Liver kinase B1 (LKB1) as a tumor suppression gene that is associated with various kinds of cancers, including lung cancer. In this study, we found that the effect of LKB1 on tumor growth was dependent on its subcellular expression in A549 and HCC827 cells. Full-length LKB1 decreased the proliferation and clonogenicity of A549-LKB1 and HCC827-LKB1 cells, but increased their apoptosis. Opposite effects were observed in A549-LKB1s and HCC827-LKB1S cells that overexpressed truncated LKB1 without the nuclear localization sequence. The truncated cytoplasmic LKB1 enhanced the growth of implanted tumors in vivo. The truncated cytoplasmic LKB1 promoted autophagy, which was independent of AMP-activated protein kinase and mTOR signaling in A549 and HCC827 cells. Further characterization indicated that higher levels of cytoplasmic LKB1 expression were associated with advanced TNM stage and reduced overall survival (OS) in 190 patients with adenocarcinoma. In contrast, high nuclear expression of LKB1 is associated with early TNM stage and longer OS. The high level of cytoplasmic LKB1 expression was an independent risk factor for poor overall survival in patients with adenocarcinoma. Together, our results revealed that cytoplasmic LKB1 promotes the growth of lung adenocarcinoma and could be a prognostic marker for lung adenocarcinoma.

**KEYWORDS**

autophagy, LKB1, lung adenocarcinoma, proliferation, subcellular expression

**INTRODUCTION**

Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors and has a high mortality rate. The incidence of NSCLC is increasing worldwide. Although significant progression has been made in the diagnosis of NSCLC, a portion of NSCLC patients are diagnosed with advanced TNM stage at the first visit. Therefore, it is important to explore the possible molecular pathogenesis and prognosis biomarkers of NSCLC.
LKB1 occurs in many types of sporadic cancers, such as lung cancer, breast cancer, and pancreatic cancer, suggesting that LKB1 acts as a tumor suppressor. Inactivation of LKB1 and altered levels of LKB1 expression are detected in NSCLC tissues. However, it is unclear what the function of different subcellular LKB1 is in NSCLC.

Autophagy is an intracellular process of cytoplasmic component degradation in the lysosome to renew some organelles and maintain metabolic homeostasis. During the process of autophagy, stimuli can initiate the formation of autophagosome, a double-membraned vesicle containing cytoplasmic contents and organelles, and its outer membrane will fuse with a lysosome to form an autolysosome to degrade the contents. Autophagy can regulate cell proliferation, apoptosis, and drug sensitivity in tumor cells. However, the precise effects underlying the regulation of autophagy on proliferation and apoptosis of NSCLC are still unclear. Furthermore, LKB1 acts as an upstream kinase for AMP-activated protein kinase (AMPK) and PI3K/AKT/mTOR signaling. It is poorly understood how the cytoplasmic and nuclear LKB1 and related signaling regulate the autophagy, proliferation, and apoptosis of NSCLC cells.

2 MATERIALS AND METHODS

2.1 Patients

One hundred and ninety patients with lung adenocarcinoma were recruited from the First Affiliated Hospital of Xi’an Jiao Tong University.

**FIGURE 1** Cytoplasmic liver kinase B1 (LKB1) promotes proliferation and clonogenicity, but inhibits apoptosis of A549 and HCC827 cells. Distribution and levels of LKB1 expression in A549-NC, A549-LKB1, A549-LKB1sh, HCC827-NC, HCC827-LKB1, and HCC827-LKB1sh cells, as well as the relative levels of cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP) in individual groups were determined by western blot analysis. The proliferation and clonogenicity of individual groups were tested by MTT and colony forming assays, respectively. The percentages of apoptotic cells were determined by flow cytometry. A, B, Expression of LKB1. C, Distribution of LKB1. D-G, Cell proliferation. H-I, Cell clonogenicity. J-K, Percentages of apoptotic cells. L-M, Levels of apoptosis-related proteins. *P < .05. NC, negative control.
Individual patients were diagnosed with lung adenocarcinoma by pathology in a blinded manner. The patients received thoracotomy or lobectomy to remove the whole tumors without adjuvant chemotherapy or radiotherapy before surgery. The tumor specimens were staged according to the 8th edition of the American Joint Committee on Cancer classification system. Those patients were followed up until December 2016.

