High Expression of SPRR1B Indicates Unfavorable Clinical Outcomes in Lung Adenocarcinoma

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Abstract

Backgrounds: Small proline-rich protein 1B (SPRR1B) was initially identified as an envelope protein of keratinocytes, which plays essential roles in squamous cell differentiation. Recently its involvement in malignancies was also explored in head and neck squamous cell carcinoma. However, whether SPRR1B participates in adenocarcinoma progression remains unknown. Here we aimed to investigate the expression and function of SPRR1B in lung adenocarcinoma (LAC).

Methods: SPRR1B expression level was examined in LAC samples and adjacent nontumorous samples through quantitative RT-PCR assay and immunohistochemistry staining. Univariate and multivariate analyses were performed to estimate the prognostic role of SPRR1B. Cellular experiments were performed to reveal the function of SPRR1B in LAC cells.

Results: The SPRR1B level in LAC samples was significantly higher compared with adjacent samples. Moreover, higher SPRR1B level was correlated with poor tumor differentiation and advanced tumor stage. LAC patients with higher SPRR1B level had worse overall survival. Moreover, SPRR1B was confirmed as an independent unfavorable prognosis factor. Cellular data indicated that knockdown of SPRR1B could attenuate the proliferation capacity of LAC cells.

Conclusions: Our results demonstrated that high SPRR1B level was significantly correlated with unfavorable clinical features and poor prognosis of LAC patients. SPRR1B might serve as a novel prognostic indicator and potential drug target for LAC treatment.

Introduction

Lung adenocarcinoma (LAC) belongs to the subtype of non-small cell lung cancer (NSCLC) and accounts for 40% of the cancers occurred in lung [1].It is one of the most prevalent malignancy in non-smokers, which becomes the leading cause of cancer-related death [2]. Current treatments options for LAC patients include surgical resection, chemotherapy, radiation therapy, targeted therapy, angiogenesis inhibitor and immunotherapy [3–5]. Despite the great improvement in the treatments, the 5-year survival rate of LAC patients is less than 16% due to the difficult in early diagnosis [6–9]. Therefore, investigating the carcinogenic mechanisms of LAC and identifying potential therapeutic target are under urgently demand for more effective treatment, as well as improving the prognosis of LAC patients.

Small proline-rich protein 1B (SPRR1B), also named as cornifin, is a cross-linked envelope precursor in keratinocytes firstly identified by K W Marvin and his colleagues in 1992 [10]. Later it was reported to participate in squamous cell differentiation including the tracheobronchial epithelial cells [11]. Interestingly, expression level of SPPR1B can be modulated by well-known oncogenic pathways such as PKC/Ras/MEKK1 signaling pathway [12]. Besides, the oncoprotein c-Jun can significantly upregulate the SPRR1B promoter activity and enhance SPRR1B protein expression in lung Clara cells [13]. Moreover, SPRR1B can be upregulated after stimulated by proinflammatory cytokines such as IL-1β and IFNγ, subsequently exerts functions in squamous metaplasia process [14]. Considering the close crosstalk

among inflammation, squamous metaplasia, and tumor development, we are interested in investigating whether SPRR1B play roles in lung cancer progression. Literature research showed that SPRR1B was indeed upregulated in lung squamous carcinoma compared with normal lung tissues according to transcriptome data, indicating its potential oncogenic functions [15]. However, the expression pattern and role of SPRR1B in lung adenocarcinoma remains unknown.

In this study, we originally demonstrated that SPRR1B was highly expressed and was an independent predictor of poor prognosis for LAC patients. We also revealed SPRR1B knockdown suppressed proliferation of LAC cells, indicating the potential treatment therapy aimed at inhibiting SPRR1B. Targeting this envelope protein might provide benefits for LAC patients with high expression of SPRR1B.

