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ORIGINAL RESEARCH

RETRACTED ARTICLE: Liver Kinase B1 (LKB1) Regulates Proliferation and Apoptosis of Non-Small Cell Lung Cancer A549 Cells via Targeting ERK Signaling Pathway

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Objective: To study the effect and potential mechanism of LKB1 on non-small cell lung cancer (NSCLC) A549 cells.

Material and Methods: A549 cells were divided into control group, LKB1 negative control (NC) group, LKB1 group, ERK inhibitor group and LKB1 + ERK activator group. Cell proliferation and apoptosis were detected by cell counting kit (CCK-8) assay and flow cytometry, respectively. Transwell assay was used to analyze the invasion ability of A549 cells. The expression of apoptosis and ERK signaling pathway-related proteins were studied by Western blot. Furthermore, a nude mouse xenograft model was constructed and treated with LKB1, ERK inhibitor and activator, respectively. The tumor volume and tumor weight were measured. Immunohistochemistry was used to test the expression of Ki-67 protein in tumor tissues, and TUNEL staining was used to test the apoptosis. Moreover, Western blot was used to detect ERK signaling pathway-related proteins in tumor tissues.

Results: Compared with control and NC groups, cell proliferation and invasion were inhibited in ERK inhibitor and LKB1 groups, while apoptosis and apoptosis-related proteins were increased ($P < 0.05$). Further study showed that ERK activator can reverse the effect of LKB1 in A549 cells. In nude mice, ERK inhibitor and LKB1 can reduce cell tumorigenicity and inhibit proliferation. Apoptosis was increased by ERK inhibitor and LKB1 treatment. Western blot showed that LKB1 and ERK inhibitor could reduce the protein expression of p-ERK1/2. However, the indicators above were the opposite in the ERK activator group.

Conclusion: LKB1 overexpression can inhibit proliferation and promote apoptosis of NSCLC A549 cells, and its mechanism may be related to inhibition of the ERK signaling pathway.

Keywords: LKB1, proliferation, apoptosis, ERK signaling pathway, lung cancer

Introduction

Lung cancer is the world's leading cause of cancer death with approximately 27% of all cancer deaths per year.[1] Lung cancer is clinically divided into two main types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Among them, 15% of lung cancer cases are SCLC, which is strongly associated with cigarette smoking.[2] NSCLC accounts for the vast majority of lung cancers, and has become resistant to anticancer drugs.[3] Although it is already known that genetic and environmental factors as well as oncological treatment are involved in the mortality rate of lung cancer, the overall survival rate of lung cancer patients is still disappointing.[4] The poor prognosis is correlated to the distant metastasis that occurs

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in NSCLC.[5] Thus, it is urgent to develop effective therapeutic approaches and targets in lung cancer therapy.

Liver kinase B1 (LKB1), also called serine–threonine kinase (STK11), was initially identified as the causal mutation in Peutz-Jeghers Syndrome (PJS).[6] PJS is a rare cancer susceptibility syndrome characterized by mucocutaneous pigmentation and the early development of multiple intestinal hamartomas.[7] LKB1 can regulate the homeostasis and immune response of hematopoietic cells through phosphorylation and activate multiple downstream substrate proteins or binding to target proteins.[8] LKB1 is a multi-functional protein ubiquitously expressed in many cell types and tissues, which plays an important role in regulating cell growth, cell metabolism, cell proliferation, cell polarity and energy balance. Alexander and Walker's study showed that LKB1 acts as a metabolic sensor to help maintain adenosine triphosphate levels during proliferation or cytokine production.[9] Pooya et al. reported that LKB1 depletion can cause delayed peripheral myelination and neuropathy,[10] and MacIver et al. indicated that LKB1 depletion can reduce regulatory T cells.[11] Loss of LKB1 triggers complex changes in the tumor microenvironment, and its inactivation is often observed in lung cancer,[12] cervical cancer[13] and hepatocellular carcinoma. However, there are few studies on the mechanism of LKB1 in lung cancer progression. In this study, the association between LKB1 and lung cancer was investigated, and the inherent molecular mechanism further explored.

Materials and Methods

Cell Culture

Human normal lung epithelial cells (BEAS-2B cells) and human non-small cell lung cancer A549 cells were purchased from Shanghai Institute of Cell Biology (Shanghai, China). Cells were cultured in RPMI 1640 medium (GIBCO, Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Solarbio, Beijing, China) and 1% penicillin-streptomycin (Solarbio, Beijing, China) at 37°C, 5% CO_2. Cells in logarithmic phase were selected for the next experiment.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by TRIzol kit (Thermo Fisher Scientific, Waltham, USA). Then, reverse transcription kit was used to transcribe it into cDNA (Baosheng Scientific, Dalian, China), and finally SYBR Green PCR Master Mix kit (Baosheng Scientific, Dalian, China) was used for PCR. The sequence of the primers are as follows: LKB1, Forward: 5′-CATGACTGTGGTGCCGTACT-3′ and Reverse: 5′-GTGACTGGCCTCCTCTTCTG-3′. β-actin, Forward: 5′-CTTCTACAATGAGCTGCGTG-3′ and Reverse: 5′-TCATGAGGTAGTCAGTCAGG-3′. The reaction was performed under the following conditions (40 cycles): 95°C for 30 s, 95°C for 5 s, and 60°C for 60 s. The data were processed with $2^{-\Delta\Delta Ct}$ method, and β-actin mRNA was used as internalreference.

