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SPARC correlates with unfavorable outcome and promotes tumor growth in lung squamous cell carcinoma

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ABSTRACT

Secreted protein acidic and rich in cysteine (SPARC) plays a crucial role in the malignant progression of a number of human cancers. However, the roles of SPARC in lung squamous cell carcinoma (LSCC) remain elusive. In this present study, we first detected SPARC expression and investigated the relationship between SPARC expression and the clinicopathological attributes of LSCC patients. Then we constructed SPARC-overexpression model in LSCC cell line to explore the characteristics of SPARC in LSCC development both in vitro and in vivo. The data demonstrated a remarkably higher level of SPARC in LSCC tissues than in corresponding non-cancerous tissues and elevated SPARC expression was significantly correlated with poor outcome in LSCC patients. Moreover, a serial of phenotypic experiments indicated that SPARC overexpression substantially facilitated the growth and inhibited the apoptosis in LSCC cells and xenografts. Taken together, our results suggest that SPARC is a novel prognostic marker for LSCC prognosis and SPARC significantly promotes LSCC tumorigenesis. Targeting SPARC may provide a novel therapeutic strategy for LSCC management.

1. Introduction

Lung cancer (LC) is the leading cause of cancer-related death worldwide, with 1.8 million new cases diagnosed annually (Siegel et al., 2019). In China, the current status of LC is remarkably terrifying because the mortality of LC has been increasing by > 400% over the past three decades (Luo et al., 2018; Wen and Dehnel, 2011). Lung squamous cell carcinoma (LSCC) is the second-largest histological subtype of non-small cell lung cancer (NSCLC) and accounts for approximately 30% of all NSCLC cases (Travis et al., 2013). In spite of great development of diagnostic and therapeutic strategies on LSCC in recent years, the prognosis of LSCC is still frustrating. A latest study reported that the 5-year survival rate of advanced LSCC was < 15% (Socinski et al., 2018). For now, large amount of studies have explored the critical molecular alterations in LSCC and provided important information that stratify patients according to the overall survival (Asai et al., 2019; Matsubara et al., 2019; Takada et al., 2019). Therefore there is an urgent need of biomarkers that could refine the prognosis and help in the choice of LSCC therapy (Travis et al., 2015).

Secreted protein acidic and rich in cysteine (SPARC) is a non-structural matricellular 32-kDa glycoprotein that is associated with cell-matrix interactions during cell adhesion, proliferation, migration, and tissue remodelling (Brekken and Sage, 2000; Chlenski et al., 2006). SPARC also plays an important role in the development of different malignancies, including LC (Clark and Sage, 2008; Wong and Sukkar, 2017). Previous researches have reported the poor prognostic role of SPARC in a number of aggressive cancers such as melanoma, glioma, colorectal cancer and breast cancer (Ikuta et al., 2009; Kim et al., 2017; Kunigal et al., 2006; Vajkoczy et al., 2000; Yoshimura et al., 2011). However, SPARC was also reported to act as the inhibitory molecular in tumorigenesis in several types of human cancer, including ovarian cancer, prostate cancer and pancreatic cancer (Sato et al., 2003; Shin et al., 2013; Yiu et al., 2001). The conflicting effects of SPARC in tumor development deserve further exploration.

In this present study, we firstly detected SPARC expression in LSCC tissue samples and cell lines. The relationships of SPARC expression with the clinicopathological attributes of LSCC patients including prognostic significance were further evaluated. Then the SPARC-

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overexpression model was constructed and a number of phenotypic experiments concerning the malignant behaviors of SPARC for LSCC development were performed.

2. Materials and methods

2.1. LSCC patients and samples collection

Tissue microarrays (TMAs) containing 90 cases of LSCC samples were purchased from Outdo Biotech Co., Ltd. (Shanghai, China), which is one of the largest institutions in China to provide TMA for scientific researches and enjoys great reputation. Several previous studies also employed the TMA products of Outdo Biotech Co., Ltd. (Chen et al., 2017; Chen et al., 2016). Related clinical data, including gender, age, tumor size, pathological grade, histological type, tumor status (T), lymph node metastasis (N), distant metastasis (M), and TNM stage, were also provided by Biotech Co., Ltd. along with TMAs and it could be download from the following website: http://www.superchip.com.cn/biology/category_264/1349.html. None of the LSCC patients received any forms of treatments (radiation therapy, chemotherapy, or immunotherapy) before surgery. Written informed consent was obtained from each patient included in this study. Ethical approval to perform this research was approved from the Human Research Ethics Committee of Nanjing Medical University.

