SPAG9/MKK3/p38 axis is a novel therapeutic target for liver cancer

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Abstract. Sperm-associated antigen 9 (SPAG9) is a biomarker and potential therapeutic target for several cancers; however, its involvement in liver cancer progression is not clear. The aim of the present study was to determine whether SPAG9 regulates proliferation of liver cancer. Immunohistochemistry and cell immunofluorescence were used to confirm the expression and the localization of SPAG9 in human liver cancer tissues and the liver cancer-derived HepG2 cells. A small interfering RNA (siRNA) designed to target *SPAG9* was transiently transfected into HepG2 cells using Lipofectamine™ 2000, and proliferation, apoptosis and cell cycle progression were analyzed using CCK-8 assay and flow cytometry; western blotting was used to detect the expression of SPAG9, JNK, p38, MKK3 and MKK6, and co-immunoprecipitation was used to assess the interaction between SPAG9 and JNK. SPAG9 was overexpressed in 16 out of 20 (80%) patients with liver cancer. The protein was localized in both the cytoplasm and nucleus of liver cancer cells obtained from patients and in HepG2 cells. Depletion of SPAG9 inhibited the proliferation of HepG2 cells, promoted apoptosis and arrested the cell cycle at the S phase. Moreover, cells deficient in SPAG9 had decreased expression of JNK, p38 and MKK3 compared to HepG2 cells not treated with an siRNA targeting *SPAG9*. In the present study, SPAG9 was revealed to regulate cell proliferation, apoptosis and cell cycle progression in liver cancer cells through the SPAG9/MKK3/p38 axis. This axis is a novel therapeutic target for liver cancer.

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Abbreviations: SPAG9, sperm-associated antigen 9; CTA, cancer testis antigen; MAPKs, mitogen-activated protein kinases; CCK-8, Cell Counting Kit-8; MAPKKKs, MAP kinase kinases kinases; MKKs, MAP kinase kinases

Key words: SPAG9, liver cancer, HepG2, MAPK, therapeutic target

Introduction

Liver cancer is the most common cancer and the second leading cause of cancer-related deaths worldwide. In China in 2015, there were 466,000 new cases of liver cancer, 422,000 patients succumbed to liver cancer (1), and the 5-year survival rate of liver cancer was only 10.1% (2). Traditional treatments such as surgery, radiotherapy and chemotherapy are not effective. Some preclinical progress has been made in the development of targeted therapies for liver cancer, however sorafenib, a Raf kinase inhibitor, is the only targeted drug for treatment of advanced liver cancer in clinical use (3). Therefore, it is necessary to explore the molecular mechanisms of development and progression of liver cancer to identify additional therapeutic targets.

Cancer-testis antigens (CTAs) are a family of tumor-associated antigens that are encoded by germ line-associated genes with expression restricted to testis in healthy subjects but ectopic expression in various tumors (4). Sperm-associated antigen 9 (*SPAG9*) is a member of the CTA family located on human chromosome 17q21.33 and normally expressed in the equatorial plate of sperm acrosome. It can be overexpressed in tumors of the ovary, breast, lung and bone (5-7), and the expression of SPAG9 in tumor tissues is correlated with progression and prognosis of cancer patients (8,9). We previously demonstrated that SPAG9 was overexpressed in liver cancer (10), however, how SPAG9 influences liver cancer progression is not yet clear. In the present study, we analyzed the function of SPAG9 in the liver cancer-derived HepG2 cells. Our data indicated that SPAG9 interacts with the MAPK pathway in liver cancer cells to enhance proliferation, providing evidence that SPAG9 and the MAPK pathway are potential liver cancer therapeutic targets.

Materials and methods

Patient samples. A total of 20 liver cancer patients were enrolled between January 2005 and January 2015 at Hunan Provincial Brain Hospital (Changsha, China). The experiments were performed according to the Medical Ethics Committee of Hunan Provincial Brain Hospital (no. L2017003). Written informed consent was obtained from all patients prior to the

collection of liver cancer tissue samples. The exclusion criteria were as follows: i) patients had distant metastasis; ii) patients had received previous radiotherapy or chemotherapy prior to hepatectomy; iii) patients had a serious infection or other malignant diseases. Cancerous tissues and adjacent non-cancerous tissues were obtained from 20 patients with liver cancer during surgical tumor resections in accordance with informed consent. The diagnosis of liver cancer was confirmed by pathobiology (11). All clinical and biological data of the patients are presented in Table I.

Cell lines and cell culture. HepG2 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 IU/ml streptomycin in an atmosphere containing 5% CO_2 at 37˚C.

