Upregulation of liver kinase B1 predicts poor prognosis in hepatocellular carcinoma

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Abstract. The majority of studies report that liver kinase B1 (LKB1) acts as a tumor suppressor by inhibiting cell proliferation and metastasis. The present study investigated the expression pattern of LKB1 in 2 cohorts of paired hepatocellular carcinoma (HCC) and analogous non-cancerous tissues (ANT). The results indicated that LKB1 was upregulated in HCC vs. ANT tissues, and that high expression of LKB1 was associated with a higher number of tumor foci, larger tumor size, poorer tumor differentiation, Edmondson-Steiner grade, Barcelona Clinic Liver Cancer grade and tumor-node-metastasis stage. Furthermore, high LKB1 expression was associated with poor overall survival (OS), shorter disease-free survival and early recurrence. Univariate and multivariate analyses demonstrated that high LKB1 expression may serve as an independent prognostic marker for OS, but not for recurrence. In addition, knockdown of LKB1 expression in HCC cell lines inhibited cell proliferation and subcutaneous tumor growth by promoting cell apoptosis. Therefore, the findings of the present study suggest a proto-oncogenic role of LKB1 in HCC.

Introduction

Liver kinase B1 (LKB1), also referred to as serine threonine kinase 11, plays diverse roles in cellular proliferation, energy metabolism, apoptosis and polarity, by regulating a variety of substrates. LKB1 activates at least 14 adenosine monophosphate (AMP)-activated protein kinase (AMPK)-associated kinases, and the most extensively investigated substrate is AMPK (1). LKB1/AMPK is activated when the AMP/ATP ratio is high under energy stress conditions, and restores intracellular ATP levels by stimulating catabolic and inhibiting anabolic pathways (2). Studies on LKB1 in cancer have demonstrated its role as a master tumor suppressor in the majority of human cancer types, including melanoma (3), non-small-cell lung carcinoma (4) and other epithelial cancer types (5). However, recent studies have reported that LKB1 acts as a proto-oncogene in certain types of cancer. Bardeesy *et al* (6) indicated that LKB1^{-/-} mouse embryonic fibroblasts were resistant to transformation by activated Ha-Ras, either alone or with immortalizing genes. Jeon *et al* (7) reported that knockdown of LKB1 and AMPK in breast cancer cells attenuated tumor development due to failure to inhibit acetyl-CoA carboxylase activity and to maintain intracellular NADPH levels. Furthermore, Martinez-Lopez *et al* (8) reported that glycine N-methyltransferase (GNMT) knockout mice may develop hepatocellular carcinoma (HCC). Reduced expression of GNMT in mouse and human HCC cells increased the activity of LKB1 and RAS. Lee *et al* (9) demonstrated that the stabilization and activation of LKB1/STE20-related kinase adaptor α (STRADA)/scaffolding mouse 25 (MO25) complex by S-phase kinase-associated protein 2-dependent ubiquitination was crucial for cell survival under energy stress conditions. They also indicated that LKB1 was highly expressed in late-stage HCC and its overexpression predicts poor survival outcomes. Furthermore, a study by Huang *et al* (10) suggested that the expression of LKB1 was decreased in HCC patients, and that low LKB1 expression predicted poor survival. Due to these contradicting results, the aim of the present study was to elucidate the role of LKB1 in HCC.

Materials and methods

Patients, specimens and follow-up. In the present study, two independent cohorts of patients who underwent curative resection at the Hepatic Surgery Center of Tongji Hospital of Huazhong University of Science and Technology (Wuhan, China) between January 2004 and January 2014 were enrolled. For cohort 1, a total of 229 HCC tissues and matched surrounding analogous non-cancerous tissues (ANT) were collected for immunohistochemical (IHC) analysis; these patients were diagnosed with liver tumors, hepatectomy was

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performed and pathological analysis confirmed the diagnosis of HCC. Complete clinicopathological data and follow-up results were acquired for this cohort. Cohort 2, lacking follow-up data, included 60 HCC samples and matched ANT for western blot analysis of LKB1 expression. Furthermore, the level of phosphorylated (p)-AMPK (Thr172) was measured to elucidate the activation of downstream signaling in 10 pairs of ANT and HCC samples. The preoperative diagnosis of HCC was performed according to the diagnostic criteria of the American Association for the Study of Liver Diseases (11). All the patients were followed-up until October 2014, with a median survival time of 23.30±0.97 months. Overall survival was defined as the time interval between the date of surgery and the date of death or the last follow-up. Disease-free survival was defined as the time interval between the date of surgery and the date of recurrence confirmed by abdominal ultrasound examinations and serum α-fetoprotein levels. If no recurrence was diagnosed, patients were censored on the date of death or the last follow-up. The median disease-free survival time was 17.02±0.98 months. The present study was approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China). The study protocol conformed to the principles outlined in the Declaration of Helsinki and written informed consent was obtained from each patient.

IHC. Formalin-fixed, paraffin-embedded tissues were sectioned at 2 μm, deparaffinized in xylene and rehydrated through a graded series of ethanol. Antigen retrieval was performed by microwave heating in 10 mM Tris base and 1 mM EDTA (pH 9.0). Endogenous peroxidase was blocked with 3% H_2O_2 in methanol. The sections were then incubated with primary antibody at 4˚C overnight (Table I). A Dako EnVision kit (Dako, Glostrup, Denmark) was used for incubation with the secondary antibody (Table I) and detection of peroxidase activity. Hematoxylin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to counterstain the nuclei for 5 min at room temperature. IHC scores were obtained by multiplying the percentage score with the intensity score of positively stained cells, as described previously (12). Scoring was performed by two certified pathologists independently, who were blinded to the patients' clinical and demographic information. The expression status is represented by the mean of several independent readings. An overall score of >6 and ≤6 was considered to indicate high and low expression, respectively. The Edmondson-Steiner, Barcelona Clinic Liver Cancer (BCLC) and tumor-node-metastasis (TNM) stages were also determined (13,14).

Cell lines and culture. The HCC cell lines MHCC97L, MHCC97H and HCCLM3 were obtained from the Liver Cancer Institute of Zhongshan Hospital (Fudan University, Shanghai, China). The HCC cell lines HLE and HLF were kindly provided by Shanshan Wang and Gang Li (Department of Molecular Biology, Peking University Health Science Center, Beijing, China). The hepatoblastoma cell line HepG2, and the HCC cell lines Hep3B, Huh7 and SK-Hep1 were purchased from the China Center for Type Culture Collection (Wuhan, China). The HCC cell line PLC/PRF-5 was purchased from the cell bank of the Chinese Academy

of Sciences (Shanghai, China). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37˚C.

