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ORIGINAL RESEARCH

The Potential of the lncRNAs ADAMTSL4-AS1, AC067931 and SOCS2-AS1 in Peripheral Blood Mononuclear Cells as Novel Diagnostic Biomarkers for Hepatitis B Virus-Associated Hepatocellular Carcinoma

Weiwei Guan[1,2], Congyue Zhang[1], Tongguo Miao[1], Chen Dong[1], Lu Li[1], Xiwei Yuan[1], Dandan Zhao[1], Rong Ai[1], Xiaoxiao Zhang[1], Mengjiao Sun[1], Haiyan Kang[2,*], Yuemin Nan[1,*]

[1]Department of Traditional and Western Medical Hepatology, Hebei Medical University Third Hospital & Hebei International Joint Research Center for Liver Cancer Molecular Diagnosis, Hebei International Science and Technology Cooperation Base, Shijiazhuang, Hebei Province, 050051, People's Republic of China; [2]Department of Liver Disease, The Fifth Hospital of Shijiazhuang, Hebei Medical University, Shijiazhuang, Hebei, 050023, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yuemin Nan, Department of Traditional and Western Medical Hepatology, Hebei Medical University Third Hospital & Hebei International Joint Research Center for Liver Cancer Molecular Diagnosis, Hebei International Science and Technology Cooperation Base, No. 139, Ziqiang Road, Shijiazhuang, Hebei Province, 050051, People's Republic of China, Tel +86 18533112266, Email nanyuemin@163.com; Haiyan Kang, Department of Liver Disease, The Fifth Hospital of Shijiazhuang, Hebei Medical University, No. 42, Tanan Road, Shijiazhuang, Hebei Province, 050023, People's Republic of China, Tel +86 13315952782, Email kanghaiyan1976@126.com

Purpose: Long noncoding RNAs (lncRNAs) might be closely associated with hepatocellular carcinoma (HCC) progression and could serve as diagnostic and prognostic markers. This study aimed to investigate lncRNA-based diagnostic biomarkers for hepatitis B virus (HBV)-associated HCC.

Materials and Methods: High-throughput transcriptome sequencing was conducted on the liver tissues of 15 patients with HBV-associated liver diseases (5 with chronic hepatitis B [CHB], 5 with liver cirrhosis [LC], and 5 with HCC). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyze lncRNA expressions. Potential diagnostic performance for HBV-associated HCC screening was evaluated.

Results: Through trend analysis and functional analysis, we found that 8 lncRNAs were gradually upregulated and 1 lncRNA was progressively downregulated by regulation of target mRNAs and downstream HCC-associated signaling pathways. The validation of dysregulated lncRNAs in peripheral blood mononuclear cells (PBMCs) and HCC tissues by qRT-PCR revealed that ADAMTSL4-AS1, SOCS2-AS1, and AC067931 were significantly increased in HCC compared with CHB and cirrhosis. Moreover, differentially expressed lncRNAs were aberrantly elevated in Huh7, Hep3B, HepG2, and HepG2.215 cells compared with LX2 cells. Furthermore, ADAMTSL4-AS1, SOCS2-AS1, and AC067931 were identified as novel biomarkers for HBV-associated HCC. For distinguishing HCC from CHB, ADAMTSL4-AS1, AC067931, and SOCS2-AS1 combined with alpha-fetoprotein (AFP) had an area under the curve (AUC) of 0.945 (sensitivity, 83.9%; specificity, 89.8%). Similarly, for distinguishing HCC from LC, this combination had an AUC of 0.871 (sensitivity, 91.1%; specificity, 68.2%). Furthermore, this combination showed the highest diagnostic ability to distinguish HCC from CHB and LC (AUC, 0.905; sensitivity, 91.1%; specificity, 75.3%). In particular, this combination identified AFP-negative (AFP < 20 ng/mL) (AUC = 0.814), small (AUC = 0.909), and early stage (AUC = 0.863) tumors.

Conclusion: ADAMTSL4-AS1, SOCS2-AS1, and AC067931 combined with AFP in PBMCs may serve as a noninvasive diagnostic biomarker for HBV-associated HCC, especially AFP-negative, small, and early stage HCC.

Keywords: ADAMTSL4-AS1, AC067931, SOCS2-AS1, HBV-associated HCC, diagnostic biomarkers

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Introduction

Hepatocellular carcinoma (HCC) is ranked as the sixth most prevalent neoplasm.[1] Moreover, HCC is the third most frequent cause of cancer-related deaths globally and severely threatens human life and health.[2,3] The risk factors of HCC include viral hepatitis, high alcohol consumption, cirrhosis, aflatoxin, smoking, and nonalcoholic steatohepatitis.[4] Chronic hepatitis B virus (HBV) infection has been identified as the major risk factor of HCC worldwide, especially in East and Southeast Asia.[5] At present, surgical resection is considered an effective therapy for HCC. Unfortunately, approximately 80%–90% of patients with HCC are not eligible for surgical intervention because of the difficulty of accurately diagnosing the condition at an early stage.[6]

Alpha-fetoprotein (AFP) is a traditional serum biomarker for HCC screening; however, it has low sensitivity (58%–68%) for detecting early stage HCC.[7] Moreover, in a previous study, elevated serum AFP levels were identified in patients with benign liver diseases, such as chronic viral hepatitis and liver cirrhosis (LC).[8] Thus, it is essential to identify novel biomarkers with high sensitivity and specificity for the early diagnosis of HCC.[9]

Long noncoding RNAs (lncRNAs) are a subtype of noncoding RNA molecules with lengths exceeding 200 nucleotides, which do not have protein-coding potential.[10] Past evidence has suggested that lncRNAs regulate multiple biological processes through mechanisms such as chromatin modification, transcription, and posttranscriptional regulation.[11] Additionally, increasing research has demonstrated that lncRNAs are involved in modulating tumorigenesis and the development of multiple cancers, including HCC.[12] Recent studies discovered that lncRNAs have aberrant expressions in tumor tissues and body fluids.[13] As peripheral blood is the most widely used specimen for tumor diagnosis, numerous studies have attempted to identify blood biomarkers for cancer diagnosis owing to easy accessibility, nonintrusiveness, and low cost.[14] Studies have reported several serum or plasma exosomal lncRNAs with the potential to be new minimally invasive markers for the early diagnosis of different cancers, including HCC.[15] However, it is unclear whether lncRNAs in peripheral blood mononuclear cells (PBMCs), which are obtained from circulating blood, can be used as diagnostic biomarkers for HCC.

We previously performed mRNA expression analysis involving high-throughput transcriptome sequencing of hepatic tissues from healthy volunteers, patients with chronic hepatitis B (CHB), patients with LC, and patients with HCC, and identified novel predictive biomarkers.[16] In this study, we performed a further analysis to characterize the aberrantly expressed lncRNAs in the CHB, LC, and HCC groups and identify the differentially expressed lncRNAs (DE-lncRNAs) in PBMCs, which could be useful as novel diagnostic biomarkers for HBV-associated HCC.