Materials And Methods

Patients and samples

The Ethic Committee of The Second Affiliated Hospital of Nanjing Medical University approved this research. 116 cases of formalin-fixed paraffin-embedded (FFPE) lung adenocarcinoma samples together with adjacent nontumorous lung samples were chosen from LAC patients who underwent surgical resection in The Second Affiliated Hospital of Nanjing Medical University during April 2011 to April 2017. All cases performed lobectomy and radical lymph node dissection. Additionally, 26 cases of fresh-frozen LAC samples and adjacent nontumorous lung samples were collected from the Department of Surgery and used for further qRT-PCR assay. None of the enrolled patients had distant metastasis. All the experiment samples used in our study has been confirmed through histopathology examination.

Consent for publication

Written informed consent documents were obtained for all enrolled patients.

RNA extraction and qRT-PCR

For mRNA analysis, we firstly extracted mRNA from fresh-frozen tissues following the manual procedure. Then, the isolated mRNA was used to perform reverse transcription through the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, USA). After reverse transcription, the cDNA products were used for RT-qPCR assay through the SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA) following the standard procedure [16]. We choose *GAPDH* as endogenous reference gene, and the used primers (ordered from ORIGENE) were as following:

SPRR1B-Forward: 5'- CTGCCCTTCAATAGTCACTCCAG -3'

SPRR1B-Reverse: 5'- CTCATACGCAGAATGGGATAGGG -3'

GAPDH-Forward: 5'- GTCTCCTCTGACTTCAACAGCG -3'

GAPDH-Reverse: 5'- ACCACCCTGTTGCTGTAGCCAA -3'

Immunohistochemistry (IHC) staining

IHC staining was performed to detect SPRR1B protein level following the manufacturer instructions. The fixed samples were firstly embedded in paraffin and sectioned at 8 μm. Slide sections were next dried and deparaffinized, then taken for antigen retrieval in the citrate buffer. Then, slide sections were blocked and incubated with the primary SPRR1B antibody (Cat. No. ab123237; Abcam) at 4°C for 12 hours. Finally, the slide sections were washed and incubated with secondary antibody and substrates were used to observe the immunoreactivities. Negative control was set up by using PBS instead of primary antibody [17].

Evaluation of IHC staining

Two independent pathologists examined the staining results at a 400 X magnification from five randomly selected fields. IHC staining intensity score was classified into four different levels as followings: 0 = negative, 1 = weak, 2 = intermediate, and 3 = strong. The proportion of positive cells was scored as followings: 0 (< 25%); 1 (25% - 50%); 2 (50% - 75%); 3 (> 75%). Finally, the total score of IHC staining (IHCS) was calculated by multiplying these two scores (ranging 0-9).

Cell line culture and siRNA transfection

Human bronchial epithelial cell (HBE) and human lung adenocarcinoma cell lines (H1299 and A549) were obtained from the American Type Culture Collection (ATCC, Rockville, USA). A549 cells were transfected with siRNA by using Lipofectamine RNAi Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions [18]. The sequences of SPRR1B-siRNAs were designed and synthesized using the BLOCK-it RNAi designer system (Life Technologies) [19] with scrambled siRNA (5'-GCAAACAUCCCAGAGGUAU -3') as control.

Western blot

Cultured cells were harvested with cold lysis buffer supplemented with protein inhibitors. Then the lysates were collected and centrifuged at 12,000 g for 30 min to obtain the supernatants. After protein quantification, the supernatant was denatured in loading buffer and same amount of proteins were resolved by SDS-PAGE and then electro-transferred onto the PVDF membrane for immunoblotting. The signals were finally detected by western blotting substrate. Primary antibodies targeting SPRR1B and GAPDH were both purchased from Abcam.

Cell proliferation assay

A549 cells were transfected with siRNA and 24 hours later the transfected cells were digested and seeded into 96-well cell-culture plates (3000 cells/well). After cultured for designated time points, the medium was removed and 100μl MTT solution were added and incubated the cells at 37°C for 4 hours. Then the solution was discarded and 200μl DMSO was added to each well. Finally, the plates were shaken for 15min and send to the microplate reader to measure absorbance at 490nm wavelength [20].

