



CaMKK β regulates proliferation, apoptosis, and glycolysis of hepatocellular carcinoma via PI3K/AKT pathway

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Background: Hepatocellular carcinoma (HCC) is the most common malignancy of liver cancer. Calcium ions/calmodulins stimulate protein kinase kinases β (CaMKK β) is a multifunctional protein kinase that is overexpressed in many types of cancer. This study aims to investigate the effect of *CaMKK β* interference on HCC in HepG2 cells and transplanted tumor mice.

Methods: *CaMKK β* gene was knocked out in HepG2 cells as an experimental group, empty vector lentivirus as a negative control (NC) group, and untreated HepG2 cells as a control group. Cell proliferation, cell cycle, apoptosis, invasion, and glycolysis potential assays were conducted, respectively. In addition, the expression of PI3K, p-PI3K, AKT, and p-AKT was quantified by Western blot. Finally, the effect of *CaMKK β* *in vivo* was investigated using a xenograft model.

Results: *CaMKK β* knockdown significantly suppressed HepG2 cell proliferation, cell cycle, invasion, EMT, and glycolysis, promoted cell apoptosis, and reduced the expression of hexokinase 2 (HK2), pyruvate kinase M (PKM2), and lactate dehydrogenase A (LDHA), p-PI3K, and p-AKT. Post the addition of AKT highly expression plasmid, glucose uptake, lactic acid production, and cell proliferation decreased, accompanied by an increase in apoptosis, which were substantially reversed. Notably, xenograft model experiments *in vivo* also confirmed that *CaMKK β* knockdown inhibited HCC growth.

Conclusions: *CaMKK β* knockdown inhibited cell proliferation, invasion, and glycolysis through the PI3K/AKT pathway, heightened apoptosis, thus promoting the development of HCC. This might be a potential target for the diagnosis and treatment of HCC.

Keywords: *CaMKK β* ; gene interference; HepG2 cells; glycolysis; hepatocarcinogenesis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer and ranks second in cancer-related deaths globally (1). In recent years, 700,000 people worldwide have died each year as a result of HCC (2). Presently, surgical resection, orthotopic liver transplantation, radiofrequency ablation, and other current methods have the limitations of high recurrence rate

and poor prognosis (3-5). Since most cases of HCC are diagnosed at an advanced stage, surgery is not a suitable treatment approach (6). Molecular targeted therapy is another treatment, but current targeted drugs, such as sorafenib, regorafenib, and cabozantinib, are limited, and all have toxic side effects (7-9). Therefore, low cytotoxic drug targets are urgently needed for HCC chemotherapy.

The second messenger in the cell is Ca^{2+} , and it plays a vital role in the signaling pathway of the cell (10).

Changes in Ca²⁺ concentration regulate biological processes such as cell proliferation and apoptosis, cell cycle, and gene transcription (11-14). When it is stimulated, Ca²⁺ concentration increases and Ca²⁺ channels are activated (15). Studies have shown that disruption of normal Ca²⁺ signaling is associated with tumor progression (16). Calmodulin (CaM) is a sensor of Ca²⁺ concentration. The Ca²⁺ binds to CaM, causing changes in the conformation of CaM and enhancing the affinity of CaM kinase (CaMKK, CaMKI, CaMKII, and CaMKIV) to CaM. The protein kinase CaMKK has a multifunction that is encoded by CAMKK1 and CAMKK2 genes to produce calcium ions/calmodulin stimulated protein kinase kinases α (CaMKK) protein or calcium ions/calmodulins stimulated protein kinase kinases β (CaMKK β) protein, respectively (17). One action of CaMKK is to phosphorylate CaMKI and CaMKIV, and AMPK and PKB/Akt (18,19). The signaling pathway formed by CaMKK, CaMKI, and CaMKIV is known as the Ca²⁺/cam-dependent kinase cascade, and is related to cell proliferation and apoptosis (20). These kinases are widely present in a variety of cancer types, and control various cancer-related functions (21). Their potential as intervention targets for anticancer therapy has been recognized.

In this study, we established stable silent CaMKK β -HCC cells and restored the function of the culture by adding AKT overexpression plasmids. Our results demonstrated for the first time that CaMKK β knockdown partially inhibited HCC cell proliferation and glycolysis and promotes apoptosis through inactivation PI3K/Akt1 signaling.

We present the following article ARRIVE in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/apm-20-1789>).

Methods

Cell culture

We purchased HepG2 cells from the Shanghai Academy of Sciences Cell Bank. The HepG2 cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) at 37 °C, 5% CO₂.

Cell transfection

The three groups of shRNA selected were shRNA1, shRNA2, and shRNA3, and were chosen to interfere with

CaMKK β gene. The interference efficiency was tested by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). The specific short hairpin RNAs (shRNAs) were cloned into the GV248 lentivirus green fluorescent overexpression vector (GenePharma, Shanghai, China). GV248 lentivirus green fluorescent empty vector (without cloning shRNAs) was used as a negative control. Lentivirus plasmids by Lipofectamine[®]2000 (Invitrogen, Carlsbad, CA, USA) were used to transfect the HepG2 cells. After transfection for 48 h, the supernatant containing lentivirus was collected and concentrated to calibrate the viral titer. The final concentration of lentivirus was 4 \times 10⁸ TU/mL, and was stored at -80 °C.

RT-qPCR

Total RNA was extracted from cells and tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The RT-qPCR reaction was performed with Quant One Step RT-qPCR Kit (TIANGEN, Beijing, China) by using the ABI7500 (Applied Biosystems, Waltham, MA, USA). The thermocycling parameters were as follows: a holding step at 95 °C 30 sec, and 40 cycles at 95 °C 5 sec and 60 °C 30 sec. Primers were as follows: CaMKK β , forward 5'-TCAAACCTTCCAACCTCCTG-3', reverse 5'-TTGCTCACACCAAGTCAGC-3'; LDHA, forward 5'-GCACGTCAGCAAGAGGGAGAAAG-3', reverse 5'-AGGTAACGGAATCGGGCTGAA-3'; β -actin, forward 5'-CTTCTACAATGAGCTGCGTG-3', reverse 5'-TCATGAGGTA GTCAGTCAGG-3'. CT value was normalized to Actin and calculated with the 2^{- $\Delta\Delta$ Ct}.

