Lamc1 promotes the Warburg effect in hepatocellular carcinoma cells by regulating PKM2 expression through AKT pathway

GuanXiong Ye, Yong Qin, Shi Wang, DeBiao Pan, ShengQian Xu, ChengJun Wu, XinMei Wang, Jun Wang, HaiLin Ye, and HeJuan Shen

Department of Hepatobiliary Surgery, People Hospital of LiShui, The Sixth Affiliated Hospital of Wenzhou Medical University, The First Affiliated Hospital of LiShui University, LiShui, Zhejiang, PR China

ABSTRACT
Hepatocellular carcinoma (HCC), the most common aggressive malignancy of the liver, is the third leading cause of cancer death across the world. Laminin gamma 1 (Lamc1), encodes laminin-γ1, an extracellular matrix protein involved in various progresses such as tumor cell proliferation and metabolism. In the present study, high expression of Lamc1 and PKM2 was observed in tumor tissues of HCC patients. In vitro, down-regulation of Lamc1 inhibited proliferation of HCC cells by promoting cell death, reduced glucose consumption and lactate production, accompanied by a decrease in the expression of glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA), and PTEN increased, as well as PTEN S380 and AKT S473/T308 phosphorylation decreased, while Lamc1 up-regulation had the opposite effect. The effects of PKM2 were similar to that of Lamc1 and markedly counteracted the effects of Lamc1 down-regulation. In addition, Lamc1-induced increase in PKM2 expression was strongly attenuated by a PI3K inhibitor, LY294002 or a si-p110 PI3K, with a significant decrease in GLUT1 and LDHA expression, as well as decreased AKT T308 phosphorylation. Thus, we speculated that Lamc1 was implicated in the progression of HCC probably by regulating PKM2 expression through PTEN/AKT pathway.

Introduction
Hepatocellular carcinoma (HCC), an aggressive malignancy of the liver, is the third leading cause of cancer death in the world and its incidence is steadily increasing across the whole world.¹ In recent years, hepatic resection or transplantation as well as radiofrequency ablation are considered to be potentially curative therapies for HCC.²,³ Although surgery is an effective method for the treatment of HCC, due to the high aggressiveness and frequent recurrence, the prognosis of HCC is still poor, and its survival rate is only 20% to 30% even after hepatic resection.⁴

Warburg effect, defined over 80 y ago, is a unique metabolic phenotype in cancer cells, through this way, glucose metabolites are preferentially converted into nucleotides, amino acid and other cell structural blocks that meet the demands of tumor metabolism and growth.⁵–⁷ Accumulated studies have shown that the enhanced Warburg effect is mainly reflected in an increase in glucose consumption and lactate production, which is observed in many human cancers including HCC, and is closely related to tumor aggressiveness and poor prognosis of patients.⁸–¹¹ Tumor-specific pyruvate kinase M2 (PKM2), one isoform of pyruvate kinase, is reported to regulate the final rate-limiting step of glycolysis. PKM2 is a special glycolytic enzyme that catalyzes the synthesis of pyruvate and ATP, thereby contributing to the Warburg effect, which contributes to the growth, survival, and metabolism of cancer cells.¹²–¹⁵ Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) as a tumor suppressor antagonizes the action of PI3K-AKT pathway in many human cancers.¹⁶ The PI3K-AKT pathway is well known to be the major signal cascades regulating glucose metabolism, and activation of AKT regulates various processes involved in cancer, such as cell cycle progression and growth, as well as controlling glucose consumption through the glucose-transporter 1 (GLUT1) transporter.¹⁷

Laminin gamma 1 (Lamc1) gene encodes laminin-γ1, an extracellular matrix protein belonging to a family of laminins and involving in the assembly of basement membranes and various progresses including the growth and metastasis of tumors.¹⁸–²⁰ Studies have closely related Lamc1 mRNA to the malignancy of HCC. Lamc1, reported to highly express in HCC tumor, promotes tumor metastasis and predicts the poor prognosis of HCC.²¹ As a trans-regulator, Lamc1 promotes the migration and invasion of HCC cells by stimulating CD151 expression through competing for microRNA-124.²² However, the effect of Lamc1 on Warburg effect in HCC remains unclear.

