

# SPAG6 promotes cell proliferation and inhibits apoptosis through the PTEN/PI3K/AKT pathway in Burkitt lymphoma

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**Abstract.** The main purpose of the present study was to elucidate the role of sperm-associated antigen 6 (SPAG6) in the occurrence and development of Burkitt lymphoma (BL) and explore the underlying molecular mechanisms. A correlation was observed between the expression of SPAG6 and the prognosis of patients with lymphoma using The Cancer Genome Atlas (TCGA) database analysis. It was demonstrated that the levels of SPAG6 in BL cells were higher compared with that in IM-9 cells by reverse transcription-PCR and western blot assays. Moreover, silencing of SPAG6 significantly decreased proliferation and increased apoptosis of Daudi and Raji cells, whereas SPAG6 overexpression exerted the opposite effects on CA46 and NAMALWA cells. When investigating the possible mechanism, it was first observed that the level of phosphatase and tensin homolog (PTEN) protein was significantly increased, while that of phosphorylated (p-)AKT protein was markedly reduced in the SPAG6-knockdown group compared with the blank control group in Daudi and Raji cells by western blot analysis. It was further ascertained whether the phosphoinositide 3-kinase (PI3K)/PTEN/protein kinase B (AKT) pathway mediates the effects of SPAG6 on cell proliferation and apoptosis, and the results demonstrated that silencing of SPAG6 suppressed the viability of Daudi and Raji cells, whereas PTEN knockdown using siRNA or SF1670 (a specific PTEN inhibitor) reversed the inhibitory effect on cell proliferation and the promoting effect on cell apoptosis induced by SPAG6 depletion *in vitro* as well as *in vivo*. These data revealed that SPAG6 may promote the proliferation and inhibit the apoptosis of BL cells via the PTEN/PI3K/AKT pathway. The results of the present study suggest that SPAG6 may play a key role in the progression of BL and may be of value as a predictive prognostic biomarker in patients with BL.

## Introduction

Lymphoma is one of the most common hematological malignancies (1). Despite it being a potentially curable disease, the survival rate of lymphoma depends on multiple factors (1). Burkitt lymphoma (BL), a B-cell tumor subtype, is characterized by aggressive proliferation, and patients with BL who have a poor survival prognosis require intensive chemotherapy (2). Thus far, no effective targeted drug has been approved for the treatment of BL (3). Therefore, most studies have focused on identifying effective targets for the treatment of BL.

Sperm-associated antigen 6 (SPAG6) was first detected in human testicular tissue (4). Its main function is to participate in the maturation of germ cells and maintain sperm viability and fertility (5,6). Recent studies have identified SPAG6 as a new cancer-testis antigen, and it is considered as a tumor marker and a potential drug candidate for the treatment of solid tumors, such as lung and breast cancer (7,8). In contrast to normal marrow, 7 genes, including Wilms' tumor 1 (*WT1*) and *SPAG6*, were found to be significantly upregulated in patients with acute myeloid leukemia (AML) through comprehensive whole-genome sequencing and gene expression analysis (9). In addition, a recent study demonstrated that SPAG6 was overexpressed in patients with myelodysplastic syndromes (MDSs) (10). Yang *et al* reported that the growth of malignant bone marrow cells SKM-1 and K562 was significantly inhibited after silencing SPAG6 expression (11). However, the roles and underlying molecular mechanisms of action in SPAG6 in other hematological malignancies, particularly lymphomas, have been less extensively investigated.

The present study examined whether there is a correlation between the expression of SPAG6 and the prognosis of patients with lymphoma via analysis of The Cancer Genome Atlas (TCGA) database, in the hope of elucidating the role of SPAG6 in the occurrence and development of BL and exploring its molecular mechanisms of action, thereby identifying new targets for the clinical treatment of BL.

## Materials and methods

**Survival of BL patients in the TCGA database.** The preprocessed level 3 RNA-seq data and corresponding clinical information of lymphoma patients were collected from TCGA database (<http://cancergenome.nih.gov/>). Data from TCGA

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were downloaded to integrate the expression of SPAG6 and survival data. Survival analysis was performed with the survival R package and using Kaplan-Meier analysis (log-rank test) (12).

**Cell culture.** All the cell lines were purchased from Nanjing Kaiji Bio-tech Co., Ltd. The human normal peripheral-blood B-lymphocyte (IM-9) and four BL cell lines (CA46, NAMALWA, Daudi and Raji) were cultured in RPMI-1640 medium (HyClone; Cytiva) supplemented with 10% FBS (HyClone; Cytiva) and 1% penicillin and streptomycin (Beyotime Institute of Biotechnology). All cells were maintained at 37°C in 5% CO<sub>2</sub> during all experiments.

**Transfection and stable cell lines.** SPAG6 shRNA and scrambled control-shRNA were purchased from Shanghai GenePharma Company. For shRNA transfection, 293T cells were grown to 30-50% confluence in 6-well culture plates and transfected with scrambled shRNA or SPAG6 shRNA using Lipofectamine™ LTX reagent with PLUS™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. Then, the supernatant from 293T cells was harvested and added to Daudi and Raji cells for 72 h, followed by selection with 1 µg/ml puromycin for 1 month. Plasmids (pcDNA3.1-SPAG6 and pcDNA3.1) were purchased from Obio Technology Co., Ltd. For plasmid transfection, CA46 and NAMALWA cells (70% confluence) were plated in 6-well culture plates. Plasmids were purified and transfected with pcDNA3.1-SPAG6 or pcDNA3.1 for 72 h using Lipofectamine™ LTX reagent with PLUS™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.), followed by selection with 1 µg/ml puromycin for 1 month.

**Cell proliferation.** Cell proliferation was tested with the Cell Counting Kit-8 (CCK-8) Assay (Dojindo Molecular Technologies, Inc.). Briefly, cells (2,000-5,000) were seeded in 96-well plates. After incubation for 24, 48, 72 and 96 h, 10 µl CCK-8 reagent was added into each well of the 96-well plates. After incubation with the CCK-8 solution for 1 h, the final optical density (OD) values at 450 nm were calculated.

**Flow cytometry assay.** Daudi and Raji cells (2x10<sup>5</sup> cells/well) were cultured in 6-well culture plates. After incubation, the cells were washed with PBS and centrifuged at 500 x g for 5 min at 4°C. Then, the cell suspension was re-suspended with binding buffer, followed by the addition of 5 µl Annexin V and 5 µl propidium iodide (PI) (Invitrogen; Thermo Fisher Scientific, Inc.) for 15 min in the dark. Cells subsequently were counted by a flow cytometer (BD Biosciences Inc.).

**Reverse transcription-quantitative PCR (RT-PCR) analysis.** Cells were harvested and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA (1 µg) was reverse-transcribed at 37°C for 15 min and 85°C for 5 sec using the PrimeScript Reverse Transcriptase system (Takara Biotechnology Co., Ltd.). qPCR amplification was performed using SYBR Green Master Mix (Takara Biotechnology Co., Ltd.) under the following conditions: 94°C for 1 min, 30 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, 72°C for 5 min. The relative expression of SPAG6 was calculated using the 2<sup>-ΔΔC<sub>q</sub></sup> method. Primers for PCR

included: SPAG6, 5'-GACGTGGTCCCAACAATTCAA-3' (forward) and 5'-ACAGCTTCTGCTAGGTCATCAT-3' (reverse); GAPDH, 5'-TGACTTCAACAGCGACACCCA-3' (forward) and 5'-ACCCTGTTGCTGTAGCCAAA-3 (reverse).

