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ORIGINAL ARTICLE

Increased Serum Levels of Trypsin Inhibitor Kazal1 in Patients with HBV-Related Hepatocellular Carcinoma Predict a Poor Prognosis

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SUMMARY

Background: **Trypsin Inhibitor Kazal1 (SPINK1) is overexpressed in various tumors, but its role in hepatitis B virus (HBV) related hepatocellular carcinoma (HCC) is unclear. The aim of this study was to investigate SPINK1 levels during the chronic progression of HBV infection and their association with the prognosis of HBV-related HCC.**

Methods: **This study enrolled 102 patients with chronic hepatitis B (CHB), 95 patients with HBV-related liver cirrhosis (LC), 104 patients with HBV-related HCC, 25 patients with intrahepatic cholangiocarcinoma (ICC), and 98 healthy controls (HCs). The serum expression of SPINK1 in each group was compared. SPINK1 levels in the supernatant of HepG2.2.15, HepG2, Huh7, and LO2 cells were determined by ELISA. The diagnostic efficacy of SPINK1 for HBV-related HCC was evaluated. Hazard ratios (HRs) for the short-term prognosis of HBV-related HCC were assessed.**

Results: **SPINK1 levels were the highest in the HBV-related HCC group compared with the HC, CHB, HBV-related LC, and ICC groups (3.19 ± 1.11 versus 1.09 ± 0.38, 1.75 ± 0.55, 2.09 ± 0.62, and 2.40 ± 0.85 ng/mL, $p < 0.01$). SPINK1 levels in the supernatant of HepG2.2.15 cells were higher than those in HepG2, Huh7, and LO2 cells (2.85 ± 0.03 versus 1.54 ± 0.04, 1.50 ± 0.04, 0.9 ± 0.04 ng/mL, $p < 0.001$). The best cutoff point for the SPINK1 level was 2.48 ng/mL. The high SPINK1 expression group (≥ 2.48 ng/mL) had a larger tumor size, poorer Child-Pugh classification and more HBV DNA than the low expression group (< 2.48 ng/mL) (all $p < 0.05$). In the HBV-related HCC group, a SPINK1 level ≥ 2.48 ng/mL along with a high alpha-fetoprotein (AFP) level, large tumor size and poor Child-Pugh grade predicted poorer overall survival (HR 4.65, 95% confidence interval (CI): 2.07 - 10.43, $p < 0.001$).**

Conclusions: **Serum SPINK1 had a high diagnostic efficacy for predicting HBV-related HCC. The presence of HBV-related HCC with a high serum SPINK1 level (≥ 2.48 ng/mL) may be associated with a poor short-term prognosis.**

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death worldwide and the second leading cause in China due to the high frequency of

heaptitis B virus (HBV) infection [1]. The prognosis of HCC has generally been thought to be bleak due to its insidious and rapid development [2]. Beasley et al. [3] first reported that HBV was one of the pathogenic factors of HCC. To date, a close association between HBV and HCC has been noted. Subsequent studies [4,5] have indicated that nearly 80% to 90% of HCC cases are connected to hepatitis viruses. Approximately 25% of patients with chronic hepatitis B (CHB) develop HCC throughout their lifetime [5]. Current consensus suggests that HBV is one of the most critical risk factors for HCC.

Detection of serum alpha-fetoprotein (AFP) levels and ultrasound scans have been extensively adopted as the mainstays of HCC screening. Nonetheless, the sensitivity of AFP detection alone was merely 60% at a cutoff point of 20 ng/mL, while ultrasonography had a sensitivity of 65 - 80% when applied as a screening test [6]. In addition, approximately 20% of patients with HCC produce little AFP [6]. Thus, both the American Society for Hepatology Studies and the European Society for Hepatology Studies have no longer suggested AFP as a screening indicator for cirrhosis-related HCC [7]. Early diagnosis and stratification are crucial for HBV-related HCC because this disease has a poor prognosis because of its low resectability. Thus, more markers in addition to AFP are urgently needed for early diagnosis and prognostic monitoring of HBV-related HCC.

Trypsin Inhibitor Kazal1 (SPINK1), also called tumor-associated trypsin inhibitor (TATI), is a gene consisting of four exons located on chromosome 5. The processed SPINK1 polypeptide has a molecular weight of 6,242 Da and consists of 56 amino acids containing 3 disulfide bonds [8]. Huhtala et al. [9] reported in 1983 that SPINK1 could be extracted from the urine of patients with ovarian cancer, suggesting that SPINK1 may be a potential tumor marker. Subsequent studies have found that SPINK1 is abnormally expressed in the serum, urine or tissues in patients with multiple tumors. SPINK1 can play the role of growth factor and inhibitor of apoptosis, thus promoting tumor progression and metastasis [10]. The amino acid sequences of SPINK1 are similar to those of epidermal growth factor, indicating that SPINK1 may play a physiological role in promoting cell growth [11,12]. Moreover, previous studies have identified that SPINK1 is a tumor suppressor gene, the inactivation of which is the basis for the tumor cell growth, proliferation, and metastasis [10,13].

Since liver repair and carcinogenesis are based on cell proliferation, overexpression of SPINK1 may lead to liver regeneration or tumor progression [14,15]. Recently, Mehner C et al. [16] reported that SPINK1 was likely a latent target for the treatment of ovarian tumors. SPINK1 can stimulate the growth and proliferation of ovarian cancer, a process that may promote resistance to anoikis by activating epidermal growth factor receptors and inhibiting the unique mechanism of protease apoptosis. Functional SPINK1 has been shown to have a pleiotropic effect in cancer, while increased expression of SPINK1 is usually correlated with a poor prognosis in most cancers [14-16]. Until now, there have been few reports on the role of serum SPINK1 in the progression of chronic HBV infection and its relationship to prognosis in HBV-related HCC.

