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Spondin-2 is a novel diagnostic biomarker for laryngeal squamous cell carcinoma☆

Haosheng Ni[a,1], Tingting Ni[b,1], Jia Feng[c], Tingting Bian[c], Yifei Liu[c,*], Jianguo Zhang[c,*]

[a] Department of Otorhinolaryngology, Affiliated Hospital of Nantong University, No. 20 Xi Si Road, Nantong, 226001, China
[b] Department of Oncology, Nantong Tumor Hospital, No. 30 Tong Yang North Road, Nantong 226001, China
[c] Department of Pathology, Affiliated Hospital of Nantong University, No. 20 Xi Si Road, Nantong, 226001, China

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ABSTRACT

Spondin-2, belongs to the SOX (SRY-related HMG box) gene family, plays a vital role in the development of malignancy, however, the role of Spondin-2 in laryngeal squamous cell carcinoma (LSCC) remains unknown. The aim of this study is to investigate the prognostic significance of and probable mechanism of Spondin-2 in LSCC. qRT-PCR, western blotting assays and IHC analysis demonstrated that Spondin-2 was significantly increased in LSCC tissues compared with adjacent non-tumorous tissues. In addition, high levels of Spondin-2 was associated with clinical stage, lymph node metastasis and pathology grade of LSCC patients ($P < 0.05$). Kaplan-Meier analysis showed that patients with high expression of Spondin-2 had a lower overall survival rate ($P < 0.05$) than that with low expression of Spondin-2. Moreover, spondin-2 silencing inhibited the proliferation of LSCC cells through inhibiting the activation of PI3K/AKT signaling. In conclusion, spondin-2 might be a novel therapeutic target and prognostic biomarker for LSCC patients.

1. Introduction

Laryngeal squamous cell carcinoma (LSCC)) is one of the most common cancers in head and neck [1]. As a frequent cancer among men, it has an incidence of 1.9% of male cancer cases and is responsible for 1.0% of cancer deaths. The treatments for LSCC mainly included surgery, radiotherapy and chemotherapy, but the efficacy of treatment depend on several factors including tumor localization, invasion and metastasis [2] Some patients in the locoregionally advanced stage still easily eventually relapsed or even died [3,4]. Therefore, the identification of molecular alterations during the progression will not only reveal the key markers but also provide beneficial treatments for LSCC.

Spondin-2Spondin-2, a member of the F-spondin family of secreted extracellular matrix proteins, was cloned firstly from noncancerous lung cells and found to be deregulated in some tumors [5,6]. Spondin-2 is a host innate immune regulator, which also can recruit inflammatory cells and develop neurons [7,8]. As previous study showed that spondin-2 was unregulated in various cancers including liver cancer [9], gastric cancer [10], ovarian [11], breast cancer [12] and so on so on. The expression of Spondin-2spondin-2 might be regulated by hormones, such as thyroid hormone [9], androgen [13], or epigenetic mechanism [14]. However, the expression and role of spondin-2 in LSCC remains inconclusive, so the aim of this study is to elucidate the role of spondin-2 in LSCC.

Spondin-2In this study, Spondin-2quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) analysis demonstrated that Spondin-2 was notably increased in LSCC tissues when compared with adjacent non-tumorous tissues. And the high levels of spondin-2 was correlated with LSCC clinical stage et al. Spondin-2Furthermore, spondin-2 silencing inhibited the proliferation and the activation of PI3K/AKT signaling *in vitro*. Spondin-2Based on the data we found, this study suggested prognostic significance and probable regulator mechanism of Spondin-2spondin-2 in LSCC, which might help us to find a novel therapeutic strategy for LSCC.

2. Materials and methods

2.1. Tissue Samples collection and cell culture

A total of 111 paraffin-embedded LSCC specimens and adjacent non-tumorous tissues were collected for immunohistochemistry analysis. A total of 15 pairs of fresh tissues from LSCC patients were

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* Corresponding authors.
E-mail addresses: ntdxliuyifei@sina.com (Y. Liu), 13815212431@163.com (J. Zhang).
[1] Contribute equally to this work.

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Table 1
Expression of Spon2 in 111 human laryngeal carcinoma tissues.

