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Original Article

The association between SLFN5 expression and the prognosis of non-small cell lung cancer

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Abstract: Schlafen-5 (SLFN5) is one member of the Schlafens (SLFNs) family, which have significant effect on cell cycle and tumorigenesis. However, it remains unclear about the function of SLFN5 in the Non-small cell lung cancer (NSCLC). The aim of this study is to explore the underlying function of SLFN5 in the NSCLC. The results demonstrated that SLFN5 was weakly expressed in almost NSCLC tissues. Low-expression of SLFN5 protein was significantly associated with a number of clinicopathological variables, including tumor diameter, T classification, N classification and clinical stage. More importantly, the overall survival was obviously improved in NSCLC patients with high-expression of SLFN5 protein. In multivariate analysis, SLFN5 expression was significantly connected with early N classification. In addition, patients with high SLFN5 expression in N0 stage had improved survival. Furthermore, our results found that the knockdown of SLFN5 could increase the expression of migration-associated protein matrix metalloproteinase 9 gene (MMP-9) and the ability of A549 cell migration. Taken together, all data suggest that SLFN5 may be an important factor to improve the prognosis in NSCLC patients. It could be a potential biomarker to identify the prognosis of NSCLC patients.

Keywords: SLFN5, NSCLE, prognosis, migration

Introduction

Lung cancer is one of most prevalent causes of cancer-related death worldwide, and the mortality for lung cancer are still augmenting yearly [1, 2]. According to 2015 Cancer Statistics, approximately 1.8 million new lung cancer cases, account for about 13% of all cancers [2]. Small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are two major pathology types of lung cancer. NSCLC accounts for over 80% of all lung cancers and has a fatal survival outcome. With poor treatment, only less than half of lung cancer patients have more than one year overall survival. Despite of almost complete and presumably curative with multimodality therapy, 5-year survival rate for patients is only approximately 15% [3]. Poor prognosis results from tumor metastasis, invasion, and recurrence. The mechanism of pneumonocyte transformation will help explore new therapies for lung cancer. Abnormal activation of signaling pathways and mutations of genetics which regulate cell proliferation, apoptosis, metastasis, invasion and so on have been deemed as an oncogenic actuator for cancer development.

Type I IFNs, and cytokines, utilized for anti-cancer treatment are known to accelerate antineoplastic [4-7], and induce upregulation of Schlafen (SLFN) [8-10]. SLFNs, a gene superfamily include 10 murine and 5 human members that share structural homology and are involved in thymocyte maturation and T cell activation mediating cell proliferation [11, 12], immune responses and viral replication [13-16], and especially retroegulation of tumorigenesis lately [10, 17, 18]. Nevertheless, the mechanisms of antineoplastic by different types of malignant human SLFNs remain unclear. Notably, human SLFN5 is reported to inhibit malignant melanoma cells after IFN treatment [19], and reduce motility and invasiveness of renal cell carcinoma (RCC) cells [9]. However, the role of SLFN5 in NSCLC is largely unknown.

Table 1. Clinicopathological features of l NSCLC patients and SLFN5 expression

Characteristics	n (%)
Gender	
Female	41 (45.6)
Male	49 (54.4)
Age (years)	
< 55	20 (22.2)
≥ 55	70 (77.8)
Tumor diameter (cm) c	
< 4	40 (44.4)
≥ 4	50 (55.6)
T classification	
T1	17 (18.9)
T2	50 (55.5)
T3	16 (17.8)
T4	7 (7.8)
N classification	
N0	39 (52)
N1	15 (20)
N2	17 (22.7)
N3	4 (5.3)
Pathological grade	
I	12 (13.3)
II	70 (77.8)
III	8 (8.9)
Clinical stage	
I	28 (32.6)
II	18 (20.9)
III	39 (45.3)
IV	1 (1.2)
SLFN5	
Low expression	34 (37.8)
High expression	56 (62.2)

T, tumour size; N, lymph node.

In the present study, we have examined the expression of SLFN5 in NSCLC tissue microarray by immunohistochemistry analysis (TMA-IHC), and focused on the correlation between SLFN5 expression and clinicopathological characteristics of NSCLC. Moreover, we also found that low-expression SLFN5 accelerated the migration in A549 cells, lung cancer cells. Therefore, our study demonstrated that SLFN5 has an important anti-tumor effect in NSCLC, which may provide a novel understanding of molecular mechanism in the migratory process of lung cells and a new target in NSCLC therapy.

