Knockdown of the Sonic Hedgehog (SHH) Gene Inhibits Proliferation of Hep3B and SMMC-7721 Hepatocellular Carcinoma Cells via the PI3K/Akt/PCK1 Signaling Pathway

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Background: The PCK1 gene encodes phosphoenolpyruvate carboxykinase (PEPCK), which has been shown have a role in metabolic events in hepatocellular carcinoma (HCC). This study aimed to investigate the role of the SHH gene and its encoded protein, sonic hedgehog (SHH), in two human hepatocellular carcinoma (HCC) cell lines.

Material/Methods: The human HCC cell lines Hep3B and SMMC-7721 were cultured. Cells were transfected with plasmids carrying specific SHH gene short-hairpin RNA (shRNA) and negative control (NC) shRNA. The effects of knockdown of expression levels of the SHH gene were studied on cell survival, cell apoptosis, the cell cycle, gluconeogenesis, and the expression of PCK1. Anchorage-independent growth, a characteristic of transformed cells, was detected by the colony formation assay. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot were performed 24 h after transfection.

Results: Knockdown of expression levels of the SHH gene reduced cell proliferation and growth of HCC cells and induced cell apoptosis and G1 cell cycle arrest in both HCC cell lines. Knockdown of the SHH gene decreased the levels of glycolysis products and increased the production of glucose and reduced the phosphorylation of PI3K and Akt but induced the expression of PCK1.

Conclusions: Knockdown of the SHH gene reduced cell survival of HCC cells by increasing apoptosis, reducing cell proliferation, inducing G1 cell cycle arrest, and restoring gluconeogenesis, and was associated with the inhibition of the PI3K/Akt axis and induced the expression of PCK1.

MeSH Keywords: Hedgehog Proteins • Liver Neoplasms • Phosphatidylinositol 3-Kinases • Phosphoenolpyruvate Carboxykinase (ATP)

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Background

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer [1] and is the second leading cause of cancer-related death, worldwide [2,3]. Chronic inflammatory diseases of the liver are known risk factors for the development of HCC [4–7], which might explain the high incidence of HCC during the past decades [8]. However, with the rapid development of treatment strategies, the incidence, morbidity, and mortality associated with HCC continue to increase. In 2017, the overall 5-year survival for patients with HCC was reported to be between 10–20% [9], which supports the need for the identification of novel therapeutic targets for HCC.

Metabolic reprogramming has been shown to be involved in oncogenesis [10]. The liver is the primary organ involved in endogenous glucose production and energy turnover, the normal function of the liver is a prerequisite for gluconeogenesis. However, during the progression of HCC, changed metabolic profiles associated with the tumor growth increase glucose uptake, glycolysis, and lactic acid production [11,12], which results in the Warburg effect, and supports the growth and survival of tumor cells [13,14]. There is increasing interest in the pathways involved in the regulation of the metabolic changes that occur during tumor progression [15]. Phosphoenolpyruvate carboxykinase (PEPCK/PCK) encoded by the PCK genes are the key enzymes regulating the process of gluconeogenesis process in the liver and govern the rate-limiting step in gluconeogenesis [15]. The activity of PEPCK is identified in the cytosol and mitochondria and two distinct isoforms exist that are encoded by different genes (PCK1 and PCK2) [16–18]. Previous studies have shown that PCK1 is a candidate target for developing treatments for HCC that act by restoring the metabolic properties of liver cells [19–21]. Khan et al. reported that the inhibition of mTOR in HCC initiated glycolytic flux in the gluconeogenesis pathway by upregulating the expression of PCK1, which suppressed the proliferation and survival of cancer cells [20]. Therefore, regulating the expression of PCK1 has been considered as a possible future targeted treatment strategy in HCC.

