# Liver kinase B1 in exosomes inhibits immune checkpoint programmed death ligand 1 and metastatic progression of intrahepatic cholangiocarcinoma

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Received March 15, 2022; Accepted June 27, 2022

DOI: 10.3892/or.2022.8367

**Abstract.** The increasing morbidity and high mortality of intrahepatic cholangiocarcinoma (ICC) has led to the urgent need for new diagnostics and therapeutics. Liver kinase B1 (LKB1) exerts a tumor suppressor role in multiple malignances, while its regulatory role in exosomes secreted by ICC cells is obscure. In the present study, exosomes were extracted from cell culture supernatants of RBE and HCCC-9810 ICC cells as well as plasma of patients with ICC by ultracentrifugation and the morphology of exosomes was identified by transmission electron microscopy. Notably, compared with that of intracellular LKB1, the protein level of exosomal LKB1 was decreased. Silencing intracellular LKB1 increased the protein levels of programmed death ligand 1 (PD-L1), Slug and phosphorylated-AKT in exosomes, accompanied by decreased expression levels of exosomal LKB1. Exosomes with lower protein levels of LKB1 promoted the expression of the immune checkpoint PD-L1, malignant phenotypes of ICC cells *in vitro*, and cancer metastasis *in vivo*. Moreover, the low level of exosomal LKB1 in plasma was tightly associated with the poor prognosis of patients with ICC. Collectively, exosomal LKB1 inhibits the immune checkpoint PD-L1 and metastasis of ICC cells. These findings may provide new methods for the diagnosis and immune therapy of ICC.

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*Abbreviations:* LKB1, liver kinase B1; PD-L1, programmed death ligand 1; ICC, intrahepatic cholangiocarcinoma; TEM, transmission electron microscopy; GFP, green fluorescence protein

*Key words:* ICC, LKB1, exosome, PD-L1, AKT, cancer metastasis

## Introduction

Intrahepatic cholangiocarcinoma (ICC) is a highly lethal hepatobiliary neoplasm with increasing incidence (1). The established standards for the treatment of ICC include first-line (gemcitabine and cisplatin), second-line (FOLFOX) and adjuvant (capecitabine) systemic chemotherapy (2,3). Due to the high aggressiveness of ICC, long-term survival is only observed in patients undergoing complete R0 surgical resection (4). Lymph node involvement is one of the most important prognostic factors. Therefore, novel and efficient methods for the diagnosis and therapy of ICC are urgently needed.

As a serine/threonine kinase, liver kinase B1 (LKB1) has been shown to be a tumor suppressor that regulates cell growth, metabolism, survival and polarity (5-7). LKB1 inactivation can also lead to centromere defects and genome instability via p53-dependent upregulation of survivin, independent of AMPK (8). LKB1 regulation of the tumor immune microenvironment is complex and has received widespread attention. For example, LKB1 deficiency promotes neutrophil recruitment and proinflammatory cytokine production to suppress T-cell activity (9). LKB1 mutations may cause PD-1 inhibitor resistance in KRAS-mutant lung adenocarcinoma (10). Loss of LKB1 silences the expression of stimulator of interferon genes, decreasing the sensitivity of KRAS-mutant lung cancer cells to cytoplasmic double-stranded DNA (11). Certain preclinical therapeutic methods have been developed to treat malignant tumors with LKB1 loss, such as dual molecular targeted therapy for mTOR (Rapamycin) and PI3K (BKM-120) (12) as well as the combination of metformin with cisplatin (13). In addition, LKB1 is involved in regulating intestinal stem cell fate, skeletal muscle development, liver regeneration and certain non-neoplastic diseases (14-17).

Exosomes, derived from the endocytic pathway, are membranous vesicles with a diameter of ~40-200 nm (18,19). In various diseases, exosomes provide a channel to view the altered cellular or tissue states, and their detection in biological fluids potentially offers a multicomponent diagnostic readout (20). Tumor-derived exosomes modulate intercellular communication between tumor and stromal cells, influencing malignant phenotypes and the tumor microenvironment (21-23). Although

the regulation of exosomes has been widely studied in multiple types of cancer, exosomes derived from and acting on ICC are rarely mentioned. Even so, exosomal circ-0000284 has been found to be a competitive endogenous RNA that promotes ICC progression and can be directly transferred from ICC cells to surrounding normal cells (24). P2RX7 may influence ICC in an exosome-related manner (25). Considering the significant role of exosomes in cancer, it was suggested that exosomes may provide a new window for the diagnosis and therapy of ICC.

Cholangiocarcinoma represents a diverse group of epithelial tumors, including intrahepatic, perihilar and distal types (26). Although the biological characteristics of the three types of cholangiocarcinoma are similar, surgical resection is more likely to be performed for ICC than for the other two types. Therefore, the present study focused on ICC research. In a recent study, the inhibitory effect of LKB1 on the transcriptional activity of the immune checkpoint PD-L1 was uncovered in ICC cells (27). However, the regulatory role of LKB1 in exosomes secreted by ICC cells remains obscure. Exosomes from cell culture supernatants of ICC cells were extracted and examined in the present study. Further study uncovered the inhibitory effect of exosomal LKB1 on PD-L1 and phosphorylated (p)-AKT expression in cells and exosomes as well as malignant phenotypes and metastasis of ICC cells. The change in exosomal LKB1 expression in plasma may be a promising target for the diagnosis and therapy of ICC in the future.

## Materials and methods

*Cell culture*. The human embryonic kidney fibroblasts 293T (RRID: CVCL_0063) were purchased from the American Type Culture Collection (ATCC). The human ICC cells RBE (RRID: CVCL_4896) and HCCC-9810 (RRID: CVCL_6908) were obtained from Wuhan Boster Biological Technology, Ltd. The culture medium for 293T cells and ICC cells was composed of DMEM, 10% (v/v) FBS and penicillin-streptomycin (all from Thermo Fisher Scientific, Inc.). Cells were passaged at an average of three days and tested routinely to avoid mycoplasma contamination.