Immunohistochemistry (IHC). The expression of SPAG9 protein in liver cancer tissues was analyzed by IHC as previously described (8). Paraffin-embedded blocks were prepared from the tissue specimens and serial sections of 4 μm were cut. Sections of tumor tissues and adjacent non-cancer tissues were incubated with anti-SPAG9 antibody (dilution 1:100; cat. no. ab12331; Abcam, Cambridge, MA, USA). Subsequently, sections were incubated with alkaline phosphatase-conjugated mouse anti-rabbit immunoglobulin G (dilution 1:1,000; cat. no. sc-2358; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactivity was visualized using 0.05% 3,3'-diaminobenzidine. Images of tissue sections were captured using an Olympus light microscope (Olympus Corp., Tokyo, Japan) after staining with hematoxylin. The assessment criteria were described in a previous study (8).

Cell immunofluorescence. Cells were washed three times with phosphate-buffered saline (PBS) and were then fixed with 4% pre-cooled paraformaldehyde for 15 min. The cells were washed with PBS and then permeabilized with PBS containing 0.1% Triton X-100 for 20 min at room temperature. The cells were washed with PBS containing 0.1% Triton X-100 and were then incubated with 5% bovine serum albumin (BSA) for 30 min at room temperature. The coverslips were incubated with anti-SPAG9 antibody (dilution 1:100; cat. no. ab12331; Abcam) overnight at 4˚C. The cells were washed with PBS containing 0.1% Triton X-100, incubated with donkey anti-rabbit secondary antibody (dilution 1:2,000; cat. no. ab150073; Abcam) at 37˚C for 1 h in the dark, and then washed with PBS containing 0.1% Triton X-100. The cells were then incubated with DAPI for 90 sec, washed with PBS, and observed under a confocal microscope (Olympus Corp.).

Western blotting. Cells were lysed for 30 min on ice with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China; cat. no. P0013B). The cell lysate was then centrifuged at 12,000 x g for 5 min at 4˚C. The supernatant was carefully collected following centrifugation. Total protein in the cell lysate was quantified with a BCA protein assay kit (CWBio, Beijing, China). Proteins (50 μg) were separated by 6 and 8% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (0.45 μm; Millipore, Billerica, MA, USA). PVDF membranes were blocked with 5% non-fat dry milk for 2 h at room temperature, and then membranes were incubated for 24 h at 4˚C with antibodies against SPAG9 (dilution 1:1,000; cat. no. ab12331; Abcam), JNK (dilution 1:1,000; cat. no. ab179461; Abcam), p38 (dilution 1:1,500; cat. no. ab170099; Abcam), MKK3 (dilution 1:5,000; cat. no. ab195037; Abcam), MKK6 (dilution 1:5,000; cat. no. ab33866; Abcam) and GAPDH (dilution 1:3,000; cat. no. LCA04; Auragene, Changsha, China). After washing with PBS containing 0.05% Tween-20, the membranes were incubated with HRP-conjugated AffiniPure goat anti-rabbit IgG (H+L) (dilution 1:4,000; cat. no. SA00001-2; ProteinTech Group, Inc., Chicago, IL, USA). The bands were visualized using SuperECL Plus Western Blotting Substrate (Pierce; Thermo Fisher Scientifc, Inc.) and were analyzed using Gel Automated Digitizing System software (version 4.0; Silk Scientific, Orem, UT, USA). GAPDH levels were used as an internal standard.

Immunoprecipitation and immunoblotting analyses. To prepare cell lysates, cells were washed in PBS, lysed in cell lysis buffer for 20 min, and centrifuged at 12,000 x g for 15 min at 4˚C to remove insoluble debris. A 50-μl aliquot of supernatant was analyzed by western blot analysis. The remainder was incubated with anti-SPAG9 antibody (dilution 1:1,000; cat. no. ab12331; Abcam) or anti-JNK antibody (dilution 1:1,000; cat. no. ab179461; Abcam) at 4˚C overnight with slow shaking. To these samples 10 μl of resuspended protein A/G-agarose beads (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added. After incubation for 4 h at 4˚C, the immobilized proteins were collected by 10,000 x g centrifugation for 1 min, washed three times with the cell lysis buffer, and solubilized by boiling for 5 min in SDS-PAGE loading buffer (10 mmol/l Tris-HCl, 2% SDS, 2 mmol/l EDTA, 0.02% bromophenol blue and 6% glycerol; pH 6.8). After separation on SDS-PAGE, proteins were transferred onto a PVDF membrane (Millipore), blocked with 5% non-fat dry milk, washed briefly, and incubated with anti-JNK antibody (dilution 1:1,000; cat. no. ab179461; Abcam) or anti-SPAG9 antibody (dilution 1:1,000; cat. no. ab12331; Abcam). Blots were washed three times with PBS containing 0.05% Tween-20 and incubated with HRP-conjugated AffiniPure goat anti-mouse IgG(H+L) (dilution 1:4,000; cat. no. SA00001-1; Proteintech Group, Inc.) or HRP-conjugated AffiniPure goat anti-rabbit IgG(H+L) (dilution 1:4,000; cat. no. SA00001-2; Proteintech Group, Inc.). The bands were analyzed using Gel Automated Digitizing System software (version 4.0; Silk Scientific).

