Int J Clin Exp Pathol 2018;11(11):5525-5535
www.ijcep.com /ISSN:1936-2625/IJCEP0083558

Original Article

Under-expression of LKB1 is associated with enhanced p38-MAPK signaling in human hepatocellular carcinoma

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Received August 4, 2018; Accepted August 26, 2018; Epub November 1, 2018; Published November 15, 2018

Abstract: The tumor suppressor liver kinase B1 (LKB1), a highly conserved and ubiquitously expressed protein kinase, plays a critical role in tumorigenesis. LKB1 has recently been identified in tumorigenesis of several cancers including lung cancer, breast cancer, and pancreatic cancer. However, the role of LKB1 in hepatocellular carcinoma (HCC) remains unclear. Herein, we examined the expression levels of LKB1 in HCC patients and cell lines by quantitative real-time PCR (qRT-PCR) and western blot analysis. Furthermore, LKB1 protein expression was analyzed in archived paraffin-embedded HCC tissues using immunohistochemistry (IHC), and its association with overall survival was shown in statistical analysis. In vitro assays, including RNAi studies, were performed to further explore the role of LKB1 in tumor progression in HCC cell lines. Our results revealed that the expression of LKB1 was lower in HCC tissue and cell lines than in corresponding adjacent normal tissue and normal human liver cell line (HL7702). Moreover, HCC patients with low LKB1 expression had advanced clinical stage and worse prognosis than those with higher LKB1 expression. Furthermore, siRNA-mediated knockdown of LKB1 resulted in enhanced cell proliferation, migration, and invasion of HCC cells. Additionally, the expression level of LKB1 positively correlated with E-cadherin levels, wherein siRNA-transfected cells exhibited significantly decreased levels of E-cadherin, while phosphorylated p38 and vimentin levels were enhanced. Inhibition of p38 MAPK signaling was capable of reversing E-cadherin up-regulation and vimentin down-regulation. In all, our results indicate that LKB1 acts as a tumor suppressor gene, which may inhibit EMT through the p38 MAPK signaling pathway involved in HCC progression.

Keywords: LKB1, hepatocellular carcinoma, invasion, migration, p38-MAPK signaling

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor and the third leading cause of cancer-related mortality worldwide [1]. Currently, surgery is still one of the most effective treatments for liver cancer patients. However, because of the high rate of recurrence and metastasis after radical surgery, the long term survival rate of patients with liver cancer is only 25-39% [2]. Furthermore, the molecular mechanisms underlying invasion and metastasis of liver cancer are not very clear; therefore, there is an urgent need to understand HCC tumor invasion and metastasis in order to improve diagnosis, treatment options, and prognosis of HCC.

Serine/threonine protein liver kinase B1 (LKB1) is classified as a tumor suppressor that activates diverse downstream kinases, thus regulating multiple biologic processes, including energy metabolism, tumor progression, cell cycle arrest, proliferation, and cell polarity. LKB1 inactivation has been associated with Peutz-Jeghers syndrome (PJS), an inherited autosomal disease characterized by gastrointestinal polyps, mucocutaneous melanin pigmentation, and multi-organ cancer susceptibility [3]. The incidence of cancer among patients with PJS has been estimated to be 18-fold higher than in the general population [4]. Mice with oncogenic KrasG12D mutant and loss of LKB1 have significantly shortened latency, increased tumor burden, and increased lung cancer invasion and distant metastasis [5]. Several studies have indicated that the under-expression of LKB1 might contribute to the progression of non-small cell lung cancer (NSCLC) [6], breast cancer, [7] endometrial cancer, pancreatic car-

cinoma [8], and liver cancer. Moreover, low expression of LKB1 in HCC may be a poor prognostic factor [9]. LKB1 directly activates AMPK and AMPK-related kinases to regulate cell metabolism, proliferation, and cell polarity [10]. Low expression of LKB1 altered cell polarity and cell adhesion, which enhanced the transformation of normal cells and the metastasis of tumor cells [11]. Moreover, Li et al. observed that cell migration increased upon LKB1 knockdown in breast cancer [7]. Although LKB1 displays some common features of tumor suppressor genes, it is unknown whether LKB1 is a liver cancer suppressor gene. To ascertain its role, our study aimed to elucidate the function of LKB1 in the growth, proliferation, invasion, and migration of HCC. Specifically, we examined LKB1 regulating mechanisms of EMT in HCC, which are currently poorly understood.

TGF-β is a key molecule that promotes EMT in tumor cells. Previous studies have indicated a positive correlation between the expression of TGF-β and the metastatic ability of tumor cells [12, 13]. TGF-β signaling involves either the Smad-dependent pathway or the Smad-independent pathway, such as p38 mitogen-activated protein kinase (p38 MAPK) and phosphoinositide-3 kinase (PI3K) [14]. In addition, p38 MAPK activation can inhibit the expression of E- cadherin [15]. LKB1 acts as an upstream regulator of AMPK and inhibits the activation of the TGF-β signaling pathway [16]. In breast cancer, LKB1 was shown to be a regulator of the p38 MAPK pathway [17]. Therefore, we speculate that LKB1 may play an important role in p38 MAPK pathway-mediated EMT in HCC.

