


RESEARCH ARTICLE

Performance of SPINK1 and SPINK1-based diagnostic model in detection of hepatocellular carcinoma

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Abstract

Objectives: To identify the SPINK1 or SPINK1-based model as a more reliable biomarker for the diagnosis of hepatocellular carcinoma (HCC).

Methods: Serum samples and related laboratory parameters were collected from 540 subjects (119 healthy donors, 113 patients with chronic hepatitis B, 122 patients with liver cirrhosis, and 186 patients with HCC). SPINK1 was determined by ELISA assay. Differences in each variable were compared by one-way ANOVA or Kruskal-Wallis test. ROC (receiver operating characteristic) curve analysis was conducted to compare the diagnostic efficiency of alpha-fetoprotein (AFP), SPINK1, and a SPINK1-based combine model constructed by binary Logistic regression.

Results: In detecting HCC using the other three groups as control, ROC curve analysis revealed that SPINK1 alone reached AUC of 0.899 (0.866–0.933), with the sensitivity of 0.812 of and specificity of 0.953. The combined model increased the AUC to 0.945 (0.926–0.964) with the sensitivity and specificity of 0.860 and 0.910, respectively. For AFP, significantly lower AUC ($p < 0.0001$) was shown, which was 0.695 (0.645–0.745) with the sensitivity and specificity of 0.634 and 0.718, respectively. In discriminating HCC from liver disease control, AUC of SPINK1 was 0.863(0.826–0.894), the sensitivity and specificity were 0.823 and 0.906, respectively. For combined model, the AUC, sensitivity, and specificity were 0.915 (0.884–0.940), 0.863, and 0.916, respectively. For detecting early-stage HCC, SPINK1 and combined model achieved the sensitivity of 0.788 and 0.818, respectively, much higher than AFP of 0.485 ($p < 0.05$); however, the difference between SPINK1 and combined model was not statistically significant ($p = 1$).

Conclusion: We provided solid evidence for SPINK1 as a robust serological tool for HCC diagnosis.

KEYWORDS

diagnostic capacity, hepatocellular carcinoma, SPINK1, tumor biomarker

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1 | INTRODUCTION

With half million newly diagnosed cases annually, hepatocellular carcinoma (HCC) is one of the six most common cancers and the fourth leading cause of cancer-related mortality worldwide.¹ Risk factors of HCC include chronic hepatitis that caused mainly by HBV or HCV infection, metabolic disorder, and alcohol consumption. In fact, nearly 85% of HCC cases around the world are attributed to chronic hepatitis.² Due to the asymptomaticity in early-stage and rapid tumor progression, HCC is usually detected at late stages, leading to a poor prognosis. Recent data show that patients who undergoing routine monitoring diagnosed with HCC are more likely to receive curative remedy than those who already developed symptoms (OR = 2.24, CI:1.99–2.52).³ The diagnosis of HCC currently relies heavily on medical imaging and histopathological approaches, which because of their obvious disadvantages, such as radioactivity and invasiveness, are hardly meet the requirements for a usual surveillance method. In this regard, serum biomarkers could be useful as complementary approach to abdomen US (ultrasound) for routine surveillance. Unfortunately, the most widely used serum biomarker, alpha-fetoprotein (AFP), is still far from satisfactory, because of its low sensitivity and specificity.⁴ Moreover, patients with chronic hepatitis (15–58%) or liver cirrhosis (11–47%) were often found with elevated AFP levels.⁵ Therefore, a more reliable indicator for the discovery of HCC, particularly in the early stage, is urgently needed. In the present study, we aimed to explore an alternative method to enhance the accuracy of serum biomarkers in the diagnosis of HCC. Through investigating HCC related gene expression datasets from the GEO (Gene Expression Omnibus), 91 upregulated genes were shared in 3 candidate studies. Of which, SPINK1 (Serine Peptidase Inhibitor Kazal type 1) was selected for further investigation. SPINK1 (also called tumor-associated trypsin inhibitor, TATI) was originally detected in the urine of an ovarian cancer patient.⁶ It was later shown to be identical to pancreatic secretory trypsin inhibitors, presented in the pancreas and pancreatic fluid with high concentrations.⁷ In addition to inhibiting the prematurely activated trypsin in the pancreas, SPINK1 was also found to be tightly connected with tumor development and progression due to its properties of growth factor⁸ and inhibitor of apoptosis.⁹ High tissue expression of SPINK1 has been widely studied in various cancers and predicts an unfavorable outcome.^{10–12} Meanwhile, high levels of SPINK1 in serum may serve as a diagnostic biomarker for tumor detection. Herein, we studied the presence of SPINK1 in serum of the HCC patients and evaluated the power of SPINK1 in discriminating HCC, especially in the early stage, from the chronic hepatitis B (CHB) and liver cirrhosis (LC) patients. In comparison with AFP, SPINK1 alone or combined with other laboratory parameters achieved a significant enhancement in the performance of HCC diagnosis.

