

# Glutamate dehydrogenase 1 mediated glutaminolysis sustains HCC cells proliferation and survival under glucose deprivation

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## Research

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# Abstract

**Background:** It is generally believed that tumor cells could sustain its proliferation and survival under different nutrient status according to a so-called metabolic flexibility. How the metabolic flexibility of glutamine metabolism of HCC cells behaves under different glucose conditions has not yet been fully elucidated. In this study, we investigated how the glutamine metabolism modulate the proliferation and survival of HCC cells in response to different glucose conditions and explored the underlying molecular mechanism.

**Methods:** Two cell lines SK-Hep-1 and PLC/PRF/5 were used to evaluate the glutamine addiction of HCC cells. Then, the cells were cultivated in high glucose medium (25mM glucose) and low glucose medium (1.0 mM glucose), respectively, to investigate whether glutaminolysis changed in response to different glucose levels. And, the underlying mechanism of glutamate dehydrogenase 1 (GDH1) sustaining HCC cells survival under glucose deprivation was explored. Additionally, the underlying correlation of GDH1 and glutamate–oxaloacetate transaminase 1 (GOT1) in glucose -poor HCC tissue was investigated.

**Results:** HCC cells were addicted to glutamine. The glutaminolysis of HCC cells was different in response to different glucose conditions. That is, glutamate transaminases GOT1 involved glutamine metabolism played a dominant role in regulating cell growth when glucose was sufficient, while deaminase GDH1 mediated glutaminolysis became dominant when glucose was limited. Mechanically, low-glucose treated HCC cells could induce an elevated expression of GDH1 to supplement the TCA cycles in respond to glucose deprivation. Additionally, we further uncovered an underlying negative association between GDH1 and GOT1 in HCC tissues with decreased glucose levels

**Conclusions:** GDH1 mediated pathway played a leading role in maintaining cell proliferation and survival under low glucose condition. By contrast, GOT1 mediated pathway was activated under high glucose condition. Mechanically, highly expressed GDH1 could drive the TCA cycle in response to glucose deprivation. Besides, there was a potential negative correlation between GDH1 and GOT1 in glucose-poor HCC tissues.

## Background

Metabolic reprogramming is a hallmark of cancer cells and plays a critical role in tumor cell survival, proliferation and migration and invasion [1]. Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China, which is characterized by insidious onset, easy metastasis, a low 5-year survival rate, and lack of diagnostic techniques to identify early symptoms. The occurrence and progression of HCC are also closely intertwined with its metabolic disorders. Similar to most other solid tumors, the metabolic process of HCC cells has undergone many significant changes compared with normal liver cells, which are manifested in abnormally elevated glycolytic activity, increased de novo synthesis of fatty acids and decreased oxidation, as well as accelerated glutamine catabolism[2–4].

Glutamine is the most abundant circulating amino acid in blood and muscle, and participates many fundamental cell functions in cancer cells, including generation of antioxidants to remove reactive oxygen species; synthesis of biological macromolecules; and activation of cell signaling. Glutaminolysis is the process by which cells convert glutamine into tricarboxylic acid (TCA) cycle metabolites through the activity of a series of enzymes. Firstly, glutamine is converted into glutamate by glutaminase (GLS). Subsequently, glutamate is converted into  $\alpha$ -ketoglutarate ( $\alpha$ -KG) via two divergent pathways. One way is via the activity of glutamate dehydrogenase (GDH). The other way is via the activity of a group of transaminases, including glutamate–oxaloacetate transaminase (GOT), glutamate–pyruvate transaminase (GPT), and phosphoserine transaminase (PSAT). Liver cancer has a metabolic dependency on glutamine[5], indicating an essential role of glutamine enzymes mentioned above in the occurrence and development of HCC. A study by Li et al. reported that GLS1 was highly expressed in liver cancer and regulated stemness properties of HCC cells via ROS/Wnt/ $\beta$ -catenin signaling[6]. However, levels of other crucial glutamine enzymes and its regulatory effect on HCC cells remain to be fully elucidated.

It is widely accepted that cancer cells usually optimize nutrient utilization when resources are scarce. Such adaptive mechanism is defined as metabolic flexibility [7, 8]. One previous study found glucose deprivation resulted in a selective pressure for KRAS mutation in colon cancer cells, consequently, the mutated KRAS rendered cells tolerant of low glucose conditions [9]. Another research uncovered that expression changes of some glutamine enzymes could lead to a pronounced suppression of pancreatic cancer cells growth in vitro and in vivo [10]. Nevertheless, how does metabolic flexibility manifest in HCC cells is not fully understood. In this study, we reported the addiction of HCC cells to glutamine, which was confirmed by the necessity of glutamine and its downstream metabolites such as nonessential amino acids (NEAAs) and  $\alpha$ -KG for the growth of HCC cells. In addition, we identified, for the first time, that under different glucose concentration levels, the major glutaminolysis pathway could change in HCC cells. That is, GOT1 mediated pathway played a dominant role in regulating cell growth when glucose was sufficient, yet GDH1 regulated pathway which mediating the transition of glutamine from transamination to deamination was activated when glucose was limited for the reason that GDH1 could drive the tricarboxylic acid (TCA) cycle in response to glucose starvation. Interestingly, we also uncovered an underlying negative relationship between GDH1 and GOT1 in HCC tissues with decreased glucose levels.

## Methods

### HCC specimens

Tumorous liver tissues and the corresponding adjacent nontumoral liver tissues were collected from 12 patients who underwent curative surgery for HCC at the Second Affiliated Hospital of Chongqing Medical University in Southwest China. The patients were not subjected to any form of chemotherapy prior to surgery. Informed consent was obtained from each patient recruited, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of Chongqing Medical University. Total RNA and proteins were acquired from these samples.

## Cell culture

SK-Hep-1 and PLC/PRF/5 cell lines were obtained from the American Type Culture Collection (VA, USA). Cells were authenticated by short-tandem repeat (STR) fingerprinting by Beijing Micro-read Genetics Company Limited recently. Indicated cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS, Hyclone) and different concentrations of glucose at 37°C in 5% CO<sub>2</sub>. The medium was refreshed every 12 h. Where indicated, cells were treated with 2 mM L-glutamine (Gibco, 210-51-024), 4 mM dimethyl-2-oxoglutarate ( $\alpha$ -KG, Sigma-Aldrich, 349631), 2 mM nonessential amino acid (NEAA, Gibco, 11140), epigallocatechin gallate (EGCG, selleckchem, S2250), and aminooxyacetate (AOA, selleckchem, S4989), respectively.