*Plasmid construction and lentiviral infection*. The 2nd generation system of lentiviruses was used in the present study. Plasmids for silencing LKB1 expression were constructed using pGreenPuro™ small hairpin (sh)RNA Cloning and Expression Lentivector (System Biosciences, LLC), and the target sequences for gene silencing are listed below: LT-shLKB1 (CCTGCTGAAAGGGATGCTT) and negative control LT-shControl (ACTACCGTTGTTATAGGTG). By mixing 8.0 $\mu$g gene silencing plasmids, 8.0 $\mu$g packaging plasmid psPAX2 and 2.7 $\mu$g envelope plasmid pMD2G with 18 $\mu$l transfection reagent Lipofectamine® 2000 in 600 $\mu$l Opti-MEM™ (both from Thermo Fisher Scientific, Inc.) for 20 min at room temperature, plasmids were co-transfected into 293T cells. After 2 days, cell culture supernatants containing lentivirus were collected every 8 h and filtered with 0.45-$\mu$m filter membranes. Cells were infected with lentivirus at different multiplicity of infection (1, 2.5, 5, 10, 20), and silencing efficiency of LKB1 lentivirus was determined by western blotting 72 h after infection. Puromycin (2 $\mu$g/ml; Thermo Fisher Scientific, Inc.) was used to screen the lentivirus-infected cells, and *in vitro* and *in vivo* experiments were conducted immediately after the puromycin selection.

*Western blot analysis*. The cultured cells were sequentially rinsed with ice-cold PBS, lysed in ice-cold RIPA lysis buffer (Thermo Fisher Scientific, Inc.) supplemented with proteinase inhibitor cocktail (Thermo Fisher Scientific, Inc.), and collected into 1.5-ml microtubes. After incubation on ice for 30 min, cell lysates were centrifuged at 16,100 x g for 15 min at 4˚C. The protein concentration was determined using a BCA protein quantification kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). An appropriate amount of BCA working solution was prepared according to the number of standards and samples and standard BSA protein solution (1 mg/ml) was dispensed into 96-well plates at 0, 1, 2, 4, 6, 8 and 10 $\mu$l and supplemented to 10 $\mu$l by adding $ddH_2O$. At the same time, 10 $\mu$l diluted protein samples were added to each well of 96-well plates. A total of 200 $\mu$l of BCA working solution was added to the sample and protein standard wells and mixed. The 96-well plates were incubated at 37˚C for 30 min and then cooled to room temperature. The absorbance was measured at 562 nm wavelength on a microplate reader SENERGY HTX (BioTek Instruments, Inc.). Densitometric analysis was conducted using software Image Lab (Version 5.2; Bio-Rad Laboratories, Inc.).

Then, 20 $\mu$g total protein was electrophoresed in 10% SDS-PAGE gels and transferred onto PVDF membranes (Merck Millipore). The PVDF membranes were sequentially blocked with 5% skim milk for 30 min at room temperature and incubated with the primary antibodies overnight at 4˚C and the secondary antibodies for 2 h at room temperature. Protein bands on PVDF membranes were detected by Supersignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Inc.).

The commercially obtained antibodies were used according to the manufacturer's protocol: LKB1 (1 $\mu$g/ml; cat. no. sc-32245, Santa Cruz Biotechnology, Inc.), E-cadherin (1 $\mu$g/ml; cat. no. 3195S), N-cadherin (1 $\mu$g/ml; cat. no. 13116S), β-Catenin (1 $\mu$g/ml; cat. no. 8480S), Slug (1 $\mu$g/ml; cat. no. 9585S), Snail (1 $\mu$g/ml; 3879S; all from Cell Signaling Technology, Inc.), PD-L1 (0.67 $\mu$g/ml; cat. no. 17952-1-AP; ProteinTech Group, Inc.), GAPDH (1 $\mu$g/ml; cat. no. MAB374; Merck Millipore), CD9 (1 $\mu$g/ml; cat. no. sc-13118; Santa Cruz Biotechnology, Inc.), TSG101 (1 $\mu$g/ml; cat. no. 102286-T38; Sino Biological), p-AKT (1 $\mu$g/ml; cat. no. 4060S), AKT (1 $\mu$g/ml; cat. no. 4691S; both from Cell Signaling Technology, Inc.), goat anti-mouse IgG (HRP-linked) (1:5,000; cat. no. 401211; Merck Millipore) and anti-rabbit IgG (HRP-linked) (1:5,000; cat. no. 7074S; Cell Signaling Technology, Inc.).

*Exosome extraction*. Exosomes in cell culture supernatants and plasma specimens of patients with ICC were extracted according to standard protocols (28,29). The simplified procedures of exosome extraction are listed below: i) To remove dead cells, cell culture supernatants and plasma were centrifuged at 2,000 x g for 20 min at 4˚C; ii) To remove cell debris, the collected supernatants and plasma were centrifuged at 10,000 x g for 30 min at 4˚C; iii) The purified supernatants were transferred to clean ultra-tubes and then ultra-centrifuged

at 100,000 x g for 70 min at 4˚C; iv) The precipitates were resuspended with ice-cold PBS and ultra-centrifuged at 100,000 x g for another 70 min at 4˚C; v) The precipitated exosomes at the bottom of the ultracentrifuge tubes were resuspended with ice-cold PBS for subsequent research. The protein markers of exosomes, including CD9, TSG101 and GAPDH, were examined by western blotting.

*Electron microscopy*. The morphology of exosomes was observed using a transmission electron microscope (TEM) JEM-3010 (JEOL, Ltd.), and the simplified protocol was as follows: i) Exosomes resuspended in 10 $\mu$l PBS were mixed with 10 $\mu$l of 4% paraformaldehyde for 20 min at room temperature and then added to the carbon films; ii) After standing for 20 min, the carbon films were rinsed with PBS twice for 2 min each time; iii) Exosomes on carbon films were then stained with 10 $\mu$l of 2% uranyl-acetate solution for 1 min at room temperature; (4) Carbon films were air-dried for 10 min at room temperature; (5) The morphology of exosomes was visualized at 100 kV.

