Sperm-Associated Antigen 5 Expression Is Increased in Hepatocellular Carcinoma and Indicates Poor Prognosis

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Background: Sperm-associated antigen 5 (SPAG5), a gene that encodes a mitotic spindle-associated protein, is closely related to tumor development and is involved in cell migration and proliferation. The objective of this research was to explore the clinical significance of SPAG5 expression in hepatocellular carcinoma (HCC) and the relationship between SPAG5 expression and HCC prognosis.

Material/Methods: Twenty pairs of fresh-frozen HCC samples and samples from 95 HCC patients in a tissue microarray were subjected to quantitative real-time reverse-transcription (qRT)-PCR and immunohistochemistry (IHC), respectively, to investigate the relationship between the expression of SPAG5 and the clinicopathological features of HCC patients.

Results: PCR data showed that the messenger RNA (mRNA) expression level of SPAG5 in HCC tissue specimens was higher than that in adjacent non-tumor tissue specimens ($p<0.05$). IHC analyses demonstrated that SPAG5 expression was significantly correlated with tumor grade ($p=0.003$), tumor number ($p=0.009$), vascular invasion ($p=0.001$), and TNM stage ($p=0.001$). Survival analysis and Kaplan-Meier curves showed that SPAG5 expression is an independent prognostic indicator for disease-free survival ($p=0.017$) and overall survival ($p=0.016$) in HCC patients.

Conclusions: Our results indicate that SPAG5 expression may be considered as an oncogenic biomarker and a novel predictor for HCC prognosis.

MeSH Keywords: Carcinoma, Hepatocellular • Prognosis • Survival

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Background

Hepatocellular carcinoma (HCC) is the 2nd and 6th leading cause of cancer-related deaths worldwide in men and women, respectively. In 2012, it was estimated that over 700 000 new cases of HCC [1] occurred. China alone accounted for approximately 50% of the worldwide morbidity and mortality [2]. Chronic hepatitis B virus and metabolic syndrome, hepatitis C virus infections, and long-term alcohol intake are major causes of HCC [3–5]. Although the number of therapeutic strategies is growing (e.g., molecular therapy, radiofrequency ablation, liver transplantation, and surgical resection) and some are currently being developed [6,7], the overall prognosis of HCC patients remains poor, with an overall 5-year survival rate of nearly 18% [8]. Currently, early-stage tumor detection and timely intervention are the best strategies to deal with HCC. However, due to the low sensitivity of monitoring tools, such as ultrasound, early diagnosis of HCC is difficult [9–11]. The practicality of using the alpha-fetoprotein (AFP) level as a means of disease detection is also being increasingly disputed [12–14]. Studies have shown that certain genes are closely associated with HCC prognosis and may serve as valuable markers for HCC treatment [15]. These genes exert critical functions in the occurrence of HCC, so they can serve as useful HCC biomarkers or therapy targets for HCC.

The sperm-associated antigen 5 (SPAG5) gene, which is located on chromosome Chl7q11.2, encodes a spindle-binding protein that regulates the assembly timing of the mitotic spindle and the separation of sister chromatids [16]. Chang et al. [17] first cloned and studied SPAG5 in 2001. Recently, it was identified as a key component required for the inhibition of apoptosis in cancer cells during cell stress [18]. It has been demonstrated that SPAG5 can promote tumor cell growth and proliferation and apoptosis [18–20]. Previous studies have shown that SPAG5 down-regulates the anti-oxidative stress response via the mammalian target of rapamycin (mTOR) signaling pathway and thus protects cells from apoptosis. This new theory further indicates that SPAG5 may act as a promoter of tumor development [18]. In recent years, many clinical studies have evaluated the expression level of SPAG5 in various malignancies and have assessed its clinical significance [19,21–23]. In patients with cervical cancer, prostate cancer, breast cancer, or lung cancer, increased expression of SPAG5 is associated with adverse prognosis [19,21,23,24]. These results indicate that SPAG5 may be an important oncogene involved in the development and progression of malignant tumors and it may affect a number of malignant behaviors of tumors. It has been reported that SPAG5 is a potential vaccine candidate target for various tumors [25,26]. However, no study has investigated the prognostic value of SPAG5 in HCC, which thus requires further elucidation.