In the present study, we examined the expression of LKB1 in HCC and investigated its clinical significance and biologic functions. First, we investigated the expression of LKB1 in HCC tissues and cell lines by using qRT-PCR and western blot. Second, we analyzed its correlations with clinicopathologic characteristics in order to determine the clinical signature of LKB1 in HCC. We found that LKB1 was underexpressed in most HCC cell lines and tissues, a pattern associated with poor prognosis in HCC. In addition, we used the RNA interference to knockdown the LKB1 expression level in the HCC cell lines and explored the impact on E-cadherin or vimentin. Our data suggested that LKB1 underexpression may partially facilitate activation of the p38 MAPK pathway and thus contribute to EMT and progression of HCC. In all, a better understanding of the molecular mechanism of LBK1 may propel its identification as a novel diagnostic biomarker and therapeutic target in HCC.

Materials and methods

Patients and surgical specimens

This study enrolled 79 HCC tissues samples for HCC patients who underwent hepatectomy at Guangxi Tumor Hospital from January 2014 to December 2014. None of the patients received chemotherapy, radiotherapy, radiofrequency ablation, target therapy prior to the operation. The exclusion criteria were a combination of another cancer and a history of liver transplantation. The 79 samples of HCC and the corresponding adjacent tissue were from the same patients. The diagnosis was confirmed by histologic examination. All of the specimens were fixed in 10% formalin and embedded in paraffin for immunochemistry. However, only 49 pair of fresh HCC tissue and ANT (adjacent noncancerous tissue), which was for quantitative PCR analysis, could be obtained from the Tumor Tissue Bank of Guangxi Tumor Hospital. Clinicopathologic data were collected from all patients, including sex, age, HBV, AFP (alpha fetoprotein), maximum size of tumors, number of tumors, tumor capsule, and tumor thrombus. In this study, each patient gave informed consent and it was approved by the ethics committee of Guangxi Tumor Hospital.

Immunohistochemistry staining

For assessment of the expression levels of LKB1 in HCC tissue, immunohistochemical analysis (IHC) was carried out in samples from all patients (n=79). The sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. Then the sections was placed at 100°C for 3 min in 10 mM sodium citrate buffer (pH=6.0) for antigen retrieval. The slides were incubated with peroxidase blocking agent for 10 min to block endogenous peroxidase activity and blocked with normal nonimmunized animal serum according the manufacturer's protocol. The sections were then incubated with anti-LKB1 antibody (1:100 dilution) for 14 h at 4°C. The primary antibody was diluted with the appropriate antibody diluent. The negative control was replaced with PBS that was not containing primary antibody.

Evaluation of immunohistochemical staining

LKB1 is predominantly staining in the cytoplasm. We used semi-quantitative analysis to analyze the immunostaining intensity of LKB1, which was determined by the percentage of positive cells and the staining intensity. Each slide was independently observed by two experienced pathologists. The proportion of positive cells was grade as follows: 0 (no positive tumor cells), 1 (1-25% positive tumor cells), 2 (26-50% positive tumor cells), 3 (51-75% positive tumor cells), 4 (76-100% positive tumor cells). Staining intensity was scored using four grades: 0 (negative), 1 (weakly positive), 2 (moderately positive), 3 (strongly positive). The staining index (range from 0 to 12) was calculated by multiplying proportion of positive cells score with score of staining intensity. Low expression of LKB1 group was for scores from 0 to 5. The ones with scores from 6 to 12 were defined as high expression of LKB1.

Cell lines

Human normal liver cell line (HL7702) and human HCC cell lines BEL-7404, Hep-G2, SSMC-7721, MHCC-97-H, Huh-7, were purchased from the cell bank of the Chinese Academy of Science. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (Gibco), 0.5% penicillin, and 0.5% streptomycin (Life Technologies Corporation, Carlsbad, CA, USA). The cell lines were cultured in a sterile incubator maintained at 37°C with 5% CO_2.

Reagents and antibodies

Rabbit anti-human LKB1 monoclonal antibody was purchased from Abcam PLC (Cambridge, UK). Rabbit monoclonal antibody against human p38 MAPK, Phospho-p38 MAPK, E-cadherin, β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit monoclonal antibody against human Vimentin was purchased from Santa Cruz Biotechnology. SB202190 was obtained from Sigma-Aldrich (St. Louis, MO, USA).

SiRNA and transfection

Three small interfering RNA sequences targeting different regions of human LKB1 mRNA were designed to exclude nonspecific effects. A scrambled siRNA was employed as a negative control (Gene Pharma, Shanghai, China). The siRNA targeting the LKB1 sequences (LKB1-siRNA1 sense 5'-CCUGCUGAAAGGGAUGCUUTT-3' and antisense 5'-AAGCAUCCCUUUCAGCAGGTT-3'; LKB1-siRNA2 sense 5'-GGAUGUGUUAUACAACGAATT-3'andantisense5'-UUCGUUGUAUAACACAUCCTT-3' LKB1-siRNA3 sense 5'-CCAACGUGAAGAAGGAAAUTT-3' and antisense 5'-AUUUCCUUCUUCACGUUGGTT-3') and the non-silencing sequence (LKB1-scrambled-siRNA sense 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUUCGGAGAATT-3'). About 24 h before transfection, cells were seeded at the density of 5 × 10*6 in six-well plates. When the cells reached confluence of 70-80%, cells were transfected with small interfering RNA following the manufacturer's protocol. After six hours of transfection, the medium was replaced by the DMEM containing 10% FBS. Transfection efficiency was evaluated by fluorescence microscopy (OLYMPUS TH4-200, Figure S1) after transfection of FAM-siRNA for six hours. The silencing effect was detected by qRT-PCR and western blotting 48 h after transfection. The small interfering RNA sequence (LKB1-siRNA1) with better silencing effect was selected for subsequent cell function experiments. For the cell proliferation assay, the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was used. After 24 hours of transfection, the cells were collected by centrifugation and resuspended. The cells were counted using a hemocytometer and seeded in a 96-well plate at a density of 5 × 10*3 cells/well with six replicates. Cells were incubation in DMEM containing 10% FBS for 6, 24, 48, and 72 h, respectively. At end of the time, the medium were added 10 μl CCK-8 reagent to each well. The cells were cultured for 2 h at cell incubator. The absorbance was measured at a wavelength of 450 nm.

