

## Original Article

# LKB1 regulates JAK2/STAT3 pathways involved in apoptosis of prostate cancer PC-3 cells

Xixi Huang<sup>1</sup>, Riyan Zhang<sup>2</sup>, Keming Wu<sup>1</sup>, Honghui Zhu<sup>1</sup>, Yue Pan<sup>1</sup>

<sup>1</sup>Department of Urology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China;

<sup>2</sup>School of Ophthalmology and Optometry, Wenzhou Medical University, Wenzhou, Zhejiang, China

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**Abstract:** Objective: The aim of this study was to investigate the effects of LKB1 on apoptosis of prostate cancer cells, examining its mechanisms. Methods: Prostate cancer PC-3 cells were transfected with LKB1 overexpression vectors (PC-3-LBK1 overexpression group) and negative controls (PC-3-vector group). Normal human prostate cells were used as control A group and untreated PC-3 cells were used as control B group. Relative expression levels of LKB1 genes were detected by quantitative real-time-PCR. Western blotting was conducted to detect relative protein expression levels of JAK2, STAT3, phosphorylated JAK2 (p-JAK2), and phosphorylated STAT3 (p-STAT3). Flow cytometry was conducted to detect apoptosis. Spearman's analysis was used to explore the correlation between relative expression levels of LKB1 genes and p-JAK2 and p-STAT3 proteins in PC-3 cells. Results: After PC-3 cells were transfected into LKB1 overexpression vectors, relative expression levels of LKB1 genes, LKB1 proteins, and p-JAK2 and p-STAT3 proteins in the PC-3-LBK1 overexpression group were significantly higher than those in the PC-3-vector group ( $P < 0.05$ ). An increase in LKB1 gene expression levels promoted PC-3 cell proliferation. Cell viability in the PC-3-vector group and PC-3-LBK1 overexpression group increased continuously over time ( $P < 0.05$ ). Cell viability in the PC-3-LBK1 overexpression group was lower than that in the PC-3-vector group. ( $P < 0.05$ ). An increase in LKB1 gene expression levels decreased apoptosis rates of PC-3 cells in the PC-3-vector group, normal human prostate cells group, and PC-3 cells group. Spearman's correlation analysis showed that LKB1, p-JAK2 ( $r = 0.984$ ,  $P < 0.001$ ), and p-STAT3 ( $r = 0.988$ ,  $P < 0.001$ ) were positively related. Conclusion: LKB1 may promote apoptosis of prostate cancer cells by activating JAK2/STAT3 signal pathways and inhibiting cancer, making this protein a potential target for treatment of prostate cancer.

**Keywords:** LKB1, JAK2, STAT3, prostate cancer, apoptosis

## Introduction

Prostate cancer is a malignant tumor that jeopardizes the life and health of men. Of all male reproductive system malignancies, prostate cancer shows the highest incidence. It is one of the leading causes of cancer-related deaths in men [1, 2]. In recent years, as the aging population has increased, incidence of prostate cancer has also increased [3]. Currently, the pathogenesis of prostate cancer is unclear. It is thought that the occurrence of prostate cancer is affected by complex and comprehensive factors, such as genes, environment, and sex hormones [4]. Early symptoms of prostate cancer are often not obvious, resulting in diagnosis at advanced stages. Current treatment methods, including surgery, radiotherapy, and chemo-

therapy, have limited curative effects of prostate cancer [5, 6]. Therefore, it is necessary to identify new therapeutic targets.

Gene therapy has received attention concerning cancer treatment and research. In recent years, the LKB1 gene has been reported to be closely related to tumors. Expression levels have been found to be decreased in patients with squamous cell lung carcinoma. Its deletion can lead to occurrence of squamous cell carcinoma [7, 8]. Previous studies have reported that LKB1 inhibits the growth of prostate cancer cells [9]. JAK2/STAT3 signal pathways are the most important target of downstream receptor cytokines, such as interleukins, chemokines, and growth factors. These include colony-stimulating factors that regulate biological

**Table 1.** Primer sequences

	Upstream	Downstream
LKB1	5'-TCTACAACATCACCAAGGGTC-3'	5'-TTCGTAAGCAAGCATCCCTTTC-3'
GAPDH	5'-CGGAGTCAACGGATTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

behaviors, such as cell proliferation, invasion, and migration. They also inhibit prostate cancer cell growth and proliferation [10, 11]. However, few studies have examined whether there is an interaction between LKB1 and JAK2/STAT3 signaling pathways.