2 | MATERIALS AND METHODS

2.1 | Subjects, serum collection, storage, and assays

Serum samples of HCC and LC patients were collected before diagnosis and receiving any form of treatments, LC was diagnosed by Color Doppler ultrasound or histopathology, HCC was diagnosed by at least two imaging approaches (Liver CT or MRI) and further confirmed by histopathology, HCC stage was determined according to CNLC (China Liver Cancer Staging) standard,¹³ and CNLC IA and CNLC IB were considered as early-stage HCC for the most of the patients in these subgroups underwent surgical resection. For CHB patients, samples were collected when they were visiting hospital for routine surveillance, all the participants were HBsAg positive for more than six months. For healthy donors, the samples were collected from the participants performing physical examination. Informed consent forms were signed by all patients and healthy donors to allow the use of their samples for experiments and the experiments were performed in accordance with the regulation of institutional ethics committee of the First affiliated hospital of Xi'an Jiaotong University. The serum was collected into 1.5 ml Eppendorf tubes and stored in -80 degree. Serum SPINK1 was measured by ELISA kit purchased from R&D Systems (Minneapolis, MN, USA) according to the users' manual. The AFP was detected by electrochemiluminescence using the Cobas 8000 e602 Analyzer (Roche Diagnostics, Germany). GGT was determined by enzymatic rate method using Hitachi Labospect 008 automatic analyzer (Hitachi High-Technologies, Tokyo, Japan). Platelet (PLT) count was measured by automated Sysmex XN-9000 hematology analyzer (Sysmex, Inc., Kobe, Japan). HBV-DNA was quantified by RT-PCR kits (DAAN gene, Guangzhou, China) using Applied Biosystems™ 7500 Thermo cyclor (Thermo Fisher Scientific, Waltham, MA, USA).

2.2 | Statistical analysis

Categorical variables were presented as percentages and continuous variables as median and quantile (P25–P75). Differences between groups or subgroups were analyzed by one-way ANOVA if the variables passed the assessment of normality distribution and homogeneity of variances. For quantitative variables that not fulfilled the requirements, Kruskal-Wallis test was used. Tables 2–4 state the results of normality distribution test, homogeneity test, and the methods for comparison, respectively. For construction of the combined diagnostic model, GGT, ALB, PLT, AFP, and SPINK1 were selected for Logistic regression using method of forward LR. The first model was built using the whole non-tumor groups as a comprehensive control.

Variables included in the equation and the results of Hosmer and Leeshawn test were listed in Tables S5 and S6. The second model was built using the liver disease groups as control, namely the CHB and liver cirrhosis. Variables included in the equation and the results of Hosmer and Leeshawn test were listed in Tables S8 and S9. The ROC curve analysis was performed to compare the performance between AFP, SPINK1, and combined model. The AUC (95% CI), sensitivity, specificity, and accuracy were calculated based on ROC curves. The Delong test was applied to compare the differences between AUC of each curve, and the results were showed in Tables S7 and S10. Finally, the confusion matrix was used for analysis of AFP, SPINK1, and combined model in detection of early-stage HCC and paired chi-square test was used for consistency check. Only the Delong test was performed by MedCalc Software 19.0.4, and the rest were all performed using IBM SPSS Software 23.

3 | RESULTS

3.1 | Baseline information of all subjects

A total number of 540 serums (including 119 healthy donors, 113 patients with CHB, 122 with LC, and 186 patients with HCC) were collected, and the median age of each group was 58, 55, 58, and 59 years, respectively. The proportion of male was slightly higher than female in all groups. Meanwhile, the laboratory parameters relating to liver disease were also listed in Table 1. For HCC group, more than 82% of the patients were infected with HBV, and only 11.83% were virus free. Liver cirrhosis was present in 80% of patients. Stage distribution showed that the early-stage HCC account for 17.4% (33 out of 186). Only 16.1% were amenable to surgical resection (Figure 1).