2.2. IHC analysis in LSCC TMA

IHC analysis was performed as previously described (Mao et al., 2019). TMAs were incubated with a primary rabbit monoclonal anti-SPARC antibody (ab225716, 1:100, Abcam, USA). The secondary antibody used was horseradish peroxidase-conjugated anti-rabbit antibody (DakoCytomation, Carpinteria, CA, USA). Phosphate-buffered saline (PBS) was used as negative control. SPARC immunostaining was evaluated by counting the intensity and percentage of SPARC-positive cells by two clinical pathologists (Li Xu and Yan Chen) independently using immunohistochemistry score (IHS) protocol. When a conclusion differed, the final decision was made by consensus (Mao et al., 2012). Staining intensity was marked as follows: 0 (negative staining), 1 (yellow staining), 2 (light brown staining), and 3 (dark brown staining). Staining percentage of SPARC was categorized as follows: 1 (0–10%), 2 (11–50%), 3 (51–80%), and 4 (81–100%). The product of the staining intensity and percentage gave rise to the IHS. The final results of IHC analysis were defined using a two level system following the IHS: < 4 indicates low expression, while 4–12 indicates high expression.

2.3. Cell lines

Four LSCC cell lines (H1703, H2170, H520, SK-MES-1) were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China) and cultured routinely by our lab.

2.4. Cell transfection to construct SPARC-overexpression model

The human LSCC cell line was cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) at 37 °C in a 5% CO_2 humidified incubator. Human SPARC cDNA was amplified by PCR using synthetic primers and was cloned into a pcDNA3.1 vector (Invitrogen, San Diego, CA, USA) to construct SPARC-overexpression model (pcDNA3.1-SPARC). Transient transfections were then performed using the liposome-mediated method (Lipofectamine 2000, Invitrogen) according to the manufacturer's instructions. The blank control, empty pcDNA3.1 vector and pcDNA3.1-SPARC transfection groups were employed for this experiment.

2.5. qPCR test and western-blotting analysis

For qPCR test, total RNA was extracted from cells using TRIzol (Invitrogen, USA) according to the manufacturer's protocol and described in our previous research(Luo et al., 2018). The primers of SPARC detection were as follows: forward primer 5′-ATG ACG ACG GCA CCT ACA G-3′; reverse primer 5′-TCG CGT TGG GGT AAC TTT TCA-3′. The GAPDH was employed as internal control, and the primers for GAPDH were as follows: forward primer 5′-AAT GGA CAA CTG GTC GTG GAC-3′; reverse primer 5′-CCC TCC AGG GGA TCT GTT TG-3′. Reactions were performed using the following conditions: 95 °C for 5 min and hen 40 cycles of 95 °C for 5 s and 60 °C for 31 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression of target genes. For western-blotting analysis, total protein from each lysate was loaded and separated by 10% SDS-PAGE and transferred onto the nitrocellulose membrane. The membranes were first incubated with a primary rabbit monoclonal anti-SPARC antibody (ab225716, 1:500, Abcam) and then a secondary antibody (Dako) and then were detected by the ECL kit and autoradiography using X-ray film.

2.6. Immunofluorescence analysis

Immunofluorescence analysis was performed as described previously (Lin et al., 2014). Briefly, the cells were incubated with a primary rabbit monoclonal anti-SPARC antibody (ab225716, 1:15, Abcam) at 4 °C overnight. The following day, the cells were washed and incubated with a secondary fluorescent antibody in the dark. Additionally, nuclei were counterstained with H33342 (Sigma-Aldrich, St. Louis, USA). Finally, the cells were visualized using a Zeiss AxioVert inverted fluorescence microscope (Carl Zeiss, Germany).

2.7. Cell proliferation, colony formation, wound healing, transwell and cell apoptosis assays in vitro

Cell proliferation was assessed by a cell counting kit-8 (CCK8) assay (Dojindo, Japan) according to the manufacturer's protocol. Colony formation assay, wound healing assay, transwell assay as well as cell apoptosis assay were performed as described previously (Lin et al., 2014; Mao et al., 2019; Zhang et al., 2019).

2.8. LSCC xenograft model construction and TUNEL detection in vivo

The animal experiment protocols were approved by the ethics committee of the Geriatric Hospital of Nanjing Medical University. A total of 5×10^6 cells were subcutaneously injected into 4-week-old BALB/c nude mice which were purchased from SLAC Laboratory Animal (Shanghai, China), Tumor volume was measured twice a week for 6 weeks by using digital callipers. On day 43, all mice were sacrificed, xenograft tumors were removed from nude mice and weighed. Then in situ cell apoptosis was evaluated by TUNEL staining assay following the protocol of TumorTACS in situ Apoptosis kit (R&D Systems, Minneapolis, MN, USA).