Materials and methods

Tissue microarray

Immunohistochemistry analysis was performed from 90 pairs of NSCLC and adjacent non-cancerous tissue specimens submitted by Outdo Biotech Co. Ltd (Shanghai, China). All of patients had a pathological diagnosis of lung adenocarcinoma without lung transplantation, radiation therapy, adjuvant chemotherapy or immunotherapy before or after surgery. Overall survival was the survival time from the surgery to death or the last follow-up interview. According to 2002 American Joint Committee on Cancer/International Union Against Cancer TNM staging system [20], all samples was identified with TNM stage. The features of lung adenocarcinoma patients are summarized in **Table 1**. Ethical approval was acquired from the Ethics Committee of Taizhou Hospital. In addition, the study got the informed consent of all patients or their relatives.

Immunohistochemistry

With streptavidin-peroxidase (SP) method, sections from NSCLC and adjacent non-cancerous tissues were deparaffinized, hydrated, and immersed in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker, followed by heat-induced epitope retrieval for 5 min. Endogenous peroxidase was quenched with 10% BSA for 30 min. Subsequently, sections were incubated with the primary antibody Schlafen5 (ab121537, 1:200; PBS as the negative control) overnight at 4°C. Then, the sections were incubated with biotinylated goat anti-mouse IgG (Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, China; 1:100) at room temperature (RT) for 20 min. Visualization of sections was completed with 3,3'-diaminobenzidine (DAB) and coun-terstaining with haematoxylin. Finally, sections were observed under a bright-field micro-scope.

Evaluation of staining

The slides with immunohistochemical staining were reviewed and scored separately by two pathologists. SLFN5 protein expression was based on the percentage of immunoreactivity and staining intensity in tissue cells [21]. Any disagreements were reinvestigated by both pathologists again. The percentage of positive-

