RESEARCH ARTICLE

Decreased Expression of LKB1 Correlates with Poor Prognosis in Hepatocellular Carcinoma Patients Undergoing Hepatectomy

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Abstract

Aim: To study any correlation of LKB1 expression with prognosis in hepatocellular carcinoma (HCC) cases. Methods: A total of 70 HCC patients and 20 primary intrahepatic stone patients in the first affiliated hospital of Wenzhou Medical College were enrolled in this study. LKB1 expression was detected by immunohistochemistry. Patients were followed-up and prognostic factors were evaluated. Result: LKB1 expression was decreased in the HCC samples. Loss of LKB1 expression in HCC was significantly related to histologic grade (*P*=0.010), vascular invasion (*P*=0.025) and TMN stage (*P*=0.011). Patients showing negative LKB1 expression had a significantly shorter disease-free and overall survival than those with positive expression (*P* = 0.001, *P*=0.000, respectively). Multivariate Cox regression analysis indicated that LKB1 expression level was an independent factor of survival (*P* = 0.033). Conclusion: HCC patients with decreased expression LKB1 have a poor prognosis. The loss of LKB1 expression is correlated with a lower survival rate.

Keywords: LKB1 - expression - hepatocellular carcinoma - survival - prognosis

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common worldwide tumor and is the third most common cause of cancer-related deaths in the world and in China (Cha et al., 2012; Takeuchi et al., 2012; Yang et al., 2012). Various factors have been associated with the initiation and development of HCC,including chronic infection of hepatitis viruses;age;sex;alcohol abuse;aflatoxin; diabetes mellitus and hereditary metabolic liver diseases (Zha et al., 2012). However,there is a lack of sensitive and specific biomarkers in the clinic, the existing early-stage screening strategies have limited benefits to the detection of HCC until multicentric recurrence and intrahepatic metastasis (Li et al., 2012). So even with advanced treatments, such as surgery, chemotherapy, and radiotherapy, HCC still presents a poor prognosis.Therefore, it is critical to identify important prognostic factors and to develop novel therapeutic strategies targeting HCC. Being similar to most other kinds of tumor,hepatocarcinogenesis is a complex process,such as the activation of oncogenes and the inactivation of tumor suppressor genes,involving genic alterations and mutations, which ultimately lead to the malignant transformation of hepatocytes (Zha et al., 2012).

The serine–threonine kinase liver kinase B1 (LKB1; also termed as STK11) encoding a serine/threonine kinase (locate on chromosome 19p13.3), is a gene of which the germ-line mutations are found in Peutz-Jeghers Syndrome(PJS) (Nakau et al., 2002; Shen et al., 2002; Shackelford al., 2009). Recently, many researches demonstrate LKB1 as one of the most commonly mutated genes in various kinds of carcinomas such as non-small cell lung carcinomas, cervical carcinomas, breast carcinomas and pancreatic carcinoma wherever in vivo and in vitro study (Shen et al., 2002; Sahin al., 2003; Fernandez al., 2004; Fenton al., 2006; Hezel al., 2008; Shackelford al., 2009). And there is a study demonstrate the functions of LKB1 correlates with angiogenesis, invasion, and metastasis (Zhuang al., 2006). Low expression of the LKB1 protein is obviously associated with a shorter survival in human breast cancer (Shen et al., 2002).

Here, we have investigated the expression and clinical significance of LKB1 in HCC.

Materials and Methods

Patient and clinicopathologic parameters

We collected cancerous tissues and surrounding noncancerous hepatic tissues from 70 consecutive patients who had undergone surgery for HCC at the first affiliated hospital of Wenzhou medical college from January 2006

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to December 2010. Normal hepatic tissues were also obtained from 20 patients who suffered from curative hepatectomy for primary intrahepatic stones from January 2011 to February 2011. The Ethics Committee of the first affiliated hospital of Wenzhou medical college approved the protocol of this study. In the 70 HCC cases, there were samples from 57 males and 13 females ranging in age from 43 to 72 years (median age 57 years). There were 4 patients with diabetes and 11 patients with hypertension. Among the 70 patients, 26 had serum α-fetoprotein (AFP) ≥ 25 μg/L, and 48 were sera positive for hepatitis B surface antigen (HBsAg). On gross examination, 35 cases had tumor sizes that were ≥ 5 cm, and 35 had tumor sizes < 5 cm (median tumor size, 4.0 cm; range, 1.2-9 cm). Histopathological diagnoses were made according to the 2002 hepatocellular carcinomas staging system of the International Union Against Cancer. Thirty cases were well differentiated; 21 cases were moderately differentiated; and 19 cases were poorly differentiated. In total, 46 HCC cases had liver cirrhosis. We defined first recurrence as evidence of a distinct new growing mass in the liver, or as distant recurrence in radiologic examinations.Seventy patients were followed up until December 2012. Total 37 death patients were included,30 patients were still alive, 3 patients were excluded ,since they were lost during following-up process.All patients had survived for at least 32 weeks after hepatectomy (median follow-up, 132 weeks; range 32-288 weeks).