2.9. Statistical analysis

Data are presented as the mean ± S.E.M. for the indicated numbers of independently performed experiments. The relationships between SPARC expression and clinicopathologic parameters were analyzed by chi-square tests. Univariate and multivariate Cox regression models were used to screen prognostic elements for overall survival. The Kaplan-Meier method was employed to demonstrate the associations between SPARC expression and the prognosis of LSCC patients. The significance level for statistical analysis was set at $p < .05$. All statistics were performed by utilizing STATA 14.0 (Stata Corporation, College Station, TX, USA) and SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

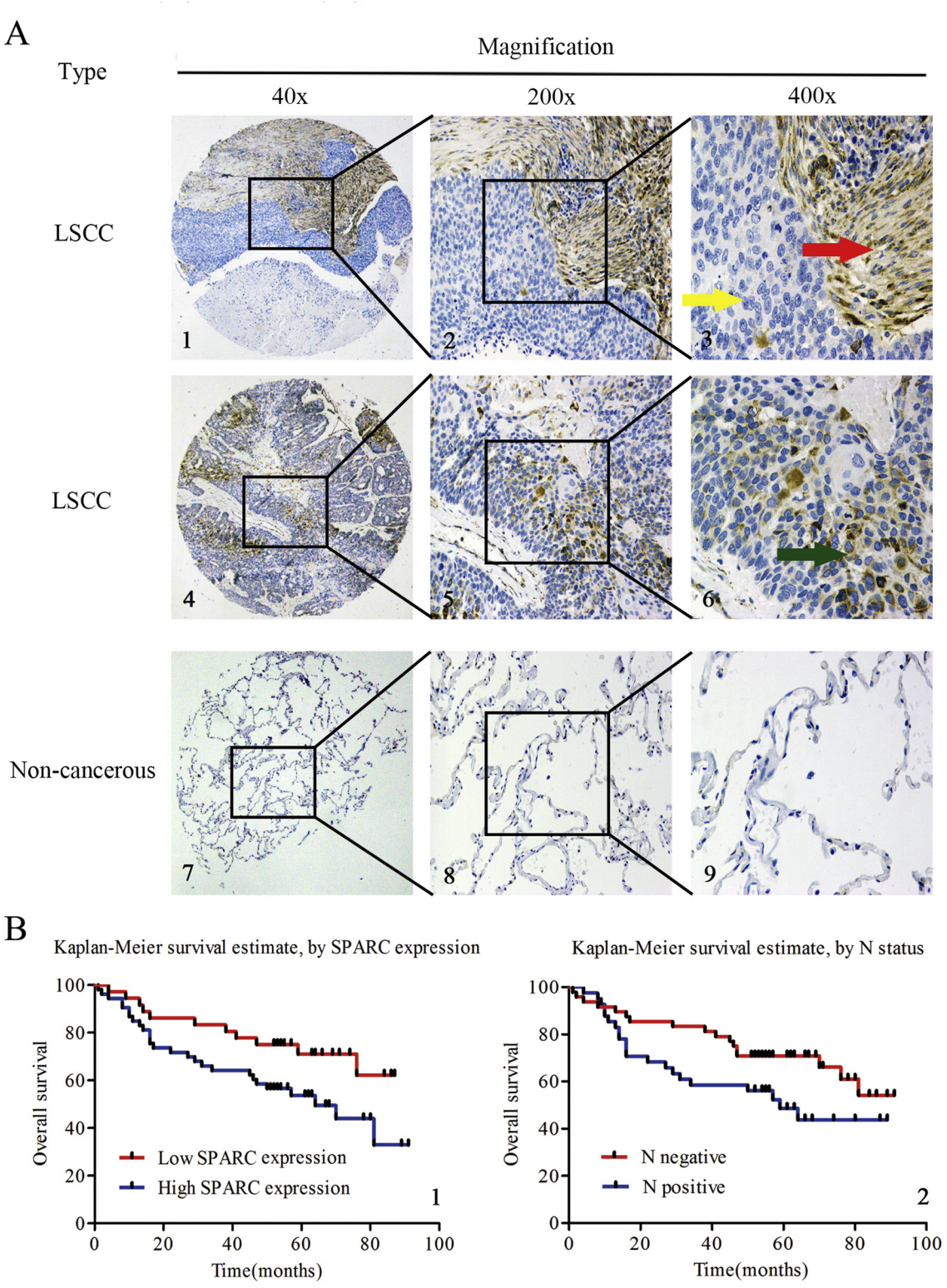

Fig. 1. A. Representative pictures of SPARC protein expression in LSCC tissue samples and corresponding non-cancerous tissue samples. A1, A2, and A3. High stromal expression of SPARC in LSCC tissue sample. Red arrow shows the positive staining in the stroma of LSCC. Yellow arrow shows the negative staining in the cytoplasm of LSCC cells. A4, A5, and A6. High cytoplasmic expression of SPARC in LSCC tissue sample. Green arrow shows the positive staining in the cytoplasm of LSCC cells. A7, A8, and A9. No expression of SPARC in non-cancerous tissue sample. Original magnification: ×40 in A1,A4 and A7, ×200 in A2, A5 and A8, ×400 in A3, A6 and A9. B. Survival analysis of LSCC patients by the Kaplan-Meier method. B1. The overall survival rate in patients with high SPARC protein expression was significantly lower than that in patients with low SPARC expression. B2. The overall survival rate in patients with positive N status was significantly lower than that in patients with negative N status. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Correlation of high SPARC protein expression with clinicopathological characteristics in 90 LSCC.