Statistical analysis

We conducted the statistical analyses using the software SPSS Statistics 19.0. The association between SPRR1B protein level and clinical characteristics were evaluated through Chi-square method. Kaplan–Meier analysis and log-rank test were used to analyze and plot the overall survival curves of enrolled LAC patients. Independent prognostic factors were identified by using multivariate analysis model [21]. $P<0.05$ was considered statistically significant.

Results

Patients' survival of entire cohort

Totally 116 LAC patients were followed up for 13-88 months and 43 participants died by the end of follow-up. The 5-year survival rate of all participants were 65.1% and median survival time was 70.0 months.

SPRR1B is highly expressed in lung adenocarcinoma tissues

To investigate the potential function of SPRR1B in LAC, we firstly tested the relative mRNA level of SPRR1B in 26 pairs of fresh LAC samples together with adjacent lung samples through qRT-PCR assay. It revealed that the relative mRNA level of SPRR1B was notably increased in LAC samples compared with adjacent noncancerous lung samples (Figure 1A). Moreover, we measured the SPRR1B protein level in another 116 cases of FFPE LAC samples and adjacent lung samples by using IHC staining. IHC data demonstrated that the protein level of SPRR1B was significantly up-regulated in some LAC samples compared with adjacent noncancerous samples. Besides, we plotted receiver operating characteristic (ROC) curve to divided enrolled patients into two categories based on the IHC score (Figure 1B), namely the high-SPRR1B expression group (IHCS $\geq$ 5, n = 55) and low-SPRR1B expression group (IHCS < 5, n = 61). The representative negative expression and positive expression of SPPR1B in LAC samples were shown in Figure 1C and 1D, respectively.

High expression of SPRR1B predicts unfavorable clinical characteristics of lung adenocarcinoma patients

To further study the clinical role of SPRR1B, we next evaluated the clinical correlations between SPRR1B and clinical features of LAC patients (Table 1). The results demonstrated that a higher level of SPRR1B was significantly associated with poorer differentiation (P=0.006), advanced TNM stage (P=0.005) and positive lymph node metastasis (P=0.006). However, no significant correlations were observed between the SPRR1B protein level and patients' sex, age, or smoking history (all P>0.05).

Higher expression of SPRR1B indicates worse prognosis of LAC patients

To understand the potential relevance between SPRR1B level and the overall survival time of LAC patients, we performed Kaplan–Meier analysis and log-rank test (Figure 2). We found that LAC patients

with higher SPRR1B level get a terrible mean overall survival time (56.2 ± 3.5 months), while patients with lower SPRR1B level is better (74.7 ± 3.2 months; P=0.001; Figure 2A). Besides, some conventional risk factors were also identified to be related to the LAC patients' overall survival time, including TNM stage (P=0.001) and lymph node metastasis (P=0.001). Of note, patients with well histological differentiation showed better clinical outcomes than those with poor differentiation or moderate differentiation, although the statistical test was not significant (P=0.074).

Additionally, we further evaluated the independent effects of those prognostic factors on the patients' overall survival time through Cox regression analysis (Table 3). We enrolled the variables with P<0.1 by univariate test, including tumor differentiation, TNM stage, lymph node metastasis, TNM stage, and SPRR1B expression.

The result indicated that SPRR1B protein level was defined as an independent prognostic element (hazard ratio = 2.116, 95% confident interval = 1.031-4.341, P = 0.041). Besides, positive lymph node metastasis (P = 0.021) and TNM stage (P = 0.001) were also identified to be an independent predictor for poor survival time of LAC patients.