Cell Transfection and Grouping

The A549 cells were grown to 70% confluence and divided into 5 groups: (i) control group (Control), no treatment; (ii) LKB1 negative control group (NC), cells were transfected with LKB1 scramble; (iii) LKB1 overexpression group (LKB1), cells were transfected with LKB1 mimics. LKB1 mimics were purchased from Guangzhou RiboBio Co., Ltd, Guangzhou, China and were transfected into the cells to construct an overexpressing LKB1 cell line using the Lipofectamine™ 2000 transfection reagent based on the manufacturer's instructions; (iv) ERK inhibitor group (PD98059), cells were treated with 100 μmol/L PD98059; and (v) LKB1 + ERK activator group (LKB1+Ani), cells were transfected with LKB1 mimics and 100 μmol/L LM22B-10.

Cell Counting Kit (CCK-8) Assay

A549 cells were cultured in 96-well plates (2×10^4 cells/mL) for 24 h, 48 h, 72 h and 96 h. Then, CCK-8 solution (Tongren Institute of Chemistry, Japan) was added to each well and incubation continued for 4 h. The optical density (OD) of each well was measured at 450 nm.

Transwell Assay

RPMI 1640 medium and Matrigel glue were mixed 1:1. Then, cell suspension (4×10^5 cells/mL) was added into the upper chamber and complete medium containing 10% FBS was added to the lower chamber. After culturing in a 37°C, 5% CO_2 incubator for 24 h, 1% paraformaldehyde was added to fix the underlying transmembrane cells and stained with crystal violet for 15 min. The invasion was photographed at ×200 magnification and quantitatively analyzed by ImageJ software.

Flow Cytometry

After collected the cells, 1× binding buffer (300 μL) (Beyotime Biotech, Shanghai, China) and 5 μL of

Annexin V-APC (Beyotime Biotech, Shanghai, China) were added to the cells. Then, 5 μL of propidium iodide (PI) was added and incubated at room temperature. 200 μL 1× binding buffer was added before using the flow cytometer (Beckman Coulter, Brea, CA, USA).

Animals and Ethics Statement

Thirty female Balb/c nude mice (4 weeks old, 16–18 g weight) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd, the animal production license number is SCXK (Jing) 20,160,006. These mice were kept in a clean environment with a relative humidity of 50–60% at 26–28°C, with free access to drinking water and food. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Pub. No. 85–23, revised 1996). The experiments have been reviewed and approved by the Animal Ethics Committee of Yantai Hospital of Traditional Chinese Medicine.

Lung Cancer Xenograft Model and Grouping

Thirty mice were divided into (i) Control group; (ii) NC group; (iii) LKB1 group; (iv) PD98059 group; and (v) LKB1+Ani group, 6 mice per group. The A549 cells (2.5×10^6 cells/mL) were inoculated into the soft skin of the right forelimb back of the nude mouse to establish the lung cancer xenograft model.

Tumor Volume Calculation

The tumors' long diameter (L) and short diameter (W) were measured every week and the tumor volume (V) = (L x W^2)/2.

Immunohistochemistry

The nude mice were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (40 mg/kg body weight) and sacrificed by cervical dislocation. The tumor tissues were dewaxed with xylene and dehydrated with ethanol. After being inactivated with 3% H_2O_2 and blocked with 5% bovine serum albumin (BSA), the slices were incubated with rabbit anti-Ki-67 antibody overnight at 4°C. After rewarming, horseradish peroxidase-labeled goat anti-rabbit IgG was used to incubate the slices. Then, the slices were stained by 3,3′-diaminobenzidine (DAB), counterstained by hematoxylin, dehydrated and sealed. Image J software was used to analyze the results.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End Labeling (TUNEL) Staining

The tumor tissue was embedded with paraffin and cut into 4 μm slices. After conventional xylene dewax and gradient ethanol dehydration, apoptosis detection kit and TUNEL assay were used to quantitatively detect apoptosis. The normal nuclei were blue, and the positive apoptotic cells were brown-yellow. AI = (number of apoptotic positive cells ÷ total cells) × 100%.

Western Blot

The cells and the tumor tissue were collected, respectively. After measured the protein concentration, each protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, USA). After blocking with 5% skim milk for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against ERK1, ERK2, p-ERK1, p-ERK2, Bcl-2, Bax and β-actin. Then, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin secondary antibody. The enhanced chemiluminescence (ECL) method was used for detecting signals, and gray scale scanning was normalized to β-actin.

Statistical Methods

All the experiments were repeated at least three times. SPSS19.0 statistical software was used for data processing. Data analysis between the two groups was done using *t*-test. One-way analysis of variance (ANOVA) was used to analysis the data between multiple groups, and subsequent analysis used LSD test. The difference was statistically significant at $p < 0.05$.

Results

The Expression of LKB1 mRNA in Different Groups

The expression of LKB1 mRNA in different groups was detected by RT-qPCR. Figure 1A showed that LKB1 had low expression in lung cancer A549 cells. Compared with Control and NC groups, the expression of LKB1 mRNA in LKB1 and LKB1 + Ani groups were significantly increased ($p < 0.05$, respectively). This result indicated the transfections were successful (Figure 1B).