Cell proliferation analysis. Cell proliferation was quantified using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were plated in 96-well plates ($5x10^4$ cells/well). At 24 and 48 h after transfection with *SPAG9*-targeted siRNA or the control siRNA, SPAG9 siRNA sequences or control siRNA were constructed: SPAG9 siRNA, TCTGGAAACGACATTTATGG; control siRNA, TGAAGGTCGGAGTCAACGGATT. The cell proliferation assay was performed by the addition of 10 μl CCK-8

Table I. Characteristics of patients with liver cancer.

Sample	Age (years)	Sex	Clinical pathological diagnosis (11)
1	62	Female	Moderately differentiated liver cancer
2	50	Female	Moderate-poorly differentiated liver cancer
3	73	Male	Moderately differentiated liver cancer
4	51	Male	Moderate-highly differentiated liver cancer
5	49	Female	Highly differentiated liver cancer
6	32	Male	Moderately differentiated liver cancer
7	62	Male	Moderately differentiated liver cancer
8	34	Male	Highly differentiated liver cancer
9	50	Male	Highly differentiated liver cancer
10	42	Male	Moderately differentiated liver cancer
11	64	Male	Moderately differentiated liver cancer
12	41	Male	Highly differentiated liver cancer
13	52	Male	Moderately differentiated liver cancer
14	49	Male	Moderately differentiated liver cancer
15	66	Male	Moderately differentiated liver cancer
16	52	Female	Moderately differentiated liver cancer
17	40	Male	Moderately differentiated liver cancer
18	49	Male	Highly differentiated liver cancer
19	45	Male	Highly differentiated liver cancer
20	59	Male	Moderately differentiated liver cancer

solution to each well, followed by incubation at 37˚C for 1.5 h. Absorbance was measured at a wavelength of 450 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell cycle assay. After transfection with *SPAG9*-targeted siRNA or the control siRNA, HepG2 cells were harvested, washed twice with PBS, and fixed in 70% ethanol at 4˚C overnight. Cells were incubated with propidium iodide (PI) at room temperature for 1 h and were analyzed by flow cytometry using a BD Biosciences flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell apoptosis assay. Apoptotic cells were distinguished from normal cells using an Annexin V-FITC/PI apoptosis kit for flow cytometry (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After transfection with *SPAG9* siRNA or siRNA control, HepG2 cells were harvested and washed twice with cold PBS and then incubated with 5 μl FITC-Annexin V and 1 μl PI working solution (100 μg/ml) for 15 min in the dark at room temperature. Cellular fluorescence was measured by flow cytometry.

Statistical analyses. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the means ± standard deviations (SD). Statistical analyses were performed with one-way ANOVA, two-way ANOVA and repeated measures ANOVA as appropriate. Bonferroni was used as a post hoc test in one-way ANOVA. The statistical significance level was set at $P<0.05$ (two-sided).

Results

SPAG9 is overexpressed in cancer tissues of liver patients and is located in the cytoplasm and nucleus of HepG2 cells. We analyzed the expression of SPAG9 protein in 20 liver cancer specimens (without consideration of the type or stage of cancer) and 10 adjacent non-cancerous tissues by immunohistochemistry. We observed the overexpression of SPAG9 in 16 out of 20 (80%) liver cancer specimens; no or weak staining was observed in adjacent non-cancerous tissues (Fig. 1). Immunofluorescence revealed that SPAG9 was expressed in the liver cancer-derived HepG2 cells. As displayed in Fig. 2, SPAG9 protein was localized in cytoplasmic and nuclear compartments of HepG2 cells.

siRNA-mediated SPAG9 depletion inhibits HepG2 cell proliferation. To explore the role of SPAG9 in cell proliferation, HepG2 cells were transfected with a *SPAG9*-specific siRNA or with a control siRNA. A CCK-8 assay was performed to evaluate the proliferation of HepG2 cells. The results revealed that the silencing of *SPAG9* expression significantly decreased proliferation compared to cells transfected with the control siRNA after 24 and 48 h ($P<0.01$ and $P<0.01$, respectively; Fig. 3).