## Small interfering RNAs and antibodies

Small interfering RNAs (siRNAs) were purchased from Shanghai Genechem Company Limited. Targeting sequence of all siRNAs were listed in Supplementary Table 1. GOT1 expression vector (#HG14196-CM) were obtained from Sino Biological Inc. (Beijing, China). Anti-GDH1 (#ab89967), Anti-GOT1 (#ab170950) were obtained from Abcam Inc. (MA, USA) and Anti- $\beta$ -actin (#9027) were purchased from Cell Signaling Technology Inc. (BOS, USA). Anti-GAPDH (sc-365062) were obtained from Santa Cruz Biotechnology (TX, USA). Horseradish peroxidaseconjugated antirabbit were purchased from GE Healthcare Life Sciences Inc. (Little Chalfont, UK). Antimouse IgG secondary antibody (#sc2005) was purchased from Santa Cruz Biotechnology, Inc. (CA, USA).

## Western blotting analysis

Cells were harvested and lysed with RIPA lysis buffer supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein quantification was evaluated by the Pierce BCA kit (Pierce; Thermo Fisher Scientific, Inc. Waltham, MA, USA). Lysate proteins were separated using 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, UK). The membranes were blocked with 5% non-fat milk for 2 h, and subsequently incubated with primary antibody at 4°C overnight. Then the membranes were incubated with anti-rabbit or anti-mouse IgG secondary antibodies. The blots were developed with ECL Western blot reagents (Millipore, Billerica, MA, USA)). The signal intensities were quantified using Image J software.

## RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was prepared using TRNzol Reagent (Tiangen Biotech Co. Ltd., Beijing, China), and cDNA was synthesized from 1  $\mu$ g of total RNA using a Fast Quant RT Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. Relative quantification of gene expression was conducted using FastStart Universal SYBR Green Master Mix (Roche Diagnostics, IN, USA) with  $\beta$ -actin mRNA as an endogenous control. The sequence of primers used in this study were listed in Supplementary Table 2. Results represented as mean  $\pm$  SD of three independent experiments. The expression values of target genes were calculated using  $2^{-\Delta\Delta Ct}$  method.

## Cell proliferation assay and colony formation assay

Cell proliferation was determined by trypan blue exclusion assay (Thermo Fisher Scientific, Waltham, MA, USA). For colony formation assay, cells were grown in puromycin-containing medium (1.0 µg/mL) for 10 days. Finally formed colonies were stained with 0.5% crystal violet.

### **Cell viability assay**

Cell viability was assayed by Cell Counting Kit-8 (CCK-8). Working solution (MedChemExpress, #HY-K0301) was added to cells at indicated times and the mixture was incubated for 3 h. The optical density (OD) values were measured with a microplate reader (Bio-Tek) at 450 nm. Cell viability was expressed as a percentage of the maximum absorbance from three replicates in three independent experiments.

### **Apoptosis assay**

The HCC cells were treated with different concentrations of EGCG or AOA and cultivated for 48 h. Cells were firstly stained using Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology) according to the manufacturer's instruction. Then, the percentage of apoptosis cells was tested by flow cytometry (BD Accuri C6).

### **Glutamine and ammonium measurements**

The amount of glutamine and ammonia in cells and supernatant were measured by L-glutamine / ammonia (rapid) assay kit from Megazyme Inc. (Wicklow, Ireland). All values were normalized to cell numbers done in parallel.

### **Glucose assessment**

The glucose level in tissues were detected using glucose assay kit (Abcam ab65333). Tissues were dissolved in double distilled water and homogenized by ultrasonic treatment. A PCA/KOH deproteinization step was performed before glucose assay. All the steps were conducted as the protocol described.

### **Energy metabolite analysis**

Metabolomic analyses were performed by multiple reaction monitoring (MRM) method. Samples were stable GDH1-silenced PLC/PRF/5 cells and negative control cells cultivated for 3days in medium containing 1.0 mM glucose, as well as negative control cells cultivated for 3days in medium containing 25 mM glucose. After ultrasound treatment, reconstitution with 1:1 (v/v) acetonitrile/water solution and centrifugation, the supernatant were used for mass spectrometry detection. Heatmaps were constructed based on the results of the metabolite levels.

### **Statistical analysis**

Data were expressed as means  $\pm$  SD from at least three independent experiments. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad

Software) and SPSS version 19.0 software (SPSS, Inc., Chicago, IL). Comparisons between two groups were performed using Mann-Whitney test. One way ANOVA followed by Dunnett's post-hoc test was

performed for comparisons among multiple groups. The value of  $P < 0.05$  was considered statistically significant.

## Results

### HCC cells were addicted to glutamine

To evaluate the glutamine addiction of HCC cells, two HCC cell lines SK-Hep-1 and PLC/PRF/5 were cultured in normal medium, medium without glutamine and medium without glucose, respectively. Undoubtedly, starvation of glucose in these cells resulted in growth arrest and cell death. In parallel, a marked reduction in cell proliferation was also observed in glutamine-deprived condition. The cell growth was almost arrested after 2 days of glutamine deprivation (Fig. 1A), indicating the glutamine dependency of HCC cells. Glutamine was coupled with glucose to maintain cell proliferation and survival. Adding additional 2 mM glutamine was able to support the growth of HCC cells in glutamine-free conditions (Fig. 1B). Furthermore, we uncovered that the growth arrest induced by glutamine deprivation could be remarkably attenuated by exogenous addition of 0.1 mM NEAAs along with 4 mM  $\alpha$ -KG, two major intermediates of glutaminolysis. However, adding additional  $\alpha$ -KG or NEAAs alone couldn't serve the same role (Fig. 1C). Together, these results indicated that glutaminolysis and its downstream metabolites were necessary for growth of HCC cells.

