed in June 2013, and ranged from 1-88 months. The median follow-up period was 18 months.

Cell lines and cell culture

Human lung ADC cell lines H322, SPC-A1, A549 and Glc-82 were obtained from a repository at the Chinese Academy of Sciences (Shanghai, China). All lines were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, USA) and 100 U/ml penicillin/streptomycin (Gibco). They were maintained in a humidified chamber at 5% CO_2 and 37°C.

Immunohistochemistry (IHC)

Immunohistochemical staining of tissue samples was performed according to previously described protocol [16]. Briefly, sections were incubated with anti-SPLUNC1 primary antibody (LifeSpan BioSciences, USA; 2.5 μg/ml) for one hour at room temperature (RT). Following incubation with the appropriate peroxidase-conjugated secondary antibody, expression patterns were visualized using the substrate diaminobenzidine (DAB) to generate a colored product. In negative control sections, the primary antibody was replaced by normal goat serum. The IHC-stained sections were observed and scored independently by two pathologists blind to the study. SPLUNC1 staining was evaluated using a previously described method [17, 18]. The following scores were assigned based on the intensity of staining- 0 (negative), 1 (weak), 2 (medium), and 3 (strong). To determine the extent of SPLUNC1 expression, the percentage of positively stained area within the entire area of the carcinoma or the entire tissue section of an unmodified sample, was calculated and scored as- 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The intensity and extent scores were summed to obtain the final staining score (0-7) for SPLUNC1 expression. Tumors with a final staining score of three or higher were considered SPLUNC1-positive.

SPLUNC1 knockdown

The following duplex siRNAs were synthesized by Gnepharma (Shanghai, China). Targeting human SPLUNC1 mRNA-5'-GCCTGAACAACAT-