Therefore, this study aimed to explore the relationship between serum SPINK1 and HBV infection in both HCC patients and human HCC cells and to evaluate the value of serum SPINK1 in the diagnosis and prediction of the short-term prognosis of HBV-related HCC.

MATERIALS AND METHODS

Subjects

From January 2016 to May 2019, a total of 326 patients from the Second Hospital of Anhui Medical University were recruited, including 102 patients with CHB, 95 patients with HBV-related liver cirrhosis (LC), 25 patients with intrahepatic cholangiocarcinoma (ICC), and 104 patients with HBV-related HCC. CHB and LC were diagnosed according to the enactments drafted by the Chinese Medical Association [17]. Summarily, the diagnosis of CHB was based on the presence of HBsAg marker sustained for not less than half a year before admission, and cases of cirrhosis or HCC were excluded. HBV-related LC was diagnosed according to the patient's medical history combined with physical examination, biochemistry, imaging radiology, endoscopy, ultrasound, and radiological evidence of cirrhosis. The diagnoses of HCC and ICC were in accordance with the criteria for primary liver cancer formulated by the Ministry of Health of the People's Republic of China (2012) [18]. The diagnosis of HBV-related HCC required HBsAg positivity, emerging characteristic imaging findings, and an AFP level over 400 ng/mL for 1 month or over 200 ng/mL for 2 months or histopathological evidence. Based on these criteria, patients were included in the HBV-HCC group if they had risk factors (HBV or cirrhosis) and at least one of the following: (i) tumor size $\leq$ 2 cm, positive imaging findings on not less than two of the four imaging studies (ultrasonography, enhanced CT or MRI, liver angiography), or satisfactory histopathological evidence; (ii) tumor size > 2 cm with at least one positive imaging finding. Ninety-eight healthy volunteers with no history of smoking, drinking, liver or angiocardiopathy, without any other acute or chronic illness were enrolled.

The Ethics Committee of the Second Affiliated Hospital of Anhui Medical University supported the study, and all participants signed an informed consent form.

Cell culture

HepG2.2.15, HepG2, Huh7, and LO2 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology. HepG2.2.15 cell was integrated with whole-HBV genome integration. HepG2, Huh7, and LO2 cells do not have HBV genome. They were preserved separately in Dulbecco's Modified Eagle's Medium and re-

plenished with 2 mmol/L L-glutamine and mixed well. Penicillin and streptomycin were added for a final concentration 50 IU/mL. An additional 500 µg/mL G418 and 5% volume foetal bovine serum was required. The cells were preserved at 37°C in a humidified incubator of 5% CO_2 and then cultivated for 3 days prior to being examined. Thereafter, the media were collected for further analysis.

Detection of serological SPINK1

Fasting peripheral venous blood were collected from subjects. High-quality serum was prepared by centrifugation (3,500 r/minute, 10 minutes) at room temperature one hour after collection. SPINK1 was detected by an ELISA kit (DY 7496/DY 008) purchased from R&D Systems (USA), and an enzyme labelling instrument (KHB ST-360) was purchased from Shanghai Kehua Experimental System.

Statistical analysis

All data were analyzed with SPSS version 23.0 (IBM Corp., Armonk, NY, USA) software. Continuous data are expressed as the average ± standard deviation. The *t*-test, one-way analysis of variance, and Mann-Whitney U test were used for statistical analysis of continuous data. Categorical data are reported as percentages and were compared with χ^2 tests. Spearman's rank correlation analysis was used to study the correlation between two variables. The highest cutoff point of serum SPINK1 was determined by receiver operating characteristic (ROC) curve analysis. Survival curves were plotted by Kaplan-Meier analysis and compared with the log-rank test. A Cox regression model was used for the univariate analysis. The multivariate analysis was performed with a Cox proportional hazards model. $p < 0.05$ was considered statistically significant.

RESULTS

General subject characteristics

The demographic baseline characteristics of all patients and controls are listed in Table 1. The average age of the patients with CHB, HBV-related LC, ICC, HBV-related HCC, and HCs were 39 ± 14, 56 ± 14, 65 ± 13, 57 ± 12, and 44 ± 5 years, respectively. Significant differences in expression of serum SPINK1 and aspartate transaminase (AST) were observed among the five groups (all $p < 0.05$). Nevertheless, there were no significant differences between the five groups in serum total bilirubin (TB), alanine aminotransferase (ALT), HBsAg, and HBV DNA expression.

Clearly, differences in serum SPINK1 levels among the five groups were statistically significant ($p < 0.001$) (Figure 1). Notably, the serum SPINK1 level in the HBV-related HCC group was obviously higher than those in the other four groups (all $p < 0.05$).

In summary, we assessed the relationship between serum SPINK1 and the progression of chronic HBV infection and found that the expression of SPINK1 increased steadily during the progression of disease. We then investigated whether SPINK1 was related to HBV viral load through the level of SPINK1 in HepG2.2.15 cells.