2.2 | Immunohistochemical staining

The freshly dissected human lung adenocarcinoma and mouse transplanted tumors were fixed in 10% buffered formalin overnight and paraffin-embedded. The tumor tissue sections (5 μm) were deparaffinized and rehydrated, followed by antigen retrieval in a microwave oven. The sections were incubated in 3% hydrogen peroxide in methanol for 10 minutes to inactivate endogenous peroxidase, blocked with 10% normal goat serum for 20 minutes at room temperature, and incubated with anti-LKB1 (1:200 dilution; D60C5F10; Cell Signaling Technology, Danvers, MA, USA) and anti-Ki67 (1:200 dilution; BD5; Cell Signaling Technology) at 4°C overnight. After being washed, the sections were incubated with secondary antibody and then with HRP-conjugated avidin for 30 minutes at room temperature, visualized with 3,3′-diaminobenzidine tetrahydrochloride, then counterstained with hematoxylin. These stained signals were photographed under a microscope (Q550CW; Leica, Manheim, Germany). The intensity of staining was evaluated semiquantitatively as scores of: 0, not staining; 1, faint staining; 2, moderate staining; and 3, strong staining. The percentages of stained cells were examined and defined as: 0, <5%; 1, 5%-25%; 2, 26%-50%; 3, 51%-75%; and 4, >75%. The final scores of each section were calculated by multiplying these two numbers, resulting in a score of 0-12. The samples with a score of 1-5 were defined as having low levels of expression; samples with a score of 6-12 were defined as having high levels of expression.

**FIGURE 2** Cytoplasmic liver kinase B1 (LKB1) promotes the growth of implanted lung tumors in mice. BALB/c nude mice were randomized and implanted s.c. with equal numbers of A549-LKB1, A549-LKB1S, A549-NC, HCC827-LKB1, HCC827-LKB1S, or HCC827-NC cells, and the dynamic growth of individual groups of tumors was monitored up to 28 days. Tumors were then dissected out and measured for their volumes and weights. Levels of Ki67 expression in individual tumors were determined by immunohistochemistry. A, Representative tumors. B, Dynamic growth of implanted tumors. C, Volumes of tumors. D, Weights of tumors. E, Levels of Ki67 expression in tumors. a, A549-NC tumor; b, A549-LKB1 tumor; c, A549-LKB1S tumor; d, HCC827-NC tumor; e, HCC827-LKB1 tumor; f, HCC827-LKB1S tumor. G, Scores of Ki-67 expression in each group. *P < .05. NC, negative control.
expression. The intensity of nuclear anti-LKB1 staining was defined as: 0, no staining; 1, staining. The percentages of nuclear-stained cells were defined as 0, <5%; 1, 5%-37%; 2, 38%-70%; and 3, >70%. A final score of <1 was defined as no nuclear LKB1 expression, whereas a final score of >2 was defined as expressing nuclear LKB1.

2.3 | Cell culture and transfection

Human lung adenocarcinoma A549 and HCC827 cells were cultured in DMEM containing 10% (v/v) FBS at 37°C in 5% CO₂. All the vectors contained the kanamycin/G418 resistance gene. When the cells reached 70% confluency, they were transfected with individual expression plasmids using the TurboFect transfection reagent (Thermo, Waltham, MA, USA). The plasmids included C-Flag pcDNA3-LKB1 for expression of the whole length of LKB1, C-Flag pcDNA3-LKB1s, for the expression of LKB1 without nuclear localization signal, or control pCDNA3 (Addgene, Cambridge, MA, USA). Two days later, these transfected cells were treated with 500 μg/mL G418 for 21 days to establish the stable expression cell lines A549-LKB1, A549-LKB1s, A549-NC, HCC827-LKB1, HCC827-LKB1s, and HCC827-NC. In addition, the different groups of cells were treated with, or without, 10 mmol/L 3-methyladenine (3-MA), 40 μmol/L compound C, or 10 μmol/L MHY1485 (Sigma, Burlington, MA, USA) to inhibit autophagy, AMPK, or mTOR, respectively.

2.4 | Western blot analysis

The different groups of cells were lysed in RIPA lysis buffer (Beyotime Chemical, Beijing, China). In addition, nuclear and cytoplasmic proteins were extracted from the different groups of cells using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Chemical). After quantification of protein concentrations, the protein samples