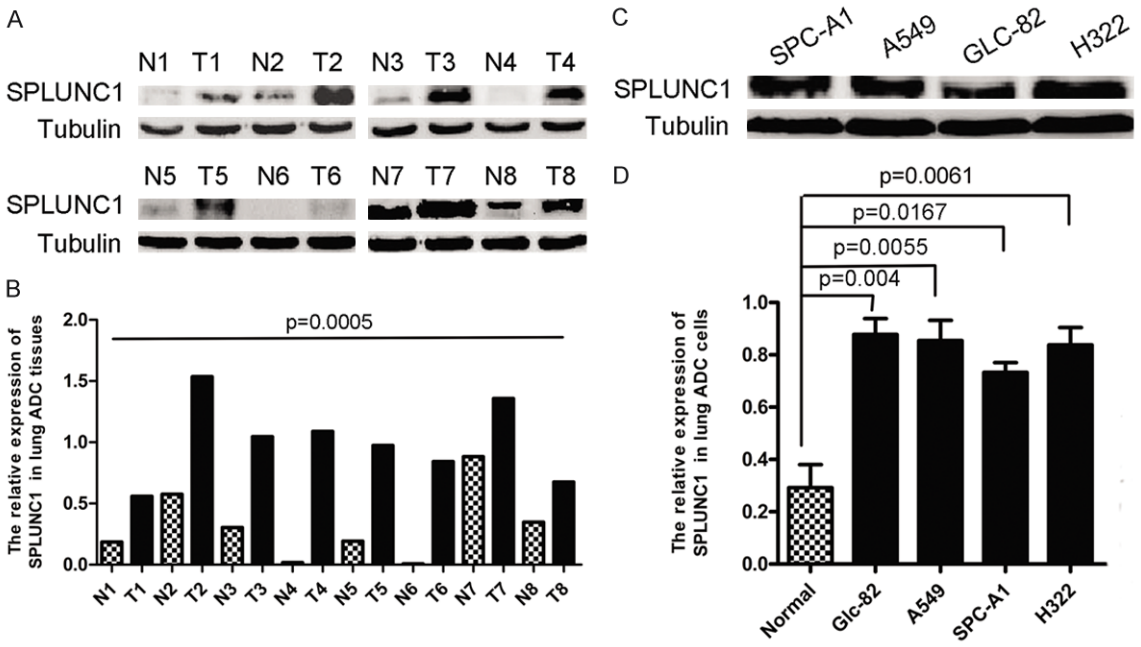

Figure 1. Expression analysis of SPLUNC1 in lung ADC tissues and cell lines by Western blotting. (A) Expression of SPLUNC1 was detected in lung ADC tissues. N=normal T=tumor. (C) Expression of SPLUNC1 was detected in lung ADC cell lines. (B and D) Signals from immunostaining were quantified by densitometric scanning. SPLUNC1 expression level was calculated as SPLUNC1 expression relative to Tubulin expression in lung ADC tissues (B) and cells (D). Data are presented as mean ± SD from three independent experiments.

CATT-GATT-3'); 2) Scrambled-(5'-TTCTCCGAACGTGTCACGTTT-3'). The scrambled sequence has limited homology to human genes, and served as a negative control. Oligonucleotides were transfected using Lipofectamine 2000 reagent (Invitrogen).

Cell proliferation assay

Cells were seeded into 96-well plates at 2×10^3 cells per well. Proliferation rate was estimated using the Cell Counting Kit-8 (CCK-8, Dojindo, Rockville, USA) assay, performed according to the manufacturer's instructions.

Wound healing and matrigel invasion assays

These assays were performed as described previously [19], and detailed in the Supplementary methods.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 statistical software package. Data from at least three independent experiments is presented as Mean ± SEM. Differences between variables were assessed using the following tests: χ^2 test, Fisher's exact test, or One-way ANOVA. In case of patients who displayed different levels of SPLUNC1 expression, survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Multivariate survival analysis was performed on all parameters that were found to be significant in univariate analysis using the Cox regression model. A *p*-value of less than 0.05 was considered statistically significant.

Results

SPLUNC1 expression is upregulated in lung ADC cell lines and tissues

To estimate the level of SPLUNC1 protein expression in lung ADC, we performed Western Blot analysis on samples obtained from four lung ADC cell lines, and eight ADC and paired normal lung tissue specimens. As shown in **Figure 1A**, we found a significant difference in relative SPLUNC1 expression level between ADC and paired normal lung tissues. We detected SPLUNC1 at significantly higher levels in tumor tissues, when compared with normal lung samples (*P<0.001*). In addition, SPLUNC1

