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Low *LKB1* Expression Results in Unfavorable Prognosis in Prostate Cancer Patients

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Statistical Analysis C
Data Interpretation D
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Background: The present study aimed to compare the expression of liver kinase B1 (*LKB1*) in prostate cancer (PCa) tissues and the paired adjacent tissues, then to evaluate the statistical relationship between *LKB1* expression and prognosis of PCa patients.

Material/Methods: The relative expression of *LKB1* at mRNA level was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The expression of *LKB1* at protein level was measured by immunohistochemistry (IHC) method. The relationship between *LKB1* expression and clinicopathologic characteristics was estimated by chi-square test. Kaplan-Meier method was used to analyze the overall survival of PCa patients with different *LKB1* expression. Cox regression analysis was performed to estimate the significance of *LKB1* expression and clinicopathologic characteristics in the prognosis of PCa patients.

Results: The relative expression of *LKB1* at mRNA level was significantly lower in PCa tissues than in the normal tissues ($P<0.001$). The *LKB1* expression was proved to be affected by clinical stage ($P=0.019$) and PSA concentration ($P=0.031$) of PCa patients. Moreover, patients with negative *LKB1* expression had shorter survival than those with positive expression. Cox regression analysis confirmed that *LKB1* could be regarded as a prognostic biomarker for PCa patients ($P=0.001$, HR=3.981, 95% CI=1.698–9.336).

Conclusions: The expression of *LKB1* was lower in PCa tissues and might be a predictor for the prognosis of PCa patients.

MeSH Keywords: Prognosis • Prostatic Neoplasms • Vascular Endothelial Growth Factor Receptor-2

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Background

Prostate cancer (PCa) is one of the most common cancers among men over 50 years old and is the sixth leading cause of cancer-related deaths world-wide [1,2]. Although its incidence is higher in developed countries than in developing counties, it is still increasing in developing areas [3–5]. There are obvious regional and ethic differences in the pathogenesy of PCa. Currently, the diagnosis of PCa is mainly based on the combination of various procedures, and PCa is usually diagnosed as a localized disease [6,7]. Treatments for PCa predominantly include surgical castration, androgen-deprivation therapy (ADT), and radiation therapy (RT) [8,9]. However, these treatments have shortcomings and there is no effective strategy to treat metastasis and recurrence of Pca that cannot be treated by surgery or radiation therapy. Therefore, an innovative biomarker for therapies and prognosis of PCa patients is urgently needed.

Liver kinase B1 (*LKB1*), a serine/threonine kinase, is located at 19p 13.3 of human chromosomes [10]. It is known as a tumor suppressor and many studies have demonstrated that *LKB1* plays an important role in regulating energy homeostasis, cell cycle progression, cell polarity, cell proliferation, senescence, DNA damage response, and differentiation [11–13]. It is also mutated in Peutz-Jeghers syndrome (PJS), which leads to an increasing risk of malignant tumors in multiple tissues [14]. In previous studies, *LKB1* was reported to be a common mutated gene in various of cancers such as non-small cell lung carcinomas, cervical cancer, breast cancer, and pancreatic carcinoma [15–18]. It was also confirmed that *LKB1* was related with prostate neoplasia and could suppress proliferation and invasion of PCa [19,20]. However, its role in the prognosis of PCa had never been determined.

This study aimed to detect the *LKB1* expression in PCa tissues and the paired adjacent normal tissues both at mRNA level and protein level. Then we attempted to further explore whether *LKB1* could serve as a prognostic factor for PCa patients, so as to understand the PCa progression, provide an efficient therapy method of Pca, and increase the survival rate of PCa patients.

Material and Methods

Patients and tissues specimens

Our study included 109 patients with PCa diagnosed at the Department of Urology Surgery of The Affiliated Hospital of Jining Medical College. None of them had ever received any chemical treatment or physical therapy before surgery. The present study was approved by the Ethics Committee of The Affiliated Hospital of Jining Medical College. All participants provided signed informed written consent in advance.

