

RESEARCH PAPER



LHPP inhibits hepatocellular carcinoma cell growth and metastasis

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ABSTRACT

Hepatocellular carcinoma (HCC) has a poor prognosis, owing to its high potential for growth and metastasis. In this study, we aimed to investigate the roles of *Phospholysine Phosphohistidine Inorganic Pyrophosphate Phosphatase (LHPP)* in human HCC cell growth and metastasis. We analyzed the *LHPP* expression level in human HCC tissues paired normal tissues in the Oncomine database, and assessed the relationship between the *LHPP* expression levels with HCC patient's overall survival and the prognostic value of *LHPP* in human HCC by Kaplan-Meier survival analysis. Real-time PCR and Western Blot were used to examine the expression levels of *LHPP* in normal liver cell line (LO2) and human HCC cell lines (SMCC-7721, HepG2, Huh7, MHCC-97 H, and LM3). Through lentivirus infection, we established human HCC stable cell lines (Huh7 and LM3) overexpressing *LHPP*. Then, we detected these cell viability, colony, and invasion. Subsequently, we performed the gene set enrichment analysis (GSEA) for the RNA-seq data of HCC patients from TCGA. Finally, we examined the expression level of several oncogenes, including *CCNB1*, *PKM2*, *MMP7*, and *MMP9*, in these cells via real-time PCR assay. Here, we found that *LHPP* is significantly downregulated in the human HCC tissues paired normal tissues. Furthermore, the high expression level of *LHPP* is associated with better clinical outcomes in human HCC. Overexpression of *LHPP* inhibits cell growth and metastasis in human HCC cells, and *LHPP* expression levels negatively correlate with cell cycle and metastasis in HCC tissues. Moreover, the level of *LHPP* is negatively correlated with *CCNB1*, *PKM2*, *MMP7*, and *MMP9* in human HCC cells and HCC tissues. These findings highlight a novel tumor suppressor in human HCC growth and metastasis, and provide a promising diagnostic and prognostic factor for human HCC.

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Introduction

Hepatocellular carcinoma (HCC), also known as hepatoma, is the most common primary liver malignancy and also the second most common cause of cancer-related death worldwide. HCC accounts for 90% of primary liver cancers [1–3]. There are diverse etiologies involved in HCC development, which is a multistep complicated process. And several crucial factors contribute to the process, such as hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, cirrhosis, diabetes mellitus, alcohol abuse, and aflatoxin [4–6]. So far, five conventional methods are still widely used in clinical: surgical resection, liver transplantation, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), and transarterial chemoembolization (TACE) [2]. Of note, due to the high incidence of recurrence, the long-term survival is still unsatisfactory and the 5-year recurrence rate

exceeds 70%. However, the molecular mechanisms for the occurrence and development of HCC are still largely unclear [7]. Therefore, it is imperative to identify novel genes or pathways that are involved during HCC progression.

Phosphohistidine phosphate inorganic pyrophosphatase (LHPP) is a member of the haloacid dehalogenase like hydrolase domain-containing (HDHD) gene family. It is also a new kind of inorganic pyrophosphatase (PPase), which is considered to be essential for life and closely related to cell survival, growth, and differentiation. Histidine phosphatase can dephosphorylate proteins containing histidine phosphate. The effect is opposite to that histidine-kinase. Their interaction plays an important role in tumor proliferation, invasion, metastasis, and poor prognosis [8–10]. Three histidine phosphatases were well known: , and *LHPP*,

and the role of the former two in tumor progression have been widely reported, but not *LHPP*. Moreover, there is still lack of evidence addressing *LHPP*'s function as a phosphatase in cancer development [11–14].

LHPP gene locates at chromosome 10 q26.13, and consists of three leucine zipper domains. It ubiquitously expresses in brain, kidney, and liver [15]. Early studies mainly focus on its psychiatric phenotypes and found an SNP at the *LHPP* gene (rs35936514/rs34997829) correlated with MDD and AD-RSB interaction [16–18]. Recently, increasing evidence showed that *LHPP* is closely linked to cancer development. Overexpression of *LHPP* is closely related to hyperthyroidism, while its low expression is related to tumor stage in Graves' disease and AFTN [19,20]. A genome-wide association study identifies risk loci in acute lymphoblastic leukemia [21]. And *LHPP* (rs201982221) acts as the significantly associated loci in oral and pharyngeal cancers [22]. Recent studies also showed *LHPP* acting as a tumor suppressor in cervical cancer and HCC (mainly by using HCC mouse model) [23–25]. Interestingly, *LHPP* overexpression can prevent liver function damage in L-dKO mice. L-dKO mice is an HCC mouse model by liver-specific deletion of *PTEN* and *TSC1*, and the L-dKO liver tumors mimicked poorly differentiated human HCC [23]. In their research, they just introduce the role of mouse *LHPP* in the HCC mouse model, but not mention humans. Yet, the role and mechanism of *LHPP* in human HCC are still largely unknown.

