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LKB1 dictates sensitivity to immunotherapy through Skp2-mediated ubiquitination of immune checkpoint proteins in HCC with python analysis

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ABSTRACT

Objective(s): Hepatocellular carcinoma (HCC) is a highly aggressive malignancy with limited treatment options, particularly in advanced stages. Immune checkpoint inhibitors (ICIs) targeting PD-1, PD-L1, and CTLA-4 have shown promise in cancer immunotherapy, but response rates in HCC remain variable.

Materials and Methods: Hep3B and HepG2 HCC cells were cultured and genetically manipulated to overexpress or deplete LKB1. Western blotting, real-time PCR, and immunofluorescence were used to assess PD-L1 expression at the protein and mRNA levels. The role of Skp2 in PD-L1 regulation was evaluated through shRNA-mediated knockdown and overexpression. Additionally, kinase-dead LKB1 mutants were expressed to determine the importance of LKB1 kinase activity in PD-L1 stability. ImageJ software and Python-based computational tools were employed for quantitative analysis of immunofluorescence and Western blot data.

Results: LKB1 overexpression up-regulated PD-L1 protein levels in HCC cells, while its depletion reduced PD-L1 expression, indicating a post-translational regulatory mechanism. Although Skp2 expression remained unchanged upon LKB1 modulation, Skp2 overexpression in LKB1-deficient cells increased PD-L1 levels, suggesting a context-dependent role for Skp2 in PD-L1 stability. Furthermore, wild-type LKB1, but not the kinase-dead mutant, restored PD-L1 expression, highlighting the essential role of LKB1 kinase activity in PD-L1 regulation.

Conclusion: This study identifies LKB1 as a critical regulator of PD-L1 stability in HCC, with implications for tumor immune evasion and immunotherapy response. While Skp2 appears to influence PD-L1 stability in specific contexts, LKB1's kinase activity is essential for PD-L1 regulation.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most challenging malignancies in oncology, characterized by its aggressive nature (1) and poor prognosis (2). As the most common form of liver cancer, HCC imposes a significant global health burden, with limited therapeutic options available for advanced stages (3). In recent years, immunotherapy has become a revolutionary strategy in cancer treatment, harnessing the body's immune system to identify and destroy cancer cells (4). Immune checkpoint inhibitors (ICIs), targeting molecules such as PD-1, PD-L1, and CTLA-4, have demonstrated remarkable success in various cancers, including HCC (5). However, the clinical response to immunotherapy in HCC remains highly variable, with a substantial proportion of patients exhibiting primary or acquired resistance (6). This heterogeneity

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Liver Kinase B1 (LKB1), a serine/threonine kinase and well-established tumor suppressor, plays a pivotal role in regulating cellular metabolism, polarity, and proliferation (7). Loss of LKB1 function has been frequently reported in various cancers, including HCC, and is associated with increased tumor aggressiveness and poor clinical outcomes (8). While LKB1's role in tumorigenesis has been extensively studied, its influence on immune regulation, particularly in the context of immunotherapy, remains poorly understood. Emerging evidence suggests that LKB1 may modulate the ubiquitination and degradation of immune checkpoint proteins, such as PD-1, PD-L1, and CTLA-4, through pathways involving S-phase kinase-associated protein 2 (Skp2) (9). Skp2, an E3 ubiquitin ligase, is known to target a wide range of substrates for ubiquitination, thereby influencing their stability and function. However, the precise mechanisms by which LKB1 and Skp2 interact to regulate immune checkpoint proteins in HCC remain largely unexplored. Skp2 promotes the cell cycle in cancer cells. The control of cell cycle stability is crucial for cancer prevention. Several molecules play a role in regulating the cell cycle, such as cell cycle proteins, cyclin-dependent kinases (CDKs), and ubiquitin ligases, among others (10). LKB1-AMPK signaling pathway plays a significant role in the regulation of cellular metabolism, proliferation and survival in cancer. To construct a LKB1-AMPK signaling-related gene signature (LRS), an ensemble of ten machine learning algorithms was applied across four datasets. Several indicators were employed to assess the effectiveness of LRS in forecasting immunological responses (10).