In this study, high expression of Lamc1 and PKM2 was detected in tumors of HCC patients. In HCC cells, Lamc1 down-regulation inhibited cell proliferation, reduced glucose consumption and lactate production, with decreased GLUT1 and LDHA, increased PTEN, as well as decreased PTEN S380 and AKT S473/T308 phosphorylation, while Lamc1 up-regulation had the opposite effect. The effects of PKM2 were similar to that of Lamc1 and markedly counteracted the

CONTACT Yong Qin lsyegx@126.com Department of Hepatobiliary Surgery, People Hospital of LiShui, The Sixth Affiliated Hospital of Wenzhou Medical University, The First Affiliated Hospital of LiShui University, The 15th Public Street of Liandu District, LiShui, Zhejiang 323000, China

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effects of Lamc1 down-regulation. In addition, Lamc1-induced increase in PKM2 expression was strongly attenuated by a PI3K inhibitor, LY294002 or a si-p110 PI3K, concurrent with decreased GLUT1 and LDHA expression, and AKT T308 phosphorylation. Thus, we speculated that Lamc1 was implicated in the progression of HCC probably by regulating PKM2 expression through PTEN/AKT pathway.

Materials and methods

Tumor tissues and adjacent normal tissues

Forty tumor tissues and 20 matched adjacent normal tissues of HCC patients who were treated at People Hospital of LiShui were collected. The tissue samples were frozen in liquid nitrogen prior to use. All clinical specimens of humans were obtained with informed consent, and all experiments of this study were approved by the Ethics Committee of LiShui University. After RNA extraction, the expression of Lamc1 and PKM2 in these tissues was detected by real-time PCR.

Cell culture

Three human HCC cell lines of HepG2, Hep3B and Huh7, as well as a normal hepatocyte cell line of LO2 were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were cultured in a 37°C, 5% CO₂ incubator with high glucose DMEM medium (HyClone, USA) containing 10% fetal bovine serum ([FBS], Gibco, USA) and 1% double-antibiotic (penicillin and streptomycin, Solarbio, China). According to the demands of the cells, the medium was refreshed everyday or every 2 d during incubation.

Construction of the lentivirus

Specific siRNA designed for the target gene is synthesized by Genewiz Company (Shanghai, China) and inserted into Angel I/EcoI I restriction sites of a pLKO.1-Puro plasmid. The coding DNA sequence (CDS) regions of Lamc1 and PKM2 were, respectively synthesized to insert into EcoR I/BamH I restriction sites of pLVX-Puro plasmids. Constructed pLKO.1-Puro-siLamc1, pLVX-Puro-Lamc1 or pLVX-Puro-PKM2 (Addgen, USA) were co-transfected with packaging plasmids psPAX2 and pMD2G (Addgen) into 293T cells by Lipofectamine 2000 (Invitrogen). After 48 h of transfection, the packaged lentiviruses in the medium were collected.

Experimental grouping

To regulate Lamc1 level in HepG2, Hep3B and Huh7 cells lines, the cultured cells were divided to infect with lentivirus of Lamc1 interference (siLamc1-1, siLamc1-2 and siLamc1-3)/negative control (Vector), Lamc1 overexpression (oeLamc1)/Vector, or PKM2 overexpression (oePKM2)/Vector, while the cells treated with medium were as Control. After 48 h of infection, the expression of Lamc1 and PKM2 were quantified by real-time PCR and western blot analysis. To explore the effect of Lamc1 on these cells, assays of cell proliferation were performed at 0, 24, 48 and 72 h. Subsequently, biochemical detections of glucose uptake and lactate level, as well as western blot for genes expression were carried out.

To investigate the effect of PKM2 on HepG2, four groups were divided to respectively infect with Vector, siLamc1, oePKM2, and siLamc1 + oePKM2. The assays of cell proliferation, biochemical detections and western blot were performed 48 h later. For further study of mechanism of Lamc1, Huh7 cells were randomly divided and respectively infected with Vector + siNC, oeLamc1 + siNC, Vector + LY294002/si-p110 PI3K (a PI3K inhibitor), and oeLamc1 + LY294002/si-p110 PI3K. Western blot analysis for associated-genes expression was performed after infection.