The function of the PCK1 gene is influenced by multiple upstream regulators and the identification of these regulators would be important to understand before considering the applications of PCK1 in the treatment of HCC. The sonic hedgehog (SHH) and PI3K/Akt axis is a well-established signaling transduction axis that has been identified in multiple cancer types, including HCC [22,23]. Therefore, the inhibition of SHH gene signaling has now been considered as a promising method to inhibit the progression of multiple cancers [24]. The PI3K/Akt pathway has been shown to promote phosphorylation of forkhead box O (FOXO) transcription factors, including FOXO1, which will result in the suppression of PCK1 gene transcription [25]. Also, the PI3K/Akt pathway is closely associated with gluconeogenesis in the liver. For example, activation of the PI3K/Akt pathway can suppress gluconeogenesis, as shown in several previous studies [26–28]. Therefore, it can be hypothesized that knockdown of the expression of the SHH gene may have a potential role in suppressing tumor cell growth in HCC associated with downstream activation of PCK1-mediated gluconeogenesis following inhibition of the PI3K/Akt pathway.

Therefore, this study aimed to investigate the role of the SHH gene and its encoded protein, SHH, in two human HCC cell lines, with the assessment of cell viability, cell apoptosis, and production of gluconeogenesis-related enzymes and PI3K/Akt and PCK1 signaling activity following SHH gene knockdown.

Material and Methods

Agents and antibodies

The following primary antibodies were used in this study: SHH (bs-1544R) and p-PI3K (bs-5538R) (Beijing Biosynthesis Biotechnology Co., Ltd., China); PCK1 (PAA936Hu01) (USCN Life Science Inc., China); cleaved caspase-3 (ab2302) and cleaved poly ADP-ribose polymerase (PARP) (ab32561) (Abcam, Cambridge, MA, USA); Bcl-2 (BA0412), Bax (BA0315), and PCK3 (BA1352) (Boster Bio, Beijing, China); p-Akt (Ser 473) (sc-8312), Akt (sc-135651) and β-actin (sc-47778) (Santa Cruz Biotechnology Inc., Dallas, TX, USA). The following secondary antibodies were used: goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (A0216) and goat anti-mouse HRP-conjugated IgG (A0208) (Beyotime, Shanghai, China).

The transfection kit (c1507) was purchased from Applygen Technologies Inc. (Shanghai, China) and the RNA extraction kit (RP1201) and reverse transcription-polymerase chain reaction (RT-PCR) kit (PR6502) were purchased from BioTeke (Beijing, China). The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay solution (M-2128) was purchased from Sigma-Aldrich, St. Louis MO, USA). Detection kits for pyruvic acid (A081), glucose (F006), citrate (A128), and lactate (A019-2) were purchased from Nanjing Jiancheng Bioengineering Institute, China. PicoProbe™ fructose-6-phosphate fluorometric assay kit (K689) and the malate colorimetric assay kit (K637) were obtained from Biovision Inc., (Milipitas, CA, USA). RIPA lysis buffer (P0013B) and the BCA protein concentration kit (P0009) were provided by Beyotime, Shanghai, China.

Cell cultures and transfection

The human hepatocellular carcinoma (HCC) cell line, Hep3B (TCHu106) was provided by the Shanghai Cell Bank of the
**Table 1.** Sequence data for the short-hairpin RNA (shRNA).

| Gene | Forward | Reverse | Sequence (5'-3') |
|------|---------|---------|-----------------|
| SHH shRNA-1 | CCCCAGATCATATTTAAGGAT | ACAGCTAGAGAGAGGAGGA | CTGTCACCTTCACCGTTCCAGTTTT |
| SHH shRNA-2 | GTTCTTGTAGATCTTTCTTC | AAGATGAGAGAGGAGGA | CTGTTGACAGCGAGACCAT |
| Control shRNA | GTGTTGACAGCGAGACCAT | - | - |

Chinese Science Academy. The human HCC cell line, SMMC-7721 (ZQ0029) was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd (Shanghai, China). Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in an atmosphere consisting of 5% CO₂ and 95% air. The cell lines were cultured to a density that allowed sufficient cells for further experiments.

Plasmids carrying specific SHH gene short-hairpin RNA (shRNA) and negative control (NC) shRNA (Table 1) were constructed by Sangon Biotech, Shanghai, China, with the sequences as previously published [29]. Each HCC cell line was divided into three groups: the parental group, the NC group, HCC cells transfected with NC plasmid, and the shSHH group, HCC cells transfected shRNA plasmid. Transfection was conducted using transfection agents according to the manufacturer’s instruction and stable knockdown of the SHH gene was screened with G418 (geneticin) (0.5 μg/μl) and was further verified using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot assays, 24 h after transfection. The off-target effect of gene knockdown was eliminated with experiments using another shRNA (Table 1, Supplemenatry Figure 1).