### Major glutaminolysis pathway of HCC cells changed under different glucose concentration levels

To investigate whether glutaminolysis changed in response to different glucose levels in HCC cells, above-mentioned two HCC cells were cultivated in high glucose medium (25 mM glucose) and low glucose medium (1.0 mM glucose), respectively, and subsequently treated with either AOA, a pan-inhibitor of transaminases [11], or EGCG, an inhibitor of GDH1 [12]. We defined medium contains 1.0 mM glucose as low glucose culture for the reason that HCC cells in media containing glucose less than 1.0 mM could barely survive (Fig. 2A). As shown in Fig. 2B and 2C, AOA treatment robustly inhibited the cells growth under high glucose conditions, while EGCG treatment had no effect on cells growth under the same conditions. Conversely, EGCG intervention markedly suppressed the HCC cells growth under low glucose conditions, yet AOA intervention didn't produce a remarkable anti-growth effect on HCC cells in the same conditions (Fig. 2D and E). On the other hand, the roles of EGCG or AOA treatment on cells apoptosis were also detected in our study. As indicated in Fig. 2F and 2G, AOA treatment robustly promoted the cells apoptosis in high glucose conditions, while EGCG treatment played no effect on apoptosis under the same conditions. Conversely, EGCG intervention markedly induce apoptosis in low glucose conditions, yet AOA intervention had no significant pro-apoptotic effect on HCC cells in the same conditions (Fig. 2H and I). These results implied that the effects of transaminases-mediated and GDH1-mediated glutaminolysis pathway on HCC cells survival altered in response to different glucose conditions, and the two changes were related.

To further identify the specific transaminase(s) involved in glutaminolysis, we impaired the activity of metabolic enzymes by siRNA individually and examined the growth of cells. The genes were successfully

silenced as detected by qRT-PCR (Supplementary Fig. S1A). Consistent with the previous results, interference of GOT1 significantly weakened HCC cells proliferation under high glucose conditions, while silencing of GDH1 did not take effect (Supplementary Fig. S1B and C). Furthermore, we also found that deletion of GDH1 significantly suppressed cells proliferation under glucose limitation, yet knock-down of GOT1 did not take effect (Supplementary Fig. S2A and B). Surprisingly, inhibiting glutamine-dependent transaminases including GOT2, GPT-1, GPT-2, PSAT-1 had essentially no impact on proliferation of cells no matter under high or low glucose conditions (Supplementary Fig. S1D, Supplementary Fig. S2C). Additionally, stable knockout cell models were generated by lentivirus expressing an shRNA targeting GOT1 and GDH1, respectively (Fig. 3A and B). As expected, GOT1 knock-down remarkably decreased the proliferation and colonies formation when HCC cells were cultivated in medium containing high glucose (Fig. 3C and D). In contrast, GDH1 depletion remarkably reduced the proliferation and colonies formation when HCC cells were cultivated in low glucose medium (Fig. 3E and F). Collectively, all the data showed that GOT1 catalyzed glutamine transamino-metabolism played a major role on HCC cells survival when glucose is high, while GDH1 mediated deamino-metabolism played a leading role on cells survival when glucose is insufficient, implying that major glutaminolysis pathways of HCC cells could change under different glucose concentration levels.

### **GDH1 mediated glutamine anaplerosis facilitated the driven of TCA cycle in response to glucose limitation**

In order to further confirm that glucose limitation promotes GDH1 mediated glutamine metabolic process, firstly, we assessed the glutamine consumption in HCC cells cultured in low-glucose medium. As shown in Fig. 4A, the levels of glutamine consumption in both two HCC cells were significantly elevated after incubation in glucose starvation condition for 24 h and 48 h. Secondly, we interrogated the levels of  $\text{NH}_4^+$ , a specific metabolite formed through a deamination reaction, under the same glucose limitation treatment. The results showed that  $\text{NH}_4^+$  levels were obviously increased after glucose starvation (Fig. 4B). Additionally, We also found significant increase of GDH1 mRNA (Fig. 4C) and protein (Fig. 4D) levels on the basis of glucose limitation. Most importantly, our mass spectrometry results demonstrated that, compared to those in high glucose conditions, levels of TCA cycle intermediates including succinate, citrate, cis-aconitate, oxaloacetate and so on, were decreased in low glucose conditions, and the decrease of which were more significantly after silencing GDH1 expression (Fig. 4E). Together, these data provided evidences that GDH1 mediated glutamine anaplerosis facilitates the driven of TCA cycle for cell survival in response to glucose limitation.

### **GDH1 expression was elevated in glucose-poor HCC tissues along with decreased expression of GOT1**

Being a most consumed nutrient, glucose, are generally shown lower expression levels in tumor tissues than in non-tumor tissues[13]. We detected glucose concentration in 12 paired HCC and adjacent nontumoral tissues, the results showed that glucose levels were strikingly decreased in HCC tissues compared to those in adjacent nontumoral tissue samples (Fig. 5A). In addition, we further examined the expression levels of GDH1 and GOT1 in above HCC tissues by western blotting. As shown in Fig. 5B, most protein levels of GDH1 were up-regulated in glucose-poor tumor tissues, simultaneously accompanied by

decreased GOT1 protein levels, suggesting an underlying negative correlation between GDH1 and GOT1. But studies with larger sample size are still needed to definitively establish the relationship between GDH1 and GOT1 in HCC with low glucose concentrations.

## Discussion

It is well established that tumor cells remodel their metabolism and energy production through aerobic glycolysis ( *Warburg effect* )[14]. Recent studies have found, in addition to glucose, that glutamine is also an important nutrient for tumor cells. Some tumor cells are more dependent on glutamine to maintain the energy requirements for their growth, exhibiting cell death follows Gln-deprivation, such phenomenon is defined as " *Gln addiction* "[15–17]. *Gln addiction* has long been determined a characteristic of tumor cells. It has been shown that malignant cells such as glioma, lung cancer and kidney cancer were addicted to glutamine. In the present study, we confirmed that liver cancer cells strongly relied on glutamine uptake, which was in line with results of other researches [18, 19]. The inhibitory effect of Gln-deprivation on HCC cells growth is consistent with the results caused by glucose-deprivation. Being an important component of glutamine metabolism, glutaminolysis also plays a critical role in cancer cell metabolism, cell signaling, and cell growth in many cancers, which suggests a critical role of glutamine related downstream metabolites in tumor cells. Our experimental data further demonstrated the necessity of NEAAs and  $\alpha$ -KG, two of the main metabolites of glutamine, on HCC cells growth. When HCC cells were supplemented with 0.1 mM exogenous NEAAs or 4 mM  $\alpha$ -KG, respectively, the number of dead cells were markedly less than those in condition of Gln-deprivation. Moreover, the cell growth inhibition was essentially completely reversed when these two metabolites were present simultaneously.

