

SPAG5 is associated with unfavorable prognosis in patients with lung adenocarcinoma and promotes proliferation, motility and autophagy in A549 cells

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Abstract. Sperm-associated antigen 5 (SPAG5) is involved in the tumorigenesis of multiple cancer types. However, the role of SPAG5 during lung adenocarcinoma (LUAD) progression remains to be fully elucidated. In the present study, the expression of SPAG5 in tumor tissues of patients with LUAD from public cancer databases was analyzed using the online software Gene Expression Profiling Interactive Analysis and University of Alabama Cancer Database. The association of SPAG5 expression levels with the prognosis of patients with LUAD was analyzed using Kaplan-Meier Plotter. In addition, the role of SPAG5 in the LUAD cell line A549 was determined by knocking down its expression with specific small interfering RNA. The results demonstrated that SPAG5 expression was upregulated in LUAD tissues and its high expression was associated with unfavorable prognosis. Furthermore, in A549 cells, SPAG5 promoted proliferation, migration, invasion and autophagy, but inhibited apoptosis. The present results suggest that SPAG5 has an oncogenic role in LUAD and may be a potential prognostic predictor and therapeutic target for LUAD.

Introduction

Lung cancer is the most common cancer type with the highest incidence and mortality among all cancers worldwide (1). In 2018, >2 million new lung cancer cases and 1.8 million lung cancer-associated deaths were estimated to have occurred worldwide (1). Lung cancer is classified into small cell lung carcinoma and non-small cell lung carcinoma (NSCLC), which includes lung adenocarcinoma (LUAD), squamous cell carcinoma and large cell carcinoma, and accounts for >80% of lung cancer cases (2). Current treatment options for NSCLC are surgery and adjuvant therapy, including radiation,

chemotherapy and targeted therapy (2). Targeted medicines, such as those against epidermal growth factor receptor and anaplastic lymphoma kinase, have been successfully applied in the clinic (3). However, it is still necessary to discover more appropriate molecular targets in order to provide personalized treatment or overcome potential drug resistance.

Sperm-associated antigen 5 (SPAG5, also known as astrin) is involved in mitotic spindle formation and chromosome segregation (4,5), and acts as an important regulator of mitosis and the cell cycle (6). Studies have revealed that SPAG5 has an oncogenic role during the tumorigenesis of multiple cancer types. It was reported to inhibit mTOR complex 1 (mTORC1) signaling to prevent apoptosis in HeLa cells (7), interact with centrosomal protein of 55 kDa to regulate PI3K/AKT signaling, and downregulate scavenger receptor class A member 5 expression via the Wnt/ β -catenin pathway in hepatocellular carcinoma cells (8,9), activate the AKT/mTOR pathway to upregulate Wnt3 expression in bladder urothelial carcinoma cells (10), and upregulate survivin expression in gastric cancer (11). It has been indicated that SPAG5 may serve as a prognostic biomarker in cervical cancer (12), breast cancer (13) and bladder urothelial carcinoma (10). A previous study reported that p53 inhibition is essential for the upregulation of SPAG5 in LUAD (14). However, the role and the underlying mechanisms of SPAG5 in LUAD remain to be fully elucidated.

In the present study, SPAG5 expression was assessed in LUAD tissues and the association of SPAG5 expression with the prognosis of patients with LUAD was analyzed. In addition, the role of SPAG5 in cellular proliferation, apoptosis, autophagy and motility of A549 cells was examined.

Materials and methods

Cell transfection and reagents. The LUAD cell lines A549, HCC827, HCC515, HCC1833, NCI-H1975, NCI-H1993, NCI-H2347 and NCI-H3255 were purchased from the Kunming Cell Bank of the Chinese Academy of Sciences. Cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an incubator with 5% CO₂. A549 cells were grown to ~75% confluence and then transiently transfected with SPAG5 small interfering RNA (siRNA; #1, 5'-GAGGAAUUGUAGAGCAUTT-3', #2, 5'-CCCACAAC

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UCACAGAGAATT-3', #3, 5'-CCCGACAACUCACAGAGA ATT-3') or scrambled siRNA (5'-UUCUCCGAACGUGUC ACGUTT-3') (purchased from Shanghai GenePharma Co., Ltd.) at a concentration of 100 pM using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Bafilomycin (used at a concentration of 100 nM) was purchased from Cell Signaling Technology, Inc. (cat. no. 54645S).

