Original Paper

Overexpression of PCK1 Gene Antagonizes Hepatocellular Carcinoma Through the Activation of Gluconeogenesis and Suppression of Glycolysis Pathways

Yufu Tang^{a,b} Yibing Zhang^a Chunhui Wang^a Zhongyi Sun^a Longfei Li^a
Shuqun Cheng^c Wenping Zhou^a

^aDepartment of Hepatobiliary Surgery, The General Hospital of Shenyang Military Area Command, Shenyang, ^bPost-doctoral Station, The General Hospital of Shenyang Military Area Command, Shenyang, ^cEastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China

Key Words

Gluconeogenesis • Glycolysis • Hepatocellular carcinoma • Phosphoenolpyruvate carboxykinase 1

Abstract

Background/Aims: Gluconeogenesis, a reverse process of glycolysis, is suppressed in neoplastic livers. Cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK-C/PCK1, encoded by *PCK1*) is a step limiting enzyme of gluconeogenesis. The induced expression of the factor is reported to initiate gluconeogenesis process and antagonize hepatocellular carcinoma (HCC). In the current study, the effect of the modulation of *PCK1* expression on HCC was assessed. **Methods:** The levels of PCK1 in clinical HCC tissues and different HCC cell lines were investigated with real time quantitative PCR, immunochemistry, and western blotting. Thereafter, the expression of *PCK1* gene was induced in two HCC cell lines and the effect of the overexpression on proliferation and migration potentials of HCC cells was detected with CCK-8 assay, flow cytometry, TUNEL staining, and transwell assay. The activities of glycolysis and gluconeogenesis pathways in *PCK1*-overexpressed HCC cell lines were detected with specific kits to underlie the mechanism by which PCK1 exerted its function. The results of the *in vitro* experiments were validated with HCC xenograft rat models. **Results:** The expression levels of PCK1 were suppressed in HCC samples and in cells derived from HCC tissues. According to the results of the *in vitro* assays, the overexpression of PCK1 decreased viability, induced apoptosis, and inhibited migration in both HCC cell lines. The effect was associated with the suppressed glycolysis and the induced gluconeogenesis pathways, represented by the enhanced production of glucose and the limited production of pyruvic acid, lactate, citrate, and malate. The results of the *in vitro* assays were confirmed in rat models in that the growth rate of solid HCC tumors was reduced in mice transplanted with *PCK1*-overexpressed HCC cells. **Conclusion:** Findings outlined in the current study demonstrated that activating gluconeogenesis process via *PCK1* overexpression was a potential treating strategy against HCC.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Introduction

During the past decades, emerging evidence demonstrates that metabolic reprogramming is a typical characteristic of cancers [1]. With the theory becoming a central subject in cancer study, metabolic pathways associated with oncogenesis, tumor progression, and tumor metastasis have been and are being discovered [2]. During the initiation of metabolic reprogramming in tumors, abnormal metabolic profiles promote glucose uptake, glycolysis, and lactic acid production even in the presence of sufficient oxygen, which supports growth, proliferation, and survival of tumor cells [3, 4]. This so-called “Warburg” effect has been validated in distinct tumor types [5, 6]. By bypassing the mitochondrial respiration in oxygen-rich conditions albeit the increased level of glycolysis, tumor cells successfully develop the key feature of metabolic reprogramming, now termed as aerobic glycolysis [2]. The “glucose addiction” endows tumor cells with enhanced metabolic flexibility for their anabolic requirements [7]. On the basis of the above studies, more attention is being paid to strategies that can restore normal metabolic conditions of cancer patients [8–11].

Gluconeogenesis, a reverse process of glycolysis, generates glucose from small carbohydrate substrates such as pyruvate, lactate, glycerol, and gluconeogenic amino acids [12]. Given that gluconeogenesis drives the metabolic flux in parallel but opposite to glycolysis, scientists believe that activating gluconeogenesis will interrupt metabolic reprogramming, initiate metabolic stress, and lead to the imbalance of energy homeostasis in cancer cells. Therefore, modalities capable of modulating gluconeogenesis are attracting lots of interest in recent years [13] and plenty effort has been made to facilitate the development of gluconeogenesis-based anti-cancer therapies [8–11]. Of types of cancers, hepatocellular carcinoma (HCC) has been proved to be sensitive to the treatments based on gluconeogenesis activation [8–11].

As the primary organ responsible for endogenous glucose production, the function of liver is closely associated with gluconeogenesis. Numerous studies indicate that the development of neoplasm in livers is always associated with the impaired gluconeogenesis [9, 10]. Thus, the restoration of gluconeogenesis has been proposed as one of the primary tasks for the management of HCC. For example, p53 tumor suppressor protein enhanced gluconeogenesis by inducing the expressions of *G6PC* and *PCK2*, which prevented glucose from being shunted to pro-cancer pathways such as glycolysis and pentose phosphate pathway (PPP) [9]. Similarly, the inhibition of mTOR2 in HCC initiated shuttle of the glycolytic flux to gluconeogenesis along with inhibited proliferation and survival in cancer cell, which was also mechanistically linked to the up-regulation of *G6PC* and *PCK1* [10].