Cell proliferation assay

The HepG2 cells were cultured in 96-well plates with 5 \times 10³ cells per well. Cells were incubated for another 48 h. Then, 10 μ L of MTT solution was added to each well and was further incubated for 4 h at 37 °C. The reaction was then finished with 100 μ L of dimethyl sulfoxide (DMSO), and the absorbance was measured at 490 nm on a Multiskan FC microplate reader (Thermo Fisher, Waltham, MA, USA).

Hoechst 33258 staining

The apoptotic morphology was observed by Hoechst 33258 (G3680, Solarbio, Beijing, China) staining. Cells were fixed with 4% formaldehyde for 10 min. Then, cells were stained

by 10 mg/L of Hoechst 33258 for 1 h. The color images were observed by an ECLIPSE Ti microscope.

Flow cytometry

The HepG2 cells were digested with trypsin (Solarbio, Beijing, China), and fixed overnight at 4 °C with 70% ethanol. Then, 10 mg/mL RNaseA (Solarbio, Beijing, China) and propidium iodide (PI) (Solarbio, Beijing, China) were added and stained overnight at 4 °C. A Cell Cycle and Annexin V-FITC Apoptosis Detection kit (Solarbio, Beijing, China) was used to detect the cell DNA content and apoptotic rate using flow cytometry (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA). Annexv-fitc(-)/PI(-) (lower left) is normal, Annexv-Fitc(+)/PI(-) cells (lower right) are early apoptotic, and Annexv-Fitc(+)/PI(+) (upper right) are late apoptotic. AnnexinV(-)/PI(+) (upper left) is necrotic cell.

Cell invasion assay

Invasion assay of HepG2 cell was handled with Trans-well chamber (6.5 mm, Corning, USA) as previous reported (22). Shortly, cell suspensions ($5 \times 10^4/100$ mL) were added to a matrigel-covered (1 mg/mL, BD, USA) membrane, and the upper and lower compartment was filled with 600 μ L NIH-3T3-conditioned medium with FBS. 24 hours later, cells were fixed with 4% paraformaldehyde and then stained with crystal violet. The number of cells was scored visually in five random fields for microscopic observation.

Detection of glucose and lactate concentrations

According to the manufacturer's instruction, the glucose and lactate concentrations in the medium were measured using the glucose detection kit (Nanjing Jiancheng, China) and the lactate detection kit (Nanjing Jiancheng, China). The absorbance glucose consumption was measured at 570 nm using a multifunctional microporous plate reader (BioTek, USA). The glucose consumption is equal to the initial glucose concentration in DMEM (450 mg/dL) minus the glucose concentration in the medium after transfection.

Enzyme activity analysis

HepG2 cells were digested with 0.25% trypsin (Sigma, USA), centrifuged for 5 minutes at 1,000 g, followed by dissolving in a 300 w ultrasonic machine. The supernatant

then was collected and the protein concentrations in each sample were quantitatively analyzed using a specific gold cholic acid (BCA) protein quantification kit (Beyotime, China). Hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) enzymatic activity were measured using an HK assay kit (A077-1, Nanjing Jiancheng, China) and PK assay kit (A076-1, Nanjing Jiancheng, China), and LDH assay kit (A076-1, Nanjing Jiancheng, China), respectively, in accordance with the manufacturer's instruction. All values were normalized to total protein level.

TUNEL assay

According to the manufacturer's instructions, a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Solarbio, Beijing, China) was used to measure the apoptosis in tumor tissues. The result was observed under an ECLIPSE Ti microscope.

Immunohistochemistry

Dewaxed and hydrated paraffin sections were sealed with 5% normal goat serum for 1 h. Then, paraffin sections were co-cultured with the antibodies Ki67 (ab15580, Abcam, Cambridge, UK) and LDHA (ab84716, Abcam, Cambridge, UK) overnight at 4 °C. Sections were then washed with tris buffered saline + tween 20 (TBST) and incubated with SignalStain[®] Boost immunohistochemistry (IHC) Detection Reagent (#8114, CST, Danvers, MA, USA) for 30 min at room temperature. The result was observed under an optical microscope.

Western blots assay

Proteins extracted from cells or tissues were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Solarbio, Beijing, China) and transferred to polyvinylidene difluoride (PVDF) membranes (Solarbio, Beijing, China). Membranes were blocked by 5% skim milk. Then, proteins were incubated with the primary antibodies overnight at 4 °C. The primary antibodies were as follows: CaMKK β (ab177255, Abcam, UK), Ki67 (ab243878, Abcam, UK), PCNA (#2586, 1:1,000, CST, USA), Survivin (#2808, 1:1,000, CST, USA), cleaved Caspase-3 (#9660, 1:1,000, CST, USA), cleaved Caspase-9 (#9508, 1:1,000, CST, USA), E-Cadherin (ab1416, 1:50, Abcam, UK); N-cadherin (ab18203, 1:600, Abcam, UK); Vimentin (ab8978, 1:600, Abcam, UK),

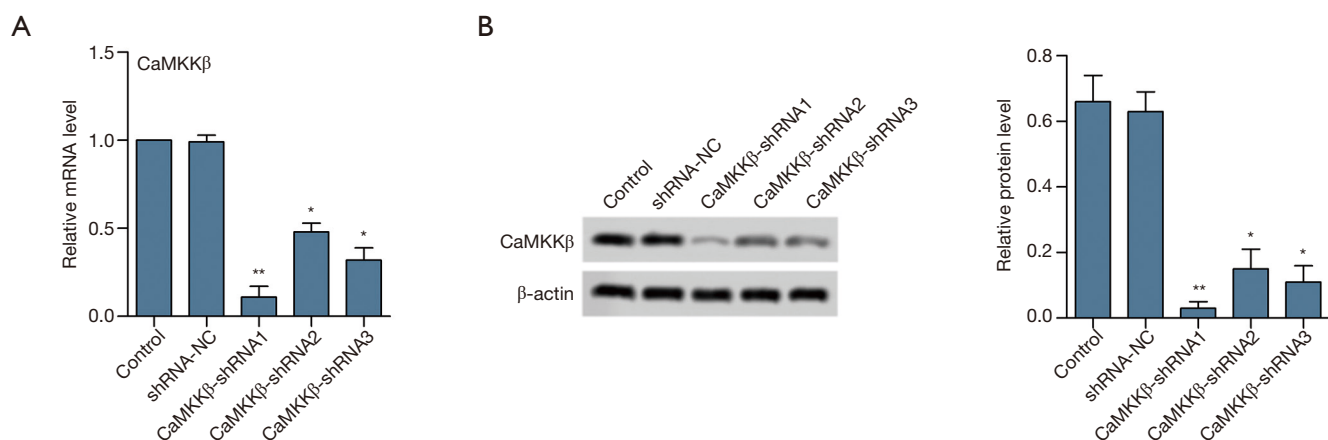


Figure 1 The interference efficiency of gene knockout. (A) RT-PCR was used to detect the mRNA level of CaMKK β in HepG2 cells with CaMKK β -shRNAs; (B) Western blotting was used to detect the protein level of CaMKK β in HepG2 cells with CaMKK β -shRNAs. The experiments were repeated three times and the data are represented as means \pm SD. *, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control.