Western blot analysis. HCC cell lines and samples were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) with proteinase and phosphatase inhibitor cocktail (Hoffmann-La Roche Ltd., Basel, Switzerland), and the protein concentration was determined by using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). A total of 20 μg of each protein was separated by 10% SDS-PAGE (Boster Biotechnology, Wuhan, China) and transferred to a polyvinylidene difluoride membrane (Hoffmann-La Roche Ltd.). The membrane was blocked with 5% skimmed milk dissolved by 1X Tris-buffered saline containing Tween-20 and incubated with specific primary antibodies at 4˚C overnight (Table I), followed by incubation with a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 37˚C for 2 h (Table I). Detection was performed using a ChemiDoc™ Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Lentivirus production, transfection and establishment of stable cell clones. The pLKO.1-TRC cloning vector (cat. no. 10878) was from Addgene, Inc. (Cambridge, MA, USA). Small hairpin (sh)RNA specific for LKB1 (shLKB1) oligos were synthesized by Tsingke Technology (Wuhan, China) and were inserted into the pLKO.1 vector, which was then transfected into 293 cells with psPAX2 and pMD2.G (cat. nos. 12260 and 12259, respectively; Addgene, Inc.) using X-tremeGENE™ HP DNA Transfection Reagent (Sigma-Aldrich; Merck KGaA). After 48 h of incubation, the virus-containing supernatant was collected and filtered through a 0.45-μm filter (PALL, Port Washington, NY, USA) (15). LKB1 overexpression lentivirus was purchased from Genecreate Technology (Wuhan, China). HCC cells were transfected with lentiviral particles in the presence of 8 μg/ml polybrene (Sigma-Aldrich; Merck KGaA) with a multiplicity of infection (MOI) ranging from 50 to 100. At 72 h after transfection, cells were selected with 5 μg/ml puromycin (Merck Calbiochem, Darmstadt, Germany) for 2 weeks. Selected pools of LKB1-knockdown or overexpressing cells were used for the subsequent experiments. The shRNA sequences are listed in Table II. HCC-LM3 shLKB1 and Huh7 shLKB1 refer to the HCC cell lines transfected with shLKB1 (LKB1 knockdown), whereas HLF LKB1 refers to the cell lines transfected with LKB1 overexpression virus. Control cells were transfected with empty vector.

Cell proliferation assay. HCC-LM3 shLKB1, HLF-LKB1 cells (1x10³ cells/well) or Huh-7 shLKB1 cells (3x10³ cells/well) and the same amount of control cells were seeded into 96-well plates. At the indicated time points, Cell Counting Kit-8 reagent (Beyotime Institute of Biotechnology) was added, followed by incubation for 1 h at 37˚C. The plate was read

Table I. Antibodies used in this study.

Antigen	Catalogue no., manufacturer	Dilution and application
LKB1	AP7239A, Abgent, San Diego, CA, USA	1:100 for IHC
LKB1	3050, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
p-AMPKThr172	50081S, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
AMPKα	5831, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
GAPDH	KC-5G4, KangChen Bio-tech, Shanghai, China	1:50,000 for WB
β-actin	sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:10,000 for WB
p21^{Cip1}	2947, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
p27^{Kip1}	3686, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
c-Myc	1472-1, Epitomics, Burlingame, CA, USA	1:2,000 for WB
PARP	9532, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
Cleaved-PARP	5625, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
Bcl-2	3498, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
Bax	5023, Cell Signaling Technology, Beverly, MA, USA	1:1,000 for WB
HRP-conjugated anti-rabbit IgG	Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA	1:3,000 for WB
HRP-conjugated anti-mouse IgG	Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA	1:4,000 for WB
Secondary antibody	Envision kit (HRP, rabbit/mouse, DAB+), Dako	Ready-to-use for IHC

LKB1, liver kinase B1; WB, western blotting; IHC, immunohistochemistry; HRP, horseradish peroxidase; p-AMPK, phosphorylated adenosine monophosphate-activated protein kinase; PARP, poly(adenosine diphosphate ribose) polymerase.

Table II. shRNA sequences used in this study.

Identifier	Sequence	Reference
shLKB1#2 sense	CCGGGCCAACGTGAAGAAGGAAATT CTCGAGAATTTCCTTCTTCACGTTGGCTTTTTG	Broad Institute http://portals.broadinstitute.org/ gpp/public/gene/search
shLKB1#2 antisense	AATTCAAAAAGCCAACGTGAAGAA GGAAATTCTCGAGAATTTCCTTCTTCACGTTGGC	
shLKB1#3 sense	CCGGGATCCTCAAGAAGAAGAAGTT CTCGAGAACTTCTTCTTCTTGAGGATCTTTTTG	
shLKB1#3 antisense	AATTCAAAAAGATCCTCAAGAAGAA GAAGTTCTCGAGAACTTCTTCTTCTTGAGGATC	

using an ELISA plate reader (Elx 800; Bio-Tek, Winooski, VT, USA) at a wavelength of 450 nm. Experiments were repeated three times.

Colony formation assay. Transfected or control cells were seeded into 6-well plates at 500 cells/well, and the medium was changed every 3 days. After 10 days of incubation, the cells were fixed with 4% formalin and stained with 0.1% crystal violet solution (ServiceBio Technology, Wuhan, China). The numbers of colonies >100 μm in diameter were quantified with a ChemiDoc™ Imaging System (Bio-Rad Laboratories, Inc.). The experiments were repeated three times.

Apoptosis assay. Huh-7 shLKB1, HCC-LM3 shLKB1 or the corresponding control cells were seeded into 6-well plates at 5x10^5 cells/well. After the cells were attached to the culture dish and had entered the logarithmic proliferation phase, they were thoroughly trypsinized, suspended, washed with ice-cold phosphate-buffered saline 3 times, re-suspended with 1X binding buffer, incubated with Annexin V and 7-amino-actinomycin D (BD Biosciences, Franklin Lakes, NJ, USA) at 37˚C for 15 min, and analyzed with a BD FACSCalibur (BD Biosciences). Experiments were repeated three times.

In vivo tumorigenicity assay. HCC-LM3 shLKB1 cells (2x10^6), Huh-7 shLKB1 cells (5x10^6), HLF-LKB1 cells (1x10^6) and equal amounts of the corresponding control cells were suspended in 100 μl DMEM and subcutaneously injected into the flank of 5-week-old male nude mice (weight, 18-19 g). All the experimental mice were purchased from HFK Technology
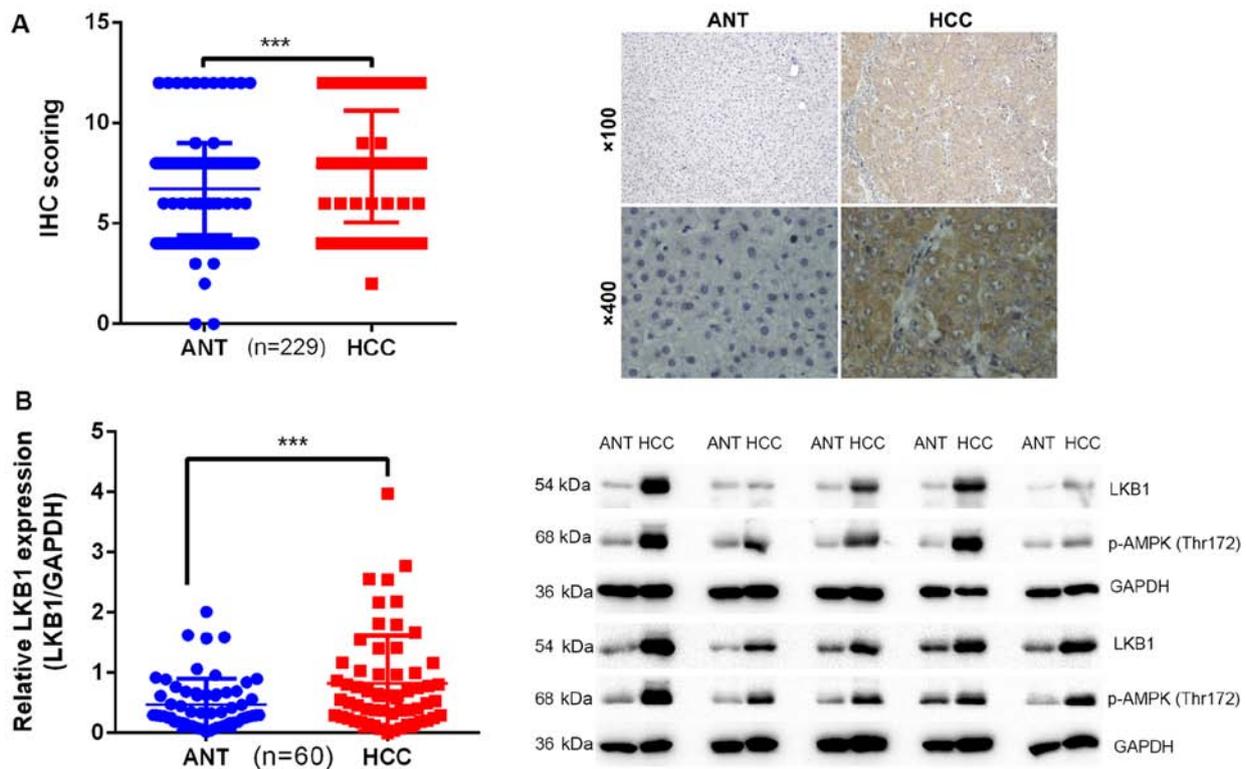
Figure 1. LKB1 is upregulated in HCC. (A) Immunohistochemical staining and (B) western blot analysis of LKB1 expression and p-AMPK (Thr172) levels in paired HCC and ANT tissues. Representative images (right panel) and quantified results (right panel) are provided. Values are expressed as the mean ± standard deviation (n=229 in A, n=60 in B). ***P<0.001. HCC, hepatocellular carcinoma; LKB1, liver kinase B1; p-AMPK, phosphorylated adenosine monophosphate-activated protein kinase; ANT, analogous non-cancerous tissues.