Western blot analysis. At 48 h after transfection, total protein was extracted from A549 cells using radioimmunoprecipitation assay lysis buffer (150 mM NaCl; 50 M Tris-HCl; pH 7.5; 1% Triton X-100; 0.1% sodium deoxycholate and 0.1% SDS) containing 0.1% proteinase and phosphatase inhibitors (Beijing Solarbio Science & Technology Co., Ltd.). Immunoblot analysis was then performed as described previously (15). The primary antibodies used were as follows: Rabbit anti-SPAG5 polyclonal antibody [cat. no. 60940; 1:1,800; Cell Signaling Technology, Inc. (CST)], rabbit anti-caspase-3 polyclonal antibody (cat. no. 9662; 1:1,000; CST), rabbit anti-cleaved caspase-3 polyclonal antibody (cat. no. 9661; 1:500; CST), rabbit anti-poly(ADP-ribose) polymerase (PARP; cat. no. 9542; 1:1,000; CST), rabbit anti-S6 kinase (cat. no. 2708; 1:1,000; CST), mouse anti-phosphorylated-S6 kinase (cat. no. 9206; 1:1,000; CST), rabbit anti-light chain (LC) 3B polyclonal antibody (cat. no. NB100-2220; 1:1,000; Novus Biologicals, LLC), rabbit anti-p62 polyclonal antibody (cat. no. 39749; 1:1,000; CST) and mouse anti- β -actin monoclonal antibody (cat. no. TA811000; 1:1,000; OriGene Technologies, Inc.). The secondary antibodies used were as follows: Horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG; cat. no. 7074; 1:5,000; CST) and HRP-conjugated anti-mouse IgG (cat. no. 7076; 1:5,000; CST). Densitometric analysis was performed using FluorChem SA 6.0.0 (Informer Technologies, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from A549 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 2 μ g RNA was reverse transcribed into complementary DNA using a reverse transcription kit (cat. no. KR103-04; Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. qPCR was performed using SYBR® Premix Ex Taq™ II (Takara Bio, Inc.) to detect SPAG5 gene expression. GAPDH was amplified in parallel as the internal control. The gene-specific primers used were as follows: SPAG5 forward, 5'-CATCTCACAGTGG GATAACTAATAAAC-3' and reverse, 5'-CAGGGATAGGTG AAGCAAGGATA-3' and GAPDH forward, 5'-CAGGAGGCA TTGCTGATGAT-3' and reverse, 5'-GAAGGCTGGGGCTCA TTT-3'. The following thermocycling conditions were used for the qPCR: Pre-denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 20 sec and extension at 72°C for 10 sec, and final extension at 72°C for 30 sec. The $2^{-\Delta\Delta C_q}$ method was used to calculate relative gene expression (16).

Flow cytometric analysis. A549 cells (1×10^5 cells/well) were seeded in 12-well plates and cultured for 17 h. Subsequently, the cells were transfected with SPAG5 siRNA or control

siRNA for 48 h. The cells were harvested and incubated with 5 μ l FITC Annexin V and 5 μ l propidium iodide (PI) for 15 min in the dark, and 300 μ l 1X binding buffer was then added. Finally, the cells were analyzed by flow cytometry (Muse™ Cell Analyzer; Merck Millipore). Cells with positive FITC Annexin V and negative PI were considered to be apoptotic.

Cell proliferation assay. A549 cells were transfected with SPAG5 siRNA or control siRNA. At 48 h post-transfection, 5×10^3 cells were seeded into 96-well plates in RPMI-1640 medium containing 2% FBS. The media was replaced every day for 4 days. Cell proliferation was determined with an MTT kit (cat. no. M1020; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's protocol. In brief, MTT was dissolved in 0.1 M PBS at a concentration of 5 mg/ml. Subsequently, 10 μ l MTT solution was added to each well, followed by incubation at 37°C for 4 h. Finally, RPMI-1640 culture medium was discarded and 200 μ l dimethyl sulfoxide was then added to each well to completely dissolve the crystals. The absorbance at a wavelength of 490 nm was measured with a microplate reader (SpectraMax® Plus 384; Molecular Devices).

Cell migration and invasion assay. Following transfection with SPAG5 siRNA or negative control for 48 h, 8- μ m pore size culture inserts (Transwell; Costar; Corning Inc.) were used for the migration assay and Matrigel Invasion Chambers (Transwell; Costar; Corning Inc.) were used for the invasion assay. The inserts were placed into the wells of 24-well culture plates, separating the plates into upper and lower chambers. A total of 600 μ l RPMI-1640 medium containing 20% FBS was added to the lower chambers. Furthermore, 3×10^4 cells in 200 μ l serum-free RPMI-1640 medium were added to each upper chamber. After incubation at 37°C in an atmosphere with 5% CO₂ for 24 h, non-invading or non-migrating cells were removed from the top wells with a cotton swab. Cells that had transgressed to the bottom of the membrane were fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 0.1% crystal violet overnight at 4°C. Randomly selected images of three independent fields of each well were captured with a phase-contrast microscope (magnification, $\times 100$; Olympus TH4-200; Olympus Corporation) and the cells were manually counted.

Colony formation assay. Following transfection with SPAG5 siRNA or negative control for 48 h, 6×10^2 cells were seeded into each well of six-well plates, and 2 ml RPMI-1640 culture medium was added into each well. Cells were cultured at 37°C in a humidified incubator with 5% CO₂ for 10-15 days. Cells were washed with PBS three times, fixed with 4% neutral paraformaldehyde solution at room temperature for 30 min, followed with three PBS washes, and 2 ml 1% crystal violet solution was added to each well and incubated at room temperature for 2 h. The cells were washed three times with PBS after the crystal violet solution was discarded. Once the plates were dry, the colonies were counted and images were captured.