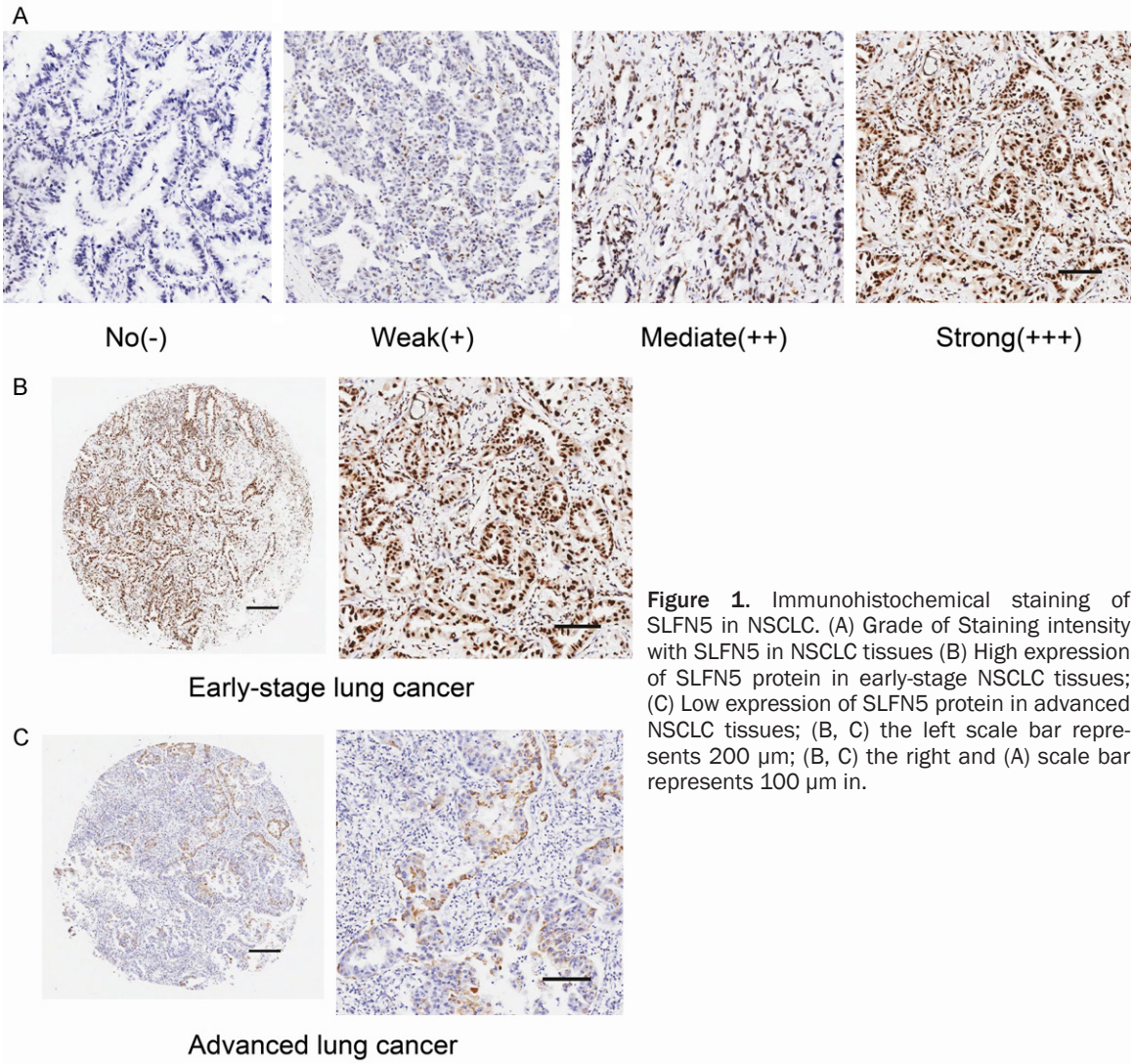

Figure 1. Immunohistochemical staining of SLFN5 in NSCLC. (A) Grade of Staining intensity with SLFN5 in NSCLC tissues (B) High expression of SLFN5 protein in early-stage NSCLC tissues; (C) Low expression of SLFN5 protein in advanced NSCLC tissues; (B, C) the left scale bar represents 200 μm; (B, C) the right and (A) scale bar represents 100 μm in.

staining cells was scored as follows: < 5% = 0; 5~25% = 1; 25~50% = 2; 50~75% = 3; ≥ 76% = 4. The staining intensity was graded as: 0 = no staining, 1 = light yellow staining, 2 = yellow staining and 3 = brown staining (**Figure 1A**). The final index of staining was defined by multiplication of proportion and intensity [22, 23]. The staining score of SLFN5 protein expression ranged from 0 to 12, and 0-4 and 6-12 represented low and high expression, respectively.

Cell culture and transfection

A549 cells were cultivated in RPMI-1640 (Gibco, BRL Co. Ltd, USA) supplemented with 10% fetal bovine serum (Gibco, BRL Co. Ltd, USA), penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C with 5% CO_2. When cells confluence reached 50%, SLFN5 siRNA (50 nM) (GenePharma, Shanghai, China) was transfected into A549 cells by Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA). After 36-48 h, all cells were executed scratch wound healing assay or western blot.

Scratch wound healing assay

Scratch wounds were scraped by a yellow pipette tip in the monolayer cells when transfected A549 cells reaching 95%-100%, then washed off cell debris with PBS. After the processing, scratch wounds were observed at different time points (12 and 24 h) using microscope and time lapse pictures were acquired with a SPOT Diagnostic CCD camera. All pictures were taken from five disparate fields in each sample and all wound pictures were mea-

Table 2. Correlation between the clinicopathologic characteristics and expression of SLFN5 protein in NSCLC patients

Characteristics	n	SLFN5		*P*
		High expression	Low expression	
Gender				
Female	41	17 (41.5)	24 (58.5)	0.509
Male	49	17 (34.7)	32 (65.3)	
Age (years)				
< 55	20	8 (40.0)	12 (60.0)	0.816
≥ 55	70	26 (37.1)	44 (62.9)	
Tumor diameter (cm)				
< 4	40	21 (40.0)	19 (60.0)	0.010*
≥ 4	50	13 (37.1)	37 (62.9)	
T classification				
T1	17	13 (76.5)	4 (23.5)	0.000*
T2-T4	73	21 (28.8)	52 (71.2)	
N classification				
N0	39	20 (51.3)	19 (48.7)	0.038*
N1-N3	36	10 (27.8)	26 (72.2)	
Clinical stage				
I	28	15 (53.6)	13 (46.4)	0.044*
II-III	58	18 (31.0)	40 (69.0)	
Pathological grade				
I	12	8	4	0.058
II-III	78	26	52	

*$P < 0.05$; T, tumour size; N, lymph node.

sured with NIH Image J software (National Institutes of Health, Bethesda, MD, USA).