HepG2 cells deficient in SPAG9 undergo apoptosis after cell cycle arrest. Flow cytometry was used to assess the effect of SPAG9 on cell apoptosis and the cell cycle. Compared with cells treated with control siRNA, apoptosis of SPAG9-depleted cells was significantly increased ($P=0.003$; Fig. 4A). Moreover, there was a significant increase in the proportion of the cell

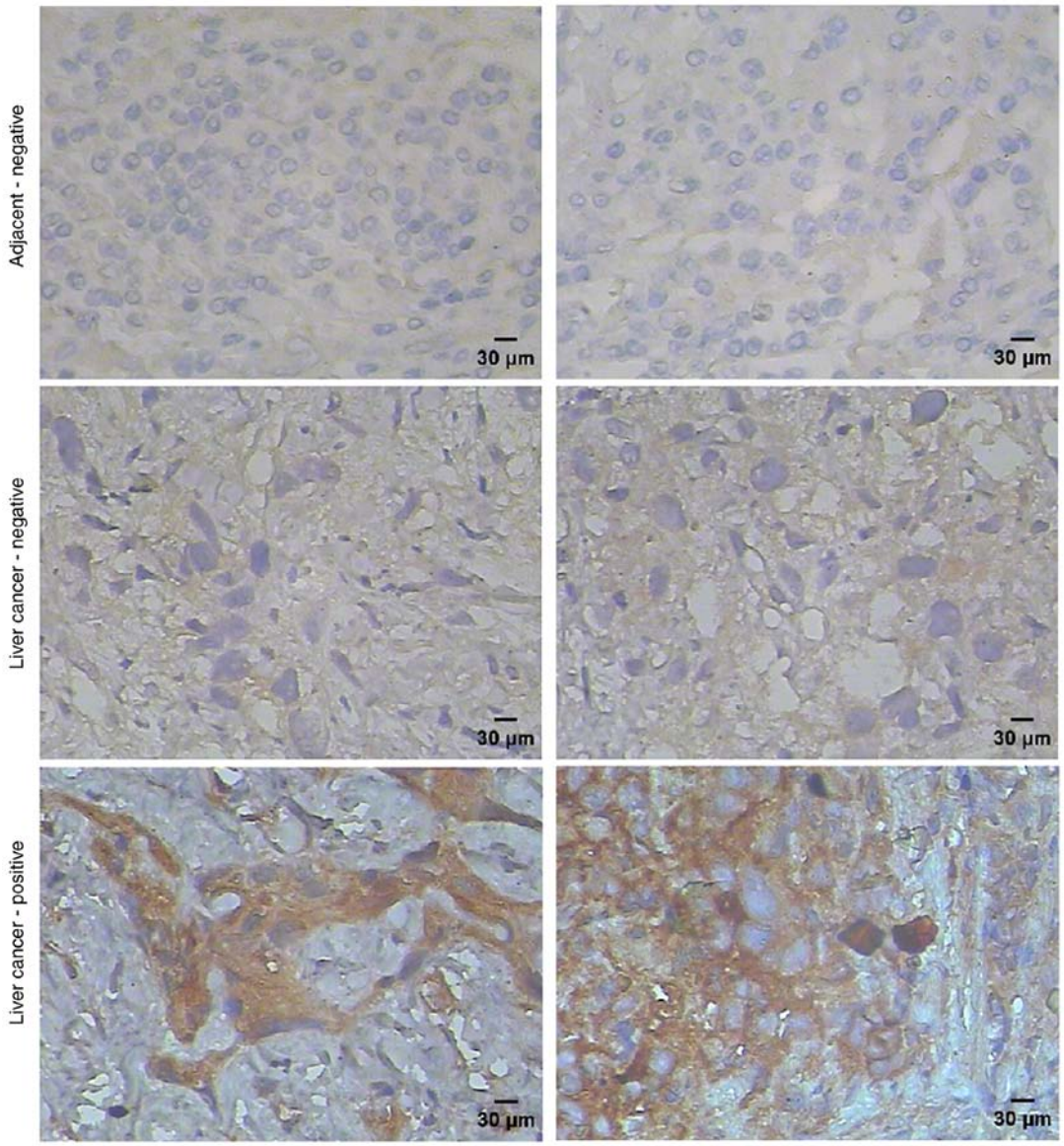

Figure 1. SPAG9 is expressed in liver tumor tissue but not in adjacent non-cancerous tissue. Brown grains indicate staining for SPAG9. Scale bars, 30 μm. SPAG9, sperm-associated antigen 9.