HK2 (ab209847, 1:1,000, Abcam, UK), PKM2 (ab137852, 1:500, Abcam, UK), LDHA (ab84716, 1:500, Abcam, UK), PI3K (ab32089, 1:1,000, Abcam, UK), p-PI3K (ab191606, 1:1,000, Abcam, UK), AKT (ab8805, 1:500, Abcam, UK), and p-AKT (ab38449, 1:500, Abcam, UK). Next, the samples were washed with PBS and incubated with anti-rabbit IgG (Solarbio, Beijing, China) and anti-biotin horseradish peroxidase (HRP)-linked antibody (Solarbio, Beijing, China) for 1 h. The bands were visualized with the enhanced chemiluminescence (ECL) detection kit (Solarbio, Beijing, China).

Animal models

We purchased 20 male, 7-week old BALB/c nude mice from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. Animals were housed in a controlled environment at 25 ± 3 °C, humidity 60%, in a cycle with 12 h light/dark. The mice had unrestricted access to food and water. Transfection of HepG2 cells with shRNA was performed by subcutaneous injection into the right thigh to form xenograft tumors. The mice were divided into two groups ($n=8$): control group, and CaMKK β -shRNA group. Tumor volume was measured every 5 days until 30 days. Mice were euthanized by intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight). Tumors were harvested for subsequent experiments. All animal experiments were approved by the ethics committee of Affiliated Hospital of North Sichuan Medical College (No. 2020053) and performed in accordance with the National Institutes of Health (NIH)

Guide for the Care and Use of Laboratory Animals.

Statistical analysis

The statistical analysis was conducted with SPSS 21.0 (SPSS, Inc., IBM, Chicago, IL, USA). Data were expressed as mean \pm SD. The data were analyzed using *t*-test and one-way analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$.

Results

shRNAs interference efficiency detection

In this study, the three kinds of shRNA, shRNA1, shRNA2, and shRNA3, were transfected into HepG2 cells to interfere with the CaMKK β gene. The results of RT-PCR results showed that the mRNA levels of CaMKK β were significantly reduced in all three shRNA groups (Figure 1A), and that CaMKK β -shRNA1 was the most efficient in this respect. Similarly, WB results showed that all three groups of shRNAs reduced CaMKK protein levels, and CaMKK shRNA1 was the most effective (Figure 1B). Since the interference efficiency of shRNA1 was the greatest, we selected CaMKK β -shRNA1 for the follow-up experiments.