Phosphoenolpyruvate carboxykinase (PEPCK/PCK, encoded by *PCK*) and glucose-6-phosphatase (*G6Pase*, encoded by *G6PC*) are key enzymes of gluconeogenesis in the liver, of which PCK governs the rate-limiting step [2]. The enzyme catalyzes the conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP) and its activity is distributed both in cytosol and mitochondria. As a result, two enzymatically indistinct isozymes, PEPCK-C (*PCK1*) and PEPCK-M (*PCK2*), encoded by different genes (*PCK1* and *PCK2*) exist [14–16]. Compared with *PCK2*, *PCK1* has been widely studied and is considered as a potential target for the development of novel treating methods for HCC [8, 10, 17]. However, most previous researches explored the function of *PCK1* in an indirect way [8, 10, 17], few studies have made attempt to elucidate the role of *PCK1* in the development of HCC by directly controlling the expression of the factor. In the current study, the expression levels of *PCK1* in clinical HCC samples were investigated. Thereafter, the expression of *PCK1* in HCC cell lines was induced and the effect on the gluconeogenesis, glycolysis, proliferation, and migration in HCC cells was assessed. Furthermore, at molecular level, the expressions of indicators related to gluconeogenesis were detected to demonstrate the mechanism through which *PCK1* exerted its anti-HCC function.

Materials and Methods

Patients and tissue specimen collection

Twelve HCC patients were included in the current study from The General Hospital of Shenyang Military Area Command. Subjects should meet the following criteria: 1) HCC samples from the hepatectomy must be identified to be primary HCC and para-tumor tissues sampled from the patients must contain no HCC cells; 2) Patients should have detailed information of clinicopathological and prognostic information. Those who had received any therapy against HCC before the operations were excluded. Tumor and para-tumor samples collected during the operations were preserved in 4°C and -80°C respectively before sent for histological and molecular analyses. The study was approved by The General Hospital of Shenyang Military Area Command ethics committee for the relating screening, inspection, and data collection of the patients. All the subjects signed written informed consent forms. All the works were undertaken following the provisions of the Declaration of Helsinki.

Chemicals and antibodies

Antibody against PCK1 was purchased from USCN Life Science Inc (Catl. No. PAA936Hu01, Wuhan, China). Antibody against G6PC was purchased from Abcam (Catl. No. ab133964, Cambridge, MA). Antibody against cleaved caspase 3 was purchased from CST (Catl. No. #9661, USA). Antibody against β -actin was purchased from Santa cruz Biotechnology, Inc (Catl. No. sc-47778, Santa Cruz, CA). Secondary goat anti-rabbit HRP IgG antibody (Catl. No. A0216, China) and goat anti-mice HRP IgG antibody (Catl. No. A0208, China), and protein concentration detection kit (Catl. No. P0011) were purchased from Beyotime Biotechnology. Pyruvic acid detection kit (Catl. No. A081), glucose detection kit (Catl. No. F006), citrate detection kit (Catl. No. A128), and lactate detection kit (Catl. No. A019-2) were purchased from Nanjing Jiancheng Bioengineering Institute, China. PicoProbe™ fructose-6-phosphate fluorometric assay kit (Catl. No. K689) and malate colorimetric assay kit (Catl. No. K637) were purchased from BioVision (USA). In situ cell death detection kit (Catl. No. 11684795910) was purchased from Roche (Switzerland).

Cell cultures and animals

Human HCC cell line CSQT-2 was a gift from Prof. Shuqun Cheng. Human HCC cell lines hep3B (Catl. No. TCHu106) and Huh7 (Catl. No. TCHu182) were purchased from Shanghai Cell Bank of Chinese Science Academy. Human HCC cell line SMMC-7721 (Catl. No. ZQ0029) was purchased from Zhongqiaoxin Zhou Biotech Company. All the cell lines were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂ and 95% air. All the cell lines were cultured at a density that allowed cell division throughout the experiments. The expression of PCK1 at mRNA and protein levels in different cells were quantified with reverse transcription quantitative PCR (RT-qPCR) and western blotting assay respectively. SPF BALB/c-nu mice were purchased from Vital River (Beijing, China) and maintained in cages at room temperature (20–25°C) with a constant humidity (55 ± 5%) with free access to food and water in a 12:12-h light-dark cycle. All the animal experiments were conducted in the accordance with the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of Laboratory Animals of The General Hospital of Shenyang Military Area Command.

Construction of PCK1 expression vector and cell transfection

The whole ORF of *PCK1* was obtained from GenBank database (Accession number: NM_002591) and ligated into pcDNA3.1 to form *PCK1* expression vector pcDNA3.1-PCK1. According to the detections of PCK1 levels in different HCC cell lines, two cell lines with relatively lower PCK1 levels, hep3B and SMMC-7721, were selected for transfection of pcDNA3.1-PCK1 using Lipofectamine 2000 (Catl. No. 1024993, Invitrogen, San Diego, CA) according to the instruction for manufacturers. Each cell line was divided into three groups: A Control group, parental cells. B NC group, cells transfected with pcDNA3.1 vector. C PCK1 group, cells transfected with pcDNA3.1-PCK1 vector. Stable transfection was selected with G418 (400 µg/mL) and verified with RT-qPCR and western blotting.