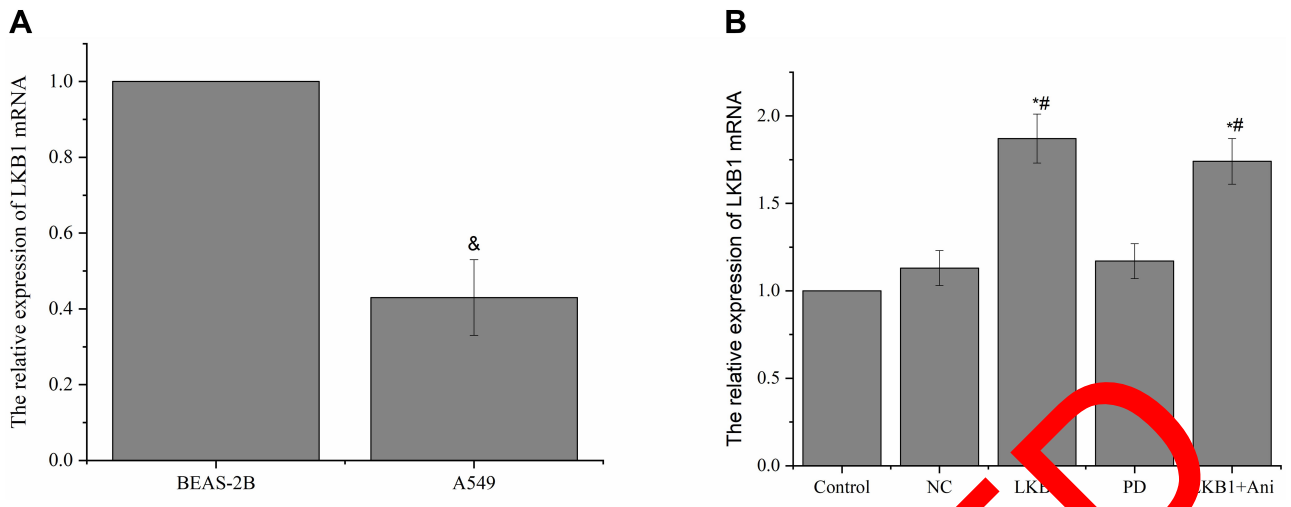

Figure 1 The expression of LKB1 mRNA. (**A**) The expression of LKB1 mRNA in BEAS-2B and A549 cells. (**B**) The expression of LKB1 mRNA in transfected cells. Values are mean ± SD. $^{\&}p < 0.05$ vs BEAS-2B group, $^{*}p < 0.05$ vs Control group, $^{\#}p < 0.05$ vs NC group.