CaMKK β knockdown interfered the proliferation and cell cycle of HepG2 cells

Results from MTT (Figure 2A) indicated that, compared

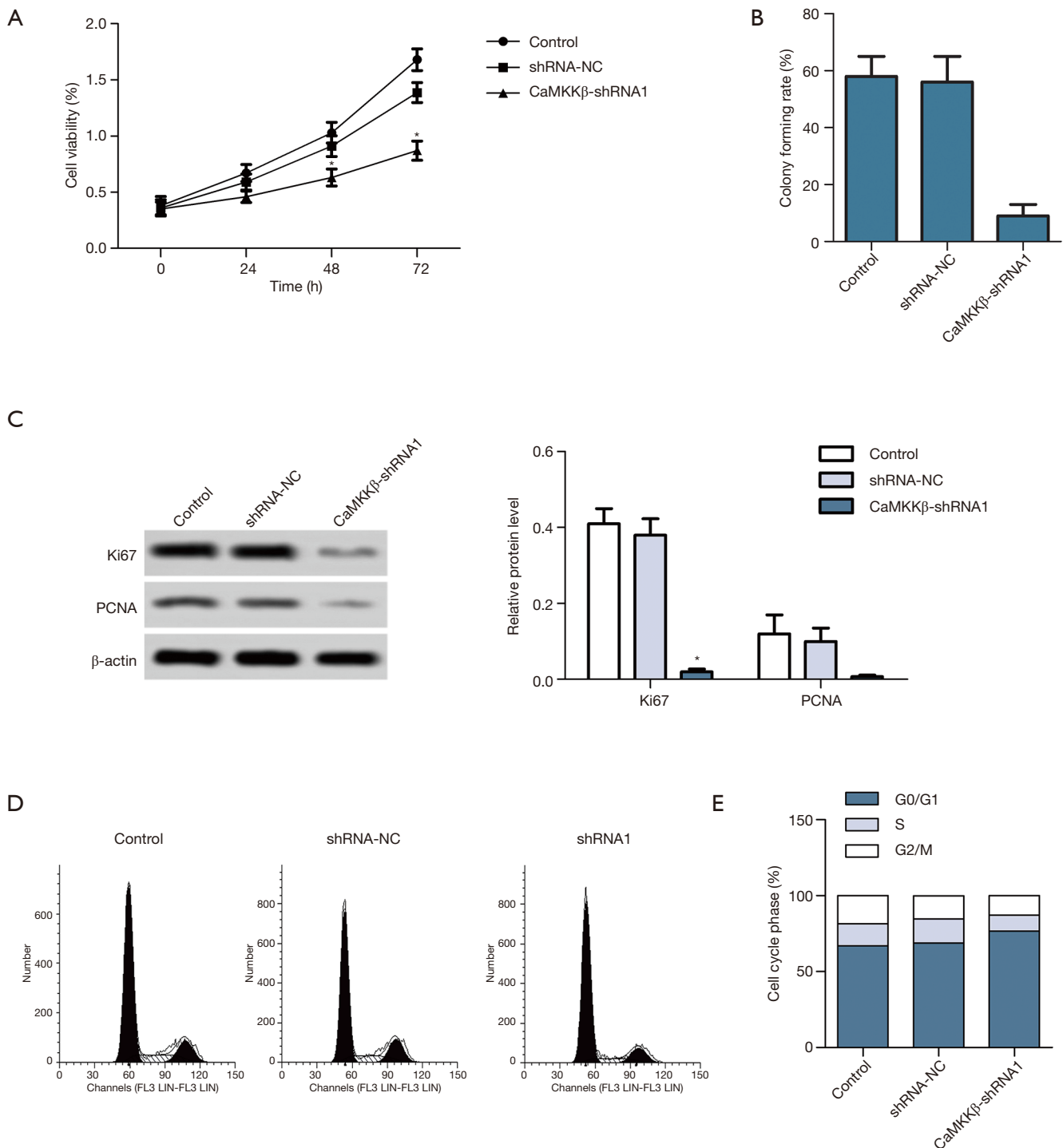


Figure 2 CaMKK β interference inhibited the proliferation and cell cycle of HepG2 cells. (A) The viability of HepG2 cells exposed to different time points (0–72 hours) was measured by MTT assay; (B) the growth of HepG2 cells with CaMKK β -shRNA1 was detected in the clone formation assay; (C) Western blotting assay was used to detect proliferation-related proteins Ki67 and PCNA in HepG2 cells with CaMKK β -shRNA1; (D) cell cycle was analysed using flow cytometry with propidium iodide staining; (E) quantification of cell percentage in cell cycle. The experiments were repeated three times and the data are represented as means \pm SD. *, $P < 0.05$ vs. control.

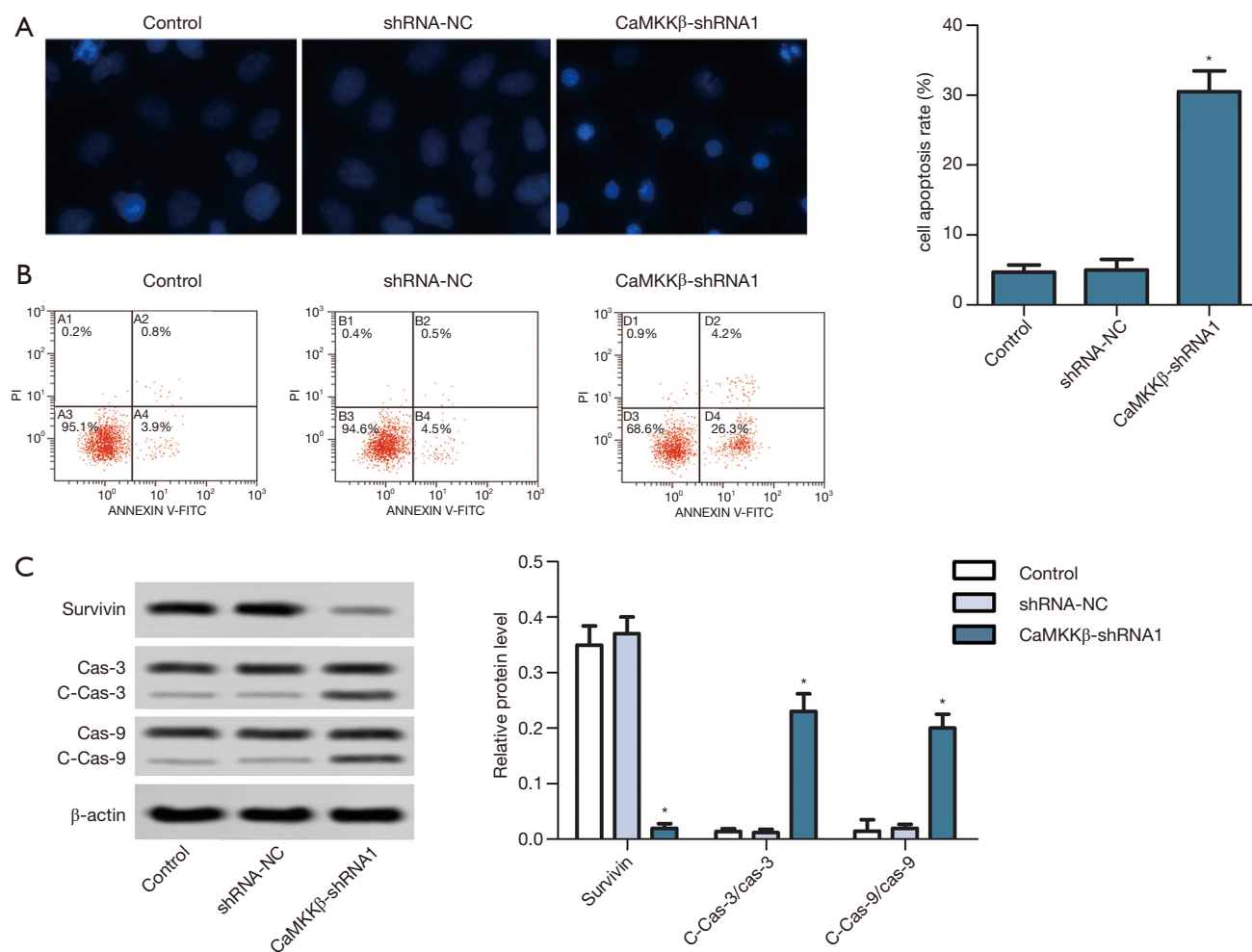


Figure 3 CaMKK β knockdown promoted the apoptosis of HepG2 cells. (A) Morphological changes of apoptotic cells were observed by Hoechst staining, the images were magnified at 400 \times ; (B) the rate of apoptotic cells was measured by flow cytometry; (C) Western blotting was used to detect the factor Survivin that blocked apoptosis and apoptotic factors caspase-3 and caspase-9. The experiments were repeated three times and the data are represented as means \pm SD. *, $P < 0.05$ vs. control.

with the control group, the cell viability of CaMKK shRNA1 group was greatly lower. And clone formation (Figure 2B) assay demonstrated that the clone formation rate of CaMKK β -shRNA1 was significantly lower than that of the control group. WB data showed that the expressions of proliferation-related proteins Ki67 and PCNA were significantly decreased in the CaMKK β -shRNA1 group compared with the control group (Figure 2C). Flow cytometry analysis showed that CaMKK β -shRNA1 treatment significantly increased the percentage of G0/G1 phase cells and decreased the percentage of S-phase cells in HepG2 cells (Figure 2D,E).