Previous studies have suggested that glucose and glutamine are the two main energy sources required for the rapid proliferation of tumor cells [20, 21]. Notably, some cancers seem to prefer aerobic glycolysis [22,23], others rely more on glutaminolysis pathway [24] Additionally, there are also some cancers tend to a combination of such two metabolic pathways[25]. Given the important role of glucose and glutamine in cell proliferation and survival, it is of great significance to clarify the relationship between glucose metabolism and glutaminolysis in HCC cells. In our study, HCC cells SK-Hep-1 and PLC/PRC/5 underwent glucose and glutamine deprivation could hardly survive, implying the synergistic effect of glutamine and glucose on HCC cells growth and survival. Besides, it is well known that glutaminolysis is a highly coordinated process catalyzed by numerous enzymes. For example, GDH catalyzes glutaminolysis to form  $\alpha$ -KG, which enters the TCA cycle. By contrast, transaminases including GOT, GPT and PSAT promote the generation of NEAAs to maintain cellular events. Therefore, regulation of such key metabolic enzymes and pathways appears particularly important in glutaminolysis. However, the interrelations between these glutaminolysis-associated metabolic enzymes and glucose metabolism in cancers has not yet been exactly elucidated. In this study, our data showed that glutaminolysis was still warranted to maintain cell growth when glucose is sufficient, no to mention glucose starvation. Interestingly, our study validated that under different glucose concentrations, the glutaminolysis pathways that played a leading role in cell survival were different. Specific manifestation was as follows: GOT1 mediated pathway played a dominant role in regulating HCC cells growth under high concentration of glucose conditions, yet

was not activated when glucose was limited. However, GDH1 mediated enzymatic reaction was activated under glucose deprivation (Fig. 5D). Surprisingly, we also reported that there was a potential negative correlation of GDH1 expression with GOT1 expression in low-glucose HCC tissues, but our research didn't reveal any obviously regulatory effect of other aminotransferases, including GOT2, GPT1, GPT2 and PSAT1, on cell growth of HCC. These results highlight the prominent places of GDH1 and GOT1 in the glutaminolysis process of HCC cells, suggesting that novel therapeutic approaches based on such two enzymes may be more beneficial to HCC treatment. Above all, the underlying opposing relationship between GDH1 and GOT1 supports the point that cancer cells adapt to nutrient-deprived tumor microenvironment during progression via adjusting the level and function of metabolic enzymes, that is, liver cancer cells maintain proliferation and survival under different nutritional conditions through the metabolic flexibility of their glutamine-related enzymes.

It has been uncovered that glucose supplement and extra-cellular glucose concentration in tumor tissues are much lower than surrounding normal tissues[13], which indicated the irreplaceable role of GDH1 in the growth and survival of cancer cells, especially under low-glucose conditions. GDH1 is a key enzyme for glutaminolysis. Several studies have reported that GDH1 provides metabolic advantages for cancer cell proliferation and tumor metastasis via regulating the production of  $\alpha$ -KG [26,27]. Nevertheless, the pathological relationship between changes in GDH1 content and occurrence and development of HCC remains to be clarified in detail. Results of an earlier study by Jin et al. revealed that GDH1 predominantly controlled intracellular  $\alpha$ -KG and subsequent fumarate levels, and contributed to redox homeostasis by activating GPx1, thereby promoting the cancer cell multiplying and tumor growth [28]. Another published research showed that phosphorylated ELK1 activated by EGFR/MEK/ERK signaling pathway enriched in the promoter of GDH1 to stimulate the transcription of GDH1, then promoted glutamine metabolism [29]. In the current study, we found an increased glutamine consumption in low-glucose cultured HCC cells, further demonstrated the necessity of glutaminolysis in HCC cells survival. It was reported that pyruvate carboxylase was highly expressed in glutamine-independent cancer cells, contributing to maintain anaplerosis under glutamine-deprivation conditions. In contrast, glutamine-dependent cell lines consume glutamine as the preferred anaplerotic substrate to drive TCA cycle[30]. Results of our study found that  $\text{NH}_4^+$ , a specific metabolite produced in a reaction catalyzed by GDH1, was markedly elevated under glucose-limiting conditions, providing evidences that up-regulated GDH1 drives increased entry of glutamine-derived carbon into the TCA cycle in response to glucose starvation. Our finding concerning the driving role of GDH1 on TCA cycle under limited glucose status agreed well with the previously reported results, which further demonstrates a critical role for GDH1 in HCC cell proliferation and tumor growth.

## Conclusions

In conclusion, our study clarified a new insight of the regulation of glutaminolysis in HCC. That is, GDH1 mediated pathway played a leading role in maintaining cell proliferation and survival under low glucose condition. By contrast, GOT1 mediated pathway was activated under high glucose condition. Moreover, highly expressed GDH1 could drive the TCA cycle in response to glucose deprivation. Interestingly, our results also suggested a potential negative correlation between GDH1 and GOT1 in glucose-poor HCC

tissues. Our data enriched the understanding of metabolic flexibility in HCC, and provided certain theory guidance for the design and development of novel therapeutic methods targeting glutamine metabolism in HCC.

## List Of Abbreviations

HCC: Hepatocellular carcinoma; TCA: Tricarboxylic acid; GLS: glutaminase;  $\alpha$ -KG:  $\alpha$ -ketoglutarate; GDH: Glutamate dehydrogenase; GOT: Glutamate–oxaloacetate transaminase; GPT: Glutamate–pyruvate transaminase; PSAT: Phosphoserine transaminase; NEAAs: Nonessential amino acids; EGCG: Epigallocatechin gallate; AOA: Aminooxyacetate; STR: Short-tandem repeat; siRNAs: Small interfering RNAs; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; CCK-8: Cell Counting Kit-8; MRM: Multiple reaction monitoring; OD: Optical density

## Declarations

### Ethics approval and consent to participate

This study was approved by the Ethics Committee and the Institutional

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

Chen J and Zheng L designed the research; Zhou YJ wrote the manuscript; Ren JH and Yu HB analyzed the data. Cheng ST and Ren F carried out cell experiments; He X carried out the detection of clinical samples. All authors have read and approved the final manuscript.