In the present study, we aimed to investigate the roles of *LHPP* in human HCC cell growth and metastasis. We showed that *LHPP* was down-regulated in human HCC tissues. Further, we demonstrated the reduction of *LHPP* in human HCC cell lines. Functional studies identified that overexpression of *LHPP* attenuated the cell viability, colony formation, and invasion in human HCC cells. Moreover, we also found that the expression of several oncogenes, including *CCNB1*, *PKM2*, *MMP7*, and *MMP9*, is suppressed by *LHPP* forceful expression in HCC.

Results

High expression of *LHPP* is associated with better clinical outcomes in HCC

Firstly, we analyzed the expression of *LHPP* from the Oncomine database (<https://www.oncomine.org/resource/main.html>) and we found that *LHPP* expression was significantly downregulated in the HCC tissues paired normal tissues (Figure 1(a,b)). Furthermore, we analyzed the

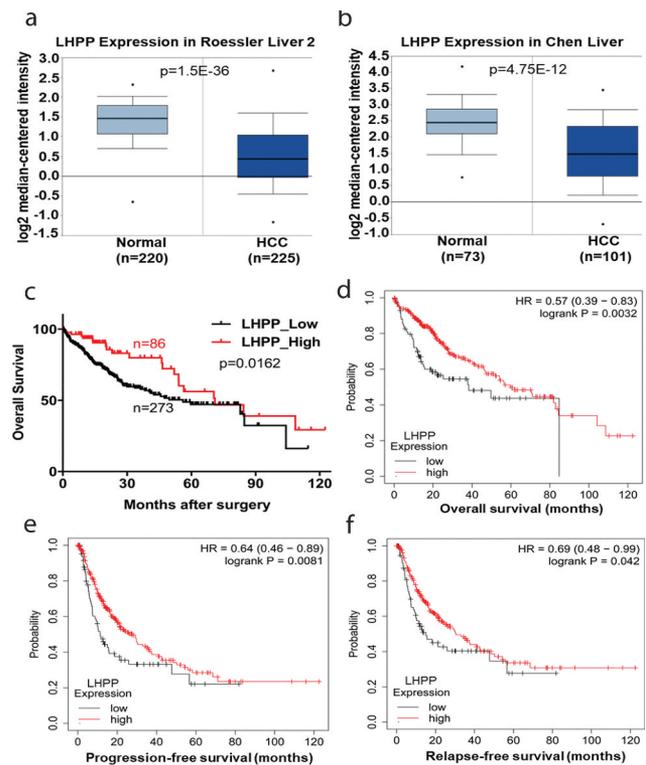


Figure 1. High expression of *LHPP* is associated with better clinical outcomes in HCC. (a–b) *LHPP* Expression in Roessler Liver 2 Statistics and *LHPP* Expression in Chen Liver Statistics. These data come from the Oncomine database (<https://www.oncomine.org/resource/main.html>). Gene expression of *LHPP* was down-regulated in the HCC tissues comparing with the normal tissues. (c) Kaplan-Meier curves of overall survival according to *LHPP* expression in HCC tissues. The overall survival was significantly reduced in the *LHPP* low expression group (*LHPP*-Low, $n = 273$) compared with the *LHPP* high expression group (*LHPP*-High, $n = 86$) (Log-Rank test, $p = 0.0162$). The HCC patients data comes from the TCGA database. (<https://tcga-data.nci.nih.gov/publications/tcga>). (d–f) The overall survival, progression-free survival and relapse-free survival probability were compared between *LHPP* high and *LHPP* low expression in the HCC patients from the Kaplan-Meier Plotter database (<https://kmplot.com/analysis/>). Log-rank p values were shown.