Groups	No.	SPARC		χ^2	*p* value
		+	%		
Age					
≥60 years	59	34	57.6	0.402	0.526
< 60 years	31	20	64.5		
Tumor diameter					
≥3 cm	79	46	58.2	0.155	0.694
< 3 cm	6	3	50.0		
Insufficient Data	5	5			
Pathological grade					
Grade I-II	64	36	56.3	1.298	0.255
Grade III	26	18	69.2		
T status					
T1-T2	64	33	51.6	7.071	0.008*
T3-T4	20	17	85.0		
Insufficient Data	6	4			
N status					
Positive	37	24	64.9	1.2069	0.272
Negative	49	26	53.1		
Insufficient Data	4	4			
M status					
Positive	1	0	0.0	1.517	0.218
Negative	89	54	60.7		
TNM stage					
Stage I-II	51	29	56.9	0.523	0.470
Stage III-IV	34	22	64.7		
Insufficient Data	5	3			

* p < .05.

Table 2
Univariate and multivariate analysis of prognostic factors for overall survival in LSCC patients.

	Univariate analysis			Multivariate analysis		
	HR	p value	95% CI	HR	p value	95% CI
SPARC expression						
High versus Low	2.11	0.037*	1.05–4.24	2.26	0.024*	1.11–4.59
Age						
≥60 years versus < 60 years	1.75	0.127	0.85–3.60	NS		
Tumor diameter						
≥3 cm versus < 3 cm	3.38	0.231	0.45–24.71	NS		
Pathological grade						
Grade I-II versus Grade III	1.03	0.942	0.51–2.06	NS		
T status						
T1-T2 versus T3-T4	0.61	0.195	0.29–1.28	NS		
N status						
Positive versus Negative	2.15	0.021*	1.12–4.14	2.15	0.022*	1.11–4.14
M status						
Positive versus Negative	5.87	0.087	0.78–44.62	NS		
TNM stage						
Stage I-II versus Stage III-IV	0.61	0.150	0.32–1.19	NS		

* p < .05; HR: hazard ration; CI: confidence interval; LSCC: lung squamous cell carcinoma, NS: not significant.

3. Results

3.1. Detection of SPARC expression by IHC analysis

High SPARC expression was observed in 54 (60.0%) of the 90 LSCC tissue samples compared with 10 (11.1%) of the 90 corresponding non-cancerous tissue samples and the difference was highly significant ($\chi^2 = 46.94$, $p = .001$). High expression of SPARC protein was mainly localized in the stroma of LSCC. Only in a few cases, SPARC protein was detected in the cytoplasm of LSCC cells (Fig. 1A). The association between SPARC expression and important clinicopathological attributes of LSCC is shown in Table 1. Positive expression of SPARC in LSCC was significantly associated with T status ($\chi^2 = 7.071$, $P = .008$). In comparison, no significant relationship was witnessed between SPARC expression and other clinical parameters, including age, tumor diameter, pathological grade, N status, M status and TNM stage (Table 1).

3.2. Survival analysis

The univariate analysis demonstrated three factors that associated with overall survival of LSCC, including SPARC expression ($p = .037$) and N status ($p = .021$). Multivariate analysis further confirmed that SPARC expression ($p = .024$) and N status ($p = .022$) were two potential prognostic predictors for 90 LSCC patients in this present study (Table 2). Kaplan-Meier survival curves were constructed to indicate that LSCC patients with high SPARC expression and positive N status suffered poor prognosis (Fig. 1B).

3.3. Construction of SPARC-overexpression model

We enrolled four LSCC cell lines (H1703, H2170, H520, SK-MES-1) to detect protein expression of SPARC. Positive SPARC expression was observed in all cell lines (Fig. 2A). Then we chose the H520 cell line with relative weak expression of SPARC to construct SPARC-overexpression model to perform the further phenotypic experiments. qPCR, Western blotting and fluorescence staining analyses confirmed that H520 cell line with SPARC-overexpression was successfully constructed. Fig. 2B, C and D demonstrated that SPARC expression was significantly upregulated in the pcDNA3.1-SPARC group compared with the blank control group and empty vector group.

