LKB1 expression reverses the tumorigenicity of L02 cells

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Abstract. The tumor-suppressor liver kinase B1 (LKB1), a highly conserved and ubiquitously expressed protein kinase, plays a critical role in tumorigenesis. In the present study, we revealed that human hepatic L02 cells had severely impaired endogenous LKB1 expression as gauged by western blot, northern blot and RT-PCR analyses. Stable ectopic expression of LKB1 in L02 cells resulted in decreased cell growth, hypophosphorylation of Rb, and marked attenuation of colony formation on soft agar. Inoculation of L02 cells into immunocompromised mice resulted in the development of subcutaneous tumors, which could be completely abrogated by ectopic LKB1 expression. The tumors that formed in the mouse model recapitulated the histopathological features of hepatocellular carcinoma under the microscope. Our results jointly suggest that severely compromised endogenous LKB1 expression in the L02 cell line may confer to L02 cells tumor-initiating capacities in vivo and in vitro, and ectopic LKB1 expression antagonizes the tumorigenic properties of L02 cells. Our findings imply that caution may be needed to interpret the results obtained on the widely used human hepatic L02 cell line. The L02 cell line may be a new model to define LKB1 signaling in L02 cells. The present study provides strong evidence that endogenous LKB1 expression is deficient in the L02 cell line, which may confer observable tumorigenicity both in vitro and in vivo initiating capacities.

Introduction

Liver kinase B1 (LKB1) also known as serine/threonine kinase 11 (STK11), is a potent tumor-suppressor gene mutationally inactivated in the autosomal dominant Peutz-Jeghers syndrome (PJS), which is characterized by the development of multiple gastrointestinal hamartomatous polyps and distinct pigmentation of the skin and mucous membranes (1-3). PJS is a cancer predisposition syndrome, with an 18-fold increased incidence of malignancy in a wide variety of tissues, including the colon, breast, stomach, uterine cervix, lung and liver (4). In addition, multiple LKB1 mutations have been identified in various sporadic cancers, particularly those of the lung and also those of the genitourinary tract and breast (5-7), further highlighting the bona fide tumor-suppressor role of the LKB1 gene.

Encoding of the serine/threonine protein kinase, LKB1, is highly conserved throughout evolution from worms to mammals and is ubiquitously expressed in both embryonic and adult tissues with a notable higher expression in the pancreas, liver and skeletal muscle (8). The universal expression of LKB1 transcripts in all mammalian tissues is consistent with the elevated risk of multiple cancer types in PJS patients, and suggests that LKB1 is important to general cell function. Genetic inactivation of LKB1 in mice leads to embryonic lethality at midgestation and a variety of developmental abnormalities including the yolk sac and placenta (9,10), indicating that LKB1 is essential for normal development. Over the past 16 years, the LKB1 tumor-suppressor kinase has been extensively studied and implicated in a broad range of cellular processes, including cell cycle arrest, energy metabolism, apoptosis, senescence, differentiation and angiogenesis (11,12). Currently, there is no doubt that LKB1 is at the center of an important signaling node affecting numerous cellular processes whose deregulation contributes to tumorigenesis, resulting in pathologies such as PJS and various sporadic cancers (4,13).

The human hepatic cell line L02 was derived from primary normal human hepatocytes and immortalized in 1980 (14). Over the past 35 years, the L02 cell line has been widely used in studies of human hepatocellular functions, particularly those related to hepatic steatosis, drug hepatotoxicity and chemical carcinogenesis (15-18), but the genetic background of LKB1 signaling in L02 cells remains unknown. In the present study, we examined endogenous LKB1 expression in the L02 cell line and investigated the effect of LKB1 expression on proliferation, anchorage-independent growth and tumorigenesis of L02 cells. The present study provides strong evidence that endogenous LKB1 expression is deficient in the L02 cell line, which may confer observable tumorigenicity both in vitro and in vivo.

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Abbreviations: LKB1, liver kinase B1; STK11, serine/threonine kinase 11; PJS, Peutz-Jeghers syndrome; HEK-293T cells, human embryonic kidney 293T cells; HUVECs, human umbilical vein endothelial cells; DIG, digoxigenin; RT-PCR, reverse transcription PCR; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma

Key words: LKB1, tumor suppressor, Peutz-Jeghers syndrome, oncogenesis, L02 cell line
and in vivo and suggests that the immortalized L02 cell line is not a normal cell line, but cancerous.