(Beijing, China) and kept under specific pathogen-free conditions with free access to food and water. The experimental mice were routinely monitored and sacrificed at the indicated time points. The length and width of the tumors was manually monitored using a Vernier caliper. Tumor volume (V) was calculated according to the following equation: V (mm³) = 0.5 x L x W², where L is the length and W the width in mm (16). The animal experiments were approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China).

Statistical analysis. Statistical analysis was performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA) or Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) software. Comparison between groups was performed by a two-tailed Student's t-test, analysis of variance with Bonferroni's post hoc test, Chi-squared test, Spearman's correlation coefficient test or a non-parametric test, including the Wilcoxon's signed-rank test. Kaplan-Meier analysis and the log-rank test were used to compare the survival between subgroups. A Cox proportional hazards model was used for univariate and multivariate analyses to determine the factors independently associated with survival and recurrence. P<0.05 was considered to indicate a statistically significant difference.

Results

LKB1 expression is upregulated in HCC. To determine the clinical significance of LKB1 in the development of HCC, the expression pattern of LKB1 was examined in two cohorts of patients. Cohort 1 included 229 patients (Table III) and cohort 2 included 60 patients. The expression of LKB1 was examined by IHC in matched pairs of HCC and ANT specimens in cohort 1. The results indicated that the expression levels of LKB1 were significantly higher in HCC tissues (8.288±2.922) compared with those in ANT tissues (6.716±2.293; Fig. 1A). This was further confirmed by western blot analysis in specimens from cohort 2: The intensity ratio (LKB1/GAPDH) in HCC tissues (0.8236±0.7931) was significantly higher compared with that in ANT tissues (0.4727±0.4279; Fig. 1B). p-AMPK (Thr172) was also upregulated in samples with high expression of LKB1 (Fig. 1B). These results suggested that LKB1 may play a proto-oncogenic role in HCC.

Upregulated LKB1 expression is correlated with numerous malignant characteristics and poor prognosis. Due to the evidence supporting the possible proto-oncogenic role of LKB1 in HCC, the present study then aimed to further elucidate the correlation between LKB1 expression and clinical characteristics. Upregulation of LKB1 was significantly (P<0.001) correlated with several clinicopathological characteristics associated with aggressive biological behavior of cancer cells, including higher number of tumor foci, larger tumor size, incomplete tumor encapsulation, vascular invasion, portal vein tumor thrombus (PVTT), poor differentiation, advanced Edmondson-Steiner grade, advanced BCLC grade and TNM stage (Fig. 2A-H). Most importantly, upregulated LKB1 expression was correlated with a shorter overall

Table III. Correlation between LKB1 expression and clinicopathological characteristics in 229 HCC patients.

Clinicopathological variables	Low expression	High expression	Percentage (%)	P-value
Sex				
Male	45	161	89.96	0.643[a]
Female	6	17	10.04	
Age, years				
≤50	28	86	49.78	0.297[b]
>50	23	92	50.22	
BMI, kg/m^2				
<25	37	124	70.31	0.763[b]
≥25	14	53	29.26	
Missing		1	0.44	
Alcohol intake				
Yes	14	61	32.75	0.469
No	37	115	66.38	
Missing		2	0.87	
Smoking				
Current, past	12	81	40.61	**0.015**
Never	39	96	58.95	
Missing		1	0.44	
HBV				
Negative	3	16	8.30	0.466
Positive	48	160	90.83	
Missing		2	0.87	
Cirrhosis				
Absent	10	39	21.40	0.724
Present	41	139	78.60	
Tumor number				
Single	44	137	79.04	0.150
Multiple	7	41	20.96	
Tumor size, cm				
≤5	25	64	38.86	**0.005**[b]
>5	26	112	60.26	
Missing		2	0.87	
Tumor encapsulation				
None	14	91	45.85	**0.009**
Complete	37	86	53.71	
Missing		1	0.44	
Vascular invasion				
Unidentified	44	113	68.56	**0.002**
Identified	7	65	31.44	
PVTT				
Unidentified	46	132	77.73	**0.015**
Identified	5	46	22.27	
Local invasion				
Unidentified	49	166	93.88	0.738[a]
Identified	2	11	5.68	
Missing		1	0.44	
Distant metastasis				
Absent	51	168	95.63	0.214[a]
Present	0	9	3.93	
Missing		1	0.44	
Differentiation				
Poor	17	64	35.371	**0.02**[b]
Moderate	23	86	47.598	
High	11	28	17.031	
Edmondson-Steiner grade				
I-II	22	63	37.12	0.313
III-IV	29	115	62.88	

Table III. Continued.