SPINK1 levels in the supernatant of human HCC cells

The concentrations of SPINK1 in the supernatant of HepG2.2.15, HepG2, Huh7, and LO2 cells were determined by antigen-specific ELISAs. Strikingly, the SPINK1 level was higher in the HepG2.2.15 cell line (2.85 ± 0.03 ng/mL) than in the other three cell lines. There was no remarkable difference between HepG2 cells (1.54 ± 0.04 ng/mL) and Huh7 cells (1.50 ± 0.04 ng/mL), but their SPINK1 levels were also increased compared with the level in LO2 cells (0.9 ± 0.04 ng/mL) ($p < 0.001$) (Figure 2). Thus, it seems that HBV may affect the expression of SPINK1, but more research on the exact mechanisms involved is needed.

Subgroup analysis of the association between serum SPINK1 and serological parameters in the HBV-related HCC group

Next, we aimed to validate whether the expression of SPINK1 was associated with related serum laboratory parameters in patients with HBV-related HCC. ELISAs revealed that the level of SPINK1 was negatively correlated with the level of albumin (ALB) in the serum ($r = -0.308$, $p = 0.001$) (Figure 3A). Furthermore, the level of SPINK1 and AST were positively correlated ($r = 0.255$, $p = 0.009$); similar trends were observed in the relationships between the SPINK1 level and TB ($r = 0.311$, $p = 0.001$), direct bilirubin (DB) ($r = 0.333$, $p < 0.001$), and log AFP ($r = 0.331$, $p < 0.001$) levels in the serum (Figure 3B - E). Likewise, serum SPINK1 levels and HBV DNA load were also positively correlated ($r = 0.211$, $p = 0.031$) (Figure 3F). Nevertheless, the correlation between serum SPINK1 levels and HBsAg levels was not statistically significant ($r = 0.008$, $p = 0.95$). Overall, serum SPINK1 was associated with clinical indicators of liver function, viral load, and AFP. Of these, the serum SPINK1 level was positively correlated with serum AST, TB, DB, log AFP, and HBV DNA level. In contrast, serum SPINK1 was negatively correlated with serum ALB. Whether the serum SPINK1 level is correlated with other parameters in the HBV-related HCC subgroup has not yet been clarified. This issue will be clarified in the next section.

Association between serum SPINK1 and clinical features in the HBV-related HCC group

In this respect, we sought to assess the diagnostic value of peripheral blood SPINK1 in different liver cancers. Subsequently, we obtained an optimal cutoff value for serum SPINK1 in HBV-related HCC cases to further explore the risk factors for the prognosis in this group. Strikingly, ROC curve analysis indicated that the detection power of serum SPINK1 was much more sensitive

Table 1. Baseline characteristics of patients from the entire study population.

Variable	HC (n = 98)	CHB (n = 102)	HBV-related LC (n = 95)	ICC (n = 25)	HBV-related HCC (n = 104)
Age (years)	44 ± 5	39 ± 14	56 ± 14	65 ± 13 [a, b, c]	57 ± 12 [a, b]
Gender (male/female)	57/41	31/71	27/68	4/41	9/95
AIB	44.07 ± 4.58	42.23 ± 4.43 [a]	33.46 ± 10.04 [a, b]	34.37 ± 7.43 [a, b]	35.6 ± 6.7 [a, b]
ALT (U/L)	19.11 ± 7.73	70.39 ± 30.51 [a]	42.80 ± 18.24 [a, b]	54.42 ± 38.77 [a]	67.8 ± 112.2 [a, b]
AST (U/L)	20.15 ± 4.12	49.31 ± 31.42 [a]	39.21 ± 19.90 [a]	51.17 ± 26.08 [a]	91.4 ± 112.5 [a]
TB (μmol/L)	11.46 ± 3.26	13.03 ± 5.40	27.91 ± 19.55 [a, b]	83.69 ± 172.79 [a, b]	52.2 ± 101.5 [a, b]
DB (μmol/L)	1.73 ± 0.58	3.02 ± 1.20 [a]	8.60 ± 6.51 [a, b]	54.19 ± 140.05 [a, b]	37.1 ± 149.9 [a, b]
PT (s)	10.75 ± 0.75	13.57 ± 1.36 [a]	13.94 ± 3.94 [a]	12.84 ± 1.84 [a]	14.1 ± 3 [a]
INR	0.85 ± 0.06	1.07± 0.10 [a]	1.24 ± 0.23 [a]	1.03 ± 0.16 [a, c]	3.8 ± 19.1 [a, c]
HBsAg (log 10 IU/mL)	-	3.67 ± 1.18	2.28 ± 1.22 [b]	-	2.10 ± 1.32 [b]
HBV DNA (log 10 IU/mL)	-	3.96 ± 1.77	1.89 ± 0.90 [b]	-	3.1 ± 1.41 [b, c]
SPINK1（ng/mL）	1.09 ± 0.38	1.75 ± 0.55 [a]	2.09 ± 0.62 [a, b]	2.40 ± 0.85 [a, b, c]	3.19 ± 1.11 [a, b, c, d]

The data are expressed as the means ± standard deviation or n (%). p-values were calculated using one-way ANOVA, followed by least-significant difference multiple comparisons test. [a]- p < 0.05 when compared with healthy controls. [b]- p < 0.05 when compared with patients with chronic hepatitis B. [c]- p < 0.05 when compared with patients with liver cirrhosis. [d]- p < 0.05 when compared with patients with intrahepatic cholangiocarcinoma.

and specific for HBV-related HCC than for primary hepatogenic cancer (PHC) and ICC, with an area under the curve (AUC) of 0.87 (95% CI: 0.832 - 0.902, p < 0.001), 0.79 (95% CI: 0.721 - 0.848, p < 0.001), and 0.84 (95% CI: 0.804 - 0.878, p < 0.001) for HBV-related HCC, ICC, and PHC, respectively (Figure 4A). The best cutoff point of serum SPINK1 was 2.48 ng/mL according to ROC curve analysis (Figure 4B).

