Journal of Hepatocellular Carcinoma

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

ZDHHC20 Activates AKT Signaling Pathway to Promote Cell Proliferation in Hepatocellular Carcinoma

Xiaoju Huang[1–3], Mengmeng Wang[1–3], Dan Zhang[1–3], Junpeng Meng[4,5], Pian Liu[1–3]

[1]Cancer Center Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, People's Republic of China; [2]Hubei Key Laboratory of Precision Radiation Oncology, Wuhan, 430022, People's Republic of China; [3]Institute of Radiation Oncology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, People's Republic of China; [4]Department of Pancreatic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, People's Republic of China; [5]Department of General Surgery, the Second Hospital of Shanxi Medical University, Taiyuan, Shanxi, 030001, People's Republic of China

Correspondence: Pian Liu; Junpeng Meng, Email liupianamazing@126.com; mengjunpeng1981@163.com

Background: Liver cancer is the sixth most common cancer worldwide, and hepatocellular carcinoma (HCC) presents one of the most challenging global health issues. ZDHHC20, a member of the ZDHHC palmitoyltransferase (ZDHHC-PAT) family, is involved in a reversible lipid modification known as palmitoylation, which contributes to the occurrence and progression of various tumors. However, the specific mechanisms underlying the involvement of ZDHHC20 in this process are unclear.

Methods: The effects of both ZDHHC20 knockdown and overexpression on hepatocellular carcinoma cell proliferation were evaluated using PCR, Western blotting, CCK-8 assay, colony formation assay, cell cycle analysis, apoptosis analysis, and EDU assay. The TCGA-LIHC dataset was analyzed bioinformatically, and the phosphorylation level of PI3K and AKT in SK-Hep1 and Huh7 cells was assessed using Western blotting. Nude mouse subcutaneous xenograft experiments were conducted to evaluate the effects of different treatment conditions on mouse tumor growth.

Results: ZDHHC20 knockdown inhibited cell proliferation and promoted apoptosis, while overexpression of ZDHHC20 promoted cell proliferation and inhibited apoptosis. Knockdown of ZDHHC20 also decreased phosphorylation of PI3K and AKT in HCC, whereas overexpression of ZDHHC20 increased phosphorylation of PI3K and AKT. The PI3K-AKT pathway inhibitors, LY294002 and MK2206, effectively inhibited the promotional effects of ZDHHC20 on the proliferation and growth of HCC.

Conclusion: High expression of ZDHHC20 promotes the proliferation and tumor growth of HCC by activating the PI3K-AKT signaling pathway. The PI3K inhibitor LY294002 and the AKT inhibitor MK2206 inhibit the promotional effects of ZDHHC20 on the proliferation of HCC and the growth of tumors.

Keywords: hepatocellular carcinoma, ZDHHC20, PI3K-AKT pathway, cell proliferation

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide. According to 2020 statistics, liver cancer is the sixth most common cancer and the third leading cause of cancer-related death.[1] HCC accounts for approximately 75–85% of primary liver cancer.[2] Unfortunately, most cases of HCC are diagnosed late due to delayed symptoms and lack of early detection, resulting in poor prognosis for patients. Therefore, more in-depth investigation on the mechanisms underlying HCC occurrence, metastasis, and recurrence, as well as the exploration of appropriate targeted interventions, is needed to improve the prognosis for patients with HCC.

Palmitoylation is a reversible lipid modification process in which palmitoyl groups are covalently bound by thioester bonds to cysteine by palmitoyl acyltransferase.[3,4] Palmitoylation is catalyzed primarily by the ZDHHC palmitoyl S-acyltransferases (PATs) family of proteins, which are known as DHHC-PAT because of their unique catalytic motif, DHHC (Asp-His-His-Cys).[5] This domain also combines two Zn2+ ions to form a zinc finger domain, resulting in

Received: 3 January 2024
Accepted: 3 July 2024
Published: 18 September 2024

a ZDHHC palmitoyltransferase. To date, 23 human ZDHHC genes (named ZDHHC1–24, without ZDHHC10) have been identified.[6] Although palmitoylation is known to regulate protein localization, stability, and function, the mechanisms by which ZDHHCs select substrate proteins for modification is not fully understood. ZDHHC20 is one of the most extensively studied members of this family and is the only DHHC enzyme with a known spatial structure.[7]

