Decreased Expression of Tumor-suppressor Gene *LKB1* Correlates with Poor Prognosis in Human Gastric Cancer

JUNJIE SUN[1,2], BINXUN LING[1], XINYU XU[3], RONG MA[4], GANG LI[5], XINGHUA CAO[6], WANG LING[6], ZHIJIAN YANG[6,7], ROBERT M. HOFFMAN[7,8] and JIANWEI LU[1]

Departments of [1]Oncology, [3]Pathology, and [5]General Surgery, and [4]Experimental Center of Clinical Oncology, Affiliated Cancer Hospital, Nanjing Medical University, Nanjing, Jiangsu, P.R. China;
[2]Department of Oncology, Wuxi No.4 Hospital, Affiliated to Jiangnan University, Nanjing, Jiangsu, P.R. China;
[6]Origin Biosciences Inc., Nanjing, Jiangsu, P.R. China;
[7]AntiCancer Inc., San Diego, CA, U.S.A.;
[8]Department of Surgery, UCSD, San Diego, CA, U.S.A.

Abstract. *Background/Aim: The present report describes the correlation of liver kinase B1 (LKB1) expression with tumorigenesis and prognosis in gastric cancer. Materials and Methods: LKB1 mRNA and protein expression was detected in gastric-cancer cell lines and patient specimens. Patients were followed-up and clinico-pathological parameters and overall survival (OS) were evaluated. Results: The expression of LKB1 mRNA and protein was lower in gastric-cancer cell lines and tumor tissues compared to normal gastric cells ($p<0.05$) and tissues ($p<0.001$). Decreased expression of LKB1 mRNA and protein in patients with gastric cancer was significantly inversely related to TNM stage, T-stage (depth of invasion), lymph-node metastasis and vascular invasion ($p<0.05$). Patients showing high LKB1 mRNA and high LKB1 protein expression had a significantly longer OS and better 5-year survival rate than those with low mRNA expression (61.3 months vs. 56.1 months and 75% vs. 58.7%, $p<0.05$, respectively) and low protein expression (64.8 months vs. 55.7 months and 72.9% vs. 64.5%, $p<0.05$, respectively). Multivariate Cox regression analysis indicated that both LKB1 mRNA and protein expression in gastric cancer were independent prognostic factors for OS. Conclusion: Patients with gastric cancer with decreased expression of LKB1 have a poor prognosis with a lower survival rate.*

Gastric cancer is the fifth most freguent type of cancer and the third leading cause of cancer-related death in the world (1). Although recent diagnostic and therapeutic strategies have gradually advanced, gastric cancer is usually not diagnosed until an advanced stage and the 5-year survival rate is still low (2, 3), Gastric cancer especially remains a challenge in East Asia, with high incidence and mortality rates persisting. Gastric cancer is a complex, multistep process involving deregulation of genetic and epigenetic alterations. Genetic alterations, such as gene amplification, mutations (4) and polymorphisms (4, 5) are associated with gastric cancer.

Liver kinase B1 (*LKB1*), also known as STK11, is a serine/threonine protein kinase. *LKB1* acts *via* the AMP-activated protein kinase (AMPK) pathway, and is frequently lost in sporadic pancreatic cancer and lung adenocarcinoma (6, 7). Inactivation of *LKB1* results in the activation of the mammalian target of rapamaycin (mTOR) pathway, which is crucial in controlling cellular energy metabolism, cell survival and growth under metabolic stress such as nutrient deficiency (8). *LKB1* has been reported to function as a tumor suppressor through the regulation of AMPK signaling in several malignancies such as lung, breast and cervical cancer (9-12). Chromosomal loss or mutation of the *LKB1* gene is associated with reduced progression-free survival in patients (13-16). However, the effect of genetic and epigenetic alterations on *LKB1* expression in gastric cancer remains unclear.

In this retrospective study, we examined mRNA and protein expression of *LKB1* in gastric cancer cell lines and tumor specimens in a large patient cohort. The relationship of *LKB1* expression with clinico-pathological parameters and overall survival was explored to demonstrate the prognostic value of *LKB1* expression in gastric cancer.