Therefore, detecting relative expression levels of LKB1 in prostate cancer PC-3 cells, the current study evaluated its mechanisms of action, aiming to identify new therapeutic targets for prostate cancer.

## Material and methods

### Research subjects

Prostate cancer PC-3 cells (PC-3[PC3]) and human prostate cells (WPMY-1) were purchased from Shanghai Enzyme Biotech Co., Ltd. (Shanghai, China). They were cultured in the RPMI1640 medium (Shanghai Rongbai Biotechnology Co., Ltd., Shanghai, China) containing 10% calf serum (Shanghai Yiwen Biological Products Co., Ltd., Shanghai, China), with a pH of 7.2-7.4, temperature of 37°C, atmosphere of 5% CO<sub>2</sub>, and humidity of 70-80%.

### Cell transfection

LKB1 overexpression vectors (cat. no. ab-150-95) were designed and synthesized by Thermo Fisher Scientific (Waltham, MA, USA). The cell suspension concentration of trypsin-digested PC-3-cells was  $5 \times 10^6$ , while the transfection concentration was 50 nM. After 24 hours of incubation, the cells were incubated with cells containing the two vectors in the RPMI1640 medium (containing 10% calf serum) for 48 hours at 37°C and 5% CO<sub>2</sub>. Afterward, they were divided into the PC-3-LBK1 overexpression group and PC-3-vector group. Quantitative real-time (qRT)-PCR was used to evaluate transfection results. For transfection, Lipofectamine™ 2000 was purchased from Shanghai Yanhui Biotechnology Co., Ltd. (Shanghai, China). Human prostate cells served as the normal human prostate cells group, while untreated PC-3 cells served as the PC-3 cells group.

### qRT-PCR

Relative expression levels of LKB1 were detected by qRT-PCR. PC-3 cells were collected after 48 hours of transfection. Cells were lysed using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) to extract total RNA from the cells. DU800 UV spectrophotometer (Beckman, Brea, CA, USA) was used to analyze concentrations and purities of extracted total RNA. The A260/A280 value was between 1.8 and 2.1, indicating that the sample was pure. Next, 1% agarose gel electrophoresis (Shanghai Jingke Chemical Technology Co., Ltd., Shanghai, China) was used to analyze RNA integrity. The reverse transcription reaction contained the following: 1 µL oligo (dT) 12-18 Primer (50 µM), 1 µL dNTP mixture, 2 µL total RNA, and ribonuclease-free distilled water to 12 µL. Reaction conditions were: 40°C for 15 minutes and 85°C for 5 seconds. The reaction system included 5 × 4.0 µL of PrimeScript Buffer, 1.0 µL of PrimeScript RT Enzyme MIX I, 1.0 µL of RT Primer Mix, and 2.0 µg of total RNA. Ribonuclease-free distilled water was added to 20 µL. After synthesis of first-strand cDNA, the amplification reaction was performed. PCR amplification conditions were as follows: pre-denaturation at 95°C for 5 minutes, denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and final melting curve at 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. The reaction system contained the following: 1 µL cDNA template, 10 µL PCR master mix, 1 µL each upstream and downstream primers, and ribonuclease-free distilled water to 20 µL. GAPDH was used as the reaction internal reference. Each sample was tested in triplicate and results were analyzed using the 2<sup>-ΔΔCt</sup> method. The qRT-PCR detection kit was purchased from Thermo Fisher Scientific. Primer sequences are shown in **Table 1**.

### Western blotting

Western blotting was conducted to detect protein expression levels of LKB1, JAK2, STAT3, phosphorylated JAK2 (p-JAK2), phosphorylated