Since the discovery of palmitoylation, various proteins have been found to undergo ZDHHC-mediated palmitoylation modification, which is implicated in the occurrence and development of various tumors. A list of 299 cancer driver genes was recently validated, of which 26% may undergo palmitoylation modification.[8] At least 15 of the 23 known human ZDHHCs are related to cancer,[9] highlighting the correlation between palmitoylation modification and cancer. However, the specific DHHC and its mediated substrates undergoing palmitoylation remain unclear. Proteins encoded by oncogenes or tumor suppressor genes can be modified by palmitoylation, affecting protein stability, membrane localization, protein interactions, and signal transduction, which regulate tumorigenesis and progression.[10–12] ZDHHC17 (also known as HIP14) is upregulated in breast and colon cancer,[13] while ZDHHC2 exhibits tumor suppressor effects in some tumors.[14] Moreover, mutated Ras is one of the most common oncogenes, and studies have found that the palmitoylation/depalmitoylation cycle can regulate the subcellular trafficking and oncogenicity of both H-Ras and N-Ras.[15,16] However, broad-spectrum protein palmitoylation inhibitors such as 2-bromopalmitate (2-BP) cannot selectively target a specific ZDHHC,[17] and there is currently no specific ZDHHC inhibitor. Therefore, targeting palmitoyltransferases or palmitoylated proteins may be a promising cancer treatment strategy.

Studies on the association between protein palmitoylation and HCC progression are relatively limited compared with other types of malignancies, such as breast, prostate, colorectal, leukemia, melanoma, pancreatic, and non-small cell lung cancer.[9] However, studies have shown that the ZDHHC2-mediated palmitoylation of CKAP4 is associated with disease progression and metastasis in patients with liver cancer.[14] Moreover, CD44 binds to Ezrin to promote cell adhesion and migration, and high levels of cholesterol promote the chelation of CD44 into lipid rafts in a palmitoylation-dependent manner, which reduces CD44-ezrin binding and inhibits HCC invasion and metastasis.[18] Given the limited research to date on protein palmitoylation in HCC progression, more in-depth studies are required to elucidate the specific mechanisms involved in protein palmitoylation affecting the occurrence and development of liver cancer.

In this study, we investigated the role of ZDHHC20 in HCC. We found that ZDHHC20 was overexpressed in HCC tumor samples, which was closely correlated with poor prognosis. Furthermore, knockdown of ZDHHC20 inhibited cell proliferation, while overexpression of ZDHHC20 increased cell proliferation in HCC. Through analysis of the TCGA-LIHC dataset, we found that ZDHHC20 was closely associated with the activation of the PI3K-AKT signaling pathway, which was confirmed in liver cancer cells. Additionally, we showed that inhibition of the PI3K-AKT signaling pathway blocked the tumor-growth promoting role of ZDHHC20 in liver cancer cells. Taken together, these results suggest that ZDHHC20 acts as a promoter of cancer cell proliferation in HCC.

Materials and Methods

Cell Culture

The hepatocellular carcinoma cell lines Huh7 and SK-Hep1 were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China). The cell lines were cultured in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell lines were routinely maintained in a 5% CO_2 incubator at 37°C.

Antibodies and Chemicals

The DMEM and 10% fetal bovine serum were purchased from Gibco Biotechnology Co. Ltd. The CCK-8 kit (CA1210) was obtained from Solarbio (Beijing, China). The RIPA Lysis Buffer (P0013B) and BCA protein assay kit (P0010) were purchased from Beyotime Biotechnology. Lipofectamine 2000 (11668030) was purchased from Thermo Fisher Scientific. Opti-MEM (31985070) was purchased from Gibco Biotechnology Co. Ltd. Puromycin (HY-K1057), MK2206 (HY-108232), and LY294002 (HY-10108) were purchased from MedChemExpress (Shanghai, China). Cell Cycle and Apoptosis Analysis Kit (40301ES60) was purchased from Yeasen Biotechnology (Shanghai) Co., Ltd. EdU cell proliferation detection kit (C0071S) was purchased from Beyotime Biotechnology (Beijing, China). The following antibodies were used: ZDHHC20 antibody

(PU805934S, 1:1000 dilution) was purchased from Abmart (Shanghai, China), Flag antibody (AE063, 1:1000 dilution), GAPDH (AC001, 1:5000 dilution), and HRP Goat Anti-Rabbit IgG (H+L) (AS014, 1:5000 dilution) were purchased from Abclonal (Wuhan, China). Additionally, Phospho-AKT (Ser473) antibody (11054, 1:1000 dilution), and AKT antibody (33748, 1:1000 dilution) were purchased from Signalway Antibody (Nanjing, China). Phospho-PI3K antibody (AF3241, 1:1000 dilution) was obtained from Affinity Biosciences (Jiangsu, China).

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (#9109, Takara Bio, Beijing, China), and the RNA samples were reverse-transcribed into cDNA using the PrimeScriptTM RT Kit (#RR037A, Takara). The genes were then amplified and detected by real-time PCR using TB Green™ Fast qPCR Mix (#RR430A, Takara) according to the manufacturer's instructions. The internal reference gene was GAPDH. The primer sequences for ZDHHC20 were F: 5'-CGCACCCACGTTTTCATACG-3'; R: 5'-TCTGGCATACTCATTCTGGTTTG-3'. The primer sequences for GAPDH were F: 5'-AATGGGCAGCCGTTAGGAAA -3'; R: 5'-GCGCCCAATACGACCAAATC-3'.