Materials and Methods

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Correspondence to: Jianwei Lu, Department of Oncology, Affiliated Cancer Hospital, Nanjing Medical University, Nanjing, Jiangsu 210009, P.R. China. E-mail: lujw@medmail.com.cn and Robert M. Hoffman, Ph.D., AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111 USA. Tel: +1 8586542555, Fax: +1 8582684175, e-mail: all@anticancer.com.

Key Words: LKB1 expression, survival, prognosis and gastric cancer.

Cell culture. Human gastric cancer cell lines NCI-N87, MKN-28, BGC-823 and MKN-45 were purchased from the Cell Resource Center of the Shanghai Life Sciences Institute, Chinese Academy of Sciences (Shanghai, China). NCI-N87 and MKN-28 are well-differentiated cell lines, while BGC-823 and MKN-45 are poorly differentiated cell lines. The immortalized normal gastric mucosal epithelial cell line GES-1 was kindly provided by the Jiangsu Institute of Pharmaceutical Research (Nanjing, China). The cells were cultured in RPMI-1640 medium (GIBCO Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO) in a humidified incubator with an atmosphere of 5% CO_2 at 37˚C.

Patients and specimens. A total of 155 formalin-fixed, paraffin-embedded gastric-cancer specimens were obtained from patients who underwent gastric resection from January 2008 to December 2009 at the Affiliated Cancer Hospital of Nanjing Medical University, Nanjing, China. All specimens were confirmed histologically, independently by two pathologists. All patients received post-operative conventional radiotherapy and chemotherapy. Ninety-five normal gastric epithelial tissue specimens from patients without gastric cancer were selected for control. Clinico-pathological data were reviewed and are listed in Table I. Tumor–node–metastasis (TNM) staging was based on the criteria of the American Joint Committee on Cancer (AJCC; fifth edition) (17). Patients were followed up, and overall survival (OS) time was recorded. All studies were approved by the Human Ethics Committee of the Affiliated Cancer Hospital of Nanjing Medical University (OR1315) and written informed consent was obtained from all patients.

Isolation of RNA and reverse transcription-polymer chain reaction (RT-PCR). Total RNA was isolated from cell lines and tumor tissues with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed using a PrimeScript RT-PCR kit (Takara, Kyoto, Japan) according to the manufacturer's instructions, followed by PCR amplification with specific primers. The following primers were used to amplify most of the coding region of *LKB1* (sense, 5' GGGATGCTTGAGTACGAACCG 3', and antisense, 5' AGTACGG CACCACAGTCATGCT 3') and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (sense, 5'-GGAAGGTGAAGGTCGGA GTC-3'and antisense, 5'-AATGAAGGGGTCATTCATGG-3').

Quantitative real-time PCR was performed using a 7300 Real-time PCR system (Applied Biosystems, Waltham, MA, USA). PCR was carried out in a volume of 20 μl containing 10 μl 2×qPCR Master Mix, 2 μl cDNA, 0.2 μl each primer and 0.4 μl ROX Reference Dye 1. Reaction conditions for amplification of target genes were one cycle of denaturation at 95˚C for 5 min, followed by 40 cycles of 5 s degeneration at 95˚C, 30 s annealing at 60˚C and 40 s prolongation at 72˚C. Data were analyzed by the relative standard curve method and normalized to *GAPDH* expression. Relative RNA expression in the gastric cancer cell lines and patient specimens was calculated using the $2^{-\Delta\Delta Ct}$ and $2^{-\Delta Ct}$ methods, respectively. All samples were performed in triplicate.

Western blotting. *LKB1* protein expression in gastric cancer cell lines was analyzed by Western blotting. Cells were lysed in 100 μl RIPA lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 1% NP-40, 150 mmol/l NaCl, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mmol/l Na_3VO_4, 1 mmol/l NaF) at 4˚C for 30 min. Cell debris was removed by centrifugation at 12,000 × *g* for 20 min at 4˚C. Protein concentrations were determined by the Bradford assay (Bio-Red, Hercules, CA, USA). An equal amount of lysate (40 μg) was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk at room temperature for 1 h and then incubated for 2 h with primary antibodies to *LKB1* and β-actin (Cell Signaling Technology, Boston, MA, USA). The membranes were then incubated for 1 h with an appropriate horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Electro-chemi-luminescence was performed according to the manufacturer's instructions using a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). Quantity One software (Bio-Rad) was used to quantify the density of bands.