3.2 | Potential biomarkers selection

For novel diagnostic biomarker selection, 3 DNA array studies concerning the HCC gene expression profile in GEO database were

analyzed.¹⁴⁻¹⁶ The baseline information of these datasets was listed in Figure 2A. The Venn diagram (Figure 2B) showed that there were 89 genes that were upregulated in tumors in all 3 studies according to the criterion of $p < 0.05$ & $\log_{2}FC > 1$ (for more specific information, see Table S1). Of which, 2 secretory proteins, SPINK1 and SPP1, aroused our attention (Figure 2C).

3.3 | Comparison of SPINK1 serum concentrations

We firstly compared the SPINK1 concentration in four major groups. No significant difference was observed between healthy donors, CHB and cirrhosis groups; however, SPINK1 concentration was markedly elevated in HCC group in comparison with each non-tumor group ($p < 0.0001$) (Figure 3A). We next compared serum SPINK1 between HCC subgroups, which were divided by stage. The concentration showed a generally ascendant trend from the earlier stage to more advanced stage. Besides, in all subgroups, SPINK1 amounts were significantly higher than that in healthy group (Figure 3B). We also analyzed the relationship between SPINK1 and HBV virus load, which was determined by serum HBV-DNA quantification using RT-PCR. The overall relationship was conducted using data from all three groups (CHB, cirrhosis, and HCC), and the data showed no significant change in SPINK1 levels along with the increase in HBV-DNA copies (Figure 3C, a, b, and c represent the SPINK1 vs. HBV-DNA in CHB, cirrhosis, and HCC, respectively).

3.4 | Performance of SPINK1 as a biomarker for the diagnosis of HCC

ROC curves were applied to determine the optimum cutoff of SPINK1 or AFP or the combination. In this step, all the non-tumor subjects, including the health, CHB, and the liver cirrhosis group were considered as a comprehensive control group. Binary logistic regression (independent variables include AFP, GGT, Alb, PLT, and SPINK1) was firstly used for construction of the combination

TABLE 1 Baseline information of all subjects

	Healthy donors (N=119)	CHB (N=113)	Liver cirrhosis (N=122)	HCC (N=186)	Sig.
Age (yrs.) ^a	58 (29-73)	55 (30-70)	58 (39-70)	59 (36-71)	n.s.
Sex, Male (%)	64 (54%)	65 (56%)	64 (52%)	99 (53%)	n.s. ^c
ALB (g/L) ^b	44.34(40.09-49.28)	44.51(36.92-49.54)	29.43 (21.39-39.63)	27.58 (19.02-37.32)	****
PLT (109/ml) ^b	193 (135-259)	189 (108-244)	123 (53-201)	130 (40-203)	****
GGT(U/L) ^b	12.52 (6.77-20.08)	67.37 (29.36-155.78)	86.34 (32.78-135.50)	97.44 (25.27-143.20)	****
ALT (U/L) ^b	23.34 (11.59-30.71)	92.14 (32.08-120.67)	85.45(39.48-116.54)	88.29 (29.96-137.20) ^d	****
AST (U/L) ^b	27.90 (15.38-34.22)	76.82 (40.08-101.26)	70.53 (33.14-112.27)	79.68(48.22-119.67) ^d	****
AFP (μg/L) ^b	6.22 (5.11-8.3)	6.4 (5.1-13.45)	9.065 (5.08-20.4)	13.04 (6.04-55.71)	****
HBsAg (+%)		103 (91.2%)	105(86.1%)	157 (84.4%)	n.s. ^c

Note: Data presented as a, median (range); b, median (25%-75% percentile). c, chi-square test. n.s, no significant, **** $p < 0.0001$. d, no significant v.s non-tumor disease group.