3.4. SPARC-overexpression facilitates malignant behaviors of LSCC cell in vitro

CCK-8, colony formation, wound healing and transwell assays showed that SPARC-overexpression significantly facilitated LSCC cell proliferation (Fig. 3A and B), migration (Fig. 3C) and invasion (Fig. 3D), respectively. Simultaneously, Annexin V/propidium iodide staining and flow cytometry assay illustrated that SPARC-overexpression led a significant decrease in cell apoptosis (blank control 8.79% vs. empty vector 10.03% vs. pcDNA3.1-SPARC 3.61%) (Fig. 3E).

3.5. SPARC-overexpression promotes tumor growth in LSCC xenograft in vivo

Three groups of xenograft were prepared in nude mice to explore the tumor-develop effects of SPARC-overexpression in vivo. As shown in Fig. 4A and B, xenograft tumors from pcDNA3.1-SPARC group grew faster than those from blank control group and empty vector group. IHC analysis further verified that SPARC protein was significantly elevated in tumors formed from pcDNA3.1-SPARC LSCC cells (Fig. 4C). In addition, TUNEL assay of xenograft tumor sections demonstrated that the number of apoptotic cells was significantly decreased in pcDNA3.1-SPARC group compared to blank control group and empty vector group (Fig. 4D).

4. Discussion

SPARC, a multicellular nonstructural glycoprotein, is well acknowledged to be involved in multiple processes of human cancers (Ahir et al., 2017; Chlenski et al., 2006). Several studies have reported that SPARC promoted invasiveness of melanoma cells (Girotti et al., 2011), accelerated epithelial-mesenchymal transition of hepatocellular