Proliferation assay

Logarithmic growth phase cells were digested with 0.25% trypsin (Solarbio) into a cell suspension of 3 × 10⁴ cells/mL. Each 100 μL of cell suspension was seeded in 96-well culture plates with three identical wells as duplicate wells, and cultured in a 37°C, 5% CO₂ humidified incubator overnight. The next day, the cells were infected with lentivirus for 0, 24, 48 and 72 h, and then 100 μL of Cell Counting Kit 8 (CCK8, CCK8: serum-free medium = 1: 10, SAB) was added to each well. After 1 h of incubation, a microplate reader (Perlong, Beijing) was used to measure the optical density (OD) of the absorbance at 450 nm.

Real-time polymerase chain reaction (RT-PCR)

The total RNA of tissues or lentivirus-infected cells was isolated by Trizol reagent (Invitrogen, 1596-026). After quantified, the integrity of RNA was confirmed by electrophoresis using 1% agarose gel. Using a reverse transcriptase kit (Fermentas, #K1622), the RNA extraction was reversed into cDNA. And then with a SYBR Green PCR kit (Thermo, #K0223), RT-PCR reactions were conducted on a Real-time detector (ABI, ABI-7300, USA). Relative to GAPDH, the mRNA levels of Lamc1 and PKM2 were analyzed using the method of 2⁻△△CT. The primers used were as follows: Lamc1, 5′ ACTCTTAATCTTGGAAGCAAGAAGGGTGTGAAC 3′ and 5′ GAATGACGCAAACAGACAGCACAACTTGAAC 3′; PKM2, 5′ AGCAAGAAGGGTGTGAAC 3′ and 5′ AATCCCATCACAGCAAGAAGGGTGTGAAC 3′; GAPDH, 5′ AGGCTGTTGTCATACTTCACTCTTC 3′ and 5′ CATCTTC 3′. In addition, the procedure of RT-PCR as follows: 95°C, 10 min (95°C, 15 s; 60°C, 45 s) × 40; 95°C, 15 s; 60°C, 1 min; 95°C, 15 s; 60°C, 1 min; 95°C, 15 s; 60°C, 1 min; 95°C, 15 s; 60°C, 1 min.

Western blot analysis

Treated-cells were lysed in RIPA buffer (Solarbio) containing protease and phosphatase inhibitors and incubated for 30 min on ice to homogenize fully. The proteins in the supernatant of lysates were collected after centrifuged for 10 min at 12000 g at 4°C, followed by quantified by a BCA protein kit (Thermo). After separation by SDS-PAGE electrophoresis, the proteins were semi-dry transferred by electroblotting onto polyvinylidene fluoride (PVDF) membranes (Millipore). Successfully transferred membranes were sheared, and then blocked in
5% skim milk (BD Biosciences, USA) at room temperature for 1 h. Following the incubation with primary antibodies against Lamc1 (1:2000, Santa Cruz, Sc-17751), PKM2 (1:100, Abcam, Ab38237), PTEN (1:1000, Cell Signaling Technology [CST], #9552), AKT (1:1000, CST, #9272), p-AKT (Ser473; 1:1000, CST, #9271), GLUT1 (1:8000, Abcam, Ab115730), LDHA (1:1000, Abcam, Ab101562), or GAPDH (1:2000, CST, #5174) overnight at 4°C with gentle shaking, the membranes were washed for 5–6 times and incubated with secondary antibodies (1:1000, Beyotime) for 1 h at room temperature. Subsequently, 5 min incubation of chemiluminescent reagent later, the target protein bands were visualized by an ECL imaging system (Tanon, Shanghai).

Biochemical detection
Logarithmic growth phase cells were inoculated in 6-well culture plates at a density of 5 × 10⁵ cells/well, and then cultured in a 37°C, 5% CO₂ incubator for 24 h. The next day, following 48 h infection of lentivirus, the cells were cultured with low glucose DMEM medium for 3 h. After washing of glucose-free Krebs-Ringer bicarbonate buffer (containing 2% BSA) at 37°C, the cells were incubated with glucose-free DMEM containing 100 µM 2-NBDG for 45 min. Finally, the cells were inoculated in 96-well plates after 3 times washing of iced PBS, and the fluorescence intensity was measured by a fluorescence microplate reader. In addition, after collected the supernatant of cultured medium, the lactate level was detected using a lactic acid kit in the guide of instructions.