**FIGURE 3** Cytoplasmic liver kinase B1 (LKB1) increases autophagy in A549 and HCC827 cells. Numbers of autophagosomes in A549-NC, A549-LKB1s, HCC827-NC, and HCC827-LKB1s cells were characterized by transmission electron microscopy (TEM), and the relative levels of autophagy-related molecule expression were determined by western blot analysis. A549 and HCC827 cells were treated with the indicated doses of 3-methyladenine (3-MA) for varying time periods, and the viability of individual groups of cells was determined by MTT. The sensitivity of A549-NC, A549-LKB1s, HCC827-NC, and HCC827-LKB1s cells to 3-MA was determined for the relative levels of autophagy-related molecule expression by western blot. A, TEM analysis of autophagosomes. a, A549-NC cells (×20 000); b, A549-LKB1s cells (×20 000); c, HCC827-NC cell (×20 000); d, HCC827-LKB1s cell (×20 000); e, autophagosome (×50 000). B, C, Autophagy-associated molecule expression. D-G, Effect of 3-MA on A549 and HCC827 cells. H-I, Western blot analysis of autophagy-associated molecule expression. *P < .05. NC, negative control
(20-50 μg/lane) were separated by SDS-PAGE on 8%-12% gels and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After being blocked with 5% fat-free milk powder in TBST, the membranes were incubated with primary antibodies against Beclin1 (ePR1733Y, 1:800 dilution; Abcam, Cambridge, UK), LC3 (L7543, 1:1000 dilution; Sigma), Atg4 (D62C10, 1:1000 dilution; Cell Signaling Technology), Atg7 (D12B11, 1:1000 dilution; Cell Signaling Technology), Atg12 (D88H11, 1:1000 dilution; Cell Signaling Technology), cleaved caspase-3 (5A1E, 1:1000 dilution; Cell Signaling Technology), caspase-3 (BG10, 1:1000 dilution; Cell Signaling Technology), cleaved poly(ADP-ribose) polymerase (PARP) (19F4, 1:1000 dilution; Cell Signaling Technology), PARP (46D11, 1:1000 dilution; Cell Signaling Technology), H3 (1B1B2, 1:1000 dilution; Cell Signaling Technology), and β-actin (sc-130301, 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After being washed, the bound antibodies were detected with HRP-conjugated secondary antibodies and visualized using the enhanced chemiluminescent reagents (Millipore). The relative levels of target protein were determined by BioImaging Systems (ChemiDoc-it 415 Imager, UVP; Upland, CA, USA).

2.5 | Cell proliferation assay
The different groups of cells (2 × 10² cells/well) were cultured in 96-well plates overnight, and the proliferation of each group of cells was determined by MTT assay. Briefly, each well of cells was exposed to 5 mg/mL MTT for 6 hours daily for 7 days and the generated formazan in individual wells was solubilized by adding 150 μL DMSO, followed by measuring the optical density at 492 nm in a microplate reader. The experiments were carried out in triplicate, and control and experimental cells were tested simultaneously.

2.6 | Colony formation assay
The different groups of cells (800 cells/well) were cultured in triplicate in 6-well plates in DMEM containing 10% (v/v) FBS at 37°C in 5% CO₂ for 21 days. The visible clones were fixed by 4% paraformaldehyde for 15 minutes and stained with 0.1% crystal violet for 30 minutes. The numbers of formed clones in individual wells were counted in a blinded manner.
2.7 | Flow cytometry

The percentages of apoptotic cells in each group were determined by flow cytometry using the Annexin V Apoptosis Detection Kit (88-8007; Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the different groups of cells (10^5/tube) were stained in duplicate with 5 μL annexin V-allophycocyanin and 5 μL 7-AAD solutions for 15 minutes at room temperature. After being washed, the percentages of apoptotic cells were determined by flow cytometry and analyzed using the FlowJo (FlowJo LLC, Ashland, OR, USA) software.

2.8 | Transmission electron microscopy

Autophagosomes in individual cells were detected by transmission electron microscopy (TEM). Briefly, the cells were harvested and the cell pellets were fixed by 2.5% glutaraldehyde for 2 hours at 4°C. After dehydration by acetone, these cell pellets were embedded and placed in 65°C for 2 hours. The cell ultrathin sections (50-70 nm) were stained by sodium acetate and lead citrate, then observed under using TEM. Autophagosomes were counted in 5 high power fields (x20,000) to obtain the average number of autophagosomes for each sample.

2.9 | Lung cancer xenograft in nude mice

Male BALB/c nude mice at 4 weeks of age were purchased from the Animal Center of Xi’an Jiaotong University and housed in a specific pathogen-free facility with free access to autoclaved food and water. To establish xenograft tumors, individual mice were randomized and implanted s.c. with 1 x 10^5 A549-NC, A549-LKB1s, A549-LKB1s, HCC827-NC, HCC827-LKB1s, or HCC827-LKB1s cells (n = 6) in 100 μL DMEM/Matrigel (No. 356234; BD, Franklin Lakes, NJ, USA) into their back. In addition, another set of mice were randomized and implanted s.c. with 1 x 10^5 A549-NC, A549-LKB1s, HCC827-
endogenous Western blot analysis indicated that A549 cells did not express LKB1 and HCC827-LKB1s group or with 5 mmol/L 3-MA (M9281; Sigma) every other day for 2 weeks. The growth of implanted tumors was monitored every other day for their volumes that were calculated using the formula: V = 0.5 x length x width^2.