STAT3 (p-STAT3), and protein lysate components, using 294  $\mu$ L RIPA, 3  $\mu$ L PMSF protease inhibitor, and 3  $\mu$ L phosphatase inhibitor. Lysate was used to lyse and extract total proteins in the cells. BCA method was used to determine extracted protein concentrations. BCA protein quantification kit was purchased from Biyuntian Company (Shanghai, China). Next, 50  $\mu$ g of the extracted protein solution was subjected to SDS-PAGE, with  $\beta$ -actin serving as an internal reference. The gel was run at a constant voltage of 80 V for 40 minutes, followed by a constant voltage of 120 V for 90 minutes. Proteins were then transferred to a membrane at 100 V for 100 minutes, followed by blocking at 37°C for 60 minutes. The membrane was incubated with the primary antibody at 4°C for 16 hours. This was followed by washing with PBS three times for 10 minutes each time, the following day. The membrane was then incubated with the secondary antibody at 37°C for 60 minutes. Signals were developed using ECL luminescence reagent. Quantity One software was used to conduct statistical analysis on the strips after film scanning. Relative expression levels of the proteins were calculated as the stripe gray value divided by the internal reference gray value. Western blot detection kit was purchased from Biyuntian Company.

## MTT cell proliferation assay

Cells were collected in the log phase. Cell suspension concentrations were adjusted to  $1 \times 10^6$ /mL and cells were routinely inoculated in 96-well plates. Five collection time points were set, including 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours. When cells were cultured at the various time points, 20  $\mu$ L MTT (5 mg/mL) solution was added. The culture was further continued at 37°C for 4 hours. The supernatant containing impurities was removed and the dimethyl sulfoxide preparation was added. It was placed on a horizontal shaking table for 10 minutes. Finally, absorbance at 570 nm was measured on a light absorption microplate reader, VersaMax (Meigu Molecular Instruments (Shanghai) Co., Ltd.). MTT assay kit was purchased from Sigma-Aldrich, USA.

## Flow cytometry

After cells were treated with trypsin (0.25%)-EDTA, they were centrifuged at 1,000 rpm and centrifuged at 25°C for 5 minutes. Cells were labeled with Annexin V-FITC and 7AAD for 20

minutes. Fluorescence (DNA content) was measured using a standard software flow cytometer. Annexin V-FITC (+) and 7AAD (-) cells and Annexin V-FITC (-) and 7AAD (-) cells are considered early and late apoptotic cells, respectively. Three sets of parallel experiments were set and performed simultaneously.

## Statistical analysis

SPSS19.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Measurement data are expressed as percentages and rates were compared using the  $\chi^2$  test. Enumeration data are expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). Comparisons between groups and pairwise comparisons were performed using one-way ANOVA combined with post-hoc Bonferroni's test. Spearman's correlation was used to analyze the correlation between relative LKB1 expression in PC-3 cells and JAK2/STAT3.  $P < 0.05$  indicates statistical significance.

## Results

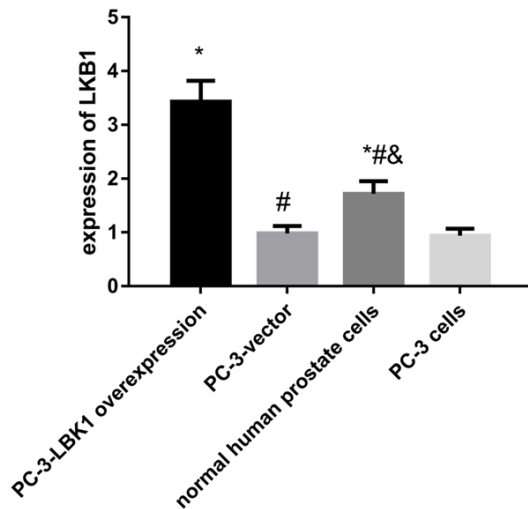
### qRT-PCR detection results

Results of qRT-PCR, measuring relative expression levels of LKB1 mRNA in the four groups, showed that relative expression levels of LKB1 in the PC-3-LBK1 overexpression group, PC-3-vector group, and normal human prostate cells group cells were  $3.43 \pm 0.39$ ,  $0.98 \pm 0.14$ , and  $1.72 \pm 0.23$ , respectively. Relative expression level of LKB1 in the PC-3 cells group was  $0.94 \pm 0.13$ . Relative expression levels of LKB1 in the four groups were significantly different ( $F = 67.430$ ,  $P < 0.001$ ). Relative expression levels of LKB1 in the PC-3-vector group ( $t = 12.212$ ,  $P < 0.001$ ;  $t = 3.688$ ,  $P = 0.006$ ) and PC-3 cells group ( $P < 0.001$ ;  $P = 0.005$ ) were lower than those in the PC-3-LBK1 overexpression group and normal human prostate cells group ( $P < 0.05$ ). Relative expression levels of LKB1 mRNA in the PC-3-LBK1 overexpression group were higher than those in the normal human prostate cells group ( $P < 0.05$ ). There were no significant differences in relative expression levels of LKB1 between the PC-3-vector group and PC-3 cells group ( $P = 0.847$ ) (**Figure 1**).