Materials and methods

Materials. Cell culture media, fetal bovine serum (FBS), G418 and Lipofectamine 2000 reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies against Rb and phospho-Rb (Ser807/811) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against LKB1 and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Unless otherwise noted, all chemicals and reagents were purchased from Sigma-aldrich (St. Louis, MO, USA). Primary antibodies against LKB1 and GaPDh were purchased from Cell Signaling Technology (Beverly, MA, USA) and were of the highest grade.

Cell culture and generation of stable L02 cell lines. Human hepatic cell line L02 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai) and cultured in RPMI-1640 medium containing 20% FBS. HEK-293T and HeLa cells, and HUVECs, were maintained in minimum essential medium (MEM) supplemented with 10% FBS. Cells were cultured in a humidified incubator at 37°C containing 5% CO₂. Cultured cells were used between passage 3 and 10. Transfection was performed using Lipofectamine 2000 according to the manufacturer’s protocols. L02 cells were transfected with either an empty mock plasmid or a plasmid expressing wild-type LKB1, as previously described (19). After selection for 16 days in G418 (600 µg/ml)-containing medium, the resistant isolated single clones were selected, expanded and propagated. LKB1 expression was verified by northern blot and western blot analyses, respectively. One maximally expressing clone from the LKB1-transfected L02 cells was obtained and maintained in the same manner as the parental cells.

Cell proliferation assay. Cell proliferation was examined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Cells (5,000/well) were plated into 96-well plates in triplicates for 96 h and incubated in culture medium containing MTS every day for 4 days. SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) was used to detect the absorbance at 490 nm. Results are presented as the average absorbance of 3-wells/experiment.

Soft agar colony formation assay. Cells were seeded in complete media at a density of 1x10⁵ cells in 60-mm dishes containing a top layer of 0.35% low melting point (LMP) agar and a bottom layer of 0.6% LMP agar. The plates were incubated at 37°C in 5% CO₂ for 4 weeks. Every 7 days, fresh medium was added to each plate. Crystal violet 0.2% was used to stain the plates. Visible colonies were photographed and counted manually.

Western blot analysis. Total proteins were analyzed by SDS-PAGE electrophoresis and blotted as previously described (19,20). Specific primary antibodies recognizing LKB1 (1:500), Rb (1:1,000), phospho-Rb (1:1,000) and GAPDH (1:1,000) were used at the indicated dilutions. HRP-conjugated secondary antibodies were used at 1:10,000 dilutions. The signals of HRP were detected by Substrate SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL, USA). The protein signal was quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

Table I. Primer pair sequences of the LKB1 gene and related information.

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<tr>
<th>Loci</th>
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<th>Annealing temp. (˚C)</th>
<th>Size (bp)</th>
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<td>R 5’-AAATGCTGGACAGCGTGC-3’</td>
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<td>F 5’-TACGGCAAGGAGAGGG-3’</td>
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<td>413</td>
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<td>R 5’-GTTGAAAGGTGCTGGA-3’</td>
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Northern blot analysis. Total RNA was isolated using TRIzol (Invitrogen Life Technologies), resolved in 1% formaldehyde-agarose gels, transferred to nylon membranes and cross-linked. Blots were probed with digoxigenin (DIG)-labeled cDNAs. The LKB1 probe was prepared using the forward primer (5’-TCGGTGTTGATGGGACACGTC-3’) and the reverse primer (5’-GGTGTGTAAGGCAGAGT-3’), and labeled with DIG using End Tailing kit (Roche Applied Science, Indianapolis, IN, USA). β-actin gene was used as a loading control. Hybridization signals were detected with chemiluminescence and recorded on X-ray film.

Reverse transcription PCR (RT-PCR) and sequencing. Total RNA (2 µg) was transcribed with M-MLV reverse transcriptase and used for subsequent PCR amplification with Taq polymerase (both from Promega). PCR products were analyzed on 2% agarose gels and verified by DNA sequencing. Housekeeping gene β-actin was amplified as an internal control. LKB1-specific primers designed to detect LKB1 transcriptional variants are described in Table I.

Tumorigenicity assay. Aliquots of the particular cell populations were counted and injected into the axillary region (2x10⁶ cells/injection site) of 6-week-old male BALB/c nude mice (Animal Center of Chongqing Medical University, Chongqing, China). The mice were housed in sterile cages under laminar flow hoods in a temperature-controlled room and were fed autoclaved chow and water ad libitum. All mice...
(n=4/group) were weekly weighed and examined every other day for morbidity and for tumor growth (measured using a digital caliper). After 6 weeks, all of the mice were euthanized with excess CO₂ and necropsied to determine possible gross metastases.