Material and Methods

Study Subjects

We obtained liver specimens from 15 patients who had HBV-associated conditions (5 with CHB, 5 with LC, and 5 with HCC) at the Third Hospital of Hebei Medical University and performed transcriptome sequencing. Simultaneously, we obtained blood samples from 49 patients with CHB, 44 with HBV-associated LC, and 56 with HBV-associated HCC at the Third Hospital of Hebei Medical University and Shijiazhuang Fifth Hospital between July 2020 and September 2023, according to our inclusion criteria. The inclusion criteria were as follows: (1) age ≥18 years; (2) seropositivity for hepatitis B surface antigen (≥6 months); (3) signing of the written informed consent form before enrollment in the group; and (4) alignment of the diagnostic criteria with universally adopted clinical guidelines for CHB,[17] LC,[18] and HCC.[19] The conditions in the patients with HCC and those with LC were confirmed by clinical findings, biochemical findings, typical pathological findings, and abdominal imaging findings, including ultrasound, computed tomography, and magnetic resonance imaging findings. The exclusion criteria were as follows: (1) other causes and other infectious diseases, such as autoimmune hepatitis and HCV coinfection; (2) any prior preoperative therapy in HCC cases, such as chemotherapy, radiotherapy, targeted therapy, ablation, and intervention; (3) pregnancy; (4) presence of another malignant tumor; and (5) presence of severe cardiovascular diseases, pulmonary diseases, diabetes, renal insufficiency, or other chronic diseases.

Our study was approved by the ethics committee of the Third Hospital of Hebei Medical University and Shijiazhuang Fifth Hospital and was conducted in accordance with the 1964 Helsinki Declaration or comparable standards.

Sample Preparation

For RNA sequencing and quantitative real-time polymerase chain reaction (qRT-PCR) assays, we used fresh-frozen liver tissues and PBMCs. PBMCs were extracted from the whole blood samples of patients with HBV-associated liver diseases through density-gradient centrifugation using Ficoll medium (Solarbio Life Sciences, Beijing, China). The obtained cells were immediately stored at −80°C until use for PCR analysis.

Cell Culture

LX2 (human normal hepatic stellate cell line) and HCC (Huh7, HepG2, Hep3B, and HepG2.215) cell lines (Procell, Wuhan, China) were cultured under a 5% CO_2 atmosphere at 37°C, using Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Biological Industries, Israel), 1% streptomycin, and 1% penicillin solution (Gibco).

RNA Isolation and qRT-PCR Analysis

Total RNA was extracted from liver tissues, PBMCs, and cells, using TRIzol (Invitrogen, Waltham, MA, USA). Reverse transcription reactions were performed using the PrimeScript RT reagent kit with gDNA Eraser and qRT-PCR was performed using TB Green Premix Ex Taq II (TaKaRa, Dalian, China), according to the manufacturer's protocol. The primer sequences are listed in Table 1. RNA expression was evaluated using β-actin as an endogenous control and quantified according to the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Continuous variables are presented as mean and standard deviation or median (interquartile range), and normality and homogeneity were tested using Shapiro–Wilk and Levene's tests. Parametric (least significant difference or *t*-test) and nonparametric (Kruskal–Wallis or Mann–Whitney *U*) statistics were used to compare the differences between the groups. Categorical data are displayed as numbers and percentages, and were analyzed using the chi-square test. A receiver operating characteristic (ROC) curve was used to evaluate diagnostic efficiency. All statistical analyses were performed using SPSS v26.0 (IBM Corp., Armonk, NY, USA), MedCalc v15.0 (MedCalc Software, Mariakerke, Belgium), and GraphPad Prism v9.0 (GraphPad Software, San Diego, CA, USA). A two-tailed *P*-value of <0.05 was considered statistically significant.

Results

Screening Novel Candidate Biomarkers for the Progression of HBV-Associated Diseases

There were 145 lncRNAs that showed significant differential expression among CHB, LC, and HCC tissues ($P \leq 0.05$ as the cutoff criterion), including upregulation and downregulation. The candidate DE-lncRNAs were described by

Table 1 Primer Sequences of the qRT-PCR Used in This Study

lncRNA Name	Forward Primer(5' to 3')	Reverse Primer(5' to 3')
ADAMTSL4-AS1	AGTCTCTTTCCTCCTGCCTTCCTG	TCTAAGCCGCCCTGTGTCTCTG
AC067931	AGTCTCTTTCCTCCTGCCTTCCTG	TCTAAGCCGCCCTGTGTCTCTG
SOCS2-AS1	TCCAGTCCTGTTTCTAGGGCTTCC	CGAACTCCTGACCTCAAGTGCTTC
AL731577	CCTGCCTGACACAGCTCACTTC	TTGGGCACGACACATAGTACACTTG
TPM1-AS	ACGCACAGACACGACTGAAAGC	GTGGTGTTGAGAAGGTTCTGGAAGG
AL356356	TCACCACCTGTGCTGTCTCCTG	CACGCTGTCCTTTCGCTGAGTC
AC037198	CAAACGCTGGGGTGAGAGGAAC	GTCATTTCTGTCAGGGGAGCCATAC
AL161773	CCTGCTTGCTGTATCTGCGTCTG	TGTCGTCACTGAGAGTCCTGGATG
AP002026	AACCAAGGCACCAATCCCAACTG	CTAGGAAGGGCAACAACCAAGTGAG
β-actin	AAAGCGGCTGTTAGTCACTGG	CGAGTCATTGCATACTGTCCAT