Western blotting

Transfected A549 cells were lysed in RIPA lysis buffer (Beyotime, Jiangsu, China), and pro-tein concentrations was measured by BCA reagent kit (Merck, Darmstadt, Germany). Equal amounts of cellular proteins (30 ug) was separated by 10% SDS-PAGE gels electrophoresis, electro-transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Subsequently membranes were blocked with 5% (w/v) no-fat milk in Tris-buffered saline with Tween-20 for 1 h at room temperature. The blots were detected with rabbit polyclonal antibody against Shlafen5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000), MMP-9 (Abcam, Cambridge, MA, USA; 1:2000) and mouse monoclonal anti- beta-actin (Santa Cruz Biotechnology; 1:3000) was used as internal control. With incubation in goat IGg anti-rabbit IgG-HRP (Santa Cruz Biotechnology) and goat Ig antimouse IgG-HRP (Santa Cruz Biotechnology), western Blot chemiluminescent were visualized using the ChemiDoc MP imaging system (Bio-Rad, Hercules, CA).

Statistical analysis

All analyses of tissue microarray were performed using SPSS for Windows Version 17.0 (SPSS Inc, Chicago, IL). The correlation between the clinicopathological features and SLFN5 expression was analyzed by χ^2 test. Overall survival rate was performed using the Kaplan-Meier method with log-rank test. Cox proportional hazards regression models were performed to analyze significant variables in the multivariate analysis. All the data of scratch wound healing assay and Western blot were presented as the mean ± SD. Unpaired two-tailed Student's *t*-test was employed for the statistical analysis of differences by GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). All the experiments were performed independently at least three times. All significant analysis was set at $P < 0.05$.

Results

SLFN5 expression in NSCLC tissues

To confirm whether SLFN5 was involved in the cancer progression of NSCLC, we examined expression of SLFN5 in 90 pairs of NSCLC and adjacent non-cancerous tissue specimens from the microarray data of Outdo Biotech Co. Ltd. Lower expression of SLFN5 was observed in advanced NSCLC tissues (**Figure 1C**). In the contrary, higher expression of SLFN5 was detected in early-stage NSCLC tissue specimens (**Figure 1B**). In order to highlight the correlation between the protein level of SLFN5 and clinicopathological characteristics of NSCLC, SPSS software was used to analyze the results of tissue microarray. As summarized in **Table 2**, there was no significant correlation among SLFN5 expression and gender ($P = 0.509$), age ($P = 0.816$) and pathological grade ($P = 0.058$).