Despite growing interest in LKB1's role in cancer biology, its contribution to immune checkpoint regulation in HCC has not been systematically investigated. In particular, the involvement of Skp2-mediated signaling in modulating immunotherapy sensitivity in HCC remains an underexplored area. Understanding the interplay between LKB1 and Skp2 in immune checkpoint regulation could provide critical insights into the molecular determinants of immunotherapy response and resistance.

In this study, we aim to investigate the role of LKB1 in dictating immunotherapy sensitivity in HCC through Skp2-mediated ubiquitination of immune checkpoint proteins. We hypothesize that LKB1 deficiency enhances the ubiquitination and degradation of immune checkpoint proteins via Skp2, thereby influencing tumor immune evasion and response to immunotherapy but the sensitivity is assumed to be more important.

The findings of this research have the potential to significantly advance the field of oncology by providing novel insights into the molecular mechanisms governing immunotherapy sensitivity in HCC Furthermore, targeting the Skp2-mediated ubiquitination pathway may offer new therapeutic avenues for enhancing the efficacy of existing immunotherapies in HCC. This study aspires to contribute to the development of more effective and tailored immunotherapeutic approaches, ultimately improving patient outcomes and addressing the unmet clinical needs in HCC treatment.

Materials and Methods

Cell culture

Hep3B and HepG2 cells were purchased from the Pasteur Institute of Iran and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin / streptomycin (pen/strep) The cells were incubated in 37 °C and under CO_2 percentage (5%) The 2x106 cells where passaged. Human HA-LKB1 plasmids in the pCMV3 vector were obtained from Sino-Biological. All plasmids used in the study were confirmed by sequencing. The cells transiently transfected. Cells transfected with the empty pCMV3 vector were used as controls in all overexpression experiments. All experiments were performed using transient transfection. Stable cell lines were not generated for this study. All assays (western blotting, qRT PCR, and immunofluorescence) were performed 48 hr post transfection.

Western blotting

Cells were harvested and centrifuged following treatment with Human HA-LKB1. They were then lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails. Protein concentrations were measured using a BCA assay kit (Thermo Scientific). Approximately 30 µg of protein was resolved using SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% non-fat milk and incubated with the primary antibody overnight at 4 °C. The following day, the membrane was washed with TBST buffer and incubated with an HRP-conjugated secondary antibody (rabbit IgG) for two hours at room temperature. Protein bands were detected using a chemiluminescence detection system (4). B-actin were used as a control.

Real-time PCR

Total RNA was extracted from cells using TRIzol and washed with RNase-free water. The RNA was then reverse-transcribed into cDNA using a reverse transcription kit. The expression level of PD-L1 mRNA was determined by qRTPCR amplification using SYBR Green-based real-time PCR with specific primers (Table 1) and optimized thermal cycling conditions (3). The B-actin was used as a control.

Immunofluorescence (IF)

Cells were treated with an anti-PD-L1 antibody and incubated for two hours at room temperature. Following a washing step, a PE-labeled secondary antibody (PE-labeled anti-human IgG) was introduced and incubated for one hour. Slides were examined and analyzed using an Olympus fluorescence microscope and ImageJ software (11). Performing at least three independent biological replicates.

Table 1. The forward and reverse primers list for real time PCR

LKB1	F	3" GGACCGAAACCTTGATCCC5"
	R	3" AAACCCTTAGGATCCAAGT5"
PDL1	F	3" GGGAAACTTAGGCCCTCCC5"
	R	3" AAATCCACTTATTTTGAGT5"
SKP2	F	3" GGATCGGGAAATTGATCCC5"
	R	3" AAACCCTTACAAGCCCTTT5"

LKB1: Liver Kinase B1; SKP2: S-phase kinase-associated protein2; SKP2: S-phase kinase-associated protein; PDL-1: Programmed death-ligand 1