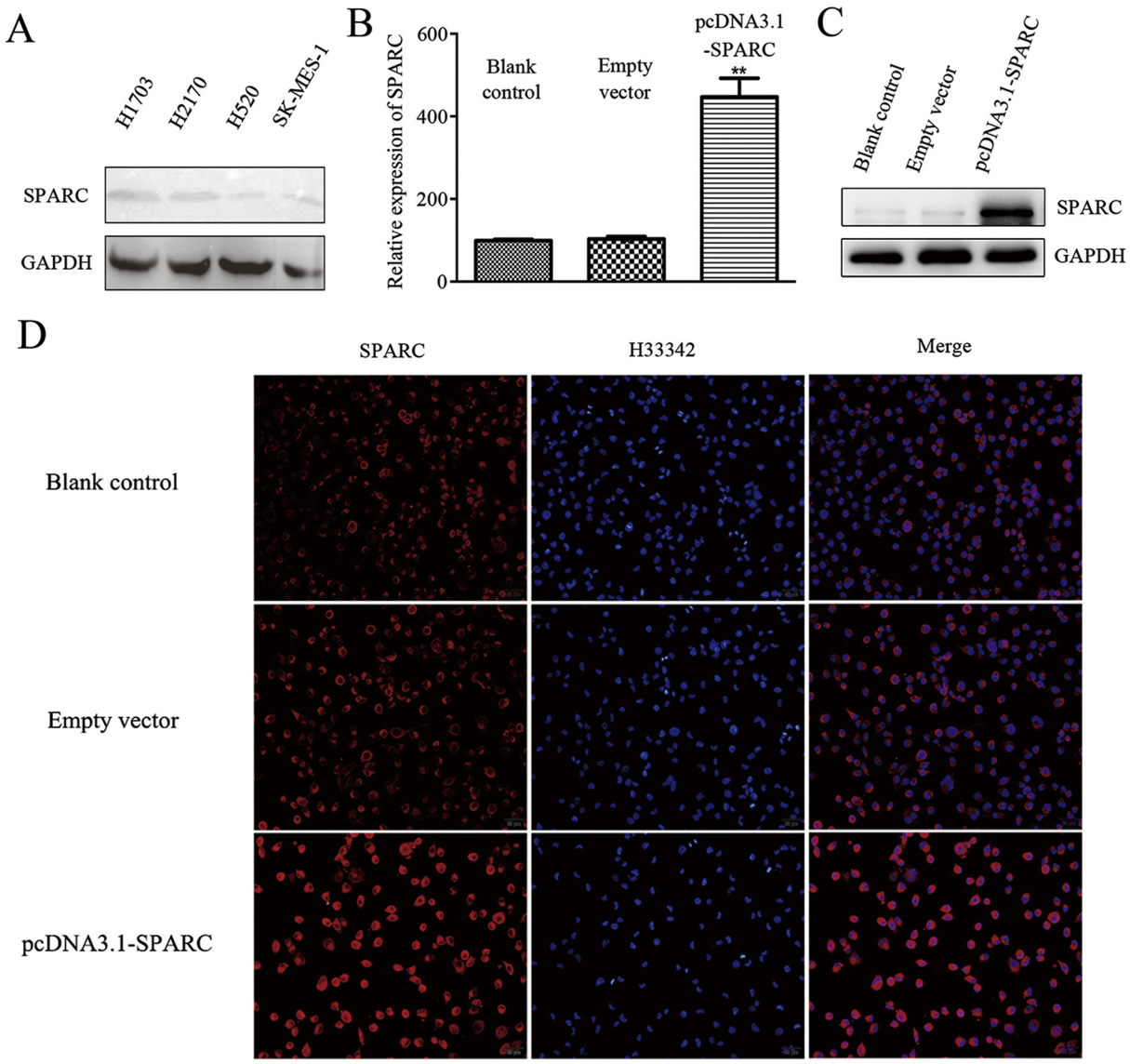

Fig. 2. A. SPARC protein expression in four LSCC cell lines (H1703, H2170, H520, SK-MES-1). Positive LSCC expression was observed in all cell lines and H520 cell line showed the most insignificant SPARC protein expression. B and C. After cell transfection in H520 cell, qPCR and Western blot analyses confirmed the successful construction of SPARC-overexpression model. The SPARC expression in pcDNA3.1-SPARC group was remarkably elevated, compared with that in blank control group and empty vector group. D. The overexpression of SPARC in pcDNA3.1-SPARC group was further confirmed by an immunofluorescence assay.

carcinoma cells (Jiang et al., 2019) and boosted migration endometrial cancer cells (Yusuf et al., 2014). However, a number of researches also stated that SPARC acted as a tumor suppressor to decrease prostate cancer proliferation (Shin et al., 2013), inhibited bladder cancer metastasis (Said et al., 2013) and induced neuroblastoma apoptosis (Sailaja et al., 2013). As for the prognostic role of SPARC in NSCLC, Huang et al. suggested that SPARC was expressed more frequently in LSCC than in adenocarcinoma and can be considered as a prognostic factor for NSCLC (Huang et al., 2012). Komiya et al. reported that stromal SPARC expression was detected in −70% of NSCLC cases and SPARC might be used as predictor for selecting patients likely to respond favorably to nab-paclitaxel treatment (Komiya et al., 2016). Kurtul et al. revealed that high SPARC expression was identified as a poor prognostic factor in cases with locally advanced NSCLC treated with concurrent chemoradiotherapy (Kurtul et al., 2014). Since SPARC exerts a complex role in different human cancers including NSCLC, the detailed function of SPARC especially its prognostic character in LSCC deserves to be further explored.

In this present study, we examined the expression of SPARC in 90 cases of LSCC and analyzed clinical significance of SPARC expression in LSCC patients. IHC analysis showed that SPARC protein was tremendously elevated in LSCC tissue samples, compared to non-cancerous tissue samples. Furthermore, overexpression of SPARC in LSCC is significantly associated with T status. Jing et al. reported similar results that SPARC protein level was upregulated in oral squamous cell carcinoma (Jing et al., 2019). In survival analysis, SPARC expression was remarkably associated with the poor overall survival of LSCC patients and was recognized as an potential indicator of LSCC prognosis. Our results are consistent with previous studies on the prognostic role of SPARC in pancreatic carcinoma (Infante et al., 2007; Sato et al., 2003; Shintakuya et al., 2018).

For further detection of the detailed activities of SPARC in LSCC development in vitro, we examined SPARC expression in LSCC cell lines and successfully developed the SPARC-overexpression model (pcDNA3.1-SPARC). CCK-8, colony formation, wound healing and transwell assays were performed and the results demonstrated showed that SPARC-overexpression significantly accelerated LSCC cell development (proliferation, migration and invasion) and inhibited LSCC cell