*Exosome treatment in cell culture*. An average of $6x10^5$ ICC cells with differential expression of LKB1 were seeded into each well of a six-well culture plate. A total of 24 h later, 2 $\mu$g exosomes resuspended in 10 $\mu$l PBS were added to each well of the cell culture supernatants. After incubation for another 24 h in cell culture incubator at 37˚C with 5% $CO_2$, total protein was collected from the cells and examined by western blotting.

*MTT assay*. A total of 500 $\mu$l of culture medium containing 10,000 ICC cells with differential expression of LKB1 was added to each well of a 24-well cell culture plate. A total of 24 h later, 0.50 $\mu$g exosomes resuspended in 2.5 $\mu$l PBS were added to each well of the cell culture supernatants. After cultivation for different times, MTT solution (Sangon Biotech) was added to each well at a final concentration of 0.50 $\mu$g/$\mu$l. After 6 h, the culture medium was carefully discarded and replaced with 150 $\mu$l of DMSO (Sangon Biotech Co., Ltd.). After 20 min of gentle shaking, the OD value at 490 nm was measured and statistically analyzed.

*Transwell assay*. Transwell experiments were conducted to determine the migratory ability of ICC cells. Before conducting experiments, cultured ICC cells in plates were starved with serum-free DMEM for 24 h. Transwell chambers with 8.0-$\mu$m PET membrane pores (Corning, Inc.) were inserted into each well of a 24-well plate. Then, 600 $\mu$l of DMEM supplemented with 10% (v/v) FBS and 0.50 $\mu$g exosomes was added to the lower chamber, and 100 $\mu$l of serum-free DMEM containing $1x10^5$ cells was added to the upper chamber. A total of 24 h later, the cells were fixed with methyl alcohol for 15 min at room temperature and stained with 0.2% (m/v) crystal violet (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. Then, the remaining cells in the upper chamber were removed with a medical cotton swab. Images of the migratory cells were captured under an inverted light microscope, and the number of migratory cells was counted and statistically analyzed.

*Animal experiment*. In total, 12 six-week-old male BALB/c nude mice (weight, ~20 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China) and fed at the animal care facility of The Central Hospital of Xiangtan. Mice were housed three per cage with free access to food and sterile water, under 12-h light/dark cycle at 25˚C and 50% humidity. To assess the regulatory effect of exosomal LKB1 on the metastasis of ICC cells *in vivo*, mice were arbitrarily assigned to four groups (n=3 each). Overall, $2.0x10^6$ ICC cells in 200 $\mu$l of sterile PBS with differential expression of LKB1 were injected into mice via the tail vein. After 7 days, 50 $\mu$g of exosomes resuspended in 100 $\mu$l of sterile PBS with differential expression levels of LKB1 were injected into mice every via the tail vein three days for five consecutive injections. Fluorescence in live mice was detected using the IVIS Lumina XR live animal imager (PerkinElmer, Inc.) according to the expression of green fluorescence protein (GFP) in lentivirus-infected ICC cells. The mice in the present study were sacrificed when they experienced a sharp decrease in activity, water and diet intake. Therefore, 40 days after cell injection, mice were sacrificed by cervical dislocation. The protocol used for animal experiments was approved (approval no. 2020073) by the Animal Care and Experiment Committee of The Central Hospital of Xiangtan (Xiangtan, China). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed in the present study.

*Patient information*. A total of 42 pairs of cancer and para-cancer tissue specimens used in the present study were obtained from The Central Hospital of Xiangtan with informed consent from 2014 to 2019. No patients received chemotherapy, radiotherapy or immune therapy before surgery. The age of the patients ranged from 37-74 years old, with 27 patients being over 60 years old. Among them, 18 patients were male and 24 patients were female. Limited by the diagnosis and treatment strategies of the hospital for ICC, gene mutational information for patients is not available. Plasma specimens of patients with ICC used in the present study were obtained with written informed consent and approved (approval no. 2019-08-001) by the institutional review boards of The Central Hospital of Xiangtan and were in accordance with the Declaration of Helsinki (2000).

*Statistical analysis*. GraphPad Prism 8 (GraphPad Software, Inc.) was used to generate statistical graphs, and statistical analyses were conducted using SPSS 22.0 (IBM Corp.). Student's two-sided unpaired t-test was used to compare the significance between two groups, and one-way ANOVA with Tukey-Kramer post hoc test was used to determine differences among multiple groups. The correlation between the protein expression of LKB1 and PD-L1 examined by western blotting was determined by Pearson's correlation analysis (two-tailed). The Kaplan-Meier method followed by log-rank test was performed to generate survival curves. Experiments were repeated two or three times with similar results. Data are represented as the means ± SD with at least three biological replicates. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*LKB1 inhibits exosomal PD-L1 in ICC cells*. Our previous research revealed that LKB1 inhibits ICC by repressing the

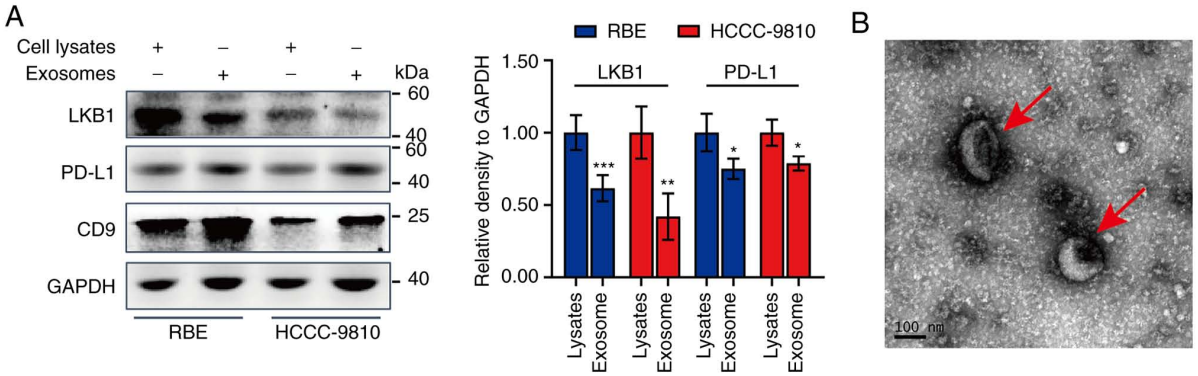


Figure 1. Identification of exosomes extracted from ICC cell culture supernatants. (A) Western blot showing the protein levels of LKB1, PD-L1, CD9 and GAPDH in cell lysates and exosomes of ICC RBE and HCCC-9810 cells. (B) Morphology of extracted exosomes from cell culture supernatants of RBE cells, as examined by transmission electron microscopy. Exosomes in the image are indicated by arrows in black. Scale bar, 100 nm. Data are represented as the means ± SD and significance was analyzed using Student's two-sided t-test. n=3 in each group. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001. ICC, intrahepatic cholangiocarcinoma; LKB1, liver kinase B1; PD-L1, programmed death ligand 1.