We first assessed the mRNA expression level of SPAG5 in fresh HCC samples using quantitative real-time reverse-transcription (qRT)-PCR and detected SPAG5 protein via an HCC tissue microarray (TMA) using immunohistochemistry (IHC) analysis. We also evaluated the relationship between SPAG5 expression and the clinicopathological features of HCC patients, with a focus on the relationship between SPAG5 expression and its prognostic characteristics.

Material and Methods

HCC tissue microarrays construction

We enrolled 95 cases of HCC to construct tissue microarrays (TMA) and the TMA were provided by the People's Hospital of Jurong Affiliated with Jiangsu University between May 2007 and July 2012 to perform IHC analysis. A number of significant clinical information were collected, such as sex, age, tumor size, tumor encapsulation, tumor number, pathological grade, hepatitis B virus infection, vascular invasion, liver cirrhosis, and Tumor Node Metastasis (TNM) stage, from the TMA data that was provided by the People's Hospital of Jurong Affiliated with Jiangsu University. None of the patients had received any form of treatments (e.g., radiation therapy, chemotherapy, or immunotherapy) before surgery. We obtained informed consent from each patient, and ethics approval to conduct the research was approved by the Ethics Research Committee of each local hospital.

qRT-PCR test in HCC samples

We enrolled 20 fresh-frozen HCC samples and matching tumor-adjacent tissues samples. Total RNA was isolated from the fresh HCC tissues by TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The protocol of qRT-PCR analysis was conducted as described before [27]. Primer sequences were as follows: SPAG5 forward primer, 5'-CTGAGCAGTAGAACTGAGGCT-3' and reverse primer 5'-TCCACATGATTGACACGGAAAT-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization control, and the primers used for qRT-PCR were as follows: forward primer 5'-GCCTCAAGATCATCAGCAAT-3' and reverse primer 5'-GGACTGTGGTCATGAGTCCT -3'. All primers were synthetized by BioTNT Co., Ltd. (Shanghai, China). The relative target gene expression of SPAG5 was calculated by using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Western blot

HCC fresh tissue was lysed by 6X sodium dodecyl sulfate (SDS) loading buffer and then fractionated by SDS-PAGE. The proteins were transferred to PVDF membranes which were then
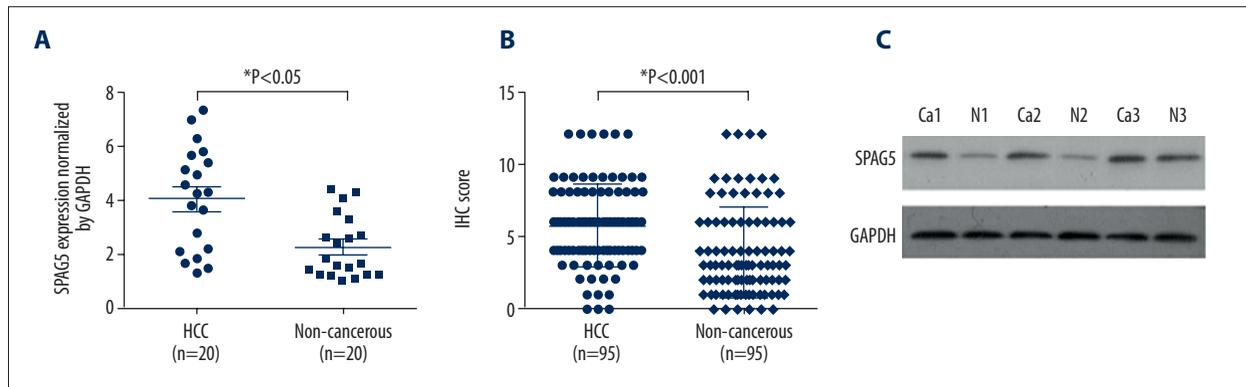
Figure 1. (**A**) SPAG5 expression is increased in fresh hepatocellular carcinoma (HCC) tissue. In total, 20 pairs of RNA derived from HCC tissues and adjacent non-tumor tissues were subjected to qRT-PCR assays to detect SPAG5 mRNA expression. The relative $2^{-\Delta\Delta CT}$ values (Wilcoxon matched paired test) are shown. (**B**) Comparison of SPAG5 expression levels between HCC tissue samples and adjacent non-tumor tissue samples. Statistical analysis was performed with a Wilcoxon signed-rank nonparametric test. (**C**) The protein levels of SPAG5 in HCC (Ca) and adjacent nontumorous tissues (N) were determined by Western blot.