All experiments using mice were in accordance with the Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) of Chongqing Medical University (Chongqing, China).

Histological analysis and immunohistochemistry. Tumor tissues were fixed in 4% paraformaldehyde and embedded in paraffin wax before sectioning at 5-μm thickness. For histopathological examinations, the sections were stained with hematoxylin and eosin (H&E). For immunohistochemistry, serial sections were deparaffinized, treated with 0.3% H₂O₂ in methanol to inactivate endogenous peroxidase, and incubated with anti-LKB1 monoclonal antibody at a 100-fold dilution for 60 min. Immunodetection of LKB1 was performed using Vector Stain Elite kit (Vector Research, Burlingame, CA, USA). A positive control was included with each batch of staining to ensure consistency between consecutive runs. Immunostaining was evaluated by a pathologist.

Statistical analysis. All cell culture experiments were repeated at least 3 times, unless otherwise indicated, and paired t-tests were used to determine statistical significance.

Results

**LKB1 expression is deficient in the L02 cell line.** We first examined the protein expression of LKB1 in the L02 cell line by western blot analysis. The HeLa cell line, which has been extensively characterized as endogenous LKB1-deficient (21), was included as a negative control. Human embryonic kidney 293T cells (HEK-293T cells) and human umbilical vein endothelial cells (HUVECs) were used as positive controls. As depicted in Fig. 1A, a specific band for LKB1 was detected in 25 µg total protein of HEK-293T cells and HUVECs, whereas the LKB1 signal was not detectable in 180 µg total protein of L02 cells and 140 µg total protein of HeLa cells, suggesting that LKB1 protein expression is severely compromised in L02 cells. In concert with the deficiency of LKB1 protein, northern blot analysis indicated that LKB1 mRNA levels were barely detectable in the L02 and HeLa cells, in contrast to the HEK-293T cells which expressed abundant LKB1 transcript (Fig. 1B), supporting the lack of LKB1 expression in the L02 cell line.

**RT-PCR and sequencing.** To better evaluate the genetic defects underlying LKB1 deficiency in the L02 cell line, we performed LKB1 mutation analysis. The transcript was screened for putative mutations by sequencing of products obtained by reverse transcription followed by polymerase chain reaction (RT-PCR) from the L02 cell line. cDNA from L02 cells was amplified with 6 sets of primers covering the entire coding region (exons 1-9) of the LKB1 gene. These primers are described in Table I. As shown in Fig. 1C, compared with the HEK-293T cells and HUVECs, LKB1 transcription was severely affected in the L02 cells particularly for the 5' end region. LKB1 mRNA expression was reduced to ~5% of the HEK-293T cells with upstream primer P1, 10% with midstream primer P2, and 25% with downstream primer P3. The majority of the LKB1 coding sequence (93%) was amplified from the L02 cells at very low levels by the primers chosen, except for the region containing the initial 89 bases, which was unable to be obtained by RT-PCR. Direct sequencing of the PCR products did not detect any abnormalities in nucleotides 90-1302 of the LKB1 open reading frame, suggesting the putative mutation region...
may be narrowed down to 89 bases downstream of the start codon. Another possibility for silencing of LKB1 expression is epigenetic inactivation caused by promoter hypermethylation (22). However, treatment with the demethylation agent 5-aza-2’-deoxycytidine could not restore mRNA or protein expression of LKB1 in our research (data not shown).

Construction of a stable L02 cell line constitutively expressing LKB1. To obtain further insights into the biological role of LKB1, we attempted to reconstruct the in vivo situation of L02 cells by stably expressing wild-type LKB1. L02 cells were transfected with an expression vector encoding both LKB1 and a neomycin resistance gene or a vector encoding the selection marker only. Transfectants were subjected to G418 selection for 16 days. After the selection, a strongly reduced number of colonies were detected in the LKB1 transfectants compared with the mock vector transfectants of the L02 cells. Individual colonies were selected and analyzed for inducible expression of LKB1 by northern blot and western blot analyses. As shown in Fig. 2, recombinant LKB1 was readily detected at high levels in the L02 cells stably transfected with the LKB1 construct, but not in the cells transfected with the control vector. Thus, the reconstruction of LKB1 gene expression was successfully achieved in the L02 cells.