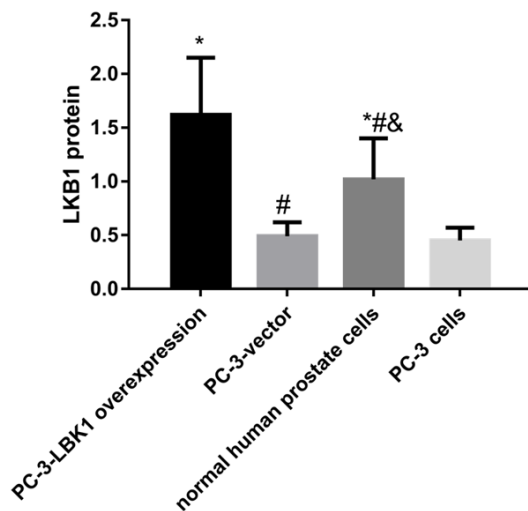
### Western blotting results

According to Western blotting results, relative expression levels of LKB1, JAK2, STAT3, p-JAK2,

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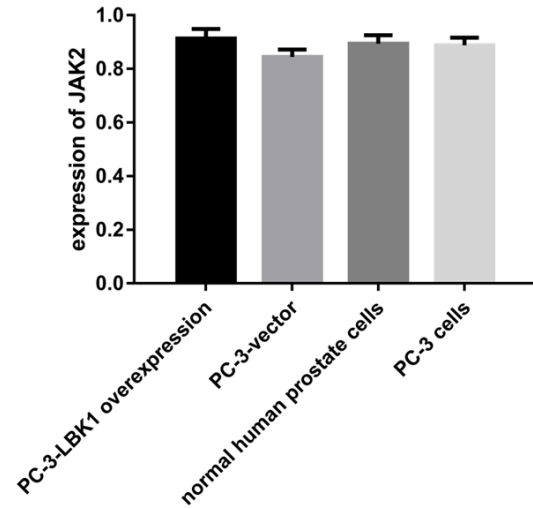


**Figure 1.** qRT-PCR detection of relative expression levels of LKB1. \* $P<0.05$  compared with the PC-3 cells group, # $P<0.05$  compared with PC-3-LBK1 overexpression group, and & $P<0.05$  compared with the PC-3-vector group.



**Figure 2.** Relative expression levels of LKB1 detected by Western blotting. \*indicates  $P<0.05$  compared to the PC-3 cells group, #indicates  $P<0.05$  compared to the PC-3-LBK1 overexpression group, and &indicates  $P<0.05$  compared to the PC-3-vector group.

and p-STAT3 proteins showed no significant differences in relative protein expression of JAK2 ( $F=2.625$ ,  $P=0.122$ ) and STAT3 ( $F=1.612$ ,  $P=0.262$ ) in the four groups ( $P>0.05$ ). Relative expression levels of LKB1 ( $F=7.913$ ,  $P=0.009$ ), p-JAK2 ( $F=72.530$ ,  $P<0.001$ ), and p-STAT3 ( $F=210.357$ ,  $P<0.001$ ) proteins were significantly different ( $P<0.05$ ). Relative expression levels of LKB1 proteins in PC-3-vectors ( $P=0.004$ ;  $P=$



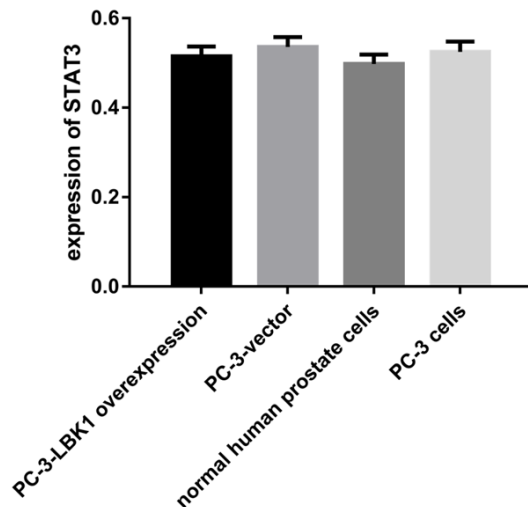
**Figure 3.** Western blot detection of JAK2 relative expression levels.