Knock down of SPRR1B suppressed tumor cell proliferation

To validate the potential function of SPRR1B in lung cancer cells, we firstly detected the expressions pattern of SPRR1B in normal lung cells (HBE) and lung adenocarcinoma cells (H1299, A549) through Western blot (Figure 3A). Consistent with clinical samples, immunoblotting data revealed that HBE cells possessed lowest SPRR1B level, while A549 expressed the highest SPRR1B expression compared to other cells. Then we knocked down the SPRR1B expression in A549 cell lines and examined the transfection efficiency (Figure 3B). Finally, we investigated the features of transfected cells and it showed that knockdown of SPRR1B could inhibit the proliferation capacity compared to control cells which transfected with scramble siRNA (Figure 3C). These data further suggested that SPRR1B might play potential role in the progression of human lung adenocarcinoma.

Discussion

Envelope proteins are emerging as a novel protein family revealed to be involved in malignancies. For example, human endogenous retrovirus type K (HERV-K) envelope protein is highly expressed in breast cancer instead of normal breast tissue, which is associated with markers of disease progression and poor clinical outcome [22]. The classic cornifin envelope protein, cornifin-α, alternatively named as SPRR1A, has also been reported to be increased in mouse skin neoplasms as early in 1996 [23]. However, few studies explored the role of SPRR1B, another cornifin subtype, in tumor development.

Here in our study, we demonstrated that the mRNA and protein level of SPRR1B was high up-regulated in LAC tissues compared with adjacent lung tissues through qRT-PCR and IHC staining. Moreover, to better analyze the clinical role of SPRR1B in LAC, we firstly divided the enrolled LAC patients into two groups according to the IHC staining results. Then we dissected the correlation between high SPRR1B expression

level and unfavorable clinical outcomes of LAC patients. We found that the high level of SPRR1B was significantly associated with positive lymph node metastasis and advanced TNM stage in those patients. In addition, we also identified SPRR1B expression level as an independent prognosis factor for LAC patients through multivariate analysis.

Consistent with its oncogenic role in LAC, previous studies proposed that overexpression of SPRR1B in bronchial epithelial cells indicates early metaplastic changes, which is mediated by ESE-1, Sp1, and AP-1 proteins [24, 25]. In addition, SPRR1B is one of the key molecular on influencing differentiation of head and neck squamous cell carcinoma (HNSCC) [26]. According to our clinical data, Chi-square test also indicated a significantly correlation between high SPRR1B protein level and poor LAC differentiation (P=0.006), further implied its potential in malignancy development.

Considering our cellular results showed SPRR1B-siRNA inhibits LAC cell proliferation, we suggested that SPRR1B plays critical roles in LAC progression. Our hypothesis is consistent with a recent study showing that SPRR1B enhances entry of cells into the G0 phase of the cell cycle in Chinese hamster ovary (CHO) cells [27]. Therefore, SPRR1B is high likely to promote lung adenocarcinoma proliferation through accelerating cell cycle, which may need further investigation.

Our study has certain limitations. Firstly, the patients were obtained from a single medical center and may thus possess regional or racial bias. Secondly, we did not assess disease-free survival of enrolled cohort due to unsatisfied patient's compliance on prescribed examination, which resulted in inaccurate recurrence time. Thirdly, we mainly focused on the potential predictive role of SPRR1B in lung adenocarcinoma without digging into its functional signaling pathway.

Conclusion

In summary, our results demonstrated that the expression level of SPRR1B was elevated in LAC tissues and significantly correlated with poor clinical outcomes of LAC patients, which can serve as an independent prognostic factor. Further studies focused on investigating the underlying molecular mechanisms of how SPRR1B promotes LAC progression will be necessary for valuable clinical treatments.

Declarations

Statement of Ethics: This research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Subjects have given their written informed consent and the study protocol was approved by The Ethic Committee of The Second Affiliated Hospital of Nanjing Medical University.

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Conflict of interest: The authors declare no conflict of interest.