Immunohistochemical analysis. *LKB1* protein expression was measured by immunohistochemical analysis in formalin-fixed, paraffin-embedded tissue sections from 155 gastric cancer and 95 normal gastric epithelial tissue specimens. Two serial sections of 4 μm were cut from each block and placed onto Super Frost Plus glass-slides (Thermo Fisher Scientific Gerhard Menzel, Braunschweig, Germany). Following deparaffinization in xylene, the slides were rehydrated and washed in Tris-buffered saline. The endogenous peroxidase activity was quenched by 10 min incubation in a mixture of 3% hydrogen peroxide solution in 100% methanol (Sigma, St. Louis, MO, USA). Slides were cleared with Tris-buffered saline and placed at room temperature for 1 h. They were then incubated with monoclonal mouse antibody to human *LKB1* protein (ab15059; Abcam, Fremont, CA, USA) at 1/100 dilution overnight at 4˚C. PBS was used instead of the primary antibody as the negative control. Immuno-staining was performed using the ChemMate EnVision Detection Kit (DAKO, Carpinteria, CA, USA) according to the manufacturer's instruction.

The sections were examined microscopically and interpreted in a blinded fashion by two pathologists. Ten areas were randomly selected and counted at a magnification of ×200. *LKB1* staining was evaluated semi-quantitatively on the basis of the percentage of positively-stained cells and classified as follows: 0, no staining or weak staining in <10% of cells; 1+, weak immuno-staining in >10% of cells; 2+, moderate immuno-staining in >10% of cells and 3+, strong immuno-staining in >10% of cells. The staining pattern was either granular or diffuse. Scores of 0 and 1+ indicate a negative tumor, while scores of 2+ and 3+ were regarded as positive.

Statistical analysis. All analyses were performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Inter-group comparisons of the clinical variables were analyzed using one-way ANOVA analysis for continuous variables and the chi-square test for discrete variables. Survival curves were calculated using the Kaplan–Meier method and compared by the log-rank test. Furthermore, Cox proportional hazard models and logistic regression models were used for multivariate analysis of survival time and the association of *LKB1* expression status with clinico-pathological variables, respectively. All *p*-values were two-sided, with $p<0.05$ considered to indicate statistical significance.

Table I. *Demographic and clinico-pathological characteristics of patients with gastric cancer (N=155).*

Characteristic	Number of patients	%
Age (years)		
>60	88	56.8
<60	67	43.2
Gender		
Male	121	78.1
Female	34	21.9
Differentiation		
Well/moderate	39	25.2
Poor	116	74.8
Histological type		
Adenocarcinoma	142	91.6
Other	13	8.4
TMM stage		
I	43	27.7
II	49	31.6
III	58	37.4
IV	5	3.3
T-Stage (invasion depth)		
T1	22	14.2
T2	38	24.5
T3	95	61.3
Lymph node metastasis		
Yes	76	49.0
No	79	51.0
Vascular invasion		
Yes	52	33.5
No	103	66.5