In addition, based on their serum SPINK1 status, we separated the cases into high- and low-level groups: ≥ 2.48 ng/mL (n = 67) and < 2.48 ng/mL (n = 37). The data implied that the high-level group had higher serum TB (p < 0.05) and DB (p < 0.05) levels than the low expression group (Table 2). Likewise, the ratio of patients in the high-level group was lower than that in the low-level group with respect to AFP level < 400 ng/mL, Barcelona Clinic Liver Cancer (BCLC) stage A disease, Child-Pugh A status and HBV-related HCC < 3 cm (all p < 0.05) (Table 2). Nevertheless, other laboratory parameters, including ALT, AST, prothrombin time (PT), international normalized ratio (INR), and HBV viral load, failed to present any significant difference between the two groups (Table 2). The age, gender, and cirrhosis ratio of patients between the two groups also showed no significant difference (Table 2).

In general, we observed that the optimal cutoff value for serum SPINK1 was 2.48 ng/mL among patients with HBV-related HCC who survived or died. Moreover, the patient ratio was lower in the high expression group than in the low expression group with respect to Child-Pugh A, BCLC stage A, and HBV-related HCC < 3 cm in HBV-related HCC. Thus, we further investigated the risk factors for the short-term overall survival (OS) rate in the HBV-related HCC group.

Subgroup analysis of the risk factors for short-term survival in the HBV-related HCC group

The subgroup analysis among patients with HBV-related HCC revealed the rate of one-year OS with respect to various factors by Kaplan-Meier survival analysis. Based on the follow-up results, among the 104 patients with HBV-rated HCC, 55/104 (53%) patients died. The median OS time in the whole cohort was 7 months (range 3 - 11 months), while the rate of one-year survival was 20.61%. There were significant differences in the OS rates of patients with distinct tumor sizes (Figure 5C). The one-year OS rates of patients with a tumor size < 3 cm, tumor size 3 - 5 cm, tumor size 5 - 10 cm, and tumor size > 10 cm were 59.10%, 30.10%, 14.10%, and 17.50%, respectively, among patients with HBV-related HCC (log-rank p < 0.001) (Figure 5C). The one-year OS rates of patients with serum SPINK1 level ≥ 2.48 ng/mL and < 2.48 ng/mL were 17.80% and 67.40%, respectively (log-rank p < 0.001) (Figure 5A). Correspondingly, the OS rate in patients with different AFP levels, BCLC stages, and Child-Pugh grades revealed distinct differences (all log-rank p < 0.001) (Figure 5B, D - F and Table 3). In the univariate analysis, serum SPINK1 was a great prognostic predictor for HBV-related HCC. High levels of serum SPINK1 (≥ 2.48 ng/mL) were associated with a poor OS rate (HR = 5.33, p < 0.001) (Figure 5A and Table 3). Table 3

Table 2. Demographic and clinical characteristics of patients according to serum SPINK1 levels.

Variable	Serum SPINK1 levels (< 2.48 ng/mL) (n = 37)	Serum SPINK1 levels (≥ 2.48 ng/mL) (n = 67)	p-value
Age (years)	55.05 ± 11.53	57.63 ± 11.91	p = 0.29
Gender			p = 0.28
Male	35 (94.6%)	59 (88.1%)	
Female	2 (5.4%)	8 (11.9%)	
ALT (U/L)	56.02 ± 74.97	74.33 ± 128.29	p = 0.43
AST (U/L)	66.78 ± 81.82	105.06 ± 124.74	p = 0.06
ALB	36.36 ± 5.89	34.83 ± 7.03	p = 0.27
TB (μmol/L)	22.64 ± 15.62	68.49 ± 123.20	p = 0.04
DB (μmol/L)	5.77 ± 5.51	54.41 ± 184.88	p = 0.04
PT (s)	13.70 ± 1.65	14.10 ± 3.83	p = 0.47
INR	1.15 ± 0.14	1.21 ± 0.29	p = 0.15
HBV DNA (log 10 IU/mL)	1.04 ± 0.32	1.07 ± 0.31	p = 0.73
HBsAg (log 10 IU/mL)	1.66 ± 1.39	1.28 ± 1.46	p = 0.20
AFP (ng/mL)			p = 0.016
< 400 ng/mL	24 (64.9%)	27 (40.3%)	
≥ 400 ng/mL	13 (35.1%)	40 (59.7%)	
BCLC stage			p < 0.001
A	27 (73.0%)	20 (29.9%)	
B	7 (18.9%)	14 (20.9%)	
C	3 (8.1%)	20 (29.9%)	
D	0 (0%)	13 (19.4%)	
Child-Pugh grade			p = 0.01
A	27 (73.0%)	34 (50.7%)	
B	10 (27.0%)	21 (31.3%)	
C	0 (0%)	12 (17.9%)	
Cirrhosis			p = 0.48
No	10 (27.0%)	14 (20.9%)	
Yes	27 (73.0%)	53 (79.1%)	
HCC tumor size			p = 0.04
HCC (< 3 cm)	19 (51.4%)	17 (25.4%)	
HCC (3 - 5 cm)	10 (27.0%)	25 (37.3%)	
HCC (5 - 10 cm)	5 (13.5%)	20 (29.9%)	
HCC (> 10 cm)	3 (8.1%)	5 (7.5%)	