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

Whole RNAs in cells under different treatments were extracted and reverse transcribed into cDNA template using specific kits, following the manufacturer’s instruction. The final qPCR reaction mixture volume of 20 μl contained 10 μl of SYBR Green Mastermix and 0.5 μl of each primer (10 μM) (Table 2), 1 μl of the cDNA template, and 8 μl of RNase-free H₂O. The amplification parameters were set as follows: 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. The relative expression level of the SHH gene was calculated with Data Assist Software version 3.0 (Applied Biosystems, Foster City, CA, USA) according to the expression of 2^{ΔΔct}.

**MTT assay**

HCC cell proliferation after knockdown of the SHH gene was detected with the MTT assay. Briefly, 1×10⁴ cells/ml were inoculated into a 96-well plate and cultured for 96 h. Every 24 h, 5 mg/ml of MTT solution was added into three randomly selected wells for each group and incubated for another 4 h. The cell viability at each time point was represented by the optical density (OD) value at 490 nm, detected using an ELX-800 microplate reader (Biotek, Winooski, VT, USA).

** Colony formation assay**

The effect of knockdown of the SHH gene on HCC cell anchorage-independent growth, a characteristic of transformed cells, was detected by the colony formation assay. Briefly, the cells were seeded into RPMI-1640 medium containing 3% methylcellulose at a concentration of 500 cells per plate. After two weeks of culture, the colonies were stained histochemically on the plates with Wright-Giemsa for 5 min. The numbers of colonies were recorded to compare the anchorage-independent growth capability between the groups. The colony formation rate was calculated as the ratio of colony number/inoculated cell number per plate ×100%.

**Flow cytometry for cell apoptosis and evaluation of the cell cycle**

Twenty-four hours after transfection, HCC cells were collected by centrifugation at 2000 rpm for 10 min. Flow cytometry was performed to determine the effect of knockdown of the SHH gene on the cell cycle distribution and cell apoptosis. For detection of cell cycle distribution, cells were incubated with 500 μl propidium iodide (PI)- fluorescein isothiocyanate (FITC) at 4°C for 30 min to determine the DNA content using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For detection of cell apoptosis, 5 μl of Annexin-V was added to the culture wells and incubated for 10 min at room temperature, followed by 5 μl of PI. Flow cytometry was performed to analyze the cell cycle distribution and cell apoptosis.

**Table 2.** Primer sequences.

| Gene   | Direction | Sequence (5’-3’) | Tm  | Product length (bp) |
|--------|-----------|-----------------|-----|---------------------|
| SHH    | Forward   | AGCAACAGCAGAGGA| 55.3| 219                 |
|        | Reverse   | AATGCAAGGAGGAGG| 53.5|                     |
| β-actin| Forward   | CTAGTTGCGTATACCC| 62  | 156                 |
|        | Reverse   | CTGTCACCTTACCGGT | 64.4|                     |
temperature before the cells were incubated with 5 μl of PI. The apoptosis rate was measured using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

**Gluconeogenesis activity**

The activity of the gluconeogenesis pathway was determined by quantifying the levels of glycolysis products, including pyruvic acid, lactate, fructose-6-phosphate, and malate, as well as the level of glucose using different detection kits according to the manufacturer’s instructions.

**Western blot**

The expression levels of components of the PI3K/Akt/PCK1 axis were detected with Western blot. Total protein was extracted using RIPA lysis buffer, and the protein concentrations were determined using a BCA protein measurement kit. Primary antibodies to SHH (1:500), cleaved caspase-3 (1:1000), cleaved PARP (1:1000), Bcl-2 (1:400), Bax (1:400), p-PI3K (1:500), PI3K (1:400), p-Akt (1:200), Akt (1:200), PCK1 (1:400), and β-actin (1:1000) were incubated with polyvinylidene difluoride (PVDF) membranes at 4°C overnight. The secondary HRP IgG antibodies (1:5000) were incubated with the membranes for 45 min at 37°C. The integrated optical density (IOD) of developed blots was detected using the gel Image-Pro system and the relative expression levels of proteins were calculated with Image-Pro analyzer (Media Cybernetics, Rockville, MD, USA).