The tumor tissues and the paired adjacent tissues were collected from PCa patients. All the specimens were biopsy materials and frozen in liquid nitrogen immediately, then the samples were stored at –80°C for RNA extraction. A follow-up of 60 months was conducted. The overall survival time was defined as the time from day of surgery to the day of death. The follow-up information was obtained via a telephone or questionnaire and was updated every 2 months. Patients who died from other disease or accident were excluded from our study.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from fresh PCa tissues and the paired adjacent tissues were extracted and purified using RNeasy Mini Kit (QIAGEN) according to the manufacturer's directions. Reverse transcription was performed with a ReverTra Ace qPCR RT Kit (Toyobo Bio-Technology, Japan) according to the manufacturer's instructions. qRT-PCR reaction was carried out in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA). The GAPDH was used as endogenous control. The relative expression of *LKB1* at mRNA level normalized to GAPDH was evaluated by comparative cycle threshold (CT) method. All the experiments were conducted under optimal conditions and in triplicate.

Immunohistochemistry assay

Immunohistochemistry (IHC) was used to examine the expression of *LKB1* at the protein level in all tissue samples. The tumor tissues and adjacent tissues were fixed in 10% formaldehyde and embedded in paraffin. Then the paraffin sections were cut into 4-μm sections, and were dewaxed and rehydrated with xylene and graded alcohol, respectively. The sections were washed with buffer solution for 5 min and then added into the primary antibody at 4°C overnight. The second antibody was added into the sections after being washed again. Finally, coloration was performed with DAB. The results are presented as the percentage of the staining cells (0 to 100%) in tissues. Staining under 20% of the tissue cells or no staining was included in the negative group (–), while the others belonged to the positive group (+).

Statistical analysis

All data processing was carried out using SPSS 18.0 software. The difference in *LKB1* expression between PCa tissues and adjacent tissues was analyzed by *t* test. The chi-square test was used to analyze the relationship between *LKB1* expression and clinicopathological characteristics. The association

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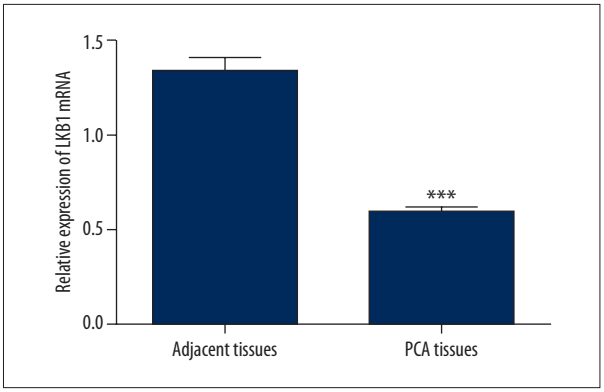

Figure 1. Relative expression of *LKB1* at mRNA level was assessed by qRT-PCR in PCa tissues and adjacent normal tissues. The *LKB1* expression was normalized to GAPDH. Expression level of *LKB1* was significantly lower in PCa tissues compared to the adjacent normal tissues ($P<0.001$).

between *LKB1* expression and overall survival, as well as the prognostic value of *LKB1,* were estimated by Kaplan-Meier and Cox regression analysis, respectively. $P<0.05$ was considered to be statistically significant.

Results

Low expression of LKB1 at mRNA level in PCa tissues

QRT-PCR was used to evaluate the expression of *LKB1* in PCa tissues and the adjacent tissues. The expression level of *LKB1* was normalized to GAPDH. The result demonstrated that the relative expression of *LKB1* at mRNA level in PCa tissues was 0.59±0.25 (mean±SD), while that in the normal tissues was 1.32±0.59 (mean ±SD). A significant decrease in the expression of *LKB1* at the mRNA level was found in PCa tissues (Figure 1, $P<0.001$).