Cell invasion assay

Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was incubated overnight at 4°C and diluted in serum-free DMEM medium. The Transwell upper chamber is a polycarbonate membrane with 8 μm pore size coated with 60 μl the Matrigel and overnight at 37°C. The cells were collected 24 hours after transfection. After the digestion, the cells were resuspended in serum-free medium and inoculated at 1 × 10*5/200 μl/well in the upper chamber. The chamber was placed in a 24-well plate, seven hundred microliters DMEM containing 10% FBS added in the lower chamber. After migrating for 24 h the

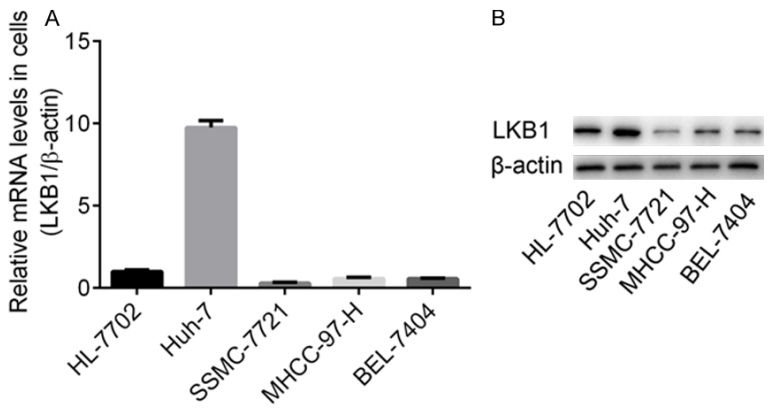

Figure 1. Expression of LKB1 mRNA and protein in HCC cell lines (Huh7, Hep-G2, SSMC-7721, MHCC-97H, and BEL7404) and HL-7702 were examined by qRT-PCR and western blot.

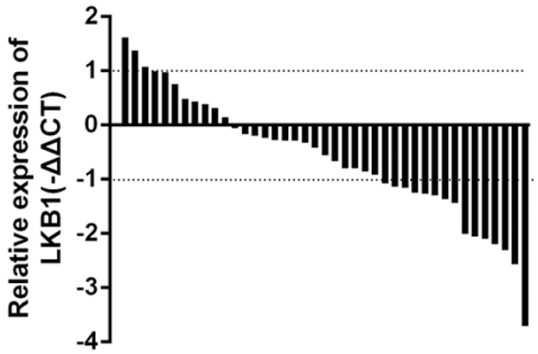

Figure 2. Waterfall plot shows expression of LKB1 in human HCC tissue (n=42), -ΔΔCt=(CtLKB1-Ctβ-actin) ANT-(CtLKB1-Ctβ-actin) HCC, Fold change=$2^{-\Delta\Delta Ct}$.

cells were fixed in methanol and then stained with Giemsa. The non-invading cells were removed from the upper chamber, and cell on the lower surface were stained. Migrated cells were calculated in five random fields (100 × magnification) for each chamber under a light microscope.

Cell migration assay

For migration assay, 2 × 10*4 cells in 200 μl serum-free DMEM were added into the upper Transwell chamber without Matrigel. The remaining experimental steps were the same as the cell invasion assay.

Quantitative real-time PCR

QRT-PCR was used to research the LKB1 mRNA levels of cell lines, HCC tissue and ANT. Total RNA was extracted from tissues and cell lines with TRIzol reagent (Invitrogen, Life Technologies, CA, USA) according to the manufacturer's instructions. The concentration of RNA was measured at A260 and A280 by Nano drop 2000 (Thermo Scientific, Waltham, MA, USA). Approximately 1 μg of RNA was used to prepare cDNA using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Q-PCR was conducted using a THUNDERBIRD qPCR Mix (TOYOBO, Osaka, Japan) in an Applied Biosystems 7500 (Applied Biosystems). The following primers were used to detect the expression of LKB1 (F, 5'-AACGGCCTGGACACCTTCT-3' and R, 5'-CCCTTCCCGATGTTCTCAA-3') and β-actin (F, 5'-AGTGTGACGTTGACATCCGT-3' and R, 5'-GCAGCTCAGTAACAGTCCGC-3'). β-actin was used as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold changes for LKB1 expression levels.

Western blotting assay

The LKB1 and E-cadherin protein levels of cells were measured by western blotting. Cells were lysed in RIPA buffer (50 mM Tris, PH 7.4, 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 1% SDS) containing 1 mmol/l PMSF and then centrifuged at 12000 rpm/min, 4°C for 10 min to collect the supernatant. BCA protein assay kit (KGPBCA, Nanjing, China) was used to measure the concentration of total protein in each sample. Fifty micrograms of total protein were separated by SDS-PAGE and then transferred to the polyvinylidene fluoride membranes. Subsequently, the membranes were blocked in TBST solution containing 5% nonfat milk for 60 min, the membranes were incubated with each primary antibody (LKB1, 1:100; E-cadherin, 1:1000; vimentin, 1:500; p38 MAPK, 1:1000; p-p38 MAPK, 1:1000; β-actin, 1:1000) and then washed and probed with respective secondary peroxidase conjugated antibodies. The detection was implemented by ECL detection reagent (BOSTER, Wuhan, China).