0.043) and PC-3 cells ( $P=0.036$ ) were significantly less than those in the PC-3-LBK1 overexpression group and normal human prostate cells group. Relative expression levels of p-JAK2 proteins in PC-3-vectors ( $P<0.001$ ;  $P<0.001$ ) and PC-3 cells ( $P<0.001$ ;  $P<0.001$ ) were significantly lower than those in the PC-3-LBK1 overexpression group and normal human prostate cells group. Relative expression levels of p-STAT3 proteins in PC-3-vectors (All  $P<0.001$ ) and PC-3 cells (All  $P<0.001$ ) were significantly lower than those in the PC-3-LBK1 overexpression group and normal human prostate cells group. Relative expression levels of LKB1 ( $P=0.038$ ), p-JAK2 ( $P=0.001$ ), and p-STAT3 ( $P<0.001$ ) in PC-3-LBK1 overexpression cells were significantly higher than those in the normal human prostate cells group. There were no statistical differences in relative expression levels of LKB1 ( $P=0.888$ ), p-JAK2 ( $P=0.363$ ), and p-STAT3 ( $P=0.085$ ) proteins in PC-3-vectors and PC-3 cells (**Figures 2-6**).

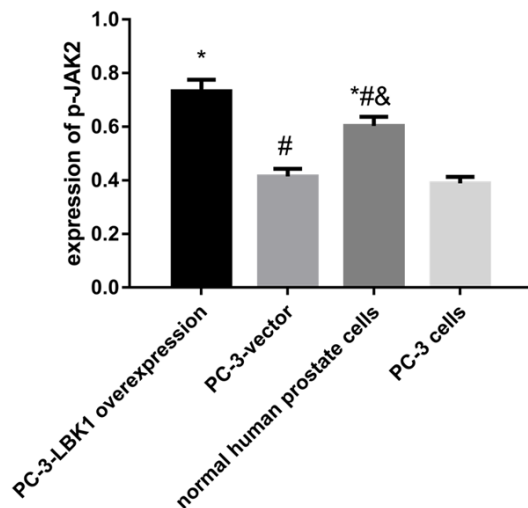
### MTT cell proliferation test results

Cell viability of PC-3-vector and PC-3-LBK1 overexpression cells groups increased with time and continued to increase ( $P<0.05$ ). Cell viability of PC-3-LBK1 in the overexpression cells group at 24 hours ( $P=0.043$ ), 48 hours ( $P=0.039$ ), 72 hours ( $P=0.034$ ), 96 hours ( $P=0.012$ ), and 120 hours ( $P=0.036$ ) was lower than that of the PC-3-vector cells group (**Figure 7**).

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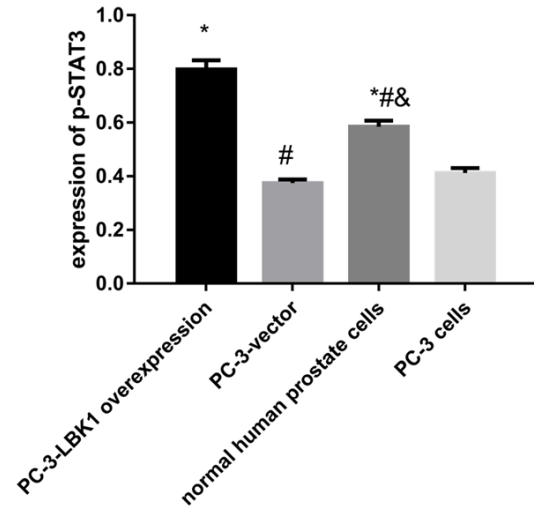
**Figure 4.** Western blot detection of STAT3 relative expression levels.