Statistical analysis
The statistical analyses of all data were conducted by the software of GraphPad prism 7.0 (GraphPad Software, USA).

Results
Lamc1 is significantly increased in tumors of HCC patients and associated with PKM2 expression
In order to explore whether Lamc1 regulates the Warburg effect, the expression levels of Lamc1 and PKM2, a key glycolytic gene, in tumors of HCC patients were detected. As shown in Figure 1, compared with adjacent nontumour tissues, the mRNA levels of Lamc1 (Figure 1(a)) and PKM2 (Figure 1(b)) were significantly increased in tumors of HCC patients. Importantly, Pearson’s analysis showed a positive correlation between Lamc1 and PKM2 (Figure 1c). It suggested that Lamc1, associating with PKM2, may be implicated in HCC progression.

High expression levels of Lamc1 and PKM2 in HCC cell lines
In vitro, LO2 (normal hepatocytes), HepG2, Hep3B and Huh7 (HCC cell lines) were applied, and the expression levels of Lamc1 and PKM2 in these cells were detected. As shown in Figure 2, both mRNA and protein levels of Lamc1 (Figure 2(a,c)) and PKM2 (Figure 2(b,c)) were much higher in HCC cells than in...
normal hepatocytes, particular in HepG2 and Hep3B. It further evidenced the close correlation of Lamc1 and PKM2 with HCC.

**Down- and up-regulation of Lamc1 in HCC cell lines by lentivirus infection**

In HepG2, Hep3B and Huh7 cells, the expression of Lamc1 was regulated through lentivirus infection. The results shown in Figure 3, Lamc1 expression were significantly down-regulated by siLamc1 lentivirus infection in HepG2 (Figure 3(a)) and Hep3B (Figure 3(b)) cells, and the effects of siLamc1-1 and siLamc1-2 were much better, while oe-Lamc1 significantly up-regulated the expression of Lamc1 in Huh7 cells (Figure 3c). Therefore, due to their effective regulation, siLamc1-1, siLamc1-2, and oeLamc1 were used for subsequent experiments.
**Lamc1 is required for the proliferation of HCC cells**

To assess the effect of Lamc1 on the proliferation of HCC cells, lentiviruses of siLamc1-1, siLamc1-2 and oeLamc1 were utilized to down- or up-regulate the expression of Lamc1. After 0, 24, 48 and 72 h of infection, the proliferation of HepG2, Hep3B and Huh7 cells were evaluated by CCK8 assays. Data shown in Figure 4, down-regulation of Lamc1 potently suppressed the cell proliferation of HepG2 (Figure 4(a) and Hep3B (Figure 4(b)), on the contrary, Lamc1 up-regulation in Huh7 significantly promoted the cell proliferation (Figure 4(c)). In addition, the rates of cell death of Lamc1-silenced HepG2 (Figure 4(d)) and Hep3B (Figure 4(e)) cells were significantly increased, whereas Lamc1 up-regulation had no significant effect on cell death rates (Figure 4(f)). It indicated that down-regulation of Lamc1 in HCC cells inhibited cell proliferation by promoting cell death. Thus, Lamc1 was required for the proliferation of HCC cells.

**Down-regulation of Lamc1 inhibits the Warburg effect in HCC cells**

We also investigate the effect of Lamc1 on Warburg effect in HCC cells to understand the mechanism of Lamc1 tumorigenic function. As shown in Figure 5, a significant decrease in percentage of glucose consumption (relative to Control, Figure 5(a,d)) and lactate production (Figure 5(b,e)) was observed in Lamc1-silenced HCC cells, while up-regulation of Lamc1 had an opposite effect (Figure 5(g,h)). It is agreement with previous studies that glucose consumption and lactate production increased significantly in cancer cells. GLUT1, a basic high-affinity glucose transporter, is critically important in malignant glucose metabolism. It is reported that increased expression of GLUT1 offers more energy to malignant tumors. Lactate dehydrogenase A (LDHA), regulating lactate production of Warburg effect, is reported to be involved in the progression of cancers. These were consistent with our observation that the expression of GLUT1 and LDHA was positively regulated by Lamc1 in HCC cells (Figure 5(c,f,i)). These indicated that Lamc1 expression was critically important for Warburg effect and down-regulation of Lamc1 potently impaired Warburg effect of HCC.