Clinicopathological parameters	No. Case	Spon2 expression, n (%)		P value
		Low	High	
Gender				
Female	4	1	3	0.542
Male	107	43	64	
Age (year)				
< 60	31	9	22	0.155
≥60	80	35	45	
Smoking				
No	27	10	17	0.751
Yes	84	34	50	
T Stages				
T1-T2	79	39	40	0.001
T3-T4	32	5	27	
Lymph node metastasis				
No	81	40	41	0.001
Yes	30	4	26	
TNM clinical stage				
I-II	57	34	23	< 0.001
III-IV	54	10	44	
Pathology grade				
Well	13	7	6	< 0.001
moderate	75	36	39	
Poor	23	1	22	
Tumor Location				
Supraglottic	31	10	21	0.568
Glottic	74	31	43	
Subglottic	6	3	3	

Annotation: Statistical analysis were performed by the Pearson χ^2 test. $P < 0.05$ was considered significant.

subjected for qRT-PCR and western blotting assays. The paraffin-embedded specimens were obtained from the LSCC patients undergone surgical excision at the affiliated Hospital of Nantong University (Nantong, China) from January 2011 to December 2016. Fresh tissues were collected from January 2016 to December 2016 and saved at −80 °C until use. None of the patients had received chemotherapy or radiotherapy before the surgery. The pathological diagnoses of the specimens were done according to the WHO criteria and TNM classification [15]. Each patient had written informed consent and the clinical process was approved by the Ethics Committee of Affiliated Hospital of Nantong University. The main clinical and pathologic characteristics of LSCC patients were as shown in Table 1.

The Hep-2 human LSCC cell line was purchased from the Procell Life Science & Technology Co., Ltd. (Wuhan, China) and the cells were authenticated by STR. Cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, MA) with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere containing 5% CO2. Cells were transfected with scrambled NC inhibitor(si-NC), Spondin-2 siRNA (si-Spondin-2, purchased from Gene Pharma Inc., Suzhou, China) or pcDNA3.1 plasmid and Spondin-2 overexpressed plasmid (Spondin-2 vector, purchased from General Biol Inc., Anhui, China) by using Lipofectamine 2000™ reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions [16]. And the sequence of scrambled NC inhibitor and Spondin-2 siRNA was shown in Table 3.

2.2. Immunohistochemistry

The paraffin-embedded LSCC specimens and adjacent non-tumourous tissues were sectioned at 10 μm thickness by standard methods, de-waxed in xylene and dehydrated in graded ethanol. Endogenous peroxidase activity was inactivated by soaking in 0.3% hydrogen peroxide for 30 min. Then these sections were heated to 121°C to retrieve the antigen. After being rinsed in phosphate buffered saline (PBS, pH 7.2) for three times, the sections were incubated with anti-Spondin-2spondin-2 (cat. no. ab171955, dilution 1:1000, Abcam, CA, USA) antibody overnight at 4 °C and then processed with the peroxidase-anti-peroxidase method (DAKO, Hamburg, Germany). Thereafter, the 3, 39-diaminobenzidine tetrachloride chromogen solution was used to visualize color reaction. Finally, the sections were rinsed with water, counterstained with hematoxylin, dehydrated and mounted. A nonspecific immunoglobulin IgG (dilution, 1: 100, Santa Cruz Biotechnology, Dallas, TX, USA) was regarded as a negative control.

The immunohistochemistry scoring evaluation was performed independently by two pathologists, who did not know the patient's clinicopathological data. Five fields were randomly selected based on each section and the expression of Spondin-2spondin-2 was evaluated by both staining-intensity and percentage of positive tumor cells. The staining-intensity of Spondin-2spondin-2 is classified into four grades: 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). The staining-extent is also divided into four grades: 0 (< 10% positive cells), 1 (10–25%), 2 (26–75%), and 3 (76–100%).The immunoreactivity score is the sum of the staining-intensity and staining-extent scores as follows:" − " (negative, score of 0), " + " (weakly positive, score of 1–2)," + + " (positive, score of 3–4), and " + + + " (strongly positive, score of 5–6). " − " and " + " are defined as low expression, and " + + " and " + + + " are defined as high expression.