**Ectopic LKB1 expression inhibits cell proliferation of L02 cells.** Downregulation of LKB1 in HeLa and G361 cells provided a growth advantage to these cells (21,23). To investigate this possibility in L02 cells, we performed MTS assay, which detects the activity of mitochondrial dehydrogenase enzymes of viable cells and is used as a measure for cell proliferation in cell cultures (24). Indeed, growth curve experiments clearly demonstrated that LKB1 expression conferred a proliferative disadvantage to L02 cells when compared with the parental L02 and mock vector-transfected L02 cells (Fig. 3A), which suggests that ectopic LKB1 expression strongly inhibited the growth of L02 cells. No difference was noted in the cellular growth between the parental L02 and the vector-transfected L02 cells. Thus, introducing wild-type LKB1 into the L02 cells led to growth suppression.

**Ectopic LKB1 expression blocks Rb phosphorylation in the L02 cells.** Cell cycle machinery controls cell proliferation, and the retinoblastoma (Rb) protein is a well-known gate keeper of cell cycle progression (25). Compatible with the decrease in cell proliferation rate in vitro, stable transfection with the LKB1 plasmid, but not with the mock vector, considerably reduced phosphorylation of Rb at Ser807 and Ser811 in the L02 cells, with no differences observed in the total Rb protein level (Fig. 3B). These results are consistent with our previous findings.
and indicate that activation of LKB1 signaling leads to hypophosphorylation of Rb, which in turn contributes to reduced cell cycle progression and cell proliferation in L02 cells.

**Ectopic LKB1 expression rescues anchorage-independent growth of L02 cells.** Anchorage-independent growth in soft agar is one of the hallmark characteristics of cellular transformation and uncontrolled cell growth, with normal cells typically not capable of growth in semi-solid matrices (26). Recent studies have provided compelling evidence that loss of LKB1 activity is a critical step in oncogenesis (27-30). To explore the oncogenic potential of LKB1-deficient L02 cells, a soft agar colony formation assay was carried out. Notably, L02 cells and the control vector transfectants grew efficiently in soft agar and formed numerous colonies, whereas the L02 cells with constitutive LKB1 expression exhibited a significant reduction in anchorage-independent growth on soft agar (Fig. 3C). These results unambiguously suggest that L02 cells gained induced tumorigenic phenotypes in vitro, and LKB1 expression is an obstacle for anchorage-independent growth of L02 cells.

**Ectopic LKB1 expression abrogates subcutaneous tumor growth.** These encouraging results prompted us to further study the potential carcinogenesis of L02 cells in vivo. A limiting dilution tumorigenicity assay was conducted utilizing subcutaneous injections into athymic nude mice without any protein support. As shown in Fig. 4A, both L02 parental cells and mock vector transfected L02 cells were capable of developing subcutaneous tumors in all of the inoculated nude mice. In contrast, L02 cells with stable LKB1 expression were unable to form any tumor in the mouse model. These data demonstrated that the L02 cell line is tumorigenic in vivo, which could be completely abolished by sustained LKB1 expression. No distant metastases to spleen, liver, kidneys, lungs or intestine in the sets of mice were observed upon necropsy.

H&E staining of tumor tissues revealed a trabecular pattern of cell growth, where tumor cells were arranged in plates of various thickness, separated by sinusoid vascular spaces. There was no discernable normal lobular architecture, although vascular structures were present. Cytologic features of malignancy were noted, with irregular nuclear contours, giant multinucleated cells, and increased nuclear/cytoplasmic ratio (Fig. 4B). These morphological appearances indicated the hepatic histogenesis of the malignant tumors in agreement with the cell lines inoculated, excluding the formation of the primary tumors. All histologic sections were analyzed by one of our expert pathologists.

A major question concerning the mechanism of tumor formation of the L02 cells was whether the tumors still expressed LKB1. Immunohistology revealed that the LKB1
protein was not detected in the subcutaneous tumors from the nude mice (Fig. 4B), with HUVECs grown on coverslips as a positive control. These results suggest that LKB1 inactivation had occurred prior to tumor emergence and loss of LKB1 expression is required as a precondition of tumor formation.

Discussion

An immortalized cell line gains the ability to overcome cellular senescence and proliferate indefinitely, which is acquired either through random mutation or deliberate modification such as ectopic expression of different genes (31). The L02 cell line was generated by Yeh et al in the early 1980's from normal adult human liver cells and is able to be spontaneously immortalized when cultured under certain conditions (14), but the underlying mechanisms have not been fully described. Tumor suppressor LKB1 is active and expressed ubiquitously in normal adult and embryonic tissues with high expression in the liver (32,33). Regarded as a normal hepatic cell line, L02 cells are not believed to be an exception to the wide spectrum of LKB1 expression. In the present study, severely reduced LKB1 mRNA and protein expression was identified in the L02 cell line, which was somewhat striking but may provide a plausible explanation for the immortalization of L02 cells, since our previous study indicated that endogenous LKB1 knockdown in normal cells accelerates cell cycle progression by a decline in the p53 and p6 pathways (20). Our results were substantiated by a previous study which showed that an immortalized hepatocyte line TPl1 beared a homozygous deletion of the LKB1 gene (34).

