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Original Article

High expression of Sp1 is significantly correlated with tumor progression and poor prognosis in patients with hepatocellular carcinoma

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Abstract: This study aimed to investigate the relationship between specificity protein 1 (Sp1) expression and clinicopathological feature and prognosis of hepatocellular carcinoma (HCC). We detected Sp1 mRNA and protein expressions in 12 fresh HCC tissues by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis, respectively. Immunohistochemical assay was performed in 127 paraffin-embedded HCC tissues. The mRNA and protein expressions of Sp1 were significantly higher in HCC tissues than in matched adjacent normal tissues. Sp1 high expression was detected in the cytoplasm and nuclei of carcinoma cells from 68 (53.5%) patients. In addition, high expression of Sp1 was significantly correlated with HBsAg infection (P = 0.036), liver cirrhosis (P = 0.024), tumor differentiation (P = 0.010), vascular invasion (P = 0.035) and metastasis (P = 0.029). Furthermore, Kaplan-Meier analysis showed that patients with high Sp1 expression had significantly lower overall survival (OS) and disease free survival (DFS) rates, compared with patients with low Sp1 expression. Sp1 is an independent prognostic factor for HCC patients. In conclusion, Sp1 expression is significantly up-regulated in HCC and could identify a subset of HCC with aggressive clinical behavior and poor clinical outcome. Sp1 is a potential independent prognostic biomarker for HCC patients after surgery.

Keywords: Hepatocellular carcinoma (HCC), specificity protein 1 (Sp1), prognostic marker, invasion

Introduction

Hepatocellular carcinoma (HCC) is one common cancer and the third leading cause of cancer-related deaths worldwide and China [1]. HCC is highly prevalent in China and its mortality rate has been increasing since the 1990s [2]. Despite the significant improvements in diagnostic and therapeutic methods in recent years, the prognosis of HCC still remains unsatisfactory, which is largely caused by its high recurrence and invasion rates [3]. The molecular mechanisms underlying HCC have not been fully known. However, the initiation and development of HCC are associated with multiple risk factors, including chronic hepatitis virus infection, aflatoxin, alcohol abuse, and diabetes mellitus [4]. These risk factors can not only facilitate deeper understanding mechanisms of HCC, but also play important roles in high-risk population screening, clinical diagnosis, prognosis prediction, and treatment efficiency evaluation. Therefore, it is important to identify novel biomarkers for HCC and help clinicians assess the clinicopathological feathers of the malignancy and decrease the rate of unfavorable outcomes.

Specificity protein 1 (Sp1) protein is a well-characterized transcription factor of Sp/Kruppel-like factor (KLF) family that can bind to sequence-specific promoter elements of its target genes through C-terminal zinc fingers domains [5]. Sp1 plays important regulatory roles in multiple biological functions, including cell cycle regulation [6], cell growth [7], apoptosis [8], metabolism [9], angiogenesis [10] and metastasis [11]. Growing evidence showed that Sp1 is overexpressed in a variety of tumors, including lung cancer [12], glioma [13], pancre-

Table 1. Association of Sp1 expression with clinicopathological features in HCC

Clinical features	Cases	Sp1 expression		*P* value
		Low level (n = 59)	High level (n = 68)	
Age (Years old)				0.183
≤50	44	24	20	
>50	83	35	48	
Gender				0.816
Male	81	37	44	
Female	46	22	24	
HBsAg				0.036
Negative	50	29	21	
Positive	77	30	47	
AFP (μg/L)				0.955
≤400	52	24	28	
>400	75	35	40	
Liver cirrhosis				0.024
No	41	25	16	
Yes	86	34	52	
Tumor size (cm)				0.230
≤5	51	27	24	
>5	76	32	44	
Tumor number				0.853
Solitary	57	27	30	
Multiple	70	32	38	
Tumor differentiation				0.010
Well + Moderate	64	37	27	
Poor	63	22	41	
Vascular invasion				0.035
No	52	30	22	
Yes	75	29	46	
Metastasis				0.029
Negative	60	34	26	
Positive	67	25	42	

HCC, hepatocellular carcinoma; AFP, AFP, α-fetal protein; HR hazard ratio; CI confidence interval; Sp1, Specificity protein 1.

atic adenocarcinoma [14], thyroid tumor [15], nasopharyngeal cancer [16] and gastric cancer [17]. However, Sp1 overexpression can lead to apoptosis in multiple cancer cells [18, 19]. The apparently opposing effects of Sp1 might be associated with its regulated multiple genes involved in essential cellular functions, including proliferation, apoptosis, differentiation, the DNA damage response, inflammation, senescence and angiogenesis [20]. Therefore, it is urgent to indentify the clinicopathological feathers and prognostic values of Sp1 in HCC.