Immunohistochemistry

Paraffin-embedded sections (3.0-3.5 μm) were deparaffinized in xylene and rehydrated through an graded series of alcohol for immunostaining. After rinsing with PBS,endogenous peroxidase was blocked with 0.3 % hydrogen peroxide for 10 min at room temperature (RT). Then the sections were immersed in EDTA and boiled for 20 min in the microwave oven for the antigen retrieval.The sections were incubated with or without the primary antibody the LKB1 antibody (1:50) in a moist chamber, overnight at 4 °C in the refrigerator. Following additional wash with PBS for three times, the sections were sequentially incubated with secondary antibody (PowerVision™ Two-Step Histostaining Reagent) at 37°C for 20min and then washed three times with PBS.3, 3-diaminobenzidinetetrahydrochloride (DAB), respectively,served as chromogens,then counterstained with hematoxylin and mounted. Omission of the primary antibody was used as a negative control for tissue sections. LKB1 (P340) pAb (Bioworld technology, co, Ltd), and PV-6001 PowerVision™ Two-Step Histostaining Reagent (Zhongshan gold bridge biological technology company, Beijing, China) were used for IHC. The sections were examined microscopically and interpreted in a blinded fashion by two pathologists. LKB1 expression in the cell cytoplasm was independently evaluated. The intensity of LKB1 was categorized as negative staining (score = 0), weakly staining (score = 1), moderately staining (score = 2), or strongly staining (score = 3). The percentage of immunoreactive cells was also assessed. The percentage of LKB1 expression was classified as 0% (score = 0), <10% (score = 1), 10%-50% (score = 2), >50% (score = 3). The staining index = intensity score×distribution score.In this research, staining index scores of ≤ 3 and ≥ 4 were defined as negative and positive.

Statistical analysis

The PASW Statistics 18 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Quantitative values were presented as median (range). The χ^2 test was used to analyze the relationship between LKB1 expression and various clinicopathological characteristics. Survival curves were calculated using the Kaplan–Meier method and compared by the log-rank test. Cox proportional-hazard analysis was used for univariate and multivariate analysis to investigate the effect of clinicopathological variables and LKB1 expression on survival. A P value of less than 0.05 was considered statistically significant.

Results

LKB1 expression in hepatocellular Carcinoma patients

LKB1 immunostaining was mostly in the cytoplasm. Overall, 39 of 70 (55.7%) cases had positive expression in liver tumor tissue, 31 of 70 (44.2%) cases had negative

Table 1. The LKB1 Expression in Normal Liver, HCC and Adjacent Non-tumorous Tissues

	Negative expression of LKB1	Positive expression of LKB1
Normal liver tissue	0	20
Hepatocellular carcinoma	31	39
Adjacent non-tumorous tissue	5	65

χ^2=34.327, *P*=0.000

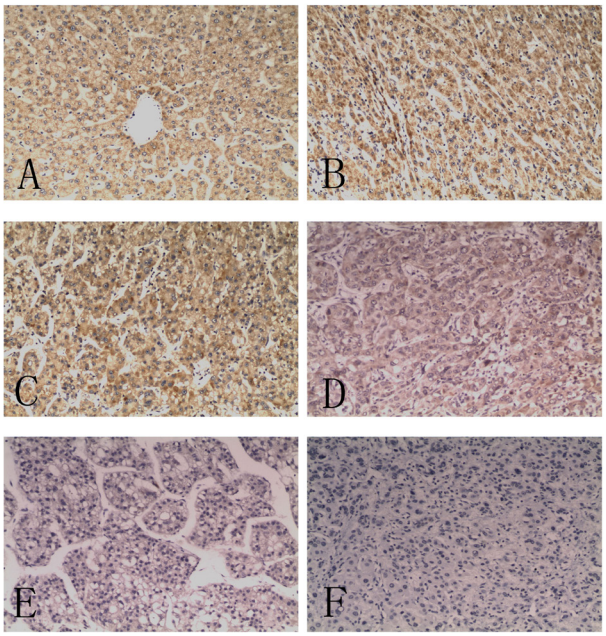

Figure 1. LKB1 Protein Showed Decreased Expression in HCC Tissues. (A) Immunohistochemical demonstration of LKB1 expression in normal livers. (B) Positive staining of LKB1 in adjacent surrounding non-tumorous tissues. (C) Strongly cytoplasmic staining of LKB1 in HCC specimens. (D) Moderately cytoplasmic staining of LKB1 in HCC specimens. (E) Weakly cytoplasmic staining of LKB1 in HCC specimens. (F) Negative staining of LKB1 in HCC specimens. All images are shown at × 100