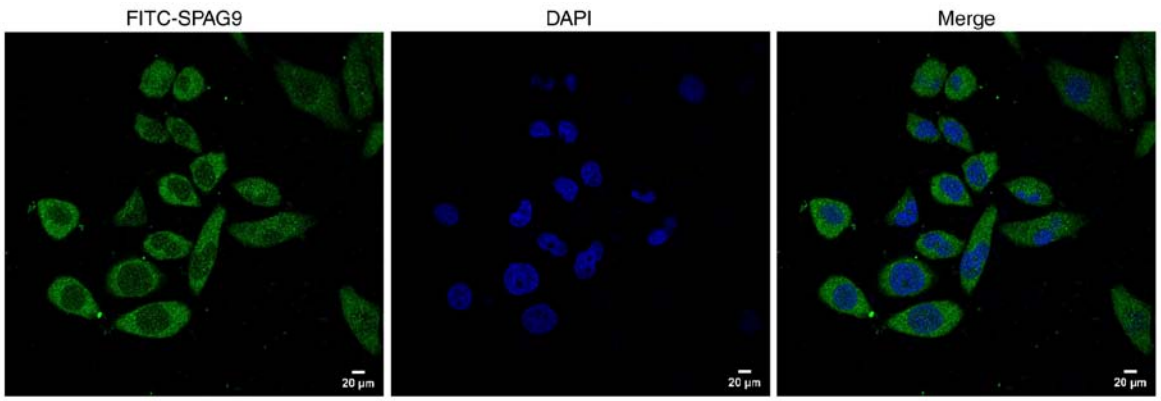

Figure 2. SPAG9 is observed in the nucleus and cytoplasm in HepG2 cells. The nucleus was identified by staining of DNA with DAPI (blue). Green fluorescence represents SPAG9 staining, blue fluorescence represents nuclear staining. Scale bars, 20 μm. SPAG9, sperm-associated antigen 9.

population in the S phase (P=0.000) and a significant decrease in the proportion of the cells in the G1 phase (P=0.004) in cells treated with *SPAG9*-targeted siRNA relative to control cells (Fig. 4B).

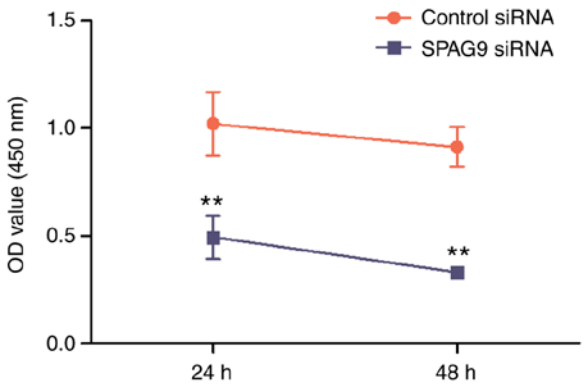

Figure 3. *SPAG9*-targeted siRNA inhibits proliferation of HepG2 cells. CCK-8 cell proliferation analysis of HepG2 cells after transfection with *SPAG9*-targeted siRNA and control siRNA. **P<0.01. SPAG9, sperm-associated antigen 9; CCK-8, Cell Counting Kit-8.

SPAG9 deficiency results in reduction in JNK levels. SPAG9 has a JNK-binding domain (12) that is predicted to regulate JNK-mediated signaling. We detected an interaction between SPAG9 and JNK in HepG2 cells by co-immunoprecipitation (Fig. 5A and B). Furthermore, the levels of SPAG9 and JNK were significantly decreased in cells transfected with *SPAG9*-targeted siRNA compared to control cells (P<0.001, P=0.024; Fig. 5C). These results indicated that SPAG9 acts upstream of JNK.

SPAG9 silencing inhibits p38 activation. p38 is another member of the MAPK signaling pathway, and MKK3 and MKK6 are kinases that act upstream of p38 (13). To determine whether SPAG9 regulates p38-mediated signaling in liver cancer cells, we examined the expression of p38, MKK3 and MKK6 in HepG2 cells transfected with *SPAG9*-targeted siRNA and control siRNA. Expression of p38 and MKK3 were significantly reduced in cells with reduced SPAG9 expression (P<0.001, P=0.016), but there was no significant change in levels of MKK6 (P=0.824; Fig. 5D).