In this study, we investigated the expression levels of Sp1 in HCC and their paired adjacent nontumor tissues, and further investigated whether the Sp1 expression levels correlate with HCC clinical parameters and prognosis.

Patients and methods

Patients and tissue samples

This study consists of 127 primary HCC tumor tissues collected from patients who underwent hepatectomy at the Affiliated Cancer Hospital of Guangxi Medical University between July 2005 and December 2010. All HCC was diagnosed by pathology based on WHO criteria, and none of these patients received chemotherapy or radiotherapy before surgery. Their complete clinicopathological data were recorded and summarized in **Table 1**. Tumor differentiation was based on the Edmondson and Steiner classification. These patients were followed up after surgery every three months, and the follow-up deadline was October 2015. Overall survival (OS) was defined as the interval between surgery and death or the last follow-up (Censored data for living patients). Disease free survival (DFS) was defined as the interval between surgery and the date of relapse. This study was approved by the Medical Ethics Committee of the Affiliated Hospital of Guangxi Medical University. Written informed consent was signed and obtained from all patients.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

A total of 12 fresh tumorous and adjacent nontumorous tissue samples were acquired and immediately frozen at -80°C after resection. Total RNA was extracted from clinical tissues using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of the total RNA using a reverse transcriptase (RT)-for-PCR Kit (Clontech Laboratories). The mRNA levels of Sp 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined using aSYBR

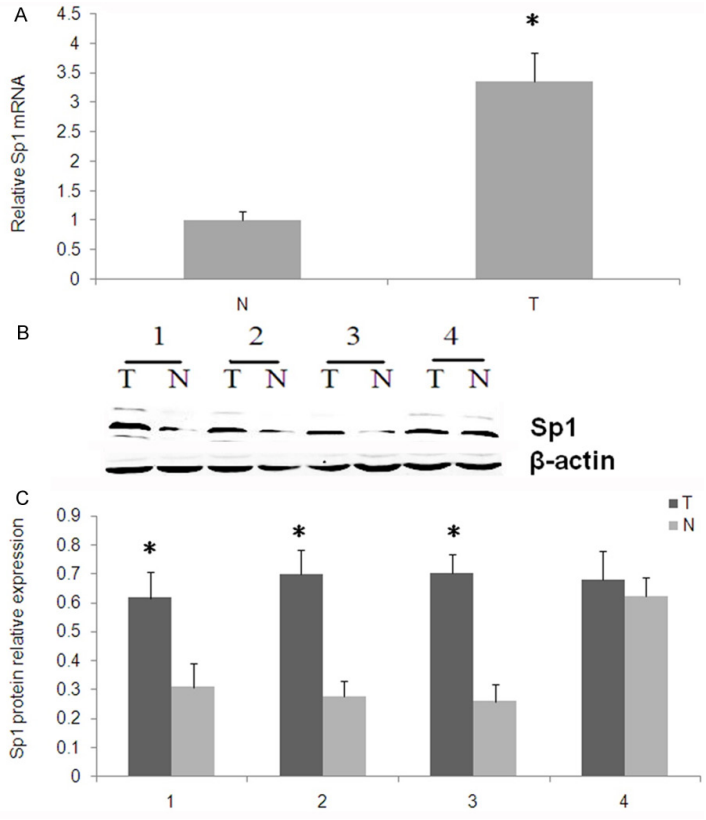

Figure 1. Sp1 was up-regulated in HCC tissues. A. Sp1 mRNA was markedly increased in tumor tissues than that in the paired adjacent nontumor tissues by qRT-PCR. B. Sp1 protein was markedly increased in tumor tissues than that in the paired adjacent nontumor tissues by western blot. β-actin protein served as an internal control. Representative pictures from three independent experiments are shown. C. Increased relative expression of Sp1 protein in HCC normalized to β-actin. Data were expressed as mean ± SD and a two-tailed, paired t-test was performed. Significant difference from the control group is denoted by ''*'' ($P<0.05$). N, normal adjacent nontumor tissues; T, HCC tumor tissue.