Analysis of SPAG5 expression in LUAD tissues. LUAD data from The Cancer Genome Atlas (TCGA) were analyzed using the Gene Expression Profiling Interactive Analysis (GEPIA; gepia.cancer-pku.cn) online tool (17). The Okayama Lung cohort (GSE31210 in Gene Expression Omnibus database) was

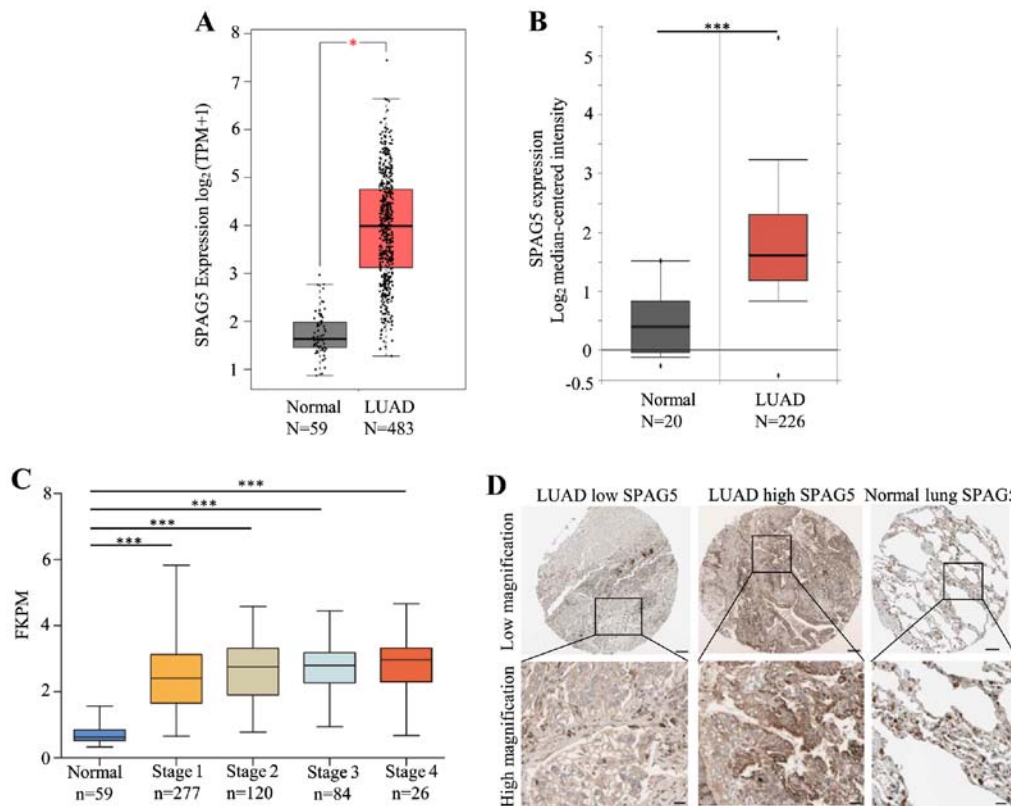


Figure 1. SPAG5 expression is upregulated in LUAD. (A) SPAG5 expression in LUAD tissues (n=483) and normal lung tissues (n=59) from TCGA database was analyzed with the Gene Expression Profiling Interactive Analysis online software. The results demonstrated that SPAG5 expression was significantly increased in LUAD. *P<0.05. (B) SPAG5 expression in LUAD tissues (n=226) and normal lung tissues (n=20) from the ONCOMINE database (Okayama Lung) was analyzed online. The results demonstrated that SPAG5 expression was significantly increased in LUAD. ***P<0.001. (C) SPAG5 expression in different stages of LUAD was significantly increased compared with in normal tissues. However, there was no significant difference between any stages. SPAG5 expression at different stages (stage 1, n=277; stage 2, n=120; stage 3, n=84; stage 4, n=26) and normal lung tissues (n=59) from TCGA database was analyzed. One-way ANOVAs and Tukey's post hoc test were used for the comparison between the groups. ***P<0.001. (D) Immunohistochemistry staining results from The Human Protein Atlas database demonstrated that SPAG5 expression was upregulated in LUAD tissues. The patient ID for the sample with low expression was 2585 and that for high expression was 3391. Scale bar, 100 μ m in low-magnification images and 25 μ m in high-magnification images. TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma; SPAG5, sperm-associated antigen 5; TPM, transcripts per million; FPKM, fragments per kilobase of transcript per million mapped reads.

analyzed using ONCOMINE (www.oncomine.org) (18,19). TCGA LUAD datasets (TCGA Pan-Cancer Atlas) were downloaded from cbiportal (<https://www.cbiportal.org/datasets>) and SPAG5 expression at different clinical stages of TCGA LUAD was analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc.) (20). Comparisons among normal groups and different clinical stages were performed using one-way ANOVA followed by Tukey's post hoc test. SPAG5 expression data by immunohistochemical (IHC) staining were obtained from The Human Protein Atlas (HPA) database (www.proteinatlas.org) (21).