incubated with a primary specific antibody for SPAG5 antibody (1: 200, ab200671, Abcam, Cambridge, MA) in 5% non-fat milk, followed by a horse radish peroxidase (HRP)-conjugated anti-Rabbit secondary antibody [28]. ECL detection reagent was used to reveal the results.

IHC analysis in HCC TMA

Immunohistochemical detection was performed as described in previous studies [29,30]. Rabbit polyclonal anti-SPAG5 antibody (1: 200, ab200671, Abcam, Cambridge, MA) was first added to incubate with TMA. After being washed with phosphate-buffered saline (PBS), the slides were incubated with anti-rabbit secondary IgG antibody. PBS was used as a negative control instead of the primary anti-SPAG5 antibody. The IHC scores ranged from 0 to 12 and were generated by multiplying the number of staining cells by staining cell intensity. The density of SPAG5 in staining cells was graded as follows: 0 (negative staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). Staining percentage of SPAG5 was categorized as follows: 0 (0%), 1 (1–25%), 2 (11–50%), 3 (51–75%), and 4 (76–100%). A score of 0–4 points was considered as low expression of SPAG5, while >4 was considered as high expression of SPAG5.

Statistical analysis

The Wilcoxon nonparametric signed-rank test was employed to detect the expression of SPAG5 in HCC tissue samples and matched non-cancerous tissue samples. The chi-square test was performed to test the correlation expression of SPAG5 with clinicopathological parameters. Univariate and multivariate analyses were conducted to screen prognostic factors of HCC. Survival rate was calculated by Kaplan-Meier method. For all tests, P-values less than 0.05 were recognized as statistically

significant. Statistical analyses were performed using STATA version 14.0 (Stata Corporation, USA) and SPSS 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

Elevated expression of SPAG5 in HCC

To examine the SPAG5 mRNA levels in HCC patients, qRT-PCR assays were performed using 20 pairs of samples of HCC tissues and matching adjacent tissue. The expression of SPAG5 mRNA level in fresh HCC tissue samples was much higher than that of non-cancerous tissue samples (p<0.05) (Figure 1A). To verify the protein level, IHC was subsequently used to evaluate the protein expression levels of the paired samples described above (Figure 1B). Similar to the qRT-PCR result, the results showed that SPAG5 expression was significantly higher in HCC tissue samples than in non-tumor tissue samples (p<0.001). Positive staining for SPAG5 was primarily localized in the cytoplasm and cell membrane. Typical IHC staining of SPAG5 expression in HCC cells is shown in Figure 2. Consistently, expression of SPAG5 protein was noticeably up-regulated in HCC samples (Figure 1C).