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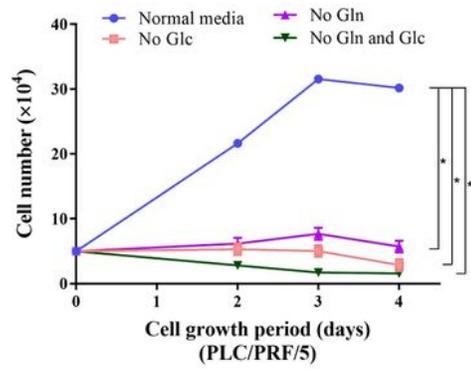
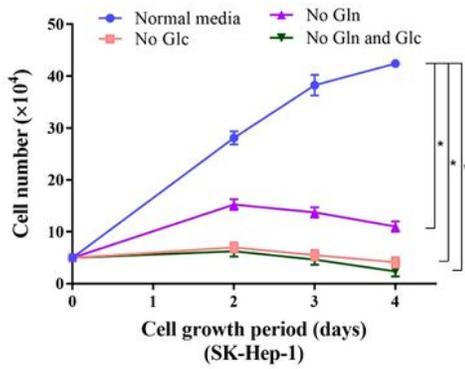
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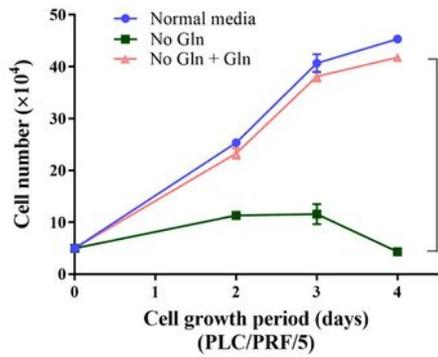
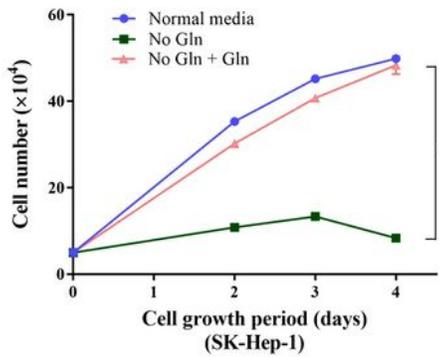
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## Figures

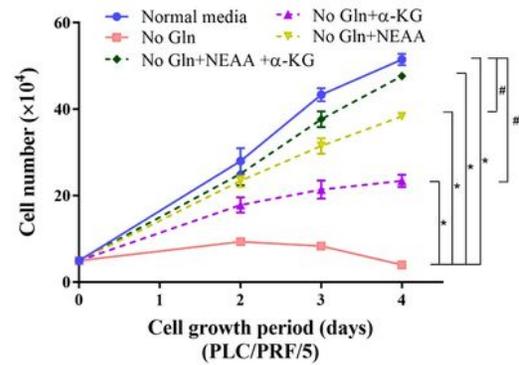
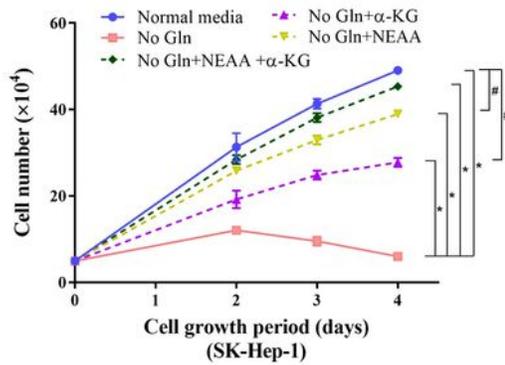
**A**



**B**



**C**



**Figure 1**

HCC cells are addicted to glutamine. A, SK-Hep-1 and PLC/PRF/5 cells were cultured in normal medium (Normal media group), normal medium containing no glutamine (No Gln group) or no glucose (No Glc group), respectively. Cells were counted at indicated time points using a trypan blue exclusion assay. \* $P < 0.05$ , when compared to that in Normal media group. B, In addition to Normal media group and No Gln group, SK-Hep-1 and PLC/PRF/5 cells were cultured in medium containing 2mM glutamine (No Gln + Gln

group). Cell numbers were counted. \*P < 0.5, when compared to that in No Gln group. C, In addition to groups mentioned above, cells were cultured in medium supplemented with  $\alpha$ -KG (4 mM) alone, nonessential amino acid (NEAA, 2 mM) alone, and a combination of  $\alpha$ -KG and NEAA, respectively. \*P < 0.5, when compared to that in No Gln group. #P < 0.5, Vs. Normal media group.