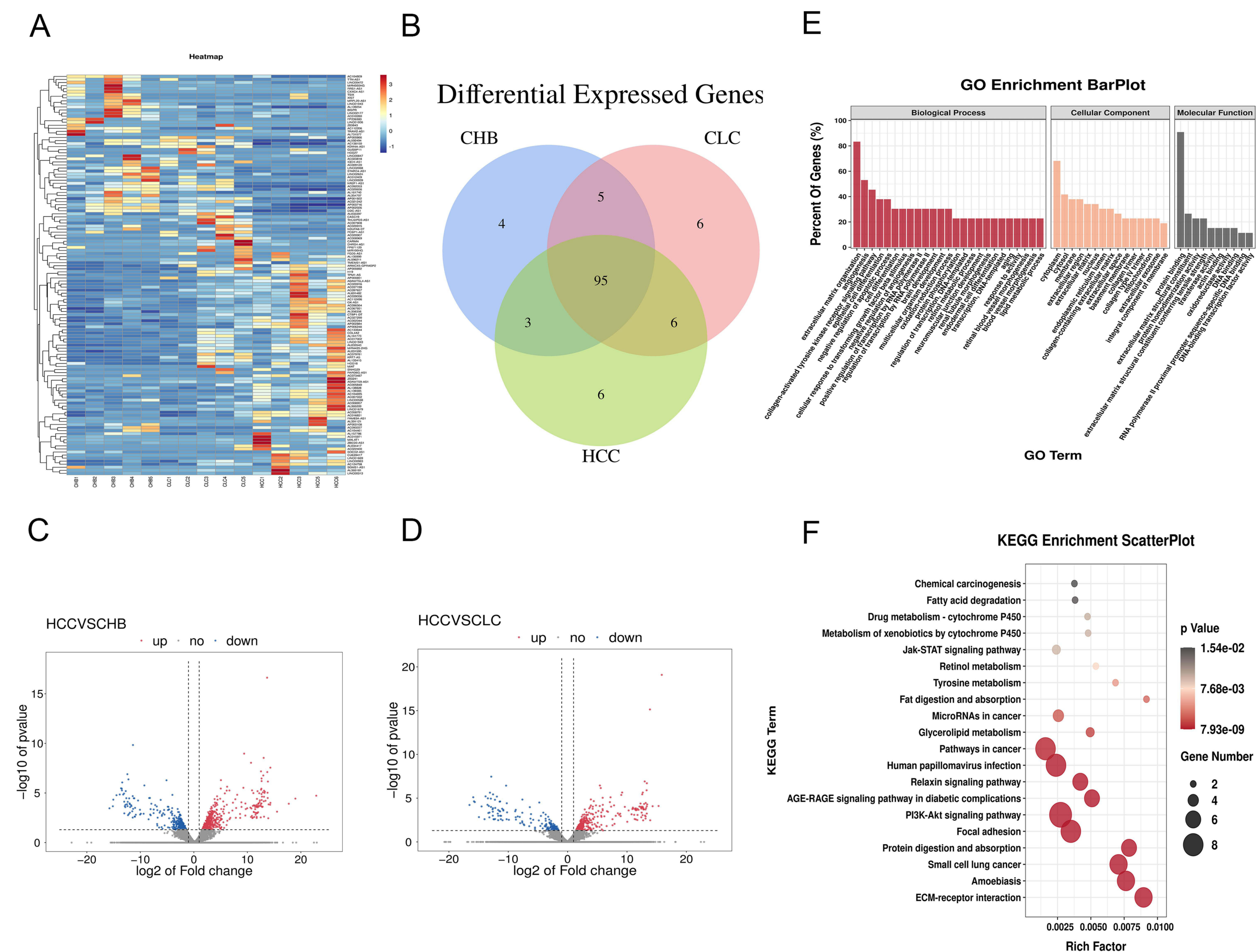

Figure 1 Bioinformatics analysis of transcriptomics data. (**A**) Clustering Heatmap of DELs among CHB, LF/LC and HCC tissues. The columns represented the clinical liver samples, and the rows indicated as DELs. Blue and red indicate down- and up-regulation, respectively. (**B**) Venn diagram showing the differential expression level of each lncRNA of the three comparison groups. Volcano plots for DELs between CHB and HCC (**C**) as well as LF/LC and HCC (**D**) groups. The red points indicated up-regulated lncRNAs, while the blue points represented down-regulated lncRNAs. The grey points represented non-significant differently expressed lncRNAs. (**E** and **F**) GO enrichment and KEGG pathway analyses of the identified lncRNAs. The depth of color and size of black spots denote the adjusted p-value and lncRNA quantities, respectively.
Abbreviations: DELs, differentially expressed lncRNAs; CHB, chronic hepatitis B; LF, liver fibrosis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; lncRNA, long non-coding RNA; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

clustering heat maps, Venn diagrams, and volcano maps (Figure 1A–D). We identified 21 DE-lncRNAs for further analyses using target gene prediction among 33 differentially expressed mRNAs. Subsequently, Gene Ontology function analysis of the predicted target mRNAs indicated that the functions were primarily related to extracellular matrix organization, cytoplasm, and protein binding (Figure 1E). In addition, Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the targeted differentially expressed mRNAs revealed 20 related pathways ($P < 0.05$), which were mainly involved in the PI3K-Akt signaling pathway, pathways in cancer, focal adhesion, and the Jak-STAT signaling pathway (Figure 1F). Finally, among the 3 HBV-associated disease groups, 8 lncRNAs with gradually increasing expression (ADAMTSL4-AS1, AC067931, SOCS2-AS1, AL731577, TPM1-AS, AL356356, AC037198, and AL161773) and 1 lncRNA with gradually decreasing expression (AP002026) were selected for further study through targeting of differentially expressed mRNAs that were enriched in the abovementioned tumor-related pathways.

Verification of Candidate lncRNAs in PBMCs, Tissues, and Cells

We performed qRT-PCR in PBMCs obtained from 49 patients with CHB, 44 patients with HBV-associated LC, and 56 patients with HBV-associated HCC. The baseline characteristics of the enrolled subjects in this validated cohort are

