LKB1 suppresses glioma cell invasion via NF-κB/Snail signaling repression

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Background: Liver kinase B1 (LKB1) is involved in various human diseases. Aberrant expression of LKB1 expression is involved in glioma progression and associated with prognosis, however, the specific mechanism involving NF-κB/Snail signaling pathways remain unknown.

Materials and methods: In the present study, quantitative real-time PCR analysis was used to investigate the expression of LKB1 tumor tissue samples and cell lines. In glioma cell lines, CCK-8 assay, transwell invasion and migration assays were used to investigate the effects of LKB1 on proliferation and invasion.

Results: We observed that LKB1 knockdown promoted glioma cell proliferation, migration and invasion. This effect was induced through NF-κB/Snail signaling activation. Also, LKB1 overexpression suppressed proliferation, migration, and invasion, which could be rescued by Snail overexpression.

Conclusion: Taken together, our results show that LKB1 knockdown promotes remarkably glioma cell proliferation, migration and invasion by regulating Snail protein expression through activating the NF-κB signaling. This may serve as a potential prognostic marker and therapeutic target for glioma.

Keywords: glioma, proliferation, migration, invasion, LKB1, NF-κB, Snail

Introduction
Gliomas are the most common primary malignant intracranial brain neoplasms in adults, accounting for >50% of the central nervous system tumor; it has the feature of rapid progression and poor prognosis.¹,² Glioma is characterized by rapid progression and poor prognosis due to its highly invasive characteristics. Therefore, elucidation of specific mechanisms underlying this invasive nature of glioma is imperative to explore novel diagnostic methods and treatments.

Liver kinase B1 (LKB1), also known as serine/threonine protein kinase 11, is located on chromosome 19p13.3 and encodes a 48-kDa serine/threonine protein kinase.³ Functionally, LKB1 plays pivotal roles in cellular processes such as apoptosis, metabolism, and cell cycle regulation. As a multifunctional molecular protein, LKB1 acts as a vital metabolic enzyme in the 5'-adenosine monophosphate-activated protein kinase (AMPK) signaling pathway,⁴ and LKB1 deactivation often mediates the mammalian target of rapamycin signaling pathway,⁵ which is critical for modulating cell proliferation, survival, and energy metabolism. Growing evidence has indicated that LKB1 functions as a tumor-suppressor gene by modulating AMPK or AMPK-related kinases, which further modulate cell cycle, apoptosis, polarity, and metabolism.⁶ Although previous studies have reported the clinical importance of LKB1 expression in gliomas and role of LKB1–AMPK pathway in suppressing glioma cell proliferation, the specific mechanisms underlying NF-κB/Snail signaling remain unknown.
NF-κB signaling pathway is activated by several signaling molecules and it is a very crucial regulatory transcription factor for cell survival, growth, and differentiation. Previous studies have demonstrated that NF-κB activation blocked the Snail degradation, subsequently promoting cancer cell migration and invasion.

In this study, we elucidated LKB1 expression and function in glioma. Our results indicated that LKB1 is down-regulated in glioma tissues. Moreover, cellular experiments revealed the pivotal role of LKB1 in inhibiting glioma cell growth, migration, and invasion, which is likely mediated through NF-κB signaling.

Materials and methods

Cell lines and reagents

HA, HM, U-87MG, H4, U-251MG, and LN-18 cells (Cellcook Biotech Co., Ltd., Guangzhou, China), which were routinely checked for contamination and authenticated by the company, were purchased. Cells were grown in DMEM (HyClone Laboratories, San Angelo, TX, USA) supplemented with 10% FBS (Gibco, Waltham, MA, USA). 4-Methyl-N-1-(3-phenylpropyl)-1, 2-benzenediamine (JSH-23), and caffeic acid phenethyl ester (CAPE) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Anti-LKB1 (3050), anti-Snail (3879), anti-Slug (9585), anti-P-IKKα/β (2697), anti-total IKKα (2682), anti-total IKKβ (8943), anti-NF-κB p65 (8242), and anti-β-actin (4970) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Patients and sample preparation

A total of 49 samples of surgically resected gliomas were collected from patients who visited Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, from September 2017 to March 2018. All patients provided written informed consent, and the study was approved by the hospital authorities. Before brain surgery, patients provided written informed consent, and the study was approved by the hospital authorities. Patients and sample preparation

