Sperm-associated antigen 9 is upregulated in hepatocellular carcinoma tissue and enhances QGY cell proliferation and invasion *in vitro*

BIQIONG REN[1-3], GUOYING ZOU[1-3], JUNYU HE[1-3], YIRAN HUANG[2], GUOAN MA[3,4], GUOFENG XU[1,2], YONG LI[1,2] and PING YU[3]

[1]Department of Clinical Laboratory, Hunan Provincial Second People's Hospital; [2]Clinical Medical School, Hunan University of Chinese Medicine, Changsha, Hunan 410007; [3]Department of Immunology, School of Basic Medicine, Central South University, Changsha, Hunan 410078; [4]Department of Hepatobiliary Disease, Tumor Hospital of Hunan Province, Changsha, Hunan 410008, P.R. China

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Abstract. The incidence and mortality rates of hepatocellular carcinoma (HCC) are higher in China compared with in other countries. Further research is required in order to improve the diagnosis and treatment of HCC. Sperm-associated antigen 9 (SPAG9) protein has been revealed to serve an important function in cancer progression; however, the underlying mechanisms remain to be elucidated. The present study investigated the expression level of SPAG9 in HCC tissues using quantitative-polymerase chain reaction, immunohistochemistry and western blotting, and the results demonstrated that SPAG9 was overexpressed in HCC tissues compared with the adjacent non-cancerous tissues. To explore the potential mechanisms underlying SPAG9 in HCC, the effect of SPAG9 on cell proliferation, cell cycle, migration and invasion capacities were investigated in the QGY HCC cell line by RNA interference. It was revealed that inhibition of SPAG9 mRNA in QGY cells significantly inhibited the expression level of SPAG9 compared with the control. Depletion of SPAG9 expression decreased cell proliferation ($P<0.01$) and increased the percentage of cells in the G_1/G_2 cell cycle phase. The percentage of cells in the S phase was decreased, and cell migration and invasion capabilities *in vitro* were reduced ($P<0.01$). In summary, the results of the present study suggested that SPAG9 was upregulated in HCC and may serve an important function in cancer cell proliferation, differentiation and invasion. Whether SPAG9 is a potential diagnostic marker and therapeutic target of human HCC requires additional study.

Correspondence to: Professor Biqiong Ren, Department of Clinical Laboratory, Hunan Provincial Second People's Hospital, 427 Furong Road, Changsha, Hunan 410007, P.R. China
E-mail: 13808481211@163.com

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Introduction

Sperm-associated antigen 9 (SPAG9) has characteristics of a scaffold protein and is involved in the c-Jun N-terminal kinase JNK signaling pathway, binding to JNK, which suggests that it is involved in physiological processes, including apoptosis, survival, proliferation and tumorigenesis (1,2). SPAG9 is a member of the cancer/testis antigen family expressed from a single copy gene located on human chromosome 17q21. Proteins in the cancer/testis antigen family are overexpressed in a variety of types of cancer (3,4). SPAG9 is also expressed in a variety of tumors (5-9). SPAG9 has been proposed as a novel biomarker for early diagnosis of multiple human tumors, including ovarian, cervical and breast cancers (10-12). Certain studies have revealed that small interfering RNA inhibits expression of SPAG9 and inhibits the growth of various types of tumor cells (13,14). SPAG9 protein has been demonstrated to be involved in cancer progression; however, the underlying mechanisms remain unknown (15,16).

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer globally and is the third most common cause of cancer-associated mortality (17). The incidence of HCC is higher in South East Asia and Africa compared with other regions of the world (18). The crucial etiological factors involved in the development of HCC include infection with hepatitis virus, the structural or functional mutation of oncogenes and tumor suppressor genes (19-21). Long non-coding RNA URHC regulates cell proliferation and apoptosis by zinc-activated channels via the extracellular signal-related kinase/mitogen-activated protein kinase (MAPK) signaling pathway in HCC (22), and microRNA-24 may modify aflatoxin B1-related HCC prognosis and tumorigenesis (23). Therefore, it is necessary to further the research in this area in order to improve the diagnosis and treatment of HCC. In the present study, a series of methods were used to evaluate the expression

level of SPAG9 in human HCC tumor tissue and its potential underlying mechanisms.

Materials and methods

Cell culture. The QGY human HCC cell line was purchased from Shanghai Institute of Pharmaceutical Industry (Shanghai, China) and cultured in RPMI-1640 (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (GE Healthcare) at 37˚C in the presence of 5% CO_2.