2.10 Statistical analysis

Data are expressed as the mean ± SD or real case number. The association between percent cases with positive staining and clinical parameters was analyzed by Pearson's χ^2 test. Overall survival (OS), defined as time from diagnosis to death or to the last follow-up, was used to draft survival curves. Survival rates were estimated by the log–Kaplan–Meier method, and the difference between subgroups was tested by the log-rank test. The potential factors for OS were determined by univariate and multivariate analyses. The difference among groups was analyzed by repeated ANOVA, and the difference between groups was analyzed by Student's t test. All statistical analyses were carried out using SPSS 22.0 software (International Business Machines Corporation, Armonk, NY, USA). P-value < .05 was considered statistically significant.

3 RESULTS

3.1 LKB1 regulates proliferation of A549 and HCC827 cells, dependent on its subcellular localization

LKB1 has been considered to be a tumor suppressor. To determine the role of different subcellular localizations of LKB1 in regulating A549 and HCC827 cell proliferation, A549 and HCC827 cells were transfected with vehicle, the plasmid for expressing full-length LKB1, or the plasmid for expressing LKB1 without the nuclear localization sequence to generate stably expressing A549-NC, HCC827-NC, A549-LKB1, HCC827-LKB1, A549-LKB1s, or HCC827-LKB1s cells. Western blot analysis indicated that A549 cells did not express endogenous LKB1 and HCC827 cell expressed low levels of endogenous LKB1 (Figure 1A). A549-LKB1, HCC827-LKB1, A549-LKB1s, and HCC827-LKB1s expressed the flag-fused LKB1 with different sizes, as expected (Figure 1B). Although LKB1 was expressed in both the cytoplasm and nucleus of A549-LKB1 and HCC827-LKB1 cells, LKB1 was only detected in the cytoplasm of A549-LKB1s and HCC827-LKB1s cells (Figure 1C). The MTT assays indicated that the proliferation of A549-LKB1 cells was weaker than that of A549-NC cells, whereas the proliferation of A549-LKB1s cells was significantly stronger than that of A549-NC (Figure 1D,E). The same results were found in HCC827 cells (Figure 1F,G). Similarly, colony formation assays revealed that the percentages of A549-LKB1 or HCC827-LKB1 cells that formed clones were significantly greater than that of A549-NC or HCC827-NC cells, respectively (Figure 1H,I). In addition, flow cytometry analysis showed that the percentages of apoptotic A549-NC or HCC827-NC cells were significantly less than that of A549-LKB1 or HCC827-LKB1 cells, but higher than that of A549-LKB1s or HCC827-LKB1s cells (Figure 1J,K).

To test the effect of LKB1 in vivo, A549-LKB1, A549-LKB1s, A549-NC, HCC827-LKB1, HCC827-LKB1s, and HCC827-NC cells were implanted s.c. into nude mice and the growth of implanted tumors was monitored for 28 days (Figure 2A). The dynamic growth of A549-NC tumors was faster than that of A549-LKB1 tumors, but slower than that of A549-LKB1s tumors. The tumor weights and volumes in the A549-NC tumors were significantly less than that of A549-LKB1s tumors, but greater than that of A549-LKB1 tumors. The same results were found in HCC827 cells (Figure 2B-E). Further immunohistochemistry revealed that the A549-NC tumors showed moderate levels of anti-Ki67 staining, whereas the levels of anti-Ki67 staining in the A549-LKB1 tumors were obviously diminished. In contrast, the levels of anti-Ki67 staining in A549-LKBs tumors were stronger than that of A549-NC tumors. A similar pattern of the relative levels of Ki67 was detected in HCC827 cells (Figure 2F,G). Collectively, such data indicated that cytoplasmic LKB1 promoted the proliferation and clonogenicity, but inhibited the apoptosis, of A549 and HCC827 cells in vitro.

3.2 Cytoplasmic LKB1 promotes autophagy and proliferation of A549 and HCC827 cells

To understand the potential mechanisms underlying the effect of cytoplasmic LKB1, we characterized autophagy in A549-LKBs, HCC827-LKB1s, and their control cells, A549-NC and HCC827-NC, by TEM. We found that the number of autophagosomes in A549-LKB1s or HCC827-LKB1s cells was significantly higher than that in A549-NC or HCC827-NC cells (Figure 3A). Further analysis indicated that the relative levels of LC3II, Beclin1, Atg4, Atg7, and Atg12 in A549-LKB1s or HCC827-LKB1s cells were significantly higher than that in A549-NC or HCC827-NC cells (Figure 3B,C). To further confirm the effect of LKB1s on cell autophagy, we used 10 mmol/L 3-MA (Figure 3D-G) to inhibit autophagy of A549-NC and HCC827-NC cells. The relative levels of LC3II, Beclin1, Atg4, Atg7, and Atg12 expression in A549-NC or HCC827-NC cells treated with 3-MA were significantly reduced compared with A549-NC or HCC827-NC cells. Compared with A549-NC or HCC827-NC cells treated with 3-MA, the relative expression levels of those molecules in the A549-LKBs cells that had been treated with 3-MA were restored (Figure 3H,I). These results suggested that cytoplasmic LKB1 promoted autophagy in A549 and HCC827 cells.