Clinicopathological variables	Low expression	High expression	Percentage (%)	P-value
Child-Pugh stage				
A	47	135	79.47	**0.016**[a]
B	4	42	20.09	
Missing		1	0.44	
TNM stage				
I-II	46	134	78.60	**0.021**[a]
III-IV	5	44	21.40	
BCLC stage				
0-A	37	80	51.09	**<0.001**[b]
B	4	17		
C	10	81		
Fasting glucose level, mM				
≤6.1	34	132	72.49	0.934[b]
>6.1	12	25	16.16	
Missing	5	21	11.35	
Diabetes				
Yes	15	23	16.59	**0.005**
No	36	155	83.41	
ALT, U/l				
≤40	31	110	61.57	0.845[b]
>40	19	63	35.81	
Missing	1	5	2.62	
AST, U/l				
≤40	32	104	59.39	0.264[b]
>40	17	66	36.24	
Missing	2	8	4.37	
TBIL, μM				
≤17.1	41	128	73.80	0.181[b]
>17.1	9	43	22.71	
Missing	1	7	3.49	
γ-GGT, U/l				
≤50	19	55	32.31	**0.007**[b]
>50	28	109	59.83	
Missing	4	14	7.86	
AFP, μg/l				
≤20	18	53	31.0	0.442[b]
>20	29	117	63.76	
Missing	4	8	5.24	
CEA (ng/ml)				
≤5.9	46	138	80.35	0.744[b]
>5.9	1	10	4.80	
Missing	4	30	14.85	
CA19-9, U/ml				
≤40	41	138	78.17	0.501[b]
>40	5	12	7.42	
Missing	5	28	14.41	
Adjuvant TACE				
Yes	4	12	6.99	0.760[a]
No	47	166	93.01	
Entecavir therapy				
Yes	26	98	54.15	0.607
No	25	80	45.85	

[a]Fisher's exact test. [b]Spearman's correlation coefficient test. Other variables, Chi-squared test. Bold print indicates statistical significance. BMI, body mass index; TNM, tumor-node-metastasis; BCLC, Barcelona Clinic Liver Cancer; PVTT, portal vein tumor thrombus; HBV, hepatitis B virus; AST, glutamic oxalacetic transaminase; ALT, glutamic-pyruvic transaminase; TBIL, total bilirubin; γ-GGT, γ-glutamyltranspeptidase; AFP, α-fetoprotein; CEA, carcinoembryonic antigen; CA199, carbohydrate antigen 199; TACE, transcatheter arterial chemoembolization; LKB1, liver kinase B1.
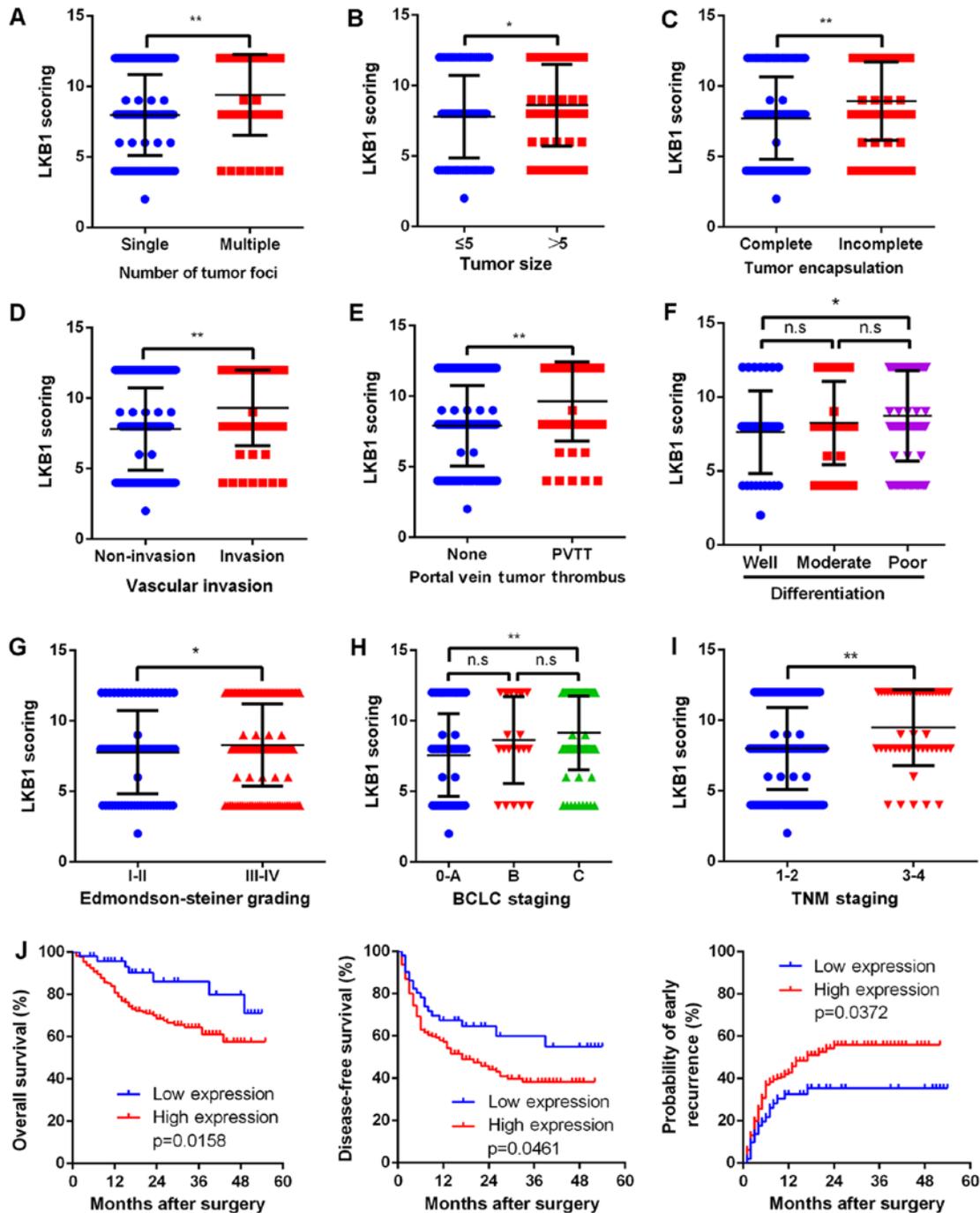
Figure 2. Upregulated LKB1 expression predicts aggressive clinicopathological characteristics and poor prognosis in HCC patients. Relative expression scores of LKB1 in 229 human HCC samples divided by (A) number of tumor foci, (B) tumor size, (C) tumor encapsulation, (D) vascular invasion, (E) PVTT, (F) tumor differentiation, (G) Edmondson-Steiner grade, (H) BCLC stage and (I) TNM stage. All values are presented as dot plots, with the middle bars representing the median and vertical bars representing the range of data. (J) Kaplan-Meier analysis of the correlation between LKB1 expression and the OS and DFS of HCC patients as well as early recurrence. **P<0.01, *P<0.05; n.s, non-significant. HCC, hepatocellular carcinoma; LKB1, liver kinase B1; PVTT, portal vein tumor thrombus; BCLC, Barcelona Clinic Liver Cancer; TNM, tumor-node-metastasis.

survival (P=0.0158), shorter disease-free survival (P=0.0461) and higher early recurrence (P=0.0372; Fig. 2J).

Furthermore, a large tumor size, multiple tumor foci, incomplete tumor encapsulation, PVTT, local invasion, vascular invasion, advanced BCLC or TNM stage were correlated with poorer survival (Fig. 3). Univariate and multivariate analyses revealed that high LKB1 expression in HCC patients may serve as an independent prognostic marker for overall survival (P=0.018 and 0.046 for uni- and multivariate

analysis, respectively), whereas it had no significant predictive value regarding recurrence (P=0.054 and 0.383, respectively; Table IV).

Knockdown of LKB1 expression inhibits HCC cell proliferation. In order to examine the role of LKB1 in HCC cell lines, LKB1 expression was knocked down in Huh7 and HCC-LM3 cells (Fig. 4A), which exhibit high and moderate endogenous LKB1 expression, respectively (data not shown). The CCK-8

Table IV. Univariate and multivariate analyses of prognostic factors in overall survival and recurrence.