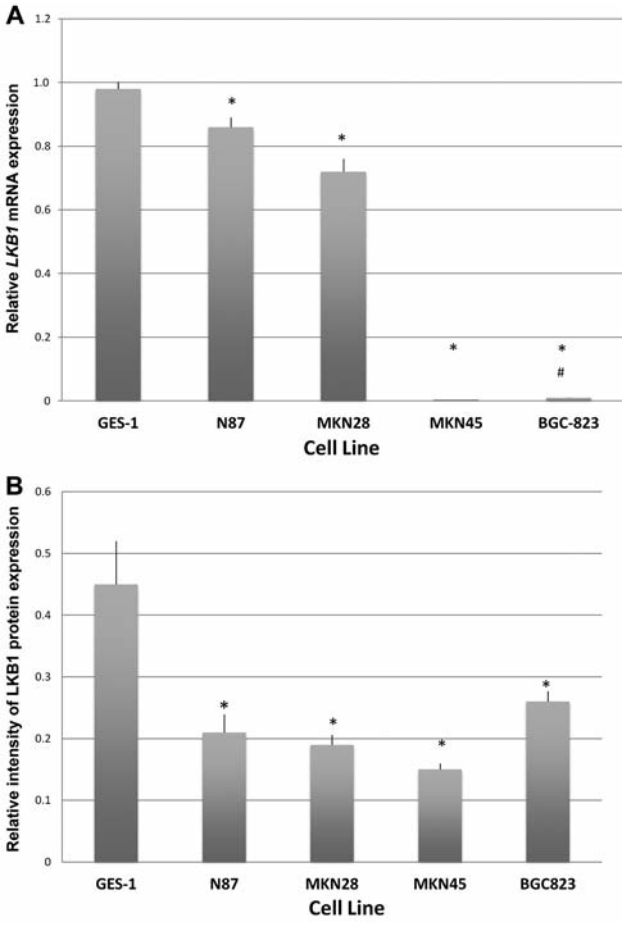

Figure 1. *Liver kinase B1 (LKB1) mRNA and protein expression is decreased in human gastric cancer cell lines. A: Decreased LKB1 mRNA expression in gastric cancer cell lines detected by real-time quantitative PCR. *p<0.05, when compared with normal gastric cell line GES-1; #p<0.001, when compared with well-differentiated cell lines NCI-N87 and MKN-28. B: Decreased LKB1 protein expression in gastric cancer cell lines detected by Western blotting. *p<0.001, When compared with normal gastric cell line GES-1.*

Results

Decreased expression of LKB1 in gastric cancer cell lines and tumor tissues. To determine the role of *LKB1* in gastric tumorigenesis, the expression level of *LKB1* was first examined in gastric cancer and normal cell lines using quantitative qRT-PCR and western blotting. As shown in Figure 1, significantly lower expression of *LKB1* mRNA and protein in gastric cancer cell lines NCI-N87, MKN-28, BGC-823 and MKN-45 was found compared to normal gastric cell line GES-1 ($p<0.05$ and $p<0.001$, respectively). It was noted that poorly-differentiated cell lines BGC-823 and MKN-45 exhibited greater down-regulation of *LKB1* mRNA expression than well-differentiated cell lines NCI-N87 and MKN-28 ($p<0.001$). However, no significant difference in *LKB1* protein expression was found between poorly- and well-differentiated cell lines ($p>0.05$).

LKB1 expression was investigated in 155 gastric-cancer and 95 normal gastric epithelial tissue specimens using qRT-PCR and immunohistochemical analysis. As shown in Table II, the expression of *LKB1* mRNA was down-regulated in gastric cancer tissues compared with normal gastric tissues ($p<0.001$). In addition, gastric cancer tissues exhibited a significantly lower expression of *LKB1* protein compared with normal gastric tissues ($p<0.001$) (Figure 2).

Association of LKB1 expression with clinico-pathological parameters. In order to better understand the clinical relevance of *LKB1* expression in gastric cancer, the association of *LKB1* expression with clinico-pathological parameters was analyzed. As summarized in Table II, we found that the expression of *LKB1* mRNA and protein were significantly inversely associated with TNM stage, T-stage (depth of invasion), lymph node metastasis and vascular invasion ($p<0.05$), but not with age, sex, differentiation and histologic type ($p>0.05$). The levels of *LKB1* expression in

Table II. *Association of liver kinase B1 (LKB1) expression with clinico-pathological characteristics in patients with gastric cancer.*

Characteristic	*LKB1* protein		*p*-Value	*LKB1* mRNA 95% CI ($\times 10^{-3}$)	*p*-Value
	+	−			
Group type			0.001*		0.001*
Normal	90	5		9.81 (8.53-11.1)	
Gastric cancer patients	48	107		3.41 (2.31-4.51)	
Age (Years)			0.862		0.782
>60	28	60		3.77 (2.44-5.10)	
<60	20	47		3.44 (1.33-5.56)	
Gender			0.062		0.439
Male	42	79		3.88 (2.50-5.27)	
Female	6	28		2.80 (1.05-4.55)	
Differentiation			0.432		0.451
Well/moderate	10	29		3.51 (2.19-4.84)	
Poor	38	78		2.61 (0.49-4.7)	
Histological type			0.235		0.740
Adenocarcinoma	46	96		3.42(2.11-4.73)	
Other	2	11		3.83(1.70-5.94)	
TMM stage			0.001*		0.039*
I,II	40	52		4.53 (2.68-6.38)	
III,IV	8	55		2.24 (1.10-3.37)	
T-Stage			0.012*		0.016*
T1,T2	26	34		5.40 (3.01-7.78)	
T3	22	73		2.54 (1.46-3.62)	
LN metastasis			0.010*		0.320
Yes	16	60		2.43 (1.45-3.41)	
No	32	47		3.48 (1.83-5.12)	
Vascular invasion			0.028*		0.032*
Yes	10	42		1.91 (1.00-2.83)	
No	38	65		4.52 (2.88-6.16)	