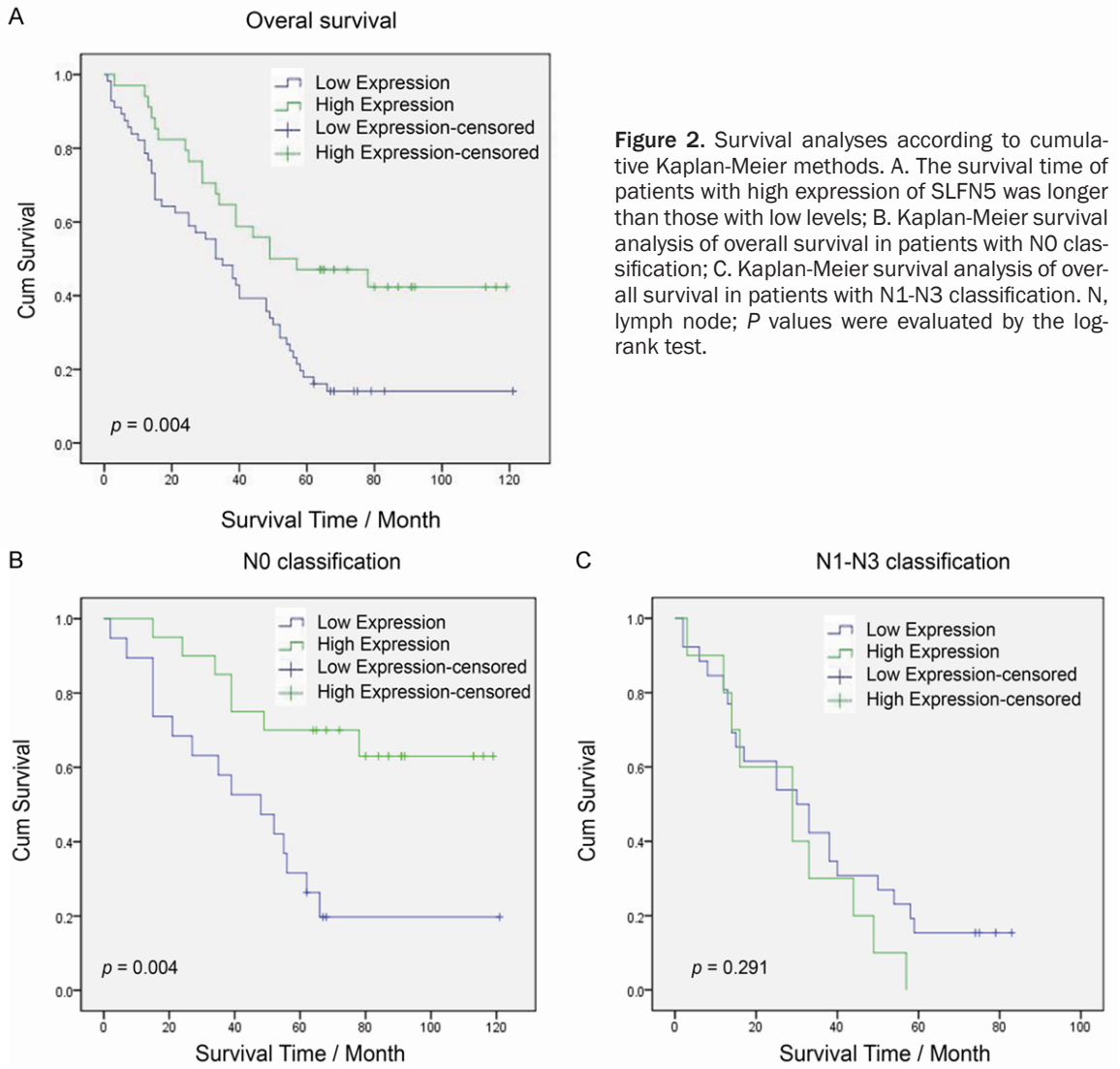

Figure 2. Survival analyses according to cumulative Kaplan-Meier methods. A. The survival time of patients with high expression of SLFN5 was longer than those with low levels; B. Kaplan-Meier survival analysis of overall survival in patients with N0 classification; C. Kaplan-Meier survival analysis of overall survival in patients with N1-N3 classification. N, lymph node; *P* values were evaluated by the log-rank test.

However, low-expression of SLFN5 protein was significantly associated with tumor diameter (< 4 cm vs. ≥ 4 cm, $P = 0.010$), T classification (T1 vs. T2-T4, $P = 0.000$), N classification (N0 vs. N1-N3, $P = 0.038$) and clinical stage (I vs. II-III, $P = 0.044$). Collectively, the expression of SLFN5 was highly correlated with clinico-pathological characteristics of NSCLC, especially among tumor diameter, T classification, N classification and clinical stage.

Association of SLFN5 expression with NSCLC prognosis

As previously statistical results noted, we speculated that SLFN5 may relate to overall survival rate of NSCLC patients. To verify this hypothesis, we explored the correlation between the level of SLFN5 protein and overall survival using Kaplan-Meier analysis with the log-rank test. The results showed that the high expression of SLFN5 protein was markedly associated with improved overall survival of NSCLC patients ($P = 0.004$, **Figure 2A**).