In the present study, we first demonstrated that LKB1 expression was downregulated at both the mRNA and protein levels in the L02 cell line, suggesting that LKB1 function could be impaired at the level of transcription. There are several potential mechanisms that may underlie the decreased LKB1 expression in L02 cells. Knudson's two-hit model explains that tumorigenesis is mainly caused by tumor-suppressor gene inactivation (35). Even though RT-PCR analysis did not show sequence variants in a sizable fraction of the LKB1-coding region, the putative mutation side may be narrowed down to the initial 89 bases in the 5' end region. The absence of mutations in the present study may be due to incomplete screening of the gene, insensitivity of our mutation-detection methods, transcript instability as a result of the presence of a mutation, or large genomic deletions that are undetectable by the PCR-based approach. Therefore, beyond the scope of the present study, further research consisting of detailed mutational analysis of LKB1 is needed to clarify the mechanisms underlying LKB1 inactivation in L02 cells.

There is ample evidence that deregulation of the LKB1 signaling pathway appears to play a major role in tumor pathogenesis, since it facilitates malignant development through suppression of cell apoptosis and growth arrest (27-30). To investigate the oncogenic state of the L02 cell line, functional assays were carried out including evaluation of the clonogenicity and tumor-initiating potential. Our results indicated that L02 cells were capable of forming colonies in soft agar with high efficiency, and developing subcutaneous tumors in immunocompromised mice. The present study provided functional evidence for the first time that the L02 cell line is tumorigenic in vivo and in vitro, and illustrated a need for cautious interpretation of previous studies in which it was assumed that L02 cells resemble typical normal human hepatic cells.

Several studies have demonstrated that re-introduction of LKB1 into various cancer cell lines lacking its expression results in G1 cell cycle arrest and cell growth suppression (21,23,36). Our previous study also showed that ectopic LKB1 expression activated the LKB1/AMPK signaling pathway and led to G1 arrest even in cells with endogenous LKB1 protein in a LKB1 kinase-dependent manner (19). In the present study, constitutive LKB1 expression was restored in the L02 cells through stable transfection, and led to significantly inhibited cellular growth, hypophosphorlation of Rb, and decreased colony formation. Subcutaneous tumorigenicity was ablated completely in nude mice by ectopic LKB1 stable expression. These results indicate that LKB1 has growth-suppressing activity when re-introduced into L02 cells, and has the capacity of reversing the tumorigenesis of L02 cells in vivo and in vitro, which is in good agreement with previous observations and further confirms the tumor-suppressor role of LKB1 in cell proliferation.

Hepatocellular carcinoma (HCC) is ranked as the fifth most common human cancer, and the third leading cause of cancer-related death worldwide, with over 500,000 new cases annually diagnosed resulting in >90% mortality (37). HCC is a very heterogeneous tumor type whose precise molecular mechanisms still await further studies. Notably, Nakau et al reported a high HCC prevalence in LKB1 (+/-) mice (38), suggestive of a strong LKB1 involvement in liver tumorigenesis. We demonstrated that the L02 cells possessed the capability to initiate tumor formation in immunocompromised mice after subcutaneous inoculation, and histology of the tumor tissues was markedly similar to the representative histopathology of human HCCs, including malignant cellular growth in trabecular patterns recapitulating liver cords (39). No expression of LKB1 protein was observed in these subcutaneous tumors, indicating that complete loss of LKB1 expression was required for the development of the carcinomas. The present study provides the first evidence that the L02 cell line is capable of initiating tumors which share histological features with human HCC. Thus, the L02 cell line may be an important and valuable model to better define the cellular and molecular mechanisms of liver transformation, and to study the mechanisms by which LKB1 exerts its growth-suppressive effect. However, the present study only crudely described the sequential cues that transform L02 cells into malignant tumors. Future research will be required to characterize in greater detail the underlying molecular mechanisms.

In summary, the results of the present study indicate that endogenous LKB1 expression is severely impaired in the L02 cell line, which, in turn, confers accelerated cell growth and malignant transformation of L02 cells, and ectopic LKB1 expression antagonized the tumorigenic properties of the L02 cells.

Acknowledgements

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References