Discussion

Numerous studies have indicated that the progression of liver cancer involves enhanced cell proliferation and resistance to apoptosis (14). Despite evidence pointing to a role for sperm-associated antigen 9 (SPAG9) as a tumor promoter in various types of cancer (15), mechanisms are still unclear. In the present study we explored the mechanism by which SPAG9 promoted liver cancer. Firstly, we observed that SPAG9 was overexpressed in 16 of the 20 (80%) liver cancer samples tested. The protein was not expressed or was expressed only weakly in adjacent non-cancerous tissues as revealed by immunohistochemistry. This finding confirms a previously proposed role for SPAG9 in liver cancer progression (16,17). It has been reported that SPAG9 is located in the cytoplasm of tumor cells (6,18); however, we observed SPAG9 in both the cytoplasm and nucleus of HepG2 cells using both immunohistochemistry and cellular immunofluorescence. Secondly, we found that silencing of *SPAG9* expression inhibited proliferation of HepG2 cells and promoted apoptosis and cell cycle arrest (Fig. 6, left panel). Notably, our previous study discovered that SPAG9 depletion caused cell cycle arrest in the G1-G2 phase in QGY-7703 cells (10); however, in HepG2 cells, the cell cycle was arrested in the S phase. We speculated that this difference is due to the different types of liver cancer from which these two cell lines were derived: QGY-7703 cells were derived from a hepatocellular carcinoma, but HepG2 cells were derived from a hepatoblastoma (19), and the specific

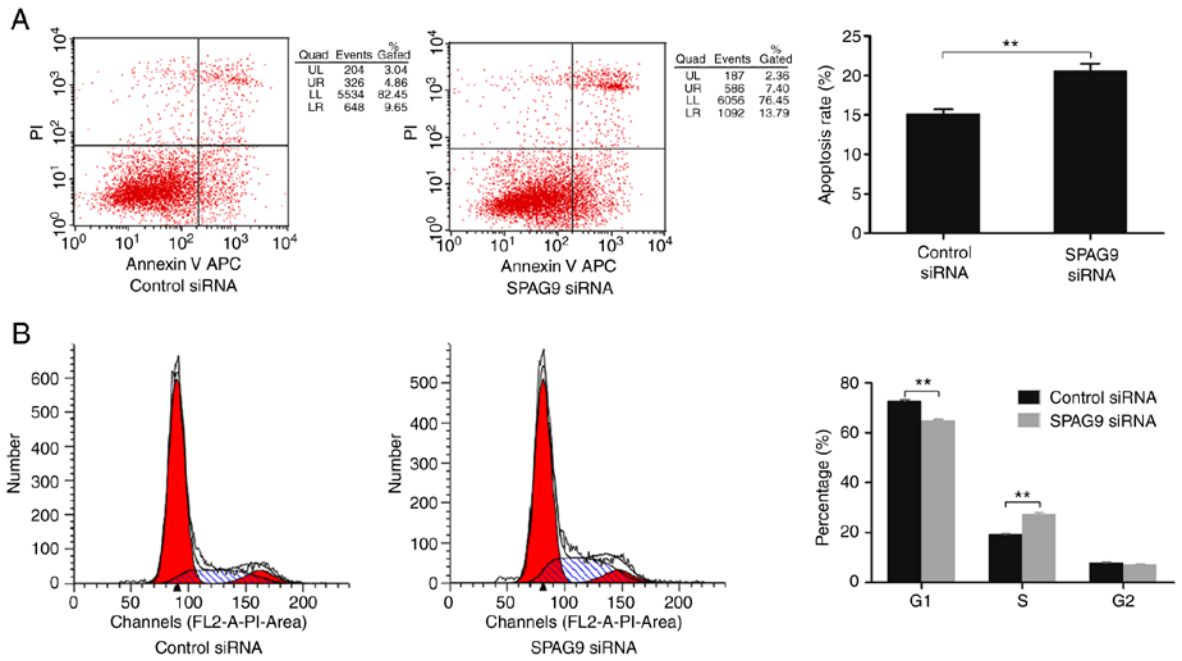

Figure 4. Silencing of *SPAG9* promotes apoptosis and arrests the cell cycle at the S phase. (A) Flow cytometric analysis of HepG2 cells transfected with *SPAG9*-targeted siRNA and control siRNA. **P<0.01. (B) Flow cytometric analysis of the cell cycle in HepG2 cells transfected with *SPAG9*-targeted siRNA and control siRNA. **P<0.01. SPAG9, sperm-associated antigen 9.