We further uncovered the effect of cytoplasmic LKB1 reduced autophagy on cell growth in A549 and HCC827 cells. We found that the number of autophagosomes in A549-LKB1s or HCC827-LKB1s were significantly greater than that of A549-NC or HCC827-NC cells, respectively (Figure 3H,I). In addition, flow cytometry analysis showed that the percentages of apoptotic A549-NC or HCC827-NC cells were significantly less than that of A549-LKB1 or HCC827-LKB1 cells, but higher than that of A549-LKB1s or HCC827-LKB1s cells (Figure 1J,K). Therefore, similar pattern of the relative levels of cleaved caspase-3 and PARP was detected in these cell lines (Figure 1L,M). Collectively, such data indicated that cytoplasmic LKB1 promoted the proliferation and clonogenicity, but inhibited the apoptosis, of A549 and HCC827 cells in vitro.
FIGURE 6 Cytoplasmic liver kinase B1 (LKB1) promotes autophagy, independent of AMP-activated protein kinase/mTOR signaling in A549 and HCC827 cells. A549-LKB1s and HCC827-LKB1s cells were treated with, or without, compound C or MHY1485, and the levels of autophagy-related molecule expression in those cells were determined by western blot analysis. A,B Relative levels of autophagy-related molecule expression in A549-NC, A549-LKB1s, HCC827-NC, and HCC827-LKB1s cells. C,D, Relative levels of autophagy-related molecule expression in A549-NC, A549-LKB1s, compound C-treated A549-LKB1s, HCC827-NC, HCC827-LKB1s, and compound C-treated HCC827-LKB1s cells. E,F, Relative levels of autophagy-related molecule expression in A549-NC, A549-LKB1s, MHY1485-treated A549-LKB1s, HCC827-NC, HCC827-LKB1s, and MHY1485-treated HCC827-LKB1s cells. *P < .05. NC, negative control; p-, phosphorylated

FIGURE 7 Immunohistochemistry analysis of liver kinase B1 (LKB1) expression in lung adenocarcinoma. A, No cytoplasmic anti-LKB1 staining. B, Faint cytoplasmic anti-LKB1 staining. C, Moderate cytoplasmic anti-LKB1 staining. D, Strong cytoplasmic anti-LKB1 staining. E, Samples with nuclear anti-LKB1 staining. F, Samples without nuclear anti-LKB1 staining
TABLE 1  Distribution of LKB1 expression in 190 lung adenocarcinoma tissues

| Intensity          | All, N = 190 |
|-------------------|-------------|
|                   | n           | %         |
| LKB1: cytoplasm staining |
| Low               | 29          | 15.2      |
| 1                 | 92          | 47.9      |
| High              | 61          | 32.1      |
| 3                 | 9           | 4.7       |
| LKB1: nuclear staining |
| 0                 | 129         | 68.2      |
| 1                 | 61          | 31.8      |

cells treated with 3-MA was less than that of A549-LKB1s or HCC827-LKB1s cells (Figure 4A). Likewise, inhibition of autophagy by 3-MA significantly inhibited the proliferation of A549-LKB1s or HCC827-LKB1s cells (Figure 4B-E). A similar pattern of clonogenicity and apoptosis was detected in different groups of cells (Figure 4F-I). Western blot analysis indicated that the relative levels of cleaved caspase-3 and PARP in the 3-MA-treated A549-LKB1s, and HCC827-LKB1s were similar to that of A549-NC and HCC827-NC cells, which were significantly lower than that in A549-LKB1s and HCC827-LKB1s cells without 3-MA treatment (Figure 4J,K).

Next, we found that, in comparison with A549-LKB1s or HCC827-LKB1s cells, inhibition of autophagy significantly inhibited the growth of implanted A549-LKB1s or HCC827-LKB1s tumors (Figure 5A-C). Inhibition of autophagy also significantly reduced the tumor volumes and weights in mice (Figure 5D,E). Moreover, immunohistochemistry revealed that inhibition of autophagy obviously decreased Ki67 expression in the tumor tissues (Figure 5F,G).

Together, these data indicated that cytoplasmic LKB1 promoted autophagy, which was associated with enhanced proliferation in A549 and HCC827 cells.