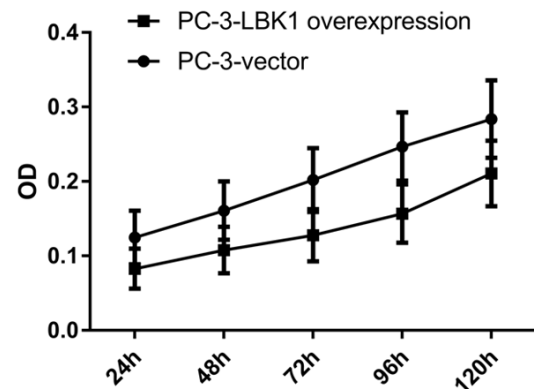
**Figure 5.** Western blot detection of p-JAK2 relative expression levels. \* $P < 0.05$  compared with the PC-3 cells group, # $P < 0.05$  compared with PC-3-LBK1 overexpression group, and & $P < 0.05$  compared with the PC-3-vector group.

### Apoptosis test results

Apoptosis rates were  $41.25 \pm 3.45\%$  in the PC-3-LBK1 overexpression group,  $11.59 \pm 1.36\%$  in the PC-3-vector group,  $23.67 \pm 1.58\%$  in the normal human prostate cells group, and  $10.33 \pm 1.24\%$  in the PC-3 cells group. Apoptosis rates of the four groups were statistically different ( $F = 138.889$ ,  $P < 0.001$ ). Apoptotic rates of PC-3-vectors ( $P < 0.001$ ), normal human prostate cells, and PC-3 cells were lower than those



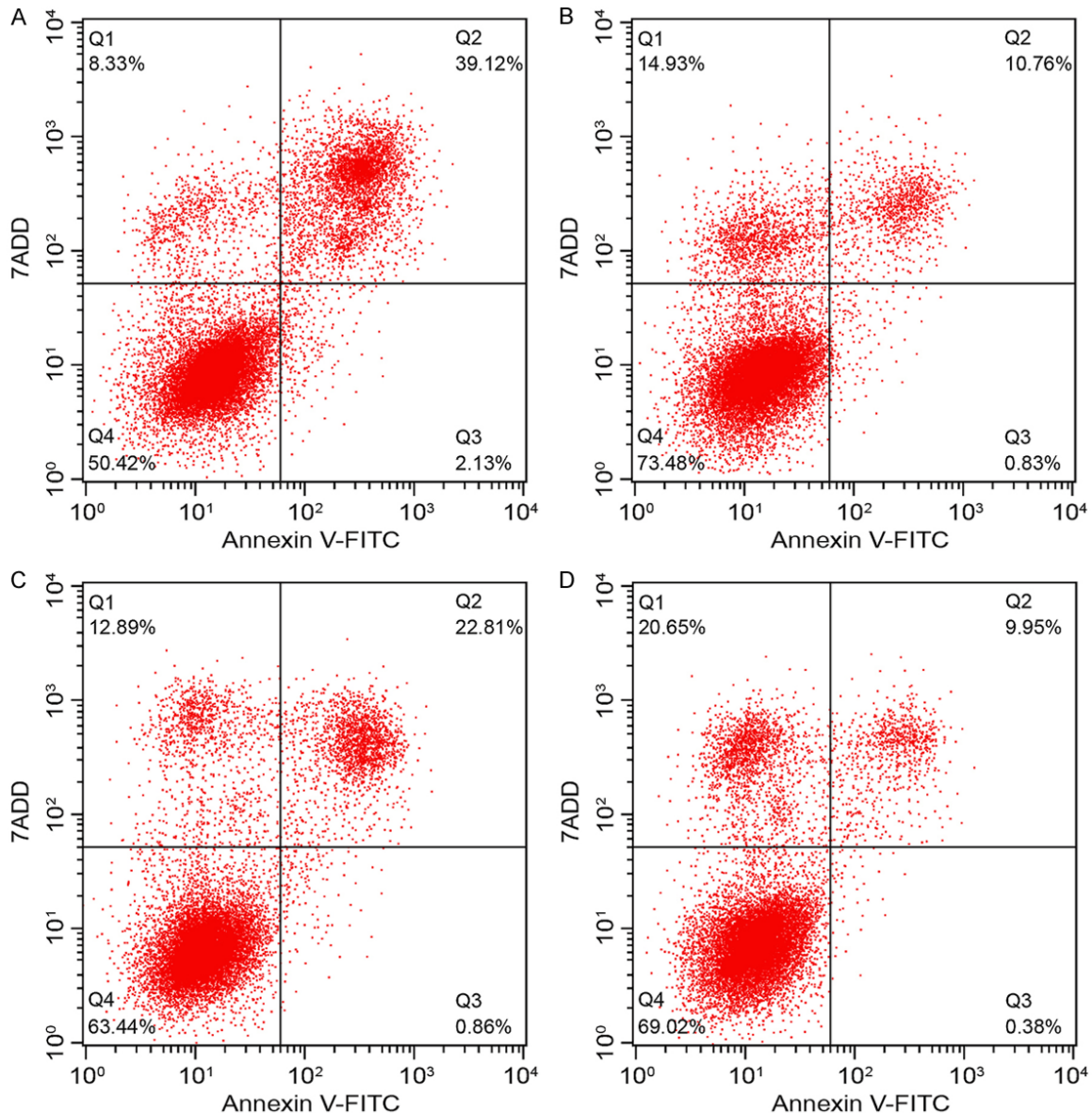
**Figure 6.** Western blot detection of p-STAT3 relative expression levels. \* $P < 0.05$  compared with the PC-3 cells group, # $P < 0.05$  compared with PC-3-LBK1 overexpression group, and & $P < 0.05$  compared with the PC-3-vector group.