Statistical analysis

All data processing was carried out using SPSS 19.0 software. The difference in LKB1 expression between HCC tissues and adjacent tissues was analyzed by *t* test. The differences between the groups were analyzed by one-way analysis of variance (ANOVA) when there were more than two groups. The chi-square test was used

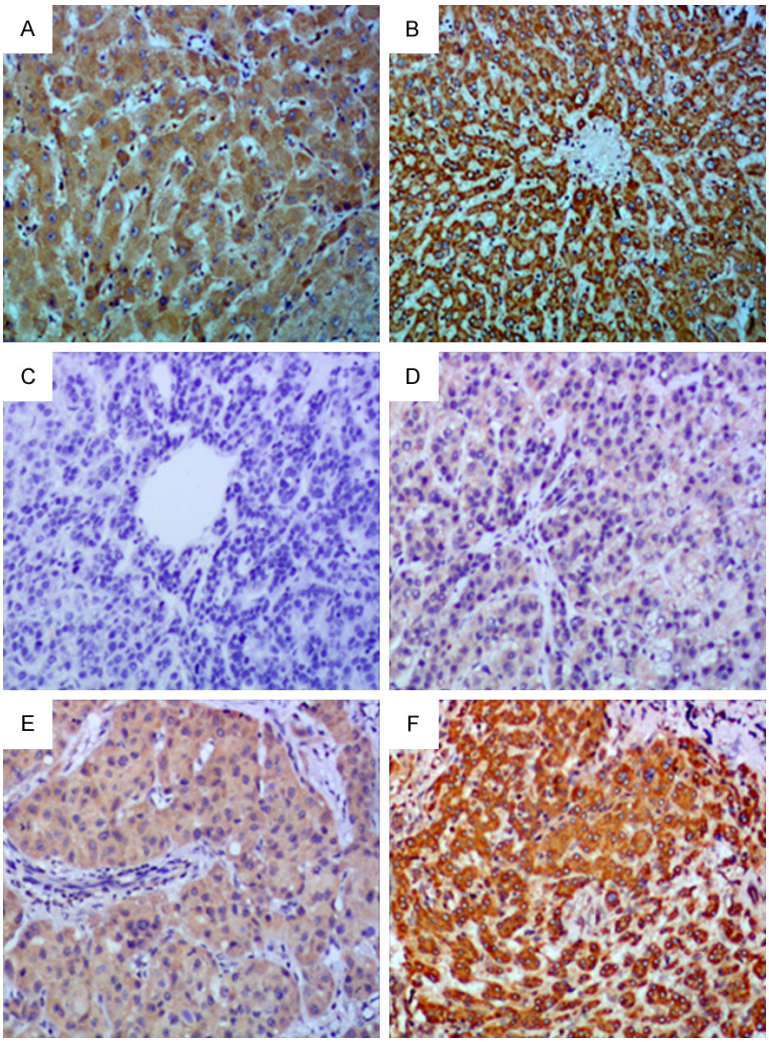

Figure 3. LKB1 protein showed decreased expression in HCC tissues. A. Immunohistochemical demonstration of LKB1 expression in normal livers. B. Positive staining of LKB1 in adjacent surrounding non-tumor tissues. C. Negative staining of LKB1 in HCC specimens. D. Weak staining of LKB1 in HCC specimens. E. Moderate staining of LKB1 in HCC specimens. F. Strong staining of LKB1 in HCC specimens.

to analyze the relationship between LKB1 expression and clinicopathologic characteristics. The association between LKB1 expression and overall survival, as well as the prognostic value of LKB1, were estimated by Kaplan-Meier and Cox regression analysis, respectively. $P< 0.05$ was considered significant.

Results

LKB1 is under-expression in HCC cell lines and HCC tissues

We used qRT-PCR and western blot analyses to measure the expression levels of LKB1 mRNA and protein in HCC cell lines, including Huh-7, BEL-7404, Hep-G2 SSMC-77-21, and MHCC-97H. Compared to the normal liver cell line (HL-7702), we found that the mRNA and protein levels of LKB1 were significantly lower in most HCC cell lines with the exception of Huh-7 cells, which showed higher expression of LKB1 (**Figure 1**). Subsequently, we verified the expression of LKB1 mRNA in HCC tissues. The results showed that the expression level of LKB1 mRNA was lower in most HCC tissues than in the corresponding adjacent tissues (**Figure 2**).

Association of LKB1 expression with clinicopathological features of HCC

We used IHC to examine the expression level of LKB1 in 79 cases of HCC samples. IHC data showed that the LKB1 protein was mainly localized in the cytoplasm in tissues (**Figure 3**). The enrolled patients were divided into two groups (low and high expression of LKB1) according to the IHC results of LKB1. Our data showed that patients in the low expression of LKB1 group were 54.4% (43 out of 79), and LKB1 IHC staining was correlated with tumor size ($P= 0.005$) and histologic grade ($P=0.024$) in HCC patients (**Table 1**). The remaining data suggested no correlation between LKB1 and age ($P=0.493$), gender ($P=0.198$), BCLC stage ($P=0.146$), number of tumors ($P=0.422$), hepatitis B ($P=0.791$), Child-Pugh stage ($P=0.503$), tumor embolus ($P=0.075$). Because LKB1 was closely related to maximum size of tumors, tumor capsule and histological grade, it can be deduced that LKB1 may be involved in HCC progression.