Establishment of knockdown and overexpression cells

To establish stable knockdown and overexpression cell lines, full-length shRNA sequences that specifically targeted LKB1, Snail, or IκBα were cloned into pLKO.1-MSCV-Puro or pcDNA3.0 vectors and were sequenced bidirectionally. Plasmid construction and lentivirus packaging were completed by Shanghai GeneChem Co., Ltd. (Shanghai, China). Cells were infected by the lentivirus according to the manufacturer’s protocol and selected by puromycin (Sigma-Aldrich Co.) after 2 weeks to obtain cell lines with stable expression. Empty vector pcDNA3.0 or pLKO.1 and scrambled shRNA were used as a negative control. The shLKB1 target sequence was 5′-CATCTACACTCAGACTTCCAC-3′, and the Snail target sequence was 5′-GCTGAGCTGTACTAGGACAA-3′. The short hairpin RNA was designed based on the LKB1 sequence: 5′-CATTGTGCACAAAGGACATCAA-3′ and 1xBtα sequence: 5′-GACGAGAAAGATCATTTGAAAT-3′. Cells infected with empty vector or scrambled shRNA were used as controls.

Transwell invasion and migration assays

Cell migration and invasion experiments were performed using the Transwell system (Corning Inc., Corning, NY, USA) based on the manufacturer’s instructions. To assess invasion, filters were precoated with Matrigel (BD Biosciences, San Jose, CA, USA). Approximately 6×10^5 cells were added to the top chamber containing serum-free DMEM. The bottom chamber contained 600 μL ofDMEM supplemented with 20% FBS. Following 24 hours of incubation, cells on the upper surface were gently removed using a cotton swab, and then the membrane was fixed with 4% formaldehyde for 20 minutes and stained with 0.1% crystal violet solution (Servicebio, Wuhan, China) for 30 minutes. Cells that migrated to the lower surface of the membrane were photographed and counted under a microscope. The same experiment was performed for the migration assay except the filters were not precoated with Matrigel.

Cell counting Kit-8 (CCK-8) assay

For CCK-8 assay, cells were counted and seeded at a density of 5,000 cells/well in 96-well plates containing complete culture medium. After treatment with 10 μL CCK-8 (Dojindo Laboratories, Kumamoto, Japan), absorbance was measured at 450 nm using a microplate reader (Spectra Max M2 reader; Molecular Devices LLC, Sunnyvale, CA, USA). Each experiment was performed in triplicate.

Western blotting

Total protein was lysed from glioma tissues or cells using RIPA lysis buffer (Boster Biological Technology, Pleasanton, CA, USA) with 1 mM phenylmethane sulfonyl fluoride (Boster Biological Technology) according to the manufacturers’ instruction. Samples were supplemented with phosphatase inhibitors (Boster Biological Technology) for phosphorylated
protein extraction. After clarification by centrifugation at 12,000xg for 20 minutes at 4°C, soluble protein concentration was measured using the bicinchoninic acid (Boster Biological Technology) method. For immunoblotting, total proteins/phosphorylated protein samples (60 µg) were incubated at 100°C for 10 minutes, separated by 12% SDS-PAGE (Boster Biological Technology) at 120 V for 2 hours, and transferred on a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA), which was incubated with 5% fat-free milk in PBS for 2 hours and incubated with primary antibodies at 4°C overnight and with secondary antibodies at room temperature for 1 hour. Finally, enhanced chemiluminescence (Boster Biological Technology) was used to visualize samples exposed to X-ray film, and β-actin (1:1,000; Cell Signaling Technology) was used as an internal control. To analyze relative protein expression, ImageJ2X analysis software package (National Institute of Mental Health, Bethesda, MD, USA) was used to measure the intensity of specific bands. All experiments were repeated thrice.

Quantitative real-time PCR (qRT-PCR)
Total RNA was lysed from previously collected glioma specimens using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis and RT-PCR were performed using SYBR® Premix Ex Taq™ Kit (Takara Bio Inc., Shiga, Japan). 18S rRNA was used as an internal control. LKB1 primer sequences were as follows: forward 5′-AGGGATGCTTGAGTACGAACC-3′ and reverse 5′-GTCTCTTCAACTACGCAC-3′. Snail primer sequences were as follows: forward 5′-TGGCGAGCTCTAATCTACGCGA-3′ and reverse 5′-AGATGAGCATTGGCAGCGAG-3′. Relative mRNA expression level was standardized as described earlier.13,14

Immunohistochemical (IHC) and immunofluorescence staining analyses
IHC analysis and semiquantitative scoring were performed as described previously.15 Cells were grown on 35 mm dishes and fixed with 4% paraformaldehyde. After fixation, cells were penetrated with Triton X-100, closed with 5% BSA in PBS for 1.5 hours at room temperature, and dyed with anti-LKB1 (1:500) with 0.3% BSA in PBS and Cy3-conjugated secondary antibodies (1:100; Cell signaling Technology, Danvers, MA, USA) with 0.3% BSA in PBS. Fluorescence was visualized under laser scanning confocal microscope (Olympus Corporation, Tokyo, Japan).