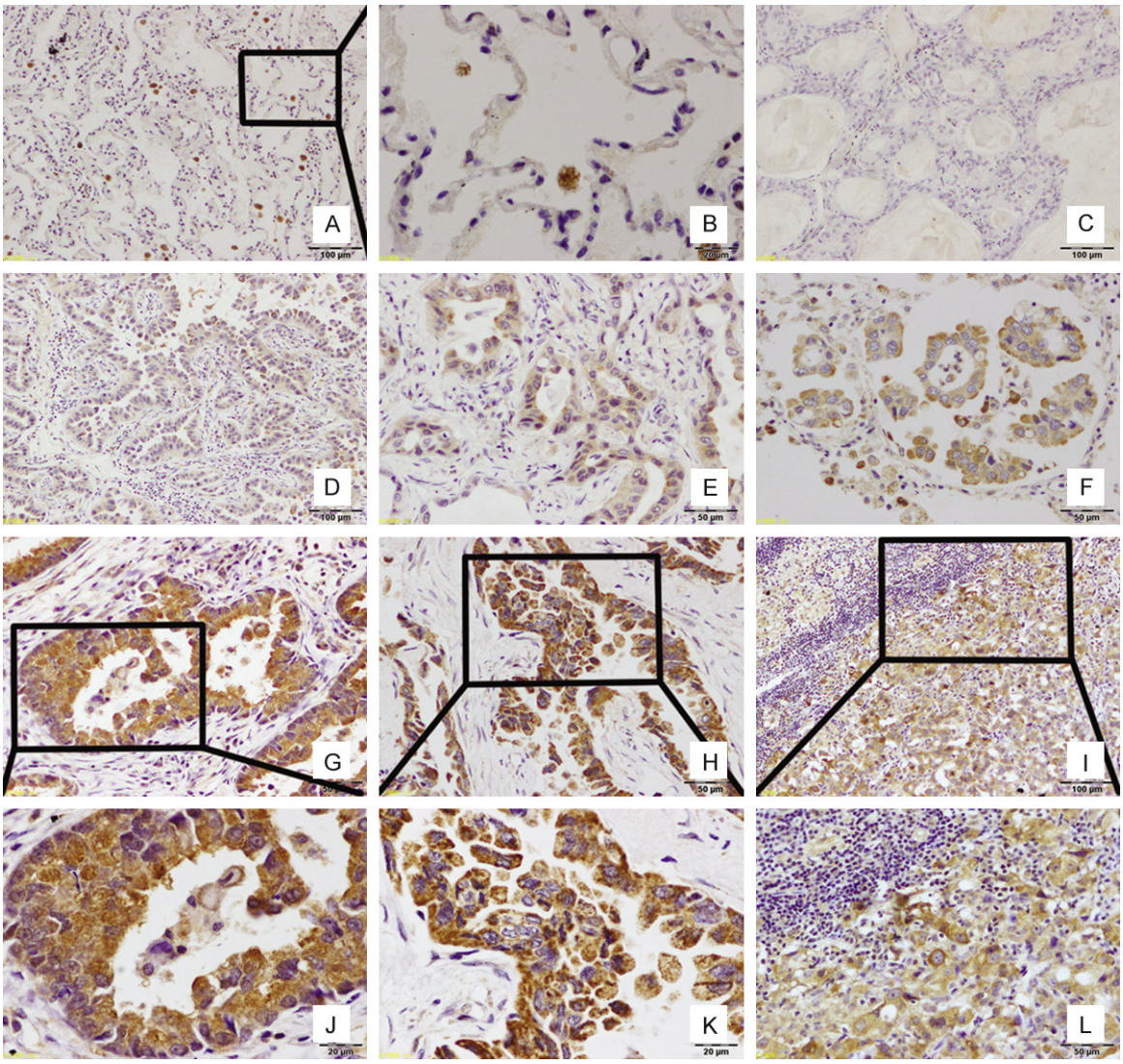

Figure 2. Expression analysis of SPLUNC1 in lung ADC tissues by immunohistochemistry. A and B. Lack of expression of SPLUNC1 in adjacent normal lung tissue. C. Lack of expression of SPLUNC1 in lung ADC tissue. D-H, J and K. Positive expression of SPLUNC1 in lung ADC tissues. I and L. Expression of SPLUNC1 in lung ADC lymph node with metastasis.

protein levels were significantly higher in four lung ADC cell lines, in comparison to all non-cancerous tissue samples (*P<0.05*, **Figure 1C**).

Upregulation of SPLUNC1 is associated with aggressive lung ADC patient phenotype

To determine the consequence of increased SPLUNC1 expression on disease pathology in lung ADC patients, we first analyzed its protein level in an independent set of 137 paraffin-embedded, archival primary lung ADC tissues using immunohistochemical staining. As shown in **Figure 2**, SPLUNC1 protein was mainly localized to the cytoplasm of cancerous cells. According to the reclassification guidelines described above, SPLUNC1 expression levels were designated as high in 54.74% (75 out of 137) lung ADC samples. While macrophages in the lung tissues also stained positive for SPLUNC1, we detected no specific expression in normal pulmonary alveolar epithelium cells (**Figure 2A**, **2B**).