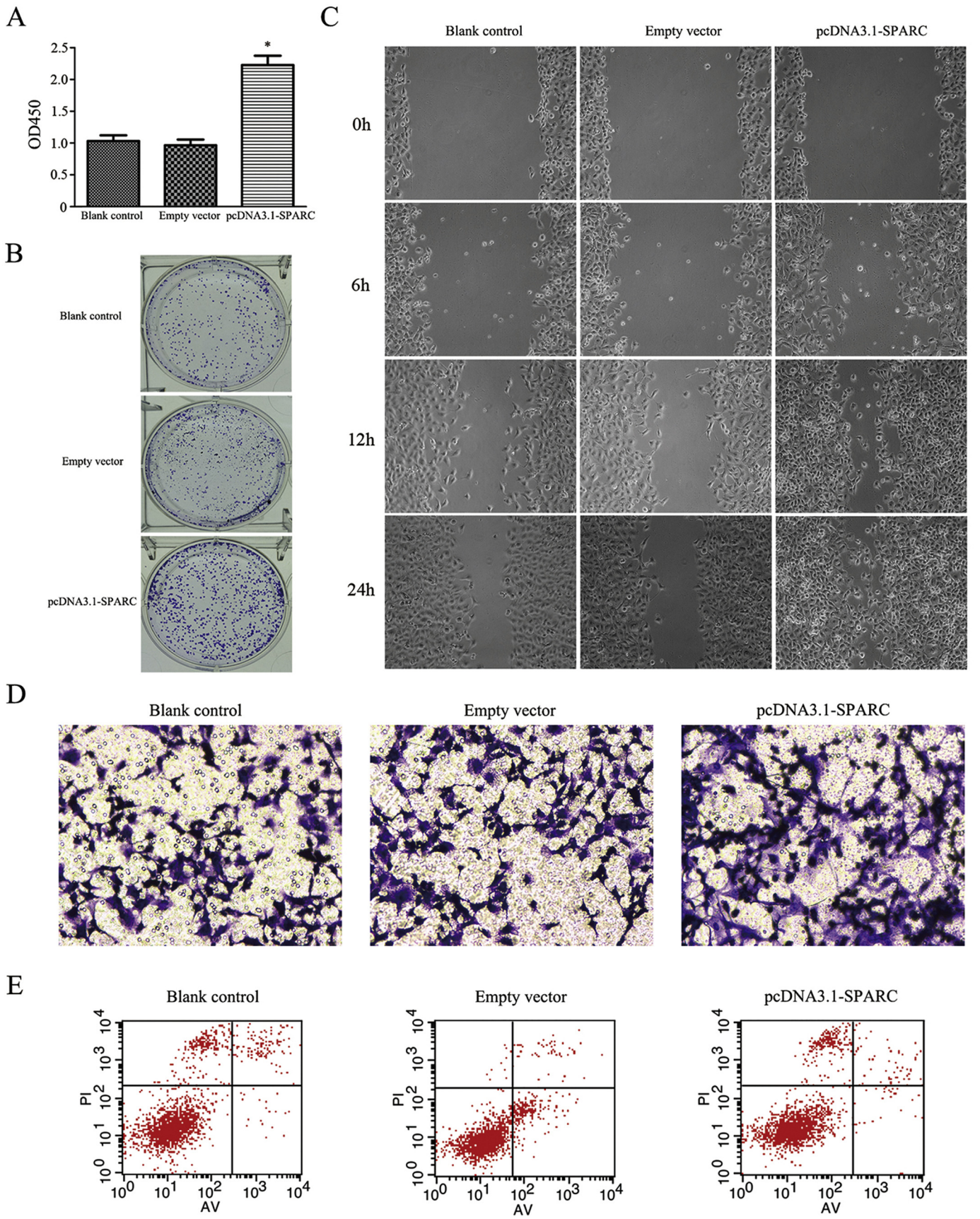

Fig. 3. Overexpression of SPARC significantly facilitated malignant behaviors of LSCC cell. A and B. CCK-8 and colony formation tests showed that SPARC-overexpression promoted LSCC cell proliferation. C. Wound healing assay revealed that SPARC-overexpression promoted LSCC cell migration. D. Transwell assay demonstrated that SPARC-overexpression promoted LSCC cell invasion. E. Annexin V/PI staining illustrated that SPARC-overexpression induced LSCC cell apoptosis. The apoptotic rate in the blank control group, empty vector group and pcDNA3.1-SPARC group were 8.79 ± 0.647%, 10.03 ± 0.914% and 3.61 ± 0.335%, respectively.