IHC analysis and semiquantitative scoring were performed as described previously.16 Scores were defined based on cell-positive dyeing intensity (0= negative; 1= weak; 2= moderate; and 3= strong) multiplied by the proportion of positively dyed cells (75%–100%=4; 50%–74%=3; 25%–49%=2; 1%–24%=1; and 0%=0), leading to scores ranging 0–12.

Dual luciferase reporter assays
U-87MG and U-251MG cells were routinely seeded in 24-well plates for 24 hours before transfection with NF-κB firefly luciferase reporter plasmid and phRL-TK (Shanghai GeneChem Co., Ltd.) using Lipofectamine™ 3000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. The Renilla luciferase expression plasmid was used as an internal control. At 24 hours after transfection, cells were harvested and lysed with 100 µL of 1× passive lysis buffer (Boster Biological Technology). Subsequently, luciferase activities were calculated using a Dual-Glo Luciferase kit (Promega Corporation, Fitchburg, WI, USA).

Statistical analysis
Data are shown as mean and SD. Statistical significance was tested using two-tailed Student’s t-test and chi-squared test. Survival analyses were performed using log-rank tests and Kaplan–Meier plots. Multivariate survival analyses were performed using a Cox regression model. Statistical analysis was performed using the Empower Stats Statistical Software Program Version 2.16.1, with P<0.05 considered significant.

Ethics approval and consent to participate
The use of human tissues was approved by the Human Research Committee of Huazhong University of Science and Technology (S304). Written informed consent was obtained from each patient.

Results
LKB1 was often downregulated in human glioma tissues
RT-PCR showed remarkable differences in LKB1 expression among human glioma specimens of different grades. Compared to low-grade gliomas, high-grade gliomas showed significantly less LKB1 gene expression (Figure 1A, P<0.001). Western blotting showed that LKB1 was often downregulated at a higher grade (Figures 1B and S1). We measured LKB1 expression levels by Western blotting in two normal brain tissues and glioma tissues of various grades (Figure S2) and checked the expression levels of LKB1 in glioma cell lines including U87MG and U251MG and in normal astrocytes (Figure S3). IHC analyses were performed to test LKB1 expression in 49 human glioma samples. As shown in Figure 1C (Figure S4), immunostaining
intensity of LKB1 evidently differed among different grades of gliomas. Compared to low-grade gliomas, high-grade gliomas showed weaker LKB1 immunostaining intensity and significantly decreased LKB1 protein expression on quantification analyses (Figure 1D, \( P < 0.01 \)). Correlation analysis of LKB1 expression with clinicopathological characteristics of 49 glioma samples with informative IHC demonstrated that downregulated LKB1 expression was significantly correlated with the Karnofsky Performance Scale (KPS) score (\( P = 0.001 \)) and recurrence (\( P = 0.01 \), Table 1); this was consistent with the results of a previous report that KPS was an independent predictor of survival.17 In addition, Kaplan–Meier analysis demonstrated that low LKB1 expression was correlated with shorter recurrence-free survival in malignant gliomas (Figure 1E, \( P < 0.05 \)). Multivariate Cox regression analysis further proved that disease-free survival was significantly higher in tumors with high LKB1 protein expression than in those with low LKB1 protein expression (HR: 2.097, 95% CI: 1.557–2.637, \( P = 0.013 \)).

**LKB1 downregulation promoted glioma cell proliferation, migration, and invasion**

To explore the role of LKB1 in glioma cell proliferation, we knocked down LKB1 in U-87 and U-251 cells. At 48 hours after transfection, the downregulation efficacy of LKB1 was measured by qRT-PCR and western blotting.
CCK-8 assay demonstrated that cell growth rates were markedly higher in the LKB1 knockdown group than in the scramble control group (Figure 2C, \(P < 0.001\)). Colony-forming assay showed that compared to the control group, the LKB1 knockdown group yielded much greater number of colonies (Figure 2D, \(P < 0.01\)). Transwell migration assay indicated that the number of migrated cells significantly increased after LKB1 knockdown (Figure 2E, \(P < 0.01\)). Invasion assay showed that compared to the control group, the LKB1 knockdown group showed more invasive cells (Figure 2F, \(P < 0.01\)).