**Figure 7.** MTT assay results of PC-3-vector and PC-3-LBK1 overexpression cell proliferation. Cell viability of PC-3-vector and PC-3-LBK1 overexpression cells increased continuously with time ( $P < 0.05$ ). Cell viability of the PC-3-LBK1 overexpression cells group was lower than that of the PC-3-vector cells group at each time point ( $P < 0.05$ ).

of PC-3-LBK1 overexpression (All  $P < 0.001$ ). Apoptotic rates of PC-3-vectors and PC-3 cells were significantly lower than those of normal human prostate cells ( $P < 0.001$ ). There were no significant differences in apoptotic rates between PC-3-vectors and PC-3 cells ( $P = 0.485$ ). The early apoptotic rate of the PC-3-LBK1 overexpression group was  $(13.13 \pm 0.58)\%$ , while the early apoptotic rate of the PC-3-vector group was  $(4.98 \pm 0.32)\%$ . The early apoptosis





**Figure 8.** Flow cytometry results of apoptosis in PC-3-LKB1 overexpression cells (A), PC-3-vector cells (B), normal human prostate cells (C), and PC-3 cells (D).

rate of the PC-3-LKB1 overexpression group was significantly higher than that of the PC-3-vector group ( $P < 0.001$ ) (**Figure 8**).

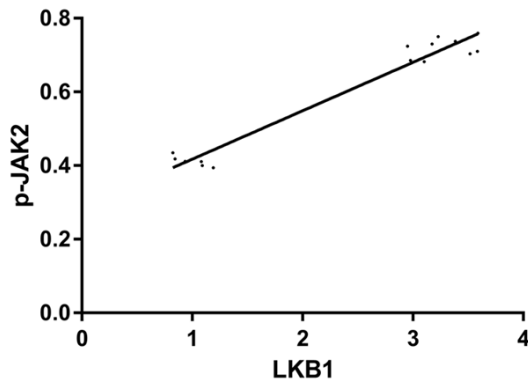
#### *Spearman's correlation analysis*

Based on relative expression levels of LKB1 in PC-3 cells and protein correlation analysis of p-JAK2 and p-STAT3, Spearman's correlation analysis indicated that LKB1 and p-JAK2 ( $r = 0.984$ ,  $P < 0.001$ ) and LKB1 and p-STAT3 ( $r = 0.988$ ,  $P < 0.001$ ) were positively related (**Figures 9 and 10**).

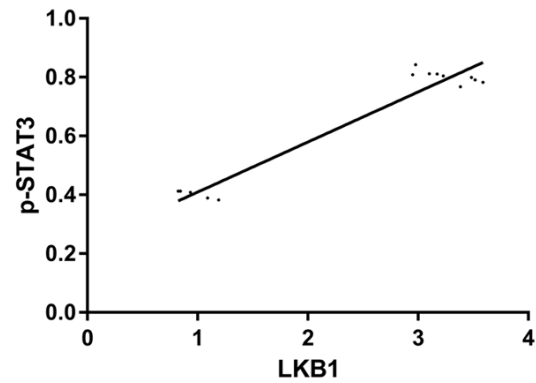
#### **Discussion**

Incidence of prostate cancer is particularly high, especially in Europe and the United States, with a mortality rate second only to lung cancer. As the worldwide population has aged, incidence of prostate cancer has increased each year [12, 13]. In recent years, the LKB1 gene has been reported to be related to certain biological behaviors, including tumor cell proliferation and invasion [14, 15]. However, its mechanisms of action remain unclear. JAK2/STAT3 signal pathways have been reported to

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**Figure 9.** Correlation analysis between relative expression levels of LKB1 and p-JAK2 proteins ( $r=0.984$ ,  $P<0.001$ ).