Transplantation of HCC cells into nude mice

In vivo HCC model was induced by transplanting different HCC cell lines (hep3B and SMMC-7721) into the back skin of nude mice. BALB/c-nu mice were randomly divided into three groups: A Control group,

BALB/c-nu mice transplanted with HCC cells. B NC group, BALB/c-nu mice transplanted with HCC cells transfected with pcDNA3.1 vector. C PCK1 group, BALB/c-nu mice transplanted with HCC cells transfected with pcDNA3.1-PCK1 vector. All the mice were raised under the same condition for 21 days to allow the formation of solid tumor. Tumor volume was measured every three days since the 7th day after the transplantation: tumor volume = [(length of major axis) × (length of minor axis)²]/2. Upon completion of the raise, mice were sacrificed and tumor specimens were collected for histological and molecular analyses.

RT-qPCR

For RT-qPCR detection, total RNA in tumor tissues and HCC cells was extracted using RNA purified total RNA extraction kit according to the manufacturer's instruction (Calt. No. RP1201, BioTeke, Beijing, China). *β-actin* was selected as the internal reference gene. The total RNA was reversely transcribed to cDNA templates using super M-MLV reverse transcriptase (Calt. No. RP6502, BioTeke, Beijing, China). The final qPCR reaction mixture of volume 20 μL consisted of 10 μL of SYBR GREEN mastermix, 0.5 μL of each primers [PCK1, forward: 5'-GCTGGTGCCCTCTAGTCTATG-3', reverse: 5'-GGTATTTGCCGAAGTTGTAG-3'; G6PC, forward: 5'-GTGCCAAGGAAATGAGGATG-3', reverse: 5'-CACGGACACCAAGATGAACC-3'; hexokinase 2 (HK2), forward: 5'-TCTACCACATGCGCTCTCT-3', reverse: 5'-GCCCATTTGTCGTTACTTTC-3'; 6-phosphofructokinase 1 (PFK1), forward: 5'-CCAAGCGTCGGGTGTTATC-3', reverse: 5'-TGCAGGTCTCGAATGGTGAAG-3'; pyruvate kinase M2 (PKM2), forward: 5'-CGCTGGATAACGCCTACAT-3', reverse: 5'-CCATTTTCCACCTCCGTC-3'; *β-actin* forward: 5'-CTTAGTTGCGTTACACCCTTCTTG-3', reverse: 5'-CTGTCACCTTCACCGTTCCAGTTT-3'], 1 μL of the cDNA template, and 8 μL of Rnase free H₂O. Amplification parameters were as following: denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s and 72°C for 30 s, and the reaction was stopped at 4°C for 5 min. Relative expression levels of the target molecules were detected and calculated with Exicycler™ 96 (BIONEER) according to the expression of 2^{-ΔΔCt}.

Western blotting assay

Total protein in tumor tissues and HCC cells was extracted using the total protein extraction kit according to the manufacturer's instruction (Calt. No. WLA019, Wanleibio, China). *β-actin* was used as the internal reference. Concentrations of protein samples were determined using BCA method. 20 μL of protein (40 μg) was subject to a 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Target proteins were transferred onto polyvinylidene difluoride (PVDF) sheets and the membranes were washed with TTBS for 5 min before incubation in skim milk powder solution for 1 h. Primary antibodies against PCK1 (1:400), G6PC (1:1000), cleaved caspase 3 (1:1000), and *β-actin* (1:1000) were incubated with membranes at 4°C overnight. After four washes using TTBS, secondary HRP IgG antibodies (1:5000) were added onto the membranes for 45 min at 37°C. After additional six washes using TTBS, the blots were developed using Beyo ECL Plus reagent and the results were detected in the Gel Imaging System. The relative expression levels of proteins were calculated with Gel-Pro-Analyzer (Media Cybernetics, USA).

Immunohistochemical detection

Sections were made from different samples, examined independently by senior pathologists, and frozen immediately for PCK1 detection. For immunohistochemical detection, tissue slides were placed in 60°C overnight before being incubated with dimethylbenzene for dewaxing. The slides were hydrated with different concentrations of alcohol (100% for 5 min, 95% for 5 min, 85% for 5 min, and 75% for 5 min.) followed by washed with H₂O₂ for 5 min. Then slides were fixed using methanol solution with 3% H₂O₂ and blocked with 1% BSA for 30 min at 37°C. They were then incubated with primary antibody against PCK1 (1:100) at 37°C for 30 min before incubated at 4°C overnight. After four cycles of 0.01 M PBS washing, 5 min for each cycle, secondary antibody (1:200) was added onto the slides and placed at 37°C for 30 min before another four cycles of PBS wash. Then diaminobenzidine (DAB) was added to the slides and reacted for 3-10 min until the reaction was stopped by ddH₂O. Slides were re-stained using haematoxylin and dehydrated. Results were detected using a microscope (BX53, OLUMPUS, Japan) at 400× magnification

Determination of activity of glycolysis pathway in PCK1-overexpressed HCC cell lines

The activity of glycolysis pathway was determined by quantifying the levels of different products (including pyruvic acid, lactate, fructose-6-phosphate, and malate) of glycolysis. The expressions of key kinases involved in the regulation of glycolysis (including HK2, PFK1, and PKM2) were detected with RT-qPCR.