LHPP expression level in the tumor tissues derived from 359 postoperative HCC patients in the TCGA data (<https://tcga-data.nci.nih.gov/publications/tcga>). These tissues were grouped under *LHPP* low expression or *LHPP* high expression according to their mRNA levels. We next assessed the relationship between the *LHPP* expression level and patient overall survival by Kaplan-Meier survival analysis. The overall survival was extremely down-regulation in the *LHPP*-Low expression group compared with the *LHPP*-High expression group (Figure 1(c)). Consistently, data analysis from the Kaplan-Meier Plotter database (<http://kmplot.com/analysis/>) also revealed lower *LHPP* expression in HCC patients showed unfavorable overall survival, progression-free survival, and relapse-free survival times compared with the higher *LHPP* expression HCC patients (Figure 1(d-f)). Taken together, these data suggest that *LHPP* may play a vital role in HCC progression.

LHPP inhibits HCC cell viability and colony formation

In order to evaluate the function of *LHPP* in HCC cell growth and metastasis *in vitro*, we firstly examined the expression levels of *LHPP* in normal liver cell line (LO2) and HCC cell lines (SMCC-7721, HepG2, Huh7, MHCC-97 H, and LM3), the Real-time PCR assay showed that the expression level of *LHPP* in the normal liver cell was significantly increased compared with the HCC cell lines (Figure 2(a)). In addition, the Western Blot assay showed a similar result in the above cells (Figure 2(b)), suggesting *LHPP*'s tumor suppressor activity in HCC progression. Lentivirus-mediated overexpression of *LHPP* in the Huh7 and LM3 cells which were performed to assess the function of *LHPP* in the HCC cell viability and colony formation. The efficiency of *LHPP* overexpression in the Huh7 and LM3 cells was assessed by Western Blot assay (Figure 2(c)). Next, assessed the *LHPP*-high expression HCC cell viability ability via the CCK8 assay. Overexpression of *LHPP* in the Huh7 and LM3 cells significantly inhibited the cell viability (Figure 2(d,e)). Also, overexpression of *LHPP* inhibited colony formation ability of the above cells (Figure

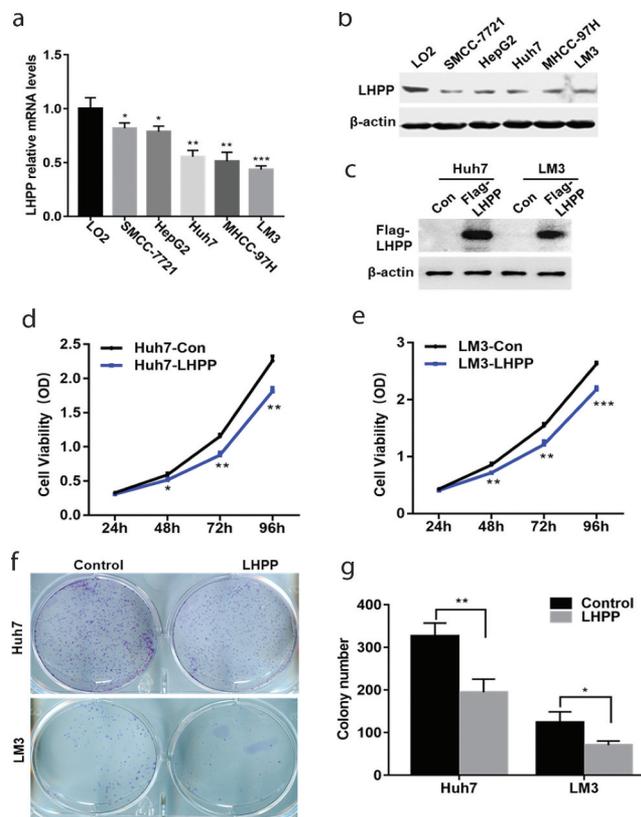


Figure 2. *LHPP* inhibits HCC cell viability and colony formation. (a-b) The expression level of *LHPP* was detected in a normal liver cell line (LO2) and HCC cell lines (SMCC-7721, HepG2, Huh7, MHCC-97 H, and LM3) using Real-time PCR assay and Western Blot. Means \pm SD from three independent experiments were presented as a relative ratios to the LO2 whose value was taken as 1.0. (c) Overexpression of *LHPP* in the Huh7 and LM3 cells infected with lentivirus. The efficiency of overexpression *LHPP* in the above cells were assessed by Western Blot. (d-e) Overexpression of *LHPP* down-regulated the cell viability in the Huh7 and LM3 cells using the CCK8 assay. (f-g) Overexpression of *LHPP* in the Huh7 and LM3 cells significantly inhibited cell colony formation assay in colony formation assay. The permeable cells were stained with Giemsa. Significant differences were determined using a one-way analysis of variance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

2(f,g)). Taken together, these results indicate that *LHPP* inhibits HCC cell growth *in vitro*.