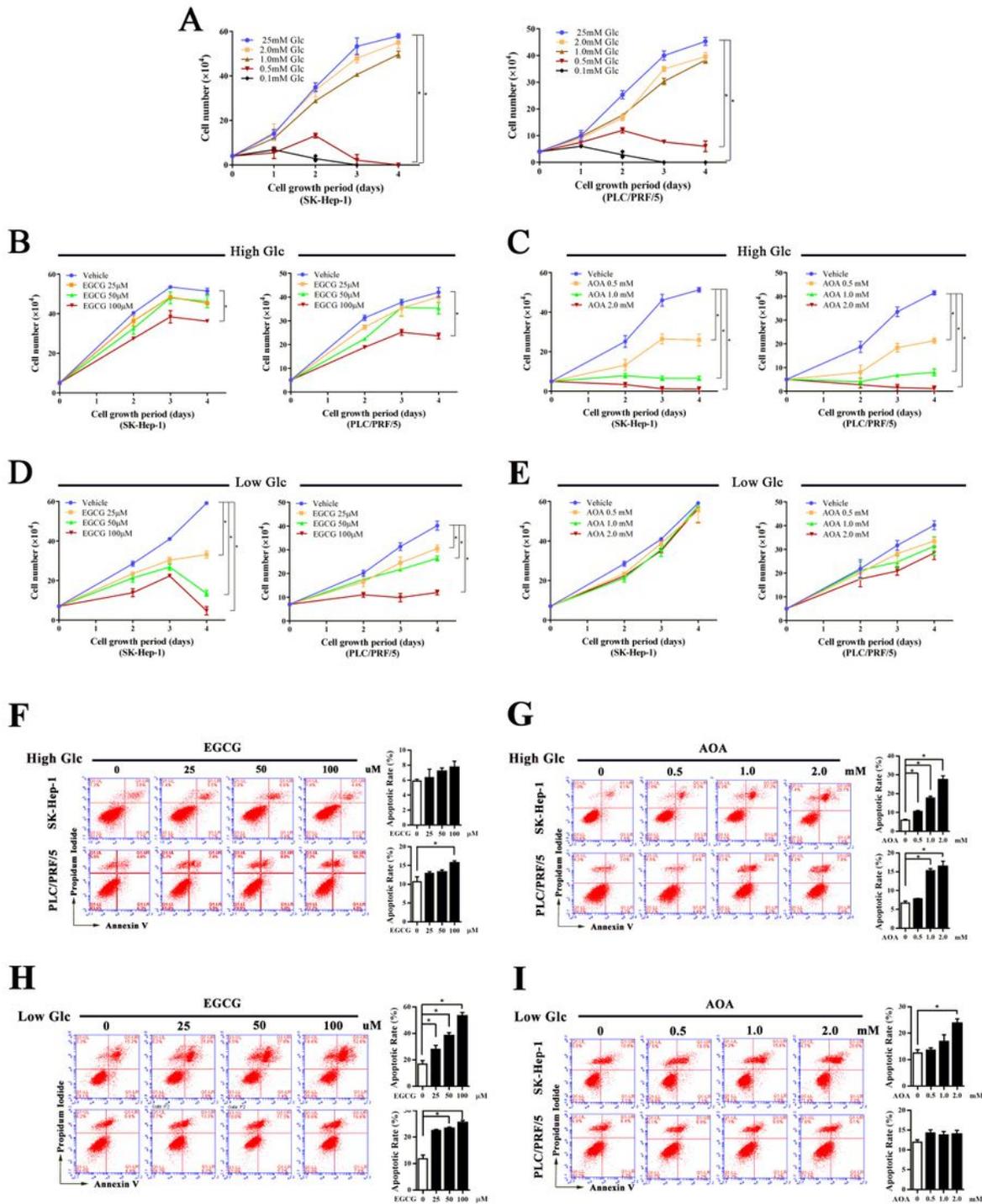
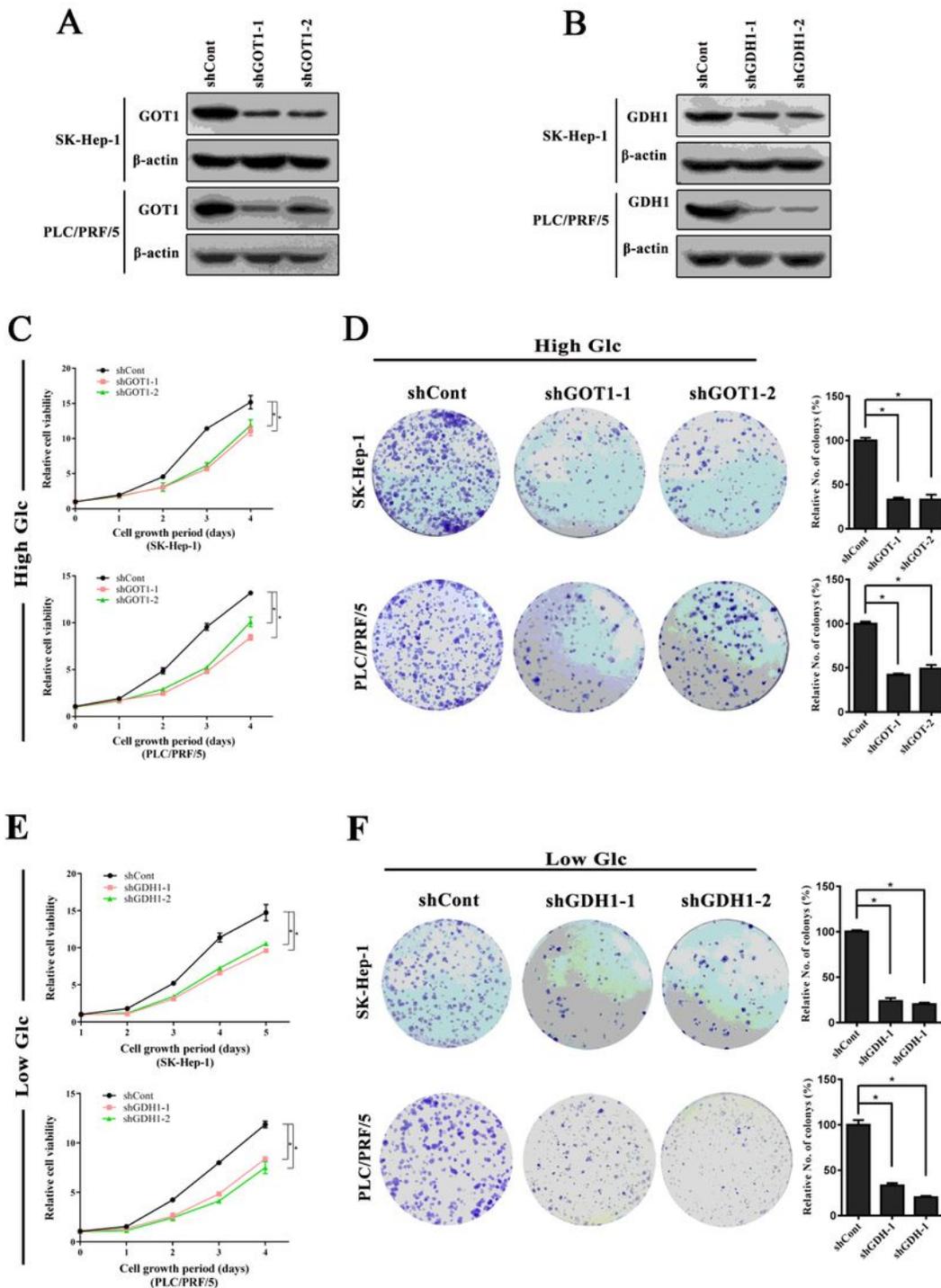