Glucose production assay

The production of glucose in cells was detected using glucose detection kit according to the manufacturers' instruction and normalized to total protein.

CCK-8 assay

Cell viabilities of *PCK1*-transfected HCC cell lines were measured using a CCK-8 method: briefly, 100 μ L CCK-8 solution was added to the cultures and incubated at 37°C for 1 h. The OD values at 450 nm of different treatments were detected using a Microplate Reader and normalized as relative cell viability (%) compared to control cells (untreated cells): $[\text{OD}_{450}]_{\text{sample}} / [\text{OD}_{450}]_{\text{control}} \times 100$ [18]

Flow cytometry assay

The apoptotic rates of HCC cells were determined with flow cytometry. Cells in different groups were collected with centrifugation at 2000 rpm for 5 min. Then the cell apoptotic rates were measured using an Annexin V-FITC Apoptosis Detection Kit (WLA001c, Wanleibio, Shenyang, China) according to the instruction for manufacturers: briefly, 5 μ L Annexin V was added to different wells. After incubation with Annexin V for 10 min at room temperature, the cells were re-suspended with 1 $\times$ Binding buffer and added with 5 μ L Propidium Iodide. The apoptotic rates were analyzed using a FACScan flow cytometry (Accuri C6, BD, USA) and the total apoptotic rate (UR+LR-all apoptosis cell percentage) was equal to the sum of the late apoptotic rate (UR, upper right quadrant-advanced stage apoptosis cell percentage) and the early apoptotic rate (LR, lower right quadrant-prophase apoptosis cell percentage).

Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL) staining

Cell apoptosis was detected using in situ cell death detection kit following the instruction for manufacturers. The apoptotic cells were stained brown and the results were detected using a microscope (BX53, OLUMPUS, Japan) at 200 $\times$ magnification.

Transwell experiment

The transwell experiment was performed to evaluate the migration ability of HCC cells: 200 μ L incubation (with 1 mM MgCl_2) medium containing 1×10^4 cells was seeded into the upper chamber of transwell chambers (Corning star, Cambridge, MA). Then cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 24 h to allow the migration through the porous membrane. Upon completion of the culture, cells remaining at the upper surface of the chamber were completely removed. The lower surfaces of the membranes were fixed with 4% paraformaldehyde for 20 min and stained in a solution containing 0.5% (w/v) crystal violet for 5 min. After being washed using ddH₂O, numbers of cells in five fields of each group were determined using Image-Pro Plus 6.0 software (Nikon) at 200 $\times$ magnification and averaged.

H&E staining

The histological changes in tumor specimens collected from nude mice were observed using Hematoxylin and eosin (H&E) staining: briefly, tissues were placed into Bouin solution (4% formaldehyde) for perfusion fixation, dehydrated using different concentrations of alcohol, and vitrified in dimethylbenzene. Afterwards, samples were embedded in paraffin, sectioned, and stained with H&E and the results were detected under a microscope at 200 $\times$ magnification. The nuclei in tumor tissue were stained blue and the cytoplasm was stained pink, and the degree of staining varied with the age of cells.

Statistical analysis

All the data were expressed in the form of mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA), ANOVA for repeated measurements, and post hoc test by Duncan method were performed using general liner model. Statistical significance was accepted with *P* value < 0.05. All the statistical analyses were conducted using SPSS version 19.0 (IBM, Armonk, NY, USA).

Results

Expression levels of *PCK1* were inhibited in clinical HCC samples and in HCC cell lines

In total, 12 HCC patients were included in the current study. At mRNA, the level of *PCK1* was highest in para-tumor samples (Fig. 1A), and decreased in tumor and tumor thrombus

tissues. Compared with tumor samples, the level of *PCK1* was even lower in tumor thrombus tissues. Based on statistical analyses, the difference between each two groups was significant (Fig. 1A) ($P < 0.05$). The changing pattern of protein was similar to that of mRNA: tissues from para-tumor samples had the highest level of *PCK1* while tumor thrombus tissues had the lowest *PCK1* level (Fig. 1B and Fig. 1C) ($P < 0.05$). Moreover, as shown in Fig. 1D, the immunohistochemical detection confirmed the results of western blotting assay: *PCK1* positive cells characterized by brownish-yellow particles were more extensively distributed in para-tumor tissues.

To verify the expression status of *PCK1* in HCC, the levels of *PCK1* in four HCC cell lines were also detected. As shown in Fig. 2, the expression of *PCK1* was lower in hep3B, Huh7, and SMMC-7721 cells which were derived from tumor tissues compared with CSQT-2 cells derived from portal vein tumor thrombus tissues. Two cell lines, hep3B and SMMC-7721 with the relatively lower levels of *PCK1* were selected for subsequent assays (Fig. 2).