Prognosis analysis. The univariate analysis of SPAG5 expression on overall survival (OS), first progression (FP) and post-progression survival (PPS) was analyzed using the online tool Kaplan Meier plotter (www.kmplot.com) (22). The cut-off values were as follows: 266 for OS, 216 for FP and 305 for PPS, which were the best threshold values automatically determined by the software. Hazard ratio (HR), 95% confidence intervals (CI) and log-rank P-values were calculated using Cox regression. The log-rank test was used to evaluate the differences between survival curves. Cox proportional hazards model was used for multivariate analysis of sex, American Joint Committee on Cancer (AJCC) stage T and stage N,

smoking history and SPAG5 expression on OS and FP. P<0.05 was considered to indicate a statistically significant difference.

Statistical analysis. SPSS 13.0 software (SPSS, Inc.) was used to perform statistical analysis. Values are expressed as the mean \pm SD. Comparisons between two groups were performed using Student's t-test and comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test. All experiments were performed at least three times independently and representative data are presented. The log-rank test was used to evaluate the differences between survival curves. Cox proportional hazards model was used for multivariate analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

SPAG5 expression is upregulated in LUAD tissues and its high expression is associated with unfavorable prognosis. To determine SPAG5 expression in LUAD tissues, the LUAD data in TCGA were analyzed using the online tool GEPIA (17). The results indicated that SPAG5 expression was significantly upregulated in LUAD tissues (n=483) compared with normal lung tissues (n=59) (P<0.05; Fig. 1A). To confirm the TCGA

results, SPAG5 expression was analyzed in LUAD tissues (n=226) and normal lung tissues (n=20) in the Okayama Lung cohort from the ONCOMINE database (18,19). The results demonstrated that SPAG5 expression was significantly increased in LUAD compared with normal lung tissues ($P<0.001$; Fig. 1B), consistent with the results of the LUAD cohort from TCGA. Furthermore, SPAG5 expression was analyzed in LUAD samples at different clinical stages from TCGA. The results showed that SPAG5 expression at stages 1-4 (stage 1, n=277; stage 2, n=120; stage 3, n=84; stage 4, n=26;) was significantly increased compared with normal lung tissues (n=59) ($P<0.001$; Fig. 1C), while there was no significant difference between any two stages of LUAD. Since the TCGA and ONCOMINE data were obtained at the RNA level, alterations in SPAG5 expression were further analyzed at the protein level by searching the HPA database (21). The results of the HPA included two datasets for IHC staining of lung cancer tissue (HPA022008 and HPA022479) comprising a total of 11 cases stained with one of two different SPAG5 antibodies. The HPA022008 dataset comprised one LUAD sample with low and five with medium SPAG5-positive staining, while the HPA022479 dataset comprised two LUAD samples with negative, two with low and one with highly positive SPAG5 staining. Representative images are provided in Fig. 1D. The IHC data confirmed SPAG5 expression in LUAD tissues at the protein level.

To reveal the potential prognostic value of SPAG5 in patients with LUAD, the association between SPAG5 expression and OS, FP and PPS was analyzed using the online tool Kaplan Meier plotter (22). The results of the univariate analysis demonstrated that high expression of SPAG5 is associated with unfavorable OS and FP (Fig. 2A and B), while there was no significant association with PPS (Fig. 2C), suggesting that SPAG5 expression may serve as a predictor of OS and FP. The results of the multivariate analysis of AJCC stage, sex and smoking history suggested that high expression of SPAG5 is an independent prognostic indicator for OS and FP (Table I).

Knockdown of SPAG5 suppresses the proliferation, migration and invasion of A549 cells. To investigate the role of SPAG5 in lung cancer *in vitro*, SPAG5 protein expression was examined in multiple LUAD cell lines by western blot analysis. The results indicated SPAG5 was expressed in all cell lines tested and its expression in A549 cells was the highest (Fig. 3A). Therefore, the A549 cell line was selected for subsequent experiments. A total of three siRNAs against SPAG5 were designed and their effects to suppress SPAG5 expression were assessed by RT-qPCR and western blot analysis. A549 cells were individually transfected with SPAG5 siRNAs#1-3 and control siRNA, and SPAG5 expression was examined 48 h after transfection. The results indicated that all SPAG5 siRNAs significantly reduced SPAG5 expression at the RNA and protein level compared with control siRNA transfection, and SPAG5 siRNA#2 had the most potent inhibitory effect (Fig. 3B). Thus, SPAG5 siRNA#2 was used in further experiments.

Since SPAG5 was indicated to be associated with unfavorable OS and FP of patients with LUAD, it was hypothesized that SPAG5 may have a role in the regulation of proliferation

Table I. Cox regression multivariate analysis on OS and FP.