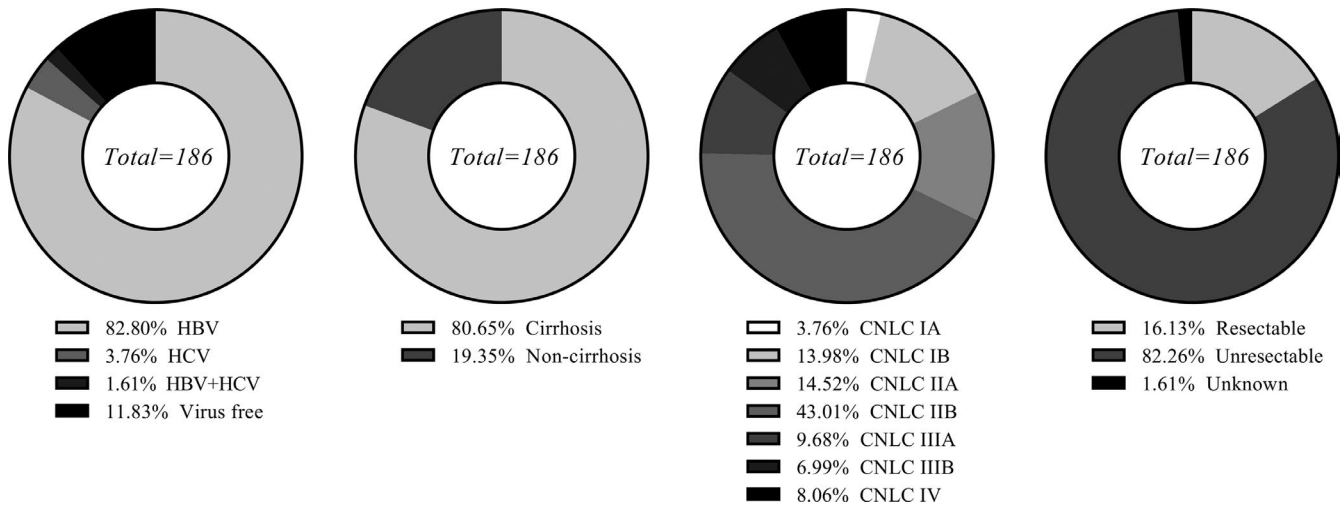


FIGURE 1 Characteristics of HCC cases

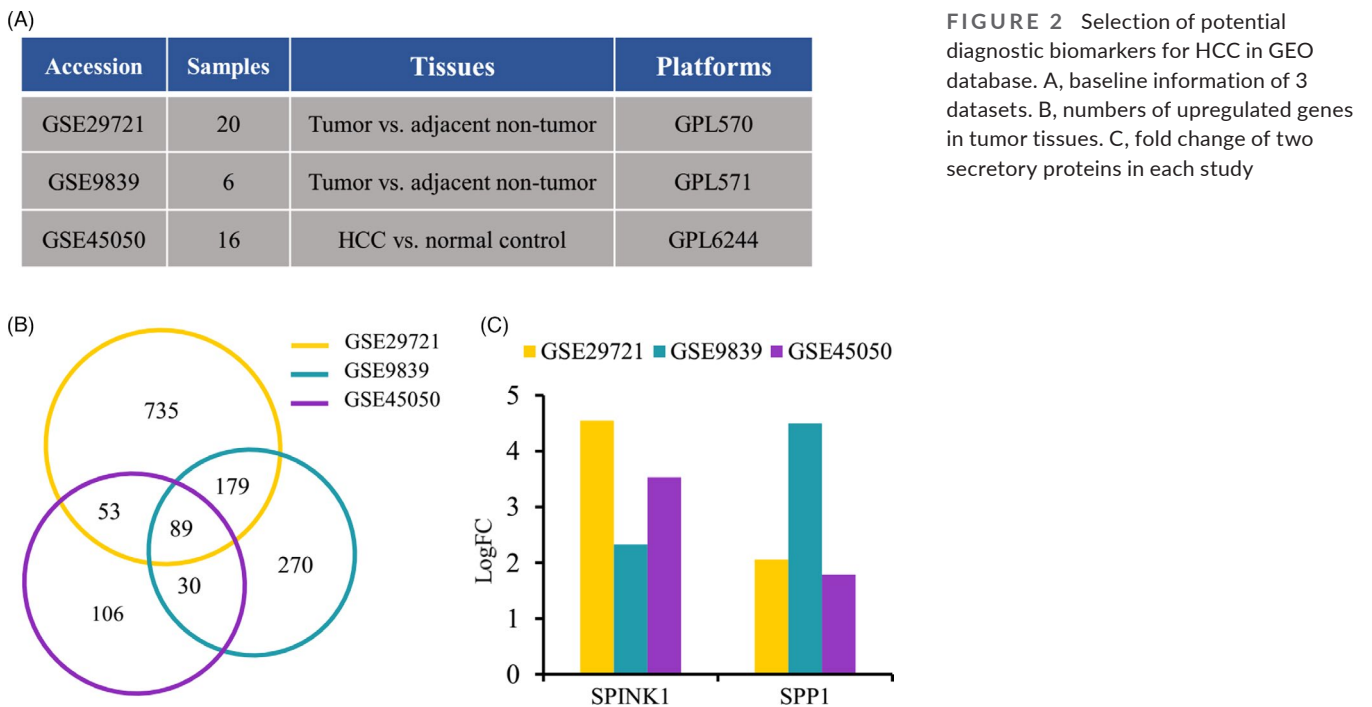


FIGURE 2 Selection of potential diagnostic biomarkers for HCC in GEO database. A, baseline information of 3 datasets. B, numbers of upregulated genes in tumor tissues. C, fold change of two secretory proteins in each study