The data are expressed as the means ± standard deviation or n (%). p-values were calculated using the independent-samples *t*-test for continuous variables and χ^2 test for categorical data. ALT - alanine transaminase, AST - aspartate aminotransferase, TB - total bilirubin, DB - direct bilirubin, PT - prothrombin time, INR - international normalized ratio, AFP - alpha-fetoprotein, BCLC stage - Barcelona Clinic Liver Cancer stage, HCC - hepatocellular carcinoma. The values indicate that p-values were < 0.05 and considered statistically significant.

shows the other prognostic variables. Among these, HBV DNA 10^3 - 10^4 IU/mL (HR = 2.81, p = 0.004) (Figure 5F and Table 3), HBV DNA 10^4 - 10^5 IU/mL (HR = 2.47, p = 0.013) (Figure 5F and Table 3), and AFP ≥ 400 ng/mL (HR = 3.46, p < 0.001) (Figure 5B and Table 3) were also related to a poor OS rate.

Cox multivariate analysis was further applied to minimize confounding factors with the risk factors for OS.

Table 3. Results of univariate and multivariate analyses in HBV related HCC patients.

	HR	95% CI		p-value	HR	95% CI		p-value
		Lower	Upper			Lower	Upper	
Age	1.292	0.775	2.154	0.327				
Gender male (vs. female)	1.656	0.786	3.490	0.185				
SPINK1 level < 2.48 ng/mL (vs. ≥ 2.48 ng/mL)	5.330	2.873	9.889	< 0.001	4.647	2.070	10.434	< 0.001
AFP levels ≥ 400 ng/mL (vs. < 400 ng/mL)	3.457	2.055	5.814	< 0.001	2.947	1.491	5.822	0.002
HCC (3 - 5 cm) (vs. HCC < 3 cm)	2.061	1.067	3.982	0.031				
HCC (5 - 10 cm) (vs. HCC < 3 cm)	3.635	1.853	7.133	< 0.001	3.612	1.562	8.350	0.003
HCC (< 3 cm) (vs. HCC < 3 cm)	4.180	1.502	11.633	0.006	4.033	1.082	15.031	0.038
Tumor numbers < 3cm (vs. ≥ 3cm)	0.588	0.353	0.979	0.042				
Child-Pugh B (vs. A)	2.858	1.628	5.018	< 0.001	3.300	1.642	6.630	0.001
Child-Pugh C (vs. A)	5.195	2.602	10.373	< 0.001				
BCLC stage B (vs. A)	1.639	0.498	5.389	0.416				
BCLC stage C (vs. A)	2.703	1.497	4.879	0.001				
BCLC stage D (vs. A)	9.986	4.704	21.199	< 0.001				
ART before HCC diagnosis (vs. non-ART)	0.469	0.246	0.896	0.022				
ART after HCC diagnosis (vs. non- ART)	0.583	0.318	1.071	0.082				
HBV DNA103 - 104 (vs. 103) IU/mL	2.813	1.404	5.638	0.004	3.136	1.222	8.043	0.017
HBV DNA 104 - 105 (vs. 103) IU/mL	2.471	1.210	5.042	0.013				
HBV DNA > 105 (vs. 103) IU/mL	1.1752	0.808	3.798	0.155	0.343	0.118	0.995	0.049

Univariate analysis was performed by the Cox regression model. Multivariate analysis was performed by the Cox proportional hazard model using an enter procedure. HR - hazard ratio, CI - confidence interval, HCC - hepatocellular carcinoma, BCLC stage - Barcelona Clinic Liver Cancer stage, AFP - alpha-fetoprotein, ART - antiviral therapy. The values indicate that p-values were < 0.05 and considered statistically significant.

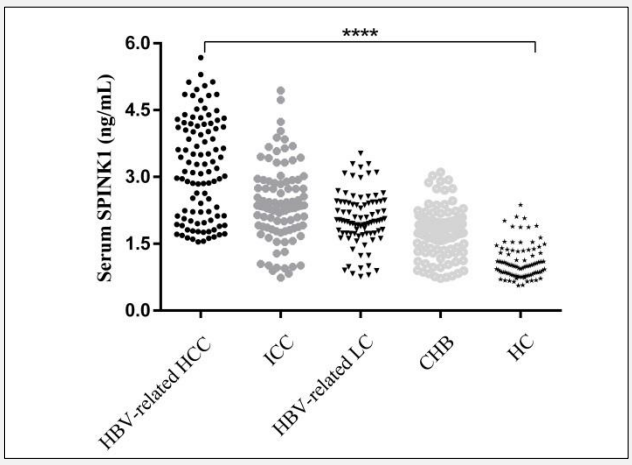

Figure 1. Concentrations of serum SPINK1 levels in patients analyzed by the χ^2 test.