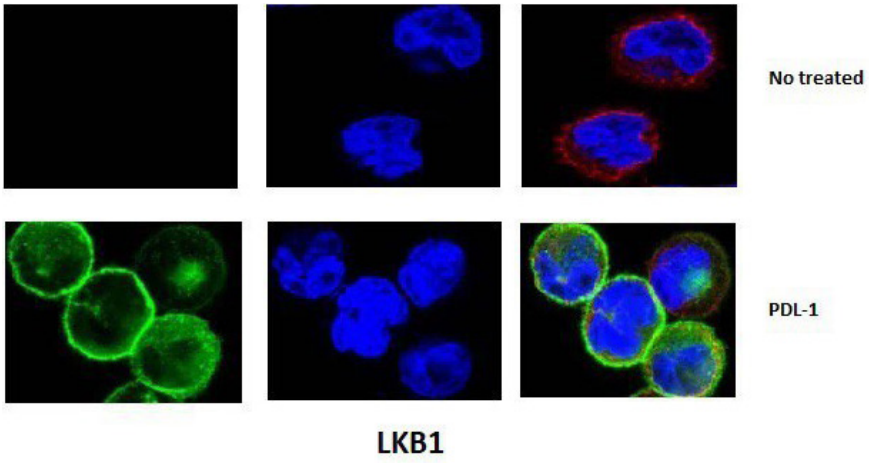

Figure 1. Immunofluorescence images of HCC cells revealed that PD-L1 levels were absent in the untreated LKB1 group, whereas PD-L1 expression increased significantly in the LKB1-treated group
LKB1: Liver Kinase B1; PDL-1: Programmed death-ligand 1

(The antibodies were obtained from Invivogen).

In this study, the Python programming language (version 3.13.0 IDLE) was utilized for the analysis of western blot images. (12) libraries were employed. Following the quantification of band intensities, the data were stored in a CSV file using Microsoft Office Excel 2021. Subsequently, the data were re-analyzed using the NumPy, pandas, and matplotlib libraries to generate the study›s graphical representations. The Python codes used in this analysis are available upon request by contacting the corresponding author via email.

Results

Investigate the effect of LKB1, by first evaluating the expression of PD-L1. Immunofluorescence images of HCC cells revealed that PD-L1 levels were absent in the untreated LKB1 group, whereas PD-L1 expression up-regulate in the LKB1-treated group. Notably, tumors lacking LKB1 showed no T-cell infiltration. These findings suggest that LKB1 acts as a potential biomarker for tumors. In tumor cells, LKB1 mutations are a determining factor and serve as a driver of PD-L1 expression (Figure 1).

The role of LKB1 in regulating PD-L1 expression in hepatocellular carcinoma (HCC) cell lines. To assess the