Correlation between SPAG5 and the clinicopathological features of HCC

In this study, we examined HCC tissues from 95 patients (85 males and 10 females), the characteristics of whom are shown in Table 1. SPAG5 expression was significantly correlated with tumor number (p=0.009), vascular invasion (p=0.001), pathological grade (p=0.003), and TNM stage (p=0.001), but not with other clinicopathological features.
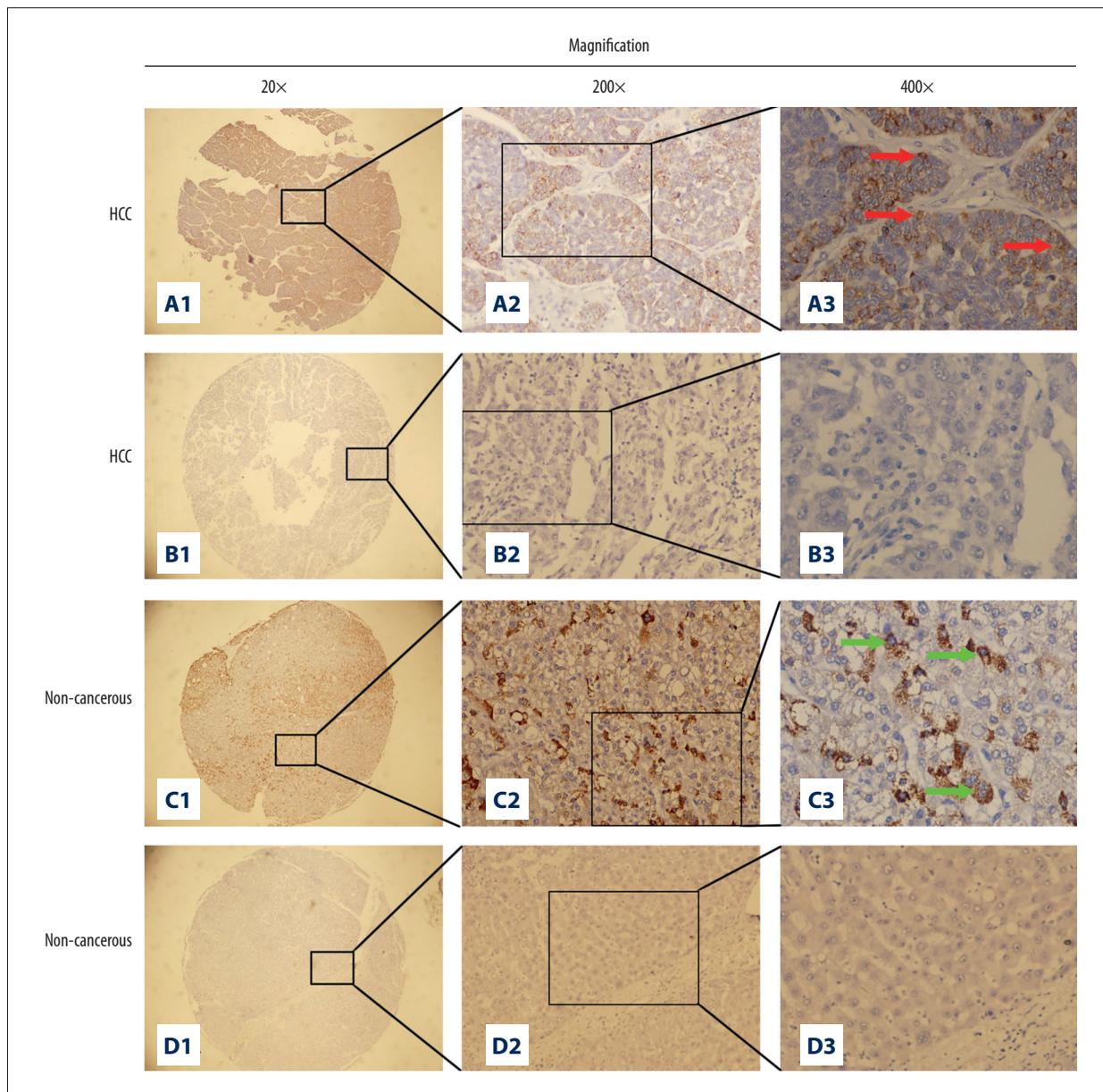
Figure 2. SPAG5 expression is significantly up-regulated in HCC. IHC detection of SPAG5 expression in the paired HCC tissues and adjacent non-tumor tissues. **A1–A3, B1–B3** show high and low levels, respectively, of SPAG5 expression in HCC tissue. **C1–C3, D1–D3** represents high and low levels, respectively, of SPAG5 expression in non-tumor tissue adjacent to tumor tissue. **Middle and right panels** contain higher-magnification images of the corresponding boxed areas in the **left panels**.