Decreased expression of LKB1 at protein level in PCa tissues

The *LKB1* protein expression of all PCa tissues and a randomly selected 70 cases of adjacent tissues were assayed by IHC method. Figure 2 shows that staining degree was obviously diminished in PCa tissues compared with adjacent tissues. To obtain an exact result of protein expression level of the *LKB1* gene in PCa patients, we analyzed the positive cell percentage

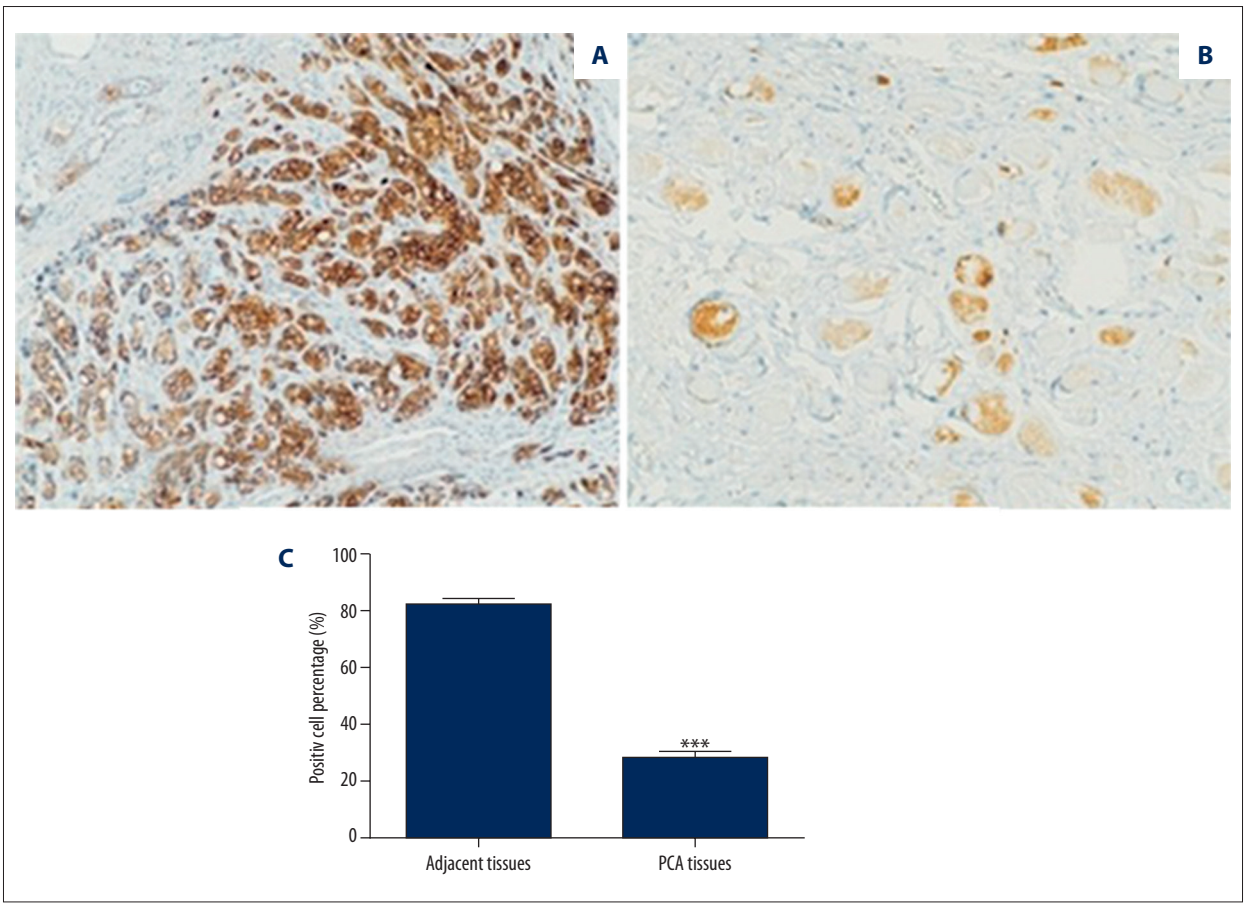

Figure 2. The expression of *LKB1* protein in PCa tissues and adjacent tissues. The *LKB1* protein expression level was lower in PCa tissues than in adjacent tissues ($P<0.001$). (**A**) Adjacent tissue; (**B**) PCa tissue; (**C**) Positive cell percentage.