LHPP prevents HCC cell migration and invasiveness

We next assessed whether *LHPP* had an inhibitory role in HCC cell migration and invasiveness ability via transwell assay. Overexpression of *LHPP* in the Huh7 and LM3 cells significantly inhibited cell

migration ability. *LHPP* overexpression displayed sharp declines in cell migration compared with the control group (Figure 3(a,b)). Moreover, *LHPP* had a similar role in the HCC cells invasiveness ability (Figure 3(c,d)). Collectively, the data showed that *LHPP* can prevent HCC cell metastasis.

***LHPP* expression levels negatively correlated with cell cycle and metastasis in human HCC tissues**

To explore the mechanism underlying *LHPP*'s suppression of HCC cell growth and metastasis, we firstly analyzed the RNA-seq data of HCC patients from TCGA-LIHC (<https://xenabrowser.net/datapages/?dataset=TCGA.LIHC>; dataset ID: TCGA.LIHC. -sampleMap/HiSeqV2), and then divided into two groups, *LHPP* low expression, and *LHPP* high expression. Next, we performed the gene set enrichment analysis (GSEA) for the above data (Figure 4(a)). Using GSEA, we showed

that cell cycle, mitotic spindle, and G2 M checkpoint pathways were found significantly enriched in HCC patients with *LHPP* low expression group (Figure 4(b-d)). Moreover, Liver metastasis and Roessler liver cancer metastasis were also highly enriched in the *LHPP* low expression group (Figure 4(e,f)), suggesting *LHPP*'s suppression in HCC metastasis. However, GSEA analysis showed that metabolic pathways such as oxidative phosphorylation and fatty acid metabolism were remarkably compromised in the *LHPP* low expression group (Figure 4(g,h)). Thus, these findings indicated that *LHPP* suppresses cell cycle and metastasis in the HCC tissues.

***LHPP* decreases the expression of *CCNB1*, *PKM2*, *MMP7* and *MMP9* in HCC cells**

Next, we wanted to investigate any possible anti-tumor mechanisms about *LHPP* in human HCC cells. We firstly detected the several oncogenes (including *CCNB1*, *PKM2*, *MMP7*, and *MMP9*) expression levels in the above *LHPP* overexpression HCC cells via Real-time PCR assay, and found that overexpression *LHPP* decreased *CCNB1*, *PKM2*, *MMP7*, and *MMP9* mRNA levels (Figure 5(a,b)). Moreover, a negative correlation was found between the expression levels of *LHPP* and *CCNB1* in human HCC tissues ($r = -0.34$, $p < 0.0001$) (Figure 5(c)). Also, the similar results could be found in the *PKM2*, *MMP7*, and *MMP9* genes (Figure 5(d-f)). These results suggested that *LHPP* constrained HCC cell growth and metastasis, likely through acting on the expression of *CCNB1*, *PKM2*, *MMP7*, and *MMP9*.