Table 2. Correlations Between LKB1 Expression and Clinicopathologic Characteristics of 70 Cases

Characteristics	Number	LKB1 expression		χ^2	*P* value
		Negative	Positive		
All cases	70	31	39		
Gender				0.219	0.639
Female		5	8		
Male		26	31		
Age				0.32	0.572
≥55 years		18	20		
<55 years		13	19		
HBV infection				2.85	0.091
Ansent		13	9		
Present		18	30		
Serum AFP				3.063	0.08
≥25 ug/L		8	18		
<25 ug/L		23	21		
Tumor size				0.058	0.81
≥5 cm		15	20		
<5 cm		16	19		
Tumor number				1.172	0.279
Single		23	33		
Multiple		8	6		
Liver cirrhosis				0.035	0.851
Ansent		11	13		
Present		20	26		
Histologic grade				6.605	0.010*
Well		8	22		
Moderate/Poor		23	17		
Vascular invasion				5.031	0.025*
Ansent		20	34		
Present		11	5		
Bile duct thrombi				3.262	0.071
Ansent		14	26		
Present		17	13		
TMN stage				6.521	0.011*
I-II		12	27		
III-IV		19	12		

*P<0.05

expression. In 70 cases with the adjacent non-tumorous tissue, 65 of its cases had positive expression. All had positive expression in normal liver tissue. The data revealed that LKB1 expression was decreased in the HCC samples (Table 1).

Correlations between the expression of LKB1 and various clinicopathologic parameters are listed in Table 2. The LKB1 expression was significantly related to vascular invasion (P = 0.025), histologic grade (P=0.010), TMN stage (P=0.011). Negative expression of LKB1 was associated with advanced clinicopathologic characteristics.However, there was no statistically significant difference between LKB1 expression and age, gender, HBV infection, serum AFP, liver cirrhosis, tumor size, tumor number, or bile duct thrombi.

LKB1 decreased-expression and survival

Patients showing negative LKB1 expression had a significantly shorter disease-free survival and overall survival than those with positive expression (P = 0.001, P=0.000, respectively, log-rank test; Figure 2). Univariate Cox regression analysis also identified that clinical variables including histologic grade, vascular invasion, bile duct thrombi, TMN stage, LKB1 expression were significantly associated with overall survival (Table 3).

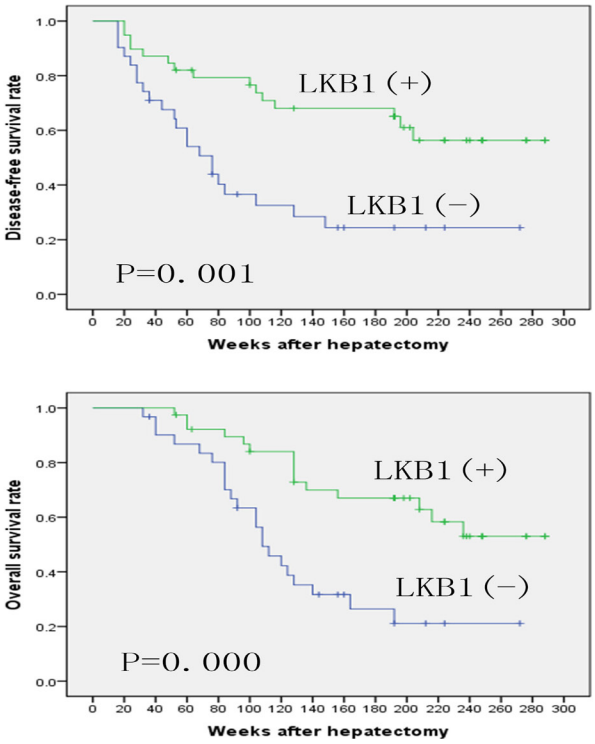

Figure 2. Kaplan-Meier Survival Analysis of Primary Hepatocellular Carcinoma Patients (n = 70) after Surgical Resection with Negative LKB1 Expression (n = 31) and Positive LKB1 Expression (n = 39). The disease-free survival rate and overall survival rate for patients in the LKB1 negative group ("-") was significantly lower than that for patients in the LKB1 positive group ("+") (log rank, P = 0.001, P=0.000, respectively).