CaMKK β knockdown prompted the apoptosis of HepG2 cells

Hoechst staining demonstrated that, the CaMKK β -shRNA1 group showed denser and heavily stained apoptotic cells compared with the control (Figure 3A). Flow cytometry showed that the apoptosis rate of CaMKK β -shRNA1 group was significantly higher than that of the control group (Figure 3B). Moreover, Western blotting showed that the expression of apoptosis suppressor Survivin in CaMKK β -shRNA1 group was inhibited, while the expression of apoptosis-related proteins Caspase-3 and Caspase-9 was significantly enhanced, compared with the control group

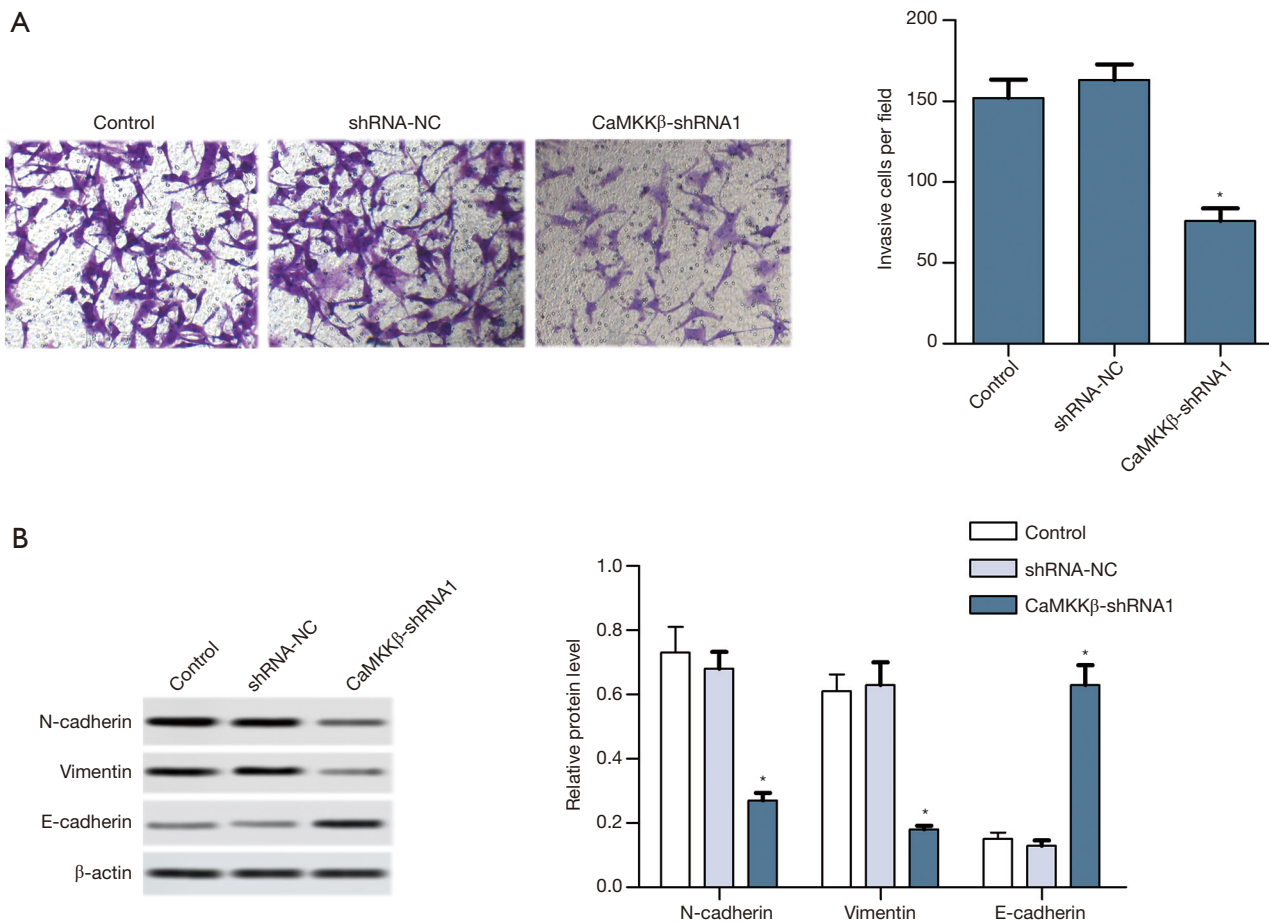


Figure 4 CaMKK β knockdown inhibited the invasion and EMT in HepG2 cells. (A) The invasion of HepG2 cells was assessed using Transwell assay, the images were magnified at 200 \times ; (B) the protein expression of E-cadherin, N-cadherin, and Vimentin were detected by western blot. The experiments were repeated three times and the data are represented as means \pm SD. *, $P < 0.05$ vs. control.

(Figure 3C). The above experimental results demonstrated that CaMKK β interference accelerated the apoptosis of Hep2 cells.

CaMKK β knockdown inhibited invasion and EMT of HepG2 cells

Next, we determined the effect of CaMKK β knockdown on cell invasion *in vitro*. Invasion assay indicated that CaMKK β knockdown significantly interfered with cell invasion relative to the untreated control group (Figure 4A). EMT is widely regarded as the key process of tumor invasion and metastasis (23), EMT-related markers (E-cadherin, N-cadherin, and Vimentin) were detected using western blot in HepG2 cells. Compared with the control group, CaMKK β knockdown remarkably increased the protein

expression of E-cadherin, but decreased the expression of N-cadherin and Vimentin (Figure 4B).

CaMKK β knockdown inhibited HepG2 cells glycolysis

By exploring the effect of CaMKK β expression on the glucose metabolism of HepG2 cells, we found that compared with the control group, CaMKK β knockdown in HepG2 cells greatly inhibited its glycolytic activity, such as reducing glucose uptake (Figure 5A) and lactate release (Figure 5B). Enzyme activity test results showed that the key glycolysis rate-limiting enzymes HK, PK and LDH are significantly lower (Figure 5C). Western blot analysis showed that the protein expression of HK2, PKM2, and LDHA in HepG2 cells were significantly reduced (Figure 5D).