2.3. Quantived real-time polymerase chain reaction assays

Total RNA from LSCC tissues and adjacent non-tumor tissues were extracted using TRIzol Reagent (Thermo Fisher Scientific,WalthamMA, USA) according to the manufacturer's instructions. Two microgram RNA were reverse transcribed into cDNA using a Quant One Step RT-PCR Kit (TIANGEN, Beijing, China). The two-step quantitative real-time PCR was then performed *via* Fast SYBGreen Master Mix (Thermo Fisher Scientific).The cycling conditions consisted as one cycle at 50 °C for 2 min and 95 °C for 2 min, 40 cycles with 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 1 min. β-actin was used as the control and the Ct-value was calculated by $2^{-\Delta Ct}$ methods. The primers was shown in Table 4: All the experiments were performed three times.

2.4. Western blotting assays

Tissues or transfected cell lines were polished with liquid nitrogen and lysed with ice-cold lysis buffer (Beyotime, Shanghai, China). The concentration of proteins was analyzed with Pierce BCA protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA). Total 50 μg proteins were separated by 12% SDS-PAGE, followed by transfer onto the PVDF membrane (Millipore, Billerica, MA, USA). The membranes were then incubated with PBS containing 5% milk for 2 h at room temperature. Incubation with the rabbit anti-Spondin-2spondin-2 antibody, rabbit anti-PI3K antibody, rabbit anti-Akt antibody, rabbit anti-Akt (phospho-T308) antibody, rabbit anti-PI3K (phospho Y464) antibody (all purchased from Abcam) and mouse anti-β-actin primary antibody (HuAn, Hangzhou, China) at 4°C overnight. After washing with TBS containing 0.1% Tween-20 for three times, the membranes were incubated with goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody (JacksonImmuno, Lancaster, PA, USA) at room temperature for 2 h. The Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific.) was used to detect signals on X-ray film. And the relative protein expression was presented as the density ratio *vs* β-actin.

2.5. CCK-8 assays

Approximately 5000 transfected cells per well was plated in 96-well plates and cultured for 24 h, 48 h, 72 h and 96 h separately, CCK-8 solution was then added to each well followed by incubation at 37°C for 4 h. The absorbance at 450 nm was determined using Multiskan FC (Thermo Fisher Scientific Inc.).

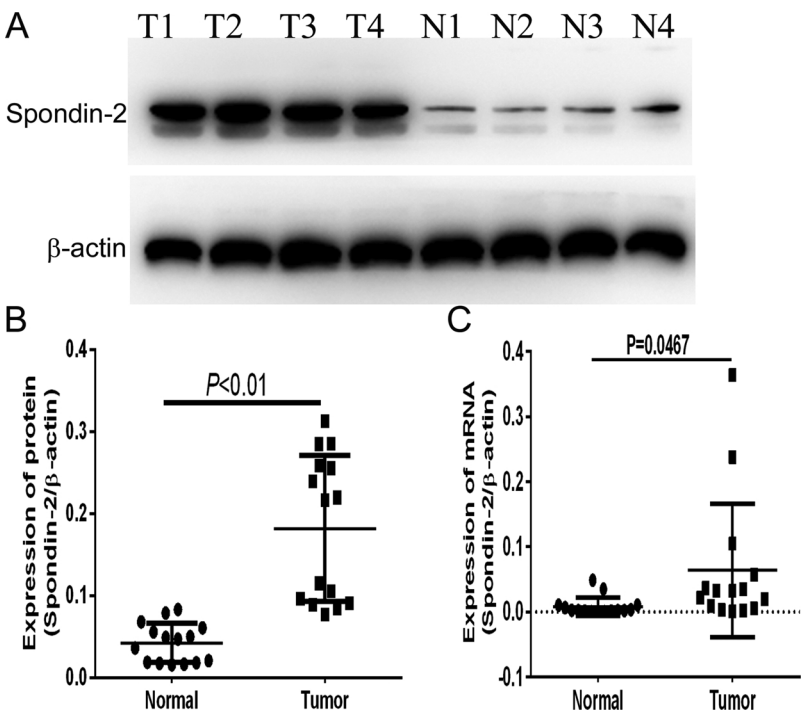

Fig. 1. Expression of spondin-2 increased in LSCC tissues. Western blotting (A) and qRT-PCR (B) analysis demonstrated the expression of spondin-2 in LSCC tissues signifantly increased compared with adjacent non-tumourous tissues (n = 15). ***P*<0.01, *vs* non-tumours tissues. N, represents non-tumours tissues; T, represents LSCC tissues.