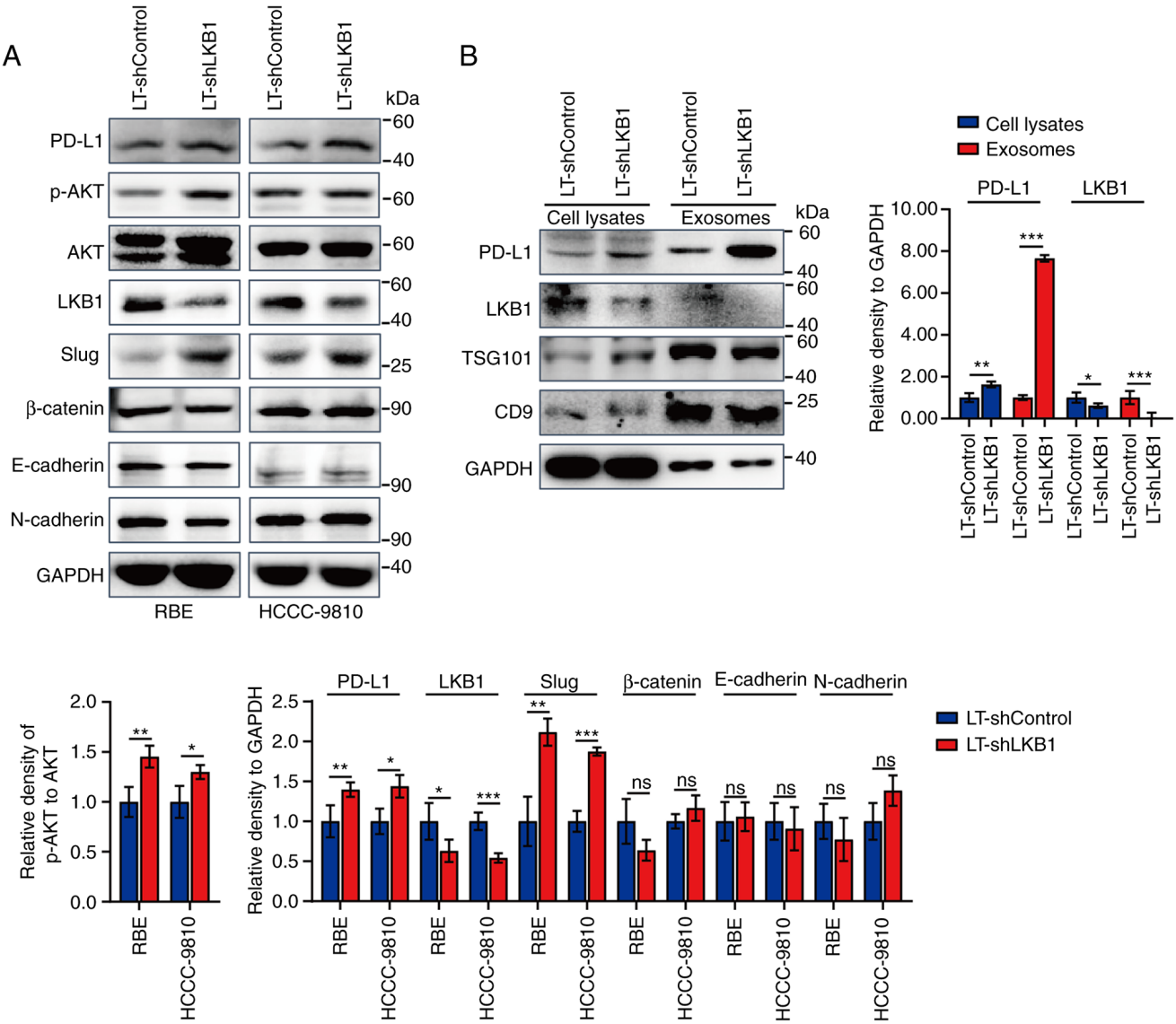


Figure 2. Negative regulation of LKB1 on exosomal PD-L1. (A) Western blot analysis for protein expression of PD-L1, p-AKT, AKT, LKB1 and epithelial-mesenchymal transition-associated markers (Slug, β-Catenin, E-cadherin, and N-cadherin) in cell lysates of RBE and HCCC-9810 ICC cells with LKB1 knockdown. (B) Western blotting for protein expression of PD-L1, LKB1, TSG101, CD9 and GAPDH in cell lysates and exosomes of RBE cells with LKB1 knockdown. Data are represented as the means ± SD and significance was analyzed using Student's two-sided t-test. n=3 in each group. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001. LKB1, liver kinase B1; PD-L1, programmed death ligand 1; p-, phosphorylated; ICC, intrahepatic cholangiocarcinoma; sh-, small hairpin; LT, lentivirus; ns, no significance.