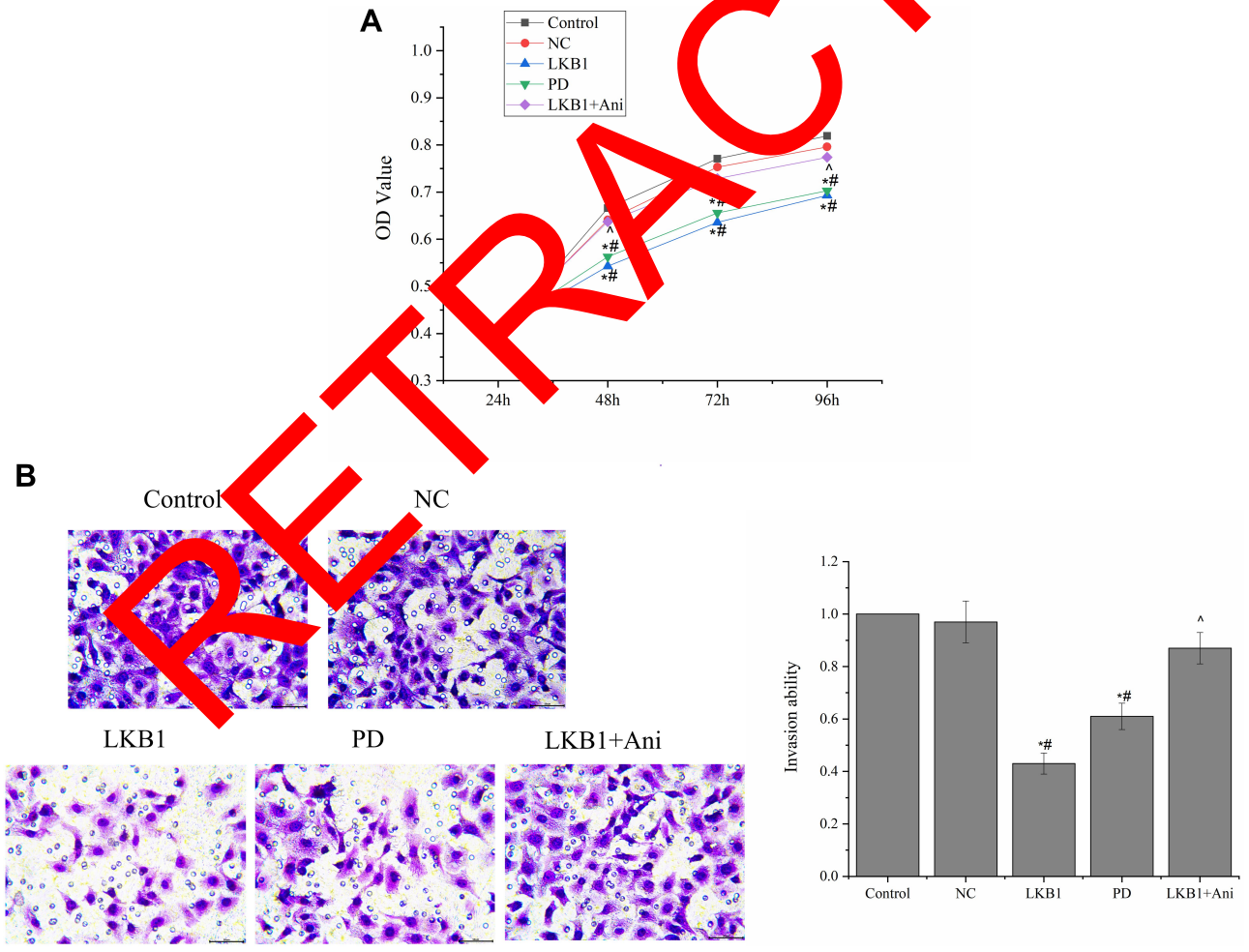

Figure 2 Effects of LKB1 on the proliferation and invasion of A549 cells. (**A**) CCK-8 assay. (**B**) Transwell assay (×200). Values are mean ± SD. $^{*}p < 0.05$ vs Control group, $^{\#}p < 0.05$ vs NC group, $^{\wedge}p < 0.05$ vs LKB1 group.

Effects of LKB1 on the Proliferation and Invasion of A549 Cells

Cell proliferation and invasion abilities were detected by CCK-8 and transwell assays, respectively (Figure 2). The LKB1 and PD groups showed decreased proliferation and invasion abilities when compared with Control and NC groups ($p < 0.05$, respectively). Moreover, A549 cells treated with LKB1 and LM22B-10 exhibited increased proliferation invasion abilities compared with the LKB1 group ($p < 0.05$). These findings suggested that LKB1 overexpression could inhibit proliferation of A549 cells through targeting the ERK signaling pathway.

Effects of LKB1 on the Apoptosis of A549 Cells

Flow cytometry analysis was used to detect the induction of cell apoptosis (Figure 3). The number of apoptotic cells in the LKB1 and PD groups (28.70±2.41% for LKB1 group and 22.63±2.07% for PD group) were significantly higher than that noted in the Control and NC groups (9.72±0.89% for Control group and 13.65 ±1.33% for NC group) ($p < 0.05$, respectively). Moreover, a significant decrease in the percentage of apoptotic cells was noted in the LKB1 + Ani group (17.82±1.57%) compared with that of the LKB1 group. These results demonstrated that LKB1 overexpression