Patient samples. A total of 16 HCC participants were enrolled between August 2010 and March 2013 at Xiangya Hospital, Central South University (Changsha, China). Written informed consent was obtained from all patients prior to enrollment in the present study. The exclusion criteria of the present study were as follows: i) Patients had distant metastasis; ii) Patients had received previous radiotherapy and chemotherapy prior to hepatectomy; iii) Patients with serious infection or other malignant diseases. The experimental protocols were approved by the Institutional Review Board of Xiangya Hospital. Carcinoma and adjacent noncancerous tissues were obtained from 16 patients with HCC during surgical tumor resections in accordance with informed consent. The present study was approved by the Ethics Committee of Hunan Provincial Second People's Hospital (Changsha, China). HCC was confirmed by pathobiology. All clinical and biological data are presented in Table I.

Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from HCC tissues and corresponding non-tumor normal tissues using TRIzol reagent (CWbio, Beijing, China) and cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis kit (CWbio), according to the manufacture's protocol. The qPCR was performed at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 60 sec and qPCR was performed using GoTaq qPCR master mix (Promega Corporation, Madison, WI, USA); BRYT Green® dye was used in the GoTaq qPCR master mix. For detection of SPAG9 mRNA expression levels, GAPDH was amplified in parallel as an internal control. The sequences of the primers used for qPCR were as follows: SPAG9 forward, 5'-AGCCGA CTTTTCAGCTCCTC-3' and reverse, 5'-AAAGCCTGCACT CTACCGTC-3'; GAPDH forward, 5'-CAATGACCCCTTCAT TGACC-3' and reverse, 5'-GACAAGCTTCCCGTTCTCAG-3' and the temperature protocol of reverse transcription was 37˚C. The mRNA expression level was evaluated using evaluated threshold cycle (Cq) values. The Cq values were normalized to the expression levels of GAPDH and the relative amount of mRNA specific to each of the target genes was determined using the $2^{-\Delta\Delta Cq}$ method (18-22). qPCR was performed with the BIO-RAD CFK96™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The data were analyzed using BIO-RADCFK Manager 2.0 software (Bio-Rad Laboratories, Inc.) for 40 cycles. Experiments were performed in triplicate.

Immunohistochemistry (IHC) and evaluation of staining. IHC was performed using the peroxidase anti-peroxidase technique as follows: Slides were incubated in citrate buffer at pH 6 and heated in a microwave for 21 min at (200 W). The antibody for SPAG9 was purchased from Abcam (cat. no. ab12331; Abcam, Cambridge, MA, USA). Antibody against SPAG9 (1:150; ab12331; Abcam) was overlaid on HCC and corresponding non-tumorous normal tissue sections (4 μm) and incubated overnight at 4˚C. Secondary antibody incubation using alkaline phosphatase-conjugated mouse anti-rabbit immunoglobulin G (cat. no. sc-2358; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was performed at room temperature for 30 min.

Tissue sections were blindly evaluated by two pathologists from the Department of Pathology, Xiangya Hospital, Central South University (Changsha, China) in an effort to provide a consensus on staining patterns by light microscopy (magnification, x100; Olympus Corporation, Tokyo, Japan). SPAG9 staining was performed according to the methods described by Kanojia *et al* (23). Each case was rated according to a score that added a scale of intensity of staining to the area of staining. At least 10 high-power fields were chosen randomly and >1,000 cells were counted for each section. The intensity of staining was graded on the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was evaluated as follows: 0, no staining of cells in any microscopic fields; 1+, <30% of tissue stained positive; 2+, 30-60% stained positive; 3+, >60% stained positive. The minimum score when determined (extension + intensity) was, therefore, 0, and the maximum was 6. A combined staining score (extension + intensity) of ≤2 was considered to be negative staining (low staining); a score of 3-4 was considered to be moderate staining; and a score of 5-6 was considered to be strong staining.