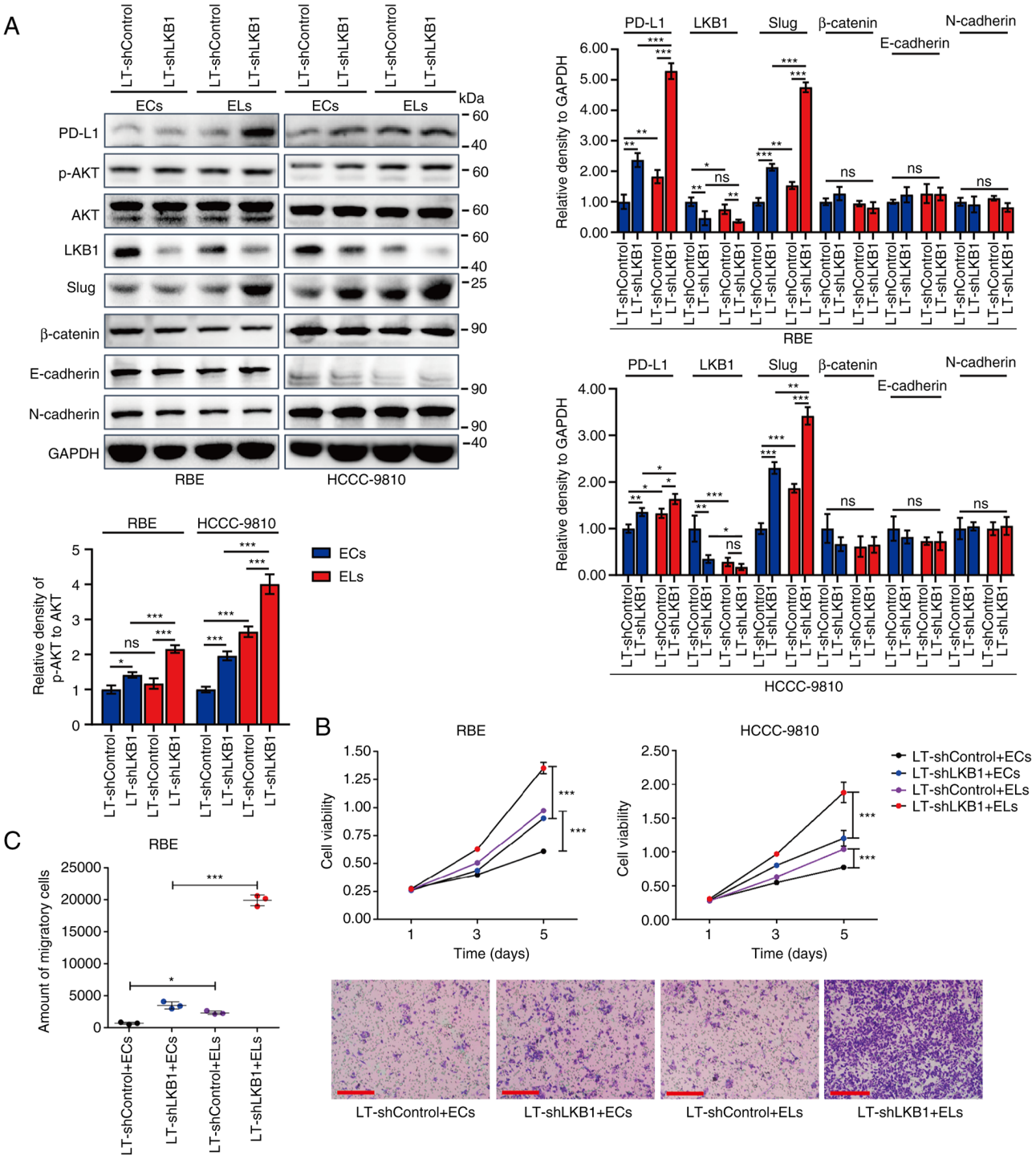


Figure 3. Regulatory effects of exosomes with differential expression of LKB1 on malignant phenotypes of ICC cells. (A) Western blotting for protein expression of PD-L1, p-AKT, AKT, LKB1 and epithelial-mesenchymal transition-associated markers (Slug, β-Catenin, E-cadherin and N-cadherin) in lysates of RBE and HCCC-9810 ICC cells. The adherent ICC cells were incubated with exosomes extracted from cell culture supernatants of corresponding ICC cells with differential expression of LKB1 for 24 h. (B) Proliferation ability of RBE and HCCC-9810 cells as assayed by MTT. The adherent ICC cells were incubated with exosomes extracted from cell culture supernatants of corresponding ICC cells with differential expression of LKB1 for different times. n=4 in each group. (C) Representative images and statistical analysis of the migratory RBE and HCCC-9810 ICC cells as examined by Transwell assays. Cells in the upper chambers of the Transwell were incubated with exosomes extracted from cell culture supernatants of corresponding ICC cells with differential expression of LKB1 for 24 h. n=3 in each group. Scale bar, 100 $\mu$m. Data are represented as the means ± SD and significance was analyzed using Student's two-sided t-test or one-way ANOVA with the Tukey-Kramer post hoc test. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001. LKB1, liver kinase B1; ICC, intrahepatic cholangiocarcinoma; PD-L1, programmed death ligand 1; p-, phosphorylated; ECs, exosomes secreted by LT-shControl cells; ELs, exosomes of LT-shLKB1 cells; sh-, small hairpin; LT, lentivirus; ns, no significance.

transcriptional activity of the immune checkpoint PD-L1 (27). However, the regulatory effect of exosomal LKB1 on ICC remains unclear. In the present study, the role of exosomal LKB1 in ICC was investigated using the RBE and HCCC-9810 cell lines. The high level of CD9 protein (Fig. 1A) and the morphology revealed by TEM (Fig. 1B) suggested the