**Statistical analysis**

Each assay was performed in triplicate and the data were expressed the mean ± standard deviation (SD). Statistical analysis and graphical data presentation were performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA). Statistical significance was considered to be a two-tailed P-value <0.05.

**Results**

**Knockdown of the SHH gene inhibited cell proliferation of Hep3B and SMMC-7721 hepatocellular carcinoma (HCC) cells**

The transfection of the SHH gene specific short-hairpin RNA (shRNA) suppressed the expression levels of SHH both at mRNA and protein levels (Figure 1). Knockdown of the SHH gene reduced Hep3B cell viability and proliferation at each time point when compared with cells in Parental and NC groups (P<0.05) (Figure 2A). Similar effects following SHH gene knockdown were also found in the SMMC-7721 cells (Figure 2B). These findings supported the role of SHH in HCC cell proliferation in vitro.

**Knockdown of the SHH gene reduced anchorage-independent growth of Hep3B and SMMC-7721 HCC cells**

Anchorage-independent growth, a characteristic of transformed cells, was detected by the colony formation assay for HCC cells after knockdown of the SHH gene. Following gene knockdown, significantly fewer colonies were detected for the Hep3B cells when compared with cells in Parental and NC groups (P<0.05) (Figure 2A) (Table 3). Inhibition of anchorage-independent growth by knockdown of the SHH gene was also found in the SMMC-7721 cells (Figure 2B) (Table 3). These findings supported the role of SHH in the maintenance of the HCC cell transformed state in vitro.
Knockdown of the SHH gene induced apoptosis and cell cycle arrest in Hep3B and SMMC-7721 HCC cells

The effect of knockdown of the SHH gene on the migration and invasion potential of HCC cells has previously been reported [22], but its effect on cell apoptosis and the cell cycle was not previously studied. In the present study, apoptosis and the cell cycle in HCC cells were studied after SHH gene knockdown, which increased the rate of cell apoptosis and the proportion of cell distributed in the G1 phase in both Hep3B and SMMC-7721 cells (Figure 4). The apoptosis rate was associated with increased expression of cleaved caspase-3, cleaved PARP, Bax, and reduced expression of Bcl-2 (Figure 4E, 4F). These results supported that inhibition of SHH gene expression reduced cell survival and increased cell apoptosis in HCC cells in vitro.
### Table 3. Data of the colony formation assays.

| Cell line | Group   | Data (%) | Rep 1   | Rep 2   | Rep 3   |
|-----------|---------|----------|---------|---------|---------|
| hep3B     | Parental| 55.67    | 45.83   | 49.00   |
|           | NC      | 49.17    | 43.97   | 42.67   |
|           | shSHH   | 25.50    | 27.33   | 22.00   |
| SMMC-7721 | Parental| 40.40    | 39.80   | 40.20   |
|           | NC      | 39.40    | 37.20   | 42.80   |
|           | shSHH   | 20.40    | 23.00   | 18.60   |

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**A**

![Image A]

**B**

![Image B]

**C**

![Image C]

**D**

![Image D]
Knockdown of the SHH gene activated gluconeogenesis in Hep3B and SMMC-7721 HCC cells

A hypothesis that drove this study was that the expression of the SHH gene might be associated with gluconeogenesis in HCC cells, and so the levels of products involved in glycolysis were measured. Knockdown of the SHH gene suppressed the production of products involved in glycolysis, including pyruvic acid, lactate, citrate, and malate in Hep3B and SMMC-7721 cells, and increased the production of glucose associated with gluconeogenesis (Figure 5) (P<0.05). However, the production of fructose-6-phosphate was not significantly affected by knockdown of the SHH gene (Figure 5), which might be attributed to the increased production of fructose-6-phosphate in gluconeogenesis.