Figure 2

Major glutaminolysis pathway of HCC cells changes under different glucose concentration levels. A, The proliferation of SK-Hep-1 and PLC/PRF/5 cells in complete medium containing 0.1mM, 0.5mM, 1.0mM, 2.0mM or 25mM glucose, respectively, was assessed using a trypan blue exclusion assay. \*P < 0.5, vs. 25mM glucose. B-E, SK-Hep-1 and PLC/PRF/5 cells were exposed to various concentrations of epigallocatechin gallate (EGCG) (B) and aminooxyacetate (AOA) (C) in high-glucose (25mM) condition for 72 h, respectively. Similarly, cells were exposed to EGCG (D) and AOA (E) respectively and cultured in low-glucose (1.0 mM) condition for 72 h. The cell numbers were obtained. \*P < 0.5, vs. vehicle. F-I, The apoptosis was analyzed by flow cytometer with Annexin V/PI after treated with EGCG (F) and AOA (G) for three days, respectively. \*P < 0.5, vs. vehicle. The effect of various concentrations of EGCG (H) and AOA (I) on cells apoptosis was analyzed by flow cytometer. Cells were cultured in low glucose (1.0 mM) media and treated for three days, respectively. \*P < 0.5, vs. vehicle.



**Figure 3**

Glutaminolysis related enzymes serve different roles in response to different glucose conditions. A-B, The GOT1 protein levels (A) and GDH1 protein levels (B) in SK-Hep-1 and PLC/PRF/5 cells transfected with shRNAs were detected by Western blot.  $\beta$ -Actin was used as an internal control. (C) The effect of GOT1 knock-down on cell viability in high glucose (25mM) medium was detected by CCK-8 assay. \* $P < 0.5$ , vs. shCont. D, The effect of GOT1 knock-down on HCC cells colonies formation viability in high glucose

(25mM) medium was detected by colony formation assay. Newly formed colonies in each well were counted and expressed as a percentage relative to the control group. \*P < 0.5, vs. shCont. E, the viability of GDH1 deficiency on cell viability in low glucose (1.0mM) medium was detected by CCK-8 assay. \*P<0.5, vs. shCont. F, The efficacy of silencing GOT1 in colony-formation viability of HCC cells in low glucose (1.0mM) medium was detected by colony formation assay. Newly formed colonies in each well were counted and expressed as a percentage relative to the control group. \*P < 0.5, vs. shCont.