Rate	Variable	Hazard ratio (95% CI)	P-value
OS	Sex	1.55 (0.86-2.79)	0.1410
	AJCC stage T	2.64 (1.34-5.21)	0.0051
	AJCC stage N	2.83 (1.69-4.75)	0.0001
	Smoking history	0.82 (0.38-1.77)	0.6138
	SPAG5	1.89 (1.01-3.53)	0.0469
FP	Sex	1.22 (0.66-2.26)	0.5333
	AJCC stage T	2.87 (1.34-6.17)	0.0068
	AJCC stage N	2.48 (1.41-4.37)	0.0017
	Smoking history	1.41 (0.7-2.84)	0.3412
	SPAG5	2.05 (1.05-4)	0.0357

OS, overall survival; FP, first progression; AJCC, American Joint Committee on Cancer.

and motility of LUAD cells. To test this hypothesis, the proliferation and motility of A549 cells transfected with SPAG5 siRNA were analyzed using MTT and Transwell assays, respectively. The results suggested that, compared with that in the control siRNA group, the proliferation (Fig. 4A), migration (Fig. 4B) and invasion (Fig. 4C) of A549 cells transfected with SPAG5 siRNA was significantly attenuated.

Knockdown of SPAG5 induces apoptosis in A549 cells. The effects of SPAG5 knockdown on A549 cell apoptosis were then examined by flow cytometry and western blot analysis. Flow cytometric analysis demonstrated that SPAG5 siRNA led to significant increase in apoptotic cells compared with control siRNA (Fig. 5A). To confirm the flow cytometry results, the activation of the apoptotic markers caspase-3 and PARP was detected by western blot analysis. The result suggested that, compared with the control siRNA group, transfection of SPAG5 siRNA resulted in a decrease of caspase-3 and PARP protein and a simultaneous increase of cleaved caspase-3 and cleaved PARP protein (Fig. 5B), indicating that SPAG5 knockdown induced apoptosis in A549 cells.

Knockdown of SPAG5 inhibits autophagy in A549 cells. As a previous study indicated that in HeLa cells, SPAG5 inhibits mTORC1, which is a vital upstream negative regulator of autophagy (7), it was hypothesized that SPAG5 may enhance autophagy. To assess this hypothesis, A549 cells were transfected with SPAG5 siRNA for 48 h and then treated with the autophagy inhibitor bafilomycin for 1 h. Subsequently, the autophagic markers LC3 and p62 were examined by western blot analysis. The results suggested that transfection of SPAG5 siRNA led to a decrease of LC3-II and a simultaneous increase of p62 compared with control siRNA (Fig. 6A), indicating that SPAG5 knockdown inhibited autophagy. In addition, mTORC1 activity was examined by detecting phosphorylation of its substrate S6K by western blot analysis. The results indicated that compared with the control siRNA group, S6K phosphorylation increased following SPAG5 knockdown (Fig. 6B), implying increased mTORC1 activity.

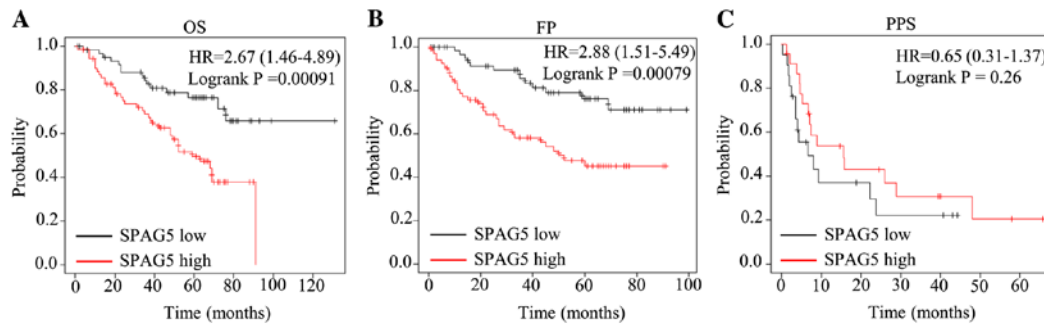


Figure 2. High SPAG5 expression is associated with unfavorable OS and FP in patients with LUAD. (A) High SPAG5 expression was associated with unfavorable OS. The OS curve of 360 patients was plotted [cut-off value, 266; HR (95% CI)=2.04 (1.38-3.01); log-rank P=0.00024]. (B) High SPAG5 expression was associated with unfavorable FP. The FP curve of 170 patients was plotted [cut-off value, 216; HR (95% CI)=2.6 (1.37-4.93); log-rank P=0.0023]. (C) High SPAG5 expression was not significantly associated with PPS. The PPS curve of 125 patients was plotted [cut-off value, 305; HR (95% CI)=1.49 (0.86-2.61); log-rank P=0.15]. The online tool Kaplan Meier Plotter was used to draw the graphs. OS, overall survival; FP, first progression; LUAD, lung adenocarcinoma; SPAG5, sperm-associated antigen 5; HR, hazard ratio; PPS, post-progression survival.