Author Contributions: Hao Cao conducted statistical analysis and wrote this manuscript; Min Li and Sen Chen collected clinical specimens and performed IHC experiments; Yi Zhu and Xueduan Chang contributed to cellular assays; Ganzhu Feng designed this project.

Availability of Data and Materials: Data will be available upon request.

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Tables

Table 1. Characteristics of the LAC patients and associations with SPRR1B expression level.

Variable	Cases	SPRR1B expression		P value
	(n = 116)	Low (n = 61)	High (n = 55)	
Sex				
Female	37	19	18	0.855
Male	79	42	37	
Age (years)				
≤ 60 yrs	65	36	29	0.496
> 60 yrs	51	25	26	
Smoking history				
Negative	51	28	23	0.658
Positive	65	33	32	
Differentiation				
Well	72	45	27	0.006*
Moderate or poor	44	16	28	
Tumor diameter				
≤ 3.0 cm	50	23	27	0.216
> 3.0 cm	66	38	28	
TNM stage				
I	54	36	18	0.005*
II-IIIa	62	25	37	
LN metastasis				
Negative	62	40	22	0.006*
Positive	54	21	33	

Table 2. Univariate analysis for the overall survival of LAC patients.

Variable	Cases	Overall survival		P value
	(n = 116)	Mean ± SD (Months)	5-year (%)	
Sex				
Female	37	70.1 ± 4.4	73.1%	0.110
Male	79	62.2 ± 3.0	61.3%	
Age (years)				
≤ 60 yrs	65	64.2 ± 3.3	66.1%	0.616
> 60 yrs	51	65.1 ± 3.5	63.8%	
Smoking history				
Negative	51	69.5 ± 3.6	76.1%	0.126
Positive	65	59.7 ± 3.1	56.1%	
Differentiation				
Well	72	68.7 ± 3.4	71.3%	0.074
Moderate or poor	44	58.8 ± 3.1	55.4%	
Tumor diameter				
≤ 3.0 cm	50	63.3 ± 3.8	66.3%	0.596
> 3.0 cm	66	64.3 ± 3.0	64.5%	
TNM stage				
I	54	74.8 ± 3.2	84.7%	0.001*
II-IIIa	62	57.7 ± 3.5	50.1%	
LN metastasis				
Negative	62	74.0 ± 3.4	77.7%	0.001*
Positive	54	56.1 ± 3.0	52.5%	
SPRR1B expression				
Low	61	74.7 ± 3.2	75.8%	0.001*
High	55	56.2 ± 3.5	53.9%	

Table 3. Multivariate analysis for the overall survival of LAC patients.

Variable	Hazard ratio	95% CI	P value
Differentiation (moderate/poor vs. well)	1.251	0.665-2.351	0.487
LN metastasis (positive vs. negative)	2.254	1.131-4.494	0.021*
TNM stage (II-IIIa vs. I)	3.722	1.715-5.841	0.001*
SPRR1B expression (high vs. Low)	2.116	1.031-4.341	0.041*