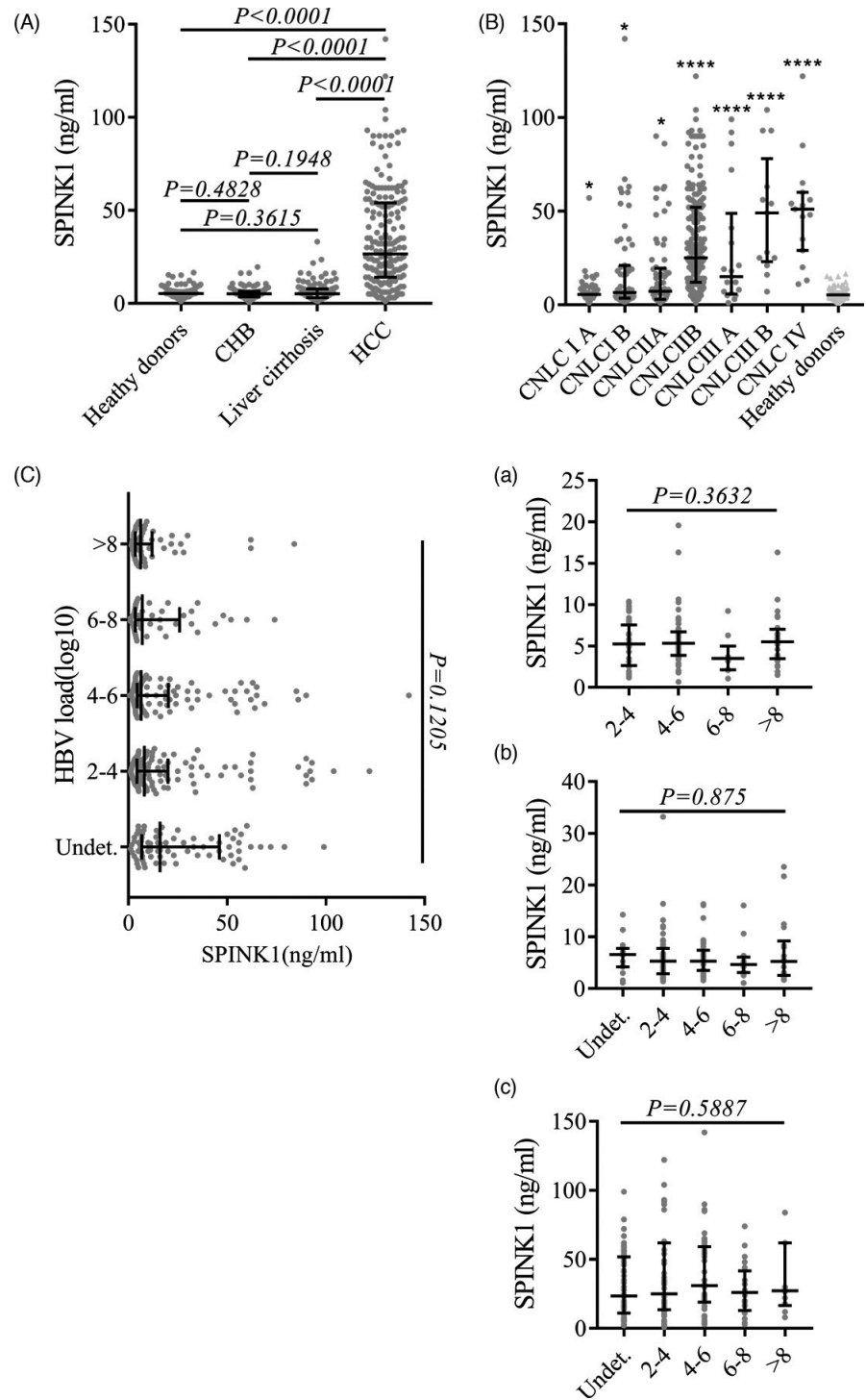
diagnostic model (Table S5 and S6). AFP and SPINK1 ($p < 0.001$) were included in the equation which was listed as following: $\text{Logit}(P) = 0.007 * (\text{AFP}) + 0.247 * (\text{SPINK1}) - 3.782$. For this model (defined as Combo), the prediction probability was used for ROC curve analysis.