**Western blot assay.** Cells were harvested and lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) with protease inhibitors (Thermo Fisher Scientific, Inc.). The protein concentration was determined by a BCA kit (Thermo Fisher Scientific, Inc.). For western blotting, samples were electrophoresed on 8-12% acrylamide gradient Tris-Tricine Ready Gels and transferred to PVDF membranes (EMD Millipore). Then, the PVDF membranes were blocked with 5% milk for 1 h at room temperature and incubated with the primary antibodies anti-SPAG6 (Abcam, ab155653, 1:1,000 dilution), anti-β-actin (Sigma-Aldrich; Merck KGaA, A5441, 1:5,000 dilution), anti-Bcl-2 [Cell Signaling Technology, Inc. (CST), cat. no. 3498, 1:1,000 dilution], anti-Bax (CST, cat. no. 5023, 1:1,000 dilution), anti-caspase-8 (CST, cat. no. 4790, 1:1,000 dilution), anti-cleaved-caspase-8 (CST, cat. no. 9748, 1:1,000 dilution), anti-poly(ADP-ribose) polymerase (PARP; CST, cat. no. 9542, 1:1,000 dilution), anti-cleaved-PARP (CST, cat. no. 52873, 1:1,000 dilution), anti-caspase-3 (CST, cat. no. 14220, 1:1,000 dilution), anti-cleaved-caspase-3 (CST, cat. no. 9664, 1:1,000 dilution), anti-phosphatase and tensin homolog (PTEN; CST, cat. no. 9188, 1:1,000 dilution), anti-AKT (CST, cat. no. 4685, 1:1,000 dilution) and anti-p-AKT (CST, cat. no. 4060, 1:1,000 dilution) overnight at 4°C, followed by incubation with secondary antibodies (CST, cat. no. 7074, 1:5,000 dilution) at room temperature for 2 h.

**Animal experiments.** Four-week-old male severe combined immunodeficiency (SCID) mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. SPAG6 stable knockdown or control Raji cell lines (5x10<sup>6</sup> cells in 50 µl DMEM) were subcutaneously injected into the left axilla of 15 SCID mice. When the tumors reached 100 mm<sup>3</sup>, the mice were randomized into three groups: i) nc/DMSO group, intraperitoneal injection of DMSO (50 µl daily) (n=5); ii) shSPAG6/DMSO group, intraperitoneal injection of DMSO (50 µl daily) (n=5); and iii) shSPAG6/SF1670 group, intraperitoneal injection of SF1670 (10 µmol/kg diluted in 50 µl DMSO; n=5) (13). The length (a) and width (b) of the tumors were calculated every 3 days and the volume (V) was calculated using the formula V=1/2ab<sup>2</sup>. After 3 weeks of treatment, the mice were euthanized by CO<sub>2</sub> asphyxiation at a flow rate of 25% volume/min of gas displacement in a chamber for 5 min in December of 2019. Death of all mice were confirmed before removing them from the chamber. Then the tumors were harvested and weighed. During the entire animal experiment, we performed this experiment following the Ethical Guidelines of Animal Experiment version 1.0 of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University released on October 28th, 2019.

**Immunohistochemistry.** The expression of proliferation-associated antigens Ki67 and proliferating cell nuclear antigen (PCNA) in tissues was measured by immunohistochemistry assay. After conventional paraffin embedding, sectioning, dewaxing and hydration, the tumor tissues were treated with