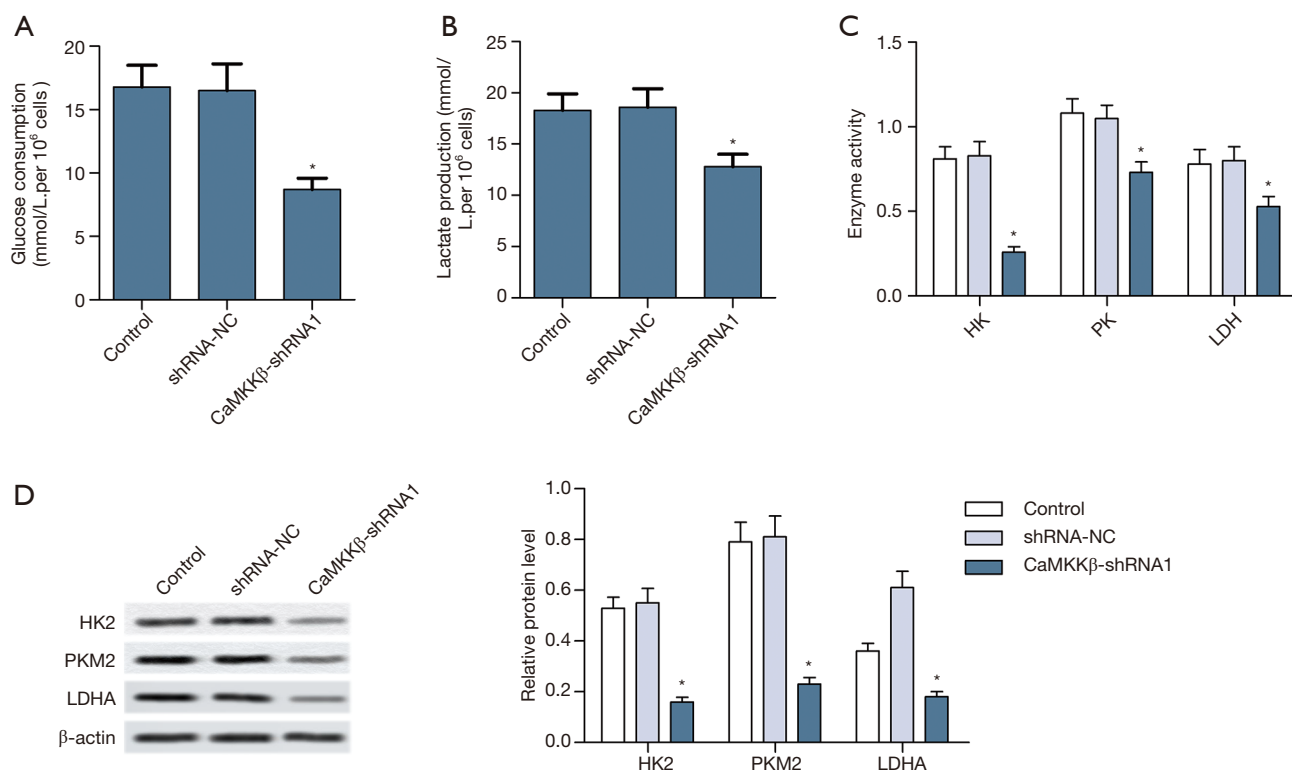


Figure 5 CaMKK β knockdown regulated glycolysis in HepG2 cells. (A) Glucose consumption and lactate production (B) were detected using designated commercial kits. (C) Hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) enzyme activity were detected using designated commercial kits. (D) Protein expression of hexokinase 2 (HK2), pyruvate kinase M (PKM2), and lactate dehydrogenase A (LDHA) by Western Blot. The experiments were repeated three times and the data are represented as means \pm SD. *, $P < 0.05$ vs. control.

CaMKK β knockdown mediated proliferation, apoptosis, and glycolysis of HepG2 cells PI3K/AKT pathways

Furthermore, the potential role of PI3K/AKT signaling in the anticancer activity of CaMKK β knockdown was examined. Western blot analysis showed that CaMKK β knockdown significantly decreased the phosphorylation levels of PI3K and AKT, compared to control group (Figure 6A). To further explore whether CaMKK β regulate proliferation, apoptosis, and glycolysis of HCC cells through PI3K/AKT pathways, AKT overexpression plasmid (pT3-myr-AKT-HA, AddGene, USA) was added to HepG2 cells to assess changes in proliferation, apoptosis, and glycolysis. The results showed that the decreased glucose consumption (Figure 6B) and lactic acid production (Figure 6C) recovered significantly after AKT plasmid transfection. Notably, overexpression of active Akt significantly increased the viability (Figure 6D) and inhibited apoptotic response (Figure 6E) and LDHA expression (Figure 6F).

CaMKK β knockdown inhibited tumor enlargement in xenograft tumor mice

In present study, we did xenograft tumor mice experiment. After 20 days of HepG2 cell injection, the tumor tissue size of the CaMKK β -shRNA1 group start to be significantly inhibited than that of the control group. And as time went on, the difference in tumor size between the two groups became more and more obvious, and the tumor weight (Figure 7A) and size (Figure 7B) of the CaMKK β -shRNA1 group was significantly blocked. The mRNA level of CaMKK β was significantly lower in the CaMKK β -shRNA1 group than that in the control group (Figure 7C). TUNEL assay showed that apoptotic cells increased more significantly in the CaMKK β -shRNA1 group than in the control group (Figure 7D). In the IHC assay, the expression of proliferation-related factor Ki67 and glycolytic restriction enzyme LDHA were decreased in the tissue of the CaMKK β -shRNA1 group, both of which compared