**Figure 10.** Correlation analysis between relative expression levels of LKB1 and p-STAT3 proteins ( $r=0.988$ ,  $P<0.001$ ).

regulate the occurrence and development of tumors. It has been reported that LKB1 and STAT3 have a mutual regulation relationship in breast cancer [16], but whether LKB1 is related to JAK2/STAT3 signal pathways requires further investigation. Therefore, this study examined expression levels of LKB1 genes in prostate cancer PC-3 cells, investigating the mechanisms of action.

This study first examined the relationship between LKB1 genes and JAK2/STAT3 signal pathways in untreated PC-3 cells and normal prostate cells. Results showed that relative expression levels of LKB1 proteins and p-JAK2 and p-STAT3 proteins in PC-3 cells were significantly lower than those in normal prostate cells. Moreover, there were no significant changes in JAK2 proteins and STAT3 proteins, as reported previously [1, 17]. Next, the current study established an LKB1 gene overexpression vector and negative control. Based on LKB1 gene expression of PC-3 cells in PC-3-LBK1 overexpression and PC-3-vector samples, as detected by qRT-PCR, relative expression levels of LKB1 genes in the PC-3-LBK1 overexpression group were significantly higher than those in untreated PC-3 cells. Negative control cells did not change significantly, indicating that establishment of the expression vector was successful. Further analysis of JAK2/STAT3 signal pathway-related proteins revealed no significant changes in JAK2 and STAT3 proteins between PC-3-LBK1 overexpression and PC-3-vector groups, while protein relative expression levels of LKB1, p-JAK2, and p-STAT3 significantly increased. Correlation an-

alysis showed that LKB1 gene, p-JAK2 protein, and p-STAT3 protein levels were positively correlated, indicating an interaction between LKB1 and JAK2/STAT3 signal pathways in prostate cancer cells. LKB1 may activate JAK2/STAT3 signal pathways. Linher-Melville et al. [18] also found that, in breast cancer cells, LKB1 can regulate STAT-mediated signal pathways involved in the invasion and inhibition of breast cancer cells, affecting the progression of breast cancer, supporting present results. LKB1 and JAK2/STAT3 signal pathways have tumor suppressor functions [19, 20]. Results of *in vitro* proliferation and apoptosis tests, in both the PC-3-LBK1 overexpression and PC-3-vector group, showed that upregulation of LKB1 gene expression levels can inhibit prostate cancer cell proliferation and promote early apoptosis of prostate cancer cells. Some studies have shown that LKB1 inhibits the growth of prostate cancer cells [9], but its mechanisms remain unclear. Based on current results, LKB1 activates JAK2/STAT3 signal pathways, promotes apoptosis of prostate cancer cells, and exhibits antitumor effects. Therefore, LKB1 is a potential target for treatment of prostate cancer. Two previous studies have reported that inhibition of LKB1 can increase the tolerance of prostate cancer to docetaxel [21, 22]. In another reported study concerning cancer treatment, LKB1 showed the ability to restore the sensitivity of non-small cell lung cancer cells to MEK inhibitors [23], which also validates the feasibility of using LKB1 as a target for prostate cancer therapy. However, there were some limitations to the current study. *In vitro* cell experiments cannot replicate complex and varied conditions

in the human body. Conditions used in this study were unable to validate that JAK2/STAT3 signal pathways are direct or indirect targets of LKB1. Additional clinical data are necessary to confirm present results. However, current results do provide a foundation for examining the roles and mechanisms of LKB1 in prostate cancer.

In summary, LKB1 may promote the apoptosis of prostate cancer cells. LKB1 plays an antitumor role by activating JAK2/STAT3 signal pathways, making it a potential target for treatment of prostate cancer.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Yue Pan, Department of Urology, The First Affiliated Hospital of Wenzhou Medical University, Shangcai Village, North Baixiang Street, Wenzhou 325000, Zhejiang, China. Tel: +86-0577-55578166; E-mail: yuepan@163.com

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