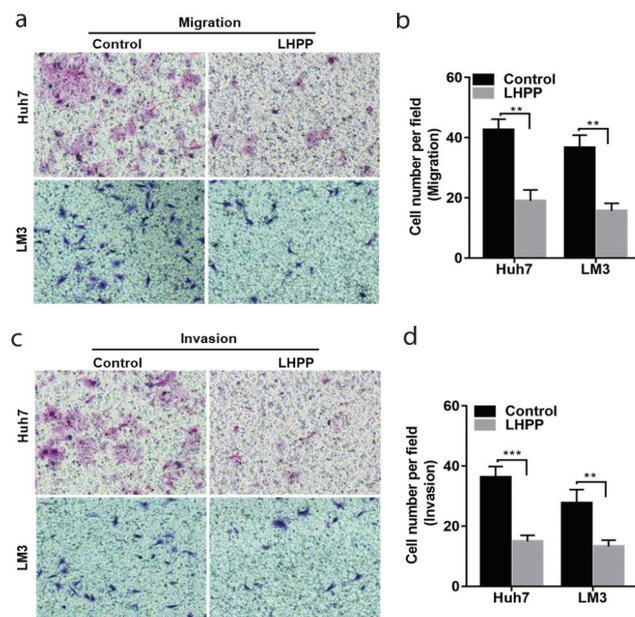
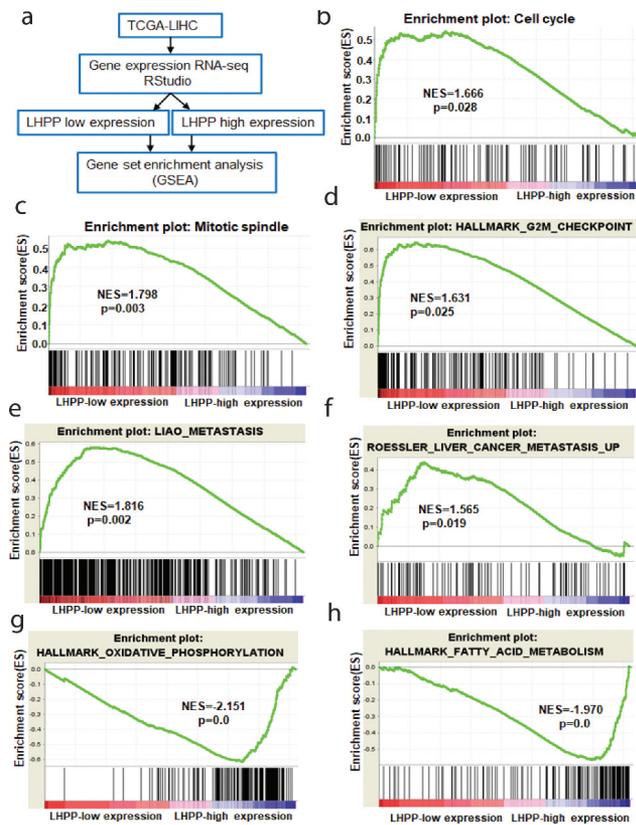


Figure 3. *LHPP* prevents HCC cell migration and invasiveness. (a-b) Overexpression of *LHPP* in the Huh7 and LM3 cells significantly inhibited cell migration ability. (c-d) Used the matrigel transwell assay to investigate *LHPP*'s role in cell invasiveness. The permeable cells were stained with Giemsa. Significant differences were determined using Student t-test, ** $P < 0.01$, *** $P < 0.001$.

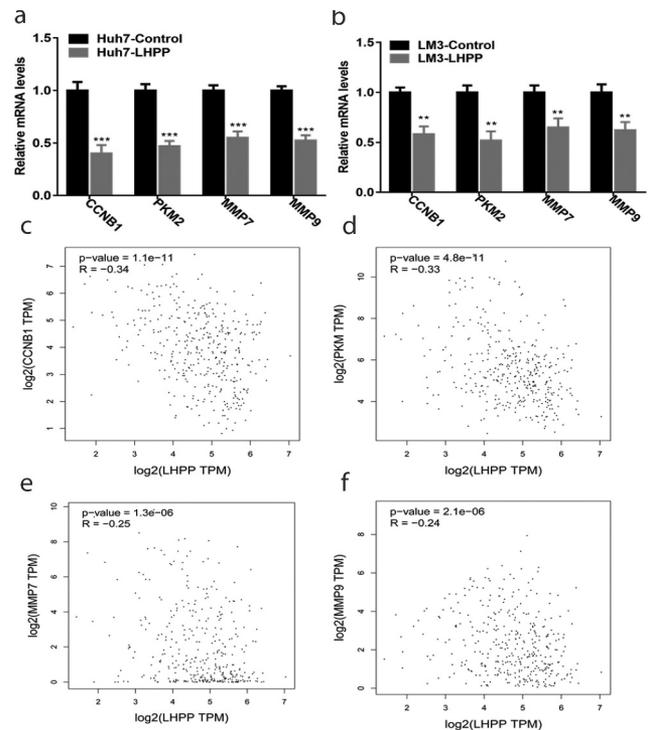
Discussion

In the present study, the TCGA database and the Oncomine database showed that *LHPP* was down-regulated in HCC tissues and high expression of *LHPP* was associated with better clinical outcomes in HCC. And then, we found that the protein and mRNA levels of *LHPP* were reduced in human HCC cells. A functional study demonstrated that *LHPP* reduction promoted the growth and metastasis of human HCC cells. In addition, we further