Knockdown of the SHH gene induced the expression of PCK1 and inhibited the PI3K/Akt pathway in Hep3B and SMMC-7721 HCC cells

This preliminary study attempted to investigate the downstream pathway that mediated the effect of knockdown of the SHH gene. Based on the findings from previous studies [22,23], the activity of the PI3K/Akt/PCK1 axis was investigated. As shown by Western blot, knockdown of the SHH gene suppressed the phosphorylation levels of PI3K and Akt in both Hep3B and SMMC-7721 cells (Figure 6). Also, inhibition of the PI3K/Akt pathway was associated with increased levels of PCK1, partially explaining the regulating sequence driving the activation of gluconeogenesis following SHH gene knockdown.
In previous studies, the role of SHH in the liver cell oncogenesis has been studied in terms of its effects on HCC cell migration and metastasis [22,23]. In this study, the effects of SHH gene knockdown on HCC cells were investigated in terms of its effects on cell proliferation and anchorage-independent growth, a characteristic of transformed cells. Gene knockdown significantly suppressed the viability of HCC cells at each sampling time point as well as inhibiting anchorage-independent growth. The finding from the present study supplement those from previous studies on the role of SHH signaling and support its role not only in metastasis but also in survival and cell proliferation in HCC.

Also, the findings of the present study identified the levels of glycolysis products following knockdown of the SHH gene. The results showed that, apart from fructose-6-phosphate, the products of glycolysis were suppressed while the production of glucose was increased, indicating inhibition of glycolysis and the initiation of gluconeogenesis. The liver is the main organ involved in the maintenance of blood glucose levels by both supplying glucose to the circulation and removing glucose from the circulation [33]. One of the most important processes during the turnover of glucose is gluconeogenesis, and when this process is disturbed due to the concomitant activation or suppression of glycolysis [15], metabolic reprogramming enhances the metabolic flexibility of cancer cells [34]. Therefore, normal gluconeogenesis in the liver might represent a strategy for the control of HCC, which is a theory that was partially supported in the current study in that induced gluconeogenesis was associated with reduced survival of HCC cells. At the molecular level, the expression levels of p-PI3K, p-Akt, and anti-apoptosis gene might activate expression of glycolysis and activated gluconeogenesis in the HCC cell lines, Hep3B and SMMC-7721. Quantitative analysis results for pyruvic acid (A), lactate (B), citrate (C), malate (D), fructose-6-phosphate (E), and glucose (F) levels in the two cell lines, Hep3B and SMMC-7721. * P<0.05 vs. the normal control (NC) group.

**Figure 5.** Knockdown the SHH gene suppressed glycolysis and activated gluconeogenesis in the HCC cell lines, Hep3B and SMMC-7721.

**Discussion**

Sonic hedgehog (SHH) signaling transduction was first identified in Drosophila melanogaster has been shown to be involved in the development and maintenance of homeostasis of normal organs. In oncogenesis, the abnormal activity of SHH signaling has been reported in several tumor types. For example, Kasperczyk et al. showed that SHH signaling promoted resistance to apoptosis and that this was mediated by NF-κB in pancreatic carcinoma and rhabdomyosarcoma [30]. Similar results were also found by Feldman et al., who showed that inhibition of SHH gene signaling reduced the metastatic potential of pancreatic cancer [31]. The role of the SHH gene in the metastatic potential of HCC was also previously reported by Sun et al., who showed the association with reduced activity of the PI3K/Akt pathway, which inhibited migration ability of HCC cells by inhibition of SHH-Gli1 signaling [22]. Based on the findings from these previous studies, the main aim of the present study was to further explore the downstream pathway mediating the function of the SHH gene in the progression of HCC. The findings showed that the SHH gene was also involved in maintaining the survival of HCC cells in vitro and that inhibition of expression of the SHH gene might activate gluconeogenesis by inducing the expression of PCK1, mediated through inhibition of the PI3K/Akt pathway.

In previous studies, the role of SHH in the liver cell oncogenesis has been studied in terms of its effects on HCC cell migration and metastasis [22,23]. In this study, the effects of SHH gene knockdown on HCC cells were investigated in terms of its effects on cell proliferation and anchorage-independent growth, a characteristic of transformed cells. Gene knockdown significantly suppressed the viability of HCC cells at each sampling time point as well as inhibiting anchorage-independent growth. The finding from the present study supplement those from previous studies on the role of SHH signaling and support its role not only in metastasis but also in survival and cell proliferation in HCC.