Serum SPINK1 levels were significantly higher in patients with HBV-related HCC than in those with ICC, LC, CHB, and HCs (** - p < 0.0001).**

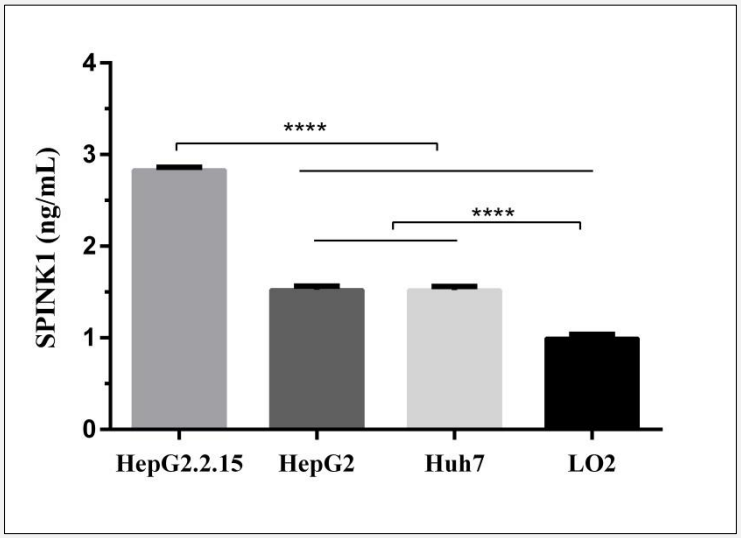

Figure 2. Concentrations of SPINK1 in human HCC cells analyzed by one-way ANOVA.

The expression of SPINK1 in HepG2.2.15 cells was significantly higher than that in HepG2, Huh7, and LO2 cells ($p < 0.0001$). The expression of SPINK1 in HepG2 and Huh7 cells was also significantly higher than that in LO2 cells (** - $p < 0.0001$).**

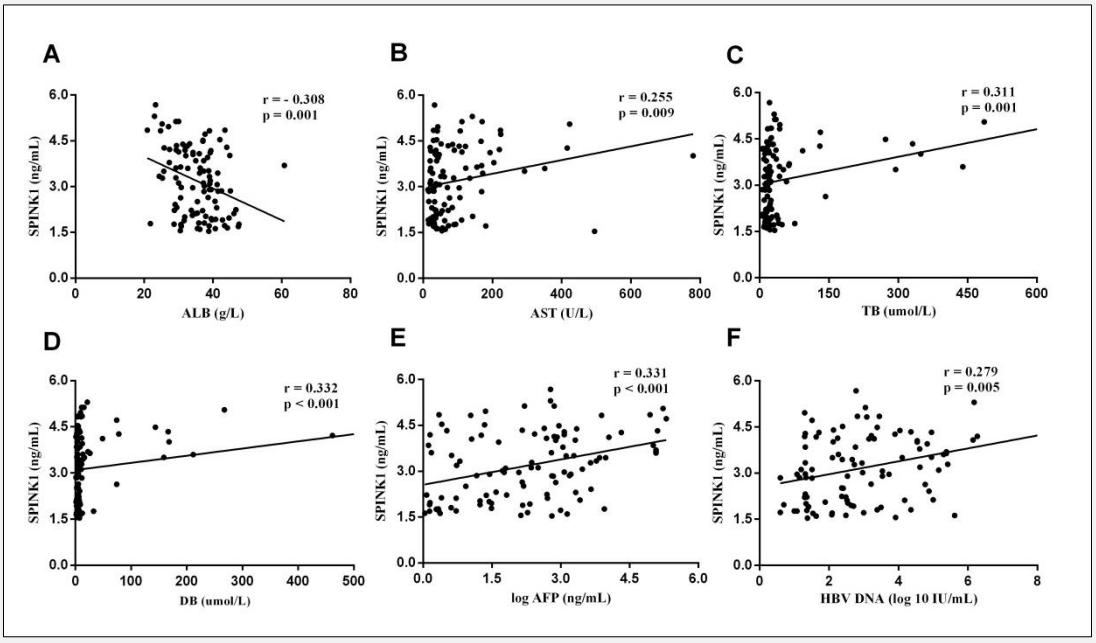

Figure 3. Serum SPINK1 levels analyzed by Spearman's rank correlation test.

A: Patients with HBV-related HCC with high ALB levels had low serum SPINK1 levels; B: Patients with HBV-related HCC with high AST levels had high serum SPINK1 levels; C: Patients with HBV-related HCC with high TB levels had high serum SPINK1 levels; D: Patients with HBV-related HCC with high DB levels had high serum SPINK1 levels; E: Patients with HBV-related HCC with high AFP levels had high serum SPINK1 levels; F: Patients with HBV-related HCC with high serum HBV DNA levels had high serum SPINK1 levels.

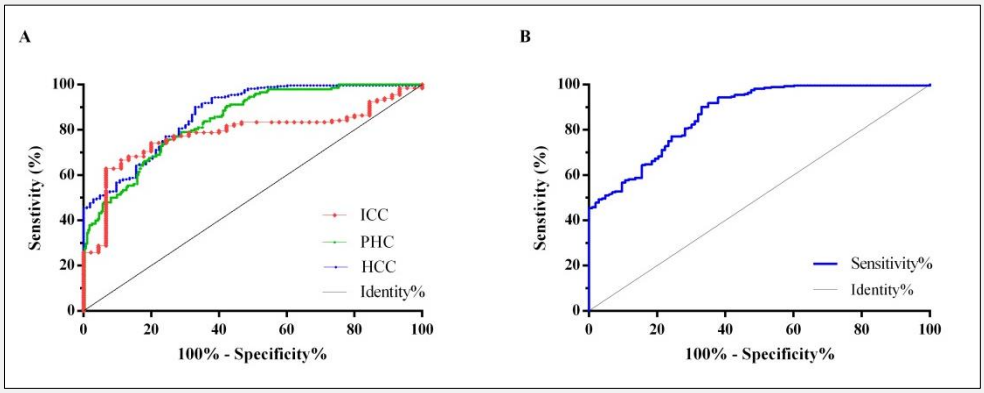

Figure 4. Comparison of serum SPINK1 diagnostic values in different liver cancers through ROC curve analysis.