CI: Confidence interval. *$p<0.05$.

patients with TMM stage III and IV were significantly lower than those in patients with TMM stage I and II ($p<0.05$). The patients who developed tumors with deeper invasion (T3), lymph-node metastasis and vascular invasion had significantly lower expression of *LKB1* than those with T1/T2, no lymph-node metastasis and no vascular invasion ($p<0.05$).

Down-regulation of LKB1 expression is associated with poor prognosis in gastric cancer. To further evaluate the associations of *LKB1* expression with survival of the patients, the Kaplan–Meier method was performed. The 5-year OS rate of the 155 patients with gastric cancer was 67% (104/155), with 51 deaths observed during the follow-up period. We categorized cases with *LKB1* mRNA-expression values higher than the median value (0.36×10^{-3}) as the high *LKB1*-mRNA expression group and the remaining as the low-mRNA expression group. Survival analysis indicated that patients who had high *LKB1* mRNA expression had a longer OS compared to patients who had low *LKB1* mRNA expression (61.3 months *vs*. 56.1 months, respectively, $p=0.028$; Figure 3A). In addition, patients with positive expression of *LKB1* protein had a longer OS than patients with negative *LKB1* protein expression (64.8 months *vs*. 55.7 months, respectively, $p=0.003$; Figure 3B). The 5-year survival rate of patients with high *LKB1* mRNA and positive *LKB1* protein expression was significantly higher than that of patients with low and negative *LKB1*-mRNA and protein expression (75% *vs*. 58.7%, respectively, for mRNA expression, $p=0.04$; 72.9% *vs*. 64.5%, respectively, for protein expression, $p=0.03$).

Multivariate analysis was performed using the Cox proportional hazards model to analyze the prognostic value of *LKB1* expression. Table III shows that *LKB1* mRNA and protein expression, TMN stage, lymph node metastasis and vascular invasion were all associated with increased OS. Age, gender and differentiation did not significantly correlate with patient survival. Both *LKB1* mRNA and protein expression in gastric cancer were independent prognostic factors for OS (*LKB1* mRNA: relative risk (RR) =3.401; 95% confidence interval=1.477-7.831, $p=0.004$; *LKB1* protein: RR=4.431; 95% confidence interval=1.363-14.407, $p=0.013$).