**LKB1 upregulation suppressed glioma cell proliferation, migration, and invasion**

LKB1 was overexpressed using lentiviruses containing shRNA to specifically target ELTD1 in the glioma cell lines U-87 and U-251. Transfection efficiency was verified using qRT-PCR and Western blotting (Figure 3A and B), and CCK-8, colony-forming, and Transwell assays were used to test effects of LKB1 on glioma cell proliferation, migration, and invasion. LKB1 overexpression significantly suppressed glioma cell proliferation, migration, and invasion (Figure 3C–F).

**Snail is involved in LKB1-regulated glioma cell proliferation, migration, and invasion**

Snail protein has been reported to regulate several cellular processes through a series of mechanisms. Previous studies have indicated that Snail overexpression promoted glioma cell proliferation, migration, and invasion. Therefore, LKB1 upregulation may repress Snail expression, thereby suppressing glioma cell proliferation, migration, and invasion. Our results showed obvious increase in Snail protein expression after LKB1 knockdown (Figure 4A). However, the Slug protein expression level remains unchanged (Figure S5). In addition, to verify whether Snail participated in LKB1-regulated glioma cell proliferation and invasion, we used lentiviruses containing shRNA to target Snail in U-87 and U-251 cells (Figure 3B). As expected, Snail overexpression abrogated the effects of LKB1 overexpression.
on inhibiting U-87MG and U-251MG cell proliferation (Figure 4C), colony formation (Figure 4D), migration (Figure 4E), and invasion (Figure 4F). Taken together, these results indicate that LKB1 inhibited glioma cell proliferation, migration, and invasion by regulating Snail protein expression.

LKB1 regulates glioma cell proliferation, migration, and invasion via the NF-κB/Snail signaling pathway

We treated LKB1-knockdown cells with two chemical inhibitors of NF-κB, CAPE, and JSH-23. First, we confirmed
the inhibition efficiency by p65 in U-87 and U-251 cells (Figure 5A). Both CAPE and JSH-23 attenuated the Snail expression induced by LKB1 knockdown in U-87 and U-251 cells (Figure 5B). Next, we confirmed whether the NF-κB signaling pathway was activated after LKB1 knockdown. After LKB1 knockdown, IκBα subunit decreased, the phosphorylation of IκB kinase added, and the phosphorylation of p65 increased (Figure 5C); all these effects were rescued by IκBα silencing. Furthermore, p65 migrated to U-87 cell nuclei after LKB1 knockdown; this effect was also rescued by IκBα overexpression (Figure 5D). Then, U-87 cells were infected with an NF-κB luciferase reporter plasmid to confirm the transcriptional activation of NF-κB. LKB1 knockdown induced NF-κB activation and simultaneous IκBα-guided

Figure 3 (Continued)
Figure 3 Upregulation of LKB1 suppresses glioma cell proliferation, migration, and invasion.

Notes: (A) The overexpression efficiency against LKB1 was verified by Western blot in U-87 and U-251 cells. (B) Growth curves between none, vector, and LKB1(OE) by CCK-8 assay. The results are shown as the mean $\pm$ SD of at least three independent experiments. (C–E) Representative images (left panels) and histogram quantification (right panels) of the (C) colony formation assay, (D) Transwell migration, and (E) invasion assays with U-87 and U-251 cells. Statistical significance was assessed using one-way ANOVA followed by Dunnett’s tests for multiple comparisons. Scale bars: 50 $\mu$m. ** $P < 0.01$.

Abbreviations: CCK-8, Cell Counting Kit-8; LKB1, Liver kinase B1; OE, overexpression.

Figure 4 (Continued)
luciferase overexpression toward the baseline (Figure 5E). Finally, we knocked down IkBα and probed Snail increased expression (Figure 5F). Collectively, these results indicate that NF-κB activation is involved in Snail expression.

Discussion

Gliomas account for ~40% of the diagnosed primary central nervous system tumors. Among gliomas, glioblastoma is the most common malignant type that is associated with very poor prognosis, with survival duration for most patients being only 15 months from diagnosis.\textsuperscript{18,19} Therefore, identification of novel and efficient molecular markers is urgent to diagnose and treat patients with glioma.