LKB1 is predictive of poor outcomes in HCC

In order to evaluate whether LKB1 affected the survival of patients with HCC, we performed survival analysis in relation to LKB1 expression. Kaplan-Meier analysis showed that patients

Table 1. Correlations between LKB1 expression and clinicopathological characteristics of HCC patients

Characteristics	Total (n = 79)	LKB1		χ^2 test, *p* value
		Low expression (n =43, 54.43%)	High expression (n =36, 45.57%)	
Age				
≥60 [n (%)]	11	5 (45.45)	6 (54.55)	0.493
<60 [n (%)]	69	39 (56.52)	30 (43.48)	
Gender				
Male [n (%)]	63	32 (50.79)	31 (49.21)	0.198
Female [n (%)]	16	11 (68.75)	5 (31.25)	
Etiology of liver disease				
Hepatitis B [n (%)]	71	39 (54.93)	32 (45.07)	0.791
Hepatitis C [n (%)]	8	4 (50.00)	4 (50.00)	
BCLC stage[a]				
A [n (%)]	37	16 (43.24)	21 (56.76)	0.146
B [n (%)]	17	10 (58.82)	7 (41.18)	
C [n (%)]	25	17 (68.00)	8 (32.00)	
Child-Pugh stage				
A [n (%)]	74	41 (55.41)	33(44.59)	0.503
B [n (%)]	5	2 (40.00)	3 (60.00)	
Maximum size of tumors				
≥5 cm [n (%)]	60	38 (63.33)	22 (36.67)	0.005*
<5 cm [n (%)]	19	5 (26.32)	14 (73.68)	
Number of tumors				
1 [n (%)]	58	30 (51.72)	28 (48.28)	0.422
≥2 [n (%)]	21	13 (61.90)	8 (38.10)	
Tumor capsule				
Complete [n (%)]	47	23 (48.94)	24 (51.06)	0.235
Incomplete [n (%)]	32	20 (62.50)	12 (37.50)	
Tumor embolus				
Positive [n (%)]	13	10 (76.92)	3 (23.08)	0.075
Negative [n (%)]	66	33 (50.00)	33 (50.00)	
Histologic grade[b]				
Well	8	1 (12.50)	7 (87.50)	0.024*
Moderate	41	22 (53.66)	19 (46.34)	
Poor	30	20 (66.67)	10 (33.33)	
AFP				
<400 [n (%)]	48	23 (47.92)	25 (52.08)	0.148
≥400 [n (%)]	31	20 (64.52)	11 (35.48)	

a&b Fisher's exact tests, and X^2 tests for all other analyses. *p<0.05.

with low LKB1 expression had a significantly shorter overall survival than those with high LKB1 expression (χ^2=6.244, P=0.012) (**Table 2**). Univariate analysis indicated that tumor size (P=0.026), number of tumors (P=0.008), histologic grade (P=0.025), LKB1 expression (P=0.012) were closely related to unfavorable outcomes in HCC patients. Multivariate analysis showed that number of tumors (HR 2.456; 95% CI 1.194, 5.051; P= 0.015), LKB1 expression (HR 0.405; 95% CI 0.188, 0.875; P=0.021) were independent prognostic predictors. In general, these results indicated that low expression of LKB1 was likely an independent indicator of poor outcome in HCC (**Table 2**; **Figure 4**).

LKB1 knockdown increase the proliferation of Huh7 cells

To further examine the mechanism underlying the potential tumor suppressor role of LKB1 in HCC, siRNA transfection was utilized. Successful siRNA-LKB1 transfection was confirmed by qRT-PCR and western blot analyses; the results showed that the expression of LKB1 mRNA decreased significantly after transfection of LKB1-siRNA in Huh7 cells, and siLKB1-1 decreased more significantly (P<0.05 vs. negative control). The expression of LKB1 protein was also significantly inhibited; therefore, siLKB1-1 was selected for subsequent studies (**Figure 5**).

Next, we examined the impact of LKB1 on the proliferation of HCC cells via the Cell Counting Kit-8 (CCK-8) assay. The results showed that siLKB1 significantly promoted proliferation of Huh7 cells (P<0.05) (**Figure 6**).

Under-expression of LKB1 enhanced HCC cell migration and invasion in vitro

We used transwell assays in order to study the regulation of LKB1 on the invasion and migration of HCC cells. We observed that the migration and invasion of cells were significantly increased after LKB1 knockdown compared to the negative control groups. These results indicated that LKB1 may inhibit the migration and invasion of HCC cells (**Figure 7**).

Table 2. Clinical variables correlated with overall survival in patients with hepatocellular carcinoma based on Cox univariate and multivariate models

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*p*	HR (95%CI)	*p*
Age: <60 vs. ≥60	0.914 (0.328-2.716)	0.914		
Sex: male vs. female	0.869 (0.376-2.010)	0.743		
Tumor size: <5 cm vs. ≥5 cm	3.034 (1.061-8.679)	0.038*		
Number of tumors: single vs. multiple	2.518 (1.230-5.156)	0.012*	2.456 (1.194-5.051)	0.015#
Tumor capsule: complete vs. incomplete	1.015 (0.504-2.043)	0.966		
Histological grade: well/moderate vs. poor	2.114 (1.066-4.190)	0.032*		
Serum AFP (ng/ml): <400 vs. ≥400	1.491 (0.749-2.970)	0.256		
HBsAg: positive vs. negative	0.960 (0.292-3.160)	0.946		
Tumor embolus: positive vs. negative	0.957 (0.394-2.325)	0.923		
BCLC stage: A vs. B vs. C	1.168 (0.800-1.706)	0.420		
Child-Pugh: A vs. B	0.701 (0.168-2.929)	0.626		
LKB1: low vs. high	0.396 (0.184-0.854)	0.018*	0.405(0.188-0.875)	0.021#

*The variables with *p* value <0.05 in univariate analysis. #The variables with *p* value<0.05 in multivariable analysis.