A: The detection power of serum SPINK1 was more sensitive and specific for HBV-related HCC than for PHC and ICC, with AUC of 0.87 (95% CI: 0.832 - 0.902, p < 0.001), 0.79 (95% CI: 0.721 - 0.848, p < 0.001), and 0.84 (95% CI: 0.804 - 0.878, p < 0.001) in patients with HCC, ICC, and PHC, respectively; B: The best cutoff point for the serum SPINK1 levels in HCC was 2.48 ng/mL (sensitivity, 70.00% and specificity, 90.14%; AUC (SE), 0.87 (0.019); 95% CI, 0.832 - 0.902, p < 0.0001).

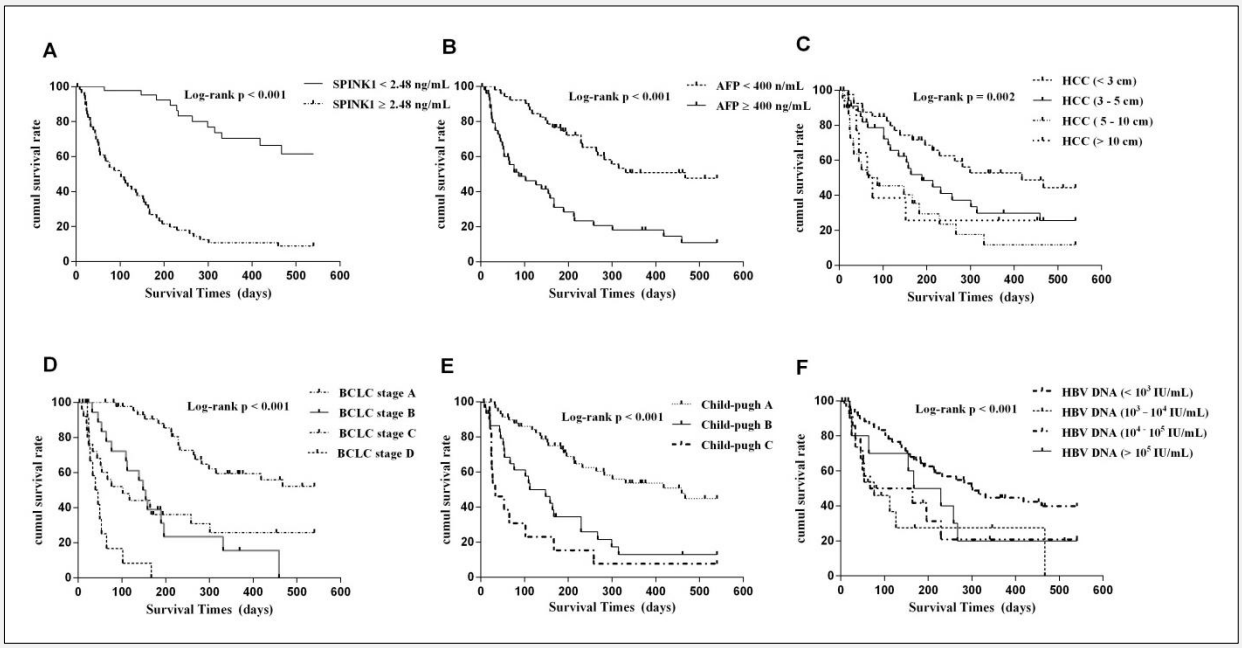

Figure 5. Cumulative survival plotted using Kaplan-Meier survival curves and compared using the log-rank test.

A: Patients with HBV-related HCC with SPINK1 < 2.48 ng/mL (solid line) had better OS than those with SPINK1 ≥ 2.48 ng/mL (dashed line) (p < 0.001); B: Patients with HBV-related HCC with AFP < 400 ng/mL (dashed line) had better OS than those with AFP ≥ 400 ng/mL (solid line) (p < 0.001); C: Patients with HBV-related HCC with a tumor size < 3 cm (dashed line) had better OS than those with a tumor size 3 - 5 cm (bold solid line), 5 - 10 cm (dotted line), or more than 10 cm (bold dashed line) (p < 0.002); D: Patients with HBV-related HCC with BCLC stage A (bold dashed line) had better OS than those with BCLC stage B (solid line), C (dashed line), or D (dotted line) (p < 0.001); E: Patients with HBV-related HCC with Child-Pugh A (dashed line) had better OS than those with Child-Pugh B (solid line) or C (dotted line) (p < 0.001); F: Patients with HBV-related HCC with HBV DNA < 10^3 IU/mL (bold solid line) had better OS than those with HBV DNA (10^3 - 10^4) IU/mL (dashed line), (10^4 - 10^5) IU/mL (bold dashed line), or > 10^5 IU/mL (solid line) (p < 0.001).

According to the results of the Kaplan-Meier survival analysis, the factors including serum SPINK1 level, AFP level, Child-Pugh grade, tumor size and HBV DNA were analyzed. A serum SPINK1 level ≥ 2.48 ng/mL was identified as an independent risk factor (HR = 4.65, 95% CI: 2.070 - 10.434, $p < 0.001$) (Figure 5 and Table 3) for the short-term prognosis of patients with HBV-related HCC. Moreover, low AFP level, small tumor size, good Child-Pugh grade, and low HBV viral load were identified as independent protective factors for the short-term prognosis.