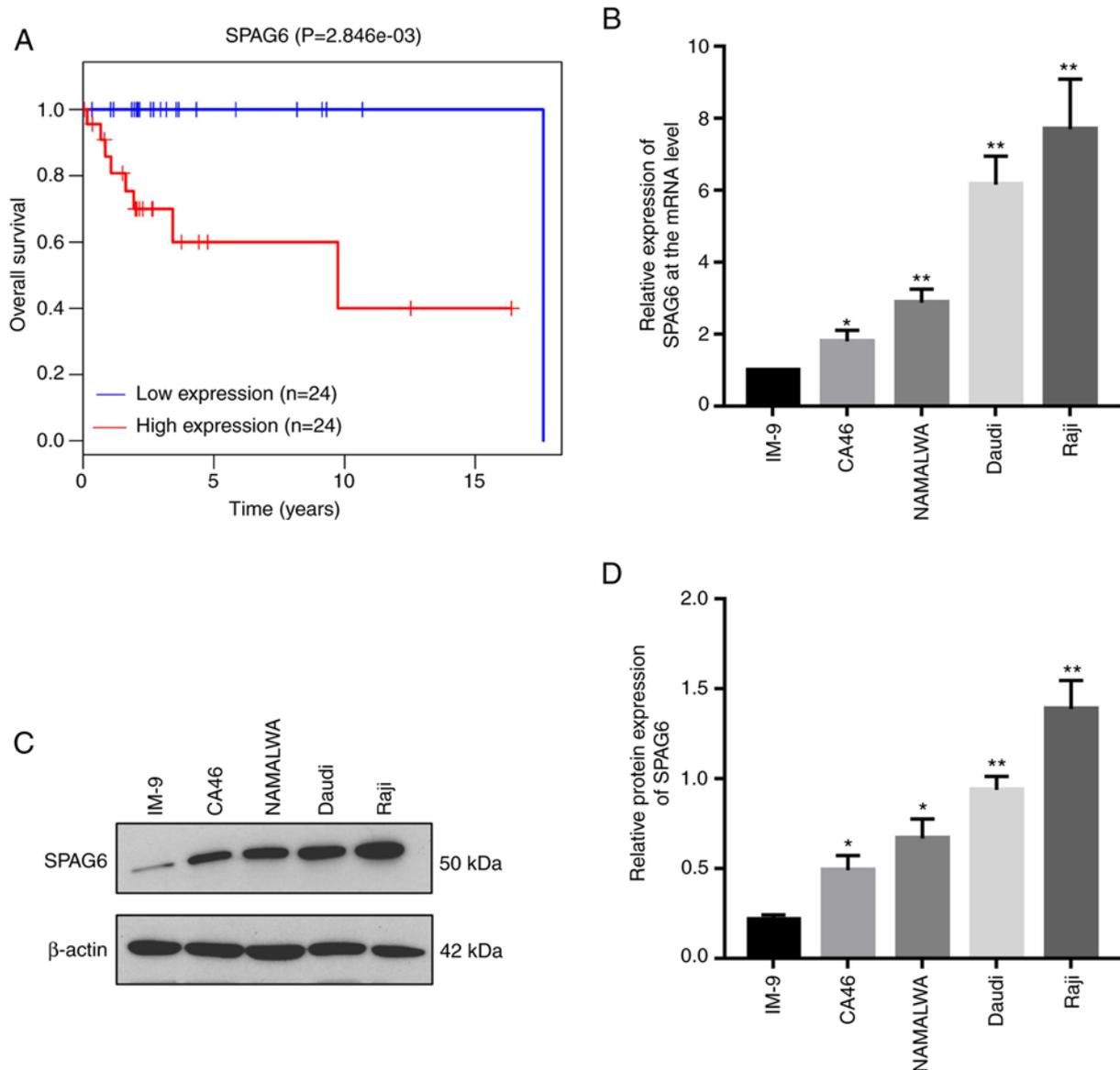


Figure 1. Increased SPAG6 expression is associated with the poor prognosis of patients with lymphoma and SPAG6 is significantly upregulated in BL cells. (A) The association between the levels of SPAG6 and the overall survival of patients with lymphoma was examined using TCGA database. Data from TCGA were downloaded to integrate the expression and survival data. Survival analysis was performed with the survival R package and using Kaplan-Meier analysis (log-rank test). (B) The mRNA levels of SPAG6 in human normal peripheral-blood B-lymphocytes (IM-9) and BL cell lines (CA46, NAMALWA, Daudi and Raji) were determined by quantitative PCR assay. (C and D) The protein levels of SPAG6 in IM-9, CA46, NAMALWA, Daudi and Raji cells were determined by western blot assay. \* $P < 0.05$ , \*\* $P < 0.01$ . The levels of SPAG6 of CA46, NAMALWA, Daudi and Raji cells were compared with IM-9 cells. SPAG6, sperm-associated antigen 6; BL, Burkitt lymphoma; TCGA, The Cancer Genome Atlas.

antigen repair buffer (pH 6.0). Following treatment with 0.3%  $H_2O_2$  for 20 min, the tumor tissue sections were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) and incubated with primary antibodies (anti-Ki-67, Abcam, ab15580, 1:200 dilution; anti-PCNA, Dako, M0879, 1:50 dilution) overnight at 4°C. On the following day, the tissue sections were washed with PBS three times and incubated with secondary antibodies at 37°C for 1 h, followed by treatment with DAB chromogen.

**TUNEL assay.** TUNEL staining was performed to assess the tumor cell apoptosis. For the TUNEL assay, tissues were fixed with 4% formalin for 24 h at 4°C and then embedded in paraffin; TUNEL staining was carried out using a TUNEL kit (Roche Diagnostics). Specifically, the tumor tissue sections

were deparaffinized in xylene and hydrated through graded alcohols. The tissue slides were blocked with PBST solution (PBS with 0.1% Triton X-100) for 20 min. After washing with PBS three times, the slides were incubated with TUNEL reaction mixture (5  $\mu$ l of enzyme solution and 45  $\mu$ l of label solution) for 2 h in the dark at 37°C. Then, the tissue sections were washed and incubated with DAPI (1:1,000 dilution, Beyotime Institute of Biotechnology) for 20 min in the dark.

**Statistical analysis.** GraphPad Prism 7.0 (GraphPad Software, Inc.) was used to create all graphs and perform statistical analyses. The two-tailed Student's t-test and one-way ANOVA were used to performed statistical analyses.  $P < 0.05$  was considered to indicate statistically significant differences.

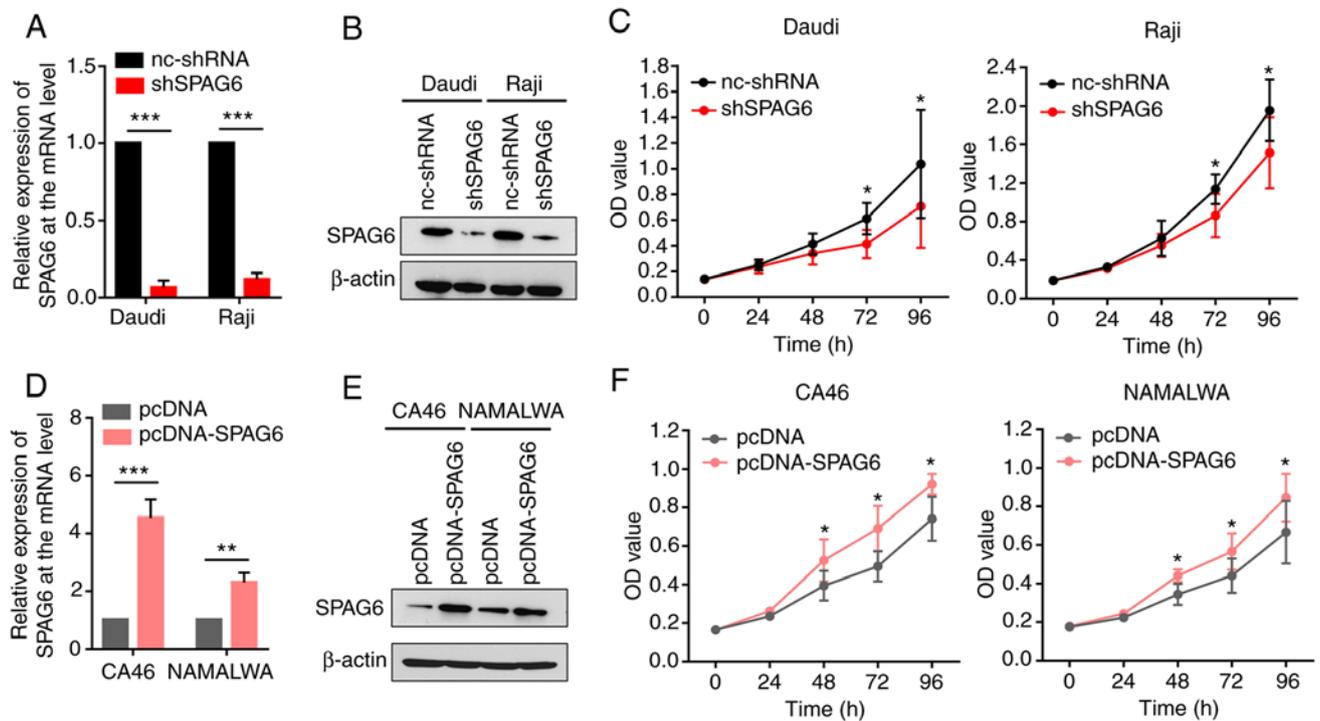


Figure 2. SPAG6 expression promotes the proliferation of BL cells. (A) Daudi and Raji cells were transfected with scrambled (control) shRNA (nc-shRNA) or SPAG6 shRNA (shSPAG6) and the knockdown efficiency was verified by quantitative PCR assay. (B) The knockdown efficiency was confirmed by western blot analysis. (C) Cell proliferation was performed by CCK-8 assay after knockdown of SPAG6 in Daudi and Raji cells. (D) CA46 and NAMALWA cells were transfected with pcDNA3.1-SPAG6 (pcDNA-SPAG6) or pcDNA3.1 (pcDNA) plasmids and the overexpression efficiency was confirmed by quantitative PCR assay. (E) The overexpression efficiency was confirmed by western blot assay. (F) Cell proliferation was examined by CCK-8 assay after overexpression of SPAG6 in CA46 and NAMALWA cells. \* $P < 0.05$ , \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Cell proliferation of SPAG6 knockdown group was compared with the nc-shRNA group; and cell proliferation of SPAG6 overexpression was compared with the pcDNA group. SPAG6, sperm-associated antigen 6; BL, Burkitt lymphoma; CCK-8, Cell Counting Kit-8.