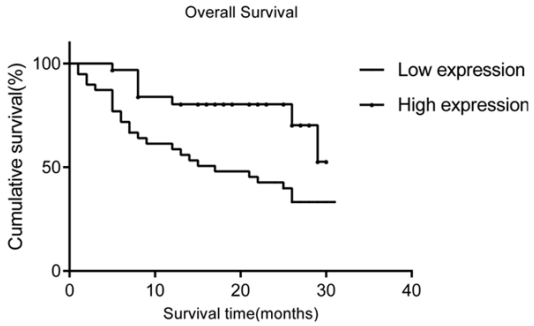

Figure 4. The survival analysis of LKB1. Patients with low LKB1 expression in tumor were closely correlated with poorer overall survival than patients with tumor with higher LKB1 expression.

Effect of LKB1 on the Expression of E-cadherin and Vimentin in Huh7 cells

The expression levels of LKB1, E-cadherin and vimentin were evaluated by western blotting to determine the correlation between LKB1 and EMT in HCC cell lines. Our results indicated that down-regulation of LKB1 resulted in upregulation of vimentin and down-regulation of E-cadherin protein expression in Huh7 cells (**Figure 8A**). These results indicated that LKB1 may play an important role in HCC progression and metastasis by regulating the EMT process.

LKB1 regulates EMT through p38 MAPK signaling pathway

Thus far, our results suggested that downregulation of LBK1 may be critical in promoting EMT. To further investigate this hypothesis, we examined the activation of p38 MAPK signaling pathway which has previously been suggested to inhibit the expression of E-cadherin. The expression of phosphorylated p38 was significantly increased following transfection of si-LKB1 in Huh7 cells (**Figure 8A**). Therefore, we hypothesized that LKB1 modulated the expression of EMT-related molecules through p38 MAPK signaling pathway. To further investigate the role of p38 MAPK in regulation of EMT, we blocked the activation of p38 MAPK by employing SB202190, a strong and highly selective inhibitor of the p38 MAPK pathway. Huh7 cells were treated with SB202190 (10 μmol/L) for 4 h, the levels of E-cadherin, vimentin, and phosphorylated p38 are presented in **Figure 8**. We observed that SB202190 treatment of siLKB1-transfected cells upregulated the expression of E-cadherin while downregulating the expression of vimentin and p-p38 (**Figure 8B**). Taken together, our data suggested that inhibition of LKB1 may affect EMTby the p38 MAPK pathway.

Discussion

Hepatocellular carcinoma (HCC) is one of the most common human malignant tumors. With a high incidence of liver cancer in China, the number of new cases and deaths in the country accounted for about 50% of the number of new cases worldwide [18]. Despite improvement in surgery for HCC treatment in recent years, late diagnosis or misdiagnosis of symptoms results

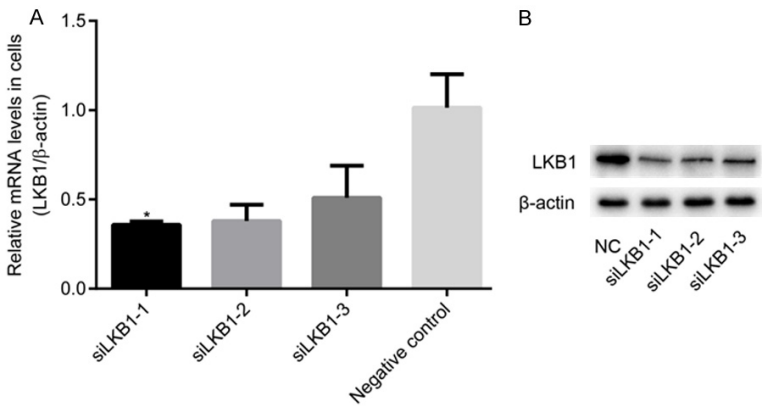

Figure 5. Knockdown of LKB1 enhanced cell proliferation in Huh7 cells. A, B. The relative mRNA and protein expression level of LKB1 in Huh7 cells was significantly decreased by siLKB1 compared with si-NC group.

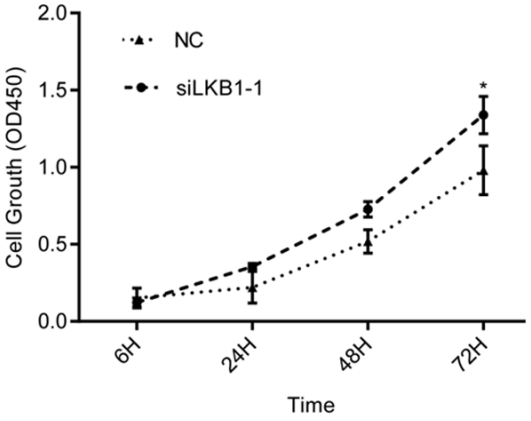

Figure 6. After transfection of 24 h, cell proliferation assays were conducted to determine the proliferation of Huh7 cells. Results are expressed as mean ± SD for three replicate determinations. Statistically significant differences between siLKB1 group and si-NC group were determined by Student's t test.

in rapid HCC progression. Typically, patients have advanced liver cancer or the emergence of extensive liver metastasis at diagnosis, thus limiting their comprehensive treatment options. Furthermore, the prognosis of HCC patients after systemic treatment still remains poor [19]. Tumor invasion and metastasis are major causes for treatment failure; therefore, the ultimate goal is to determine the molecular mechanism of metastasis and target critical pathway inhibition of this process. Tumor metastasis and invasion are complex biologic processes, which are impacted by both oncogene activation and tumor suppressor gene inactivation. Therefore, understanding the molecular mechanisms of tumor invasion and metastasis with the aim of exploring effective drug targets and biomarkers for the diagnosis and treatment of cancer is of great significance.