A, Overall survival

Factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (>50 vs. ≤50 years)	0.990	0.775-1.265	0.938			
Sex (male vs. female)	1.528	1.115-2.092	0.008	1.155	0.290-4.604	0.302
Diabetes mellitus (yes vs. no)	1.57	0.677-3.641	0.293			
Blood glucose (>6.1 vs. ≤6.1 mM)	0.923	0.453-1.882	0.826			
Tumor foci (multiple vs. single)	0.850	0.641-1.128	0.26			
Tumor size (>5 vs. ≤5 cm)	3.270	1.779-6.011	<0.001	4.161	1.587-10.910	0.004
Tumor encapsulation (none vs. complete)	0.432	0.263-0.711	0.001	1.403	0.708-2.779	0.332
Differentiation (poor vs. high + moderate)	2.224	1.246-3.970	0.007	0.397	0.110 -1.430	0.158
Edmondson-Steiner grade (III+IV vs. I+II)	0.832	0.639-1.804	0.173			
TNM stage (III+IV vs. I+II)	0.732	0.557-0.961	0.025	0.591	0.227-1.538	0.281
Child-Pugh stage (B vs. A)	1.387	0.786-2.448	0.259			
BCLC stage (B+C vs. 0+A)	0.596	0.460-0.772	<0.001	1.772	0.541-5.803	0.344
PVTT (identified vs. unidentified)	2.314	1.407-3.805	0.001	2.291	0.778-6.750	0.133
Vascular invasion (identified vs. unidentified)	0.514	0.401-0.685	<0.001	1.155	0.290-4.604	0.838
Local invasion (identified vs. unidentified)	2.661	1.211-5.847	0.015	3.126	0.880-11.102	0.078
Distant metastasis (identified vs. unidentified)	1.446	0.453-4.613	0.533			
HBV (positive vs. negative)	0.939	0.405-2.176	0.883			
Cirrhosis (present vs. absent)	0.795	0.567-1.114	0.182			
AST (>40 vs. ≤40)	2.168	1.135-3.574	0.002	1.295	0.660 -2.540	0.452
γ-GGT (>50 vs. ≤50)	1.994	1.090-3.648	0.025	1.204	0.507 -2.862	0.674
AFP (≥20 vs. <20)	2.196	1.170-4.124	0.014	1.605	0.781-3.297	0.198
CA199 (>40 vs. ≤40)	2.752	1.344-5.634	0.006	7.273	3.079-17.177	<0.001
LKB1 expression (high vs. low)	2.617	1.179-5.808	0.018	2.372	1.014-5.550	0.046

B, Recurrence

Factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (>50 vs ≤50 years)	0.855	0.595-1.23	0.399			
Sex (male vs. female)	0.912	0.502-1.657	0.762			
Diabetes mellitus (yes vs. no)	0.569	0.313-1.035	0.065			
Blood glucose (>6.1 vs. ≤6.1 mM)	0.828	0.492-1.392	0.476			
Tumor foci (multiple vs. single)	1.988	1.337-2.956	0.001	1.295	0.610-2.749	0.500
Tumor size (>5 vs. ≤5 cm)	2.983	1.937-4.595	<0.001	2.599	1.539-4.391	<0.001
Tumor encapsulation (none vs. complete)	2.220	1.533-3.215	<0.001	1.520	0.969-2.383	0.068
Differentiation (poor vs. high + moderate)	0.046	0.218-0.755	0.004	1.212	0.867-1.692	0.260
Edmondson-Steiner grade (III+IV vs. I+II)	1.325	0.902-1.948	0.152			
TNM stage (III+IV vs. I+II)	2.510	1.694-3.719	<0.001	0.994	0.751-1.315	0.964
Child-Pugh stage (B vs. A)	1.146	0.736-1.785	0.546			
BCLC stage (B+C vs. 0+A)	2.385	1.637-3.475	<0.001	0.825	0.587-1.160	0.269
PVTT (identified vs. unidentified)	0.369	0.249-0.547	<0.001	0.673	0.456-0.994	0.047
Vascular invasion (identified vs. unidentified)	2.045	1.407-2.971	<0.001	1.256	0.827-1.909	0.285
Local invasion (identified vs. unidentified)	3.619	1.975-6.632	<0.001	2.367	1.072-5.228	0.033
Distant metastasis (identified vs. unidentified)	1.593	0.699-3.632	0.268			
HBV (positive vs. negative)	0.848	0.466-1.540	0.588			
Cirrhosis (identified vs. unidentified)	1.234	0.776-1.963	0.374			
AST (>40 vs. ≤40)	1.593	1.097-2.313	0.014	1.363	0.862-2.155	0.185

Table IV. Continued.

Factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
γ-GGT (>50 vs. ≤50)	1.861	1.203-2.800	0.005	1.706	0.946-3.076	0.076
AFP (≥20 vs. <20)	1.352	0.897-2.038	0.150			
CA199 (>40 vs. ≤40)	1.644	0.877-3.082	0.121			
LKB1 expression (high vs. low)	1.622	0.992-2.652	0.054	1.263	0.747-2.133	0.383

TNM, tumor-node-metastasis; BCLC, Barcelona Clinic Liver Cancer; PVTT, portal vein tumor thrombus; HBV, hepatitis B virus; AST, glutamic oxalacetic transaminase; γ-GGT, γ-glutamyltranspeptidase; AFP, α-fetoprotein; CA199; carbohydrate antigen 199; LKB1, liver kinase B1.
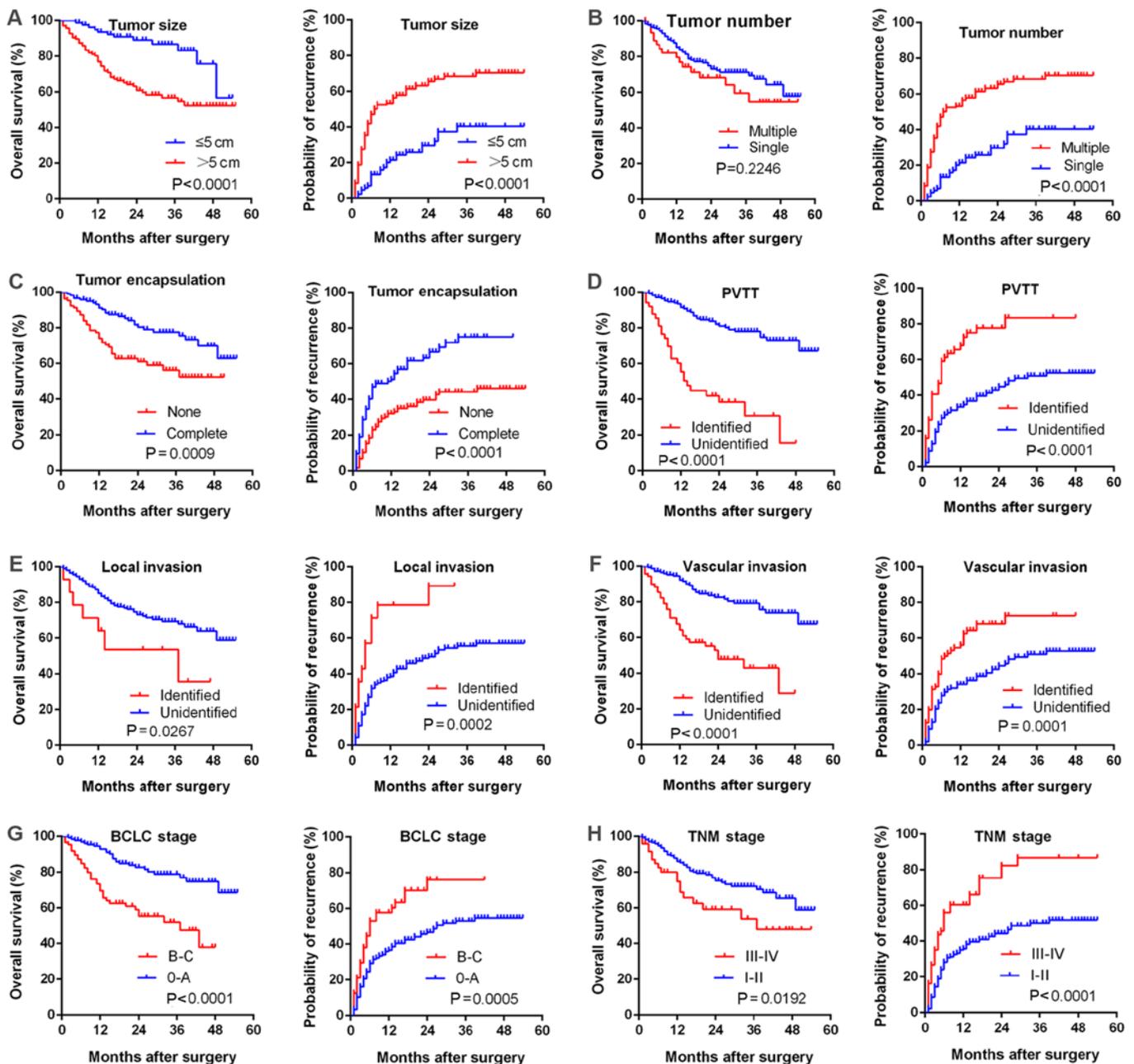
Figure 3. Aggressive pathological characteristics are correlated with poor prognosis. A total of 229 human hepatocellular carcinoma samples were stratified by (A) tumor size, (B) number of tumor foci, (C) state of tumor encapsulation, (D) presence of PVTT, (E) local invasion, (F) vascular invasion, (G) BCLC stage and (H) TNM stage. All the pathological characteristics associated with aggressiveness predict shorter overall survival and earlier recurrence. PVTT, portal vein tumor thrombus; BCLC, Barcelona Clinic Liver Cancer; TNM, tumor-node-metastasis.