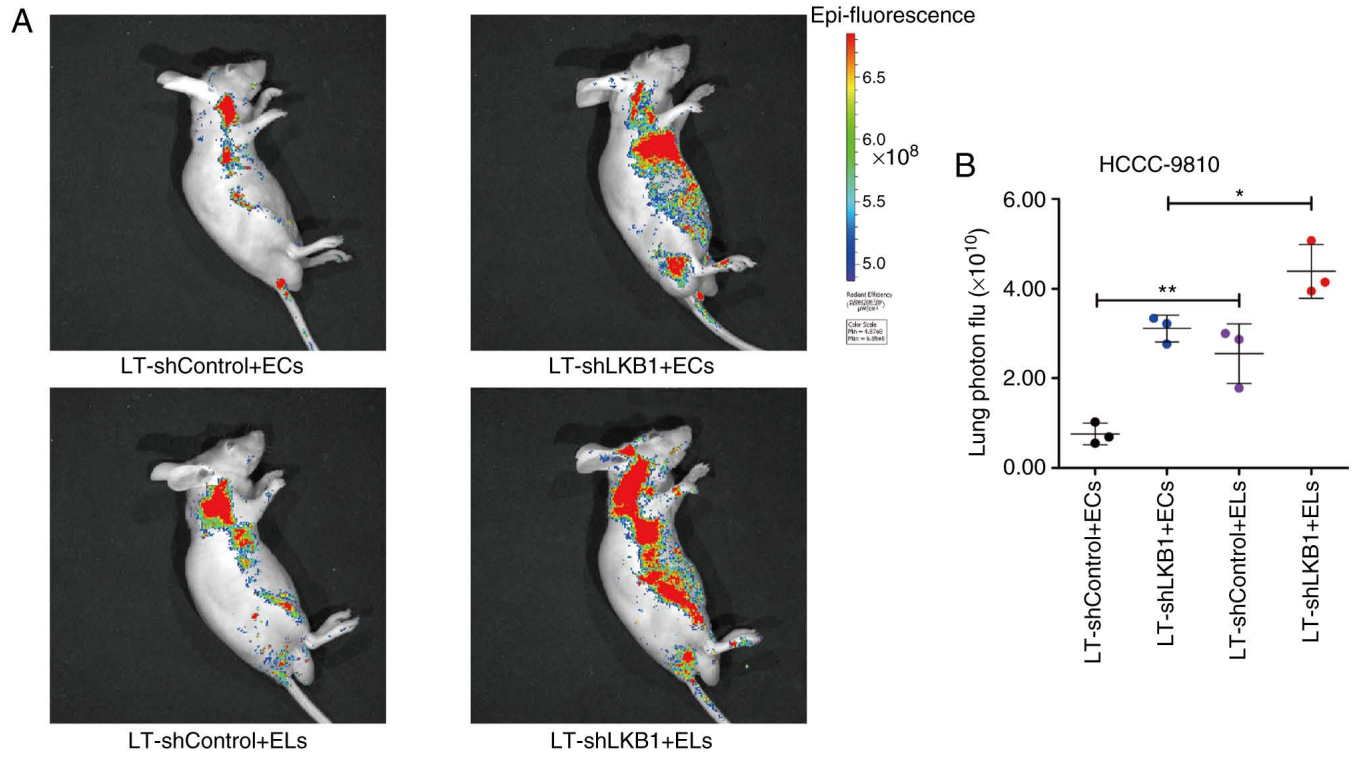


Figure 4. Regulation of exosomes with differential expression of LKB1 on cancer metastasis *in vivo*. (A) Representative images and (B) statistical analysis of GFP fluorescence in BALB/c nude mice 40 days after tail vein injection of HCCC-9810 cells. Exosomes extracted from cell culture supernatants of HCCC-9810 cells were administered to mice by tail vein injection every three days for five consecutive injections. Data are represented as the means ± SD and significance was analyzed using one-way ANOVA with the Tukey-Kramer post hoc test. n=3 in each group, $^{*}$P<0.05 and $^{**}$P<0.01. LKB1, liver kinase B1; ECs, exosomes secreted by LT-shControl cells; ELs, exosomes of LT-shLKB1 cells; sh-, small hairpin; LT, lentivirus.

successful extraction of exosomes from cell culture supernatants of ICC cells. The diameter of the captured exosomes in the image was ~150 nm (Fig. 1B). Notably, the protein level of LKB1 in exosomes secreted by RBE and HCCC-9810 cells was lower than that in cell lysates (Fig. 1A). Meanwhile, the PD-L1 level in exosomes was higher than that in cell lysates. These findings indicated that exosomes secreted by ICC cells may exert a tumor-promoting role and that exosomal LKB1 may suppress ICC.

To verify this hypothesis, ICC cells stably infected with lentivirus for silencing LKB1 expression were constructed for the following research. Silencing LKB1 expression increased the PD-L1 level in ICC cells as well as that of p-AKT and the transcription factor Slug (Fig. 2A). Consistent with our previous research, LKB1 did not affect the protein expression of epithelial-mesenchymal transition-associated markers, including β-Catenin, E-cadherin, and N-cadherin (27). Exosomes were extracted from the cell culture supernatants of ICC cells RBE and HCCC-9810 with differential expression of LKB1. Exosomal PD-L1 and LKB1 levels were examined by western blotting. Silencing intracellular LKB1 downregulated the protein level of exosomal PD-L1, accompanied by a decrease in the protein level of exosomal LKB1 (Fig. 2B).

*Exosomal LKB1 inhibits the immune checkpoint PD-L1 and malignant phenotypes of ICC cells*. Next, the inhibitory effect of exosomal LKB1 on ICC was confirmed *in vitro*. Notably, compared with exosomes secreted by LT-shCtrl cells, ICC cells incubated with exosomes secreted by LT-shLKB1 cells expressed lower levels of LKB1 protein and higher levels of PD-L1, p-AKT and Slug (Fig. 3A). These findings suggested that exosomal LKB1 inhibits the immune checkpoint PD-L1 and tumor signal transduction of ICC cells. Thus, exosomal PD-L1 plays a tumor suppressor role in ICC.

Next, the malignant phenotypes of ICC cells were examined after treatment with exosomes with differential expression of LKB1. Compared with exosomes derived from ICC cells with high levels of LKB1, exosomes secreted by ICC cells with low levels of LKB1 significantly promoted the proliferation of ICC cells RBE and HCCC-9810 (Fig. 3B). Moreover, exosomes with low levels of LKB1 exhibited a significant effect on enhancing the migratory ability of ICC cells (Fig. 3C). Therefore, it was identified that exosomal LKB1 inhibits the malignant phenotypes of ICC cells.