Recent research shows that the invasion and metastasis of cancer is a multistep process. EMT, which was first discovered at key transition steps during embryogenesis, is thought to be a key phenotypic change in the ability of tumor cells to acquire invasion and metastatic features and to promote malignant progression. The classic description of EMT is the transformation of epithelial cells into mesenchymal cells. The expression of epithelial maker E-cadherin was down-regulated and mesenchymal maker vimentin was up-regulated in the present study. The epithelial cells lose cell polarity and cell adhesion, and acquire mesenchymal-like characteristics in order to migrate away from the original tissue. E-cadherin has been recognized as a very important marker for EMT, and its under-expression has been strongly associated with cancer progression and poor prognosis [20]. Vimentin is an intermediate filament protein is mainly expressed in mesenchymal cells, and in a variety of epithelial tumors. Vimentin is closely related to the differentiation, invasion, and metastasis of tumor cells, and can be used as a prognostic indicator of malignant tumor. Increasing evidence suggests that EMT plays an extremely important role during the process of the spread of cancer of HCC [21]. In agreement with the results of previous research, we found that knockdown of LKB1 decreased E-cadherin and increased vimentin expression, enabling in the spread of HCC in distant metastatic sites.

In the current study, to interrogate the role of LKB1 in HCC, we detected the expression levels of LKB1 mRNA and protein in HCC tissues and cell lines. The expression of LKB1 mRNA in HCC tissues was significantly lower compared to the adjacent tissues. Moreover, the relative expression of LKB1 mRNA in most cell lines was lower than in normal liver cell line (HL-7702), in all but one cell line (Huh-7). Furthermore, we used IHC analysis of 79 cases and examined the relationship between LKB1

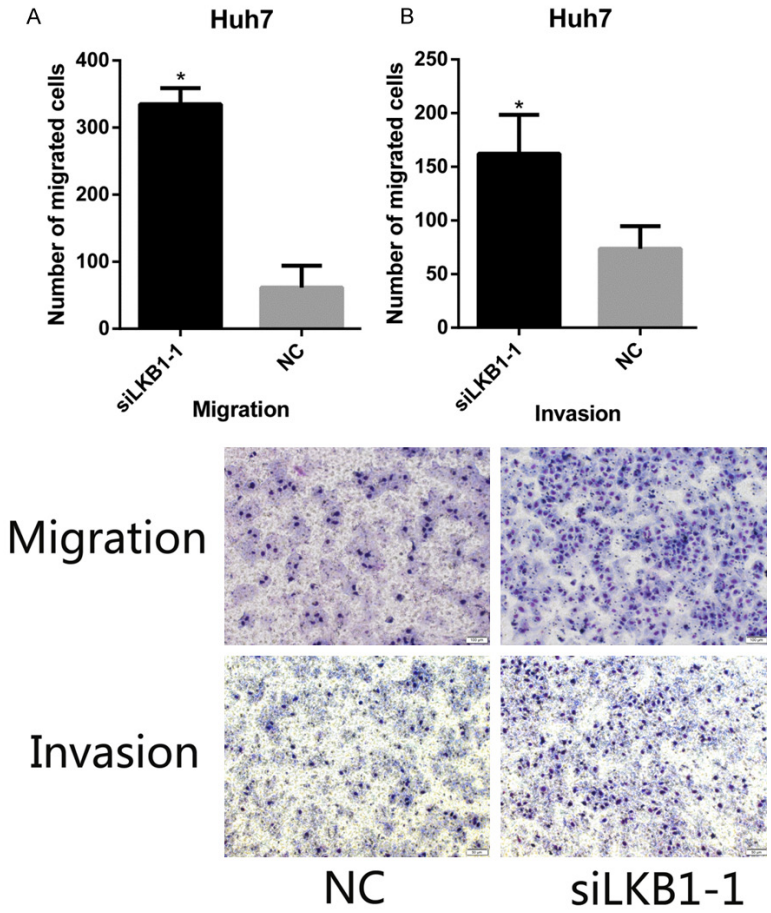

Figure 7. Knockdown LKB1 expression was enhanced cell migration and invasion of Huh7 cells. Trans-well assay showed Huh7 cells infected with si-LKB1 displayed significantly higher migration capacity compared with the si-NC group. Cell invasion assay showed Huh7 cells infected with si-LKB1 displayed significantly higher invasion capacity compared with the si-NC group.