Green PCR Kit (Applied Biosystems) and LightCycler480 384-well PCR system (Roche Diagnostics). The GAPDH was used as an internal control for ITPKA. Primers for Sp1 were 5'-TCCAGACCATTAACCTCAGTGC-3' (forward) and 5'-TGTATTCCATCACCACCAGCC-3' (reverse). Primers for GAPDH were 5'-CTCCTCCTGTTCGACAGTCAGC-3' (forward) and 5'-CCCAATACGACCAAATCCGTT-3' (reverse). The value of relative expression for each sample was determined using the Ct method, and was normalized to the average expression value of 12 adjacent nontumorous tissues.

Western blot analysis

Protein expression of Sp1 was determined by western blot analysis in fresh tumorous and adjacent nontumorous tissues. Protein was extracted using cell lysis buffer and their concentrations were determined by BCA method (Pierce, Rockford, IL, USA). The protein (50μg) was electrophoretically separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Then PVDF membrane was blocked with 3% BSA and incubated with primary mouse anti-human monoclonal antibody Sp1 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37°C overnight. After washing, the PVDF membrane was incubated with horseradish peroxidase (HRP)-conjugated second rabbit anti-mouse monoclonal antibody (1:1000 dilutions) to for 2 h at room temperature. The signal was detected by enhanced chemiluminescence (Pierce® ECL Plus Western Blotting Substrate, Pierce Biotechnology, IL, USA). β-actin was used as an internal control. To confirm equal loading of the samples, the same membrane was stripped and incubated with mouse monoclonal antibodies against β-actin.

Immunohistochemical (IHC) assay

Immunohistochemical staining was performed as previously described with slight modifications [16]. Briefly, paraffin-embedded samples were cut into 5 μm sections and placed on polylysine-coated slides. Paraffin sections were baked for 2 h at 58°C, dewaxed in xylene and rehydated with ascending graded ethanol. Endogenous peroxidase activity was quenched by incubating sections in 0.3% hydrogen peroxide for 10 min. Antigen retrieval was performed by high pressure cooking (700 W microwave oven) sections in 10 mM citrate antigen retrieval solution (0.1 mM citric acid, 0.1 M sodium citrate; pH = 6.0) for about 10 min. Sections were washed with PBSTX (0.05 M PBS, 0.1% Triton-X 100) and incubated with primary antibody against human Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A) at 4°C overnight, and then incubated with HRP-conjugated IgG secondary antibo-