Figures

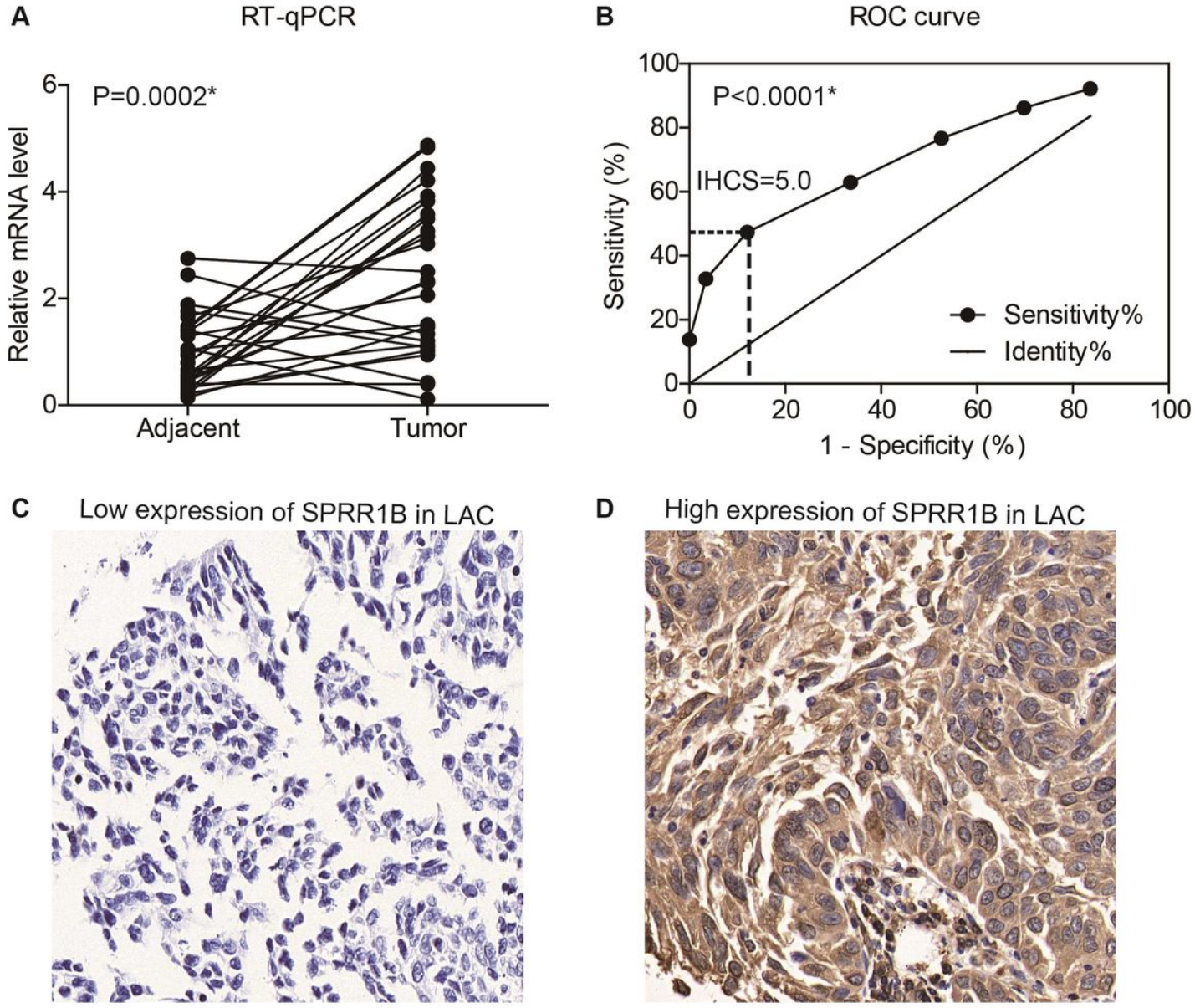

Figure 1

Expression of SPRR1B in LAC tissue. (A). Quantitative RT-PCR showed the mRNA level of SPRR1B in LAC samples was elevated significantly compared to adjacent nontumorous samples. * indicates $P < 0.05$ by paired student's t-test. (B). ROC curve of IHC staining results was plotted according to the IHC score (IHCS). The cut-off value was IHCS=5.0, with area under curve (AUC) = 0.7149 ($P<0.001$). According to the cut-off value, we divided patients into low-SPRR1B group and high-SPRR1B group. (C). Representative negative expression of SPRR1B in lung adenocarcinoma tissue by IHC staining (IHCS=0). (D) Representative high expression of SPRR1B in lung adenocarcinoma tissue by IHC staining (IHCS=9).