Table 2 Clinical and Laboratory Characteristics of the Paticipants

Variables	CHB	LC	HCC	F/Z/X2	P
	(N=49)	(N=44)	(N=56)		
Age(years)	41.4±9.8	52.5±11.4*	56.5±9.6*	44.512	<0.001
Gender(male), n(%)	35(71.4)	33(75)	48(81.4)	1.396	0.498
AFP(ng/mL)	3.3 (2.1–4.9)	2.9 (1.8–5.6)	14.2 (3.2–421.8)*#	30.011	<0.001
HBsAg(log_{10} IU/mL)	3.4(2.8–3.8)	3.0(2.8–3.1)*	3.0(2.5–3.2)*	11.047	0.004
HBeAg	0.7(0.4–9.5)	0.6(0.0–3.8)	0.5(0.0–1.1)*	7.928	0.019
HBVDNA(log_{10} IU/mL)	1.3(1.3–1.4)	1.3(1.3–1.3)	2.4(1.3–5.0)*#	22.331	<0.001
RBC(10^{12}/L)	4.6±0.5	3.5±1.0*	3.9±0.7*	38.267	<0.001
HGB(g/L)	144.0(130.6–153.0)	101.5(79.3–140.5)*	128.0(104.0–135.0)*	34.407	<0.001
WBC(10^9/L)	5.3(4.2–6.5)	3.5(1.9–5.0)*	4.6(3.5–6.5)#	15.595	<0.001
PLT(10^9/L)	197.0(154.3–235.4)	70.5(44.3–103.3)*	115.0(65.0–150.0)*#	61.588	<0.001
INR	1.1(1.0–1.1)	1.3(1.2–1.7)*	1.2(1.1–1.3)*#	50.548	<0.001
PT(s)	12.0(11.2–12.8)	16,4(14.0–19.5)*	13.6(12.5–14.9)*#	61.751	<0.001
ALT(U/L)	22.0(16.5–37.5)	24.0(14.0–32.8)	41.3(25.0–73.0)*#	18.339	<0.001
AST(U/L)	20.0(18.0–28.5)	31.0(22.8–38.5)*	39.0(29.5–101.0)*#	41.463	<0.001
ALP(U/L)	75.0(62.5–85.0)	70.0(54.0–101.5)	118.0(85.0–185.0)*#	38.257	<0.001
GGT(U/L)	26.0(17.0–36.0)	30.0(14.5–54.0)	73.0(33.4–235.0)*#	31.588	<0.001
TBIL(μmol/L)	15.9(11.2–20.2)	32.2(16.0–50.5)*	20.4(12.9–33.5)*#	23.846	<0.001
DBIL(μmol/L)	4.1(3.0–5.1)	10.2(5.8–30.3)*	9.3(5.2–18.5)*	48.538	<0.001
TP(g/L)	73.9(70.7–76.7)	63.4(54.8–70.6)*	67.1(61.9–74.7)*#	32.694	<0.001
ALB(g/L)	46.7(44.0–48.8)	37.6(33.5–43.3)*	36.3(32.0–40.5)*	59.000	<0.001
CEA(ng/mL)	1.7(1.1–2.5)	2.9(1.7–4.4)*	3.1(1.8–4.3)*	25.349	<0.001
CA199(U/mL)	13.1(5.5–15.8)	18.2(10.6–34.8)*	39.8(20.6–92.1)*#	34.645	<0.001
CA125(U/mL)	11.2(8.9–14.2)	21.1(16.2–33.3)*	32.4(12.0–151.5)*	33.798	<0.001

Notes: Date are presented as means(SD) or median(interquartile range) or percentage. *P<0.05 vs chronic hepatitis B; #P<0.05 vs liver cirrhosis.

Abbreviations: CHB; chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; AFP, alpha fetoprotein; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBVDNA, hepatitis B virus deoxyribonucleic acid; RBC, red blood cell; HGB, hemoglobin; WBC, white blood cell; PLT, platelet; INR, international normalized ratio; PT, prothrombin time; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; TBIL, total bilirubin; DBIL, direct bilirubin; TP, total protein; ALB, albumin; CEA, carcinoembryonic antigen; CA199, carbohydrate antigen 199; CA125, carbohydrate antigen 125.

shown in Table 2. There were significant differences in biochemical, hematological, and coagulation parameters and HBV DNA levels among the 3 HBV-associated liver diseases. Additionally, the levels of AFP, carcinoembryonic antigen, CA199, and CA125 were significantly higher in the HCC group than in the non-HCC group. Moreover, the expression levels of the lncRNAs ADAMTSL4-AS1, AC067931, SOCS2-AS1, and AL731577 significantly and gradually increased in the 3 groups (Figure 2A–D). On the other hand, the expression levels of the lncRNAs TPM1-AS, AL356356, AC0137198, and AP002026 either had inconsistent trends or lacked statistical significance. The expression level of