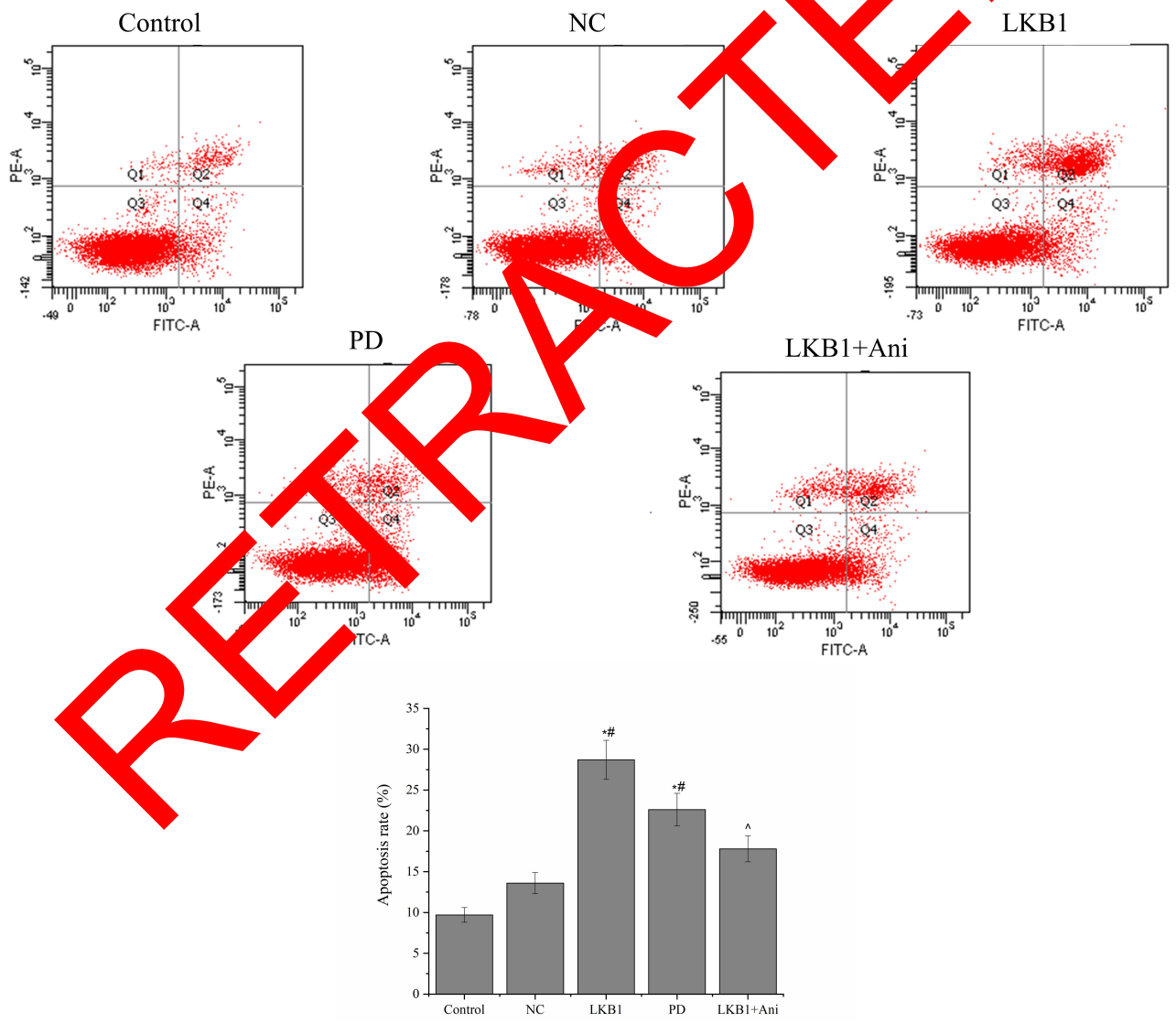

Figure 3 Effects of LKB1 on the apoptosis of A549 cells. Values are mean ± SD. *p < 0.05 vs Control group, #p < 0.05 vs NC group, ^p < 0.05 vs LKB1 group.

could promote apoptosis of A549 cells through targeting the ERK signaling pathway.

The Expression of Apoptosis and ERK Signaling Pathway-Related Proteins in A549 Cells

As shown in Figure 4, compared with Control and NC groups, the expression of apoptosis-related proteins in LKB1 and PD groups were significantly increased ($p < 0.05$, respectively). However, compared with LKB1 group, the protein expression of Bcl-2 in LKB1 + Ani group was increased ($p < 0.05$), while the protein expression of Bax was significantly decreased ($p < 0.05$). On the other hand, the protein expression of p-ERK1/2 in LKB1 and PD group was down-regulated compared with that of the Control and NC groups ($p < 0.05$, respectively), and the protein expression of total ERK1/2 was not significantly changed, that is, overexpression of LKB1 inhibited ERK1/2 phosphorylation levels to reduce the activity of the ERK pathway.

Effect of LKB1 on Tumor Growth in Mice

As shown in Figure 5A and B, compared with Control and NC groups, the tumor growth rate in LKB1 and PD groups was significantly slowed, whereas in LKB1 + Ani group, the tumor growth rate was significantly accelerated ($p < 0.05$, respectively). Moreover, immunohistochemistry was used to detect the expression of Ki-67 in tumor tissue (Figure 5C). The Control and NC groups showed decreased percentage of Ki-67 positive cells when compared with Control and NC groups, while in the LKB1 +Ani group, the percentage of Ki-67 positive cells in the tumor tissue were significantly increased ($p < 0.05$, respectively). These results demonstrated that LKB1