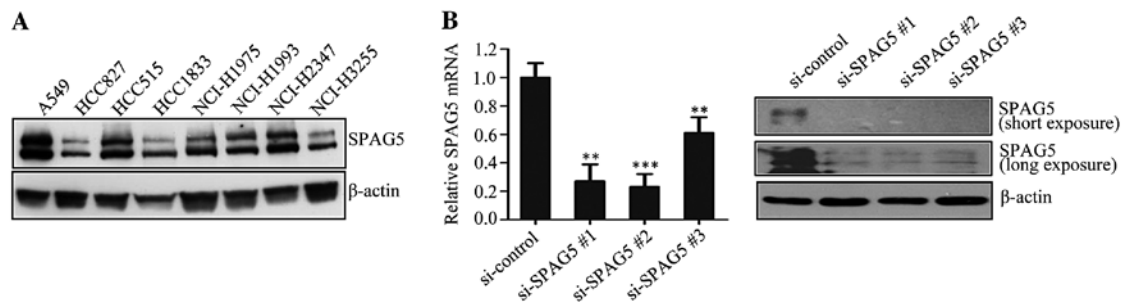


Figure 3. SPAG5 is expressed in multiple lung adenocarcinoma cell lines. (A) SPAG5 protein levels were determined by western blot analysis. The results showed that A549 cells had the highest SPAG5 expression among the cell lines tested. (B) A549 cells were treated with SPAG5 siRNA for 72 h and SPAG5 mRNA (left) and protein (right) levels were then examined. The results indicated that SPAG5 siRNA #2 had the highest efficacy. **P<0.01 and ***P<0.001 vs. si-control. SPAG5, sperm-associated antigen 5; siRNA, small interfering RNA.

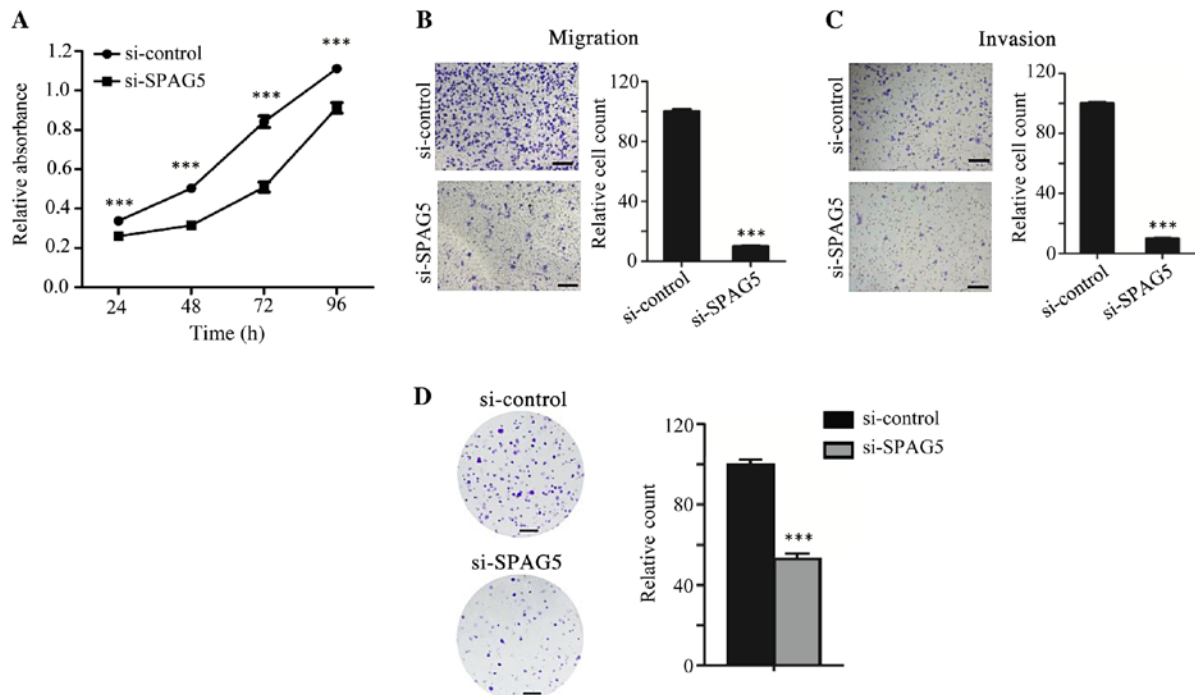


Figure 4. SPAG5 knockdown inhibits proliferation, migration, invasion and colony formation of A549 cells. (A) A549 cells were transfected with SPAG5 siRNA and the proliferation was then determined by MTT assay. (B) Migration and (C) invasion of A549 cells transfected with siRNA was measured using a Transwell assay without or with Matrigel, respectively. Representative images of filter membranes are shown. Magnification, x200. Scale bar, 200 μ m. (D) Colony formation assay results of A549 cells transfected with SPAG5 siRNA or control siRNA. Scale bar, 5 mm. ***P<0.001 vs. si-control. SPAG5, sperm-associated antigen 5; siRNA, small interfering RNA.