3.3 | Autophagy enhanced by cytoplasmic LKB1 is independent of AMPK/mTOR signaling in A549 and HCC827 cells

It is well known that AMPK/mTOR signaling regulates autophagy, particularly in a nutrition-deficient condition.13 To further understand the regulation of cytoplasmic LKB1 on autophagy, the relative levels of AMPK and mTOR were determined by western blot analysis. We found that there were similar levels of AMPK and mTOR expression and phosphorylation between A549-NC or HCC827-NC and A549-LKB1s, or HCC827-LKB1s cells (Figure 6A,B), suggesting that induction of unipolar cytoplasmic LKB1 expression did not alter AMPK and mTOR signaling in A549 and HCC827 cells. Furthermore, treatment with compound C (an AMPK inhibitor) or with MHY1485 (an mTOR inhibitor) did not change the relative levels of LC3II, Atg4, Atg7, Atg12, or Beclin1 expression in A549-LKB1s, or HCC827-LKB1s cells (Figure 6C-F). Hence, autophagy enhanced by cytoplasmic LKB1 was independent of AMPK/mTOR signaling in A549 and HCC827 cells.

3.4 | Expression of LKB1 in human lung adenocarcinoma

We finally examined LKB1 expression in 190 lung adenocarcinoma tissues by immunohistochemistry. There were 162 (84.8%) of 190 lung adenocarcinoma tissues with cytoplasmic LKB1 expression and 61 (31.8%) with nuclear LKB1 expression (Figure 7, Table 1). The frequency of cancers with nuclear LKB1 and low levels of cytoplasmic LKB1 expression was significantly lower than those without nuclear LKB1 and high levels of cytoplasmic LKB1 expression (Table 2). Stratification analysis indicated that cytoplasmic LKB1 expression was positively associated with T stage (P = .01) and TNM stage (P = .045; Table 3) in this cohort. Furthermore, the nuclear LKB1 expression was associated inversely with T stage (P = .028) and TNM stage (P = .023, Table 3) in this cohort. More importantly, patients with higher levels of cytoplasmic LKB1-expressing lung cancer had significantly shorter OS than those with lower levels of cytoplasmic LKB1-expressing cancers (Figure 8A, P < .001). In contrast, patients with negative nuclear LKB1-expressing lung cancers had significantly shorter OS than those with nuclear LKB1-expressing cancers (Figure 8B, P < .001). Further analysis indicated that the patients with nuclear and lower levels of cytoplasmic LKB1-expressing lung cancers had the longest OS and the patients with either lower levels of cytoplasmic LKB1, but without nuclear LKB1, or nuclear and higher levels of cytoplasmic LKB1-expressing lung cancers had similar OS, whereas those with higher levels of cytoplasmic LKB1, but without nuclear LKB1-expressing lung cancers had the shortest OS in this population (Figure 8C, P < .001). Finally, univariate analysis indicated that age at diagnosis (<60 years old), lepidic lung adenocarcinoma, and nuclear LKB1 expression were associated with a longer OS, whereas advanced T and TNM stages, lymph node metastasis, and cytoplasmic LKB1 expression were associated with a shorter OS in this cohort (Table 4). Multivariate analysis revealed that age at diagnosis (<60 years old), lepidic lung adenocarcinoma, and nuclear LKB1 expression were independent factors for a longer OS, whereas advanced TNM stages and cytoplasmic LKB1 expression were independent risk factors for a shorter OS in this cohort (Table 4). Collectively, our data indicated that subcellular localization of LKB1 was an independent biomarker for prognosis of patients with lung adenocarcinoma.
**TABLE 3** Stratification analysis of the distribution of LKB1 expression with demographic and clinical characteristics of patients with lung adenocarcinoma