In conclusion, a serum SPINK1 level ≥ 2.48 ng/mL was identified as a significant risk factor in the course of HBV-related HCC, together with high AFP level, large tumor size, and poor Child-Pugh grade.

DISCUSSION

HCC is the second leading cause of cancer-related death worldwide. China alone accounts for approximately 50% of all cases and deaths [19]. Many studies have identified that HBV is one of the most significant pathogenic factors involved in the course of HCC, and even chronic asymptomatic HBV carriers without cirrhosis still have a 5 times higher risk of developing HCC than normal patients [20-22]. Considering the high risk of mortality, it is of great clinical and public health significance to identify effective, reliable biomarkers for the management of patients with HBV-related HCC. The emerging indicator SPINK1 plays an important role in varied physiological metabolic and disease progression. In addition to protecting tissue organs from invasion by inhibiting trypsinogen activation, it also inhibits apoptosis and promotes tumor progression. SPINK1 is increased in the serum and tumor tissue of patients with non-small cell pulmonary cancer, bladder cancer, colon cancer, breast cancer, and other tumors [13].

Mounting evidence [23,24] suggests that increased SPINK1 possibly takes part in the carcinogenesis of hepatocirrhosis with HBV infection; therefore, it has the potential to be a biomarker in the early diagnosis of HBV-related HCC. Recently, it was reported that SPINK1 is overexpressed in patients with viral hepatitis, especially in HBV-related HCC [23,25], and enhanced SPINK1 is associated with an adverse prognosis [26]. Lee Y C et al. [27] reported that SPINK1 played a major part in cell migration and inflammatory signaling and resulted in terminal HCC with portal vein invasion. In line with these experimental observations, the present study highlighted that SPINK1 expression was enhanced in patients with HBV-related HCC, especially in those with early mortality. In addition, we further confirmed that HBV can facilitate the expression of SPINK1, whereby SPINK1 expression in HepG2.2.15 cells was obviously higher than that in non-HBV-infected cells, which is similar to Zhu's study [25]. In order to investigate the possible mechanisms by which HBV enhances SPINK1 expression, Zhu C et al. [25] combined the SPINK1 promoter with luciferase reporter gene, then the complex was co-transfected into HepG2 cells with HBV-infected cloned pHBV1.3 and each gene of the HBV genome (pCMV-S, pCMV-E, pCMV-C, pCMV-X, and pCMV-P). The expression of SPINK1 was measured by luciferase activity. They have demonstrated that HBV X protein (HBx) could up-regulate the expression level of SPINK1 by activating its promoter. Nevertheless, the principles and mechanisms of SPINK expression in HBV-related HCC remain poorly defined, and further studies are imperative.

We were the first to explore the diagnostic significance of SPINK1 in HBV-related HCC. In this regard, ROC curve analysis was used to compare the diagnostic value of SPINK1 for HCC, PHC, and ICC. Serum SPINK1 was efficient to diagnose PHC, with an AUC of 0.84 ($p < 0.001$), consistent with previous reports [28]. The AUC in HBV-related HCC (0.87, $p < 0.001$) was even larger than that in PHC, which further demonstrated the specific diagnostic value of the serum SPINK1 level for HBV-related HCC. Intriguingly, the overexpression of SPINK1 was related to a low proportion of patients with Child-Pugh A, BCLC stage A, and HBV-related HCC < 3 cm.

Furthermore, we discovered that serum SPINK1 in HBV-related HCC patients can act as a useful biomarker for adverse outcomes. Earlier studies have recounted that a high AFP level, tumor size and high HBV DNA load are related to a poor prognosis in early-stage HCC [29]. In this study, a serum SPINK1 level ≥ 2.48 ng/mL was identified as an independent risk factor for short-term prognosis in patients with HBV-related HCC. Moreover, a high AFP level, a large tumor volume, high Child-Pugh grade, and HBV viral load were identified as important risk factors for the short-term prognosis in patients with HBV-related HCC. Furthermore, a high HBV DNA load was identified as one of the risk factors for HBV-related HCC.

To sum up, the present report was the first to identify that serum SPINK1 could be a reliable, reproducible, and easily applicable noninvasive hematological marker of prognosis in HBV-related HCC. Otherwise, this study also suggested an optimal cutoff point for serum SPINK1, enabling serum SPINK1 to be applied as a prognostic indicator in clinical practice. The study also provided some new clues into the identification of tumor indicators for HBV-related HCC surveillance. However, there are still several limitations. First, the sample size was relatively small, and the results must be further confirmed in a large-center, randomized, controlled study. Second, the specific mechanism underlying HBV infection and SPINK1 expression remains to be investigated. This is the starting point for our further research. Finally, the patients with HBV-related HCC selected in this cohort received different treatments, including surgical therapy, interventional therapy, and chemotherapy. It is of great importance to further prove the prognostic worth of serum SPINK1 for patients with HBV-related HCC receiving different treatments.

In summary, the serum SPINK1 level may be a sensitive, noninvasive index for HBV-related HCC and an effective indicator for the prognostic stratification of patients with HBV-related HCC. Additionally, HBV has some effects on the expression of SPINK1 in both patients and cells. However, further research needs to be performed to clarify the mechanism by which HBV regulates the expression of SPINK1.

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The authors declare that they have no competing interest.

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