## Results

*SPAG6 is significantly upregulated in BL cells and increased SPAG6 expression is associated with poor prognosis.* In order to examine the effects of SPAG6 and its clinical relevance in BL patients, the association between the levels of SPAG6 and the survival of BL patients was investigated in the TCGA database (12). The result indicated that higher expression of SPAG6 is associated with worse prognosis of lymphoma patients (Fig. 1A), indicating that SPAG6 expression is closely linked to the development and progression of lymphoma.

BL, a germinal center B-cell-derived tumor, is the most frequently occurring non-Hodgkin lymphoma (NHL), accounting for 40% of childhood NHLs (14,15). Despite the fact that a high-dose combination of chemotherapy is an effective treatment strategy for BL, a poor prognosis is unavoidable. Furthermore, the exact mechanism underlying the development and progression of BL remains unclear. Hence, BL was selected as the study focus to assess the role of SPAG6. The expression of SPAG6 was analyzed in human normal peripheral blood B-lymphocytes (IM-9) and BL cell lines (CA46, NAMALWA, Daudi and Raji). The RT-PCR result revealed that the mRNA levels of SPAG6 in CA46, NAMALWA, Daudi and Raji cells were  $1.80 \pm 0.26$ ;  $2.88 \pm 0.31$ ;  $6.17 \pm 0.65$  and  $7.70 \pm 1.14$  times higher, respectively, compared with that noted in the IM-9 cells ( $P < 0.05$ ; Fig. 1B). Moreover, the protein levels of SPAG6 were significantly increased in the BL cell

lines compared with this level in the IM-9 cells ( $P < 0.05$ ) as detected by western blot analysis (Fig. 1C and D).

*SPAG6 expression promotes the proliferation of BL cells.* As shown in Fig. 1B-D, the expression of SPAG6 was the highest in the Daudi and Raji cells. To ascertain whether SPAG6 expression is associated with the proliferative activity of BL cells, SPAG6 expression was first knocked down in Daudi and Raji cells using shRNAs. The results revealed that the expression of SPAG6 was significantly reduced in the knockdown groups compared with the control groups in both Daudi and Raji cells, as shown by RT-PCR and western blot assays (Fig. 2A and B). Furthermore, a proliferation assay was performed to determine whether SPAG6 depletion could affect the proliferation ability of Daudi and Raji cells. As shown in Fig. 2C, cell viability was markedly decreased at 72 h in the shSPAG6 groups compared with the control groups, as shown by the CCK-8 assay.

Based on the expression of SPAG6 in BL cell lines, CA46 and NAMALWA cells were selected to construct cell lines stably overexpressing SPAG6 (Fig. 1B-D). Using plasmid transfection, CA46 and NAMALWA cell lines that stably overexpressed SPAG6 were generated and the transfection efficiency testing indicated that the levels of SPAG6 were significantly higher in the SPAG6 overexpression groups compared with those in the control groups, as shown by RT-PCR and western blot assays (Fig. 2D and E). By contrast, the CCK-8 assay revealed that overexpression of SPAG6 significantly enhanced cell proliferation from 48 h (Fig. 2F). These results

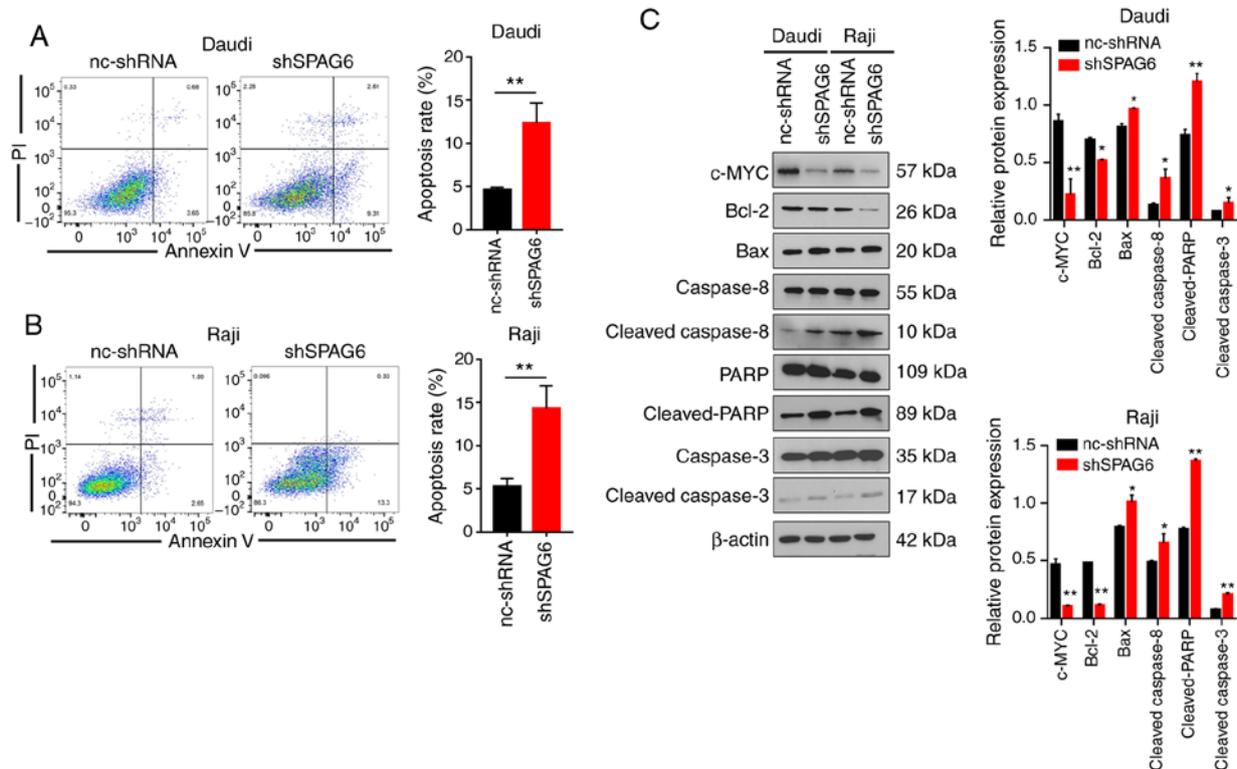


Figure 3. SPAG6 expression inhibits the apoptosis of BL cells. (A and B) Annexin V-PI staining was used to detect the apoptosis rate of control groups (nc-shRNA) and SPAG6 knockdown (shSPAG6) groups by flow cytometry assay in Daudi and Raji cells. (C) The levels of apoptosis-related proteins (c-MYC, Bcl-2, Bax, caspase-8, cleaved-caspase-8, caspase-3, cleaved-caspase-3, PARP and cleaved-PARP) were examined by western blot analysis after knockdown of SPAG6 in Daudi and Raji cells. \* $P < 0.05$ , \*\* $P < 0.01$ . The relative expression of apoptosis-related proteins of shSPAG6 group was compared with the nc-shRNA group. SPAG6, sperm-associated antigen 6; BL, Burkitt lymphoma; PARP, poly(ADP-ribose) polymerase.

suggest that the expression of SPAG6 is correlated with the proliferation of BL cells.