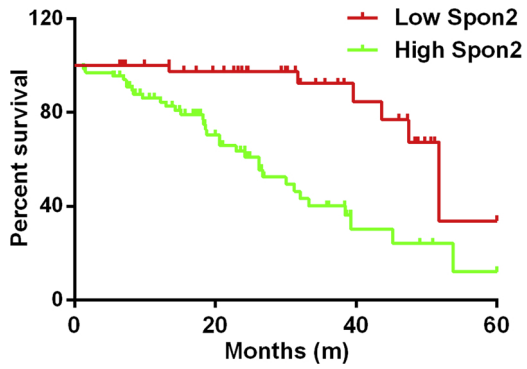

Fig. 2. Kaplan–Meier survival curves of LSCC patients. Patients with high spondin-2 had a poorer prognosis than those with low expression.

2.6. Statistical analysis

All the experiments were performed three times and the data were presented as mean ± standard deviation (SD). Statistical analysis was carried out using SPSS 19.0 software. The correlation between the expression levels of Spondin-2and clinicopathological parameters was assessed by Pearson Chi-square. The data of qRT-PCR and western blotting assays were analyzed by Student's *t*-test The survival data were calculated using Kaplan-Meier curves and the log-rank test. The univariate and multivariate analyses were performed using Cox's proportional hazards regression model. The *P* value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Spondin-2was highly expressed in LSCC

To investigate the expression of Spondin-2 in LSCC, we firstly performed qRT-PCR and western blotting analysis to determine the expression of Spondin-2 in 15 pairs of LSCC tissues and the adjacent non-tumor tissues. As shown in Fig. 1A, the mRNA and protein levels of spondin-2 were both increased in LSCC tissues compared with the non-tumor tissues. Also immunohistochemistry analysis demonstrated spondin-2 was significantly increased in paraffin-embedded LSCC samples (Fig. 3), which suggested spondin-2 may participate in the progression of LSCC.

3.2. Correlations of spondin-2 with clinicopathologic characteristics in patients with LSCC

To further evaluate the clinical significance of spondin-2 in LSCC, we analyzed the relationship between spondin-2 expression and patients' clinicopathological characteristics by Chi-square test. As shown in Table 1, high expression of spondin-2 was significantly correlated with clinical stages, lymph node metastasis and pathology grade ($P < 0.05$). However, high levels of spondin-2 had no statistically significant correlations with other characteristics as gender, age, smoking or tumor location ($P > 0.05$).

3.3. Spondin-2 expression was correlated with overall survival rate of LSCC

Kaplan-Meier analysis was used to assess the association of spondin-2 expression with patients' survival. The results showed that patients with high expression of spondin-2 had the lower overall survival rate compared with patients with low expression of spondin-2 ($P < 0.05$) (Fig. 2).

3.4. Prognostic significance of spondin-2 in LSCC

Moreover, we used the univariate and multivariate analysis to identify potential prognostic factors for LSCC patients. The univariate analysis revealed that clinical stages, lymph node metastasis, pathology grade and spondin-2 expression were associated with patients' overall survival ($P < 0.05$) (Table 2). We also used the multivariate analysis to evaluate the independent prognostic factors. The results demonstrated that spondin-2 expression were independent prognostic indicators for LSCC patients.