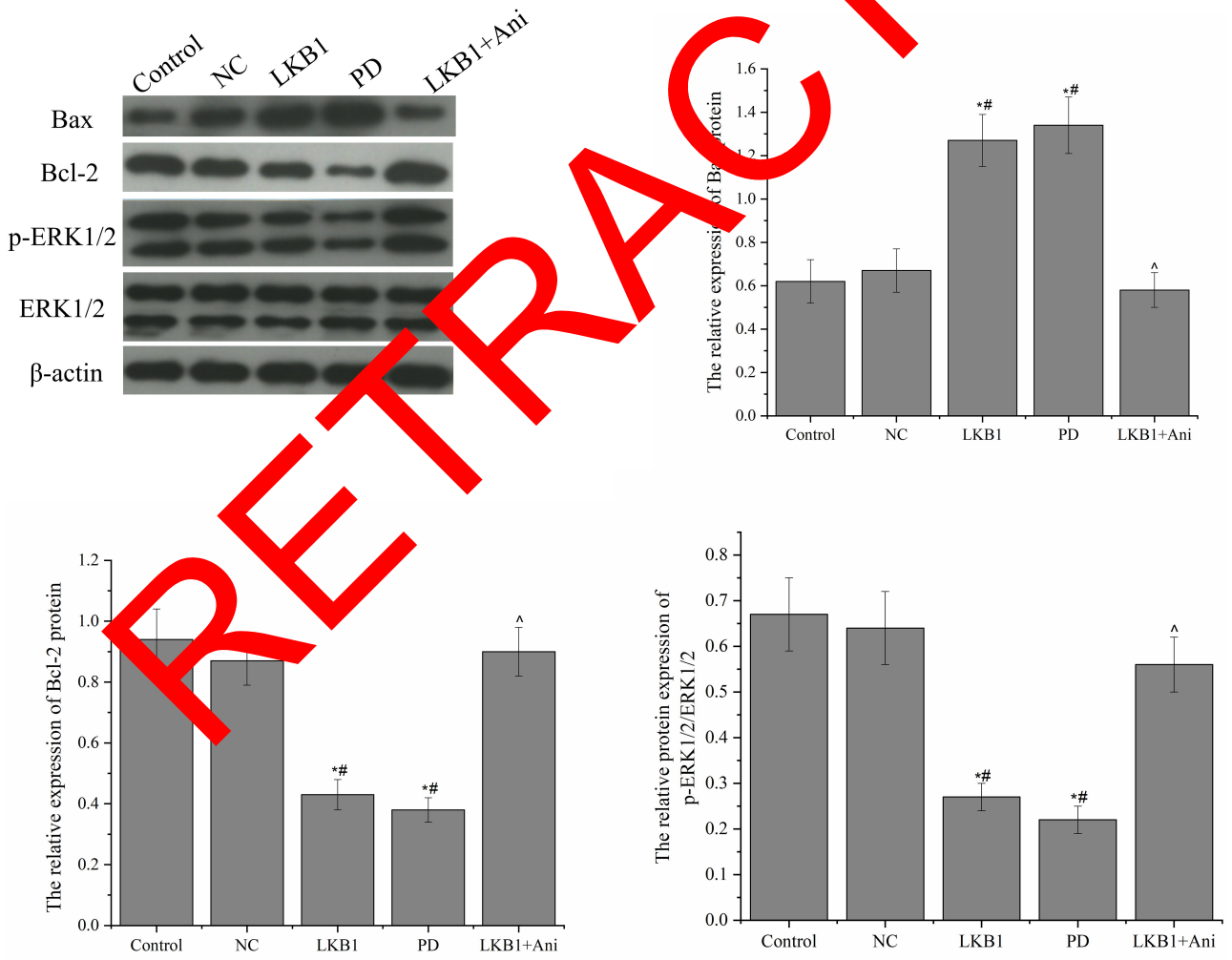

Figure 4 Effects of LKB1 on apoptosis and ERK signaling pathway related proteins in A549 cells. Values are mean ± SD. *p < 0.05 vs Control group, #p < 0.05 vs NC group, ^p < 0.05 vs LKB1 group.

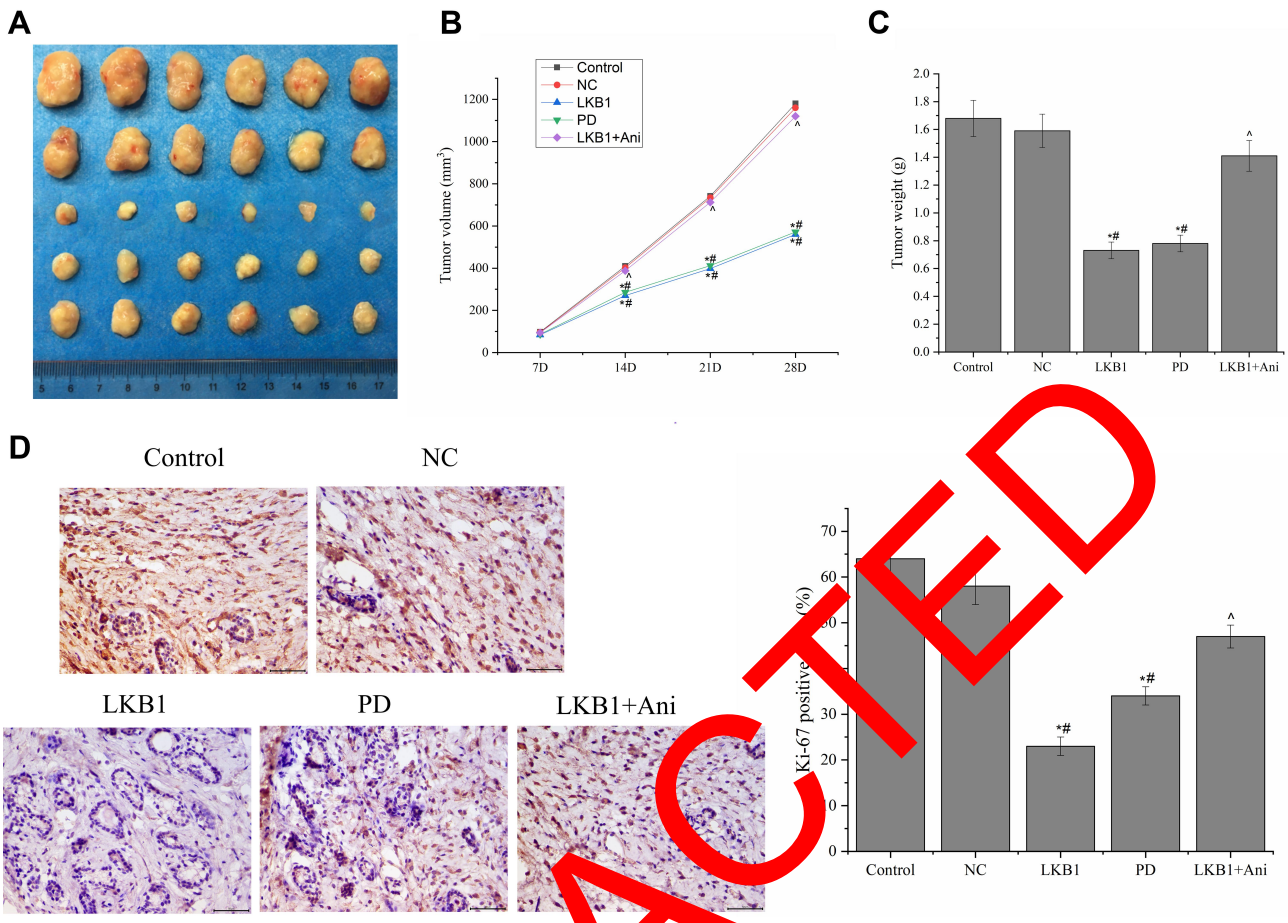

Figure 5 Effect of LKB1 on tumor growth in mice. (**A**) Tumor ...aph; (**B**) ...or volume; (**C**) Tumor weight; (**D**) Immunohistochemistry (×400). Values are mean ± SD. *p < 0.05 vs Control group, #p < 0.05 vs NC group, ^p < ...05 vs L...l group.