Overexpression of *PCK1* blocked the glycolysis process in HCC cell lines

The transfection of expression vector increased the expression of *PCK1* both at mRNA and protein levels (Fig. 3) in HCC cells. The overexpression of *PCK1* suppressed the production of pyruvic acid, lactate, citrate, and malate in HCC cells, representing the blockade of glycolysis process due to the induced expression of *PCK1* (Fig. 4A) ($P < 0.05$). However, the production of fructose-6-phosphate was not significantly influenced by the overexpression of *PCK1* (Fig. 4A), which might be attributed to the increased production of fructose-6-phosphate in gluconeogenesis induced by *PCK1* overexpression. The results of biochemical detection were further validated by RT-qPCR. As shown in Fig. 4B, the expressions of *HK2* and *PKM2* were inhibited, representing an impaired glycolysis process by *PCK1* overexpression. It was also found that the expression of *PFK1* was not influenced by *PCK1* overexpression, partially explaining the production pattern of fructose-6-phosphate in cells of different groups.

Overexpression of *PCK1* decreased viability, induced apoptosis, and suppressed migration ability in HCC cell lines

The viabilities of both HCC cell lines were detected with CCK-8 assay and the results showed that the overexpression of *PCK1* markedly reduced cell viability (Fig. 5A) ($P < 0.05$). Correspondingly, the apoptotic process in both cell lines were initiated by *PCK1* overexpression as detected by flow cytometry (Fig. 5B) ($P < 0.05$). For hep3B cells, the late apoptotic rate was higher than the

Fig. 1. Expression of *PCK1* was suppressed in clinical HCC tissues. A, quantitative analysis results of RT-qPCR detection for *PCK1* gene with clinical samples. B, representative images of western blotting assay for *PCK1* with clinical samples. C quantitative analysis results of western blotting assay for *PCK1* with clinical samples. D, representative images of immunochemical detection of *PCK1* expression in Patient158802 and *PCK1* positive cells were stained brownish-yellow. N represents para-tumor tissues. T represents tumor tissues. Scale bar, 100 μ m. P represents tumor thrombus tissues. “*”, significantly different from N samples, $P < 0.05$. “#”, significantly different from T samples, $P < 0.05$. Each assay was represented by 12 replicates.

Fig. 2. Selection of HCC cell lines with lowest expression level of PCK1 gene. The expressions of PCK1 were lower in hep3B, Huh7, and SMMC-7721 cells which were derived from HCC tissues compared with CSQT-2 cells which were derived from portal vein tumor thrombus. Based on the detecting results, hep3B and SMMC-7721 were selected for further experiments. Each assay was represented by three replicates.

early apoptotic rate, but for SMMC-7721 cells, a reserve pattern was observed. The effect of *PCK1* overexpression on cell apoptosis was further assessed by TUNEL staining and western blotting detection of cleaved caspase 3: the induced expression of *PCK1* was associated with higher apoptotic rates (Fig. 5C) and higher cleaved caspase 3 levels (Fig. 5D), confirming the pro-apoptosis function of *PCK1* in HCC cells. Except for the influence on survival of HCC cells, the overexpression of *PCK1* also impaired migration potential of HCC cells. In transwell assays, the average numbers of cells penetrating the membranes were significantly smaller in *PCK1* groups compared with Control or NC group (Fig. 5E) ($P < 0.05$).

Antagonizing effect of *PCK1* on HCC cells was exerted by activating process

To confirm the increased level of gluconeogenesis in HCC cells, the expression of another step limiting enzyme in gluconeogenesis, G6PC, was also detected. Both at mRNA and protein levels, the overexpression of *PCK1* induced the expressions of G6PC (Fig. 6A and 6B). Moreover, the production of glucose in *PCK1*-overexpressed cell lines was increased as well, confirming the activation of gluconeogenesis (Fig. 6C). The initiation of gluconeogenesis process was concomitant with the inhibiting effect of *PCK1* overexpression on HCC cells, which implied that the function of *PCK1* in HCC was related to the activation of gluconeogenesis.

Fig. 3. Levels of PCK1 was increased in hep3B and SMMC-7721 cell lines after transfection of expression vector. A, quantitative analysis results of RT-qPCR detection for PCK1 gene with HCC cell sample. B, representative images and quantitative analysis results of western blotting assay for PCK1 with HCC cell samples. “*”, significantly different from Control group, $P < 0.05$. “#”, significantly different from NC group, $P < 0.05$. Each assay was represented by three replicates.

Fig. 4. Overexpression of PCK1 gene suppressed glycolysis process. A, quantitative analysis results of detection of pyruvic acid, lactate, citrate, malate, and fructose-6-phosphate production in different cell lines. B, quantitative analysis results of RT-qPCR detection for HK2, PKM2, and PFK1 expression with HCC cell samples. “*”, significantly different from Control group, $P < 0.05$. “#”, significantly different from NC group, $P < 0.05$. Each assay was represented by three replicates.