Also, the findings of the present study identified the levels of glycolysis products following knockdown of the SHH gene. The results showed that, apart from fructose-6-phosphate, the products of glycolysis were suppressed while the production of glucose was increased, indicating inhibition of glycolysis and the initiation of gluconeogenesis. The liver is the main organ involved in the maintenance of blood glucose levels by both supplying glucose to the circulation and removing glucose from the circulation [33]. One of the most important processes during the turnover of glucose is gluconeogenesis, and when this process is disturbed due to the concomitant activation or suppression of glycolysis [15], metabolic reprogramming enhances the metabolic flexibility of cancer cells [34]. Therefore, normal gluconeogenesis in the liver might represent a strategy for the control of HCC, which is a theory that was partially supported in the current study in that induced gluconeogenesis was associated with reduced survival of HCC cells. At the molecular level, the expression levels of p-PI3K, p-Akt, and anti-apoptosis gene might activate expression of glycolysis and activated gluconeogenesis in the HCC cell lines, Hep3B and SMMC-7721. Quantitative analysis results for pyruvic acid (A), lactate (B), citrate (C), malate (D), fructose-6-phosphate (E), and glucose (F) levels in the two cell lines, Hep3B and SMMC-7721. * P<0.05 vs. the normal control (NC) group.
factors were inhibited, while the level of pro-apoptosis factors was increased after SHH gene knockdown.

As previously reported, several factors can suppress the function of PCK1 via the activation of PI3K/Akt signaling [25]. The interaction between PCK1 and PI3K/Akt was also supported by the findings of the current study, as inhibition of the PI3K/Akt axis was associated with increased expression of PCK1, which confirmed the regulatory sequence from PI3K/Akt to PCK1. Although previous studies also showed that the function of SHH gene expression was modulated by the PI3K/Akt axis, the current study showed that knockdown of the SHH gene impaired the function of the PI3K/Akt pathway, representing the formation of a regulating loop between SHH and PI3K/Akt. These results demonstrated that the inhibition of SHH signaling inhibited oncogenesis in HCC cells studied in vitro by suppressing the PI3K/Akt pathway and inducing the PCK1 pathway.

Figure 6. Knockdown of the SHH gene inhibited PI3K/Akt activity and increased PCK1 expression in the HCC cell lines, Hep3B and SMMC-7721. (A) Quantitative analysis results and representative images of the Western blot results for PI3K, Akt, and PCK1 in Hep3B cells. (B) Quantitative analysis results and representative images of the Western blot results for PI3K, Akt, and PCK1 in SMMC-7721 cells. * P<0.05 vs. the normal control (NC) group.
Conclusions

This study aimed to investigate the role of the sonic hedgehog (SHH) gene and its encoded protein, SHH, in two human hepatocellular carcinoma (HCC) cell lines, Hep3B and SMMC-7721. Knockdown of the SHH gene reduced cell survival of HCC cells by increasing apoptosis, reducing cell proliferation, inducing G1 cell cycle arrest, and restoring gluconeogenesis, and was associated with the inhibition of the PI3K/Akt axis and induced the expression of PCK1. The findings from this preliminary study supplement the findings from previous studies that have demonstrated a role for SHH in invasion and metastasis of HCC and have shown that the effects of inhibition of the SHH gene activated gluconeogenesis, was associated with the activation of PCK1, which might depend on the inhibition of PI3K/Akt pathway. These findings require further study but indicate the possibility that the future metabolic treatment approaches for HCC might focus on modulating the expression and function of the SHH gene.

Supplementary Figure

**Figure 1.** Transfection of specific shRNA-2 suppressed the level of sonic hedgehog (SHH) at the mRNA and protein level and reduced proliferation of Hep3B and SMMC-7721 cells. (A) Reverse transcription quantitative polymerase chain reaction (RT-qPCR) detection of the SHH gene in Hep3B and SMMC-7721 cells. (B) Quantitative analysis results and representative image of Western blot detection of the SHH protein in Hep3B and SMMC-7721 cells. (C) Quantitative analysis results of the MTT assay for cell proliferation in Hep3B cells. (D) Quantitative analysis results of the MTT assay for cell proliferation in SMMC-7721 cells. * P<0.05 vs. the normal control (NC) group.

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