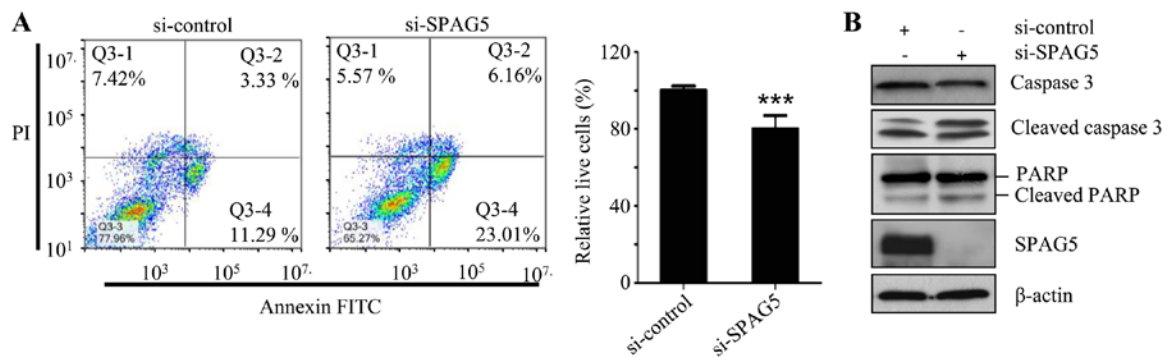


Figure 5. SPAG5 knockdown induces apoptosis. A549 cells were transfected with SPAG5 siRNA for 72 h. (A) Apoptosis was detected by flow cytometry. Representative flow cytometry dot plots (Annexin V⁺/PI⁺ cells were considered apoptotic) and quantitative results are shown. (B) The apoptotic markers caspase-3 and cleaved caspase-3 were detected by western blot analysis. Representative western blot images are shown. Data are expressed as the mean \pm standard deviation. ***P<0.001 vs. si-control. SPAG5, sperm-associated antigen 5; siRNA, small interfering RNA; PI, propidium iodide; PARP, poly (ADP-ribose) polymerase.

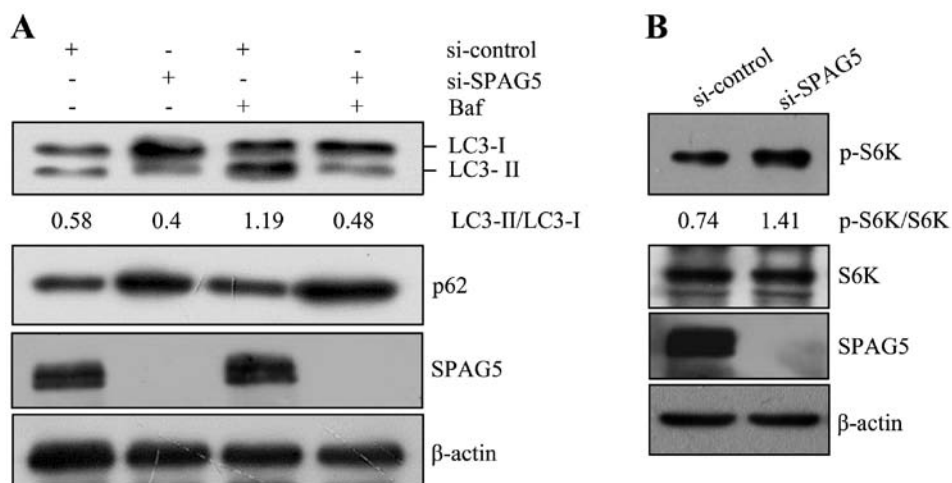


Figure 6. SPAG5 knockdown suppresses autophagy by activating mTORC1. A549 cells were transfected with SPAG5 siRNA for 48 h, followed by bafilomycin treatment for 1 h. The autophagic markers LC3 and p62 and S6K phosphorylation were detected by western blot analysis. (A) The levels of LC3-II were reduced and p62 protein levels were increased by SPAG5 siRNA, implying inhibition of autophagy. Bafilomycin treatment did not result in any obvious accumulation of LC3. (B) Phosphorylation of S6K was increased by SPAG5 siRNA, implying increased mTORC1 activity. SPAG5, sperm-associated antigen 5; siRNA, small interfering RNA; LC3, light chain 3; mTORC1, mTOR complex 1, Baf, bafilomycin.