*Exosomal LKB1 suppresses ICC cell metastasis in vivo*. Considering the inhibitory effect of exosomal LKB1 on the migration ability of ICC cells, it was hypothesized that exosomal LKB1 may influence ICC metastasis. Therefore, animal experiments were conducted to explore the regulatory effect of exosomal LKB1 on ICC metastasis. The wild-type ICC cell line HCCC-9810 was administered to male BALB/c nude mice through tail vein injection. After 7 days, exosomes of HCCC-9810 cells with differential expression of LKB1 resuspended in sterile PBS were administered to mice by tail vein injection. Through tracing the GFP expression of lentivirus-infected HCCC-9810 cells *in vivo*, it was found that mice injected with exosomes with lower levels of LKB1

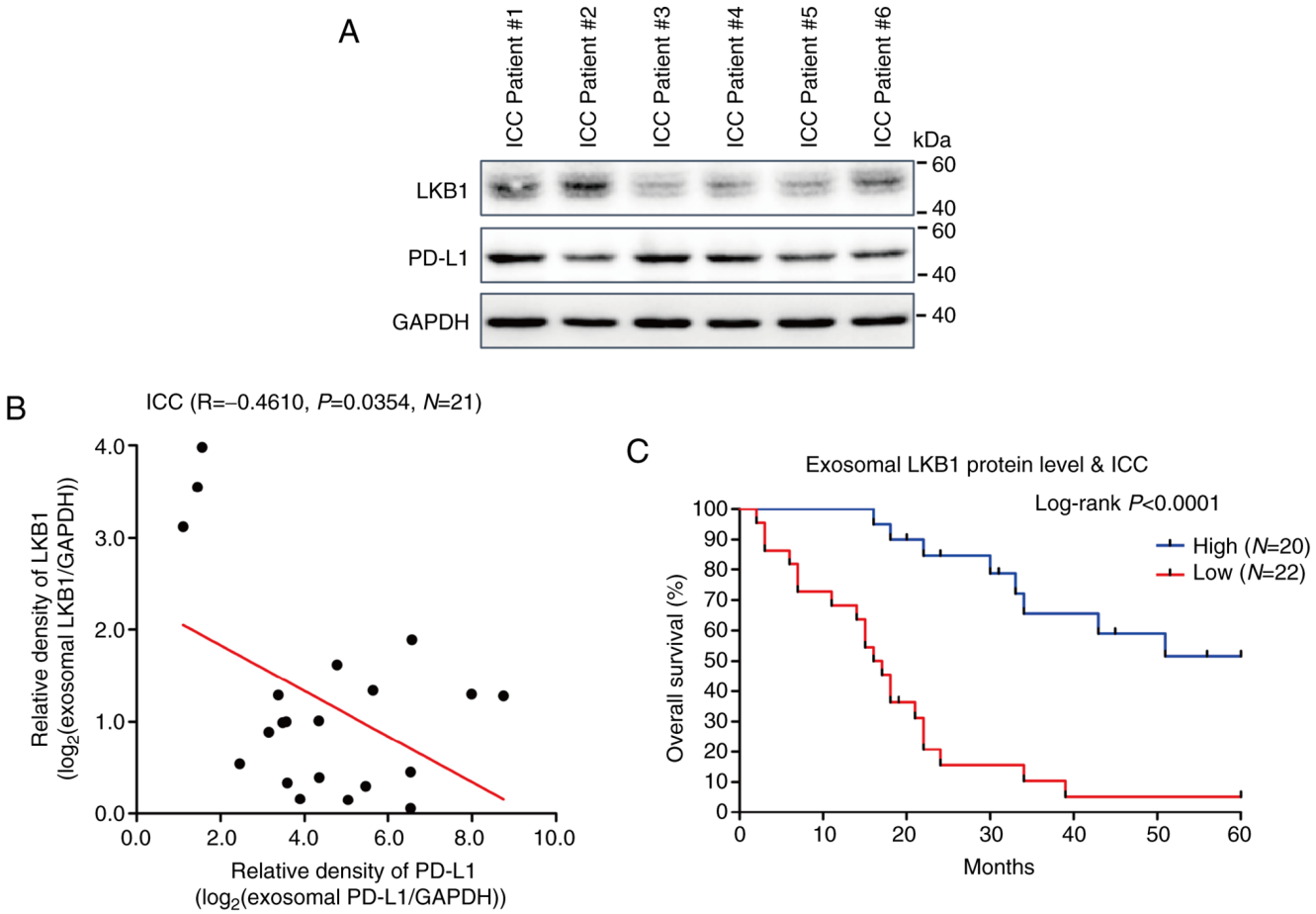


Figure 5. Correlation between the protein level of exosomal LKB1 derived from plasma specimens and survival prognosis in ICC. (A) Western blotting showing the protein levels of LKB1, PD-L1 and GAPDH in exosomes derived from the peripheral blood of patients with ICC. (B) Pearson's correlation coefficients for the relative protein levels of LKB1 and PD-L1 in exosomes derived from the plasma of patients with ICC (n=21). (C) Kaplan-Meier analysis for the overall survival of patients with ICC presenting high and low levels of PD-L1 protein in exosomes derived from plasma, as analyzed using the log-rank test (n=42). LKB1, liver kinase B1; ICC, intrahepatic cholangiocarcinoma; PD-L1, programmed death ligand 1.

exhibited a significantly stronger intensity of luciferase, while mice injected with exosomes with higher levels of LKB1 protein exhibited a significantly weaker intensity of luciferase (Fig. 4A). The statistical analysis of luciferase intensity showed a significant difference among the groups (Fig. 4B). These findings suggested that exosomal LKB1 inhibits ICC metastasis.

*Low levels of exosomal LKB1 may predict poor prognosis of ICC*. To further evaluate the clinical importance of exosomal LKB1 in cancer research, exosomes were extracted from plasma specimens of patients with ICC. Western blotting revealed that the expression trends of LKB1 and PD-L1 protein in exosomes in the plasma of patients with ICC were almost opposite (Fig. 5A). Pearson's correlation (R) analysis revealed a negative correlation between the protein levels of LKB1 and PD-L1 in exosomes of plasma specimens (Fig. 5B). These findings are consistent with the aforementioned observations in cell culture supernatants of ICC cells. Moreover, the Kaplan-Meier analysis for the overall survival of ICC indicated that a low level of exosomal LKB1 may predict poor prognosis of ICC (Fig. 5C). These experimental results from clinical samples suggested the inhibitory effect of LKB1 on exosomal PD-L1 and demonstrated the tumor suppressor role of exosomal LKB1 in ICC.