analyzed the RNA-seq data of HCC patients from TCGA using GSEA, and found that *LHPH* expression levels negatively correlated with cell cycle, mitotic spindle, G2 M checkpoint pathway, and metastasis in human HCC tissues. Furthermore, we showed that *LHPH* decreased the expression of *CCNB1*, *PKM2*, *MMP7*, and *MMP9* in human HCC cells.

Currently, it has been reported that *LHPH* knockdown can accelerate cell proliferation, metastasis, and apoptosis by modulating AKT in cervical cancer [24]. Consistent with this finding in bladder cancer, knockdown of *LHPH* can enhance the cell viability and colony formation via inactivating AKT/p65 signaling [25].



However, there are few studies available reporting on the roles of *LHPH* in human HCC cells. The present study showed that *LHPH* is down-regulation in HCC cells and overexpression of *LHPH* inhibits the growth and metastasis of HCC cells. However, there are still few studies that describe the precise role of *LHPH* in human HCC and its underlying mechanisms still unclear. In this study, we found that *LHPH* expression levels negatively correlated with cell cycle, mitotic spindle, G2 M checkpoint pathway and metastasis in human HCC tissues. Since the *CCNB1*, *PKM2*, *MMP7*, and *MMP9* are the key genes in involving the above pathways, we next detected these oncogenes expression in the *LHPH* overexpressing HCC cells. Also, our data

showed that overexpression *LHPP* decreased CCNB1, PKM2, MMP7, and MMP9 expression. Thus, we conclude that there is a certain relationship between *LHPP* and these oncogenes, which may represent a new therapeutic target for HCC.

In this context, we mainly elucidate the effect of *LHPP* on the growth and metastasis of human HCC cells. Consequently, we found that *LHPP* negatively correlates with cell cycle, mitotic spindle, G2 M checkpoint pathway, and metastasis in human HCC tissues. Importantly, we further demonstrated that *LHPP* reduces the expression levels of CCNB1, PKM2, MMP7, and MMP9 genes. This provides a new idea for the follow-up study of human HCC.

Materials and methods

LHPP expression analysis from the TCGA database

The transcriptional level of *LHPP* in HCC tissues was analyzed from the Oncomine database (<https://www.oncomine.org/resource/main.html>). A total of 329 HCC specimens and 226 normal tissues were available from the *LHPP* Expression in Roessler Liver 2 Statistics and *LHPP* Expression in Chen Liver Statistics.

Kaplan-Meier survival analysis of the *LHPP*

HCC tissues contain patient survival information coming from the TCGA database (<https://tcga-data.nci.nih.gov/publications/tcga>). According to the expression and median value of the *LHPP* gene in HCC patients, they were divided into two groups: high expression group and low expression group. The survival curve was drawn from the Kaplan–Meier Plotter database (<http://kmplot.com/analysis/>).

The gene set enrichment analysis (GSEA) of the *LHPP*

GSEA was performed using RNA-seq sets from TCGA-LIHC (<https://xenabrowser.net/datapages/>

`dataset=TCGA.LIHC; datasetID:TCGA.LIHC.sampleMap/HiSeqV2`). According to the mRNA level of the *LHPP* gene in HCC patients, they were divided into two groups: high expression group and low expression group.

Cell culture and stable cell lines

Human HCC cell lines (SMCC-7721, Huh7, HepG2, MHCC-97 H, and LM3) and normal liver cells (LO2) were from Cell Bank of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All cells were passaged in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, HyClone), 1x Pen/Strep, and 4 mM L-glutamine and incubated at 37°C in 5% CO₂.

For over-expression of *LHPP*, Flag-tagged full-length *LHPP* cDNA was cloned in the pCDH-CMV-MCS-EF1-Puro plasmid. For lentivirus production, HEK293 T cells transfected with the lentiviral vectors and helper plasmids DR8.91 and VSVG. Forty-eight-hour and 72 h after transfection, virus-containing media were collected and concentrated by centrifugation at 10,000 g for 18 h. Cells were transduced using lentivirus with 8 µg/ml polybrene and then selected with the appropriate antibiotics.