**Lamc1 suppresses the cell proliferation and Warburg effect via regulating PKM2**

Studies have closely related a constitutively low PKM2 activity to Warburg effect. Here, lentiviruses of siLamc1 and oePKM2 were applied to explore the possible relationship between PKM2 and Lamc1. As shown in Figure 6, the expression of PKM2 was significantly up-regulated by oePKM2 infection in HCC cells (Figure 6(a,b)), and PKM2 up-regulation significantly promoted the cell proliferation (Figure 6(c) and elevated glucose consumption (Figure 6(e)) and lactate production (Figure 6(f)), but the cell death rates was less changed (Figure 6(d)). In addition, we found that the effects of Lamc1 down-regulation on the proliferation, cell death and glucose metabolism of HCC cells was potently counteracted by PKM2 up-regulation. Similarly, PKM2 up-regulation counteracted the inhibition of GLUT1 and LDHA expression in Lamc1-silenced HCC cells (Figure 6(g)). It further confirmed that Lamc1 regulated the cell proliferation and Warburg effect of HCC probably by modulating PKM2 expression.

![Figure 4](image_url)

**Figure 4.** Lamc1 is required for the proliferation of HCC cells. After infection with siLamc1 or oeLamc1 lentivirus, (a–c) the proliferation of HCC cells was evaluated by CCK8 assays at 0, 24, 48 and 72 h. (d–f). The cell death rates of HCC cells were evaluated by Trypan blue staining. With three repeated independent experiments, the data were shown as mean ± SD, *P < 0.05, **P < 0.01, and ***P < 0.001 compared with Vector.
Figure 5. Down-regulation of Lamc1 inhibits the Warburg effect in HCC cells. After down- and up-regulation of Lamc1, through biochemical detections, glucose consumption (a,d,g) and lactate production (b,e,h) of these cells were measured. Percentage of glucose consumption is relative to Control. (c,f,i). The protein levels of GLUT1 and LDHA was detected by western blot. With at least three independent experiments, the data were presented as mean ± SD, **P < 0.01, and ***P < 0.001 compared with Vector.

Figure 6. Lamc1 suppresses the cell proliferation and Warburg effect via regulating PKM2 (a,b). The efficiency of oePKM2 regulating PKM2 expression in HepG2 cells was determined by RT-PCR (a) and western blot (b). After co-infection of lentiviruses siLamc1 and oePKM2 in HCC cells, (c) the cell proliferation were assessed by CCK8 assays at 0, 24, 48 and 72 h. (d) The cell death rates in HepG2 were evaluated by Trypan blue staining. (e,f) The glucose consumption (e) and lactate production (f) of HCC cells were detected by biochemical detections. (g) The protein levels of PKM2, GLUT1 and LDHA were detected by western blot. With at least three independent experiments, the data were expressed as mean ± SD, **P < 0.01 and ***P < 0.001 compared with Vector, ##P < 0.01 and ###P < 0.001 compared with siLamc1.
**Lamc1 regulates PKM2 expression through the phosphorylation of PTEN/AKT pathway**

We then investigated the potential molecular mechanism by which Lamc1 regulates HCC cells by regulating PKM2 expression. As shown in Figure 7, we found that down-regulating Lamc1 in HCC cells significantly inhibited PKM2 expression, decreased AKT S473/T308 phosphorylation and PTEN S380 phosphorylation, and increased AKT, while AKT was unchanged (Figure 7(a,b)). On the contrary, up-regulation of Lamc1 had an opposite effect (Figure 7(c)). In addition, inhibition of PI3K by LY294002 or a si-p110 PI3K prevented Lamc1-induced increase in the protein levels of PKM2, GLUT1 and LDHA in HCC cells, accompanied by decreased AKT S473/T308 and unchanged AKT (Figure 7(d,e)). PTEN as a tumor suppressor is capable to counteract PI3K-AKT action. PTEN is the major substrate of PTEN, which is activated to form PIP3, and S380 is one of the phosphorylation sites of PTEN, regulating the stability of PTEN. The phosphorylation of PTEN S380 inhibits the phosphorylation of PIP2 to PIP3, and dephosphorylation of PIP3 inhibits PI3K/AKT signaling pathway. These results indicated that PTEN/AKT functioned upstream of PKM2 and was responsible for PKM2 expression, and through inhibition of PTEN/AKT pathway, Lamc1 suppressed the expression of PKM2 to further inhibit Warburg effect in HCC.