overexpression can inhibit tumor ...owth ...ugh targeting the ERK signaling pathway.

Effect of LKB1 o... Tumor A...optosis in Mice

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The Expression of ERK Signaling Pathway-Related Proteins in Mice Tumor Tissues

Compared with Control and NC groups, the protein expression of p-ERK1/2 in LKB1 and PD groups were significantly down-regulated, while p-ERK1/2/ERK1/2 was significantly increased in LKB1 + Ani group compared with that in LKB1 group (p < 0.05, respectively). The results indicated that LKB1 could inhibit the ERK signaling pathway (Figure 7).

Discussion

Due to smoking, air pollution, diet, genetics and other factors, lung cancer has become the most common cancer in the world.[1] With high mortality and poor prognosis, there is an urgent need to develop effective colon and lung treatments and methods. LKB1 is located at the position of human chromosome 19p 13.3, which is widely expressed in a variety of human tissues. Inactivating mutations of the tumor suppressor gene LKB1 are frequently detected in NSCLC and cervical carcinoma.[15] In NSCLC, the most frequent oncogenic mutation in western countries is KRAS.[16] KRAS-mutant cancers are the epitome of malignant tumors recalcitrant to targeted therapy efforts

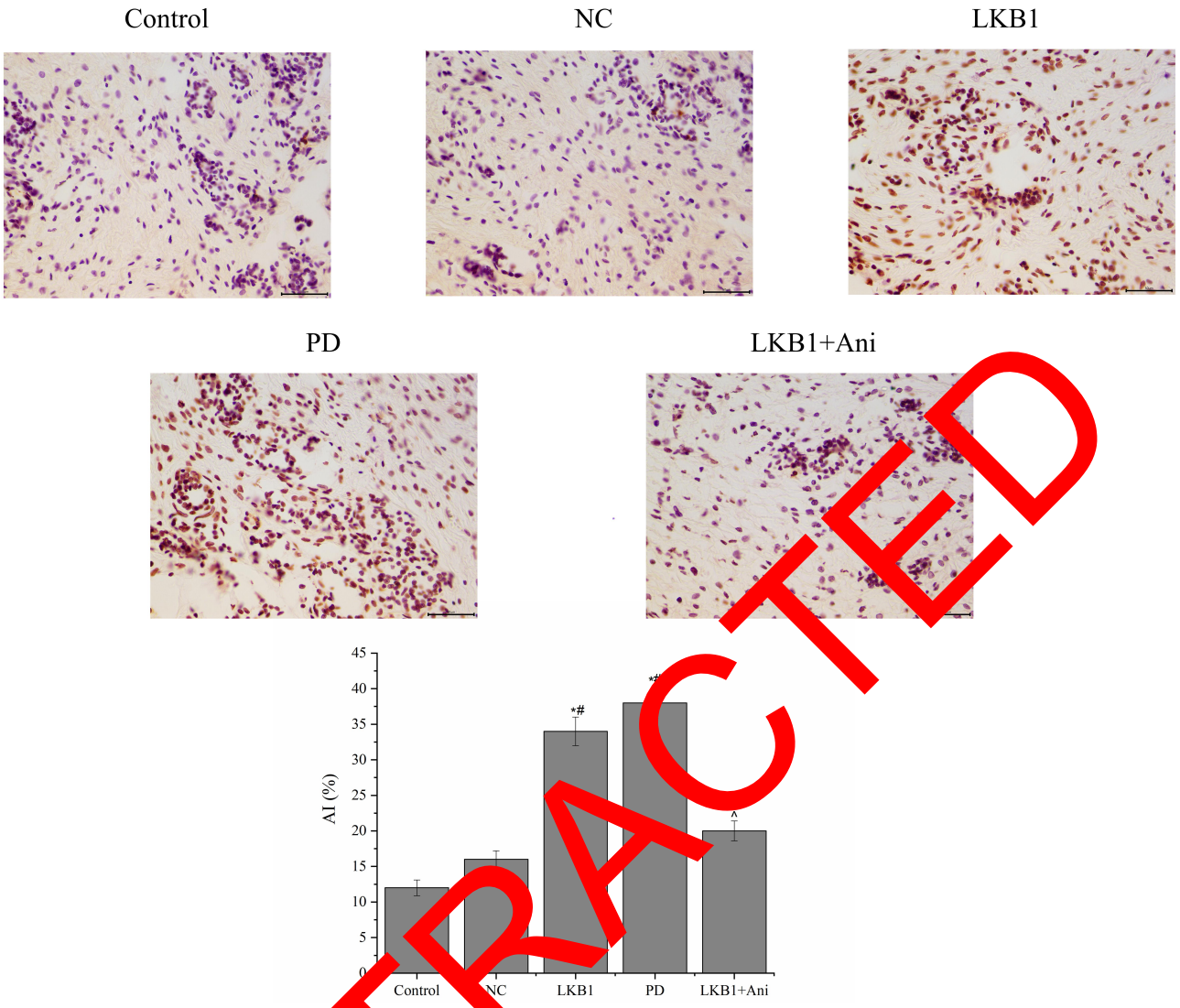

Figure 6 Effect of LKB1 on tumor apoptosis in mice. Values are mean ± SD. *p < 0.05 vs Control group, #p < 0.05 vs NC group, ^p < 0.05 vs LKB1 group.