*SPAG6 expression inhibits the apoptosis of BL cells.* Abnormal apoptosis, including resistance to apoptosis, is considered to be an important mechanism underlying tumorigenesis (16). Previous studies have found that, in most types of lymphoma, apoptosis is significantly reduced and abnormal or reduced apoptosis is the main cause behind the occurrence and development of lymphoma (17). Therefore, promoting the apoptosis of lymphoma cells is one of the strategies employed for treating lymphoma. We next sought to investigate whether the expression of SPAG6 is also associated with reduced apoptosis of BL cells. The cells were stained with Annexin V-PI solution to evaluate the apoptosis rate by flow cytometry. Specifically, the percentage of apoptotic cells in the blank control and SPAG6-knockdown groups of Daudi cells was  $4.62 \pm 0.28$  and  $12.32 \pm 2.34\%$ , respectively ( $P < 0.01$ ) (Fig. 3A). An increased apoptosis rate was also observed in the SPAG6-silenced group compared with the non-transfected group of Raji cells ( $P < 0.01$ ; Fig. 3B).

c-MYC is an established indicator of BL (18), and it also plays an important role in numerous biological processes, including cell growth and proliferation, cell cycle progression and apoptosis (19). The expression of c-MYC was reduced after the knockdown of SPAG6 in Daudi and Raji cells, as shown by western blot analysis (Fig. 3C). In addition, the western blot results demonstrated that the expression of Bcl-2 (a well-known

anti-apoptotic protein) in the SPAG6-knockdown group of Daudi and Raji cells was significantly downregulated, while the expression of Bax (a well-known pro-apoptotic protein) was markedly enhanced (Fig. 3C). Moreover, the protein levels of cleaved-caspase-8, cleaved-caspase-3 and cleaved-PARP were obviously increased in the SPAG6-knockdown groups compared with the control groups in Daudi and Raji cells (Fig. 3C). In summary, the aforementioned data indicate that SPAG6 expression is correlated with the apoptosis of BL cells.

*SPAG6 promotes proliferation of BL cells through the PTEN/phosphoinositide 3-kinase (PI3K) protein kinase B (AKT) pathway.* The PTEN/PI3K/AKT signaling pathway plays a key role in the development of various cancers and the regulation of essential tumor cell functions (20). In particular, PTEN, a tumor suppressor, can negatively regulate the PI3K/AKT signaling pathway (21). To confirm whether the effects of SPAG6 on the viability of BL cells were mediated via the modulation of the PI3K/AKT pathway, AKT, p-AKT and PTEN were tested in SPAG6-depleted or SPAG6-overexpressed cells by western blotting. The experimental results uncovered that the level of PTEN protein was significantly increased, while p-AKT protein was markedly reduced in the SPAG6-knockdown group (shSPAG6) compared with the blank control group (nc-shSPAG6) in Daudi and Raji cells, as shown by western blot analysis (Fig. 4A). Conversely, the expression of PTEN was obviously decreased and the protein levels of p-AKT was significantly increased in the SPAG6-overexpression group

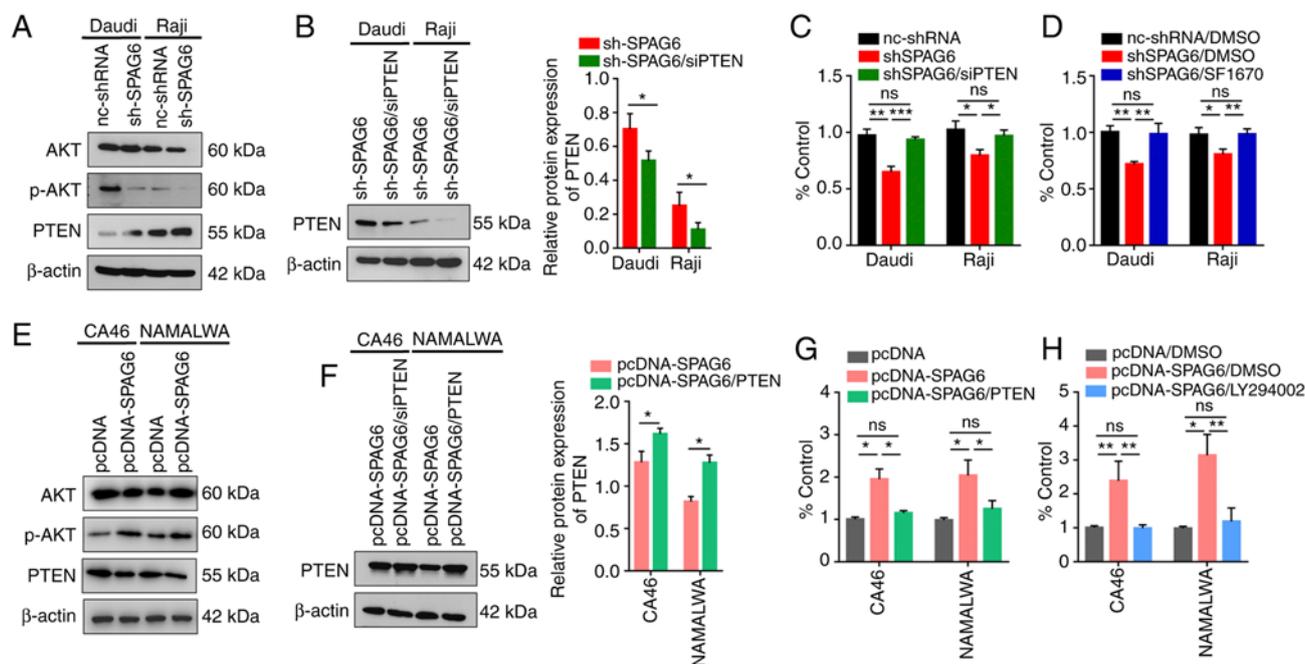


Figure 4. SPAG6 promotes proliferation of BL cells through the PTEN/PI3K/AKT pathway. (A) The levels of PI3K/AKT pathway-related proteins (AKT, p-AKT and PTEN) were examined in SPAG6-depleted (shSPAG6) or control (nc-shRNA) Daudi and Raji cells by western blot analysis. (B) Stable SPAG6-knockdown Daudi and Raji cells were transfected with PTEN siRNA (siPTEN) and the knockdown efficiency was confirmed by western blot assay. (C) Cell viability in the nc-shRNA, shSPAG6 and shSPAG6/siPTEN groups was examined using the CCK-8 assay. (D) Cells were treated with DMSO or SF1670 (10  $\mu$ M) for 24 h and cell viability in the nc-shRNA/DMSO, shSPAG6/DMSO and shSPAG6/SF1670 groups was examined using the CCK-8 assay. (E) The levels of PI3K/AKT pathway-related proteins (AKT, p-AKT and PTEN) were examined in SPAG6-overexpressing (pcDNA-SPAG6) or control (pcDNA) CA46 and NAMALWA cells by western blot analysis. (F) Stable SPAG6-overexpression CA46 and NAMALWA cells were transfected with PTEN overexpression plasmid (PTEN) and the overexpression efficiency was confirmed by western blot analysis. (G) Cell viability in the pcDNA, pcDNA-SPAG6 and pcDNA-SPAG6/PTEN groups was examined using the CCK-8 assay. (H) Cells were treated with DMSO or LY294002 (20  $\mu$ M) for 24 h and cell viability in the pcDNA/DMSO, pcDNA-SPAG6/DMSO and pcDNA-SPAG6/LY294002 groups was examined using the CCK-8 assay. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ns, not significant. SPAG6, sperm-associated antigen 6; BL, Burkitt lymphoma; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; CCK-8, Cell Counting Kit-8.