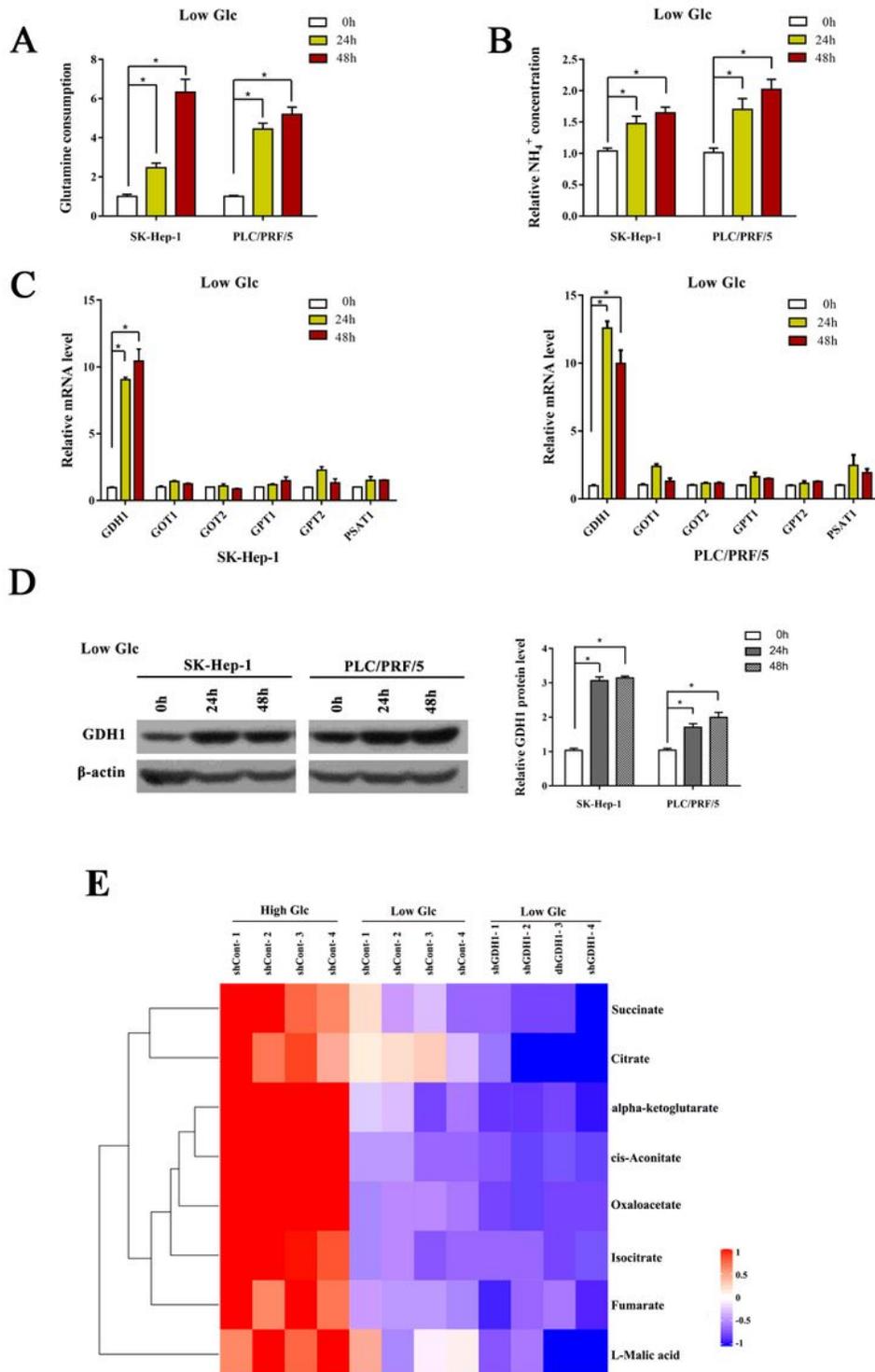
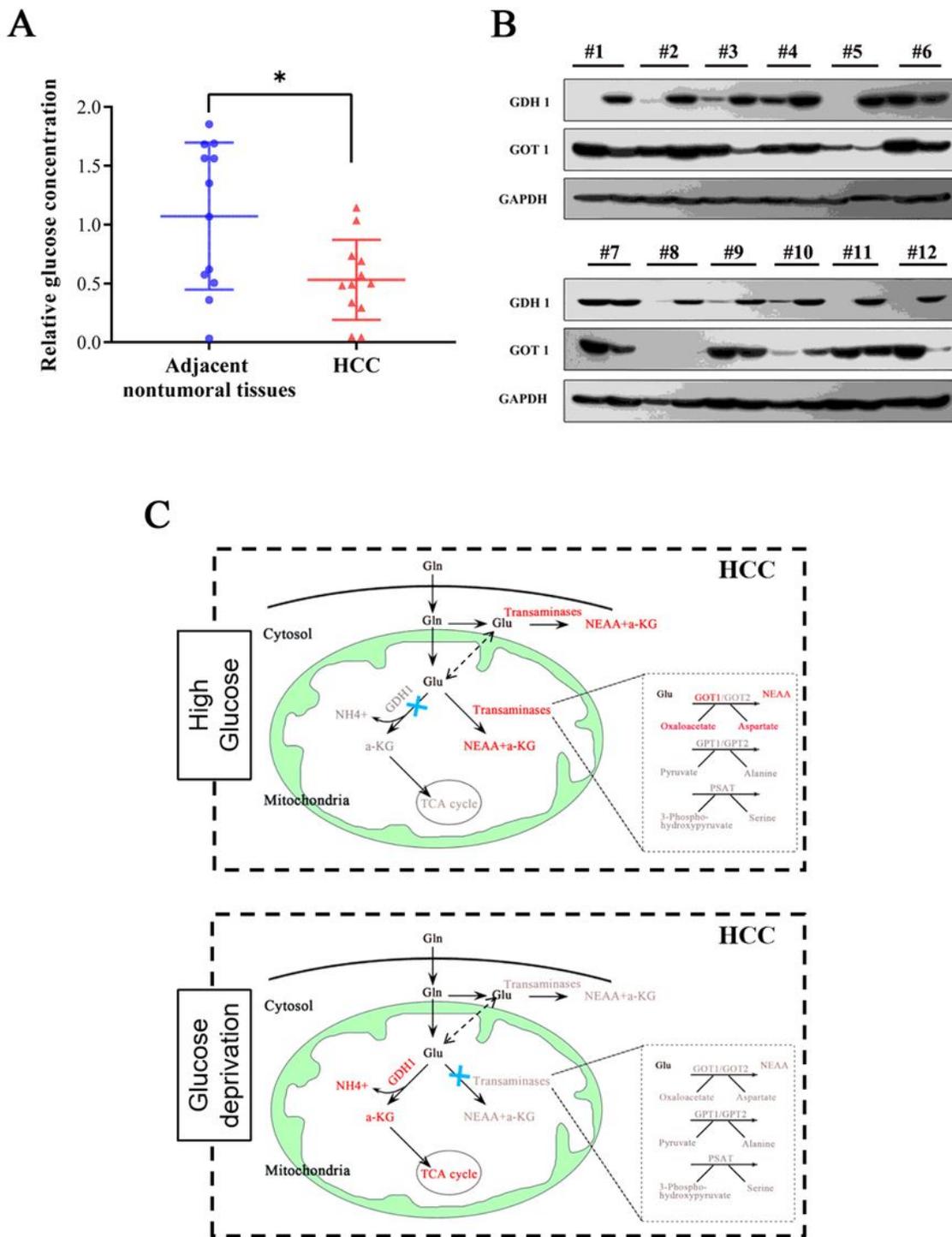


Figure 4

GDH1 mediated glutamine anaplerosis prompted the driven of TCA cycle in response to glucose limitation. A, Fold change in glutamine consumption in SK-Hep-1 and PLC/PRF/5 cells after cultivation in low-glucose medium (1.0 mM glucose) for 24 h and 48 h, respectively. \*P < 0.5, when compared to the 0 h group. B, Fold change in relative NH<sub>4</sub><sup>+</sup> concentration in HCC cells after cultivation in low-glucose medium (1.0 mM glucose) for 24 h and 48 h, respectively. \*P < 0.5, when compared to the 0 h group. C, Enzymes mRNA levels in SK-Hep-1 and PLC/PRF/5 cells cultured in low glucose conditions were detected by RT-qPCR. \*P < 0.5, vs. 0 h group. D, Enzymes protein levels in SK-Hep-1 and PLC/PRF/5 cells cultured in low glucose conditions were detected by Western blot. The signal intensities were quantified using Image J software. \*P < 0.5, vs. 0 h group. E, Heat map comparing relative levels of TCA intermediates in GDH1 knock-down PLC/PRF/5 cells after 3 days of low-glucose culture when compared with control (n = 4 independent samples per condition). Blue and red indicates down- or up-regulation, respectively.



**Figure 5**

GDH1 was upregulated in glucose-poor liver cancer tumor, accompanying with decreased GOT1. A, relative glucose levels in 12 paired clinical liver cancer tissues. The central line represents the mean and the error bars indicate the standard deviation. \* $P < 0.5$ , vs. adjacent nontumoral tissue group. B, images of Western blot of GDH1 and GOT1 protein levels in glucose-poor liver cancer specimens. C, a schematic diagram of GDH1 and GOT1 mediated pathways in different glucose conditions in HCC.

## Supplementary Files

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