We also investigated independent prognostic markers of NSCLC. As presented in **Table 3**, univariate analysis indicated that T classification (T1 vs. T2-T4, $P = 0.006$), N classification (N0 vs. N1-N3, $P = 0.003$) and expression of SLFN5 (low vs. high staining, $P = 0.003$) were significant factors for the survival time of NSCLC patients. T classification [95% confidence interval (CI) 0.069-0.644] and N classification (95% CI 0.048-0.546) were negatively associated with overall survival. There was a positive correlation between SLFN5 and overall

Table 3. Univariate and multivariate analysis of overall survival in NSCLC patients

Factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Gender						
Female vs. Male	1.429	0.553-3.697	0.461	0.908	0.279-2.950	0.872
Age						
< 55 vs. ≥ 55	1.038	0.3330-3.264	0.949	0.918	0.221-3.804	0.906
T classification						
T1 vs. T2-T4	0.211	0.069-0.644	0.006*	0.473	0.094-2.385	0.364
N classification						
N0 vs. N1-N3	0.162	0.048-0.546	0.003*	0.239	0.062-0.920	0.037*
SLFN5						
Low vs. high expression	4.737	1.727-12.995	0.003*	2.186	0.645-7.411	0.209

*$P < 0.05$; T, tumour size; N, lymph node; HR: hazard ratio; 95% CI, 95% confidence interval.

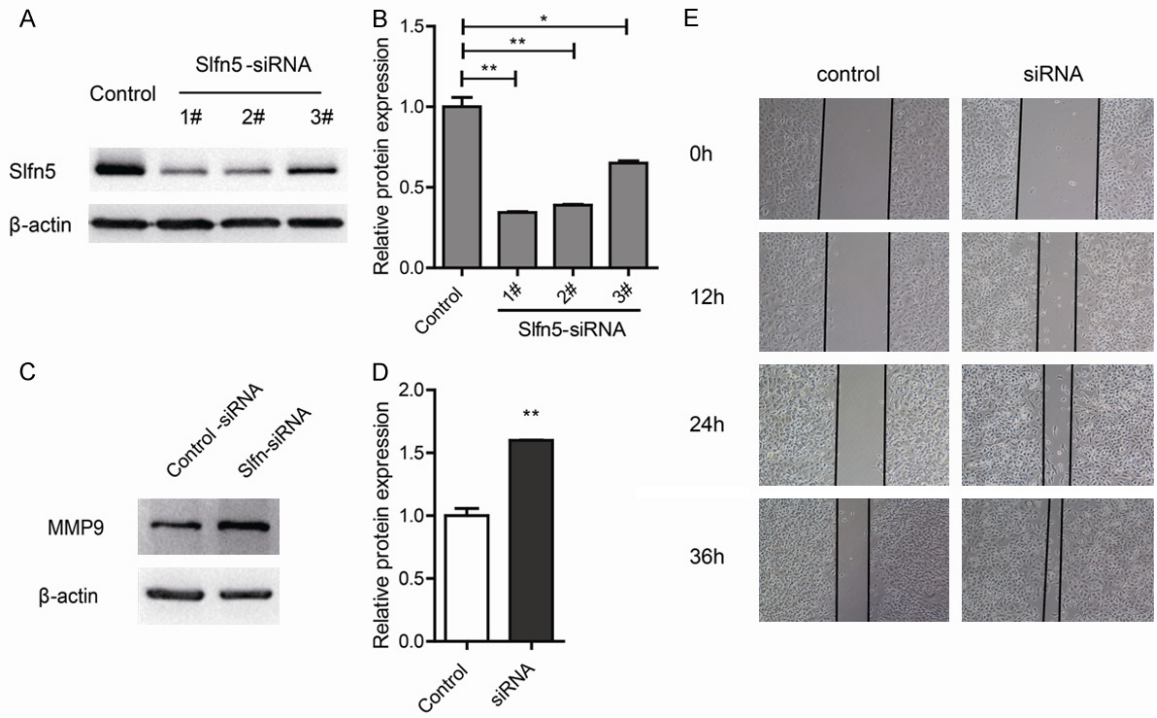

Figure 3. SLFN5 inhibits A549 cells migration. A. Control-siRNA, SLFN5-siRNA #1, SLFN5-siRNA #2 and SLFN5-siRNA #3 were transfected into A549 cells. Western blotting analysis reveals a decrease in SLFN5, β-actin served as loading control; B. Statistical analysis of relative protein expression levels of SLFN5 after normalization with β-actin; C. Knockdown SLFN5 in gain of cell migration associated protein MMP-9, β-actin served as loading control; D. Statistical analysis of relative protein expression levels of MMP-9 after normalization with β-actin; E. SLFN5 significantly inhibits A549 cells migration. Data were collected at 12, 24 and 36 h (original magnification, ×100). All data (mean ± SD) are representative of three independent experiments. *$P \leq 0.05$, **$P \leq 0.01$ indicates highly statistically significant differences compared with control-siRNA group.