**Discussion**

HCC is the most common liver cancer causing death, and its incidence in both developing and developed countries is increasing. It has been reported that due to its high incidence and high mortality, the prognosis of HCC is poor. In the present study, we found that Lamc1 was highly expressed in HCC tumors, and may regulate the cell proliferation and Warburg effect in HCC by regulating PKM2 expression through inactivation of PTEN/AKT pathway, which may be a new potential therapeutic target for HCC.

Lamc1, one of the extracellular matrix proteins, is a part of the tumor microenvironment and essential for growth, metastasis and survival of tumors. Studies have reported that Lamc1 is an independent predictor of liver cancer prognosis, which is consistent with our observation that Lamc1 was significantly elevated in HCC tissues or cell lines. For further study, the expression of Lamc1 was down- and up-regulated by lentivirus in HCC cells. We confirmed that down-regulation of Lamc1 significantly inhibited the proliferation of HCC cells by promoting cell death, and inhibited the Warburg effect, accompanied by decreased GLUT1 and LDHA, two key factors involved in Warburg effect, whereas Lamc1 up-regulation was opposite. In addition, PKM2 up-regulation potently enhanced the proliferation and Warburg effect of HCC cells, and the effects of Lamc1 down-regulation on HCC cells were counteracted by PKM2 up-regulation. It is consistent with the previous observations that PKM2 promotes tumor angiogenesis, the expression of PKM2 coincides with cancer cell glycolysis addiction and is associated with chromosome segregation and mitosis in tumor cells, decreasing PKM2 can partly inhibit the growth of liver cancer cells. Furthermore, significantly decreased PKM2 expression was observed in Lamc1-silenced HCC cells, as well as decreased AKT S473/T308 and PTEN S380 phosphorylation and increased AKT S473/T308 and PTEN S380 phosphorylation.
PTEN. These showed that the expression of PKM2 was regulated by Lamc1 and there was a possible relationship between PKM2 and PTEN/AKT pathway. Inhibition of PI3K by LY294002 or a si-p110 PI3K significantly reduced Lamc1-induced increases in PKM2 expression, concurrent with decreased GLUT1 and LDHA expression, and AKT S473/T308. It demonstrated that PTEN/AKT functioned upstream of PKM2 and was responsible for PKM2 expression, and through the inhibition of PTEN/AKT pathway, Lamc1 suppressed the expression of PKM2 to further inhibit Warburg effect in HCC. Interestingly, it has been reported that through suppression of PKM2 activity, PARP14 promotes the Warburg effect in HCC, which is inconsistent with our results. This may be due to a negative correlation between PKM expression and PKM2 activity; however, further validation is still needed. And MEG3 inhibits the growth of HCC cells through the negative regulating PKM2 and β-catenin activity. PI3K-AKT pathway is a major signal cascade regulating glucose metabolism in human cancers. Through activation of PI3K-AKT pathway, Twist, a key regulator of EMT, promotes the glucose metabolism in breast cancer cells. And Lamin A/C proteins are reported to positively involve in malignant behavior through PI3K-AKT pathway. 14-3-3-β promotes HCC cell metastasis via PI3K-AKT-NF-κB pathway. Based on these, we inferred that Lamc1 down-regulation inhibited the proliferation and Warburg effect of HCC cells probably by regulating PKM2 expression through inactivation of PTEN/AKT pathway.

In summary, we demonstrated that down-regulation of Lamc1 significantly suppressed proliferation and Warburg effect of HCC cells by regulating PKM2 expression through inactivation of AKT pathway. Targeting Lamc1 may provide a new potential therapeutic target for HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Funding
This study was supported by Public Technology Research and social development project of Science and Technology Department of Zhejiang Province (2017C33184).

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