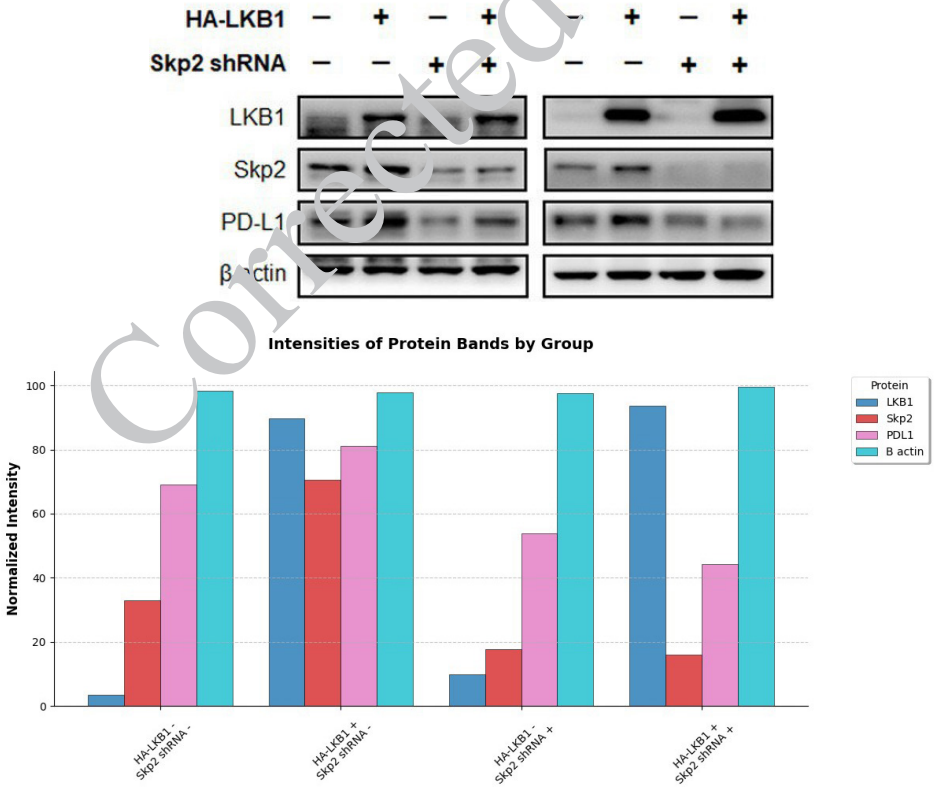

Figure 2. LKB1 had a minimal effect on PD-L1 transcript levels in Hep-B3 and HepG2 cells
In contrast, Western blot analysis demonstrated a significant increase in PD-L1 protein abundance upon LKB1 expression, suggesting that LKB1 selectively modulates PD-L1 at the post-transcriptional level.
LKB1: Liver Kinase B1; SKP2: S-phase kinase-associated protein; PDL-1: Programmed death-ligand 1 (PD-L1); Hep-B3: Hepatitis-B cell line name; HepG2: Hepatitis cell line name

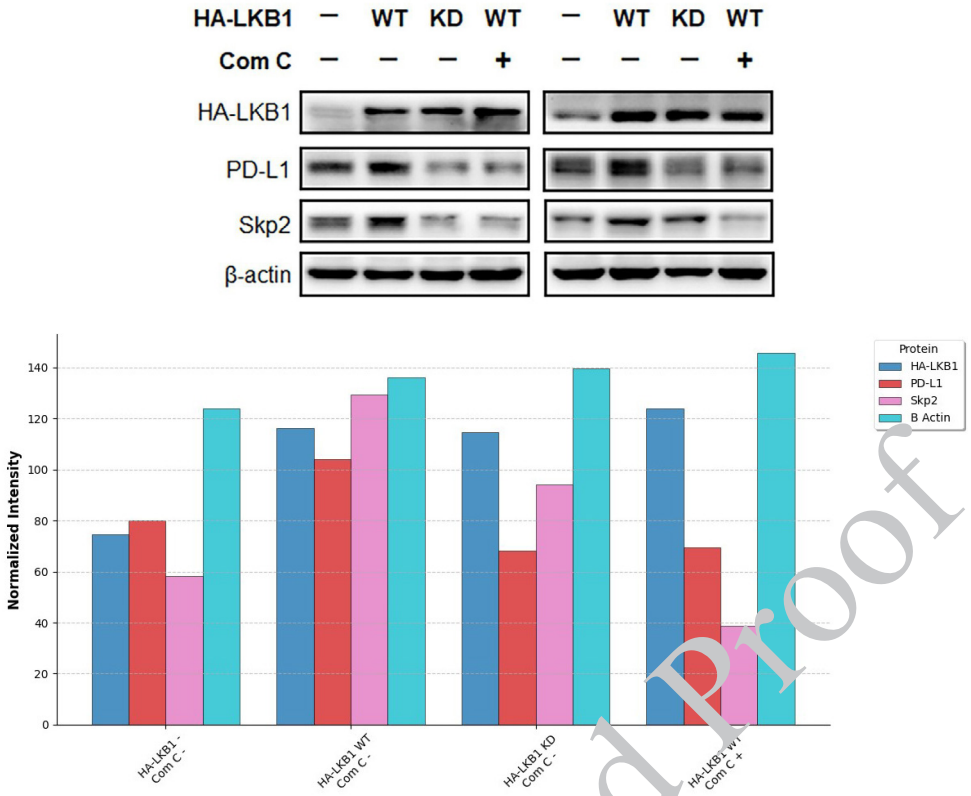

Figure 3. The role of LKB1-AMPK signaling in PD-L1 protein abundance was investigated
Given that LKB1 has kinase activity, we examined whether this kinase activity is essential for maintaining high PD-L1 expression. To address this, HA-tagged LKB1 and a kinase-dead mutant were generated, and these plasmids were transfected into Hep-B3 and HepG2 cells. The results showed that overexpression of wild-type LKB1 (LKB1 WT) significantly increased PD-L1 protein abundance in both cell lines. B-actin was used as an internal control.
LKB1: Liver Kinase B1; SKP2: S-phase kinase-associated protein; PDL-1: Programmed death-ligand 1 (PD-L1); Hep-B3: Hepatitis-B cell line name; HepG2: Hepatitis cell line name
AMPK: Adenin- Manose protein kinase