Overexpression of PCK1 inhibited the formation of solid tumor *in vivo*

To validate the inhibiting effect of PCK1 on tumorigenicity of HCC, BALB/c-nu mice were transplanted with HCC cells to induce *in vivo* HCC models. As shown in Fig. 7A and Fig. 7B, volume of solid tumors in different groups kept increasing until the completion of house. For mice transplanted with hep3B cells, the average tumor volume in PCK1 group was significantly smaller than Control and NC groups, and the differences since the second sampling time point were statistically significant (Fig. 7A; Table 1) ($P < 0.05$). A similar growth pattern was also detected for assays with SMMC-7721 cells (Fig. 7B; Table 1) ($P < 0.05$). Illustrated by H&E staining, in samples overexpressing PCK1, cell integrity was impaired, cytoplasm spread in wider range, and adhesion between cells were broken (Fig. 7C and Fig. 7D). Corresponding to the RT-qPCR and western blotting detection results of the *in vitro* experiments, the expressions of PCK1 and G6PC were both up-regulated, representing the initiation of gluconeogenesis *in vivo* (Fig. 7E-7H).

Discussion

Liver plays a determinant role in the maintenance of blood glucose concentration by supplying glucose to circulation and by removing glucose from circulation after meal ingestion [19]. One of the critical processes involved in the regulation of blood glucose by liver is gluconeogenesis. The process is described as the production of glucose from non-carbohydrate carbon sources and a reverse glycolysis pathway [2]. It is well recognized

that the concomitant activation or suppression of glycolysis and gluconeogenesis will cause a metabolic stress [2]. The metabolic reprogramming in cancer cells is mainly attributed to the “aerobic glycolysis” process which enhances the metabolic flexibility of cancer cells [7]. Thus, the modulation of glycolysis/gluconeogenesis has reasonably become an attractive therapeutic strategy for treatments of cancers.

In the present study, the expression and function of PCK1, a step limiting enzyme of gluconeogenesis, was detected *in vivo* and *in vitro* and employed as a target for the regulation of gluconeogenesis in HCC cells. The enzyme allows the conversion of small carbon metabolites and tricarboxylic acid cycle metabolites into glycolysis intermediates [9]. Based on the investigation with clinical HCC samples and different HCC cell lines, the expression of PCK1 was clearly suppressed in HCC. The overexpression of PCK1 in two HCC cell lines with relatively lower PCK1 levels inhibited cell viability and migration. Moreover, the antagonizing effect of PCK1 overexpression on HCC cells was concomitant with the suppression of glycolysis and the induction of gluconeogenesis, confirming the conclusion that the promoted gluconeogenesis in HCC would detrimentally affect cancer cell survival [10].

The suppression of gluconeogenesis in HCC has been reported by previous studies [9, 10, 20, 21]. Thus, exploring factors activating gluconeogenesis has become a central subject for the development of anti-HCC therapies. As mentioned above, p53 and mTOR2 have exhibited their potential to modulate gluconeogenesis both through the regulation of PCKs [9, 10]. However, the functions of these indicators are not restricted to the influence on glucose metabolism. Their complicated functions in diverse biological processes constrain their potent as targets for the development of anti-HCC therapies by modulating gluconeogenesis [9, 22-24].

For gluconeogenesis, the synthesis of PEP from OAA is an absolutely required process. Generally, this reaction strictly depends on the activity of PCK1 [25]. Mice with homozygous deletion of PCK1 gene are impaired by severe hypoglycemia and profound changes in lipid and amino acid metabolism after birth [26]. Therefore, it was expected in the current study that the overexpression of PCK1 would counteract the pro-survival function of metabolism reprogramming in HCC cells. The results of our experiments were promising in that PCK1 overexpression not only inhibited cell growth and migration *in vitro* but also restricted

Fig. 5. Overexpression of PCK1 gene reduced cell viability, induced cell apoptosis, and attenuated cell migration potential in hep3B and SMMC-7721 cell lines. A, quantitative analysis results of CCK-8 assay with different cell lines. B, representative images and quantitative analysis results of cell apoptotic rate in different cell lines. For hep3B cells, the proportion of late apoptotic rate was higher than that of early apoptotic rate. For SMMC-7721 cells, the proportion of late apoptotic rate was lower than that of early apoptotic rate. C, representative images and quantitative analysis results of TUNEL staining. D, representative images and quantitative analysis results of western blotting assays of cleaved caspase 3. E, representative images and quantitative analysis result of transwell assay with different cell lines. F, representative images and quantitative analysis result of transwell assay with different cell lines. “*”, significantly different from Control group, $P < 0.05$. “#”, significantly different from NC group, $P < 0.05$. Magnification, 200 $\times$. Each assay was represented by three replicates.

Fig. 6. Overexpression of PCK1 gene initiated the gluconeogenesis process in hep3B and SMMC-7721 cell lines. A, quantitative analysis results of RT-qPCR detection for G6PC gene with HCC cell samples. B, representative images and quantitative analysis results of western blotting assay for G6PC with HCC cell samples. C, quantitative analysis results of glucose production with HCC cell samples. “*”, significantly different from Control group, $P < 0.05$. “#”, significantly different from NC group, $P < 0.05$. Each assay was represented by three replicates.