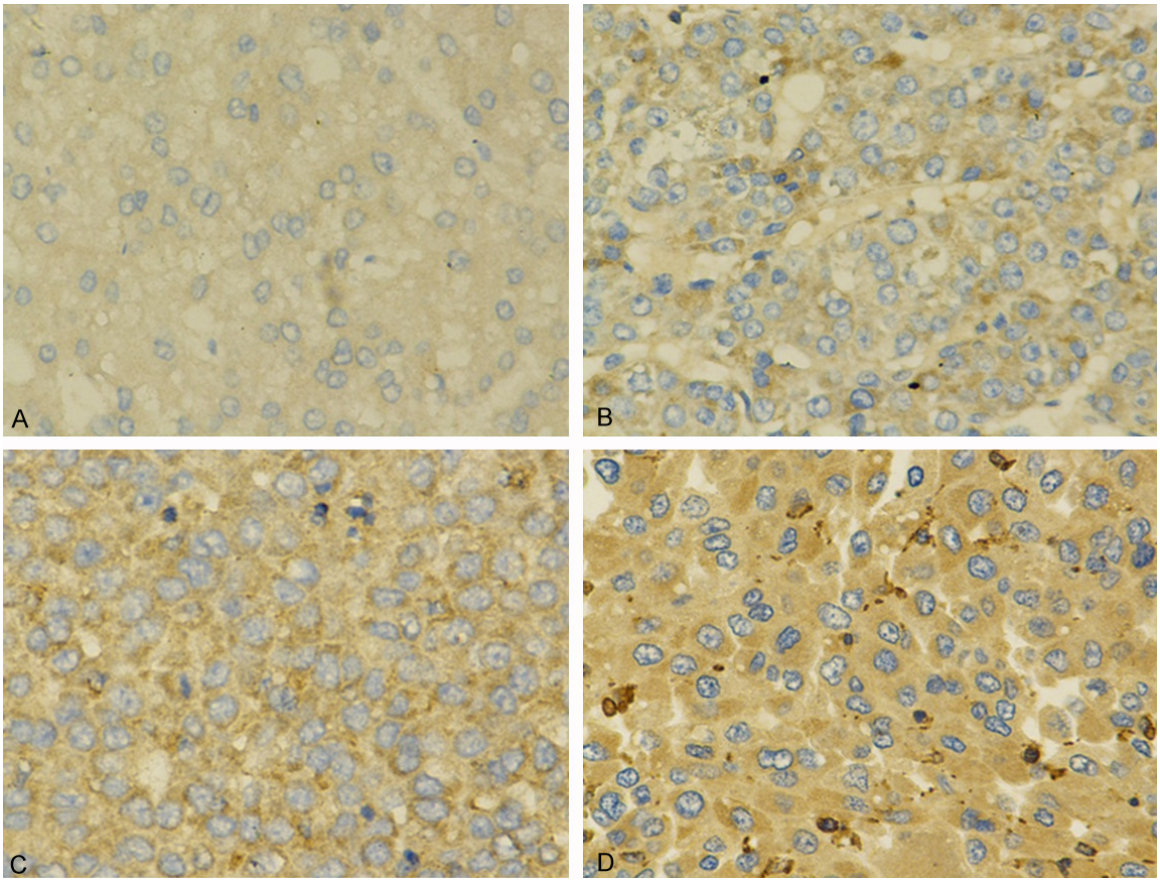

Figure 2. Typical immunohistological features of Sp1 protein expression in hepatocellular carcinoma (HCC). A. Low expression of Sp1 in normal adjacent nontumor tissues. B. Low expression of Sp1 in HCC. C. Moderate expression of Sp1 in HCC. D. High expression of Sp1 in HCC. Magnifications: × 400.

dy (Concentrations 1:100) (Medical Biological Laboratory, Nagoya, Japan) at 37°C for 30 min. Then sections were incubated with complex/horseradish peroxidase (1: 200 dilution) at 37°C for 30 min, and visualized by immersion in diaminobenzidine solution (10×DAB, TBS, 30% H_2O_2). The brown-colored precipitate indicates the cellular site of Sp1 protein. Subsequently, sections were counterstained with hematoxylin (Zymed Laboratories, South San Francisco, CA), followed by dehydration and mounting in a non-aqueous medium. Background staining control was performed by incubating slides with PBS solution other than primary antibody. The immunohistochemically stained tissue sections were read and scored blinded by two pathologists. Positive staining was defined as strong brown granules in nucleus and cytoplasm. Negative staining was defined as no staining or weak diffuse background granules. All Sp1-positive cells showed similar average staining intensity. Therefore, the expression of Sp1 was evaluated according to the percentage of staining from >1000 carcinoma cells for each case. For statistical analyses, the cases with percentage of staining of >20% were considered as high Sp1 expression [14].

Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD). All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 19.0 (SPSS Inc, Chicago, IL). The mRNA and protein expressions of Sp1 were compared between primary HCC tumors and adjacent nontumorous tissues using unpaired and paired two-tailed student's t test, respectively. The association between Sp1 expression and clinicopathological parameters was analyzed by chi-square test or Fisher's exact test. The survival curve OS and DFS were calculated by the Kaplan-Meier analysis with log-rank test. Univariate and multivariate survival analysis was performed using the Cox proportional hazard model with a forward stepwise procedure (entry