impact of LKB1 on PD-L1, we expressed LKB1 in a panel of HCC cells and measured PD-L1 expression at both the protein and mRNA levels. Real-time PCR analysis revealed that LKB1 had a minimal effect on PD-L1 transcript levels in Hep3B and HepG2 cells. In contrast, Western blot analysis demonstrated a up-regulation in PD-L1 protein abundance upon LKB1 expression, suggesting that LKB1 selectively modulates PD-L1 at the post-transcriptional level.

Furthermore, we assessed the levels of SKP2 (S-phase kinase-associated protein 2), a key regulator of protein ubiquitination, and found no significant changes between the groups, indicating that LKB1's effect on PD-L1 is not mediated through alterations in SKP2 expression. B actin was used as an internal control to ensure consistent loading across samples. The levels of mRNA undetectable by IF but present at basal levels by qPCR.

The data presented in Figure 2 highlight that LKB1 plays a crucial role in modulating PD-L1 protein levels, likely through post-translational mechanisms such as ubiquitination, which is mediated by Skp2. These findings provide a quantitative basis for understanding how LKB1 influences immune checkpoint regulation and contributes to the observed differences in immunotherapy response in HCC.

Investigation of the role of LKB1-AMPK signaling in PD-L1 protein abundance. Given that LKB1 has kinase activity, we examined whether this kinase activity is essential for maintaining high PD-L1 expression. To address this, HA-tagged LKB1 and a kinase-dead mutant were generated, and these plasmids were transfected into Hep3B and HepG2 cells. The results showed that overexpression of wild-type LKB1 (LKB1 WT) up-regulated PD-L1 protein abundance in both cell lines. B actin was used as an internal control. These findings suggest that LKB1 plays a crucial role in regulating PD-L1 at the protein level, potentially through post-translational mechanisms such as ubiquitination mediated by Skp2. This provides a quantitative basis for understanding the molecular mechanisms underlying the observed differences in immunotherapy response in HCC (Figure 3).

To further investigate the role of LKB1 in regulating PD-L1 expression, we analyzed protein band intensities in two hepatocellular carcinoma (HCC) cell lines, Hep3B and HepG2. Figures 4A and Figure 4B present the normalized intensity measurements of key proteins, including PD-L1, Skp2, and B actin, in HepG2 and Hep3B cells, respectively.

In HepG2 cells (Figure 4A), the overexpression of LKB1 up-regulated PD-L1 protein levels, while Skp2 expression remained relatively unchanged. Similarly, in Hep3B cells (Figure 4B, LKB1 expression led to a notable up-regulation of PD-L1 protein abundance, with no significant alteration in Skp2 levels. B actin was used as an internal control to ensure consistent loading and accurate quantification across all samples.Figure 4C represents quantitative densitometric analysis of PD L1 protein levels normalized to B actin. Normalized intensity was calculated using ImageJ by dividing target protein band intensity by the corresponding B actin intensity.