Fig. 7. Overexpression of PCK1 gene restricted growth of solid HCC and activated the gluconeogenesis process animals models. A, quantitative analysis result of tumor volume changes in animals transplanted with hep3B cells. B, quantitative analysis result of tumor volume changes in animals transplanted with SMMC-7721 cells. C, representative images of H&E staining with tumor tissues in animals transplanted with hep3B cells. D, representative images of H&E staining with tumor tissues in animals transplanted with SMMC-7721 cells. E, quantitative analysis results of RT-qPCR detection for PCK1 gene with tumor samples. F, quantitative analysis results of RT-qPCR detection for G6PC gene with tumor samples. G, representative images and quantitative analysis results of western blotting assay for PCK1 with tumor samples. H, representative images and quantitative analysis result of western blotting assay for G6PC with tumor samples. “*”, significantly different from Control group, $P < 0.05$. “#”, significantly different from NC group, $P < 0.05$. Scale bar, 50 μ m. Each assay was represented by six replicates.

Table 1. Average tumor volume in mice transplanted with different HCC cell lines* - *Analyses based on ANOVA for repeated measurements showed that Group was an independent factor influencing the tumor volume. *, significantly different from Control group, $P < 0.05$. #, significantly different from NC group, $P < 0.05$. Each assay was represented by six replicates

Volume of tumor	Cell Line					
	Control	hep3B NC	PCK1	Control	SMMC-7721 NC	PCK
7 th day	18.59±4.7	23.3±10.4	21.6±8.1	20.0±5.6	14.2±1.9	16.8±2.3
10 th day	93.7±16.6	88.7±14.4	58.9±21.9*	83.7±39.3	71.2±24.2	70.4±40.9
13 th day	204.7±65.0	216.8±50.3	147.1±68.1*	278.5±81.4	169.1±50.0	159.7±83.9*
16 th day	340.1±121.9	461.9±102.6	238.6±87.7*	603.7±200.5	406.3±80.4	282.8±102.6*
19 th day	642.1±239.4	751.0±137.3	325.3±98.0*	838.5±223.9	913.2±267.8	410.7±133.8*
22 nd day	968.2±287.3	1021.7±192.3	434.5±126.3*	1318.7±335.0	1484.8±447.3	545.4±135.6*

growth and invasiveness of solid HCC tumors in animal models without evident side effects on animal survival. To further determine the effect of *PCK1* overexpression on glycolysis and gluconeogenesis, the levels of products and enzymes in glycolysis were measured. The results showed that in *PCK1*-overexpressed HCC cells, glycolysis process was evidently suppressed while gluconeogenesis process was initiated. The up-regulation of G6PC and PCK via mTOR2 inhibition induced the glucose production in cancer cells [10]. However, rather than increasing the mitochondrial oxidative phosphorylation, such redundant production of glucose only led to glucose consumption that was shunted off into gluconeogenesis [10]. Thus, the activation of *PCK1* and G6PC in cancer cells created a “futile” cycle where glucose consumption was increased but only to have it to be broken down and then recycled through gluconeogenesis. Moreover, distinct from factors such as p53 and mTOR2, the function of *PCK1* is highly related to glucose metabolism especially in liver tissues [27]. The intended modulation of *PCK1* gene has little influence on other biological processes, which can be employed as a highly specific candidate for the development of anti-HCC therapies.

In conclusion, the overexpression of *PCK1* showed lethal effect on HCC cells. The induced expression of *PCK1* not only inhibited cancer cell proliferation and migration *in vitro* but also restricted growth of solid tumors *in vivo*. Advantageous over the previous reported factors, the modulation of *PCK1* takes its action on the metabolism reprogramming feature of cancer cells and has little effect on other biological pathways. With more comprehensive studies in the future, the potential of *PCK1* as a highly specific and effective target for gluconeogenesis-based anti-HCC therapies will be fully explored.

Acknowledgements

The study was supported by National Natural Science Foundation of China (No. 81602571), Doctoral Scientific Research Foundation of Liaoning Province (No. 201603197), and Postdoctoral Science Foundation of China (No: 2015M572798).

Disclosure Statement

The authors disclose no conflict of interests.

References

- 1 Butler EB, Zhao Y, Munoz-Pinedo C, Lu J, Tan M: Stalling the engine of resistance: Targeting cancer metabolism to overcome therapeutic resistance. *Cancer Res* 2013;73:2709-2717.
- 2 Khan MW, Chakrabarti P: Gluconeogenesis combats cancer: Opening new doors in cancer biology. *Cell Death Dis* 2015;6:e1872.