SPINK1 alone reached sensitivity of 0.812, specificity of 0.935, and total accuracy of 0.904 at the cutoff of 10.835 ng/ml, far better than AFP alone which revealed 0.634, 0.718, and 0.690, respectively, at the cutoff of 9.450 $\mu\text{g/L}$. For the combination model, sensitivity, specificity, and the total accuracy were 0.860, 0.910, and 0.893 at the cutoff of 0.313. The AUC of ROC of AFP, SPINK1, and the combination model were 0.695, 0.899, and 0.945, respectively (Figure 4. A; Table 2). Meanwhile, the differences in AUC between

each indicator were compared, and the results showed all of that were statistically significant ($p < 0.0001$) (Table S7), indicating that the combination model was the best option for HCC diagnosis.

Our etiological data showed that HBV infection has been the major cause of HCC; thus, it was urgently in need of a more accurate indicator than AFP which was currently used for the routine monitoring of HCC in this population. We next compared the three indicators for their capacity to discriminate HCC cases from HBV-related liver disease groups. Therefore, in this step, healthy donors were excluded, and CHB and liver cirrhosis patients with HBV infection were included as comprehensive control group; for HCC group, patients of virus free were excluded. Combination model was rebuilt in this scenario. Three variables, AFP, SPINK1, and Alb

FIGURE 3 Comparison of serum SPINK1, in 4 major groups (A), HCC subgroups by stage (B). C, analysis of correlation between serum SPINK1 and HBC load, subjects were the sum of (a) CHB, (b) liver cirrhosis, and (C) HCC undet. represented the individuals who had been infected historically, but the results turned negative with the present samples according to the limitation of reagent. Undet., undetected. * $p < 0.05$, **** $p < 0.0001$



($p < 0.001$), were included. $\text{Logit}(P) = 0.006 * (\text{AFP}) + 0.208 * (\text{SPINK1}) - 0.032 * (\text{Alb}) - 2.181$ (Tables S8 and S9). The prediction probability was used as abovementioned.

Similar results were obtained, as the combination model (Combo_CHB&LC) showed the best performance with sensitivity of 0.823, specificity of 0.906, and total accuracy of 0.869 at the cutoff of 0.384. At the same cutoff, the capacity of SPINK1 alone was slightly lower. Sensitivity, specificity, and accuracy were 0.812, 0.902, and 0.862, respectively. For AFP, at the same cutoff, the

sensitivity remained 0.634, the specificity was slightly elevated into 0.753, and the total accuracy reached 0.700. The AUC of ROC of AFP, SPINK1, and the combination model were 0.703, 0.863, and 0.915, respectively (Figure 4B; Table 3). The differences were statistically significant (Table S10).

We also investigated the performance in discriminating early-stage HCC (CNLC IA and IB), in which, most cases were resectable. The confusion matrix in Table 4 showed that out of 33 early-stage HCC, 16, 26, and 27 were predicted by AFP, SPINK1, combo model,