(pcDNA-SPAG6) compared with the control group (pcDNA) in CA46 and NAMALWA cells (Fig. 4E). We further explored whether the PI3K/PTEN/AKT pathway is implicated in the SPAG6-mediated enhancement of cell proliferation. For that purpose, PTEN was knocked down by siRNAs in Daudi and Raji cells (Fig. 4B). Interestingly, silencing of SPAG6 suppressed the proliferation of Daudi and Raji cells, whereas PTEN knockdown (siPTEN) rescued the tumor-inhibiting effect of SPAG6 depletion (Fig. 4C). Similarly, SF1670, a specific PTEN inhibitor, also reversed the anti-proliferative effect exerted by SPAG6 knockdown (Fig. 4D).

Next, PTEN overexpression was achieved using plasmids in CA46 and NAMALWA cells (Fig. 4F). SPAG6 overexpression promoted the proliferation of CA46 and NAMALWA cells, whereas PTEN overexpression rescued the SPAG6 overexpression-mediated tumor-promoting effect (Fig. 4G). Furthermore, stably overexpressing SPAG6 cell lines were treated using the PI3K-specific inhibitor LY294002, and reversal of the proliferative effect of SPAG6 overexpression on CA46 and NAMALWA cells was observed by inhibiting the PI3K pathway (Fig. 4H). Overall, these data indicate that SPAG6 may promote the proliferation of BL cells via the PTEN/PI3K/AKT pathway.

*SPAG6 inhibits apoptosis of BL cells via the PTEN/PI3K/AKT pathway.* It was verified that SPAG6 induced growth

promotion in human BL cells by activating the PTEN/PI3K/Akt signaling pathway. We further examined whether SPAG6 promotes the AKT pathways associated with apoptosis. Raji cells had highest expression of SPAG6 in the BL cell lines (Fig. 1C and D), so this cell line was chosen for the subsequent apoptosis experiments. Annexin V-PI staining was performed to detect the apoptosis rate in four groups of Raji cells (nc-shRNA, shSPAG6, shSPAG6/siPTEN and shSPAG6/SF1670) by flow cytometry assay. It was demonstrated that the apoptotic rates in the four groups were  $4.91 \pm 0.98$ ,  $11.34 \pm 2.31$ ,  $6.22 \pm 1.81$  and  $5.63 \pm 0.73$ , respectively (Fig. 5A). SPAG6 knockdown enhanced the apoptosis of Raji cells, while PTEN knockdown by siRNA or inhibitor reversed the apoptosis activation caused by SPAG6 depletion. The apoptosis-related protein levels detected by western blotting confirmed these results. As shown in Fig. 5B, the protein levels of cleaved-caspase-3 and cleaved-PARP were obviously increase in the SPAG6-knockdown group compared with the control group, while the increased protein levels induced by SPAG6 knockdown were partly reversed after silencing of PTEN. These results indicated that SPAG6 may inhibit the apoptosis of BL cells through the PTEN/PI3K/AKT pathway.

*SPAG6 promotes tumor growth via the PTEN/PI3K/AKT pathway in vivo.* As mentioned above, SPAG6 was shown