| Characteristic | n    | %     | LKB1 expression |          |       |          | Nuclear |          |       |
|---------------|------|-------|-----------------|----------|-------|----------|---------|----------|-------|
|               |      |       | Cytoplasm       |          |       |          |         |          |       |
|               |      |       | Low             | High     | P-value|          | Low     | High     | P-value|
|               |      |       | n   | %  | n   | %  | n    | %   | n    | %   |
| All cases     | 190  |       |     |     |     |     |     |     |     |     |
| Gender        |      |       |     |     |     |     |     |     |     |     |
| Male          | 103  | 54.21 | 67  | 65.05 | 36  | 34.85 | 0.557  | 71  | 68.93 | 32  | 31.07 | 0.739 |
| Female        | 87   | 45.79 | 53  | 60.92 | 34  | 39.08 | 0.557  | 58  | 66.67 | 29  | 33.33 |        |
| Age, years    |      |       |     |     |     |     |     |     |     |     |
| 60            | 104  | 54.74 | 61  | 58.65 | 43  | 41.35 | 0.157  | 68  | 65.38 | 36  | 34.62 | 0.385 |
| ≥60           | 86   | 45.26 | 59  | 68.60 | 27  | 31.40 | 0.157  | 62  | 72.09 | 25  | 27.91 |        |
| Histological subclassification |      |       |     |     |     |     |     |     |     |     |
| Lepidic       | 34   | 17.89 | 24  | 70.59 | 10  | 29.41 | 0.633  | 21  | 61.76 | 13  | 38.24 | 0.598 |
| Acinar        | 123  | 64.74 | 77  | 62.60 | 46  | 37.40 | 0.633  | 85  | 69.11 | 38  | 20.89 |        |
| Papillary     | 22   | 11.58 | 14  | 63.64 | 8   | 36.36 | 0.633  | 14  | 63.64 | 8   | 26.36 |        |
| Micropapillary| 3    | 1.58  | 1   | 33.33 | 2   | 66.67 | 0.633  | 3   | 100.00| 0   | 0.00  |        |
| Solid         | 8    | 4.21  | 4   | 50.00 | 4   | 50.00 | 0.633  | 6   | 75.00 | 2   | 25.00 |        |
| T stage       |      |       |     |     |     |     |     |     |     |     |
| T1 + T2       | 154  | 81.05 | 104 | 67.53 | 50  | 32.47 | 0.010  | 99  | 64.29 | 55  | 35.71 | 0.028 |
| T3 + T4       | 36   | 18.95 | 16  | 44.44 | 20  | 55.56 | 0.010  | 30  | 83.33 | 6   | 16.67 |        |
| Lymph node metastasis |      |       |     |     |     |     |     |     |     |     |
| No            | 102  | 53.68 | 68  | 66.76 | 34  | 33.33 | 0.280  | 69  | 67.65 | 33  | 32.35 | 0.937 |
| Yes           | 88   | 46.32 | 52  | 59.09 | 36  | 40.91 | 0.280  | 60  | 68.18 | 28  | 31.82 |        |
| TNM stage     |      |       |     |     |     |     |     |     |     |     |
| Stage I-II    | 118  | 62.11 | 81  | 68.64 | 37  | 31.36 | 0.045  | 73  | 61.86 | 45  | 38.14 | 0.023 |
| Stage III-IV  | 72   | 37.89 | 39  | 54.17 | 33  | 45.83 | 0.045  | 56  | 77.78 | 16  | 22.22 |        |

*P*-value < 0.05 are highlighted in bold.

**FIGURE 8** High levels of cytoplasmic liver kinase B1 (LKB1) expression are associated with poor overall survival in lung adenocarcinoma. Patients with lung adenocarcinoma were stratified, based on the distribution and levels of LKB1 expression, and their overall survival was established by the Kaplan-Meier method, followed by log-rank test. A, overall survival of low cytoplasmic LKB1 group and high cytoplasmic LKB1 group. B, overall survival of positive nuclear LKB1 group and negative nuclear LKB1 group. C, overall survival of low cytoplasmic LKB1 with nuclear LKB1 group, low cytoplasmic LKB1 without nuclear LKB1 group, high cytoplasmic LKB1 with nuclear LKB1 group and high cytoplasmic LKB1 without nuclear LKB1 group.
4 | DISCUSSION

In this study, we found that cytoplasmic \textit{LKB1} promoted the growth of lung adenocarcinoma by enhancing autophagy. First, we showed that induction of truncated \textit{LKB1} was exclusively expressed in the cytoplasm, and full-length \textit{LKB1} was expressed in both cytoplasm and nucleus of A549 and HCC827 cells. \textit{LKB1} promoted the proliferation, clonogenicity, and growth of implanted tumors, accompanied by the inhibition of apoptosis in A549 and HCC827 cells. \textit{LKB1} had opposite effects in A549 and HCC827 cells. Second, we detected higher levels of autophagy in A549-LKB1S or HCC827-LKB1S than the control A549-NC or HCC827-NC cells. Inhibition of autophagy abrogated the effect of LKB1S on the proliferation and clonogenicity of A549 and HCC827 cells. Such data clearly indicated that cytoplasmic \textit{LKB1}, promoted autophagy and growth of lung adenocarcinoma cell.