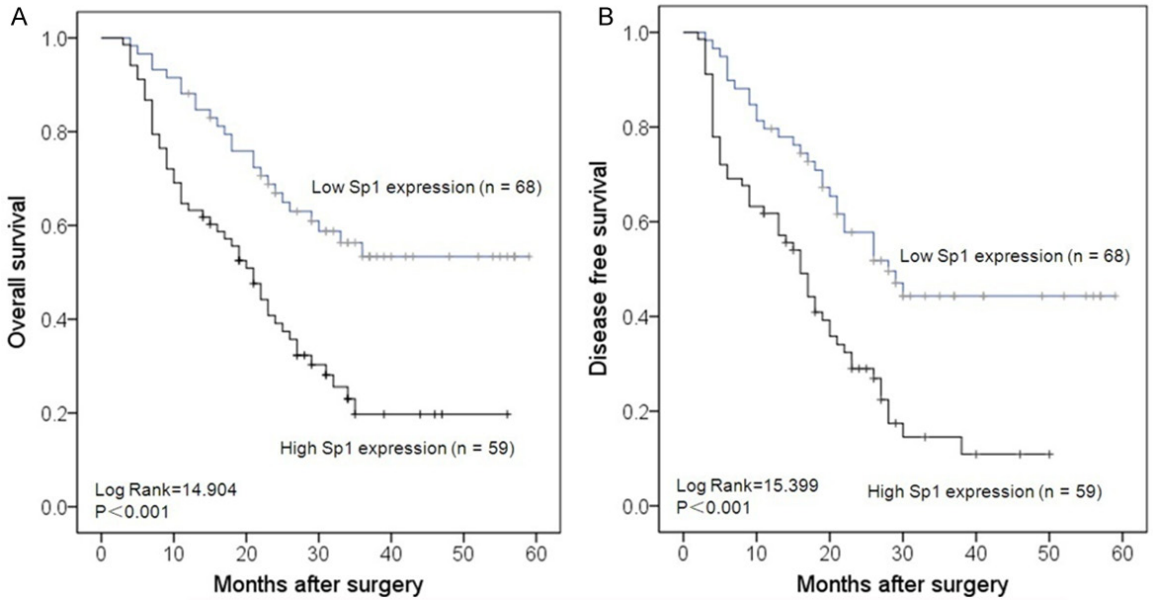

Figure 3. Kaplan-Meier analysis of survival for Sp1 expression in 127 cases of HCC. *P* value was obtained using the log-rank test of the difference. A. Overall survival (OS) differences between patients with high and low levels of Sp1 protein expression (P<0.001, log-rank test). B. Disease free survival (DFS) differences between patients with high and low levels of Sp1 protein expression (P<0.001, log-rank test).

Table 2. Univariate Cox regression analyses for overall survival and disease free survival in HCC

Clinical feathers	Overall survival		Disease free survival	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age	0.909 (0.548-1.508)	0.712	0.782 (0.483-1.265)	0.782
Gender	1.118 (0.668-1.872)	0.671	1.068 (0.661-1.727)	0.788
HBsAg	1.157 (0.633-2.114)	0.635	1.120 (0.635-1.977)	0.695
AFP	1.083 (0.656-1.790)	0.754	1.302 (0.810-2.093)	0.275
Liver cirrhosis	0.970 (0.508-1.851)	0.926	0.984 (0.539-1.794)	0.957
Tumor size	1.860 (1.041-3.324)	0.036	1.488 (0.871-2.542)	0.145
Tumor number	1.008 (0.612-1.659)	0.976	1.090 (0.680-1.747)	0.720
Tumor differentiation	1.752 (1.020-3.009)	0.042	1.548 (0.937-2.555)	0.088
Vascular invasion	1.821 (1.050-3.157)	0.033	1.910 (1.141-3.198)	0.014
Metastasis	1.926 (1.144-3.244)	0.014	1.964 (1.201-3.211)	0.007
Sp1 expression	1.705 (1.011-2.876)	0.046	1.697 (1.041-2.766)	0.034

HCC, hepatocellular carcinoma; AFP, α-fetal protein; HR, hazard ratio; CI, confidence interval; Sp1, Specificity protein 1.

probabilities: 0.05; removal probabilities: 0.10). *P* value of <0.05 was considered statistically significant difference.

Results

Sp1 expression was up-regulated in HCC

To explore the expression of Sp1 in HCC, the mRNA and protein levels of Sp1 were examined in the primary hepatic epithelial tissues and tumor tissues. qRT-PCR showed the mRNA level of Sp1 was significantly higher in fresh HCC tissues than in adjacent nontumorous tissues (P<0.05) (**Figure 1A**). We further performed western blot in 4 paired normal (N) and tumor (T) tissues from HCC patients. Compared with non-tumor tissues, up-regulation of Sp1 protein was detected in tumor tissues of all 4 HCC patients (P<0.05) (**Figure 1B**, **1C**).