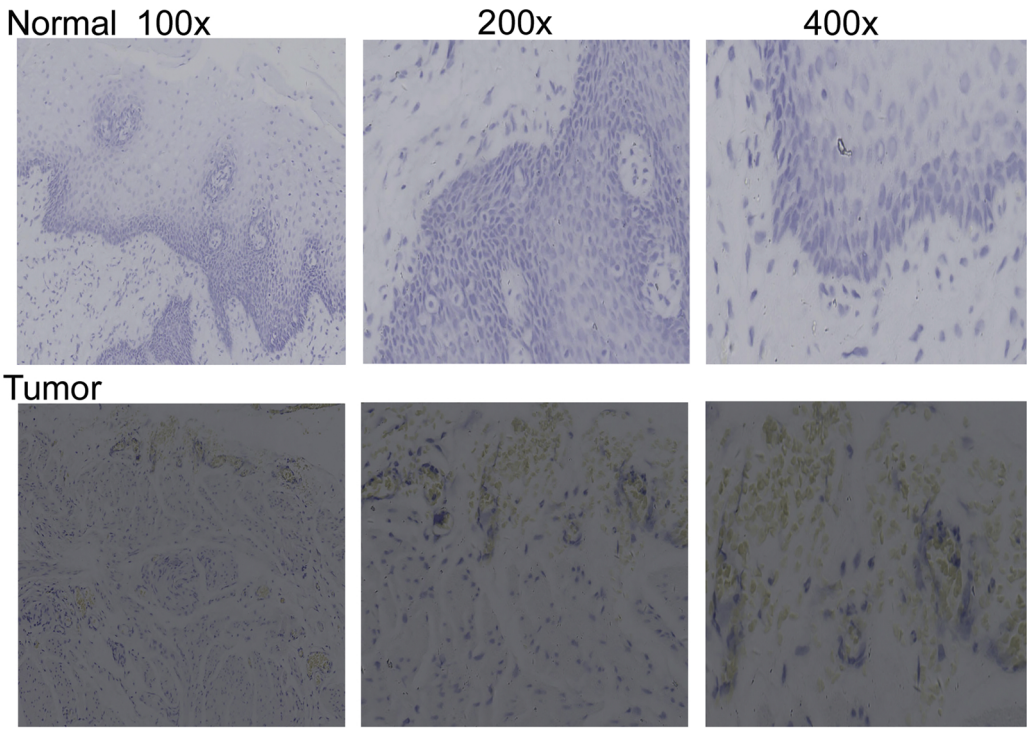

Fig. 3. Immunochemistry analysis demonstrated the expression of Spondin-2 in LSCC tissues significantly increased compared with normal tissues, also the expression of spondin-2 correlated with the pathology grade.

Table 2
Survival status and clinicopathological parameters in 111 human laryngeal carcinoma tissues.

Clinicopathological parameters	Total	Survival status, n (%)		P
		Dead	Alive	
Gender				
Female	4	2	2	0.526
Male	107	37	70	
Age, year				
< 60	31	12	19	0.623
≥60	80	27	53	
Smoking				
No	27	8	19	0.491
Yes	84	31	53	
T Stages				
T1-T2	79	18	61	< 0.001
T3-T4	32	21	11	
Lymph node metastasis				
No	81	26	55	0.217
Yes	30	13	17	
TNM clinical stage				
I-II	57	10	47	< 0.001
III-IV	54	29	25	
Pathology grade				
Well	13	5	8	0.847
moderate	75	25	50	
Poor	23	9	14	
Tumor Location				
Supraglottic	31	15	16	0.053
Glottic	74	24	50	
Subglottic	6	0	6	
Spon2				
Low expression	44	6	38	< 0.001
High expression	67	33	34	

Annotations: Statistical analyses were performed by the Pearson χ [2] test. $P < 0.05$ was considered significant.

3.5. Downregulation of spondin-2 inhibits the proliferation of LSCC through PI3K/AKT signaling

As spondin-2 was significantly upregulated in LSCC tissues, so *in vitro* Hep-2 cells were transfected with spondin-2 siRNA (si-Spondin-2) or spondin-2 overexpressed plasmid (Spondin-2 vector) to knockdown or restored its expression. As indicated, Spondin-2 siRNA significantly decreased but Spondin-2 vector increased the Spondin-2 levels compared with those in the control group. The CCK-8 assay also revealed that Spondin-2 silencing caused a significant decrease in the proliferation of Hep-2 cells until 48 h incubation, and Spondin-2 overexpressed promoted proliferation of Hep-2 cells, when compared with that in the control group (si-NC and pcDNA3.1 groups) (Fig. 4B).

In addition, sicnc the PI3K/AKT pathway is critical for the proliferation and invasion of LSCC cells [17–19]. We then examined the levels of phosphorylated (active) forms of AKT and PI3K in LSCC cells transfected with Spondin-2 siRNA, si-NC, pcDNA3.1 or Spondin-2 overexpressed plasmid. As shown in Fig. 4, Spondin-2 depletion significantly decreased both phosphorylated levels of PI3K and AKT, but not the expression of total PI3K and AKT in Hep-2 cell. These results suggested that Spondin-2 silencing inhibited proliferation of Hep-2 cells partly through inactived PI3K/AKT signaling pathway.