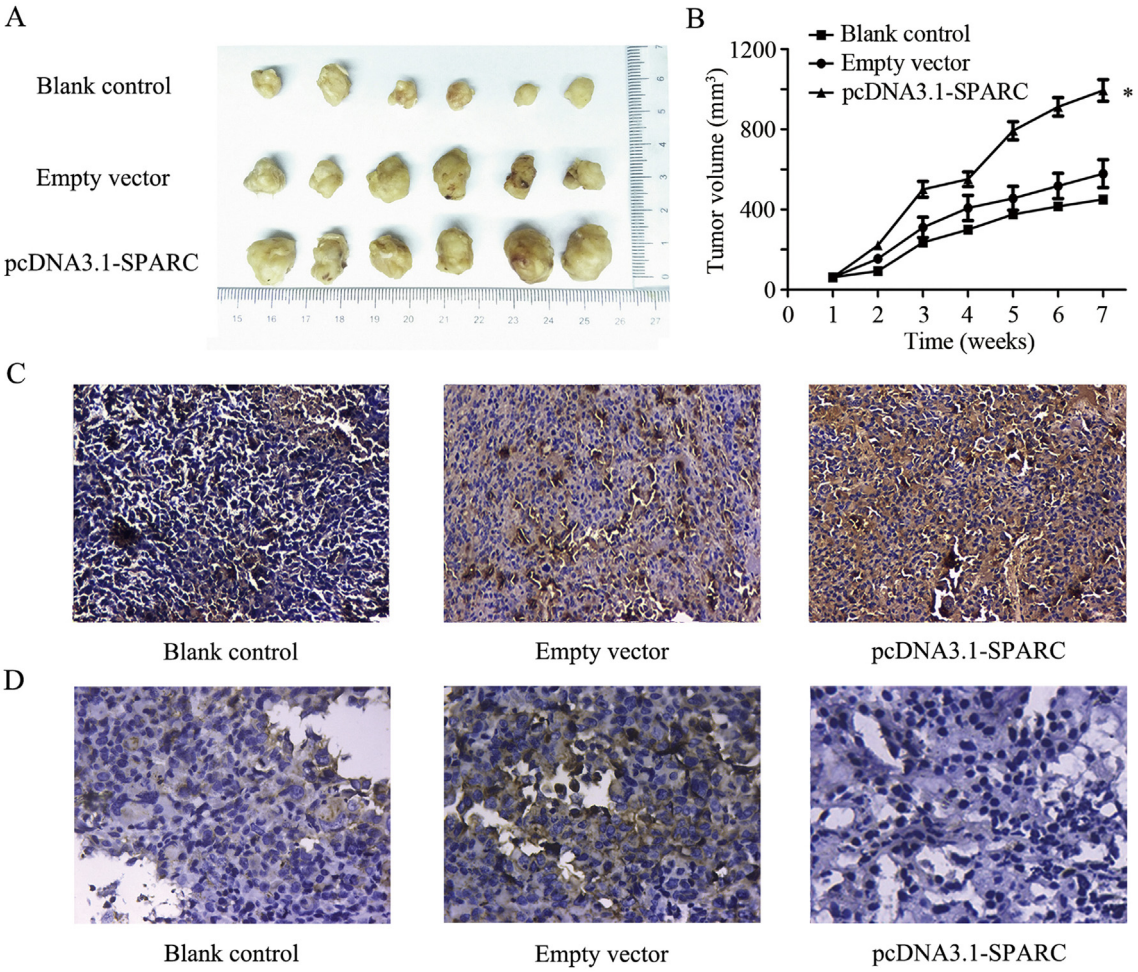

Fig. 4. A and B. Overexpression of SPARC significantly accelerated xenograft tumor growth in nude mice. The tumor size was monitored and recorded every 7 days. C. SPARC expression was confirmed by IHC analysis and the xenograft from pcDNA3.1-SPARC group showed the dramatically elevation of SPARC expression. D. TUNEL staining demonstrated significantly lower number of apoptotic cells in pcDNA3.1-SPARC group than in blank control and empty vector groups.

apoptosis. Furthermore, we employed nude mice to explore the characteristic of SPARC in LSCC xenograft in vivo. SPARC-overexpression dramatically facilitated tumor growth and downregulated tumor apoptosis in LSCC xenograft nude mice. Hung et al. reported that SPARC could induce cell migration and epithelial mesenchymal transition through WNK1/snail in non-small cell lung cancer (Hung et al., 2017); Sun et al. described that SPARC acted as a mediator of TGF-β1 in promoting EMT in lung cancer cells (Sun et al., 2018); Grant et al. found that SPARC-dependent manner took great part in promoting cell invasion in NSCLC pathogenesis (Grant et al., 2014). These above data highly enlightened the oncogenic role of SPARC in LSCC and targeting SPARC might be a promising therapeutic strategy for LSCC management.

However, there are several limitations in this present study. Firstly, we did not construct SPARC-knockdown model to explore the characteristics of SPARC in LSCC development. The reason was that we did not identify a LSCC cell line with high SPARC expression, even the SPARC expression in H1703 was moderate. So we only chose the H520 cell line with the minimum SPARC expression to develop the SPARC-overexpression model. Although the data from the SPARC-knockdown model can provide stronger evidence, we reckon that the present results with SPARC-overexpression model in this study are acceptable. A previous study also followed the similar protocol to investigate the function of MAGEC2 in breast cancer metastasis (Yang et al., 2014). Secondly, the TMA quality is unexceptional because the numbers of distant metastases are small, and some important clinical information of survival are missing (i.e., DFS, PFS). Thirdly, the mechanism of SPARC function in LSCC development was still elusive. As a matter of fact, the role of SPARC in NSCLC was diverse. For example, high SPARC expression in NSCLC tumors was associated with longer survival, while its absence was a negative prognostic factor (Huang et al., 2012). In comparison, patients with SPARC-positive stroma expression encountered significantly poorer overall post-operative survival (Wong and Sukkar, 2017). Future researches that enroll larger LSCC samples and elaborate experimental design are necessary to further explore how SPARC affects the malignant behaviors of LSCC.

To sum up, we showed for the first time that high SPARC expression was significantly associated with unfavorable outcome in LSCC and that SPARC might be identified as an potential prognostic indicator for LSCC. Moreover, SPARC-overexpression could facilitate LSCC development in vitro as well as in vivo. These findings underline that targeting SPARC may be a potential strategy for LSCC treatment.

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Declaration of Competing Interest

The authors declare that they have no competing interest.

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