Next, all lung ADC patients were divided into two groups according to their IHC evaluation score. The relationship between the clinicopathologic features exhibited by these patients and SPLUNC1 expression levels in their lung tissues, is summarized in **Table 1**. We found no

Table 1. Correlation between the clinicopathogic features and expression of SPLUNC1

Characteristics	n	SPLUNC1 expression		
		Low (%)	High (%)	*P* value
Gender				
Male	82	38 (46.34)	44 (53.64)	0.755
Female	55	24 (43.64)	31 (56.36)	
Age (years)				
<50	26	9 (34.62)	17 (65.38)	0.226
≥50	111	53 (47.75)	58 (52.25)	
Smoking status				
Smokers	63	27 (42.86)	36 (57.14)	0.603
Non-smokers	74	35 (47.30)	39 (52.70)	
Pathologic classification				
Adenocarcinoma in situ	8	6 (75.00)	2 (25.00)	0.124
Minimally invasive adenocarcinoma	5	3 (60.00)	2 (40.00)	
Invasive ADC, lepidic predominant	6	4 (66.67)	2 (33.33)	
Invasive ADC, acinar predominant	99	42 (42.42)	57 (57.58)	
Invasive ADC, papillary predominant	3	3 (100.00)	0 (0.00)	
Invasive ADC, micropapillary predominant	4	1 (25.00)	3 (75.00)	
Invasive ADC, solid predominant	6	1 (16.67)	5 (83.33)	
Invasive mucinous ADC	6	2 (33.33)	4 (66.67)	
T-stage				
1-2	98	50 (51.02)	48 (48.98)	0.032
3-4	39	12 (30.77)	27 (69.23)	
N-stage				
Negative	79	46 (58.23)	33 (41.77)	<0.001
Positive	58	16 (27.59)	42 (72.41)	
Distant metastasis				
Negative	95	50 (52.63)	45 (47.37)	0.009
Positive	42	12 (28.57)	30 (61.43)	

significant association between SPLUNC1 expression and the age, gender, smoking status, or histological classification of lung ADC patients ($P>0.05$). Interestingly however, SPLUNC1 expression positively correlated with T stage ($P=0.032$), N stage ($P<0.001$), and distant metastasis (M classification, $P=0.009$) in patients.

Upregulation of SPLUNC1 is correlated with poor prognosis of lung ADC patients

To evaluate the prognostic value of SPLUNC1 in lung ADC patients, we next analyzed the association between SPLUNC1 expression level and duration of patient survival using Kaplan-Meier analysis and the log-rank test. Our analysis shows that there is a significant correlation between SPLUNC1 protein expression in lung ADC tissues overall survival of patients (log-rank test statistic= 10.002, $P=0.002$, **Figure 3A**). Similarly, SPLUNC1 expression also significantly correlated with overall survival of invasive ADC patients (log-rank test statistic=10.703, $P=0.001$) and acinar predominant invasive ADC patients (log-rank test statistic=8.441, $P=0.004$; **Figure 3B**, **3C**). The log-rank test indicated that the length of patient survival differed significantly between groups that displayed high *versus* low levels of SPLUNC1 protein. This shows that increased SPLUNC1 expression is positively correlated with a shorter survival period of lung ADC patients.

Next, we asked whether SPLUNC1 expression could serve as an independent prognostic factor of disease outcomes. To address this question, we performed multivariate survival analysis including the parameters, gender, age, smoking status, histological classification, T stage, N stage, M stage and SPLUNC1 expression level. Based on the results of this analysis, we identified SPLUNC1 protein level as a reliable and independent prognostic factor of disease outcome in lung ADC patients (**Table 2**). Altogether, our findings strongly suggest that SPLUNC1 protein expression correlates significantly with prognosis of lung ADC, especially its invasive form.