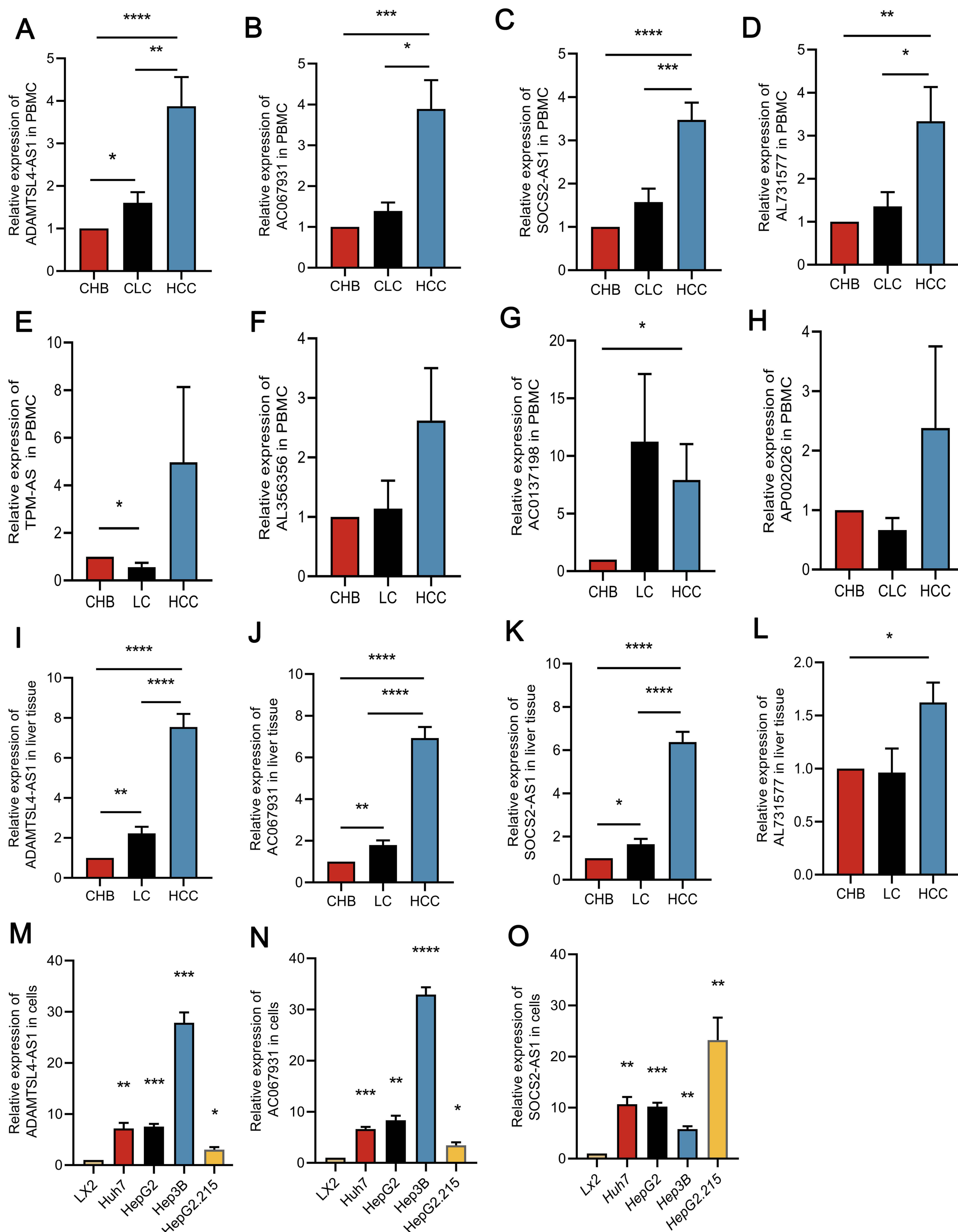

Figure 2 The relative expression profile of the selected lncRNAs in CHB patients, HBV-associated LC patients, and HBV-associated HCC patients. Relative lncRNA expression levels in PBMC samples were detected by qRT-PCR. (**A**) ADAMTSL4-AS1, (**B**) AC067931, (**C**) SOCS2-AS1, (**D**) AL731577, (**E**) TPM-AS, (**F**) AL356356, (**G**) AC037198, (**H**) AP002026. The expression levels of related lncRNAs in liver tissues were detected by qRT-PCR. (**I**) ADAMTSL4-AS1, (**J**) AC067931, (**K**) SOCS2-AS1, (**L**) AL731577. Relative expression levels of lncRNAs were upregulated in four human HCC cells(Huh7, HepG2, Hep3B, HepG2.215) comparison with Lx2 cells determining by qRT-PCR. (**M**) ADAMTSL4-AS1, (**N**) AC067931, (**O**) SOCS2-AS1. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

AL161773 in PBMCs was too low to be detected (Figure 2E–H). The expression levels of the candidate lncRNAs ADAMTSL4-AS1, AC067931, and SOCS2-AS1 were further confirmed to be increased in liver tissues on qRT-PCR (n = 5 per group), however, AL161773 was excluded due to inconsistent trends and lack of statistical significance (Figure 2I–L). Moreover, the expression levels of these 3 candidate lncRNAs were remarkably higher in Huh7, HepG2, Hep3B, and HepG2.215 cells than in LX2 cells (normal hepatic stellate cell line) (Figure 2M–O). Collectively, these results indicated that ADAMTSL4-AS1, AC067931, and SOCS2-AS1 should be further studied as biological markers for HCC.

Relationship Between ADAMTSL4-AS1, AC067931, and SOCS2-AS1 Expressions in PBMCs and Clinical Characteristics in Patients with HBV-Associated HCC

To assess the correlation between DE-lncRNA expressions and various clinicopathological data, we divided patients with HCC into a high expression group (n = 28) and low expression group (n = 28) based on the median expression values of ADAMTSL4-AS1, AC067931, and SOCS2-AS1 separately. We found that the expression of ADAMTSL4-AS1 in the PBMCs of patients with HBV-associated HCC was not significantly correlated with various clinicopathologic features ($P > 0.05$). However, the expression of AC067931 was closely associated with clinical stage ($P = 0.033$). Moreover, the expression of SOCS2-AS1 in PBMCs was distinctly relevant to vascular invasion ($P = 0.011$). Nevertheless, other characteristics did not show any significant differences or associations (Table 3).

Table 3 Correlation of Clinicopathological Variables with the Expression of ADAMTSL4-AS1, AC067931 and SOCS2-AS1 in PBMCs from HBV-Associated HCC Patients

Variables	N	ADAMTSL4-AS1		P	AC067931		P	SOCS2-AS1		P
		Low	High		Low	High		Low	High	
Age										
≤50y	12	6	6	1.000	7	5	0.515	7	5	0.515
>50y	44	22	22		21	23		21	23	
Gender										
Male	47	25	22	0.467	24	23	1.000	23	24	1.000
Female	9	3	6		4	5		5	4	
AFP										
>7ng/mL	35	16	19	0.408	18	17	0.783	16	19	0.408
≤7ng/mL	21	12	9		10	11		12	9	
Tumor size										
>5cm	21	11	10	0.783	11	10	0.830	12	10	0.584
≤5cm	35	17	18		16	18		16	18	
Clinical stage										
I-II	28	13	15	0.593	10	18	0.033	12	16	0.285
III-IV	28	15	13		18	10		16	12	
Vascular invasion										
Yes	22	11	11	1.000	13	9	0.274	14	8	0.011
No	34	17	17		15	19		14	20	
Tumor number										
=1	22	10	12	0.584	10	12	0.584	10	12	0.584
>1	34	18	16		18	16		18	16	
Lymph node metastasis										
Yes	5	2	3	1.000	3	2	1.000	3	2	1.000
No	51	26	25		25	26		25	26	
Distant metastasis										
Yes	4	1	3	0.604	1	3	0.604	2	3	0.656
No	52	27	25		27	25		26	26	

Abbreviation: AFP, alpha fetoprotein.

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Diagnostic Performance of ADAMTSL4-AS1, AC067931, and SOCS2-AS1 in HBV-Associated HCC

ROC curve analysis was used to assess the potential diagnostic performance of the 3 lncRNAs ADAMTSL4-AS1, AC067931, and SOCS2-AS1, and AFP for HBV-associated HCC. As shown in Figure 3A–C and Table 4, ADAMTSL4-AS1, AC067931, and SOCS2-AS1 exhibited area under the curve (AUC) values of 0.833 (95% CI: 0.747–0.898), 0.819 (95% CI: 0.732–0.887), and 0.841 (95% CI: 0.757–0.905), respectively, for distinguishing patients with HBV-associated HCC from those with chronic HBV infection, and the corresponding sensitivities were 92.9%, 92.9%, and 91.1% and specificities were 61.2%, 61.2%, and 65.3%, respectively. The combination of the 3 lncRNAs showed better diagnostic power than that of the individual lncRNAs as the AUC reached 0.865 (95% CI: 0.785–0.924), with a sensitivity of 94.6% and specificity of 63.3%. However, the widely used HCC biomarker AFP showed limited diagnostic efficacy as the AUC was 0.769 (95% CI: 0.676–0.845), with a low sensitivity of 55.4% and specificity of 98%. The accuracy for differentiating patients with HBV-associated HCC from patients with CHB was higher when using the 3 lncRNAs alone or in combination than when using AFP, with obviously increased sensitivity and decreased specificity. Furthermore, we analyzed the combination of the 3 lncRNAs with AFP for HBV-associated HCC, and noted better diagnostic performance when using this combination than when using the 3 lncRNAs or AFP alone as the AUC reached 0.945 (95% CI: 0.883–0.980), with a high sensitivity of 83.9% and high specificity of 89.8%. Thus, the combination of ADAMTSL4-AS1, AC067931, and SOCS2-AS1 with AFP may be a novel biomarker of HBV-associated HCC.