Western blot analysis. The HCC tissues, corresponding non-tumor normal tissues and QGY cells were lysed in RIPA buffer at 4˚C for 5 min (CWbio) and total protein concentration was determined using a Pierce® BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Extracts containing 50 μg protein were separated in 10% SDS-PAGE gels and electroblotted onto nitrocellulose membranes (HyClone; GE Healthcare Life Sciences). The membranes were blocked using Tris-buffered saline/Tween-20 (25 mM Tris-HCl, 150 mM NaCl, pH 7.5 and 0.05% Tween-20) supplemented with 5% non-fat milk at room temperature for 3 h, followed by overnight incubation at 4˚C with primary antibodies (rabbit anti-SPAG9 antibody; Abnova, Tapai, Taiwan; cat. no. PAB8794; dilution, 1:500). Following three washes with Tris-buffered saline/Tween-20 (25 mM Tris-HCl, 150 mM NaCl, pH 7.5 and 0.05% Tween-20), the membranes were incubated with mouse anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-2491; Santa Cruz Biotechnology, Inc.; dilution, 1:5,000) at room temperature for 1 h and the specific signals were visualized using an ECL detection system. Anti-GAPDH antibody (Santa Cruz Biotechnology, Inc.; cat. no. Sc-25778; 1:3,000) was used as a loading control at 4˚C overnight. The bands were analyzed using Gel Automated Digitizing System software (version 4.0; Silk Scientific, Orem, UT, USA). The relative expression levels (fold) were evaluated

Table I. Characteristics of patients with HCC.

Samples	Age (years)	Sex	Tumor size (cm x cm x cm)	Pathological diagnosis and classification (Edmondson grading system) (30)
1	42	Male	5.0x4.0x3.5	2-3 grade well differentiated HCC
2	40	Female	5.0x5.0x4.5	2 grade moderately differentiated HCC
3	39	Male	7.0x5.0x5.0	2 grade moderately differentiated HCC
4	44	Female	3.5x3.0x0.5	2-3 grade well differentiated HCC
5	45	Male	4.0x3.0x3.0	2-3 grade well differentiated HCC
6	48	Male	4.0x3.5x2.0	2-3 grade well differentiated HCC
7	58	Male	6.0x6.0x4.5	2 grade moderately differentiated HCC
8	56	Male	3.0x2.5x2.0	1-2 grade well differentiated HCC
9	50	Male	3.0x4.0x4.0	2-3 grade well differentiated HCC
10	43	Male	3.5x4.0x2.0	2 grade moderately differentiated HCC
11	57	Female	5.5x5.0x4.0	1-2 grade well differentiated HCC
12	51	Male	3.5x3.0x3.0	2-3 grade well differentiated HCC
13	64	Male	6.0x3.5x4.0	2 grade moderately differentiated HCC
14	58	Female	5.0x3.5x3.0	2-3 grade well differentiated HCC
15	48	Male	4.0x5.0x3.5	2 grade moderately differentiated HCC
16	54	Male	4.0x3.5x3.0	1-2 grade well differentiated

HCC, hepatocellular carcinoma.

by normalizing the integrated optical density (IOD) for each band to that of the corresponding GAPDH band.

Design and synthesis of SPAG9 hairpin-like small interfering (si)RNA, and construction of recombinant eukaryotic expression plasmid. According to the SPAG9 gene sequence (NM_001130527.2), the oligo DNA single strand 1, 2 was designed and synthesized by Ambion; Thermo Fisher Scientific, Inc., and was used as the target of RNAi. The control siRNA oligonucleotides 3 and 4 were also designed and led to the formation of double-stranded DNA with annealed oligonucleotides. EcoRI and BamH RV restriction sites were introduced at the end of the terminal. The lentivirus pWPT-green fluorescent protein (GFP) was used as the carrier; the viral vector was purchased from Clontech Laboratories, Inc. (Mountainview, CA, USA). A lentivirus vector containing GFP, SPAG9 RNAi sequences or control siRNA was constructed: SPAG9 siRNA, TCTGGAAACGACATTTATGG; control siRNA, TGAAGG TCGGAGTCAACGGATT.

siRNA virus infection of QGY cells. QGY HCC cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C in 5% CO_2. There were three groups assessed in the present study: An empty vector group, a control siRNA group and a SPAG9 siRNA group. At 60% confluence, the lentivirus vector was added at a multiplicity of infection of 10 at 37˚C. Following 48 h, infection efficiency (based on GFP expression level) was determined using an inverted fluorescence microscope (Leica DMI3000B; Leica Microsystems GmbH, Wetzlar, Germany).