SPLUNC1 promotes proliferation, migration and invasion of lung ADC cells in vitro

To further explore the function of SPLUNC1 in lung ADC pathology, we examined changes in the aggressive phenotypic properties of lung ADC cells following knockdown of SPLUNC1 expression. To achieve knockdown, we trans-

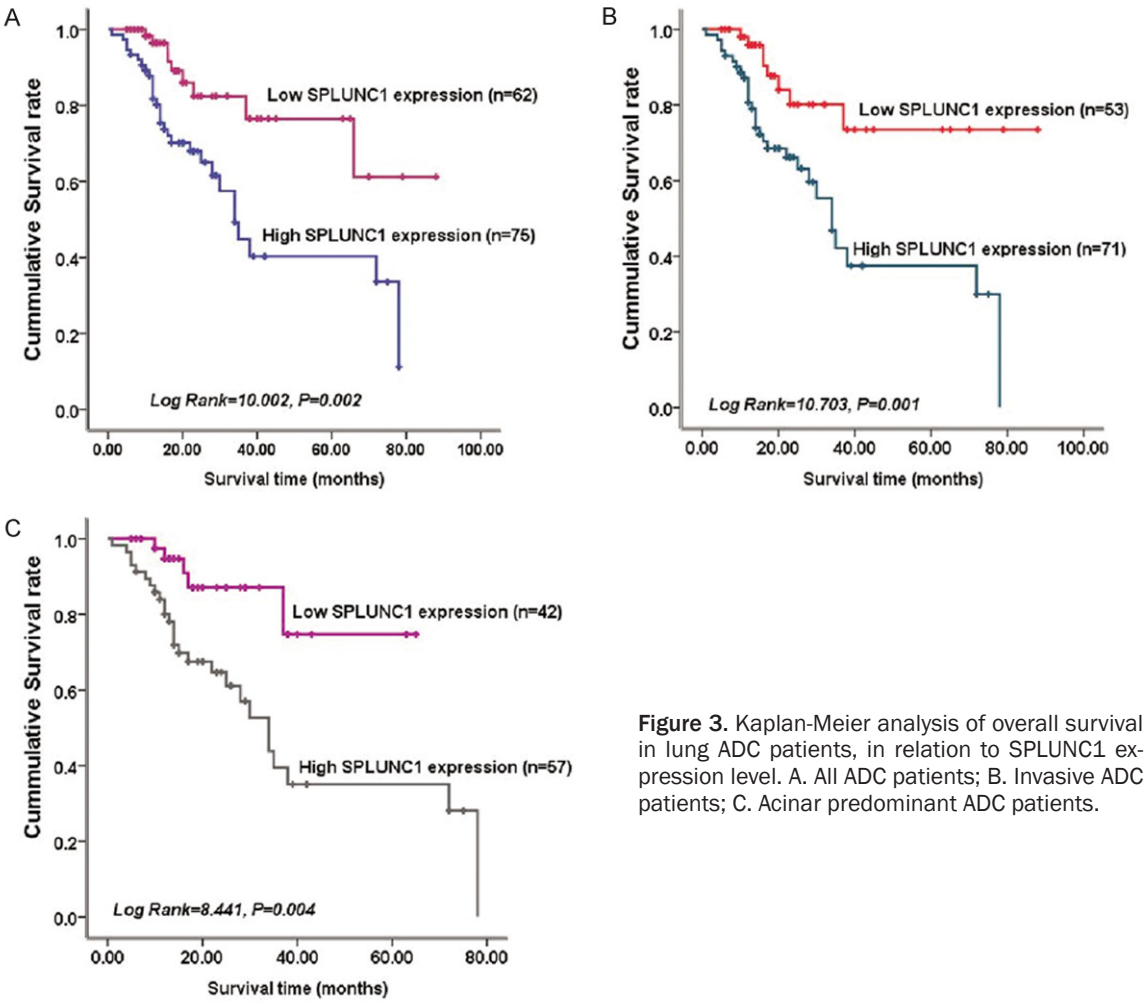

Figure 3. Kaplan-Meier analysis of overall survival in lung ADC patients, in relation to SPLUNC1 expression level. A. All ADC patients; B. Invasive ADC patients; C. Acinar predominant ADC patients.