Table 3. Overall Survival Cox Regression Analysis

Variables	Relative risk(95% CI)	*P*-value
Univariate		
Gender	0.960(0.421-2.191)	0.922
Age	1.230(0.645-2.346)	0.53
HBV infection	0.851(0.431-1.682)	0.643
Serum AFP	2.019(0.993-4.107)	0.052
Tumor size	0.895(0.469-1.708)	0.738
Tumor number	1.380(0.627-3.036)	0.423
Liver cirrhosis	1.932(0.929-4.015)	0.078
Histologic grade	2.828(1.380-5.795)	0.005*
Vascular invasion	4.130(2.024-8.431)	0.000*
Bile duct thrombi	2.737(1.425-5.256)	0.002
TMN stage	3.811(1.939-7.491)	0.000*
LKB1	0.317(0.161-0.624)	0.001*
Multivariate		
Vascular invasion	2.528(1.215-5.258)	0.013*
Bile duct thrombi	1.995(1.023-3.890)	0.043*
TMN stage	3.232(1.573-6.641)	0.001*
LKB1	0.459(0.225-0.938)	0.033*

*P<0.05

Furthermore, to evaluate the potential of LKB1 expression as an independent predictor for overall survival of HCC, multivariate Cox regression analyses (Forward: LR) were performed. While the others failed to demonstrating independence, vascular invasion, bile duct thrombi, TMN stage, LKB1 expression may play a role in predicting the overall survival in HCC (P = 0.013, 0.043, 0.001 and 0.033, respectively, Table 3).

Discussion

Hepatocellular carcinoma is one of the most common malignant tumors and has an obviously poor prognosis. The lack of effective screening indicator of early hepatic carcinoma is one of the reasons for poor prognosis. When patients with typical symptoms or findings obviously serologic abnormalities, most patients have advanced hepatic carcinoma or have been transferred. most patients have advanced hepatic tumor or have been metastasized. In this study, we reported that LKB1 expression which maybe a better predictor for HCC were associated with prognosis of HCC patients.The liver kinase B1 (LKB1) which was mapped to chromosome 19p13.3 and was found to encode a serine/threonine kinase by Hemminki et al.in 1998 (Avizienyte al., 1999; Su al., 1999; Esteller al., 2000). LKB1 is central in regulating cellular metabolism and cell growth by integrating information which included the oxygen status,energy, the presence of growth factors and nutrient availability.Furthurmore, many researches showed frequent LKB1 allelic loss in various human cancers, including hepatocellular carcinoma (HCC), non-small cell lung carcinomas, cervical, breast carcinomas, pancreatic carcinoma and epithelial carcinoma (Avizienyte al., 1999; Shen et al., 2002; Kim al., 2007; Gao al., 2011; Herrmann al., 2011). Animal experiments showed that the loss of LKB1 gene attributes to hepatocarcinogenesis (Miyoshi al., 2009). In breast cancer cells study showed overexpression of LKB1 protein can result in growth inhibition of breast tumor cells and less expression of LKB1 protein contribute to tumor cell cycle progression (Shen et al., 2002; Zhuang al., 2006). Suppress LKB1–AMPK signaling such as hyperglycaemia and overnutrition, which may result in an increased cancer risk in patients who are diabetic or obese. Conversely, activation of LKB1–AMPK signalling might contribute to a decreased cancer risk (Shackelford al., 2009). Honokiol via an LKB1-dependent pathway activates AMP-activated protein kinase in breast cancer cells and inhibits breast carcinogenesis (Nagalingam et al., 2012). And use of metformin in diabetic patients,in a dosedependent manner, is associated with a decreased risk of HCC via induction of cell cycle arrest at G0/G1 phase and inhibition of hepatoma cells proliferation (Chen et al., 2013). By activating the LKB1-AMPK signaling pathway may become the trend of targeted therapy.

What is the role of LKB1 in HCC patients?In this study, we also investigated LKB1 protein expression in 70 cases of human HCC samples.We found that in a total of 70 cases, there were 31 (44.3%) cases showing the loss expression of LKB1. We also indicated that LKB1 protein expression levels were significantly associated with vascular invasion (P = 0.025), histologic grade (P=0.010), TMN stage (P=0.011). Furthermore, we demonstrated that the loss expression of LKB1 protein shows a significant association with a shorter disease-free survival and overall survival (P = 0.001, P=0.000, respectively, log-rank test; Figure 2). To our discover, this is the first study that demonstrates a possible prognostic value for low LKB1 expression in human HCC. Of course, further researches are needed to confirm our findings.

In conclusion, our results may indicate that LKB1 plays a role in tumor suppressor function in human hepatocellular carcinoma. Decreased expression of the LKB1 protein in human hepatocellular carcinoma is significantly associated with a shorter survival.LKB1 expression may be a valuable prognostic marker in human hepatocellular carcinoma in further clinical examination.

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