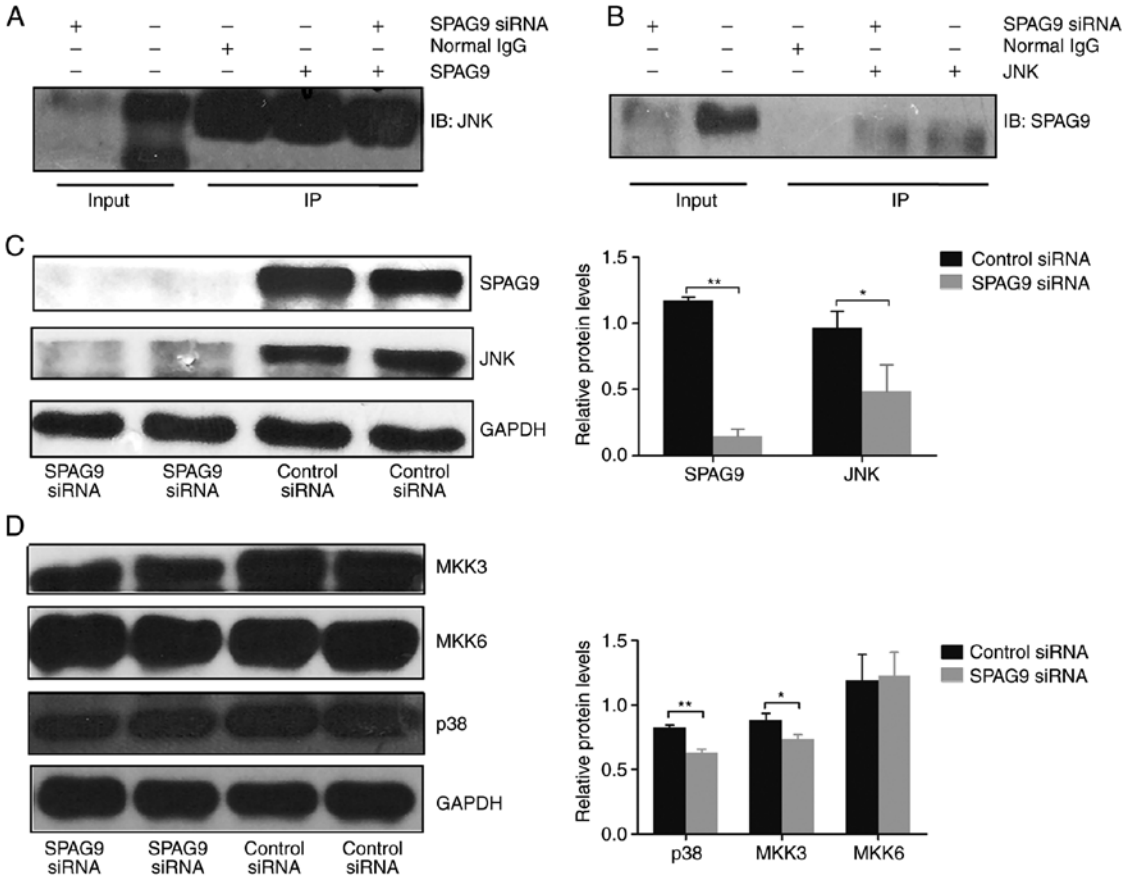

Figure 5. SPAG9 regulates liver cancer progression through the MAPK signaling pathway. (A) Western blot analysis for SPAG9 and JNK in HepG2 cells transfected with *SPAG9*-targeted siRNA and control siRNA. (B) Western blot analysis for SPAG9 and JNK of extracts of HepG2 cells transfected with *SPAG9*-targeted siRNA or control siRNA and immunoprecipitated with anti-SPAG9. (C) Western blot analysis for SPAG9 and JNK of extracts of HepG2 cells transfected with *SPAG9*-targeted siRNA or control siRNA and immunoprecipitated with anti-JNK. GAPDH was used as a loading control. **P<0.01, *P<0.05. (D) Western blot analysis for p38, MKK3 and MKK6 in extracts of HepG2 cells transfected with *SPAG9*-targeted siRNA or control siRNA. **P<0.01, *P<0.05. SPAG9, sperm-associated antigen 9; MAPK, mitogen-activated protein kinase.