Discussion

Patients with lung cancer have a poor prognosis and the 5-year survival is <18% (2). Therefore, it is urgent to discover novel biomolecules that may be used as diagnostic markers and/or therapeutic targets. Vålík *et al* (23) analyzed the gene expression profiles of 81 tissue samples of patients with NSCLC, revealing that SPAG5 is upregulated in NSCLC and therefore suggested that SPAG5 may be a biomarker in this cancer type. However, the underlying mechanisms of SPAG5 in LUAD have remained elusive. The present study demonstrated that SPAG5 expression was upregulated in LUAD by analyzing its expression in two different large LUAD cohorts from two databases. However, SPAG5 expression did not change significantly from LUAD stage 1 to stage 4. These results indicated that SPAG5 upregulation occurs early during the tumorigenesis of LUAD, implying that SPAG5 may be a potential diagnostic marker for LUAD. In addition, high expression of SPAG5 was indicated to be associated with unfavorable OS and FP, which may also suggest that SPAG5 may be used as a prognostic marker in LUAD.

Dysregulation of proliferation is a hallmark of malignant tumor growth (24). It was hypothesized that inhibiting SPAG5 expression may suppress certain cancerous features of LUAD cells. Thus, this possibility was tested *in vitro* using LUAD A549 cells, which exhibited high endogenous SPAG5 expression. The present study demonstrated that, in A549 cells, knockdown of SPAG5 inhibited cell proliferation and induced apoptosis, indicating that SPAG5 may have a role in the regulation of cell proliferation and death. These results are in agreement with those of previous studies on cervical cancer (12), bladder urothelial carcinoma (10) and gastric cancer (11). Although the apoptosis of A549 cells was analyzed upon SPAG5 knockdown by flow cytometry to detect annexin and by western blotting to examine the apoptotic markers caspase-3 and PARP, it would be useful to confirm the results in cells treated with caspase inhibitor when performing further experiments. Certain carcinomas arise from epithelial tissues and may subsequently obtain the capability of migration and invasion to progress to higher pathological grades of malignancy due to aberrations in the expression of relevant genes associated with invasion and metastasis, such as N-cadherin (24). The present study

indicated that knockdown of SPAG5 in A549 cells inhibited cell migration and invasion, suggesting that SPAG5 may have a role in tumor metastasis. As SPAG5 may act via the Wnt/ β -catenin and AKT/mTOR pathways (8,10), both of which are crucial pathways in a variety of cancers, it is necessary to explore which mechanism enables LUAD cells to obtain those features in the future. As epidermal growth factor receptor (EGFR) acts upstream of the AKT/mTOR pathway, it was questioned whether there is any association between EGFR and SPAG5. To address this, the correlation between EGFR and SPAG5 expression in LUAD tissues was analyzed with GEPIA. However, a weak but not significant correlation was observed (data not shown), indicating they do not significantly affect the expression of each other.

Autophagy is a lysosomal degradation pathway through which cells are able to recycle unwanted or damaged proteins or organelles to maintain cellular homeostasis or in response to microenvironmental stress (25). Accumulating evidence has indicated that autophagy may serve as a tumor-suppressing or tumor-promoting process, depending on the context or tumor type (26). A previous study reported that SPAG5 inhibits mTORC1 activity by binding to Raptor to prevent mTORC1 assembly in HeLa cells (7). Given that mTORC1 acts as a suppressor of autophagy, it was hypothesized that SPAG5 may have a role in the regulation of autophagy. Thus, the effect of SPAG5 knockdown on autophagy was examined in the present study. The results indicated that knockdown of SPAG5 inhibited autophagy accompanied by increased S6K phosphorylation, implying SPAG5 promoted autophagy through regulating the activity of mTORC1 in A549 cells. Therefore, SPAG5 may promote tumor cell survival through enhancing autophagy to recycle cellular components in the microenvironment lacking oxygen and nutrients. To the best of our knowledge, the present study was the first to reveal the role of SPAG5 in the regulation of autophagy.

Since high SPAG5 expression was observed in LUAD tissues and may contribute to cancerous features, including excessive proliferation, evasion of apoptosis and enhancement of autophagy, it is important to uncover how SPAG5 expression is regulated in LUAD. Wang *et al* (14) observed that SPAG5 expression in LUAD cells is suppressed by p53. Song *et al* (27) reported that microRNA (miR)-1179 inhibited SPAG5 expression in NSCLC cells. Wang *et al* (28) revealed that long non-coding RNA LINC00875 may sponge miR-1179 to regulate SPAG5 expression in LUAD cells. As the *in vitro* experiments in the present study were only performed in A549 cells with siRNA to knock down SPAG5, further experiments including knockdown and overexpression of SPAG5 should be performed in other LUAD cell lines in order to confirm the conclusions.

In summary, the present study demonstrated that the expression of SPAG5 was upregulated in LUAD tissues and its high expression is associated with unfavorable clinical outcomes. It may promote the proliferation, motility and autophagy, and inhibit apoptosis of LUAD cells. The present results indicated that SPAG5 has an oncogenic role in LUAD and may be a potential prognostic predictor and therapeutic target for LUAD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AL conceived the study. RH performed the bioinformatical analysis and AL performed the experiments. RH and AL analyzed and interpreted the data. AL and RH wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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