RNA isolation and quantitative real-time PCR

Total RNA extraction of the HCC cells was extracted using TRIzol (Invitrogen) and 1.5 µg RNA was reverse transcribed by the First Strand cDNA Synthesis Kit (Marligen Biosciences). Quantitative real-time PCR was performed by SYBR Green PCR Master Mix (Applied Biosystems) in the ABI 7300 Detection System (Applied Biosystems). PCR reactions were done in triplicates with following conditions: 95°C/30 s, 40 cycles of 95°C/10 s, 55°C/10 s and 72°C/10 s on Roche cycler and repeated at least three times. Relative mRNA levels were calculated using

Table 1. Primer sequences for qRT-PCR.

Resource	Gene name	Primer sequence (5' 3')
Human	LHPP	Forward GCTGGACGTTTGTCCCTACA Reverse GTGGGCTTCCACTCCTATCG
Human	CCNB1	Forward GCAGCAGGAGCTTTTGTCTT Reverse CCAGGTGCTGCATAACTGGA
Human	PKM2	Forward GCCTGCTGTGTCGGAGAAG Reverse CAGATGCCTTGCGGATGAATG
Human	MMP-7	Forward TTGATGGGCCAGGAACACG Reverse AGACTGCTACCATCCGTCCA
Human	MMP-9	Forward CTGGAGGTTGACGTGAAGGC Reverse GGCTTCTCTCGGTACTGGAAG
Human	β-actin	Forward TCCCTGGAGAAGAGCTACG Reverse GTAGTTTCGTGGATGCCACA

the $-\Delta\Delta C_t$ method using β -actin as control and expressed as $2^{(-\Delta\Delta C_t)}$. See Table 1 for the complete list of qPCR primer sequences.

Western blot analysis

Cells were lysed in RIPA buffer (Beyotime) containing protease inhibitors (KeyGen Biotech) for 30 min on ice and centrifuged at 4°C at $16,000 \times g$ for 15 min to collect the supernatant. The protein concentrations were measured by Bradford assay (Bio-Rad Labs) and mixed with loading buffer before boiled. Equal amounts of protein samples were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were then blotted with primary antibodies overnight at 4°C, followed by incubation with the secondary antibody (1:5000). The proteins were then developed using enhanced chemiluminescence (ECL, Thermo Fisher Scientific). Antibody used for western blot: LHPP (Proteintech), β -actin (Proteintech).

Cell viability analysis

Cell viability was measured with a CCK-8 kit (Boster). Huh7 and LM3 cells stably expressing vector or LHPP (2,500 cells/well) were seeded into 96-well plates. The above cells were cultured at 37°C in 5% CO₂ for 24, 48, 72, and 96 h, the medium of each well was replaced with 10% CCK-8 solution in fresh medium. The above cells were then incubated for 2 h, afterward,

absorbance at 450 nm was measured using a microplate reader.

Colony formation assay

The above HCC cells were seeded, respectively, in six-well plates at a density of 500 cells/well at 37°C in a 5% CO₂ humidified environment. After incubation about 2 wk, the plates were washed by PBS and fixed with methanol for 1 h, and then stained with Giemsa solution (Solarbio) for 30 min. Subsequently, the plates were washed with clean water and the number of the above cell colonies was counted.

Transwell migration and invasion

For transwell migration and invasion assays, matrigel (BD Biosciences) was just used in the invasion assay. Ten percent matrigel allowed to polymerize at the base of the top chamber of a 24 well transwell plate for 30 min at 37°C. The above HCC cells (2×10^5 cells/well) were starved in serum-free medium for 16 h and added to the top chambers. The bottom chambers were filled serum-containing medium. After 48 h, we used the cotton swabs to remove the non-invading cells from the upper surface of the membrane. The above cells that invaded were fixed in 4% paraformaldehyde for 30 min and then stained with 0.1% crystal violet for 30 min at room temperature. Excess dye was washed off with water.

Statistical analysis

Statistical analysis was performed with SPSS 24. The Kaplan–Meier survival analysis, Student t-test, the Pearson test, and one-way analysis of variance were used to compare the difference of the variables. P-value <0.05 was considered statistically significant.

Disclosure statement

The authors declare no conflict of interest.

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