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Table 1. The relationship between *LKB1* expression and clinicopathological characteristics of PCa patients.

Clinical features	Case (n)	*LKB1* expression		χ^2	*P*
		Negative (n)	Positive (n)		
Age				0.714	0.398
≤55	39	26	13		
>55	70	52	18		
Hematuria				0.265	0.607
Yes	57	42	15		
No	52	36	16		
Urine retention				2.212	0.137
Yes	58	45	13		
No	51	33	18		
Creatinine (μmol/L)				4.469	0.035
≤110	46	28	18		
>110	63	50	13		
Clinical staging				5.468	0.019
T_1+T_2	51	31	20		
T_3+T_4	58	47	11		
PSA (ng/ml)				4.645	0.031
≤6	56	35	21		
>6	53	43	10		

in the 2 tissues. We found that positive cell percentage was 28.4% in PCa tissues and 81.4% in normal tissues. Both the staining degree and positive cell percentage indicated that the *LKB1* protein expression in PCa tissues was significantly lower than that in the normal tissues (Figure 2, $P<0.001$).

Correlation between LKB1 expression and clinicopathological characteristics

The association between *LKB1* expression and clinicopathological characteristics, including age, hematuria, urine retention, creatinine (μmol/L), clinical staging, and PSA (ng/ml), were evaluated to determine whether *LKB1* participates in the development of PCa. The results showed that creatinine level ($P=0.035$), advanced clinical stage ($P=0.019$), and high concentration of PSA ($P=0.031$) were all related to low *LKB1* expression (Table 1). However, no clinical relevance was observed between *LKB1* and age, hematuria, or urine retention (Table 1, $P>0.05$).

Association between LKB1 expression and overall survival of PCa patients

During the follow-up, 47 of 78 (60.3%) patients with negative *LKB1* expression died, whereas only 7 (22.6%) patients with positive *LKB1* expression died. Kaplan-Meier analysis exhibited

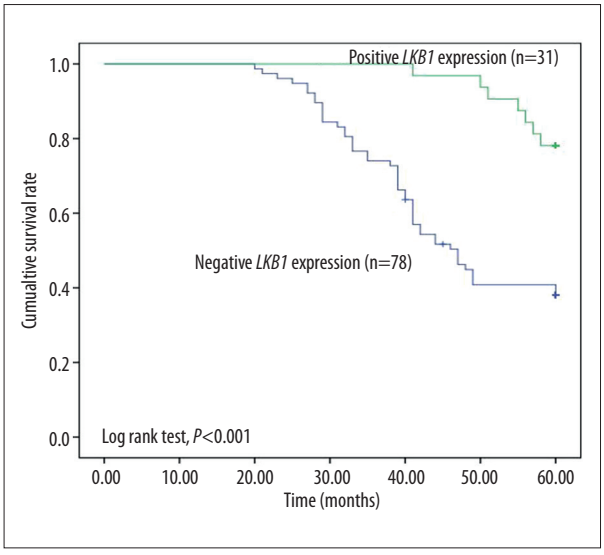

Figure 3. Kaplan-Meier analysis with the expression of *LKB1* showed that patients with negative expression of *LKB1* had a significantly shorter overall survival time than those with positive *LKB1* expression (log-rank test, $P<0.001$).

that patients with negative *LKB1* expression had significantly lower overall survival than those with positive *LKB1* expression (Figure 3, log rank test, $P<0.001$). Multivariate Cox

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Table 2. Multivariate analysis for prognostic factors in prostate cancer via cox regression analysis.