## Discussion

Accumulating evidence reflects LKB1 regulation of the immune checkpoint PD-L1. In KRAS-mutated lung cancer, the LKB1 co-mutation is associated with a reduced level of PD-L1 protein, resulting in therapeutic resistance to PD-1/PD-L1 inhibitors (30). Compared with patients with non-squamous LKB1-mutant non-small-cell lung cancer, patients with wild-type LKB1 present improved response to anti-PD-L1 immunotherapy. Therefore, loss of LKB1 clearly leads to upregulated levels of PD-L1 (27,31). In the present study, a low level of exosomal LKB1 was uncovered in the cell culture supernatants of ICC cells and the plasma of patients with ICC. At present, no P53 mutations have been reported in ICC cells RBE or HCCC-9810, while the IDH1 mutation (R132S) exists in RBE cells (32). Both RBE and HCCC9810 cells exhibit strong migratory and clonogenic abilities (33), while HCCC9810 cells exhibit stronger resistance to drugs, such as anlotinib and gemcitabine, than RBE cells (34). HCCC-9810 cells have the ability to form tumors *in vivo* (35,36), while RBE cells cannot form tumors in athymic nude mice (37). Based on the aforementioned findings, it was considered that HCCC-9810 cells may have stronger aggressive and metastatic ability than RBE cells. These two differential cell lines, RBE and

HCCC-9810, were selected to study the regulation of exosomal LKB1 on ICC. The downregulation of intracellular LKB1 led to the downregulation of exosomal LKB1 and the upregulation of PD-L1, Slug and p-AKT in exosomes. Exosomal PD-L1 contributes to immunosuppression and is associated with the anti-PD-1 response (38). Thus, LKB1 may be involved in immunosurveillance of ICC by suppressing exosomal PD-L1. Exosomal LKB1 was revealed to downregulate PD-L1 levels in ICC cells, which may provide new insights into the immune evasion of ICC cells by modulating exosomes.

Moreover, exosomes secreted by ICC cells with low levels of LKB1 promoted the metastasis of ICC cells. PD-L1 and p-AKT are both closely related to malignant transformation and poor prognosis of malignances (39-41). The crosstalk between the PI3K-AKT-mTOR and LKB1-AMPK signaling pathways is critical for modulating cancer metastasis, metabolism and prognosis (42,43), and Slug functions greatly in cancer development (44). Although the present study did not show direct evidence of the involvement of AKT and Slug in the inhibitory effect of LKB1 on ICC metastasis, it could still be concluded that LKB1 may suppress metastasis via an exosome-mediated mechanism. These regulatory factors may be the key points for LKB1 to inhibit immune evasion and metastasis of ICC. However, the direct mechanism by which exosomal LKB1 regulates ICC metastasis still needs to be further explored.

Exosomes secreted by cancer cells can be used as warehouses to transfer biologically active molecules, such as RNA and proteins, thereby regulating the occurrence and development of malignances (45,46). Except for CD9, CD63 and TSG101, both GAPDH and β-actin have been detected in exosomes (47-49), all of which may be exosomal markers. The abundant level of GAPDH protein in exosomes secreted by RBE and HCCC-9810 cells suggested the role of GAPDH as a loading control for exosomes at least. Limited by experimental conditions and wide spread of COVID-19, nanoparticle tracking analysis on the extracted exosomes was not performed, which may need to be further improved in future studies. Notably, exosomal PD-L1 promotes the immune evasion of cancer cells and reduces the response efficiency to PD-1 inhibitors (38). The regulatory role of LKB1 in exosomes remains obscure. Consistent with the inhibition on p-AKT and Slug, LKB1 was revealed to decrease exosomal PD-L1 in ICC cells in the present study, providing a new mechanism for understanding the anticancer effect of LKB1. These findings may provide new methods for inhibiting the immune evasion of ICC cells by targeting exosomal LKB1 and PD-L1.

Liquid biopsy has gained momentum in clinical cancer research. Continuously released by living cells, exosomes contain DNA, RNA and proteins, providing direction for clinically relevant diagnosis (50). Owing to their non-invasive nature and real-time assessment, exosome-based diagnostics are more readily available to track patients over time and monitor potential disease progression and therapeutic intervention in an improved way (51). Through conducting small RNA sequencing and proteomics evaluation, certain miRNAs and proteins have been identified with elevated or attenuated expression in plasma specimens of cancer patients, acting as potential diagnostic markers for multiple types of cancer (52-54). In the present study, it was revealed that low expression of exosomal LKB1 in the plasma of patients with ICC may predict poor prognosis, further emphasizing the clinical significance of LKB1 research in ICC.

In summary, the significance and innovations of the present study are mainly reflected in the following aspects. First, LKB1 was shown to inhibit exosomal PD-L1 in ICC cells. Second, *in vitro* and *in vivo* experiments revealed the inhibitory effect of exosomal LKB1 on ICC. Third, the low expression level of exosomal LKB1 was found to be tightly associated with the metastasis and poor prognosis of ICC. Exosomal LKB1 exerts a tumor suppressor role in ICC and may be an important biomarker for the diagnosis and immune therapy of ICC.

**Acknowledgements**

Not applicable.

**Funding**

The present study was supported by the Hunan Natural Science Foundation (grant no. 2019JJ50626) and the Project of Health and Family Planning Commission of Hunan Province (grant no. B20180832).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

ZL, KZ and TM conceived and designed the experiments. ZL, KZ, JZ, XZ, HL, KP, XL, FL, BJ and MZ performed the experiments. ZL, KZ, HL, XL and TM managed data. ZL, KZ, JZ, XZ and TM analyzed the data. KP and TM contributed to reagents, materials and analysis tools. ZL and TM acquired funding. ZL, KZ and TM wrote the paper. ZL and KZ confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

The protocol used for animal experiments was approved (approval no. 2020073) by the Animal Care and Experiment Committee of The Central Hospital of Xiangtan (Xiangtan, China). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed in the present study. Informed consent was obtained from all patients for collection of plasma specimens. Patient studies were approved (approval no. 2019-08-001) by the institutional review boards of The Central Hospital of Xiangtan (Xiangtan, China) and were in accordance with the Declaration of Helsinki.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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