Table 2. Summary of Overall survival analyses by univariate and multivariate COX regression analysis

Variables	Univariate analysis			Multivariate analysis		
	P value	HR	95% Confidence interval	*P* value	HR	95% Confidence interval
Gender	0.331	1.364	0.730-2.549			
Age	0.589	0.805	0.366-1.771			
Smoking	0.874	1.052	0.564-1.963			
Pathologic classification	0.982	0.998	0.821-1.213			
T-stage	0.051	1.871	1.087-3.794			
N-stage	0.036	2.025	1.047-3.915	0.377	1.377	0.677-2.801
M-stage	0.018	2.275	1.148-4.505	0.175	1.648	0.810-3.395
SPLUNC1 expression	0.003	3.088	1.467-6.500	0.015	2.603	1.206-5615

fected A549 and SPC-A1 cells with siRNA targeting SPLUNC1 transcripts. The efficiency of knockdown and the subsequent decrease in SPLUNC1 protein levels was confirmed by western blot analysis (**Figure 4A**).

Next, we subjected knockdown and scrambled-transfected control cells to CCK-8 assay, in order to measure differences in the rate of proliferation. We found that transient transfection with SPLUNC1-targeted siRNA, leads to a decrease in the growth rate of A549 and SPC-A1 cells. We estimated a significantly slower proliferation rate in SPLUNC1 knockdown cells when compared with scrambled cells (**Figure 4B**, **4C**). Next, we determined the effect of