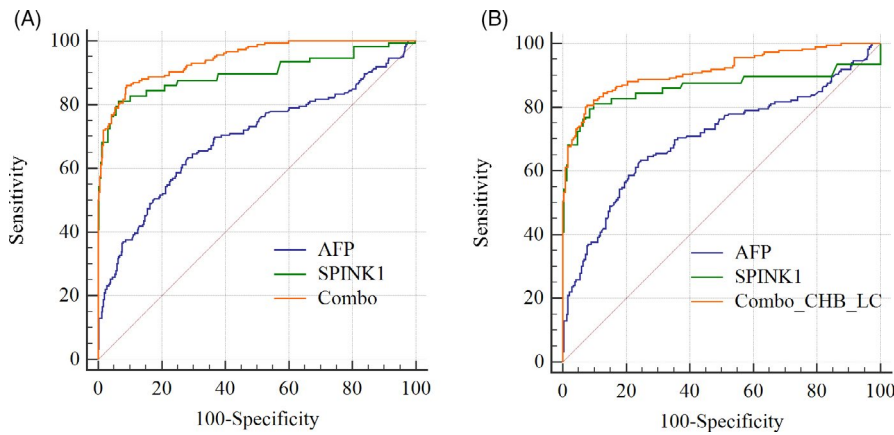


FIGURE 4 ROC curves of AFP, SPINK1, and Combo model for diagnosis of HCC using comprehensive controls (Health +CHB + liver cirrhosis) (A) or liver disease controls (B).

TABLE 2 Diagnostic performance of AFP, SPINK1, and combination model for discriminating HCC from comprehensive controls (Related to Figure 4A)

	Cutoff	Sensitivity	Specificity	Accuracy	AUC	SE	Sig.	CI 95%
AFP	9.440	0.634	0.718	0.690	0.695	0.026	0.000	0.645–0.745
SPINK1	10.672	0.812	0.935	0.904	0.899	0.017	0.000	0.866–0.933
Combo	0.313	0.860	0.910	0.893	0.945	0.010	0.000	0.926–0.964

TABLE 3 Diagnostic performance of AFP, SPINK1, and combination model for discriminating HCC from CHB/LC controls (Related to Figure 4B)

	Cutoff	Sensitivity	Specificity	Accuracy	AUC	SE	Sig.	CI 95%
AFP	9.440	0.634	0.753	0.700	0.703	0.027	0.000	0.657–0.746
SPINK1	10.672	0.812	0.902	0.862	0.863	0.022	0.000	0.826–0.894
Combo_CHB&LC	0.384	0.823	0.906	0.869	0.915	0.015	0.000	0.884–0.940

respectively. The sensitivity was 0.485, 0.788, and 0.812. The paired chi-square test (McNemar) was applied to compare the agreement of test. Both SPINK1 and Combo model showed better performance than AFP alone for their sensitivity were significantly higher than AFP ($p = 0.041$ and $p = 0.019$). However, Kappa value of 0.904 and $P = 1$ indicated high consistency between SPINK1 and combo model, and the difference in sensitivity between SPINK1 and Combo (0.788 vs. 0.818) was not significant (Table 4).

Considering that cirrhosis is the principal risk factor for HCC development, we finally rebuild the model using liver cirrhosis alone as non-tumor control for detection of eHCC, in which, only SPINK1 was included (Table S11). Then, we compared the power of SPINK1 with AFP for detection of eHCC from LC, SPINK1 achieved sensitivity, specificity, and accuracy of 0.788, 0.893, and 0.871 at cutoff of 14.32 ng/ml (Table 5).

4 | DISCUSSION

Hepatocellular carcinoma is the fourth most lethal cancer around the world. After decades of intensive study, risk factors, such as CHB, cirrhosis, alcohol consumption, and metabolic disorders, are well identified, early detection of HCC from high-risk population

remains a challenge due to the lack of effective tools. Regular surveillance programs based on abdominal ultrasound (US) examination and plasma AFP determination are recommend, patients at high risk should undergo this examination once for every six months. However, limitations are obvious by using these methods. For US tests, accurate judgement is dependent heavily on operators' rich experience, thus, varied skills among sonographers are likely to cause less objectiveness,¹⁷ and the AFP, the most widely used serological indicator in HCC diagnosis, showed an optimum sensitivity of 65% for early-stage HCC discrimination.^{18–20} In line with these studies, our data revealed the sensitivity of 63.4% and only 48.5% for detecting HCC and early-stage HCC, respectively. As such, identification of a novel serological biomarker or the marker panel for detection of HCC, particularly, in early stage, is urgently necessary.