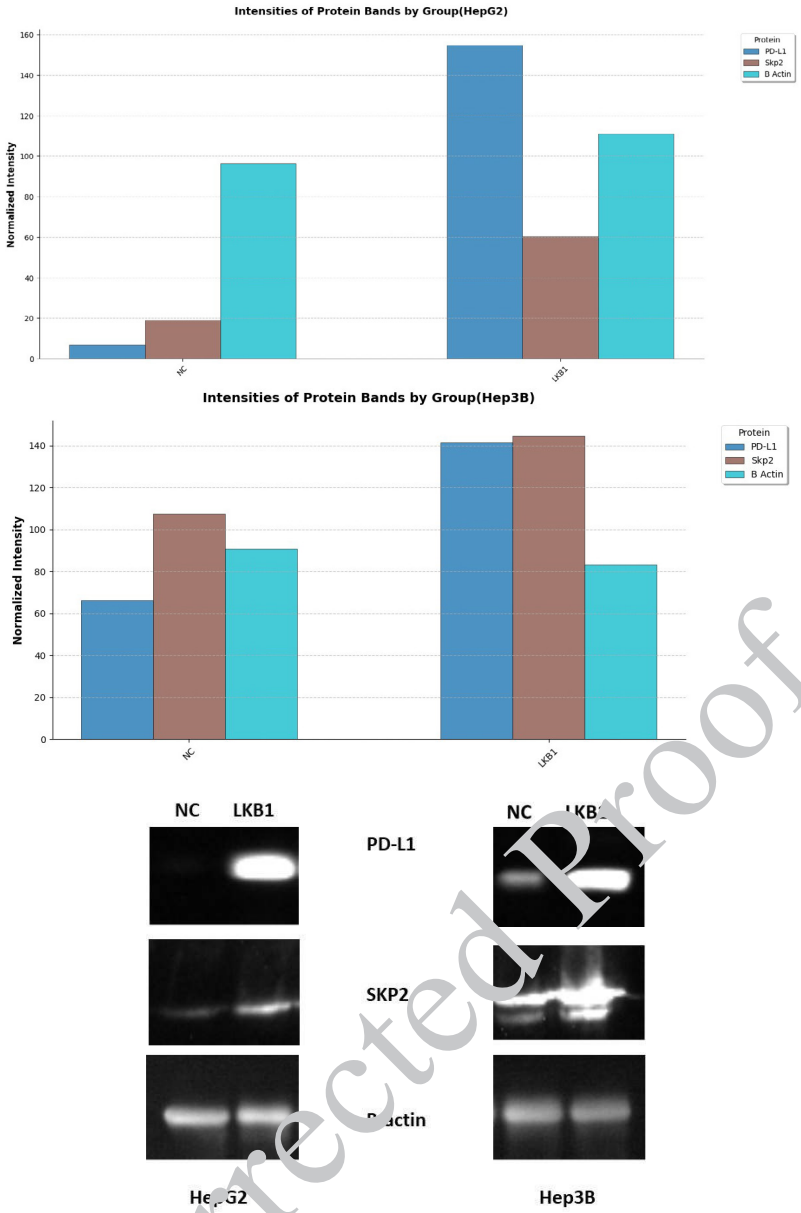

Figure 4. In HepG2 cells (Figure 4A), the overexpression of LKB1 resulted in a significant increase in PD-L1 protein levels, while Skp2 expression remained relatively unchanged. Similarly, in Hep3B cells (Figure 4B and 4C, LKB1 expression led to a notable up regulation of PD-L1 protein abundance, with no significant alteration in Skp2 levels.
LKB1: Liver Kinase B1; SKP2: S-phase kinase-associated protein; PDL-1: Programmed death-ligand 1 (PD-L1); Hep-B3: Hepatitis-B cell line name; HepG2: Hepatitis cell line name AMPK: Adenin- Manose protein kinase; NC: Normal control

Discussion

Recent studies showed that the critical role of LKB1 in regulating immune checkpoint proteins, particularly PD-L1, and its implications for immunotherapy sensitivity in HCC. By exploring the interplay between LKB1, Skp2, and PD-L1, we demonstrate that LKB1 deficiency significantly alters PD-L1 expression, potentially influencing tumor immune evasion and response to ICIs (8).

A key finding of this study is that LKB1 enhances PD-L1 protein expression in HCC cells without significantly affecting its mRNA levels. This suggests that LKB1 primarily regulates PD-L1 through post-translational mechanisms rather than transcriptional activation. These results align with existing literature highlighting the importance of post-translational modifications, such as ubiquitination, in immune checkpoint regulation (15). The observed increase in PD-L1 protein levels upon LKB1 overexpression, coupled with the lack of change in Skp2 expression, indicates that LKB1 may stabilize PD-L1 independently of Skp2-mediated ubiquitination (16, 17).