4. Discussion

In the present study, we analyzed the spondin-2 expression in Laryngeal squamous cell carcinoma (LSCC) and assessed its clinical diagnosis significance. The results revealed that the spondin-2 was highly expressed in LSCC tissues compared with paired non-tumor tissues, which suggested spondin-2 might play an important role in LSCC, as well as be an independent prognostic factor and a potential target. Moreover, it is among the first studies to demonstrate the expression of spondin-2 in LSCC.

Laryngeal squamous cell carcinoma (LSCC) is a common type of tumor in the head and neck [20]. Despite numerous therapeutic

Table 3
Contribution of various potential prognostic factors to survival by Cox regression analysis on 111 human laryngeal carcinomas.

	P value	95% confidence interval
T Classification		
T1 + T2 vs T3 + T4	0.165	0.785–4.105
TNM clinical stage		
I-II *vs* III-IV	0.018	1.238–9.909
Spon2 expression		
Low *vs* High	< 0.001	2.149–13.793

Table 4
Sequences for qRT-PCR analysis.

	Sense (5' to 3')	Antisense (5' to 3')
Spondin-2	GGAGGGATAGGACAGACAGACA	GCAGATAGGACGTGGGAGAAA
β-actin	GATGAGATTGGCATGGCTTT	GTCACCTTCACCGTTCCAGT

interventions, the 5-year overall survival (OS) rates are still low because of local recurrence and metastasis [21,22]. Early diagnose and treatments are significantly in improving patients' survival. So, it is urgent to identify biomarkers in LSCC and develop novel therapeutic strategies.

Spondin-2, as an extracellular matrix protein, is also known as spondin 2 and M-spondin or DIL-1 andwas previously identified by mRNA differential display screeningof cancerous and noncancerous lung cells [20]. Liao CH et al. [9] has found that Spondin-2 protein has been upregulated in hepatocellular carcinoma tissues using IHC method, while the expression was not related to pathological stages. Zhang Q et al. [5] reported that the upregulation of Spondin-2 mRNA expression was substantially correlatedd with stage, T stage, Dukes stage and tobacco consumption time of colorectal carcinoma patients, suggesting Spondin-2 as a histological diagnostic biomarker. Giuseppe Lucarelli et al. [6] confirmed that Spondin-2 were significantly overexpressed in prostate cancer patients than in healthy individuals, and serum Spondin-2 had better diagnostic value than serum sarcosine. Spondin-2 was also higher expressed in ovarian tumors compared with normal tissues and serum Spondin-2 could be as a diagnostic biomarker [11].Our result was in accordance with previous reports, which further confirmed the significance of spondin-2 in cancers.

Although Spondin-2 was reported to have numerous biological functions on immune response, inflammatory cells recruitment and neurons development, its role in human carcinogenesis remains unclear [6]. Spondin-2 is showed to be regulated by metastasis associated in