Detection of cell growth by MTT. Cells in the logarithmic growth phase were seeded into 96-well plates at $1x10^3$ cells per well in a 200 μl volume. A total of 20 μl MTT (5 mg/ml) was added to each well and plates were incubated at 37˚C for 4 h. The liquid was removed from each well and 150 μl dimethyl sulfoxide was added at 37˚C for 5 min. Plates were rapidly oscillated at room temperature at 300 x g on a microplate reader (MK3; Thermo Fisher Scientific, Inc.) for 60 sec to fully dissolve the precipitate, and absorbance of each well at 490 nm was evaluated.

Cell cycle analysis by flow cytometry. The QGY cells were cultured in serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2 for 24 h and digested with 0.25% trypsin at room temperature for 2 min, washed once with PBS, centrifuged at 476 x g for 5 min and resuspended in 70% ethanol. Cell density was adjusted to $1x10^6$ cells/ml and cells were fixed with 4% formaldehyde at 4˚C overnight. Cells were washed with cold PBS and 100 μl RNase A (0.5 mg/ml) was added. After 30 min at 37˚C, 400 μl propidium iodide (10 μl/ml) was added and the samples were incubated at 4˚C in the dark for 30 min. A FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was then used to detect the cell cycle distribution.

Transwell chamber assay to investigate the migration ability of cells. Cells in the logarithmic growth phase were digested with trypsin at room temperature for 2 min. A total of 600 μl 10% FBS supplemented with RPMI-1640 was added to the lower Transwell chamber and $3x10^5$ cells in 300 μl serum-free medium was added to the upper chamber. Following a 48 h incubation at 37˚C in 5% CO_2, the chamber was washed with PBS and a cotton swab was used to remove the cells that did not pass through the basal membrane of the invasion chamber. Cells that had adhered to the surface of the inferior chamber