Correlation between Sp1 expression wand clinicopathologic features in HCC patients

We performed immunohistochemical analysis to measure Sp1 expression in 127 retrospec-

Table 3. Multivariate Cox regression analyses for overall survival and disease free survival in HCC

Clinical feathers	Overall survival		Disease free survival	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Tumor size	1.919 (1.101-3.347)	0.022		
Tumor differentiation	1.797 (1.061-3.043)	0.029	1.826 (1.151-2.897)	0.011
Vascular invasion	1.881 (1.095-3.230)	0.022	1.911 (1.156-3.157)	0.011
Metastasis	1.942 (1.177-3.202)	0.009	2.077 (1.312-3.287)	0.002
Sp1 expression	1.732 (1.053-2.851)	0.031	1.724 (1.085-2.740)	0.021

HCC, hepatocellular carcinoma; HR, hazard ratio; CI, confidence interval; Sp1, Specificity protein 1.

tive HCC cases. Sp1 was found nuclear and cytoplasmic localization, but primarily in the nucleus, in most HCC tissues. On the contrary, most adjacent non-tumor tissues showed little Sp1 staining (**Figure 2**). Among all 127 HCC patients, 68 (53.5%) cases showed Sp1 high expression with at least 20% positive cells, and the remaining 59 cases showed Sp1 low expression. The correlation between Sp1 staining and clinicopathological features of primary HCC was summarized in **Table 1**. High expression of Sp1 was significantly positively correlated with HBsAg infection (P = 0.036), liver cirrhosis (P = 0.024), tumor differentiation (P = 0.010), vascular invasion (P = 0.035) and metastasis (P = 0.029). However, no correlation was observed between Sp1 expression and age, gender, α-fetal protein (AFP) levels, tumor size or tumor number.

Correlation between Sp1 expression and survival of HCC patients

Kaplan-Meier analysis was used to investigate the impact of Sp1 expression on survival of HCC patients. The 5-year OS and DFS rates were significantly lower in high Sp1 expression group (OS: 27.9%; DFS: 22.1%) than those in the low Sp1 expression group (OS: 57.6%; DFS: 49.2%) (**Figure 3**). Univariate analysis showed that tumor size, tumor differentiation, vascular invasion, metastasis, and Sp1 expression were prognostic factors for OS and DFS (**Table 2**). Multvariate analysis demonstrated that tumor size, tumor differentiation, vascular invasion, metastasis, and Sp1 expression were also independent prognostic predictors for OS and DFS (**Table 3**).

Discussion

Sp1 has been extensively investigated and found to be overexpressed in multiple cancers [12-17]. However, the expression and significance of Sp1 have rarely been explored in HCC. In this study we used qRT-PCR and western blot and found higher expression of Sp1 mRNA and protein in HCC tissues than in adjacent nontumorous tissues. Moreover, immunohistochemical assay showed positive staining in the cytoplasm and nuclei of HCC cells, which represents aberrant expression of Sp1 protein. We adopted 20% positive cells as a cutoff value and divided all 127 cases into high expression group (n = 68) and the low expression group (n = 59). High expression of Sp1 was significantly correlated with HBsAg infection, liver cirrhosis, tumor differentiation, vascular invasion and metastasis. Kaplan-Meier analysis showed that the OS and DFS rates were significantly lower in high Sp1 expression group (OS: 27.9%; DFS: 19.7%) than those in the low Sp1 expression group. Furthermore, Cox proportional hazard model was used and found Sp1 expression was an independent prognostic predictor for OS and DFS of HCC. This indicates Sp1 may be one oncoprotein in the development and progression of HCC and could act as a prediction marker for prognosis of HCC patients.

Sp1 play key roles in multiple biological functions and was supposed to act as both oncogene and tumor suppressor gene. In our study, Sp 1 was overexpressed in HCC tissues compared with adjacent nontumorous tissues, which indicates it act as one oncoprotein. Moreover, high Sp1 expression was positively correlated with HBsAg infection and liver cirrhosis. Human hepatitis B virus (HBV) is one causative factor in the formation of HCC. A functional Sp1 binding site was found in the promoter of HBV large surface antigen [21], which suggests Sp1 may be involved in the coordinate regulation of expression of HBV genes [22]. On the contrary, HBV-X antigen could phosphorylate