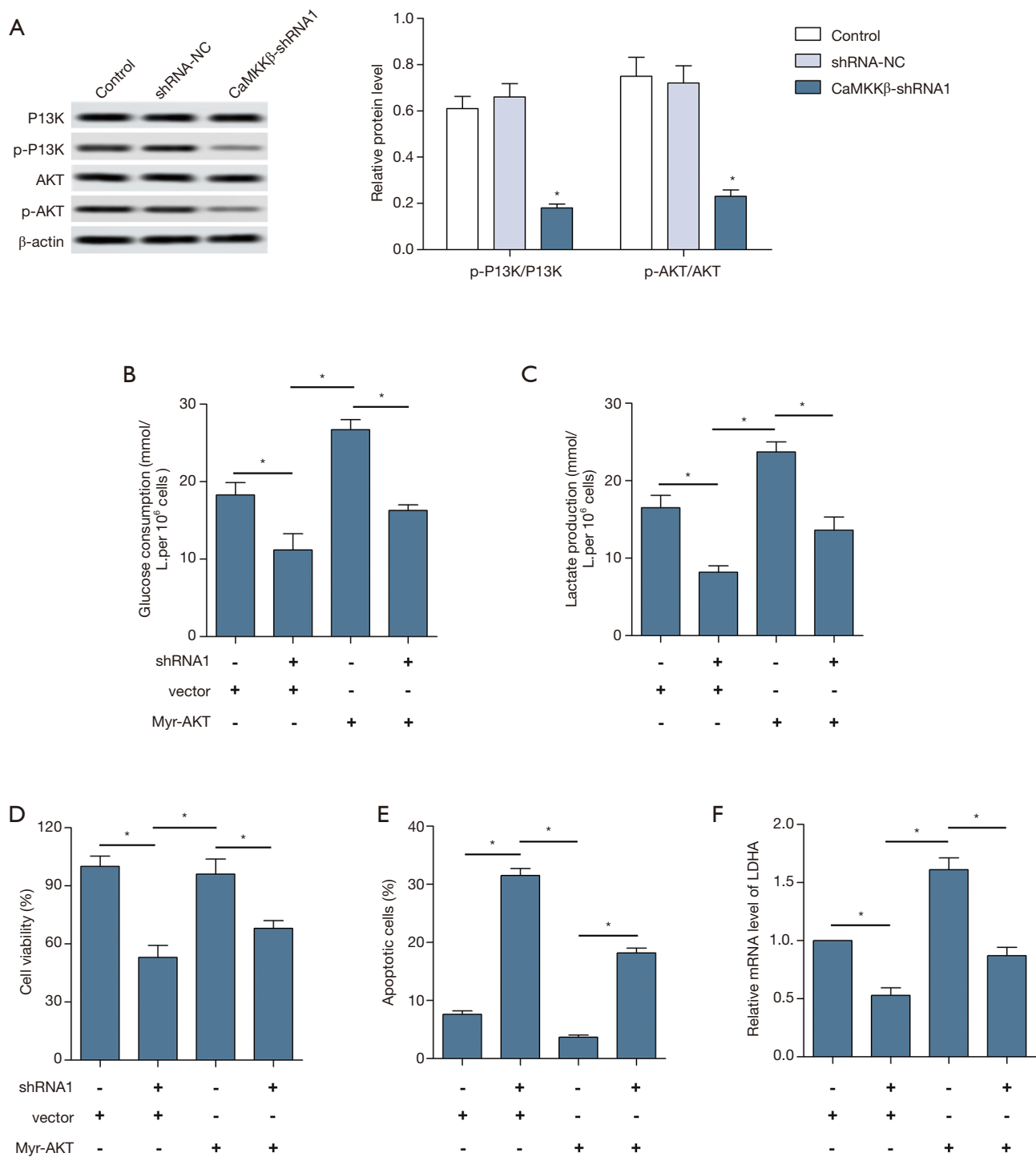


Figure 6 PI3K/AKT signaling mediated CaMKK β knockdown-induced anticancer effects. (A) Protein expression of PI3K, p-PI3K, AKT and p-AKT by Western Blot. HepG2 cells were transfected with active Akt-expressing plasmid or empty vector for 48 h. (B) Glucose consumption and lactate production (C) were detected using designated commercial kits. (D) Cell viability was tested by MTT assay. (E) Apoptosis was analysed by flow cytometry. (F) The mRNA level of LDHA was detected using RT-PCR. The experiments were repeated three times and the data are represented as means \pm SD; *, $P < 0.05$ for indicated comparisons.