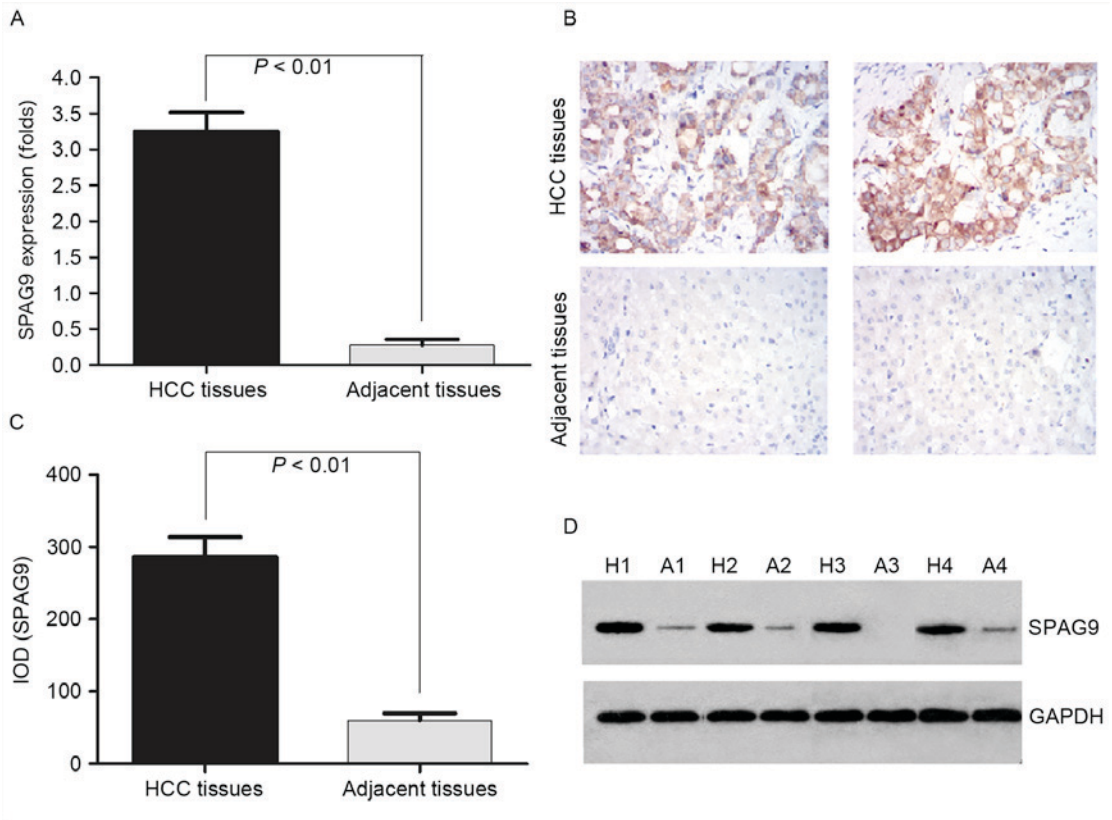

Figure 1. Detection of SPAG9 expression levels in the HCC tissues and the adjacent non-cancerous tissues by RT-qPCR, IHC and western blotting. (A) RT-qPCR was performed to validate the expression level of SPAG9 in HCC tissues and the adjacent non-cancerous tissues. GAPDH was used as an internal control and for normalization of the data. (B) IHC analysis of the expression level of SPAG9 protein in HCC and adjacent non-cancerous tissues. Brown grains indicate a positive signal. Original magnification, x400. (C) The protein expression levels of SPAG9 were significantly higher in the HCC tissues compared with in adjacent non-cancerous tissues, as assessed by western blot analysis. (D) SPAG9 protein expression levels in 4 tissues used in the detection of mRNA expression levels by RT-qPCR. SPAG9, sperm-associated antigen 9; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; IHC, immunohistochemistry; H, HCC tissues; A, adjacent non-cancerous tissues; IOD, integrated optical density.

were fixed with 100% methanol at room temperature for 30 min and the chamber was immersed in Giemsa (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) staining solution at room temperature for 20 min. Excess dye was removed with PBS and the chambers were dried in air. Various fields of view (magnification, x100) were randomly captured by light microscope (Olympus Corporation) and cells were counted. The number of assessed fields of view was 200.

Statistical analyses. The Pearson's χ^2 test, Fisher's exact test, unpaired Student's t-tests, Wilcoxon signed-rank tests, Mann-Whitney U tests and Kruskal-Wallis one-way analysis of variance tests were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). The Bonferroni post hoc test used for the χ^2 tests and the Kruskal Wallis tests. Results were expressed as the mean ± standard deviation. All P-values were two-tailed, and P<0.05 was considered to indicate a statistically significant difference.

Results

SPAG9 is highly expressed in HCC tissues. In order to detect the mRNA expression levels of SPAG9 in HCC and the adjacent non-cancerous tissues, 16 samples of each were selected to perform RT-qPCR of the SPAG9 gene. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method and the fold change in the expression levels of these genes relative to the internal control gene, GAPDH, were analyzed. The expression level of the SPAG9 gene was higher in the HCC samples compared with in the adjacent non-cancerous tissues, and the normalized SPAG9 gene expression level in HCC was upregulated by 3.35-fold (P=0.003; Fig. 1A).

To confirm the pattern of SPAG9 expression in HCC, IHC was performed with antibodies against SPAG9 protein in HCC and adjacent non-cancerous tissues. SPAG9 was identified as differentially expressed between HCC tissues vs. the adjacent non-cancerous tissues. IHC demonstrated a similar pattern in protein expression level to the western blotting results. There was a 75.0% (12/16) high score of SPAG9 expression level in HCC tissues and 0% (0/16) in the adjacent non-cancerous tissues. The distribution of a low score was 0% (0/16) and 62.5% (10/16) in HCC and the adjacent non-cancerous tissues, respectively (P=0.0001; Fig. 1B; Table II). This corresponded with the RT-qPCR results.

To determine whether the SPAG9 protein was expressed at a higher level in HCC compared with adjacent non-cancerous tissues, the protein expression levels of SPAG9 were further examined by western blotting in 1 to 4 samples (Fig. 1C and D). SPAG9 protein high expression level was detected in cancerous tissue (IOD 286.84±75.91) and at lower levels in the adjacent noncancerous tissues (IOD 29.86±34.91; P<0.01; Fig. 1C), which corresponded with the RT-qPCR results.

Table II. Expression levels of SPAG9 in HCC tissues compared with adjacent non-cancerous tissues, as assessed by immunohistochemistry.

Tissue	No. patients	Score			χ^2	P-value
		Low (%) (0-2)	Moderate (%) (3-4)	High (%) (5-6)		
HCC	16	0 (0)	4 (25.0)	12 (75.0)	22.40	0.0001
Adjacent tissues	16	10 (62.5)	6 (37.5)	0 (0)		

Pearson's χ^2 test was used for statistical analysis. SPAG9, sperm-associated antigen 9; HCC, hepatocellular carcinoma.