In addition, the diagnostic roles of ADAMTSL4-AS1, AC067931, SOCS2-AS1, AFP, and the combination of the 3 lncRNAs were further investigated for discriminating patients with HCC from patients with cirrhosis and those with the combination of chronic HBV infection and cirrhosis. The AUC values of ADAMTSL4-AS1, AC067931, SOCS2-AS1, AFP, and the combination of the 3 lncRNAs between patients with HCC and those with cirrhosis were 0.752 (95% CI: 0.656–0.833), 0.761 (95% CI: 0.665–0.840), 0.757 (95% CI: 0.661–0.860), 0.783 (95% CI: 0.690–0.860), and 0.762

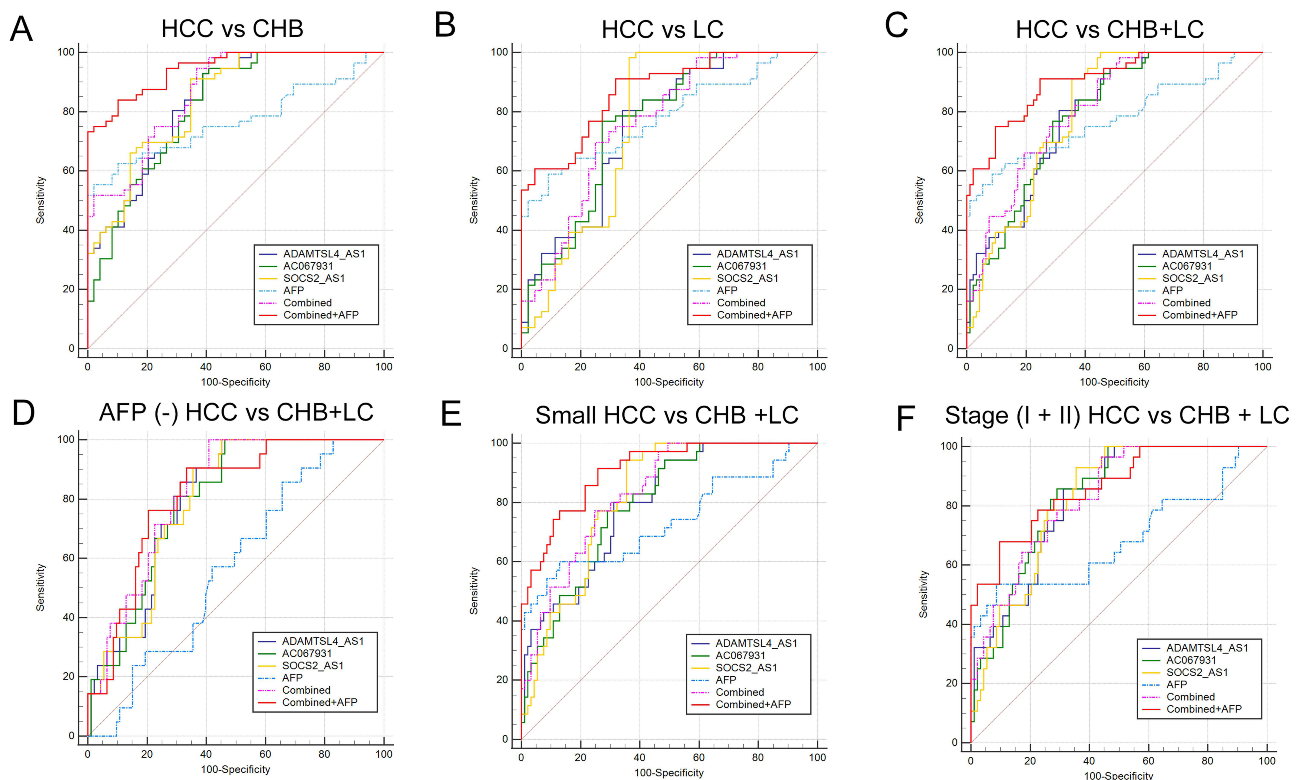

Figure 3 Area under receiver operating characteristic curves (AUROCs) of lncRNAs and AFP for differentiating the HBV-related HCC group from the HBV-associated with non-HCC groups. (**A**) HCC from CHB (**B**) HCC from LC (**C**) HCC from CHB+LC. ROC curve analysis for the individual lncRNAs or AFP or their combinations in the diagnosis of (**D**) AFP negative HCC patients, (**E**) small HCC patients and (**F**) early-stage HCC patients from CHB+LC.

Table 4 Differential Diagnostic Efficacy of PBMC ADAMTSL4-AS1, AC067931, SOCS2-AS1, AFP, the Combined and the Combined+AFP

Group	Marker	AUC	P value	95% CI	SEN(%)	SPE(%)
HCC vs CHB	ADAMTSL4-AS1	0.833	<0.001	0.747–0.898	92.9	61.2
	AC067931	0.819	<0.001	0.732–0.887	92.9	61.2
	SOCS2-AS1	0.841	<0.001	0.757–0.905	91.1	65.3
	AFP	0.769	<0.001	0.676–0.845	55.4	98
	Combined	0.865	<0.001	0.785–0.924	94.6	63.3
	Combined+AFP	0.945	<0.001	0.883–0.980	83.9	89.8
HCC vs LC	ADAMTSL4-AS1	0.752	<0.001	0.656–0.833	80.4	65.9
	AC067931	0.761	<0.001	0.665–0.840	76.8	72.7
	SOCS2-AS1	0.757	<0.001	0.661–0.837	98.2	63.6
	AFP	0.783	<0.001	0.690–0860	58.9	90.9
	Combined	0.762	<0.001	0.666–0.841	69.6	75.0
	Combined+AFP	0.871	<0.001	0.789–0.929	91.1	68.2
HCC vs CHB+LC	ADAMTSL4-AS1	0.795	<0.001	0.721–0.856	80.4	68.8
	AC067931	0.791	<0.001	0.717–0.853	76.8	71.0
	SOCS2-AS1	0.802	<0.001	0.729–0.862	91.1	64.5
	AFP	0.776	<0.001	0.700–0.840	58.9	91.4
	Combined	0.813	<0.001	0.741–0.872	75.0	72.0
	Combined+AFP	0.905	<0.001	0.846–0.947	91.1	75.3

Abbreviations: AFP, alpha fetoprotein. CHB; chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; AUC, area under the curve; CI, confidence interval; SEN, sensitivity; SPE, specificity.