and first-line chemotherapy.[17] In this study, LKB1 overexpression significantly inhibited the proliferation and invasion of A549 cells and induced apoptosis of A549 cells. Moreover, overexpression of LKB1 inhibited ERK1/2 phosphorylation levels to reduce the activity of ERK pathway. All the results verified that LKB1 overexpression inhibits NSCLC by inhibiting the ERK signaling pathway.

Cell proliferation is one of the important physiological functions of viable cells. However, when cells are affected by carcinogenic factors, they cannot complete cell differentiation normally. Instead, they become malignant, proliferating cells that continuously divide and are not controlled by organic matter.[18–20] The results from the CCK-8, transwell and immunohistochemistry assays indicated that LKB1 overexpression could inhibit the proliferation of tumor cells. Apoptosis is a basic biological phenomenon of cells that is required to maintain the stability of the intracellular environment.[21] Our study showed that LKB1 overexpression could increase the apoptotic index of A549 cells and tumor. The cell apoptosis always linked with genes under physiological and pathological conditions. Both Bcl-2 and Bax are Bcl-2 family members; Bcl-2 plays an important role in the maintenance of cell survival, while the main role of Bax is to accelerate apoptosis.[22] In the present study, the apoptosis-related proteins in A549 cells and tumor tissues were increased after LKB1 mimic interference.

Protein kinases (ERKs), including ERK1 and ERK2, are considered to be the convergence point of many

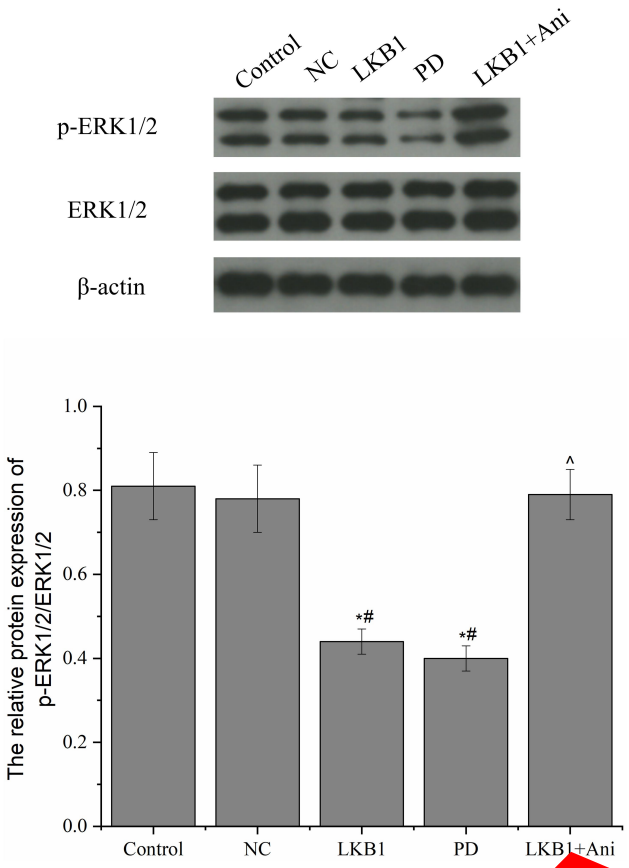

Figure 7 Effects of LKB1 on ERK signaling pathway related proteins in mice. Values are mean ± SD. *p < 0.05 vs Control group, #p < 0.05 vs NC group, ^p < 0.05 vs LKB1 group.

signaling pathways. The ERK pathway plays an important role in cell development, colonization, apoptosis, and malignant transformation.[23–25] Phosphorylated ERKs are transferred to the nucleus by the cytoplasm, which participates in various biological reactions.[26] The activation of ERKs by phosphorylation is a prerequisite for the function of the ERK pathway. Western blot indicated that the protein expression of p-ERK1 and p-ERK were decreased in LKB1 overexpression group. In order to further verify whether LKB1 affects the activity of lung cells by regulating the ERK signaling pathway, the ERK inhibitor PD98059 and LKB1 overexpression in combination with ERK activator LM22B-10 were used in the experiment. The results showed that the malignant biological behavior of A549 cells was inhibited and the phosphorylation level of ERK was significantly reduced after being treated with PD98059.

In sum, LKB1 played an anti-tumor role in lung cancer progression through inhibiting cell tumorigenicity and proliferation and invasion, and accelerating apoptosis. The possible mechanism is related to inhibition of the ERK signaling pathway. Therefore, the data provided novel evidence on the role of LKB1 as a new therapeutic target for lung cancer.

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Disclosure

The authors declared no conflicts of interest.

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