Impact of SPAG5 proteins on the survival of HCC patients

Cox regression univariate analysis showed that SPAG5 expression, tumor size, pathological grade, and TNM stage had an unfavorable effect on DFS and OS (Tables 2, 3). High SPAG5 expression was associated with a poor prognosis and shorter DFS ($p<0.001$) (Figure 3A). In addition, SPAG5 expression was associated with a lower OS rate in HCC patients ($p<0.001$) (Figure 3B). Multivariate analysis showed that high SPAG5 expression tended to be an independent risk indicator for DFS ($p=0.017$) (Table 2) and OS ($p=0.016$) for HCC (Table 3).

Discussion

SPAG5 is recognized as an important promoter of tumor progression. Numerous studies have shown that compared with that of non-cancerous tissue, the median expression of SPAG5 in head and neck cancer, prostate cancer, breast cancer, and

Table 1. The relationship between SPAG5 expression and the clinicopathological features of hepatocellular carcinoma patients.

Groups	No.	SPAG5		χ^2	p Value
		+	%		
Total	95	54	56.8		
Gender				0.79	0.374
Male	85	47	55.3		
Female	10	7	70.0		
Age (years)				2.00	0.157
≥60	23	16	69.6		
<60	72	38	52.8		
Tumor size (cm)				0.37	0.542
>5	31	19	61.3		
≤5	64	35	54.7		
Tumor encapsulation				1.09	0.579
None	49	29	59.2		
Complete	45	24	53.3		
Insufficient data	1	1			
Tumor number				6.79	0.009*
Multiple	12	11	91.2		
Solitary	83	43	51.8		
Hepatitis B virus infection				1.54	0.464
Yes	74	40	54.1		
No	20	13	65.0		
Insufficient data	1	1			
Liver cirrhosis				2.92	0.232
Yes	85	50	58.8		
No	9	3	33.3		
Insufficient data	1	1			
Pathological grade				8.78	0.003*
Grade 1–2	46	19	41.3		
Grade 3	49	35	71.4		
Vascular invasion				21.1	0.001*
Present	24	22	91.7		
Absent	59	23	39.0		
Insufficient data	12	9			
TNM stage				22.9	0.001*
Stage I	60	23	38.3		
Stage II	32	28	87.5		
Stage III	3	3	100.0		

* $p<0.05$.

bladder cancer tissues is critically up-regulated [25]. SPAG5 expression increases with the progression of prostate cancer (PCa), and its expression level is very strongly correlated with clinical stage, Gleason score, lymph node metastasis, and biochemical recurrence [24]. High SPAG5 expression in breast cancer tissue is associated with histological grade, P53 mutations, and HER2 amplification [21,31]. Down-regulation of SPAG5 inhibits the proliferation of tumor cells and hinders tumor cell migration and invasion [19]. Fredlund et al. [20] confirmed

that abnormalities in mitotic checkpoints and mitotic progression are involved in breast cancer cell proliferation. SPAG5 is involved in the metastasis of metastatic breast cancer (MBC) and is a potential driver of MBC cell proliferation [32]. SPAG5 maps to chromosome 17q11.2 and encodes a protein named astrin, which is associated with the mitotic spindle machinery [33]. Moreover, SPAG5 promotes cell apoptosis through the mTOR pathway to increase the sensitivity of tumor cells to paclitaxel. Therefore, the increased sensitivity of breast cancer

Table 2. Univariate and multivariate analyses on the disease-free survival of 95 cases of hepatocellular carcinoma.