In the present study, we explored the performance of SPINK1 as a tumor marker in the diagnosis of HCC. The biological function of SPINK1 (also named tumor-associated trypsin inhibitor, TATI) has been widely studied since its original isolation as a secreted proteins from urine of ovarian cancer patients.²¹ SPINK1 is Physiologically synthesized and secreted by pancreatic acinar cells, forming the first line of barrier to prevent the trypsinogen from premature activation.²² Therefore, markedly increased serum SPINK1 levels were observed in patients with acute pancreatitis,^{23,24} which

TABLE 4 Comparison of three methods in detection of early-stage HCC

		Predicted						Total
		AFP		SPINK1		Combo		
		eHCC	Ctrl	eHCC	Ctrl	eHCC	Ctrl	
Actual	eHCC	16	17	26	7	27	6	33
	Ctrl ^a	100	254	23	331	32	322	354
Sensitivity		0.485		0.788		0.818		
Specificity		0.718		0.935		0.910		
Accuracy		0.698		0.930		0.902		
Kappa				-0.191 ^b		-0.13 ^a ; 0.904 ^c		
Sig ^d .				0.041 ^b		0.019 ^a ; 1 ^c		

Note: a, Comprehensive control (Health+CHB+ LC); b, SPINK1 vs. AFP; c, Combo vs. SPINK1; d, significance of McNemar Test; eHCC, early-stage HCC.

TABLE 5 Diagnostic performance of AFP, SPINK1 for discriminating eHCC from LC controls

	Cutoff	Sensitivity	Specificity	Accuracy	AUC	SE	Sig.	CI 95%
AFP	11.27	0.455	0.795	0.723	0.597	0.063	0.123	0.516–0.675
SPINK1	14.32	0.788	0.893	0.871	0.791	0.067	0.000	0.718–0.852

was probably caused by leakage from the pancreas. Beyond basal expression in pancreatic acinar cells, elevated SPINK1 expression was also found in multiple types human cancer, including cancers of the gastrointestinal tract, lung, bladder, kidney, prostate, testis, ovary, cervix, and breast.²⁵ Earlier study showed that elevation of serum SPINK1 levels in non-hepatic cancer patients was seen, only in metastasis-carrying patients with terminal-stage cancers who showed sub-fever or inflammation, such as carcinomatous peritonitis or tumor invasion. In these patients, the serum SPINK1 may have reflected a synthesis in the liver in response to inflammation.²⁶ In HCC, global gene expression profiling demonstrated that SPINK1 was the most strongly upregulated gene.²⁷ Our results from GEO dataset analysis also revealed that SPINK1 was at the top of upregulated gene lists. All these findings implied a promising diagnostic role for SPINK1 in HCC. Our data, indeed, provided solid evidence for the potential application of SPINK1 clinically. A recent study documented that expression of SPINK1 was associated with hepatitis virus infection²⁸; however, we did not find significant elevation of serum SPINK1 levels in CHB and liver cirrhosis cohorts compared with healthy controls. Moreover, serum SPINK1 levels did not vary in the light of virus load. We assumed that the actual reason for SPINK1 overexpression in HBV-related HCC should be attributed to the tumor cells themselves. On the one hand, SPINK1 was reported as acute-phase proteins,²⁹ it is reasonable to speculate that virus infection caused SPINK1 elevation may be the consequence of activation of host immune system and the presence of inflammation. On the other hand, HCC cells have high levels of NFIL-6,³⁰ a transcriptional factor, which is likely to augment SPINK1 gene transcription by interacting with IL-6-responsive element, key *cis-acting* element upstream the initiation start site of the SPINK1 gene.²⁶ The possibility that high level of

serum SPINK1 originated from liver cancer cells would improve the specificity of SPINK1, especially in CHB-related HCC.

Although the SPINK1 or SPINK1-based model in our study showed powerful capacity as an indicator for HCC, we have to mention that only one marker is hard to cover all the HCC cases considering the biological heterogeneity of tumor. Furthermore, most of the subjects in CHB and cirrhosis groups in our study were outpatients who were attending hospital for regular surveillance with recessive symptoms of inflammation. For further studies, more effects should be made to distinguish the cancer from immune response when the patients were suffering severe liver damage which may cause elevation of SPINK1 as acute-phase protein. Finally, a more effective diagnostic tool should highlight the combination of multiple biomarkers. Similar to SPINK1, secreted phosphoprotein 1 (SPP1) as another secretory factor in the overlay upregulated gene list of the GEO datasets. Therefore, it would be intriguing to study the combination of SPINK1 and SSP1 in our future research.

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AUTHOR CONTRIBUTIONS

Fang Wang designed the research and wrote the article. Hui Liu, Youxi Bai, and Hui Li performed the statistical analysis. Fang Wang conducted the ELISA assay. Zhonglin Wang collected the laboratory parameters and patients' information. Xin Xu drew the figures.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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