Table III. Proliferation of QGY cells.

Group	Incubation period (h)			
	24	48	72	96
SPAG9 siRNA	0.294±0.007	0.376±0.021[a]	0.584±0.063[a]	0.662±0.127[a]
Control siRNA	0.316±0.031	0.506±0.063	0.812±0.064	0.946±0.127
Empty vector	0.294±0.035	0.518±0.042	0.825±0.076	0.982±0.135

Proliferation was analyzed using an MTT assay (n=10). [a]P<0.01, SPAG9 siRNA vs. control siRNA vector. SPAG9, sperm-associated antigen 9; siRNA, short interfering RNA.

SPAG9 effects the proliferation of HCC cells. QGY human HCC cells were infected with lentiviral vectors engineered to express siRNA targeting SPAG9 or a control siRNA with an empty vector. Green fluorescence due to a virally expressed GFP was observed in cells following infection for 12 h using an inverted fluorescence microscope. Fluorescence increased with infection time. The same vision field was randomly selected and observed under fluorescence (73 perspectives) and white light (76 perspectives) to determine infection efficiency. At 48 h, the infection efficiency was ~92% (Fig. 2A). The expression level of SPAG9 protein in the SPAG9 siRNA cells was significantly lower compared with in cells in the control siRNA group (Fig. 2B). These results revealed that the silencing of SPAG9 expression was successful in QGY cells.

To investigate the effect of silencing of SPAG9, the present study evaluated the proliferation of QGC cells between the siRNA group and control group by MTT assay. The results demonstrated that SPAG9-deficient cells did not proliferate as well as cells treated with empty vector or cells that expressed the control siRNA (Table III). Compared with the control siRNA-expressing cells, proliferation of cells that expressed SPAG9 siRNA were inhibited by 32.6% at 96 h.

SPAG9 promotes cell cycle progression. Flow cytometry was performed to detect the cell cycle distributions of QGY cells (Fig. 3). In cells that expressed control siRNA, 41.6% were in the G_0/G_1 cell cycle phase, whereas 58.7% of cells that expressed the SPAG9 siRNA were in the G_0/G_1 cell cycle phase. Compared with the control siRNA-treated cells, the percentage of cells

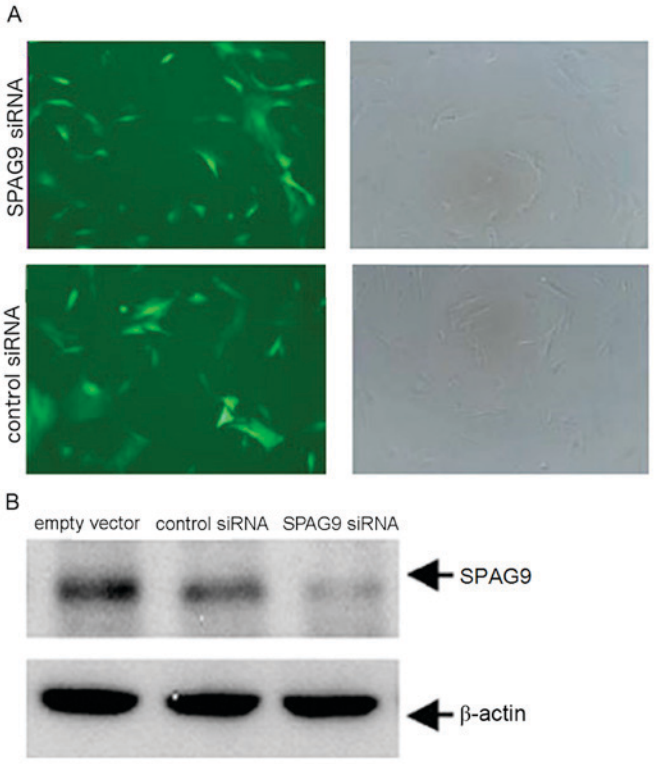

Figure 2. Microscopic analysis of QGY cells 48 h following infection with lentivirus designed to express SPAG9 siRNA or control siRNA (magnification, x100). (A) Fluorescence and white light images of QGY cells that express SPAG9 siRNA or control siRNA. SPAG9 protein expression levels were analyzed by (B) western blotting in QGY cells infected with an empty vector or vectors containing control siRNA or SPAG9 siRNA. SPAG9, sperm-associated antigen 9; siRNA, small interfering RNA.