	Univariate analysis			Multivariate analysis		
	HR	p >|z|	95% CI	HR	p >|z|	95% CI
SPAG5 expression High versus low	4.37	0.001*	1.798–10.606	3.34	0.017*	1.236–9.036
Gender Male versus Female	3.58	0.209	0.489–26.199			
Age (years) ≥60 versus <60	1.28	0.533	0.593–2.746			
Tumour size (cm) >5 versus ≤5	2.24	0.022*	1.126–4.462	1.87	0.083	0.922–3.813
Tumor encapsulation None versus complete	1.76	0.118	0.866–3.586			
Tumor number Multiple versus solitary	2.07	0.108	0.852–5.027			
Hepatitis B virus infection Yes versus no	1.05	0.905	0.456–2.426			
Liver cirrhosis Yes versus no	1.69	0.474	0.403–7.050			
Pathological grade Grade 1 and 2 versus Grade 3	0.32	0.005*	0.152–0.710	0.50	0.093	0.221–1.122
Vascular invasion Present versus absent	2.04	0.061	0.966–4.327			
TNM stage Stage I versus Stage II versus Stage III	0.46	0.012*	0.250–0.845	0.88	0.737	0.431–1.813

*$p<0.05$.

patients with high SPAG5 expression to anthracyclines may be related to activation of the mTOR signaling pathway [34]. The ectopic overexpression of miR-539 significantly inhibits SPAG5 expression, while the rescue of SPAG5 expression can reverse the inhibitory effect of miR-539 on PCa cell proliferation and metastasis [24]. Zhong et al. [35] demonstrated that ORP8, via SPAG5, can mediate the interfering effect of oxysterol on HepG2 (a human liver cancer cell line) cell cycle by causing accumulation of G2/M phase cells. These data indicate the promoting role of SPAG5 in cancer development, which can thus be considered a new and useful biomarker for human malignancies. However, its significance with respect to the clinicopathological features of HCC patients, especially its role in HCC prognosis, has not yet been explored. The use of SPAG-5 as a potential therapeutic target of HCC requires further investigation.

In this study, we first tested the expression of SPAG5 mRNA, and the qRT-PCR data indicated that the expression of SPAG5 in fresh HCC tissue samples was far higher than that of non-cancerous tissue samples. Western blot and IHC assay showed consistent results in that the protein expression of SPAG5 was higher in HCC samples than in non-cancerous tissue. The results

of the qRT-PCR and IHC analyses in this study are in accordance with the findings of previous studies on human cancers with a high level of SPAG5 expression [19,21,23,24]. Moreover, high protein expression of SPAG5 was correlated with 4 important pathological parameters: tumor number, TNM stage, vascular invasion, and pathological grade. The above data are also in agreement with the data reported in previous studies [21,24], which supports the oncogenic role of SPAG5 in tumorigenesis.

In survival analysis, the assessment of survival in HCC patients was performed according to the univariate model. The data showed that SPAG5 expression, tumor size, pathological grade, and TNM stage were significantly correlated with DFS and OS. A multivariate analysis further showed that SPAG5 expression may be an independent prognostic factor that affects DFS or OS. Kaplan-Meier curves indicated that the prognosis of patients with high SPAG5 expression was significantly worse than that of patients with low SPAG5 expression. These results were also consistent with previous studies showing that high SPAG5 expression is as a predictor of poor survival in breast tumor patients [21].

Table 3. Univariate and multivariate analyses on the overall survival of 95 cases of hepatocellular carcinoma.