TAN *et al*: LKB1 PREDICTS POOR PROGNOSIS IN HCC
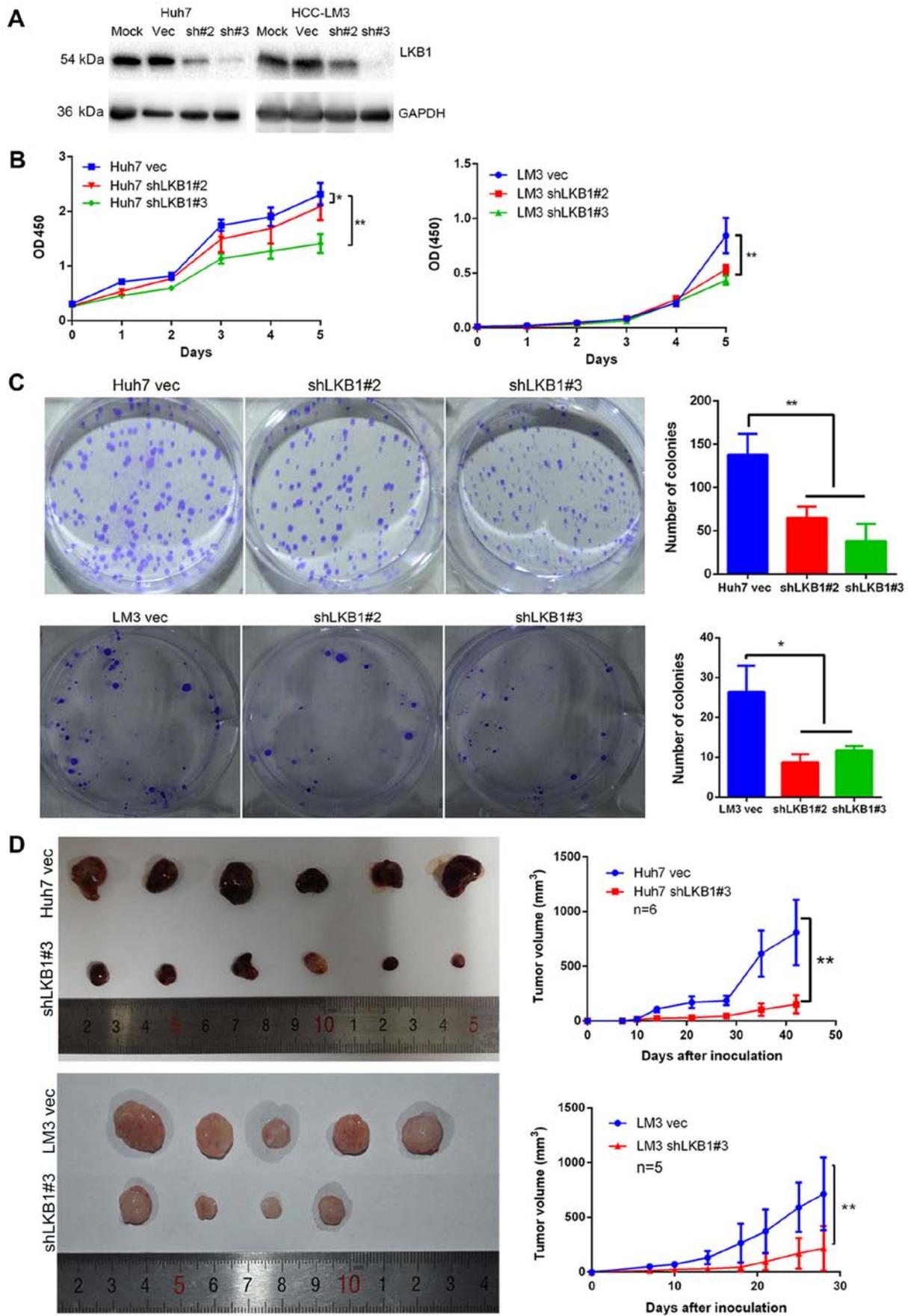
Figure 4. Knockdown of LKB1 expression suppresses the proliferation of HCC cells. (A) Western blot analysis confirmed knockdown of LKB1 expression and downregulation of p-AMPK (Thr172) by small hairpin RNAs in Huh7 and HCC-LM3 cells. (B and C) The effect of LKB1 on HCC cells was evaluated by (B) Cell Counting Kit-8 and (C) colony formation assay. Colony formation images representative of 3 independent experiments are provided. (D) Knockdown of LKB1 expression suppressed tumor growth by subcutaneously injected cells (n=6 and n=5 in the Huh7 and HCC-LM3 groups, respectively). **P<0.01, *P<0.05. HCC, hepatocellular carcinoma; LKB1, liver kinase B1; p-AMPK, phosphorylated adenosine monophosphate-activated protein kinase.
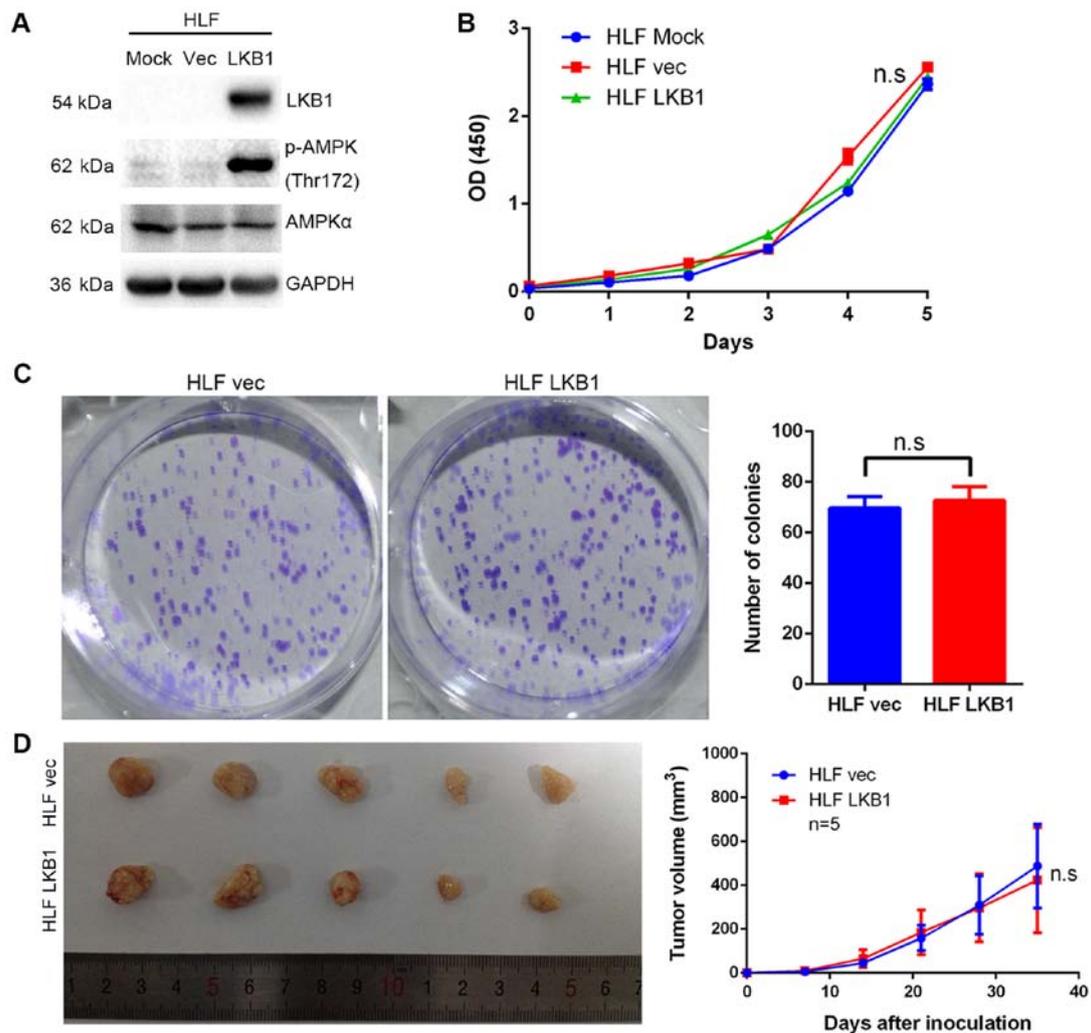
Figure 5. LKB1 overexpression did not affect the proliferation of HCC cells. (A) Western blot analysis confirmed the overexpression of LKB1 and upregulation of p-AMPK (Thr172) in HLF cells. (B and C) LKB1 overexpression exerted no effect on the proliferation of HCC cells as detected by (B) Cell Counting Kit-8 and (C) colony formation assay. Colony formation images representative of 3 independent experiments are provided. (D) LKB1 overexpression did not affect the growth of subcutaneously injected HLF cells (n=5). n.s, non-significant; HCC, hepatocellular carcinoma; LKB1, liver kinase B1; p-AMPK, phosphorylated adenosine monophosphate-activated protein kinase.