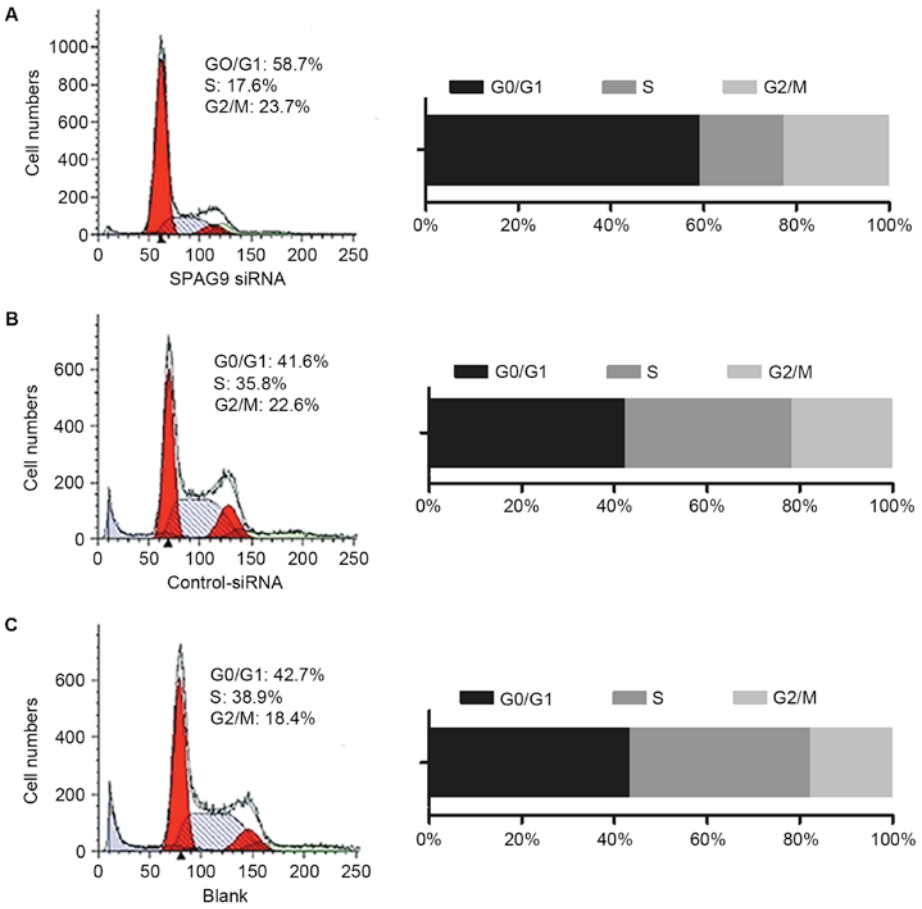

Figure 3. Flow cytometry analysis of the cell cycle in QGY cells infected with (A) lentivirus for expression of SPAG9 siRNA, (B) lentivirus for expression of control siRNA and (C) empty vectors. SPAG9, sperm-associated antigen 9; siRNA, small interfering RNA.

in G_0/G_1 cell cycle phase were increased by 17.1% in SPAG9 siRNA-expressing cells and the percentage in the S phase was reduced by 18.2% (Fig. 3). There was no significant difference between cells that expressed control siRNA and cells infected with the empty vector, suggesting that inhibition of SPAG9 expression may inhibit the cell cycle in the G_1 and G_2 phases.

SPAG9 enhances cell migration. Finally, the migration of cells deficient in SPAG9 was compared with that of control cells using a Transwell chamber assay. Compared with cells that expressed control siRNA, the percentage of cells that expressed the SPAG9 siRNA that migrated toward rich medium was significantly decreased ($P<0.01$). There was no difference in migration of cells that expressed control siRNA and cells infected with an empty vector ($P>0.05$; Fig. 4). The results of the present study indicated that inhibiting SPAG9 expression levels reduced cell migration.