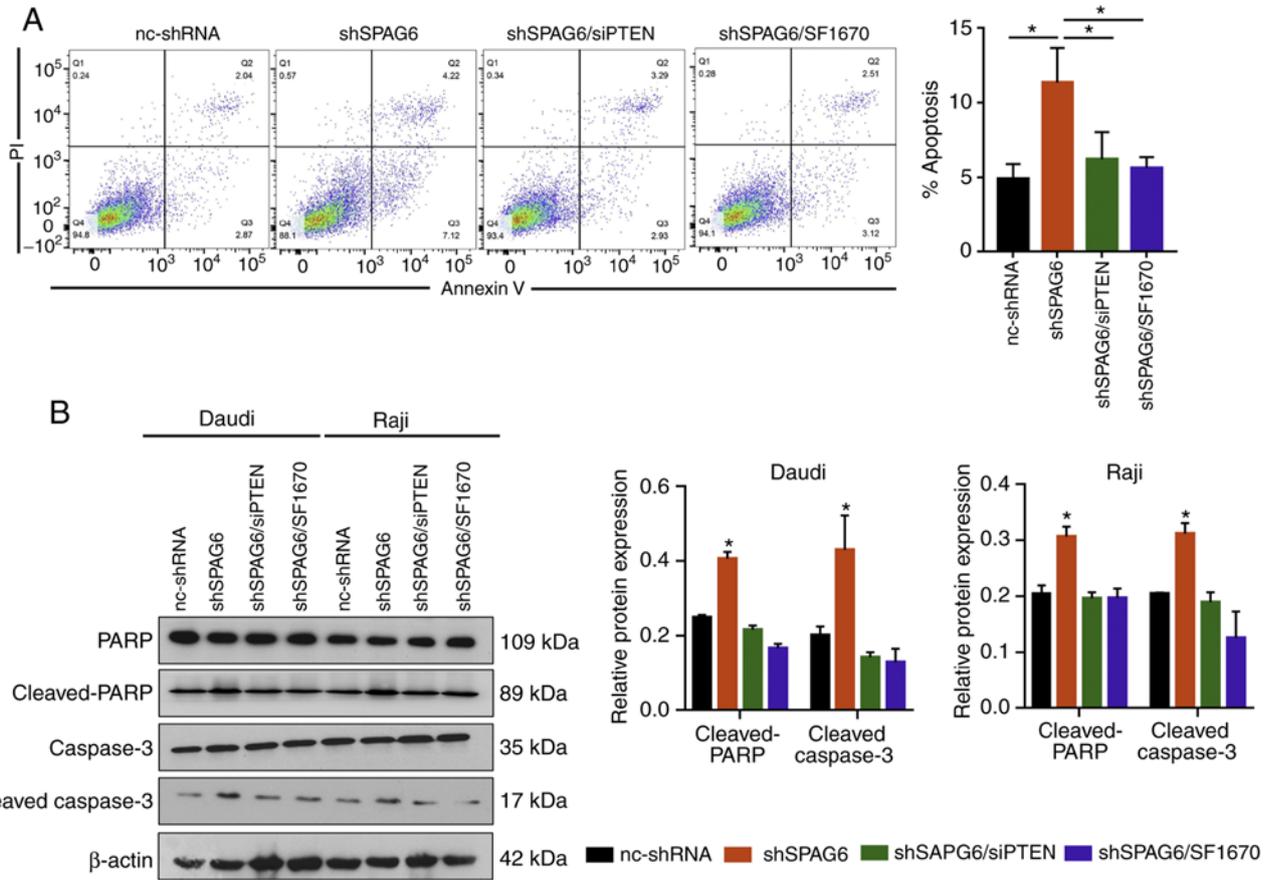


Figure 5. SPAG6 inhibits apoptosis of BL cells via the PTEN/PI3K/AKT pathway. (A) The apoptosis rate in the nc-shRNA, shSPAG6, shSPAG6/siPTEN and shSPAG6/SF1670 groups was examined using flow cytometry assay. (B) The levels of apoptosis-related proteins (caspase-3, cleaved-caspase-3, PARP and cleaved-PARP) were examined by western blot assay in the nc-shRNA, shSPAG6, shSPAG6/siPTEN and shSPAG6/SF1670 groups. \*P<0.05. SPAG6, sperm-associated antigen 6; BL, Burkitt lymphoma; PARP, poly(ADP-ribose) polymerase; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase.

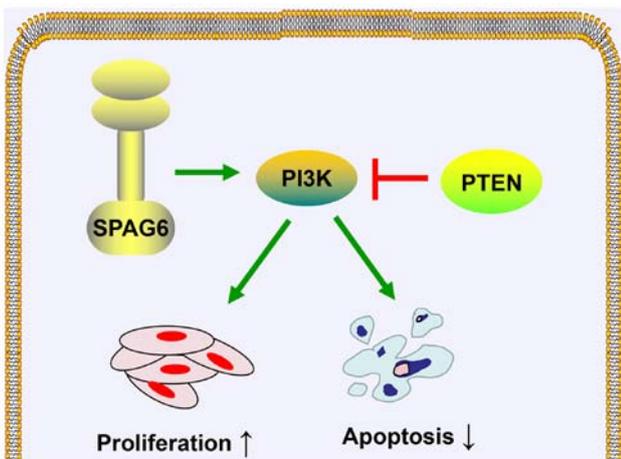


Figure 6. Schematic representation. SPAG6 promotes cell proliferation and inhibits cell apoptosis via the PTEN/PI3K/AKT pathway in BL cells. SPAG6, sperm-associated antigen 6; BL, Burkitt lymphoma; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B.

to promote cell proliferation and inhibit cell apoptosis via the PTEN/PI3K/AKT pathway in BL cells *in vitro* (Fig. 6). To observe whether SPAG6 exerts functional effects on BL progression *in vivo*, a Raji BL cell xenograft mouse model was established. The mice were divided into three groups:

nc-shRNA/DMSO group (injection with control cells and treatment with DMSO); shSPAG6/DMSO group (injection with SPAG6 stable knockdown cells and treatment with DMSO); and shSPAG6/SF1670 group (injection with SPAG6 stable knockdown cells and treatment with SF1670). It was confirmed that the tumor size and weight in the tumor tissues from the SPAG6-knockdown (shSPAG6/DMSO) group were smaller compared with those in the control tissues (nc-shRNA/DMSO group) (Fig. 7A-C). Consistently, the expression of the proliferation-related proteins Ki67 and PCNA in BL tumor tissues was reduced following SPAG6 knockdown compared with the blank group, as demonstrated by immunohistochemistry (Fig. 7D). Furthermore, TUNEL assay revealed that the apoptotic cell numbers were obviously increased in the shSPAG6/DMSO group (17.64±1.76) compared with the nc-shRNA/DMSO group (7.27±2.77; P<0.05; Fig. 7E). Interestingly, silencing of PTEN using the inhibitor SF1670 (shSPAG6/SF1670 group) reversed the tumor growth induced by SPAG6 depletion compared with the shSPAG6/DMSO group (Fig. 7A-C). Moreover, there was no difference in the expression of Ki67 or PCNA between the shSPAG6/SF1670 and the nc-shRNA/DMSO groups, whereas the expression of Ki67 and PCNA was higher in the shSPAG6/SF1670 group compared with the shSPAG6/DMSO group (Fig. 7D). In addition, PTEN knockdown in the shSPAG6/SF1670 group (10.06±2.96) partly