In this study, we measured LKB1 expression in 49 glioma samples using RT-PCR and in 16 glioma samples using Western blotting and found that LKB1 was often downregulated in high-grade gliomas. Subsequently, we analyzed the clinical relevance of LKB1, which highlighted the potential of LKB1 as a novel clinical prognostic index. Huang et al have reported that LKB1 downregulation promoted tumor progression and predicted unfavorable prognosis in patients with glioma.\textsuperscript{20} However, precise mechanisms underlying this role remain largely unknown. In this study, we observed that the LKB1 protein modulated tumor cell proliferation, migration, and invasion, thereby, playing an important role in tumor progression. Through gain and loss of function experiments, we found that LKB1 inhibited glioma cell proliferation, migration, and invasion. LKB1 knockdown markedly promoted tumor cell proliferation, whereas ELTD1 overexpression inhibited tumor cell growth.

Furthermore, by screening for possible signaling pathways using a dual luciferase reporter assay (Figure S6), we
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Figure 5 LKB1 regulates glioma cell proliferation, migration, and invasion via the NF-κB/Snail signaling pathway.

Notes: (A and B) U-87 and U-251 cells transfected with LKB1 plasmid and co-cultured with NF-κB signaling inhibitors were reaped, and the lysates were immune-blotted for (A) p-P65 Tot.P65, (B) Snail, Slug, and β-Actin. (C) LKB1 knockdown decreased the protein level of iκBα and increased the protein level of p-IKK and p-P65. Other proteins remain unchanged. (D) The p65 migrated to the nuclei of U-87 cells after LKB1 knockdown and its effects were abrogated by iκBα overexpression. (E) LKB1 knockdown induced NF-κB activation and iκBα overexpression abrogated its effects. (F) iκBα knockdown increased the protein level of Snail. Slug remains unchanged. Statistical significance was assessed using one-way ANOVA followed by Dunnett’s tests for multiple comparisons. Scale bars: 50 µm. **P<0.01.

Abbreviations: LKB1, liver kinase B1; NS, no statistical significance.
found that the LKB1 gene regulated NF-κB signaling, which plays important roles in cancer growth and metastasis. P65 is a regulatory transcription factor particularly vital in cell survival, growth, and differentiation.21,22 NF-κB activation blocked Snail degradation, subsequently promoting cancer cell migration and invasion.23,24 Moreover, LKB1 knockdown markedly enhanced NF-κB reporter activity. In particular, the expression of tumor-related gene Snail was upregulated at the mRNA and protein levels when LKB1 expression was disrupted in glioma cells, suggesting that Snail is the downstream effector molecule of LKB1.

Conclusion

In summary, we demonstrated the function and expression patterns of LKB1 in glioma and frequently downregulated LKB1 expression in glioma tissues. Our findings indicated that the tumor suppressor role of LKB1 is possibly conferred via NF-κB/Snail signaling; therefore, targeting NF-κB/Snail signaling may inhibit tumor cell growth. Understanding the precise role of LKB1 in the NF-κB/Snail axis will improve our knowledge of LKB1-induced tumor growth inhibition and enable the development of novel molecular therapeutic strategies against glioma.

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Disclosure

The authors report no conflicts of interest in this work.

References

**Supplementary materials**

**Figure S1** LKB1 expression levels in two NBTs and glioma tissues of various grades. 
**Abbreviations:** LKB1, liver kinase B1; NBT, normal brain tissue.

**Figure S2** Expression levels of LKB1 in glioma cell lines including U87MG and U251MG and in normal astrocytes. 
**Abbreviation:** LKB1, liver kinase B1.

**Figure S3** The Slug protein expression level remains unchanged in U-87 and U-251. 
**Notes:** ***P*** < 0.001. 
**Abbreviations:** LKB1, liver kinase B1; KD, knockdown.

**Figure S4** LKB1 level in low grade and high grade glioma. 
**Abbreviations:** HGG, high grade glioma; LGG, low grade glioma; LKB1, liver kinase B1.
Figure S5 Immunostain for LKB1 in LGG and HGG.
Notes: Magnification ×20.
Abbreviations: HGG, high grade glioma; LGG, low grade glioma; LKB1, liver kinase B1.

Figure S6 Luciferase reporter assay to screening signaling pathway.
Abbreviations: HGG, high grade glioma; LGG, low grade glioma; LKB1, liver kinase B1; KD, knockdown.