(95% CI: 0.666–0.841), respectively, with sensitivities of 80.4%, 76.8%, 98.2%, 58.9%, and 69.6% and specificities of 65.9%, 72.7%, 63.6%, 90.9%, and 75.0%, respectively. Moreover, the AUC values of ADAMTSL4-AS1, AC067931, SOCS2-AS1, AFP, and the combination of the 3 lncRNAs between patients with HCC and those with the combination of chronic HBV infection and cirrhosis were 0.795 (95% CI: 0.721–0.856), 0.791 (95% CI: 0.717–0.853), 0.802 (95% CI: 0.729–0.862), 0.776 (95% CI: 0.700–0.840), and 0.813 (95% CI: 0.741–0.872), respectively, with sensitivities of 80.4%, 76.8%, 91.1%, 58.9%, and 75.0% and specificities of 68.8%, 71.0%, 64.5%, 91.4%, and 72.0%, respectively. ADAMTSL4-AS1, AC067931, SOCS2-AS1, and the combination of the 3 lncRNAs were potentially better diagnostic tools than AFP for distinguishing patients with HCC from patients with chronic HBV infection and those with the combination of chronic HBV infection and cirrhosis when compared to discriminating patients with HCC from patients with cirrhosis. The diagnostic accuracy of the combination of the 3 lncRNAs with AFP was further analyzed for discriminating patients with HCC from patients with cirrhosis and those with the combination of chronic HBV infection and cirrhosis. The combination of the 3 lncRNAs with AFP exhibited an AUC of 0.871 (95% CI: 0.789–0.929) for discriminating patients with HCC from those with cirrhosis, and the corresponding sensitivity and specificity were 91.1% and 68.2%, respectively. Moreover, the combination of the 3 lncRNAs with AFP exhibited good diagnostic value with an AUC of 0.905 (95% CI: 0.846–0.947) for distinguishing patients with HCC from those with the combination of chronic HBV infection and cirrhosis, and the corresponding sensitivity and specificity were 91.1% and 75.3%, respectively. Thus, the combination of the 3 lncRNAs with AFP could be a valid diagnostic marker for HBV-associated HCC.

Diagnostic Performance of ADAMTSL4-AS1, AC067931, and SOCS2-AS1 in AFP-Negative, Small, and Early Stage HCC

The diagnostic performances of ADAMTSL4-AS1, AC067931, SOCS2-AS1, AFP, the combination of the 3 lncRNAs, and the combination of the 3 lncRNAs with AFP were further investigated in AFP-negative HCC (AFP < 20 ng/mL), small HCC (size < 5 cm), and early stage HCC. As shown in Figure 3D–F and Table 5, for distinguishing patients with AFP-negative HCC from those with the combination of chronic HBV infection and cirrhosis, the AUC values of

Table 5 Diagnostic Efficacy of Individual PBMC lncRNAs or AFP or Their Combinations in AFP(-), Small, and Early-Stage HCC Patients

Group	Marker	AUC	P value	95% CI	SEN(%)	SPE(%)
AFP (-) HCC vs CHB+LC	ADAMTSL4-AS1	0.800	<0.001	0.715–0.869	100	54.8
	AC067931	0.797	<0.001	0.712–0.867	100	53.8
	SOCS2-AS1	0.795	<0.001	0.709–0.865	90.5	64.5
	AFP	0.570	0.2508	0.474–0.662	85.7	34.4
	Combined	0.825	<0.001	0.743–0.890	100	59.1
	Combined+AFP	0.814	<0.001	0.730–0.881	90.5	66.7
Small HCC vs CHB +LC	ADAMTSL4-AS1	0.797	<0.001	0.716–0.863	80.0	68.8
	AC067931	0.794	<0.001	0.714–0.861	77.1	71.0
	SOCS2-AS1	0.819	<0.001	0.742–0.882	94.3	64.5
	AFP	0.736	<0.001	0.651–0.810	60.0	87.1
	Combined	0.833	<0.001	0.757–0.893	77.1	75.3
	Combined+AFP	0.909	<0.001	0.845–0.952	91.4	74.2
Stage (I + II) HCC vs CHB + LC	ADAMTSL4-AS1	0.820	<0.001	0.740–0.884	85.7	68.8
	AC067931	0.831	<0.001	0.752–0.893	85.7	71.0
	SOCS2-AS1	0.825	<0.001	0.745–0.888	92.9	64.5
	AFP	0.685	0.0060	0.594–0.766	53.6	91.4
	Combined	0.831	<0.001	0.752–0.893	96.4	55.9
	Combined+AFP	0.863	<0.001	0.789–0.919	67.9	90.3

Abbreviations: AFP, alpha fetoprotein. CHB; chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; AUC, area under the curve; CI, confidence interval; SEN, sensitivity; SPE, specificity.

ADAMTSL4-AS1, AC067931, SOCS2-AS1, AFP, the combination of the 3 lncRNAs, and the combination of the 3 lncRNAs with AFP were 0.800 (95% CI: 0.715–0.869), 0.797 (95% CI: 0.712–0.867), 0.795 (95% CI: 0.709–0.865), 0.570 (95% CI: 0.474–0.662), 0.825 (95% CI: 0.743–0.890), and 0.814 (95% CI: 0.730–0.881), respectively, with sensitivities of 100%, 100%, 90.5%, 85.7%, 100%, and 90.5% and specificities of 54.8%, 53.8%, 64.5%, 34.4%, 59.1%, and 66.7%, respectively. Moreover, for distinguishing patients with small HCC from those with the combination of chronic HBV infection and cirrhosis, the AUC values of ADAMTSL4-AS1, AC067931, SOCS2-AS1, AFP, the combination of the 3 lncRNAs, and the combination of the 3 lncRNAs with AFP were 0.797 (95% CI: 0.716–0.863), 0.794 (95% CI: 0.714–0.861), 0.819 (95% CI: 0.742–0.882), 0.736 (95% CI: 0.651–0.810), 0.833 (95% CI: 0.757–0.893), and 0.909 (95% CI: 0.845–0.952), respectively, with sensitivities of 80.0%, 77.1%, 94.3%, 60.0%, 77.1%, and 91.4% and specificities of 68.8%, 71.0%, 64.5%, 87.1%, 75.3%, and 74.2%, respectively. Furthermore, for distinguishing patients with early stage (stage I + II) HCC from those with the combination of chronic HBV infection and cirrhosis, the AUC values of ADAMTSL4-AS1, AC067931, SOCS2-AS1, AFP, the combination of the 3 lncRNAs, and the combination of the 3 lncRNAs with AFP were 0.820 (95% CI: 0.740–0.884), 0.831 (95% CI: 0.752–0.893), 0.825 (95% CI: 0.745–0.888), 0.685 (95% CI: 0.594–0.766), 0.831 (95% CI: 0.752–0.893), and 0.863 (95% CI: 0.789–0.919), respectively, with sensitivities of 85.7%, 85.7%, 92.9%, 53.6%, 96.4%, and 67.9% and specificities of 68.8%, 71.0%, 64.5%, 91.4%, 55.9%, and 90.3%, respectively. Obviously, the diagnostic accuracy of the combination of the 3 lncRNAs with AFP was significantly better than that of each lncRNA alone or AFP alone for the diagnosis of AFP-negative, small, and early stage HCC related to HBV infection.