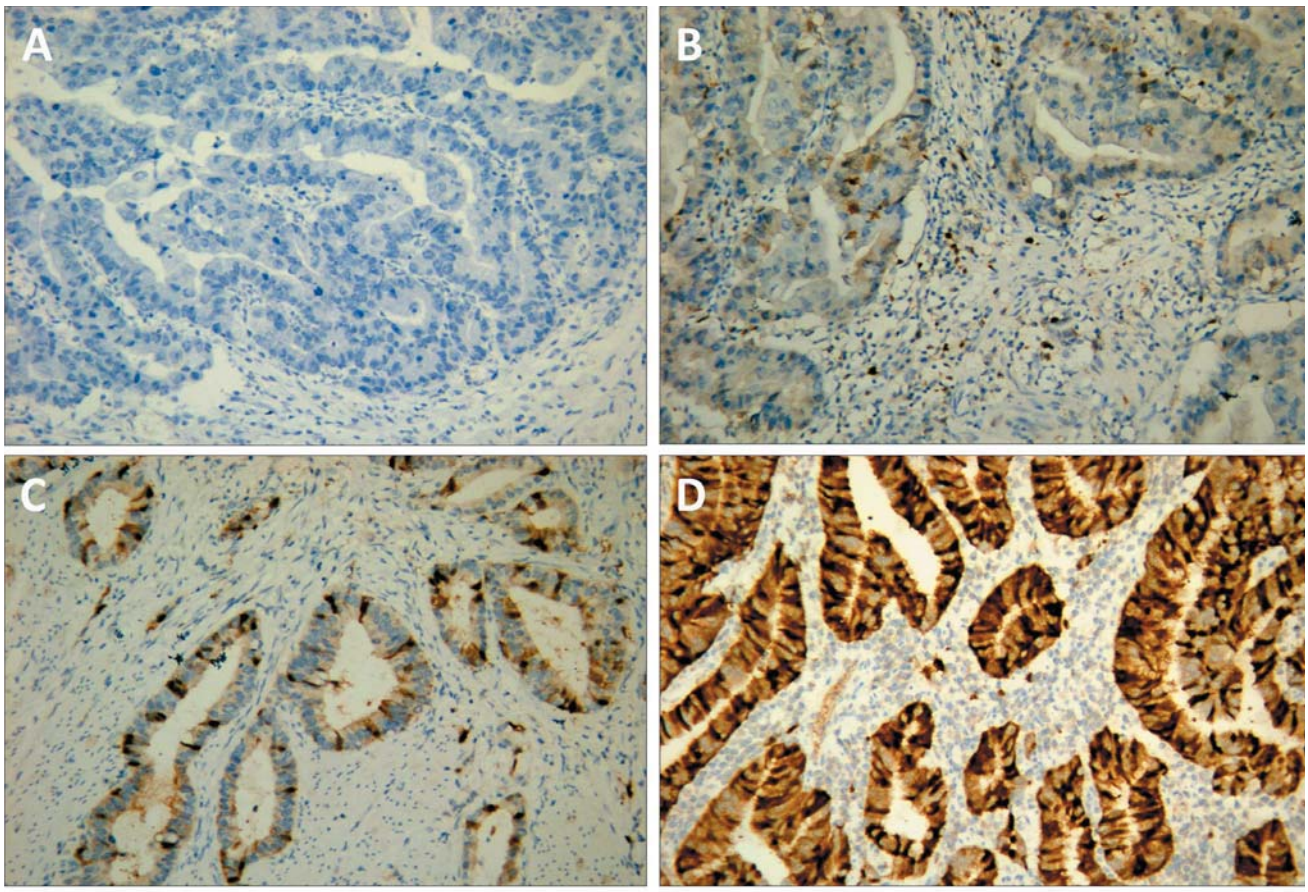

Figure 2. *Representative immunohistochemistry staining for liver kinase B1 (LKB1) protein expression in gastric cancer and normal tissue samples. A: LKB1 protein expression scored as 0. B: LKB1 protein expression scored as 1+. C: LKB1 protein expression scored as 2+. D: LKB1 protein expression scored as 3+. LKB1 expression is observed in the cytoplasm and nucleus of immuno-reactive cells. Original magnification: ×200.*