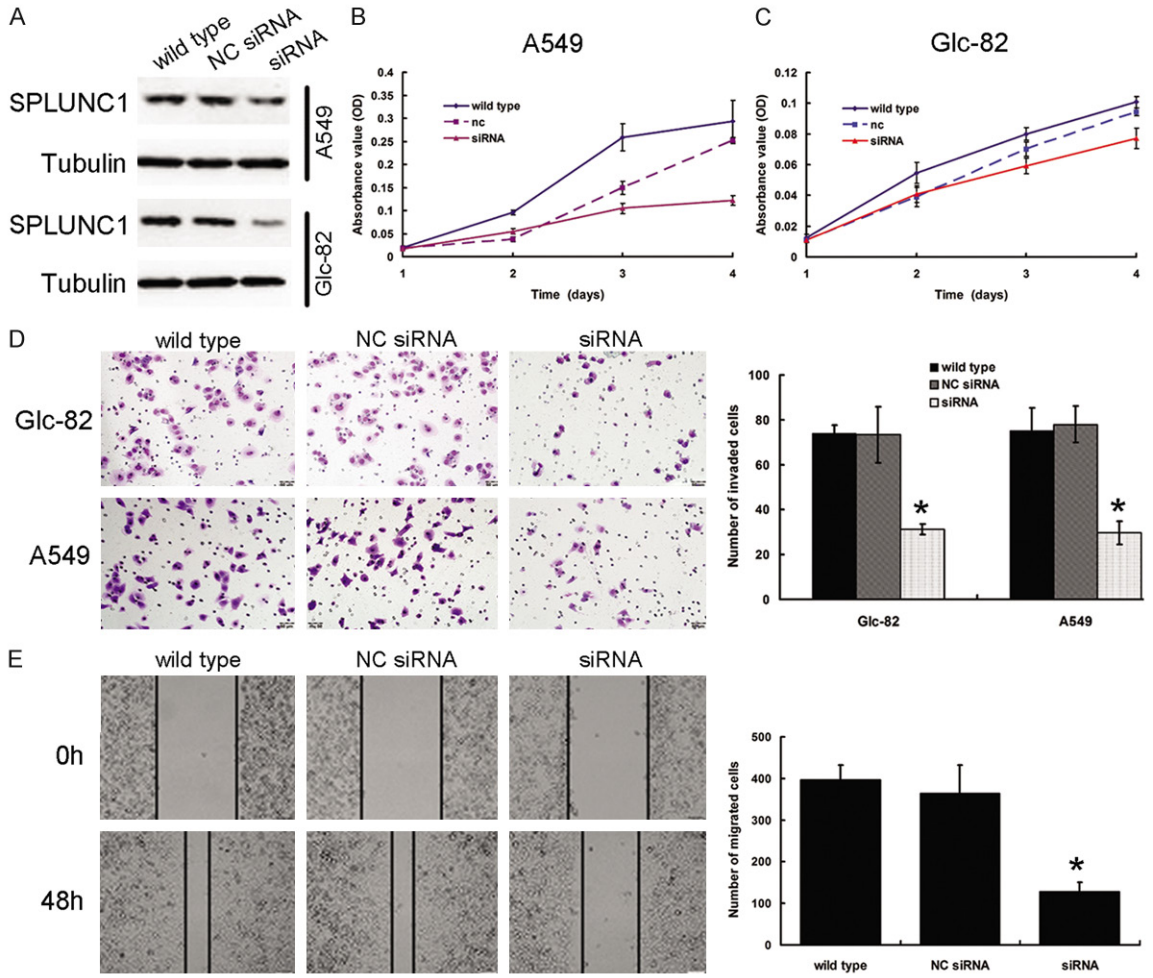

Figure 4. SPLUNC1 promotes aggressive phenotypic behavior in lung ADC cells. (A) The efficiency of siRNA-mediated knockdown of SPLUNC1, compared to scrambled negative control (NC) and healthy cells, is determined by western blot analysis. (B and C) The rate of cell proliferation in vitro estimated by CCK-8 assay. (D and E) Effects of SPLUNC1 downregulation on cell motility and invasiveness determined using the matrigel invasion assay (D) and wound healing assay (E). Data are presented as mean ± SD. Results were reproducible in three independent experiments. **P*<0.05.

SPLUNC1 on the invasive capacity of lung ADC cells, using the wound-healing and matrigel invasion assays. As shown in **Figure 4D**, results of the matrigel invasion assay indicated that downregulation of SPLUNC1 markedly reduced the invasiveness of lung ADC cells (A549: *P=0.0124*; SPC-A1: *P=0.0084*). Similarly, the wound-healing assay also demonstrated that functional deficiency of SPLUNC1 strongly reduced the migration of cancer cells (*P=0.0138*, **Figure 4E**). Altogether, our findings suggest that SPLUNC1 function is essential for cell migration and invasion during lung ADC.

Discussion

SPLUNC1 was first identified as a protein with widespread expression in the palate, nasal septum, nasal conchae, adult trachea, and bronchi of the lung [20]. It encodes a secreted protein found in abundance in the human sputum and tracheobronchial secretions, saliva [21], nasal lavage fluid [9], and apical secretions of cultured human tracheobronchial epithelial cells [12, 22]. In the past, various antimicrobial activities have been attributed to SPLUNC1, in addition to evidence that it might function as a host defense protein [7-12]. However, the role of SPLUNC1 in tumorigenesis is not fully known or understood. Its expression has been detected in colorectal cancer, esophageal cancer, breast cancer, hepatocellular carcinoma, and lung cancers with a glandular phenotype [13, 23]. Using semi-quantitative RT-PCR, Iwao et al. [13] showed that SPLUNC1 expression was

enhanced in 84% (26 out of 31) NSCLC tumors, relative to the surrounding normal tissues. The authors also detected SPLUNC1 in 16 out of 20 (80%) lymph node specimens obtained from NSCLC patients showing metastasis. In contrast, none of the healthy control lymph nodes (0 out of 16) expressed this protein, suggesting that SPLUNC1 could serve as a potential molecular marker for detecting and monitoring the spread of cancer cells to lymph nodes in NSCLC patients. SPLUNC1 was also shown to be a marker that could reliably distinguish between gastric hepatoid adenocarcinoma and primary hepatocellular carcinoma [14]. Interestingly, studies on nasopharyngeal carcinoma identified SPLUNC1 as a tumor suppressor protein that inhibited tumor growth *in vitro* [24]. Others have shown that overexpression of SPLUNC1 in deficient cancer cells leads to downregulation of miRNA-141 [25, 26]. Altogether, these findings indicate that the role of SPLUNC1 in cancer progression is not completely clear. In the present study, we demonstrate a significantly higher expression of SPLUNC1 in lung ADC cell lines and clinical patient samples, when compared with control groups. Importantly, we found that SPLUNC1 protein expression in all healthy lung tissue specimens was either absent or low. In contrast, we frequently detected upregulation of SPLUNC1 in most lung ADC specimens, which indicated a potentially important role in lung carcinogenesis. Statistical evaluation further showed that enhanced expression of SPLUNC1 is closely associated with an aggressive lung ADC patient phenotype, those diagnosed with T-stage, lymph node metastasis and distant metastasis. Our results also suggest that the level of SPLUNC1 protein expression is closely correlated with overall survival of lung ADC patients. Altogether, we show that high SPLUNC1 expression is a reliable and significant predictor of poor prognosis in lung ADC patients.