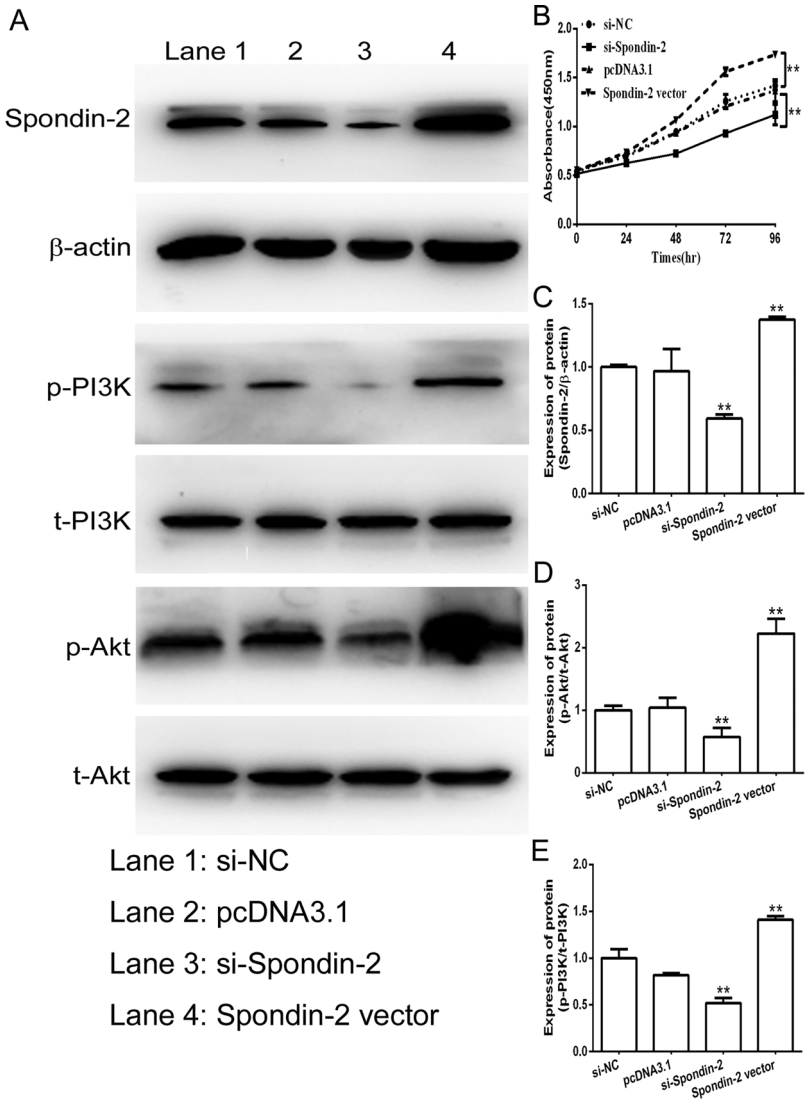

Fig. 4. Spondin-2 silencing inhibited the proliferation of LSCC through PI3K/AKT signaling pathway. (A) Presentative images protein levels in Hep-2 cells transfected with spondin-2 siRNA (si-Spondin-2), spondin-2 overexpressed vector (Spondin-2 vector) or negative control (si-NC and pcDNA3.1). (B) CCK-8 assays for proliferation of Hep-2 cells when transfected with indicated molecular. (C–E) Density analysis was performed to examine the protein levels of p-PI3K, t-PI3K, p-AKT and t-AKT. $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* si-NC.

colon cancer 1 (MACC1) to promote cell viability, migration and invasion of colorectal carcinoma cells [20]. In our study, we found spondin-2 silencing inhibited the proliferation of LSCC cells *in vitro*, which is consent with the results reported, but the mechanism is still unclear.

Previous studies revealed that laryngeal squamous cell carcinoma progression is associated with many signaling pathway, such as NF-κB signaling pathway [23], AKT signaling pathway [18,24], Notch2 signaling pathway [25] and so on. Also Spondin-2 is involved in the activation of many signaling pathway, such as Wnt signaling pathway [26,27]. However, no evidence demonstrated whether Spondin-2 could regulate the activation of PI3K/AKT signaling pathway in LSCC. So, in this study, we detected the activation of PI3K/AKT signaling pathway when transfection of Spondin-2 siRNA or Spondin-2 overexpressed plasmid. The results demonstrated that Spondin-2 silencing triggered the inactivation of AKT and PI3K in LSCC cells, which suggested that the activation of PI3K/AKT is partly regulated by Spondin-2 in LSCC cells. It is well known that the activated PI3K/AKT pathway directly modulates cell growth, migration and invasion of many types of cancer cells, including LSCC cells [28,29]. Therefore, it is reasonable to speculate that the decreased cell proliferation observed in Spondin-2 siRNA-transfected LSCC cells is partly due to the inactivation of PI3K/AKT signaling pathway.

Based on the data above, we think our study also has several limitations. Firstly, it is a retrospective study which means our study is limited by the bias of patients, samples and clinical data. Secondly, we did not provide a deep investigation on the mechanism of spondin-2 in development and prognosis of LSCC, which needs our further study to confirm.

5. Conclusion

In conclusion, our results demonstrated that spondin-2 plays an important role as a tumor promoter in LSCC. And the expression of spondin-2 is associated with pathological characteristics of LSCC. spondin-2 might be an independent prognostic factor as well as a probable novel therapeutic target molecular for LSCC.

Competing interests

All the authors have no competing interests.

Ethical approval

The study was approved by the Ethics Committee of Affiliated Hospital of Nantong University.

Informed consent

All patients provided written informed consent.

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