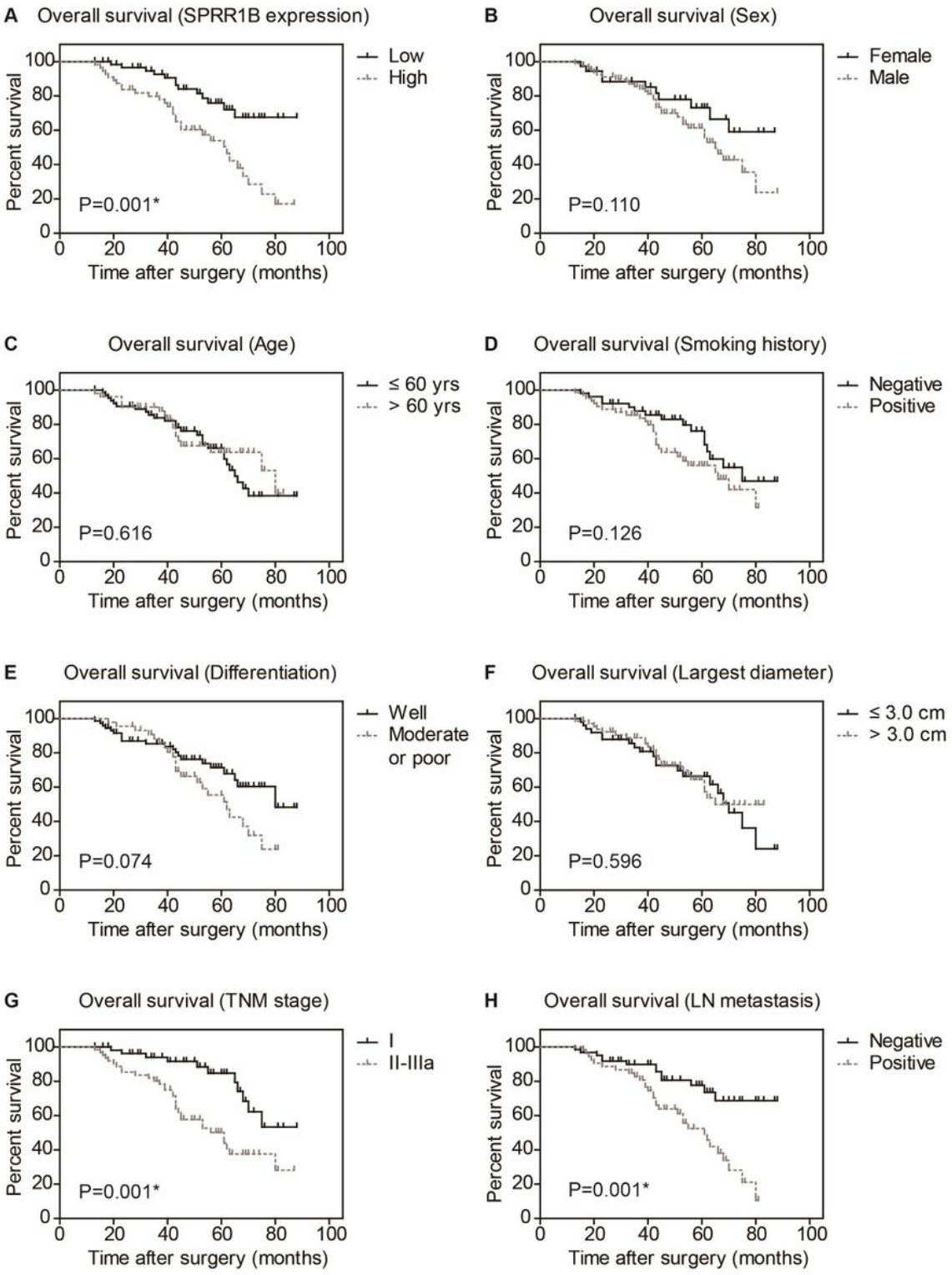

Figure 2

Kaplan-Meier analyses of the overall survival for enrolled LAC patients. Kaplan-Meier analysis of the patients' overall survival according to SPPR1B expression (A), sex (B), age (C), smoking history (D), tumor differentiation (E), tumor largest diameter (F), TNM stage (G) and lymph node metastasis (H).