The clinical implications of our findings are significant. Tumors lacking LKB1 exhibited low PD-L1 expression and minimal T-cell infiltration, suggesting that these tumors may be less responsive to PD-1/PD-L1 blockade therapies. This is consistent with clinical observations that tumors with low PD-L1 expression often exhibit resistance to ICIs (18, 19). Future studies should explore whether restoring LKB1 function or targeting Skp2-mediated pathways could enhance the efficacy of immunotherapy in LKB1-deficient tumors (20). Some of these studies with a same result with our study in different cancers (15, 16, 18).

Another critical aspect of our study is the demonstration

that LKB1 kinase activity is essential for PD-L1 regulation (21). Overexpression of wild-type LKB1 up-regulated PD-L1 protein levels, whereas a kinase-dead mutant failed to do so. This suggests that LKB1-AMPK signaling may play a role in stabilizing PD-L1. Given AMPK's established role in metabolic stress and immune responses (22), its involvement in PD-L1 modulation represents a promising area for further research. Sympathetic the metabolic-immune crosstalk mediated by LKB1-AMPK signaling could provide new therapeutic avenues for enhancing immunotherapy efficacy in HCC (23). While our study provides compelling evidence for the role of LKB1 in immune checkpoint regulation, several questions remain unanswered. First, the precise molecular mechanisms by which LKB1 modulates PD-L1 stability require further investigation. Specifically, the potential involvement of other E3 ubiquitin ligases or cofactors in this process needs to be explored. Second, *in vivo* validation using HCC mouse models is essential to confirm our findings and assess the therapeutic potential of targeting the LKB1-Skp2 axis. Additionally, integrating machine learning-based predictive models, leveraging Python-driven computational analysis, could refine patient stratification strategies based on LKB1 and PD-L1 expression patterns. The Figure 4 demonstrate that LKB1 plays a critical role in modulating PD-L1 expression at the protein level in both HepG2 and Hep3B cell lines. The consistent increase in PD-L1 levels upon LKB1 overexpression, without corresponding changes in Skp2, suggests that LKB1 regulates PD-L1 through mechanisms independent of Skp2 expression. This provides further evidence that LKB1-mediated regulation of PD-L1 may involve post-translational modifications, such as ubiquitination, which could influence the sensitivity of HCC cells to immunotherapy. These results underscore the importance of the LKB1 pathway in immune checkpoint regulation and its potential as a therapeutic target in HCC.

While our study provides compelling evidence for the role of LKB1 in immune checkpoint regulation, several questions remain unanswered. First, the precise molecular mechanisms by which LKB1 modulates PD-L1 stability require further investigation. Specifically, the potential involvement of other E3 ubiquitin ligases or cofactors in this process needs to be explored. Second, *in vivo* validation using HCC mouse models is essential to confirm our findings and assess the therapeutic potential of targeting the LKB1-Skp2 axis. Additionally, integrating machine learning-based predictive models, leveraging Python-driven computational analysis, could refine patient stratification strategies based on LKB1 and PD-L1 expression patterns.

Conclusion

In summary, our study identifies LKB1 as a key regulator of PD-L1 stability in HCC and highlights its potential role in dictating immunotherapy sensitivity. By elucidating the interplay between LKB1, Skp2, and immune checkpoint proteins, we provide a foundation for future therapeutic strategies aimed at enhancing immune checkpoint blockade efficacy in LKB1-deficient tumors. These findings underscore the importance of post-translational modifications in immune regulation and open new avenues for targeting LKB1-Skp2 signaling in HCC treatment.

Acknowledgment

This study was down with authors self-found.

Ethics

IR.TMU.RIGLD.REC.1401.002.

Authors' Contributions

M K, H J and F Y are done the western blot and real time and IF. N S and T M are done a python analysis. A D and H R and K M and D A write the draft of article. MA M, N SM, S D, F M and A Y and M M edit the article. A J and Z H and Z F and P K edite second draft. S H and SS are corresponding the article.

Conflicts of Interest

No Conflict of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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