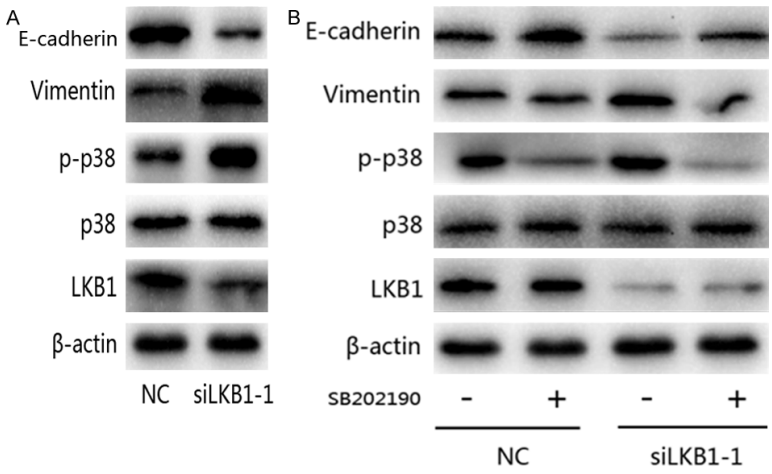

Figure 8. A. Effects of knockdown of LKB1 on expression of E-cadherin and vimentin and p-p38 in Huh7 cells. B. Effects of knockdown of LKB1 on p38 MAPK signaling and treated with SB202190.

expression and clinicopathologic features. The results showed that the relative expression level of LKB1 was significantly correlated with tumor size and histologic grade in HCC patients. However, LKB1 expression was not associated with age, gender, BCLC stage, number of tumors, hepatitis B, Child-Pugh stage, or tumor embolus. Furthermore, we demonstrated that low expression of LKB1 protein exhibited a significant association with a shorter overall survival (P=0.021). These data indicated that the low expression of LKB1 was a possible independent prognostic factor in HCC. Our study was consistent with a previous report that found that the expression of LKB1 was lower in gastric tissue cell lines and tumor tissues (155 gastric cancer) compared to normal gastric cells and tissues (95 normal gastric epithelial tissue specimens) [22]. Therefore, we speculate that LKB1 may also regulate the growth and metastasis of HCC cells.

To determine the biological function of LKB1 in HCC, we silenced its expression by siRNA transfection. Following the inhibition of LKB1, the proliferation, invasion, and migration of cells were significantly enhanced compared with the control group. In addition, the expression of E-cadherin and vimentin was examined, and we found that down-regulation of E-cadherin and increased expression of vimentin in the siLKB1 group suggesting that down-regulation of LKB1 in Huh7 cells may induce EMT to regulate invasion and migration of tumor

cells. We also found that phosphorylated p38 was significantly increased in the LKB1-silenced group. Because the activation of p38 MAPK signaling pathway can down-regulate the expression of E-cadherin, we hypothesized that LKB1 low expression leads to the progression of tumor by modulating EMT by p38 MAPK signaling pathways and thus affecting the malignant progression of HCC. Furthermore, we found that with the inhibition of the p38 MAPK pathway, phosphorylated p38 levels were significantly decreased, E-cadherin expression increased, whereas vimentin expression decreased. These data suggested that the activation of p38 MAPK signaling pathway is one of the mechanisms leading to tumor invasion and migration when LKB1 is silenced. However, investigation of the detailed molecular regulation mechanism requires further study. Nonetheless, our data suggests that LKB1 may play an important role in the progression, tumorigenesis, and development of HCC as a tumor suppressor gene.

Our research has several limitations: our studies on cell growth and metastasis were only carried out in vitro, thus need to be verified in in vivo studies. Secondly, only one HCC cell line was employed in majority of this study; therefore, additional research in HCC cell lines that overexpress LKB1 are required to support our findings. Finally, the amount of clinical sample data was small (n=79), and follow-up time (32 months) was too short; thus, larger studies with longer follow-up are needed to confirm our views.

In conclusion, our results suggest that LKB1 is a tumor suppressor gene with low expression in hepatocellular carcinoma and may regulate the progression of HCC through the p38 MAPK signaling pathway. Further studies are needed to understand the mechanisms underlying the effect of LKB1-induced cell polarity on liver cancer metastasis and invasion.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Grant nos. 81460426 and 81360315), the Natural Science Foundation of Guangxi Province (Grant no. 2014GXNSFAA118191).

Disclosure of conflict of interest

None.

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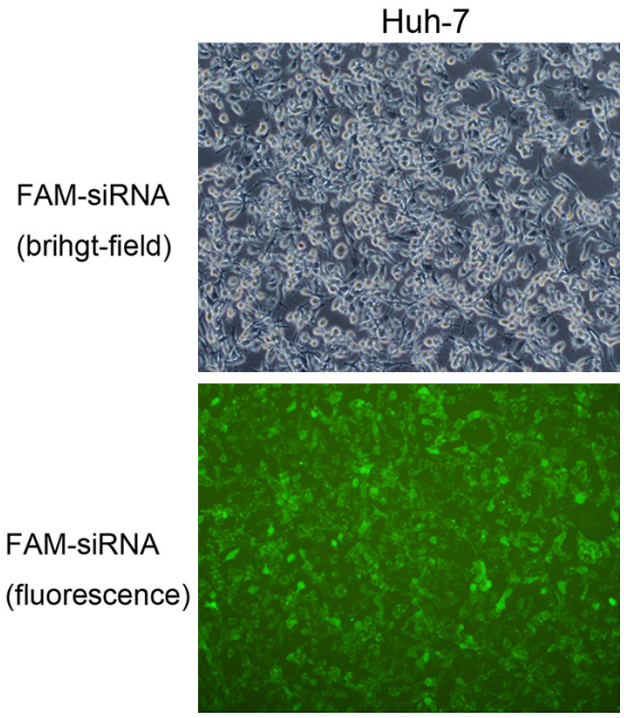

Figure S1. Efficiency of transfection in Huh-7 cells.