	Univariate analysis			Multivariate analysis		
	HR	p >|z|	95% CI	HR	p >|z|	95% CI
SPAG5 expression High versus low	4.29	0.001*	1.773–10.373	3.31	0.016*	1.245–8.748
Gender Male versus Female	1.97	0.351	0.473–8.246			
Age (years) ≥60 versus <60	1.15	0.712	0.537–2.484			
Tumour size (cm) >5 versus ≤5	2.13	0.029*	1.083–4.206	1.70	0.132	0.851–3.411
Tumor encapsulation None versus complete	1.96	0.063	0.964–3.990			
Tumor number Multiple versus solitary	1.91	0.129	0.828–4.395			
Hepatitis B virus infection Yes versus no	1.11	0.806	0.475–2.603			
Liver cirrhosis Yes versus no	1.73	0.452	0.414–7.235			
Pathological grade Grade 1 and 2 versus Grade 3	0.34	0.004*	0.159–0.713	0.48	0.067	0.217–1.054
Vascular invasion Present versus absent	2.01	0.062	0.967–4.178			
TNM stage Stage I versus Stage II versus Stage III	0.50	0.014*	0.283–0.869	0.92	0.805	0.477–1.775

*$p<0.05$.
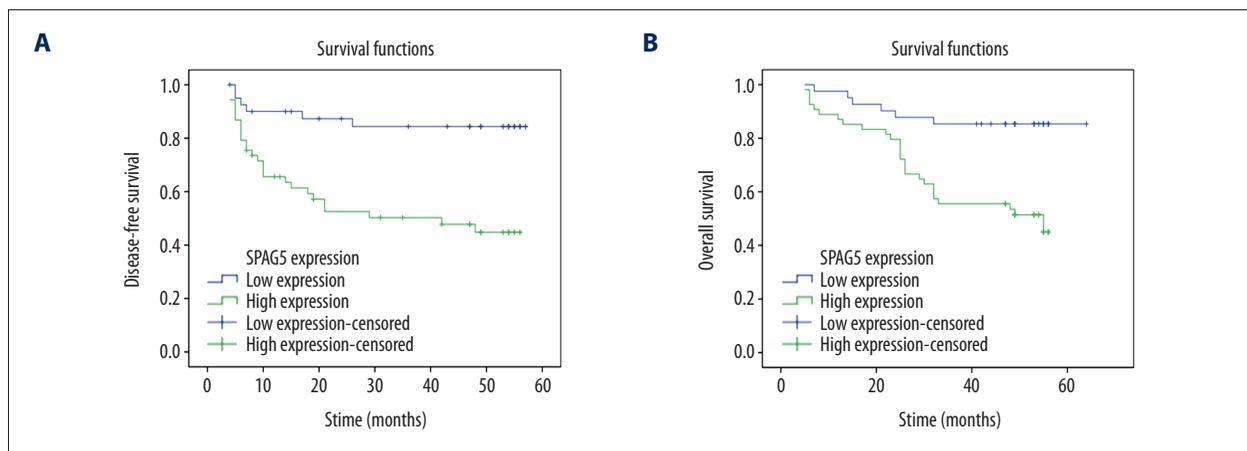
Figure 3. Disease-free and overall survival curves of hepatocellular carcinoma (HCC) after hepatectomy were assessed by Kaplan-Meier analysis according to protein SPAG5 expression. Patients with higher expression of SPAG5 were significantly associated with poorer disease-free (DFS) (**A**, P<0.001) and overall survival (OS) (**B**, P<0.001).

One limitation of this study was that we did not collect clinical data on HCV infection or the history of alcohol consumption of the HCC patients, and these factors are considered important in the etiology of HCC. In future studies, we will attempt to collect clinical samples and the corresponding data.

Conclusions

In conclusion, we report the differential SPAG5 expression at both the protein and mRNA levels in HCC. Moreover, for the first time, the association of SPAG5 expression and clinical

attributes of HCC patients, especially the prognostic function of SPAG5, was assessed. SPAG5 may be considered a new prognostic marker for HCC patients, and targeting SPAG5 could provide new treatment strategies for HCC.

Conflict of interest

None.

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