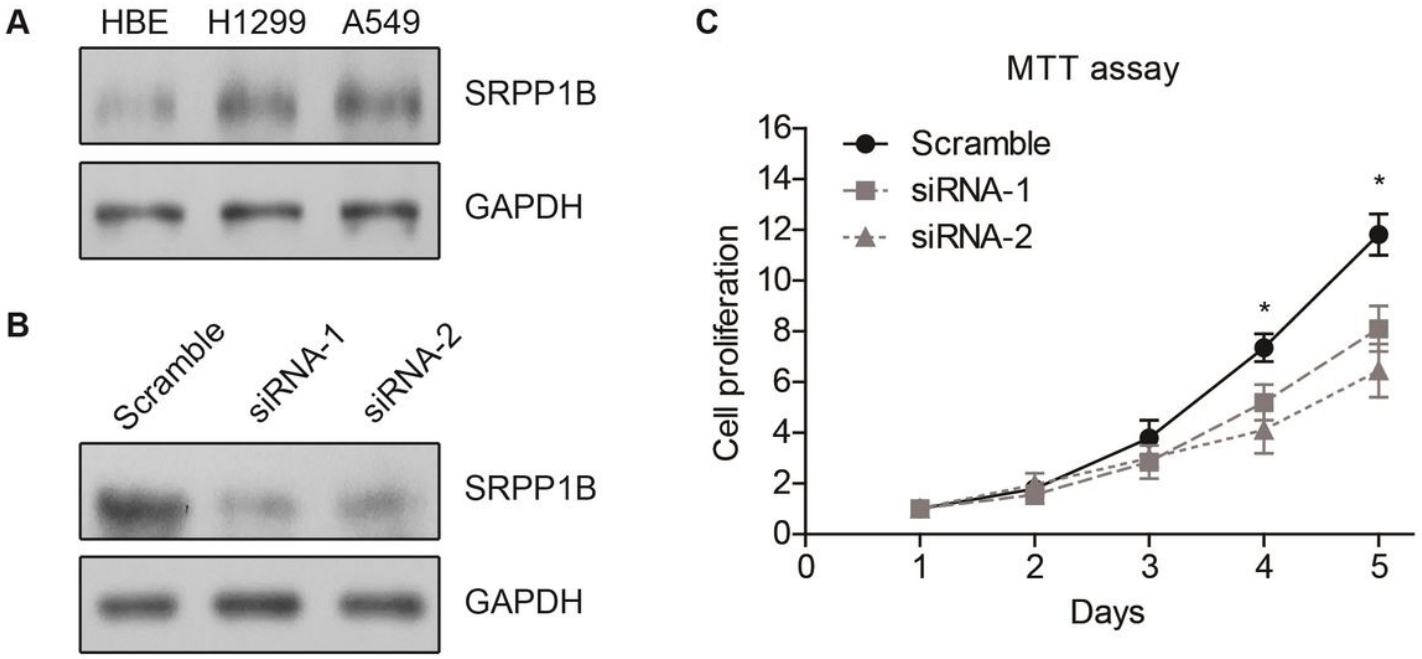

Figure 3

Effect of SPRR1B on the proliferation of LAC cells (A) Western blot presented the expression of SPRR1B in different cell lines, including nontumorous lung bronchial epithelial (HBE) cells and two LAC cell lines (H1299, A549). (B) Transfection efficiency of siRNAs in A549 cells were tested by immunoblotting, showing that SPRR1B-siRNA can efficiently downregulate its protein level. (C) MTT assays were conducted to explore the effects of siRNAs on A549 cell proliferation, which confirmed an anti-proliferation role of SPRR1B-siRNA. Data was obtained from three independent repeats. * indicates $P < 0.05$ by One-way ANOVA test.