and colony formation assays indicated that knockdown of LKB1 significantly inhibited cell proliferation (Fig. 4B and C). Furthermore, LKB1 was found to be ectopically overexpressed in HLF cells (Fig. 5A), which have no detectable LKB1 expression (data not shown). However, overexpression of LKB1 exerted no effect on the growth of HLF cells (Fig. 5B and C). The *in vivo* tumorigenicity assay indicated that the volume of tumors grown from subcutaneously injected cells was smaller in the LKB1 knockdown groups compared with that in the control groups (Fig. 4D). However, no significant difference in volume was observed between the tumors derived from LKB1-overexpressing HLF and those from control cells (Fig. 5D).

LKB1 knockdown inhibits tumor cell proliferation by promoting cell apoptosis. Since knockdown of LKB1 inhibited Huh7 and HCC-LM3 cell proliferation, flow cytometric analysis was performed to determine whether this anti-proliferative effect was due to cell cycle arrest. No significant differences in the distribution of cells in each phase of the

cell cycle were observed (data not shown). However, the cell apoptosis assay indicated that, in LKB1-knockdown cells, the apoptotic rate was higher compared with that in the control cells (Fig. 6A). Western blot analysis further confirmed an increased amount of cleaved caspase-3 and PARP in the knockdown group (Fig. 6B). In addition, reduced c-Myc expression and elevated expression of p21[Cip1] and p27[Kip1] were observed in LKB1-knockdown cells, suggesting that p21[Cip1] and p27[Kip1] affect cell proliferation via other mechanisms (Fig. 6B).