**TABLE 4** | Univariate and multivariate analyses of factors affecting overall survival in lung adenocarcinoma patients

| Characteristic          | n     | Univariate analysis | Multivariate analysis |
|-------------------------|-------|---------------------|-----------------------|
|                         |       | HR (95% CI)         | P-value               | HR (95% CI)         | P-value   |
| Gender                  |       |                     |                       |                       |
| Male                    | 103   | 1.03 (0.74-1.41)    | 0.879                 | –                     | –         |
| Female                  | 87    |                     |                       |                       |           |
| Age, years              |       |                     |                       |                       |           |
| 60                      | 104   | 0.69 (0.50-0.96)    | 0.027                 | 0.60 (0.42-0.84)     | 0.003     |
| ≥60                     | 86    |                     |                       |                       |           |
| Histological subclassification |       |                     |                       |                       |           |
| Lepidic                 | 34    | 2.03 (1.29-3.19)    | <0.001                | 2.15 (1.36-3.40)     | <0.001    |
| Acinar/papillary        | 145   |                     |                       |                       |           |
| Micropapillary/solid    | 11    |                     |                       |                       |           |
| T stage                 |       |                     |                       |                       |           |
| T1 + T2                 | 154   | 1.57 (1.03-2.38)    | 0.035                 | –                     | –         |
| T3 + T4                 | 36    |                     |                       |                       |           |
| Lymph node metastasis   |       |                     |                       |                       |           |
| No                      | 102   | 1.53 (1.11-2.11)    | 0.009                 | –                     | –         |
| Yes                     | 88    |                     |                       |                       |           |
| TNM stage               |       |                     |                       |                       |           |
| Stage I-II              | 118   | 2.22 (1.59-3.01)    | <0.001                | 2.02 (1.15-3.56)     | 0.015     |
| Stage III-IV            | 72    |                     |                       |                       |           |
| Cytoplasmic LKB1        |       |                     |                       |                       |           |
| Low                     | 120   | 3.89 (2.72-5.56)    | <0.001                | 4.27 (2.88-6.33)     | <0.001    |
| High                    | 70    |                     |                       |                       |           |
| Nuclear LKB1            |       |                     |                       |                       |           |
| Low                     | 129   | 0.31 (0.21-0.46)    | <0.001                | 0.27 (0.17-0.40)     | <0.001    |
| High                    | 61    |                     |                       |                       |           |

*P*-value < .05 are highlighted in bold.

–, not applicable; CI, confidence interval; HR, hazard ratio.
The relationship between autophagy and cancer is complicated. Previous studies have found low levels of autophagy in cancer cells due to the mutation, deletion, and functional inhibition of autophagy-related genes. Autophagy can inhibit the process of carcinogenesis by degrading harmful proteins and damaged organelles, reducing the instability of the genome, or converging inflammatory responses induced by necrosis. Inhibition of autophagy is associated with promoting cell differentiation, protein catabolism, and autophagic cell death. However, our findings could be surprisingly against the traditional dogma. It is possible that autophagy induced by LKB1, may help cancer cell survival under a nutrition deficiency circumstance to overcome metabolic stress and maintain energy balance.

It is well known that LKB1 can activate AMPK. Activation of AMPK can inhibit mTOR and further enhance autophagy. In this study, we found that cytoplasmic LKB1, enhanced autophagy, independent of AMPK and mTOR signaling. The full-length LKB1 localizes in the nucleus. It interacts with STc20 Related Adaptor in the nucleus to form a dipolymer complex and the complex relocates from the nucleus to the cytoplasm where the complex binds to MO25 and forms a tripolymer complex, leading to AMPK activation. As the short form of LKB1 has no normal nuclear localization signal, so it cannot form a tripolymer complex and fails to activate AMPK. Although mTOR signaling is an important negative regulator of autophagy and the AMPK/mTOR pathway can be activated by LKB1, the independence of cytoplasmic LKB1, promoting autophagy suggests that cytoplasmic LKB1 might enhance autophagy by other pathways or stimuli. Actually, previous studies have shown that the Bcl-2 family, p53 protein, oxidative stress and endoplasmic reticulum stress can enhance autophagy. We are interested in further investigating the precise mechanisms underlying the action of LKB1, in promoting cell autophagy and the growth of lung adenocarcinoma cells.

To look for clinical significance, we characterized LKB1 expression in 190 lung adenocarcinoma samples. We found that LKB1 was expressed in both the nucleus and cytoplasm of lung adenocarcinoma. Interestingly, the levels of nuclear LKB1 were inversely associated with cytoplasmic LKB1 in lung adenocarcinoma. Furthermore, cytoplasmic LKB1 was associated with poor OS in this population and was an independent risk factor for prognosis.

In summary, our data indicated that cytoplasmic LKB1 promoted A549 and HCC827 cell survival in vitro and in vivo. Furthermore, cytoplasmic LKB1 was associated with poor prognosis in lung adenocarcinoma cancer. Therefore, our findings could provide new insights into the pathogenesis of lung adenocarcinoma and novel biomarkers for prognosis of lung adenocarcinoma.

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CONFLICT OF INTEREST
The authors have no conflict of interest.

5 | ETHICS APPROVAL
All protocols were approved by the Ethics Committee of Xi’an Jiaotong University, and informed consent was obtained from all patients before surgery. All in vivo protocols were approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University.

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