Lung ADC tissues are characterized by complex histological heterogeneity, leading to different prognosis in different histological subtypes. Because pre-invasive lesions (associated with minimally invasive ADC) are associated with favorable overall survival, which is different from invasive lung ADC, we also analyzed the merits of SPLUNC1 as a predictor of overall survival of patients diagnosed with invasive lung ADC. Moreover, since the primary histological subtype in the cases examined in this study is acinar predominant ADC, we also investigated the relationship between SPLUNC1 expression and the duration of survival of acinar predominant lung ADC patients. Our analysis revealed that the survival period is significantly different between patient groups displaying high *versus* low expression level of SPLUNC1. Specifically, we found that high expression is correlated with shorter duration of survival of invasive ADC patients, especially those diagnosed with the acinar predominant subtype. Our findings are consistent with a previous study on gastric cancer, where upregulation of SPLUNC1 was found to be correlated with an advanced stage and/or poor prognosis [14].

Thus far, no studies have reported the function of SPLUNC1 in lung cancer. However, it has been shown to inhibit cell proliferation in nasopharyngeal carcinoma by modulating the mitogen-activated protein kinase (MAPK) pathway [24]. In the present study, we provide the first evidence of the role of SPLUNC1 in regulating the behavior of lung ADC cells *in vitro*. Specifically, we show that knockdown of SPLUNC1 inhibits cell proliferation, migration and invasion, suggesting its role in tumor progression *in vivo*. Our *in vitro* functional assays, combined with IHC analysis, strongly suggest that enhanced SPLUNC1 expression promotes tumor progression in lung ADC, and is consequently, a novel predictor of poor prognosis.

Finally, some investigators have addressed the role of Toll-like receptor (TLR) signaling on SPLUNC1 expression [27-29]. For example, they showed that SPLUNC1 expression is significantly upregulated in nasal polyp epithelial cells after stimulation with TLR agonists [29]. TLR2 was shown to induce MAPK/activator protein-1 (AP-1) signaling leading to increased SPLUNC1 expression in human lung epithelial cells [28]. TLRs are important components of innate immunity, and function as pivotal elements in mucosal host defense. Moreover, tumor cells express TLRs on their surface and are therefore, directly activated by endogenous or pathogen-derived ligands to secrete inflammatory cytokines and chemokines. These small molecules can further induce tumor cell proliferation, promote angiogenesis, and facilitate invasion and metastasis [30-32]. Based on the findings that TLRs affect tumor progression, and that activation of TLRs could lead to SPLUNC1 expression, we propose the following

hypothesis-Induction of SPLUNC1 following TLR activation could play an important mechanistic role in the initiation and progression of tumorigenesis in lung ADC. However, further studies are needed to explore the underlying molecular mechanisms mediated by SPLUNC1, and clarify its involvement with the onset and development of lung cancer, and possibly other forms of cancer in future.

Our findings have extended previous findings about the role of SPLUNC1 in cancer progression. We show that SPLUNC1 expression is upregulated in lung ADC, which is in turn significantly associated with tumor progression in patients, leading to poor clinical outcome. We have identified SPLUNC1 as a novel and reliable prognostic factor for lung ADC patients. Our findings further suggest that SPLUNC1 could be investigated as a potential therapeutic target against lung ADC.

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Disclosure of conflict of interest

None.

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