Discussion

HCC is highly invasive and metastatic, and has a low survival rate and high recurrence rate.[20] Thus, the early diagnosis of HCC is essential to improve prognosis and long-term survival.[21,22] Currently, there is no effective diagnostic marker in the clinic for HBV-associated HCC, and the screening of small, early stage, and AFP-negative tumors remains a challenge.[23] AFP remains the most commonly used biomarker for HCC, despite its inadequate sensitivity and

specificity.[24] Thus, it is imperative to identify novel diagnostic markers for HBV-associated HCC that have higher sensitivity and specificity.

With the rapid development of bioinformatics and sequencing technology, lncRNAs have drawn increasing attention as molecular markers owing to their roles in the progression of many tumors, including HCC.[25] Wang et al showed that Lnc-MyD88 had great diagnostic value for HBV-associated HCC and AFP-negative HCC, with higher efficacy when combined with AFP.[26] Mo et al found that LINC01793 could distinguish patients with HCC from healthy controls, patients with CHB, and patients with LC, and mentioned that it could be a noninvasive biomarker of HCC and AFP-negative HCC, especially when combined with AFP.[27] Wang et al showed that the serum levels of LINuc001ncr and LINAX800134 can diagnose HBV-associated HCC and early stage HCC.[28] In summary, increasing numbers of lncRNAs in the serum or plasma have been shown to be diagnostic biomarkers for HCC; however, lncRNAs in PBMCs have not been well understood as diagnostic biomarkers for HCC. Compared with other human organs, the liver has a unique immune structure. It has been verified that PBMCs, including lymphocytes and monocytes, are strongly connected with the occurrence, development, and malignancy of tumors caused by immune dysfunction.[29] Thus, the gene expression of PBMCs has recently been shown to have important clinical value in diagnosing HCC. For instance, Kunadirek et al reported that MIR4435-2HG, lnc-POLD3-2, and their combination were sensitive biomarkers for discriminating HBV-associated HCC from non-HCC, particularly among individuals with normal serum AFP levels.[30] Hu et al showed that CLDN18 in PBMCs was a potential prognostic marker and immunotherapeutic target for HCC.[31] Moreover, lncRNAs are emerging as essential regulators involved in *immune checkpoint regulation and* resistance to *immunotherapy*.[32] Thus, lncRNAs in PBMCs have been receiving increasing attention from researchers. In *our study, we attempted to* identify lncRNAs as diagnostic biomarkers for HBV-associated HCC using PBMCs obtained from 3 groups of patients.

Through bioinformatics analysis, we identified 8 progressively elevated lncRNAs and 1 gradually downregulated lncRNA during the progression of HBV-associated liver diseases. We also discovered that these lncRNAs were associated with several signaling pathways, such as the PI3K-Akt signaling pathway, pathways in cancer, focal adhesion, and the Jak-STAT signaling pathway, indicating that lncRNAs may contribute to the occurrence and progression of tumors. Moreover, data from qRT-PCR demonstrated for the first time that ADAMTSL4-AS1, AC067931, and SOCS2-AS1 were highly expressed in liver tissues and PBMCs over the course of chronic HBV infection, ranging from cases of CHB to HCC, and in HCC cell lines. To our knowledge, ADAMTSL4-AS1 and AC067931 have never been studied in HCC previously. In our study, we demonstrated for the first time that ADAMTSL4-AS1 and AC067931 were upregulated in HCC tissues, cells, and PBMCs in patients with HBV-associated HCC. Previous studies have found that SOCS2-AS1 was upregulated in many tumor tissues and promoted tumor progression by enhancing cell proliferation, *migration, and* invasion, *and inhibiting cell apoptosis*. Wu et al showed that the lncRNA SOCS2-AS1 was significantly upregulated in gliomas and that it promoted the progression of gliomas by regulating ITGB1 expression.[33] Zhang et al demonstrated that the lncRNA SOCS2-AS1 was highly expressed in papillary thyroid cancer and that patients with a high level of SOCS2-AS1 had a poor survival rate, with enhanced PTC cell proliferation through the destabilization of p53 protein.[34] Misawa et al demonstrated that SOCS2-AS1 contributed to the development of castration-resistant prostate cancer by inhibiting apoptosis.[35] Recently, Zhong et al reported that the expression of SOCS2-AS1 was low in NANOG+ HCC stem cells and that patients with a high level of SOCS2-AS1 had a poor prognosis through the regulation of miR-454-3p/CPEB1.[36] Although this result is not consistent with our finding, our study performed assessments in tissues, liver cells, and PBMCs, rather than in NANOG+ HCC stem cells.

Further ROC curve analysis confirmed that ADAMTSL4-AS1, AC067931, SOCS2-AS1, and the combination of the 3 lncRNAs had better diagnostic efficiency than AFP for distinguishing patients with HCC from patients with chronic HBV infection and those with the combination of chronic HBV infection and cirrhosis when compared to discriminating patients with HCC from patients with cirrhosis, with obviously better sensitivity and slightly worse specificity. In terms of clinical diagnosis, a single tumor biomarker has some limitations owing to the complicated *pathological* alterations associated with *the onset and progression of* cancer.[37] Thus, to reduce the drawbacks of the low sensitivity and specificity of a single lncRNA for diagnosis, we further explored the diagnostic value of the combination of ADAMTSL4-AS1, AC067931, SOCS2-AS1 with AFP. This combination

of the 3 lncRNAs with AFP could distinguish between patients with HCC and those with other benign liver diseases. Furthermore, this combination had high diagnostic accuracy for the detection of small, early stage, and AFP-negative HCC. As the aim of this study was to explore novel diagnostic markers for HBV-associated HCC, all included patients with LC and HCC had a history of chronic HBV infection. In summary, the combination of ADAMTSL4-AS1, AC067931, SOCS2-AS1, and AFP may serve as a potential noninvasive biomarker for the diagnosis of HBV-associated HCC.

Our study has several limitations. First, the clinical sample size was small. We need to recruit more patients, and the diagnostic efficacy of the identified lncRNAs should be confirmed in another validation set with more subjects. Second, the expression levels of DE-lncRNAs in the PBMCs of patients with HBV-associated HCC were not clear before and after surgical resection. We should analyze these expression levels in further studies. Moreover, further confirmation is needed through in vitro and in vivo experiments to study the immunomodulatory effects of ADAMTSL4-AS1, AC067931, and SOCS2-AS1 on HCC, and the mechanism in the context of the tumor immune environment needs to be further explored.

Conclusion

Our study revealed for the first time that ADAMTSL4-AS1, AC067931, and SOCS2-AS1 were significantly upregulated in the PBMCs and tumor tissues of patients with HBV-associated HCC, and that the combination of the 3 lncRNAs with AFP could be an innovative and promising diagnostic biomarker for HBV-associated HCC, especially small, early stage, and AFP-negative HCC.

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Disclosure

The authors declare no conflicts of interest in this work.

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