Discussion

LKB1 has been reported to act as a tumor suppressor in the majority of published studies. LKB1 suppresses cell growth and viability through the LKB1/AMPK/mammalian target of rapamycin signaling pathway (17). However, certain studies suggested that LKB1 exerts a proto-oncogenic effect through modulating cellular metabolism and resistance to oncogenic transformation (8,9). Therefore, it is of paramount importance
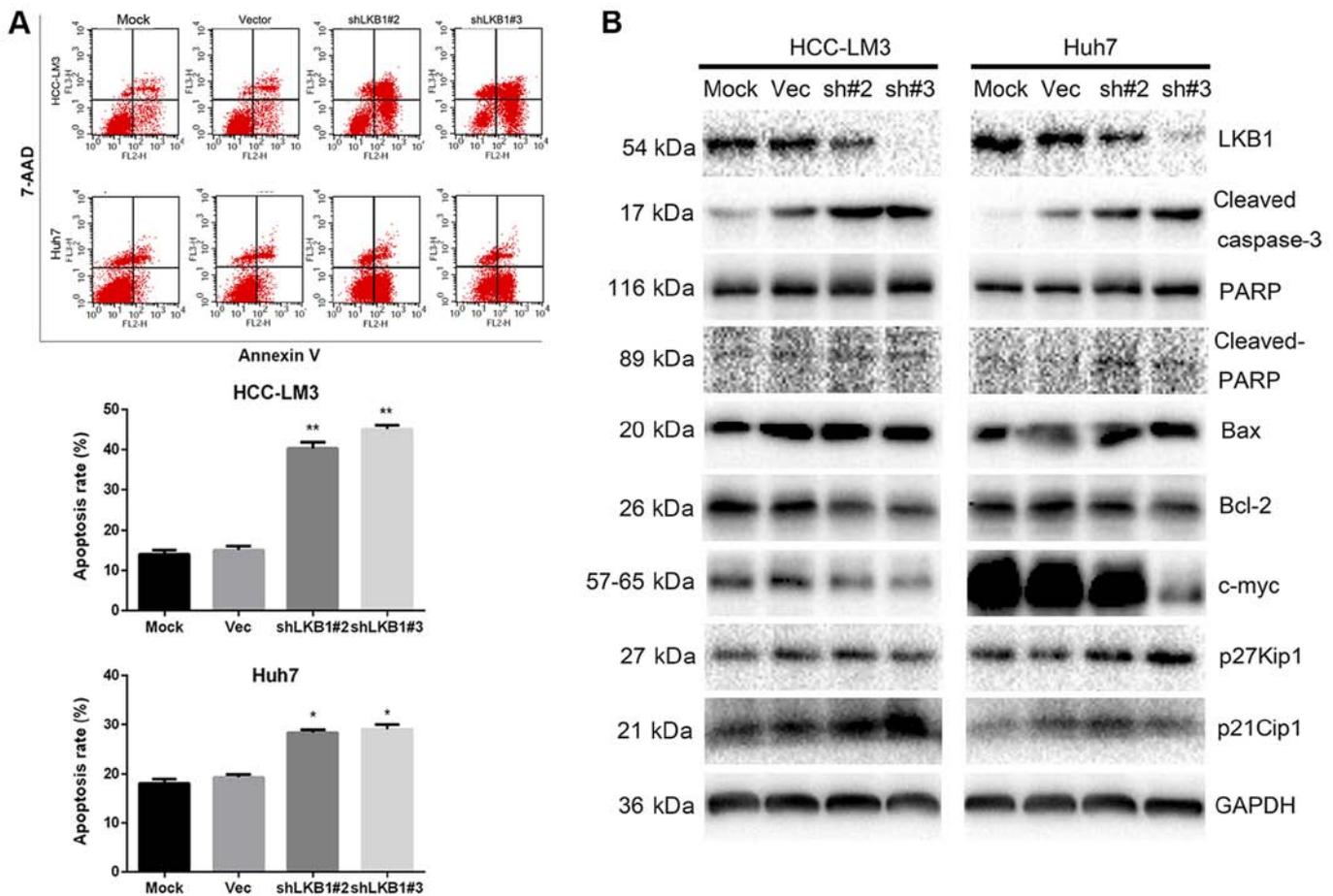
Figure 6. LKB1 knockdown promotes cell apoptosis and regulates proliferation-associated genes. (A) Knockdown of LKB1 promotes cell apoptosis. (B) The expression of cleaved caspase 3 and cleaved PARP was increased as detected by western blot analysis in LKB1-knockdown cells. The expression of the indicated proteins was detected by western blot analysis. **P<0.01, *P<0.05. LKB1, liver kinase B1; PARP, poly(adenosine diphosphate ribose) polymerase.

to elucidate the function of LKB1 in different types of cancer. In the present study, the expression pattern of LKB1 was detected in two cohorts of HCC and paired ANT specimens. The results demonstrated that LKB1 was frequently upregulated in HCC tissues, and the high expression of LKB1 was correlated with numerous malignant characteristics, shorter overall survival and earlier recurrence. It was also revealed that a large tumor size, multiple tumor foci, incomplete tumor encapsulation, PVTT, local invasion, vascular invasion, and advanced BCLC or TNM stage were associated with a worse prognosis. Knockdown of LKB1 inhibited cell proliferation by promoting apoptosis and regulating proliferation-associated genes, but overexpression of LKB1 exerted no effect on the proliferation of HCC cells. It is well-known that, under quiescent conditions, LKB1 is localized to the nucleus and activation of LKB1 requires translocation from the nucleus to the cytoplasm by forming a heterotrimer with the proteins STRADA and MO25 (18,19). It may be hypothesized that enhanced LKB1 expression in HCC cells does not affect STRADA and MO25 and, accordingly, LKB1 translocation to the cytoplasm remains unchanged.

HCC is one of the leading causes of cancer-associated mortality worldwide, and its incidence is increasing (20). The Asia-Pacific area is the region with the highest prevalence of HCC (21,22), and a large number of patients are first diagnosed

with HCC at an advanced stage. Therefore, the therapeutic efficacy is not optimal, and mortality due to cancer recurrence or metastasis is common. In the present study, the proto-oncogenic role of LKB1 in HCC was demonstrated. Whether and how LKB1 affects HCC metastasis, and the possible therapeutic approaches based on LKB1, remain to be further investigated in future studies.

Germline mutation of LKB1 is responsible for a pre-cancerous condition referred to as Peutz-Jeghers syndrome, which is characterized by the development of benign hamarto-matous polyps in the gastrointestinal tract and hyperpigmented macules on the lips and oral mucosa. Patients with Peutz-Jeghers syndrome develop gastrointestinal hamartomas and have a markedly increased risk for developing gastrointestinal, breast and gynecological cancers (23). Dahmani *et al* (24) reported a novel LKB1 isoform, which lacks the N-terminal region and a portion of the kinase domain, named ΔN-LKB1. This enhances the metabolic activity of AMPK in HeLa cells and NCI-H460 lung cancer cells and has intrinsic oncogenic properties. In order to explore the possibility of mutated LKB1 in HCC tissues and cell lines used in the present study, the literature on LKB1 mutation in HCC was reviewed. Kim *et al* (25) collected 80 HCC samples and 7 dysplastic nodules to investigate potential mutations in all 9 exons of LKB1. The results revealed the presence of only one missense mutation of CCG→CTG (Pro→Leu)

among the 80 HCC cases, whereas no mutation was identified among the 7 dysplastic nodules. Pineau *et al* (26) collected 57 hepatobiliary cancer cell lines for detection of homozygeous deletions, and no homozygous deletion of LKB1 was detected in the HCC cell lines used in their study. Therefore, the effect of LKB1 observed in the present study was likely exerted by a non-mutated protein.

Activation of LKB1 by phosphorylation at the Ser428, Ser307 and Ser399 sites is required for translocation from the nucleus to the cytoplasm (27-29). It has been reported that LKB1 regulates glucose metabolism and suppresses gluco-neogenesis in the normal liver (30), and knockout of LKB1 in mouse livers leads to the inability to use glucose, resulting in severe hyperglycemia (31). Apoptosis is a type of programmed cell death under various types of stress (32,33). It is reasonable to hypothesize that LKB1-knockdown cells underwent apoptosis due to inability to use glucose. LKB1 may be used as a potential therapeutic target in HCC treatment by agents suppressing phosphorylation at Ser428, Ser307 and Ser399, thereby inhibiting nuclear export of LKB1.

The *in vivo* tumor inhibitory effect of LKB1 was previously investigated by knockout of LKB1 in mice (34,35), and the most recent study indicated that LKB1 acts as a master gatekeeper of liver regeneration (36). Another previous study indicated that LKB1 was downregulated in HCC and that low expression is correlated with poor prognosis (10). This conclusion was made based on IHC staining analysis of 70 cases. In the present study, in which the scale of samples included was enlarged, different conclusions were reached. Along with the results of previous studies (8,9,37), the present study suggests that LKB1 plays a proto-oncogenic role in HCC. It is suggested that the function of LKB1 varies between different cancer types and pathological conditions. Therefore, the heterogeneity of cancers should be taken into consideration in cancer therapy.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article. The authors declare that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Authors' contributions

XT and LC designed the experiments, XT and ZL performed the experiments. ZL collected clinicopathological data. XT, BZ, XC and LC analyzed the results. XT and ZL generated the data, prepared the panels and assembled the figures and tables. XT and LC wrote the manuscript. All authors have reviewed and approved the final version of the manuscript.

Ethics approval and consent to participate

The protocol of the present study, involving both human clinical samples and animal experimentation, was approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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