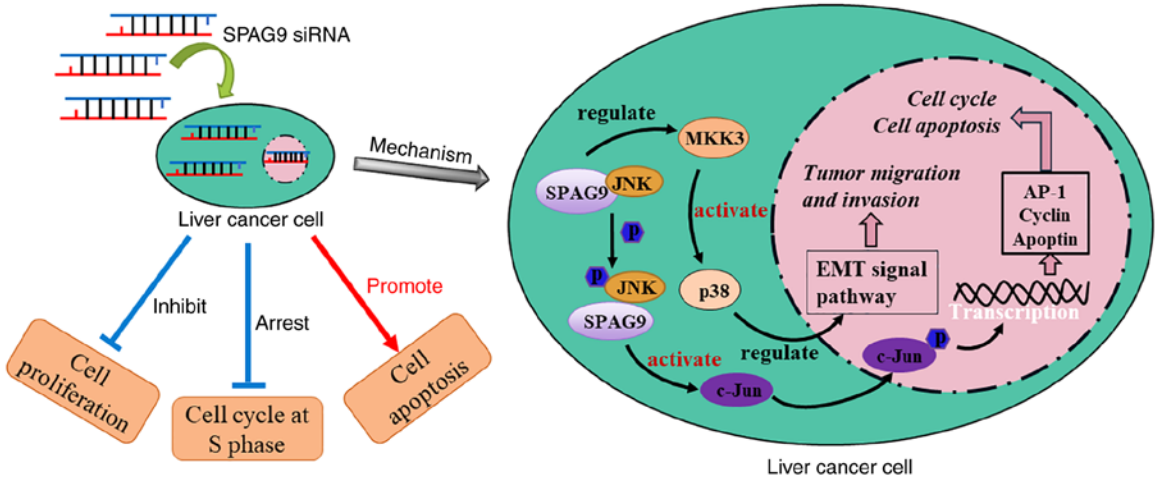

Figure 6. The role of SPAG9 in the progression of liver cancer. SPAG9, sperm-associated antigen 9.

regulatory mechanism of SPAG9 in the cell cycle and apoptosis requires further exploration. Thus, SPAG9 was highly expressed in tissues of patients with liver cancer and in cell lines derived from liver tumors; therefore, the biological function and potential clinical value of SPAG9 warrants further study.

Mitogen-activated protein kinase (MAPK) is a signaling pathway associated with cell proliferation, differentiation, migration, invasion and apoptosis (20). Scaffold proteins can recruit MAPK cascade components to direct phosphorylation and pathway activation (21), SPAG9 can act as a scaffold protein by tethering JNK and other p38 signaling modules through its

JNK-binding domain (22), and it has been revealed to participate in the activation of the MAPK signaling pathway (23). In a previous study, SPAG9 was revealed to be involved in osteosarcoma proliferation and invasion by positive regulation of JNK-mediated signaling (7). Our results demonstrated the involvement of SPAG9 in liver cancer. We also revealed that silencing of *SPAG9* significantly downregulated the expression of JNK. In the liver cancer-derived HepG2 cells, SPAG9 interacted with JNK; this interaction was previously demonstrated in an ovarian cancer cell line (5). JNK is a kinase that phosphorylates scaffold proteins and protein kinases, and its upregulation contributes to cancer growth and apoptosis (24,25). Our data suggest that SPAG9 directly interacts with JNK to regulate the cell growth and apoptosis of liver cancer cells.

p38 is a MAP kinase that regulates a stress-activated signaling pathway. It can be activated by various extracellular stimuli such as oxidative stress, ischemia hypoxia, UV irradiation and cytokines, and it regulates cell survival and death during normal development and during tumorigenesis (26). p38 plays a role in the progression of liver cancer, and increased phosphorylation of p38 is a predictor of poor survival of patients with liver cancer, and inactivation of p38 can lead to apoptosis in liver cancer cells (27,28). SPAG9 may regulate tumor cell survival and apoptosis through p38 signaling (29), and SPAG9/p38 signaling pathway activation has been shown to cause changes in expression of proteins that regulate the cell cycle and that activate the epithelial-mesenchymal transition (30,31). The activation of p38 requires three-tiered signaling that involves MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MKKs) and MAP kinases (MAPKs). MKK3 and MKK6 are kinases that act upstream of p38 (32).

In the present study that focused on liver cancer, we found that silencing of *SPAG9* significantly decreased the expression of p38 and MKK3 but did not cause a significant change in the expression of MKK6. Stramucci *et al* proposed that blocking upstream kinases could interfere with p38-mediated signaling resulting in blocking of pro-tumorigenic signals and leaving tumor suppressive signals unaffected (32). Indeed, MKK3 is regarded as a target for tumor therapy (33). Our results indicated that in liver cancer SPAG9 regulated the activation of p38 through the SPAG9/MKK3/p38 axis (Fig. 6, right panel), and factors involved in this novel pathway are possible therapeutic targets for liver cancer.

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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

SL conceived and designed the study, performed the experiments and drafted the manuscript. BR analyzed the data, revised the manuscript, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work. GZ provided experimental guidance, analyzed the data and revised the manuscript. JL, WC, YH, XC and YF performed the experiments, acquired and interpreted the data, 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 experiments were performed according to the Medical Ethics Committee of Hunan Brain Hopsital (no. L2017003). Written informed consent was obtained from all patients prior to the collection of liver cancer tissues.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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