Variable	*P* value	HR	95%CI
Hematuria	0.188	0.659	0.354–1.227
Urine retention	0.155	0.655	0.365–1.173
Clinical staging	0.442	1.255	0.703–2.240
LKB1 expression	0.001	3.981	1.698–9.336

regression analysis showed that clinical features had no significant relationship with the prognosis of PCa, but *LKB1* expression (*P*=0.001, HR=3.981, 95%CI=1.698–9.336) was associated with the prognosis of PCa patients (Table 2). Therefore, we inferred that these clinical features could not act as markers for PCa prognosis, but *LKB1* might be a novel indicator for the prognosis of PCa patients.

Discussion

PCa is a malignant tumor that presents in the prostatic tissues of the males and is the result of disordered growth of prostatic vesicle cells. Up to now, pathogens that induce PCa are still indefinite, which may be associated with the alteration of gene, such as the change of androgen receptor relative genes. It is usually a fatal disease for most patients diagnosed in advanced stages. Therefore, it is of great significance to explore effective diagnostic and prognostic markers for PCa.

Several molecular markers have been investigated in PCa tissues as predictive biomarkers [21–26]. For example, Rajal et al. reported that ERG was overexpression in PCa and Xu et al. also verified that ERG played a prognostic role in prostatic acinar adenocarcinoma [27,28]. Zheng et al. demonstrated that *SFRP1* could be a prognostic biomarker in PCa patients [29]. In addition, *LKB1* is a tumor suppressor gene with a molecular weight of 50 KD. It contains 10 exons and consists of kinase domain, N terminal regulatory domain, and C terminal regulatory domain. It has been studied in various diseases and was confirmed to play crucial roles in different cell processes. For instance, in the study of Inge et al. the inactivation of *LKB1* could sensitize NSCLC to pharmacological aggravation of ER stress, and another report by Inge et al. found that *LKB1* expression in NSCLC determined the sensitivity to 2-deoxyglucose [30,31]. Moreover, many studies suggested that the *LKB1* gene may have a role in PCa, including the precursor lesions, as well as proliferation and invasion of PCa [19,20,32]. However, the association of the *LKB1* gene with PCa development remains unclear. In this study, we detected the expression of *LKB1* in PCa tissues and adjacent normal tissues both at mRNA level and protein level. Our study results demonstrate that *LKB1* expression was reduced in PCa tissues and it might be a tumor suppressor in PCa. This result was consistent with the trend in lung cancer [33].

As *LKB1* expression was linked with various cancers, thus it might be a potential prognostic marker. A previous study found that loss of *LKB1* protein expression may be useful as a prognostic marker for breast carcinoma [34]. To clarify whether *LKB1* had been involved in the development of PCa and its prognostic value in PCa patients, we analyzed the relationship between *LKB1* expression and clinicopathologic characteristics as well as the overall survival of patients. It was shown that the *LKB1* expression was related to clinical staging and PSA concentration tightly. In addition, Kaplan-Meier analysis shoed that the overall survival time of patients with low *LKB1* expression was shorter than in those with high *LKB1* expression. Cox regression analysis determined that there was a statistically significant relationship between *LKB1* expression and the prognosis of PCa patients, indicating that *LKB1* could be a prognostic biomarker for PCa patients.

As with other tumor suppressor genes, it is also difficult to identify the patients with or without low *LKB1* expression, and potential mechanisms of *LKB1* in various tumors are still unknown. Many studies have demonstrated that *LKB1* functions in various cancers through the *LKB1/AMPK* signaling pathway [35–37]. Young-Ok Son et al. illustrated that cadmium induced autophagy through *LKB1- AMPK* signaling in skin epidermal cells [38]. Brown et al. showed that *LKB1* expression was inhibited by estradiol-17β in MCF-7 cells [39]. Therefore, we presumed that the effect of *LKB1* on PCa might be related to the *LKB1/AMPK* signaling pathway or estradiol-17β approach, but this hypothesis must be verified in further research.

Conclusions

In conclusion, *LKB1* is a tumor suppressor in PCa via its decreased expression in PCa tissues. Statistical significance was found between *LKB1* expression and the prognosis of PCa patients, suggesting that *LKB1* may be a candidate prognostic marker for PCa patients.

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