Sp1 and enhance its DNA-binding activity, and upregulate expression of the IGF-II and Lin28A/Lin28B, thereby promoting cell proliferation during hepatic carcinogenesis [23, 24]. This indicates there may be positive reciprocal regulation between Sp1 and HBV antigens expression. Liver fibrosis is one important mechanism underlying cirrhosis and is another causative factor in the formation of HCC, with characterized excessive accumulation of extracellular matrix (ECM). Sp1 could participate in the HBV-associated fibrogenesis initiated by transforming growth factor-β1 (TGF-β1) by binding to the CC (GG)-rich TGF-β1 responsive element of the collagen I gene promoter [25]. Sp1 could also upregulate α-SMA, collagen I and TGF-β1 synthesis in the TGF-β1-CD147 positive feedback loop during liver fibrosis [26]. Moreover, silencing Sp1 showed anti-fibrotic effects in liver cirrhosis through blocking Sp1 binding to the promoter regions of fibrotic genes, such as TGF-β1, Platelet-derived growth factor (PDGF)-BB, α-SMA, collagen I and tissue inhibitor of metalloproteinases-1 (TIMP-1) [27, 28]. Therefore, Sp1 plays cancer-promoting role in multiple steps of HCC formation, which may be mediated by transcriptional activation of its downstream genes associated with HBV infection and liver cirrhosis.

We also provide evidence that high expression of Sp1 is associated with the presence of vascular invasion and metastasis. Our results are in accordance with other reports that high Sp1 expression is associated with tumor invasion and metastasis in lung cancer [12], glioma [13], pancreatic adenocarcinoma [14], nasopharyngeal cancer [16] and gastric cancer [17]. These clinical effects on tumor invasion and metastasis by Sp1 were further confirmed by reports that Sp1 overexpression can induce invasion in cultured cancer cells [29, 30]. Furthermore, Sp1 also promote tumor invasion and metastasis by forming Fyn-SP1-SATB1 axis or Sp1-CD147 positive feedback loop [31, 32]. On the contrary, downregulating Sp1 can inhibit tumor invasion and metastasis in HCC [33], as well as gastric cancer [34], prostate cancer [35] or Non-small cell lung cancer (NSCLC) [36]. Therefore, Sp1 is a promising therapeutic target for a variety of cancers, including HCC [37].

We also have an interesting finding that Sp1 overexpression is positively correlated with well and moderate tumor differentiation of HCC. However, other report showed that Sp1 overexpression is positively correlated with poor differentiation of pancreatic ductal adenocarcinoma [14]. This discrepancy may be caused by the fact that Sp1 demonstrates multiple biological functions and acts as oncogene or tumor suppressor gene in various types of cancer [20]. Reports have shown that Sp1 is a differentiation inducer in various cell types. Sp1 overexpression could induce differentiation of squamous cell carcinoma cell line [38]. Sp1 also participated in terbinafine-induced human epithelioid squamous carcinoma (A431) cells differentiation [39], 4-hexylresorcinol (4-HR)-induced tongue squamous cell carcinoma (SCC-9) cells differentiation [40], and all-trans retinoic acid (RA)-induced human neuroblastoma (SH-SY5Y) cells neuronal differentiation [41]. Further investigation is needed on the effect of HCC cells differentiation by Sp1.

Our study demonstrated that high expression of Sp1 is an independent prognosis factor for HCC patients in the multivariate Cox proportional hazards regression analysis. The OS and DFS of patients with high Sp1 expression were markedly shorter than that with low Sp1 expression. HCC is a heterogeneous disease and patients may have quite different prognosis despite with the same TNM stage, histopathologic features and treatment strategy [42]. Our study shows that Sp1 may act a prediction marker for HCC prognosis and patients with Sp1 overexpression can be identified at initial diagnosis of HCC. Therefore, Sp1 can help clinicians evaluate the clinicopathological feathers and prognosis in these high-risk populations, and choose more efficient therapeutic strategy and decrease the rate of unfavorable outcomes.

In conclusion, this is the first study to systemically evaluate the correlations of Sp1 expression with clinicopathological feathers and prognosis of HCC. Our results demonstrated that Sp1 is up-regulated in HCC, and expression of Sp1 is correlated with aggressive tumor behaviors and poor clinical outcome. Our study identified Sp1 as an independent prognostic marker for HCC patients.

Disclosure of conflict of interest

None.

Authors' contribution

Wei Sun analyzed the data and wrote the manuscript; Ren-Fei Shan designed the study and revised the manuscript; Yan-An Zhu and Jie Qin performed all the experiments and collected all the data; Jian-Ping Chen analyzed the data.

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