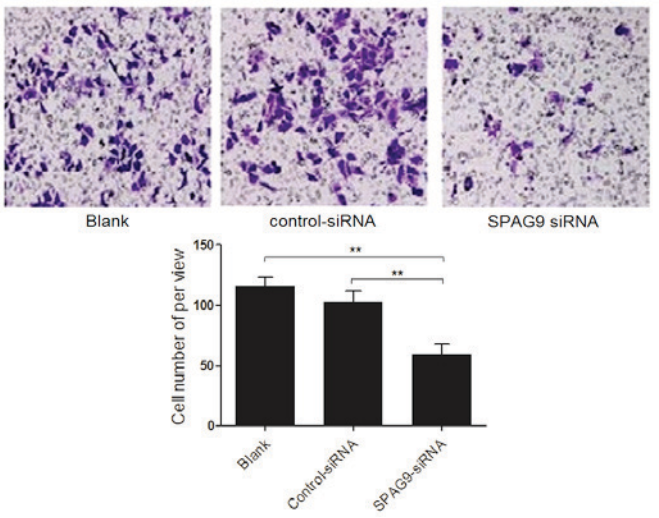

Figure 4. Results of the Transwell chamber assay. Compared with cells that expressed control siRNA and blank cells, the percentage of cells that expressed the SPAG9 siRNA that migrated toward rich medium was significantly decreased (**P<0.01). There was no significant difference in migration of cells infected with control siRNA compared with cells infected with empty vector (P>0.05). SPAG9, sperm-associated antigen 9; siRNA, small interfering RNA.

Discussion

The occurrence and development of HCC are complex processes associated with a variety of oncogenes, tumor suppressor genes and certain cell cycle regulation factors (24,25). Therefore, further investigation is required to fully understand and improve treatments for HCC. Attempts to diagnose HCC early in order to decrease the mortality rate and prolong the survival time and quality of life have been made (26); however, there have been no major breakthroughs at present. Cancer/testis antigens expressed in a variety of human malignant tissues provide a

novel direction for the study of HCC, and SPAG9 is one of these cancer/testis antigens (27). A previous study concerning ovarian cancer suggested that SPAG9 regulates the MAPK signaling pathway (28). The MAPK signaling pathway is mediated by various stimuli, and the selectivity and specificity of these reactions depends on scaffold proteins (29). SPAG9 interacts with JNK in the A549 lung cancer cell line and SPAG9 contributes to invasive abilities via a mechanism mediated by matrix metalloproteinase 9 and the activation of JNK (14).

The present study revealed that the expression level of the SPAG9 gene and protein was upregulated in the HCC tumor tissues compared with in adjacent non-cancerous tissues ($P<0.01$). The analysis of HCC tissues from patients in the present study was consistent with previous studies that evaluated SPAG9 expression levels in the QGY hepatoma cell line (11,27). RNAi technology was used to inhibit the expression level of SPAG9 in cultured cells and the results revealed that SPAG9 deficiency was able to affect the proliferation and progression of HCC QGY cells. The present study performed an MTT assay and demonstrated that, compared with the control siRNA-expressing cells, proliferation of cells that expressed SPAG9 siRNA was significantly inhibited from 48 h and the rate of inhibition achieved was 32.6% at 96 h. The number of QGY cells in the G_0 phase increased and the number in the S phase decreased following SPAG9 gene silencing. A Transwell chamber assay was performed and revealed that inhibiting the expression level of SPAG9 reduced the migratory ability of QGY cells; however, the specific mechanism underlying this effect requires further study. Inhibiting SPAG9 expression may effectively decrease the differentiation and proliferation of OGY cells, indicating that SPAG9 may serve a specific function in accelerating the cell cycle, promoting proliferation and migration of HCC. This revealed that downregulation of scaffold proteins may induce inactivation of numerous signaling pathways. Therefore, blocking the tumor signaling pathway by knocking out the tumor scaffold proteins is a novel concept for cancer treatment.

The present study evaluated a limited number of patients and only one immortalized cell line. Due to the heterogeneities of HCC, the expression levels of SPAG9 may differ. SPAG9 protein levels were increased in the adjacent tissues of patients 4 and 7 relative to the adjacent tissues, as assessed by western blotting and IHC, but in the other patients the expression level was low relative to that of the tumor tissues. The present study observed an increased expression level of SPAG9 in the tumor samples evaluated compared with adjacent non-cancerous samples and SPAG9 expression was decreased in adjacent non-cancerous samples compared with tumor samples. The results of the present study suggested that SPAG9 was upregulated in HCC and may serve an important function in cancer cell proliferation, differentiation and invasion. Furthermore, that downregulation of SPAG9 expression levels may inhibit the proliferation and invasion of HCC. Whether SPAG9 is a potential diagnostic marker and therapeutic target of human HCC requires additional investigation.

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