survival (95% CI 1.727-12.995). In multivariate analysis, we found the the independent prognostic marker of NSCLC was only the N classification (N0 vs. N1-N3, $P = 0.037$). We next confirmed the prognostic value of SLFN5 expression on N classification of clinical stage using Kaplan-Meier analysis. Patients with high SLFN5 expression in N0 classification had a longer survival time than those with low SLFN5 expression ($P = 0.004$, **Figure 2B**), but this was not consistent with later N classification (N1-N3, **Figure 2C**). Taken together, our data demon-

strated that high SLFN5 expression will lengthen overall survival of NSCLC patients, especially in the patients with N0 classification.

SLFN5 inhibits A549 cells migration

Then we verified whether the change of SLFN5 affected the migratory regulation of A549 cells, we separately transfected three siRNA of SLFN5 into A549 cells. The western blot indicated that SLFN5 was successfully silenced in A549 cells after transfection, especially in SLFN5-siRNA 1# (**Figure 3A**). We further detected MMP9 protein, cell migration-associated protein in SLFN5-silenced cells. MMP9 proteins was observably increased after SLFN5 silence. Scratch wound healing assay was employed to further explore the role of SLFN5 in the process of migration in A549 cells. As shown in different time point, silenced SLFN5 obviously enhanced the ability of A549 cells compared with control group (**Figure 3B**). Consequently, our results showed that SLFN5 inhibited the migration in A549 cells.

Discussion

Migration is a prominent feature of almost all kinds of progressive tumor metastasis [24]. Although the functions and mechanisms of Schlafen family in cell proliferation, immune responses and viral replication are established unequivocally, human SLFNs are reported to inhibit tumourigenesis and the migration in NSCLC [13-16]. Antonella et al. found that SLFN5 could downregulate the expression of matrix metalloproteinases (MMP)-1 and MMP-13, cell migration associated proteins to reduce malignant melanoma cell motility [9]. Sassano et al. also reported SLFN5 had negative influence on the motility and invasiveness of RCC cells [19]. As demonstrated in our study, SLFN5 can inhibit the migration ability in lung cancer, and SLFN5 silence aggrandizes migration ability, and enhances MMP-9 protein, cell migration associated protein in A549 cells. Thus, SLFN5 may be involved in the tumorigenesis and migration of NSCLC. However, further researcher are needed to explore the underlying molecular mechanisms of SLFN5 on the migration in NSCLC.

In this study, SLFN5 is less in almost advanced NSCLC tissues than early-stage NSCLC tissue specimens. Our work has explored the correlation between the expression of SLFN5 protein and clinicopathological characteristics of NSCLC. Lung cancer tissue with low-expression of SLFN5 protein is significantly associated with tumor diameter, T classification, N classification and Clinical stage. In addition, NSCLC patients with high-expression of SLFN5 protein have better overall survival than those with low-expression of SLFN5 protein (P = 0.004). All these results suggest that the progress and poor prognosis of NSCLC is highly associated with the low expression of SLFN5.

T classification (P = 0.006), N classification (P = 0.003) or expression of SLFN5 (P = 0.003) are significant factors to evaluate the survival time of NSCLC patients, and N classification (P = 0.037) can serve as an independent prognostic marker of NSCLC as confirmed by multivariate analysis. Then we find that the SLFN5 expression is highly associated with N0 classification in NSCLC. High expression of SLFN5 indicates long survival in patients with the early stage of NSCLC. Therefore, SLFN5 is a potential biomarker for patients with early-stage of NSCLC.

SLFN5 has an important role in NSCLC, and inhibit tumor metastasis by reducing the migration of tumor cells. In addition to overall survival, our studies have demonstrated the expression of SLFN5 is markedly associated with clinicopathological characteristics of NSCLC, especially N0 classification. However, the accurate mechanisms between SLFN5 expression and NSCLC metastasis inhibition remain elusive.

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Disclosure of conflict of interest

None.

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