- 3 Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000;100:57-70.
- 4 Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB: Brick by brick: Metabolism and tumor cell growth. *Curr Opin Genet Dev* 2008;18:54-61.
- 5 Leithner K, Hrzenjak A, Trotschmuller M, Moustafa T, Kofeler HC, Wohlkoeing C, Stacher E, Lindenmann J, Harris AL, Olschewski A, Olschewski H: Pck2 activation mediates an adaptive response to glucose depletion in lung cancer. *Oncogene* 2015;34:1044-1050.
- 6 Ward PS, Thompson CB: Metabolic reprogramming: A cancer hallmark even warburg did not anticipate. *Cancer Cell* 2012;21:10.
- 7 Cairns RA, Harris IS, Mak TW: Regulation of cancer cell metabolism. *Nat Rev Cancer* 2011;11:85-95.
- 8 Arai T, Kano F, Murata M: Translocation of forkhead box o1 to the nuclear periphery induces histone modifications that regulate transcriptional repression of pck1 in hepg2 cells. *Genes Cells* 2015;20:340-357.
- 9 Goldstein I, Yizhak K, Madar S, Goldfinger N, Ruppini E, Rotter V: P53 promotes the expression of gluconeogenesis-related genes and enhances hepatic glucose production. *Cancer Metabol* 2013;1.
- 10 Khan MW, Biswas D, Ghosh M, Mandloi S, Chakrabarti S, Chakrabarti P: Mtorc2 controls cancer cell survival by modulating gluconeogenesis. *Cell Death Discovery* 2015;1.
- 11 Sheikh TI, Adam T, Qadri I: Upregulated hepatic expression of mitochondrial pepck triggers initial gluconeogenic reactions in the hcv-3 patients. *Asian Pac J Trop Med* 2015;8:618-623.
- 12 Bhalla K, Liu W-J, Thompson K, Anders L, Devarakonda S, Dewi R, Buckley S, Hwang B-J, Polster B, Dorsey SG: Cyclin d1 represses gluconeogenesis via inhibition of the transcriptional coactivator pgc1 α . *Diabetes* 2014;63:3266-3278.
- 13 Ma R, Zhang W, Tang K, Zhang H, Zhang Y, Li D, Li Y, Xu P, Luo S, Cai W: Switch of glycolysis to gluconeogenesis by dexamethasone for treatment of hepatocarcinoma. *Nat Commun* 2013;4.
- 14 Chang HC, Lane MD: The enzymatic carboxylation of phosphoenolpyruvate. II. Purification and properties of liver mitochondrial phosphoenolpyruvate carboxykinase. *J Biol Chem* 1966;241:2413-2420.
- 15 Nordlie RC, Lardy HA: Mammalian liver phosphoenolpyruvate carboxykinase activities. *J Biol Chem* 1963;238:2259-2263.
- 16 Hanson RW, Garber AJ: Phosphoenolpyruvate carboxykinase. I. Its role in gluconeogenesis. *Am J Clin Nutr* 1972;25:1010-1021.
- 17 Kim H-J, Jee HJ, Yun J: DNA damage induces down-regulation of pepck and g6p gene expression through degradation of pgc-1 α . *Acta Bioch Bioph Sin* 2011:gmr053.
- 18 Agirre M, Zarate J, Puras G, Ojeda E, Pedraz JL: Improving transfection efficiency of ultrapure oligochitosan/DNA polyplexes by medium acidification. *Drug Deliv* 2014;22:100-110.
- 19 Shao J, Qiao L, Janssen RC, Pagliassotti M, Friedman JE: Chronic hyperglycemia enhances pepck gene expression and hepatocellular glucose production via elevated liver activating protein/liver inhibitory protein ratio. *Diabetes* 2005;54:976-984.
- 20 Gold J: Cancer cachexia and gluconeogenesis. *Ann NY Acad Sci* 1974;230:103-110.
- 21 Bongaerts GP, van Halteren HK, Verhagen CA, Wagener DJ: Cancer cachexia demonstrates the energetic impact of gluconeogenesis in human metabolism. *Med Hypotheses* 2006;67:1213-1222.
- 22 Sahin E, Colla S, Liesa M, Moslehi J, Muller FL, Guo M, Cooper M, Kotton D, Fabian AJ, Walkey C, Maser RS, Tonon G, Foerster F, Xiong R, Wang YA, Shukla SA, Jaskelioff M, Martin ES, Heffernan TP, Protopopov A, Ivanova E, Mahoney JE, Kost-Alimova M, Perry SR, Bronson R, Liao R, Mulligan R, Shiriha OS, Chin L, DePinho RA: Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* 2011;470:359-365.
- 23 Wang S-J, Yu G, Jiang L, Li T, Lin Q, Tang Y, Gu W: P53-dependent regulation of metabolic function through transcriptional activation of pantothenate kinase-1 gene. *Cell Cycle* 2013;12:753-761.
- 24 Sabatini DM: Mtor and cancer: Insights into a complex relationship. *Nat Rev Cancer* 2006;6:729-734.
- 25 Stark R, Guebre-Egziabher F, Zhao X, Feriod C, Dong J, Alves TC, Ioja S, Pongratz RL, Bhanot S, Roden M, Cline GW, Shulman GI, Kibbey RG: A role for mitochondrial phosphoenolpyruvate carboxykinase (pepck-m) in the regulation of hepatic gluconeogenesis. *J Biol Chem* 2014;289:7257-7263.
- 26 Hakimi P, Johnson MT, Yang J, Lepage DF, Conlon RA, Kalhan SC, Reshef L, Tilghman SM, Hanson RW: Phosphoenolpyruvate carboxykinase and the critical role of cataplerosis in the control of hepatic metabolism. *Nutr Metab* 2005;2:33.
- 27 Beale EG, Harvey BJ, Forest C: Pck1 and pck2 as candidate diabetes and obesity genes. *Cell Biochem Bioph* 2007;48:89-95.