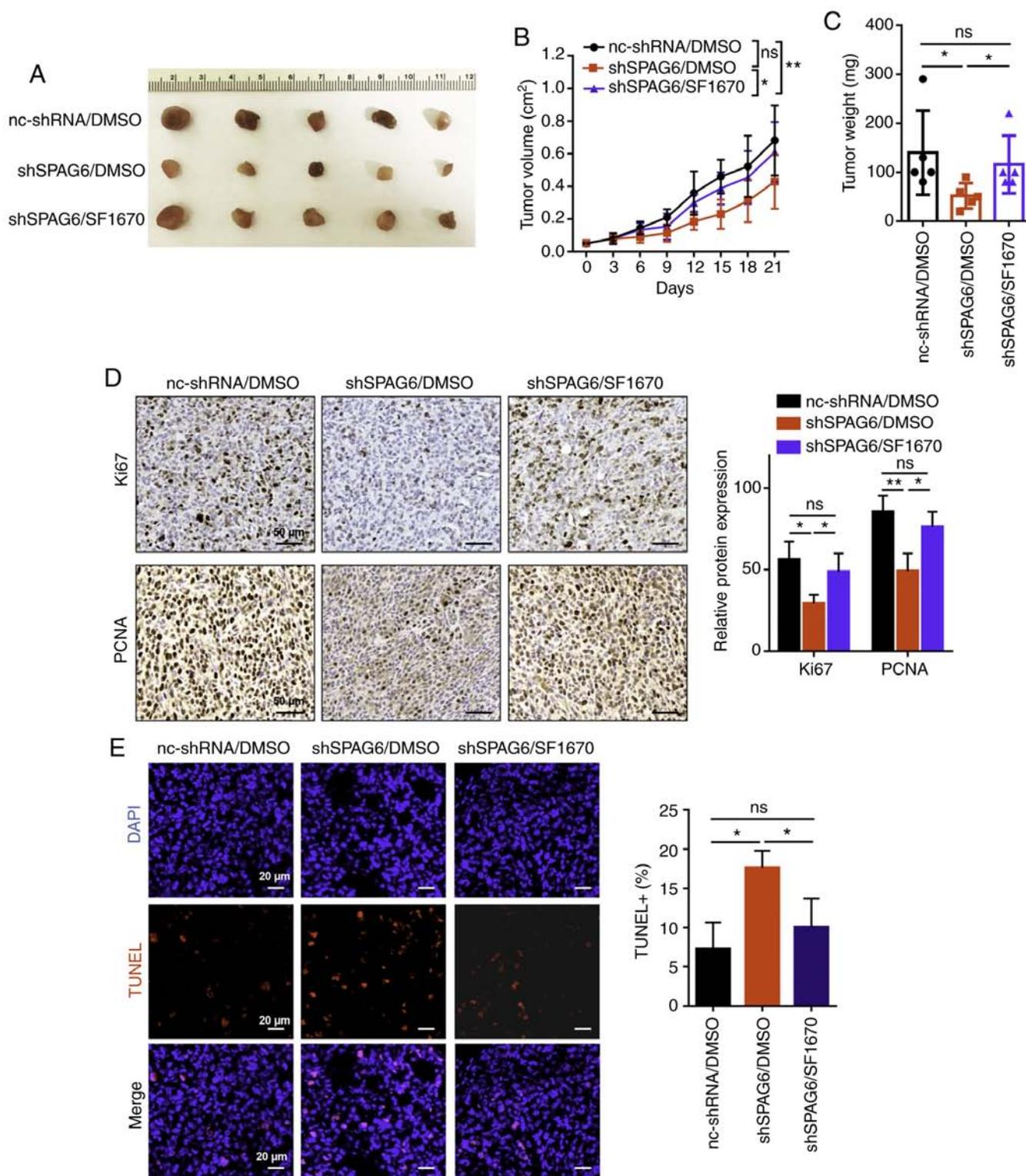


Figure 7. SPAG6 promotes tumor growth via the PTEN/PI3K/AKT pathway *in vivo*. (A) SPAG6 stable knockdown (shSPAG6/DMSO) or control (nc-shRNA/DMSO) Raji cell lines were injected into 15 SCID mice. The tumor sizes (B) and tumor weights (C) in the nc-shRNA/DMSO, shSPAG6/DMSO and shSPAG6/SF1670 group. (D) The expression of Ki-67 and PCNA in tumor tissues was determined by immunohistochemistry assay. Scale bar, 50  $\mu$ m. (E) TUNEL staining was performed to assess the apoptosis in the tumor tissues. Scale bar, 20  $\mu$ m. \* $P$ <0.05; \*\* $P$ <0.01; ns, no significant. SPAG6, sperm-associated antigen 6; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; PCNA, proliferating cell nuclear antigen.

rescued the apoptotic ratio mediated by SPAG6 depletion compared with the shSPAG6/DMSO group ( $17.64 \pm 1.76$ ), as shown by the TUNEL assay (Fig. 7E). Collectively, these results indicate that SPAG6 promotes tumor growth via the PTEN/PI3K/AKT pathway *in vivo*.

## Discussion

Sperm-associated antigen 6 (SPAG6) is an ortholog of chlamydia PF16, located at the chromosomal region 10p12.2 (5). SPAG6 was originally identified in human testicular tissues

and plays a role in regulating germ cell maturation and flagellar movement (4,6). Subsequently, it was found to be expressed in cilia-containing tissues, such as the lung and brain, and highly expressed in the embryonic spinal cord (22). SPAG6 also plays an important role in neuronal migration in mice (23), as well as in the process of neuronal proliferation and differentiation (24). Over the past decade, the amount of research focusing on the association between SPAG6 and cancer has markedly increased. Recent studies have demonstrated that abnormal expression of SPAG6 is associated with the clinical outcome of breast and lung cancer (7,8). Mulaw *et al* reported that SPAG6 is highly expressed at the chromosomal regions t(10;11)(p12;q14) of CALM/AF10-positive acute myelogenous leukemia (AML) (25). Furthermore, lower expression of SPAG6 was found to be associated with longer relapse-free survival in patients with AML (26). These studies suggested that SPAG6 may act as an oncogene and may be a useful prognostic marker and a therapeutic predictor.

To the best of our knowledge, the present study was the first to examine the prognostic value of SPAG6 in lymphoma patients according to the TCGA database. A poor prognosis of lymphoma was found to be significantly correlated with over-expression of SPAG6. These data suggest that the SPAG6 may, at least in part, contribute to the progression of lymphoma. Moreover, high levels of SPAG6 were observed in Burkitt lymphoma (BL) cell lines compared with those in normal human peripheral-blood B-lymphocytes. It was next demonstrated that SPAG6 enhanced cell proliferation and repressed cell apoptosis, *in vitro* as well as *in vivo*.

There are several pathways through which SPAG6 can affect cell proliferation and apoptosis. First, TRAIL is a member of the tumor necrosis factor family and it can mediate apoptosis (27). Li *et al* found that the levels of FADD and TRAIL were increased in SPAG6-knockdown SKM-1 cells in myelodysplastic syndromes (MDSs), suggesting that SPAG6 may modulate TRAIL activity to regulate cell apoptosis (28). Furthermore, modulation of DNA methylation has been implicated in cell proliferation. Altenberger *et al* reported that SPAG6 expression is regulated by DNA hypomethylation, which may lead to the proliferation of non-small cell lung cancer (NSCLC) cells (8). In addition, AKT is an important mediator of the cell cycle in malignant tumors, and cell cycle arrest is a key event in repression of cell proliferation (29). FoxO is a major downstream molecule of the PI3K/Akt signaling pathway and was found to be primarily regulated by AKT (30). A previous study reported that SPAG6 knockdown could downregulate the phosphorylation of AKT and FoxO, resulting in inhibition of cell proliferation and cell cycle arrest in MDS, indicating that SPAG6 may participate in cell cycle regulation through the AKT/FoxO pathway (31).

Phosphatase and tensin homolog (PTEN), as a negative regulator of the PI3K/AKT pathway, has been shown to be missing or inactivated in various tumors (20,21). It has been demonstrated that PTEN also plays a key role in regulating cell viability and apoptosis, not only in solid tumors, but also in MDS and chronic myeloid leukemia cells (32-34). It was previously demonstrated that PTEN is a key factor that may regulate PI3K activity in BL (35). AKT acts as a downstream factor of PI3K, and the observed inhibition of AKT phosphorylation

suggests activation of the PI3K/AKT signaling pathway (36). To investigate whether the effects of SPAG6 on the proliferation and apoptosis of BL cells were associated with the activation of the PI3K/AKT pathway, the present study demonstrated that the PTEN protein was significantly upregulated and p-AKT protein was significantly downregulated after the knockdown of SPAG6 expression in Daudi and Raji cells. The results of the present study also uncovered that silencing of SPAG6 suppressed viability and promoted apoptosis of BL cells, whereas PTEN knockdown rescued the tumor-inhibiting effects of SPAG6 depletion, *in vitro* as well as *in vivo*. These results suggested that SPAG6 may exert tumor-promoting effects on BL cells through the PTEN/PI3K/AKT pathway.

In conclusion, the present study demonstrated that the expression of SPAG6 was correlated with the prognosis of patients with BL. It was also observed that SPAG6 promotes the proliferation and inhibits the apoptosis of BL cells via the PTEN/PI3K/AKT pathway *in vitro* and *in vivo*. These results indicate that SPAG6 plays a key role in the development of BL and may be of value as a predictive and prognostic biomarker in patients with BL.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Authors' contributions

RZ and ZT designed the study. RZ, HZ and YY performed the experiments. RZ and YW analyzed the data. RZ and ZT discussed the project. RZ and ZT drafted, and HZ proofread and revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

The animal experiments conducted in the present study were approved by the Animal Care Committee of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University (approval no. DWP201900101).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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