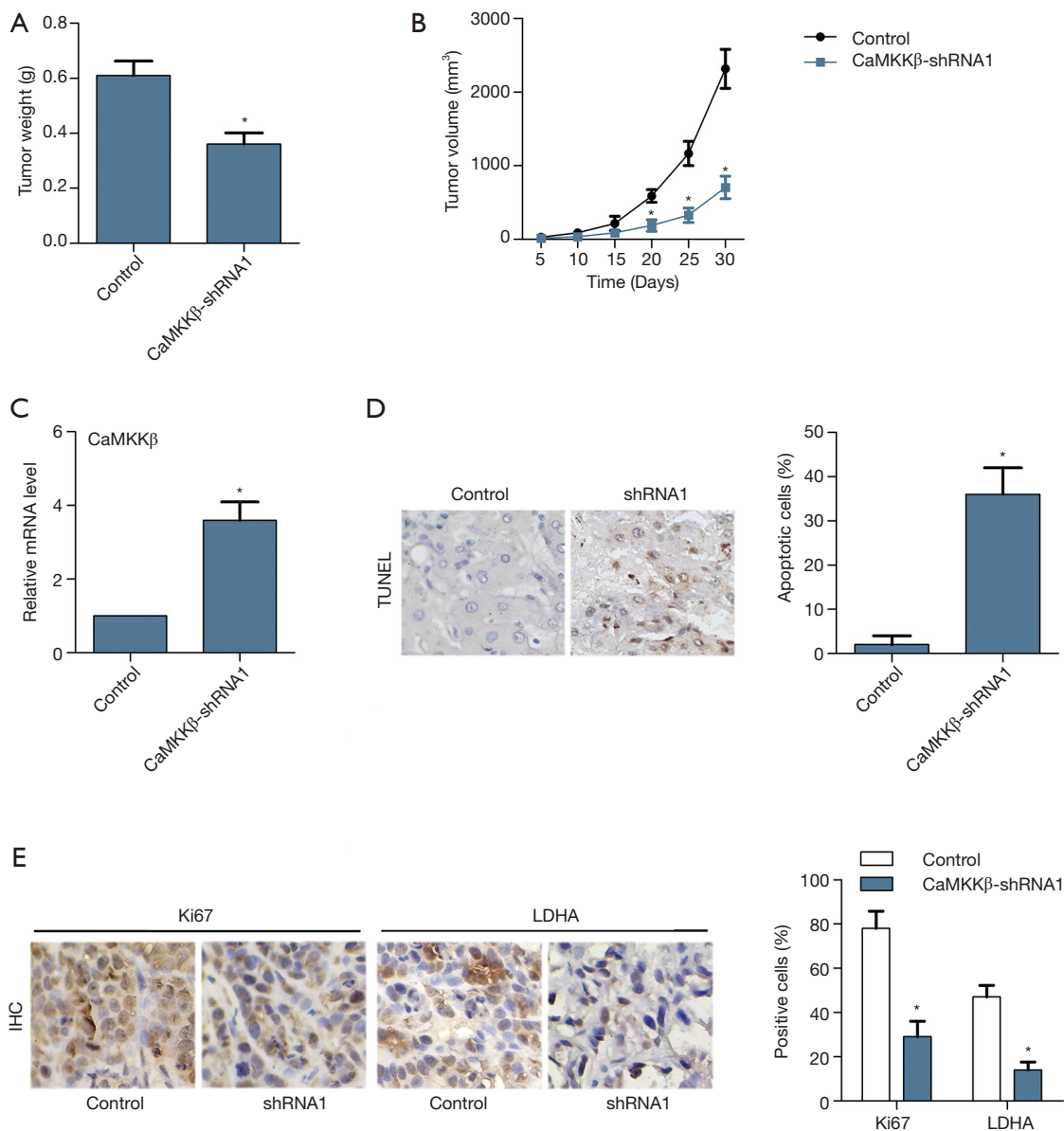


Figure 7 CaMKK β knockdown significantly inhibited tumorigenesis in xenograft tumor mice. (A) The tumor weight of the xenograft mice tumor model was measured; (B) the tumor size of the xenograft mice tumor model was measured; (C) the mRNA levels of CaMKK β in mice tumor tissues were detected by RT-PCR; (D) the TUNEL assay measured the number of apoptotic cells in mice tumor tissue, the images were magnified at 200 \times ; (E) immunohistochemical assay was used to detect the expression of proliferation-related proteins and glycolytic restriction enzyme in tumor tissues, the images were magnified at 400 \times . The experiments were repeated three times and the data are represented as means \pm SD. *, $P < 0.05$ vs. control.

with that in the control group (Figure 7E).

Discussion

Studies have shown that CaMKK β expression is limited in

normal tissues, but it is overexpressed in tumor tissues of certain cancers (24-26). The expression of CaMKK β was significantly up-regulated in HCC and negatively correlated with the survival of HCC patients. CaMKK β protein is highly expressed in a variety of HCC cell lines and is

significantly up-regulated compared to normal primary HCC (27). To assess how CaMKK β regulate important biological processes of HCC, we knock out CaMKK β expression in HepG2 cells. we found that CaMKK β knockdown promoted glycolysis in HepG2 cells through PI3K/AKT pathways and regulated cell proliferation and apoptosis. Furthermore, the *in vivo* experiment also showed that CaMKK β knockdown limited glycolysis and HCC growth. This finding confirmed the results of previous studies that CaMKK β might inhibit the occurrence of HCC.

CaMKK β was overexpressed in a variety of tumor cell types *in vitro*, which affected the proliferation, migration and invasion of tumor cells. It has previously been reported that CaMKK β regulates cell proliferation in high-grade glioma samples (28). CaMKK β was overexpressed in LNCaP of prostate cancer cells to increase cell migration (24). In contrast, the down-regulation of CaMKK expression induced by RNA interference or drug inhibition inhibited the proliferation, migration and invasion of prostate cancer cells (29). By inhibiting the high expression of CaMKK β in HepG2 cells, we found that the down-regulation of CaMKK β resulted in the inhibition of cell proliferation, and the expression of proliferation-related proteins Ki67 and PCNA were significantly inhibited. Similarly, inhibition of CaMKK β activity in HepG2 cells suggested that down-regulation of CaMKK β expression promoted apoptosis. Survivin expression, which inhibits apoptosis, was down-regulated, while the pro-apoptotic Caspase-3 and Caspase-9 expressions were up-regulated. *In vivo* experimental results of transplanted tumor mice further confirmed that CaMKK β interference blocked the proliferation of HCC and promoted its apoptosis.

The increase of glycolysis in tumor cells provides energy for the growth of tumor cells, and the intermediate metabolites of glycolysis can provide raw materials for tumor cell proliferation (30). Therefore, glycolysis is closely related to cell proliferation and apoptosis (31). In this study, we found that CaMKK β knockdown in HepG2 cells not only led to changes in glycolysis, also cell proliferation and apoptosis. Many enzymes in the glycolytic pathway are upregulated by carcinogens, which play an important role in triggering aerobic glycolysis and its toxicity. Rády *et al.* reported that CFLP mice treated with aflatoxin B1 enhanced intraperitoneal HK, PFK, PK and LDH activities 28 days after administration (32). In addition, the food-borne mycotoxin ferric acid up-regulates several glycolytic enzymes including PDK1, PKM2 and LDHa to convert

energy metabolism from mitochondrial processes to glycolysis in human HepG2 cells (33). Like this, our results also showed that, after treating HepG2 cells for 72 hours, CaMKK β knockdown increased the activity and protein levels of HK, PK and LDH.

The PI3K/AKT signaling pathway is related to a variety of human malignant tumor biological processes, such as cell adhesion, growth, invasion, and angiogenesis (34,35). The PI3K/AKT pathway is one of the most common activation pathways in cancers, and it is of great significance in regulating glucose metabolism, proliferation and apoptosis of tumor cells (36). Akt plays a role in the anti-apoptotic pathway through phosphorylation of downstream target proteins. Continuous activation of AKT can prevent PTEN-mediated apoptosis by phosphorylation of Bad (Bcl-2 family member) and protease Caspase-9. in addition, AKT also regulates cell proliferation by mediating the cell cycle. Nevertheless, it has not been tested whether CaMKK β regulates glycolysis in HCC cells through the PI3K/AKT pathway. Our research showed that, after establishing silenced CaMKK β -HepG2 cells, CaMKK β knockdown inhibited glycolysis in HepG2 cells through the PI3K/AKT pathway, and regulated cell proliferation and apoptosis.

In summary, CaMKK β knockdown regulates cell proliferation, apoptosis, and glycolysis in HepG2 cells through the PI3K/AKT pathway, accordingly inhibiting the occurrence and development of HCC. This study has some limitations, such as upstream events leading to CaMKK β down-regulation in HCC and the specific molecular binding mechanism of CaMKK β to regulate PI3K/AKT pathway is still unclear and needs further study.

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Footnote

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Data Sharing Statement: Available at <http://dx.doi.org/10.21037/apm-20-1789>

Conflicts of Interest: All authors have completed the ICMJE

uniform disclosure form (available at <http://dx.doi.org/10.21037/apm-20-1789>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal experiments were approved by the ethics committee of Affiliated Hospital of North Sichuan Medical College (No. 2020053) and performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

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