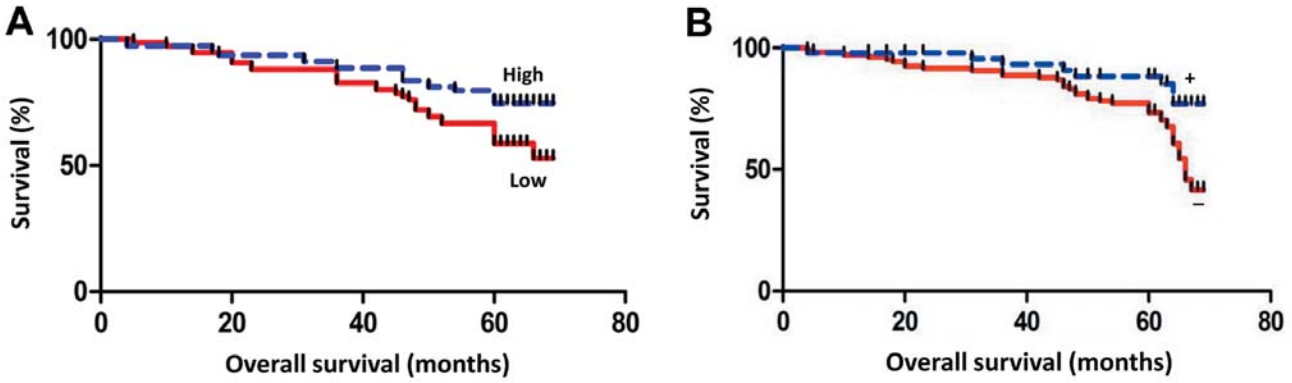

Figure 3. *Kaplan–Meier survival analysis of patients with gastric cancer (n=155) according to the level of liver kinase B1 (LKB1) expression, using the median level as cut off. A: Overall survival of the patients with low LKB1 (n=75) and high (n=80) LKB1 mRNA expression. B: Overall survival of the patients with negative (–) (n=107) and positive (+) (n=48) expression of LKB1 protein. The overall survival rate for patients in the low LKB1 mRNA and protein-negative group was significantly lower than that for patients in the high LKB1-mRNA (log-rank, p=0.04) and protein-positive group (log-rank, p=0.03).*

Table III. *Cox multivariate analysis of the association of liver kinase B1 (LKB1) expression with overall survival (OS) in patients with gastric cancer.*

Variable	RR (95% CI)	*p*-Value
LKB1 mRNA expression		
High *versus* low	3.401 (1.477-7.831)	0.004*
LKB1 protein expression		
Positive *versus* negative	4.431 (1.363-14.407)	0.013*
Vascular invasion		
Yes *versus* no	4.203 (1.377-12.834)	0.012*
TMN stage		
I/II *versus* III/IV	0.494 (0.41-0.528)	0.039*
LN metastasis		
Yes *versus* No	3.953 (2.137-7.300)	0.001*
Differentiation		
Well/moderate *versus* poor	1.345 (0.466-9.151)	0.451
Age (years)		
>60 *versus* <60	0.773.51 (0.358-1.668)	0.512
Gender		
Male *versus* female	1.371 (0.662-2.842)	0.396

RR: Relative risk; CI: confidence interval. *p<0.05.

Discussion

To our knowledge, this is the first study to describe a possible prognostic value for low *LKB1* expression in human gastric cancer. In this study, we found *LKB1* expression was significantly reduced in gastric-cancer cell lines and tissues compared with normal gastric cells and tissues at both mRNA and protein levels. These results indicated that *LKB1* might function as tumor suppressor in gastric cancer. Interestingly, our data demonstrated that poorly-differentiated cell lines exhibited more down-regulation of *LKB1* at the mRNA level than well-differentiated cell lines. The tumors from the patients with poorly-differentiated gastric cancer also had a lower *LKB1* mRNA level compared with well-differentiated gastric cancer, although the statistical analysis was not significant. This needs to be clarified with additional analysis of larger cohorts of patients and gastric cancer cell lines.

It is well described in the literature that *LKB1* loss confers poor clinical outcome in many different types of cancer (18, 19). In this study, the relationship between *LKB1* expression levels and certain clinico-pathological parameters of gastric-cancer was evaluated. Previous research has shown that in lung adenocarcinoma, *LKB1* loss at the transcriptional level promotes tumor malignancy, consequently resulting in poor patient outcomes. Our study revealed a similar phenomenon in which reduced *LKB1* expression in patients with gastric cancer was correlated with higher clinical stage, T-stage, lymph-node metastasis and vascular invasion. Importantly, patients with a low level of *LKB1* expression had significantly shorter survival times and worse 5-year survival rates compared to those with a high level of *LKB1* expression.

Multivariate analysis indicated that *LKB1* expression was an independent risk factor for prognosis for patients with gastric cancer. These results suggest that *LKB1* might be used as a novel prognostic marker for gastric cancer. Of note, *LKB1* detection assays in mouse and human tissues have been used in multiple investigations, revealing that *LKB1* could be a potential predictor of clinical prognosis in diverse human malignancies (20).

In conclusion, our results indicate that *LKB1* has a tumor-suppressor function in human gastric cancer. Low expression of the *LKB1* protein in human gastric cancer is significantly associated with a shorter survival. *LKB1* expression may be a useful prognostic marker in human gastric cancer and a potential molecular target for treatment of this disease.

Conflict of Interest

None of the authors have any conflict of interest in regard to this study.

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