Plasmids and Transfection

The overexpression plasmid FLAG-ZDHHC20 (GV657 vector) was purchased from Shanghai Gene Chem Co., Ltd. The small hairpin RNA (shRNA) targeting ZDHHC20 (shZDHHC20) was purchased from RuiBo Biotechnology Co., Ltd (Guangzhou, China). Huh7 and SK-Hep1 cells were seeded in 6-well plates and transfected with shZDHHC20 and the ZDHHC20 overexpression plasmid using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Shanghai, China) and Opti-MEM (Gibco, Shanghai, China) at 37°C for 8 hours. After transfection, Opti-MEM was replaced with complete DMEM medium. Stable expression cell lines were constructed using an appropriate concentration of puromycin (MedChemExpress, Shanghai, China) for screening transfected cell lines after transfection for 72 hours. The sequences for shZDHHC20-1 were: 5'-GAACAAGCTTCTGTTACAA-3'. The sequences for shZDHHC20-2 were:5'-GCAAGAGCTTTACCTATCT-3'.

Western Blot Analysis

Cells were collected and lysed with RIPA lysis buffer (Beyotime Biotechnology, Beijing, China) containing 1% phosphatase and protease inhibitors for 30 minutes. The concentration of target proteins was determined using the BCA protein assay kit (Beyotime Biotechnology, Beijing, China). Equal amounts of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred to PVDF membranes (Millipore, Shanghai, China). After blocking in 5% skimmed milk, these membranes were incubated with specific antibodies at 4°C overnight. The next day, the PVDF membranes were washed three times using TBST and then were incubated for 1 hour with the secondary antibody. Finally, protein bands were visualized using the enhanced chemiluminescence ECL substrate, and protein band intensities were determined using Image Lab software.

Colony Formation Assay

Cells were cultured to the logarithmic growth phase and digested into single-cell suspensions with trypsin. Cells were plated into 6-well plates at 500 cells per well. The medium of the 6-well plate was changed every 3 days. After 14 days, the cell colonies were fixed using 4% paraformaldehyde for 30 minutes at room temperature and stained with crystal violet solution for 15 minutes. Subsequently, colonies were photographed and counted.

CCK-8 Assay

To evaluate cell growth ability, a CCK-8 Kit (Solarbio, Beijing, China) was utilized according to the manufacturer's instructions. After transfection, 96-well plates were loaded with 2000 cells per well, and MK2206 or LY294002 was added. Four replicate wells were set up for each group. Specifically, 10 μL of CCK-8 was added to each well at the designated time points, followed by incubation at 37°C for 1 hour. The absorbance (450 nm) of different groups of cells was detected using an enzyme labeling instrument at specific time points.

Flow Cytometry

After 72 hours of cell transfection, cells were inoculated in 6-well plates at 5×10^5 cells/well. When confluency reached around 70%, the cells were digested using trypsin, centrifuged and washed twice with PBS. Cells were then centrifuged again and collected. Data were analyzed using FlowJo V10 software. To determine cell cycle distribution, cell cycle distribution was analyzed using ModFit LT5.

EdU Assay

Cells were transfected with shZDHHC20 or ZDHHC20 overexpression plasmid for 72 h. Cells in the logarithmic growth phase were inoculated in 96-well plates. Cell proliferation was assessed using the EdU Cell Proliferation Assay Kit. Cells were observed after staining using the EdU kit as directed by manufacturer's instructions. Photographs were taken using a fluorescence microscope. EdU-positive cells were counted using ImageJ software.

Mouse Study

Nude BALB/c-nu mice (male, 4–5 weeks old, 18–20 g) were purchased from Beijing HFK Bio-science (Beijing, China). The sex of the mice did not influence the results of this study. Liver cancer cells (5×10^6) subjected to different treatments were suspended in 100 μL PBS and inoculated in the left flanks of mice to observe tumor growth. The MK2206 (120 mg/Kg, p. o.) was administrated once every three days. The width and length of the tumor were measured every 3 days with a caliper, and the tumor volume was evaluated with the formula: Volume (mm^3) = $L \times W^2/2$. After 21 days, the mice were euthanized, and the tumors were dissected, weighed, and fixed in 4% paraformaldehyde. All animal experiment procedures were approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (3385).

Immunohistochemistry (IHC)

The tissue microarray slide was purchased from Zhongke Guanghua (Xi'an) Intelligent Biological Technology (D097LV01), and the tissue chip was stained with ZDHHC20 antibody (Abmart, 1:2000 dilution) using an IHC staining kit (Bios Biological Technology, Wuhan, China).

Statistical Analysis

GraphPad Prism 8.0 Software was used. Differences between the two groups were tested for significance using the student *t*-test. One-way analysis of variance (ANOVA) followed by Turkey's multiple comparisons post-hoc test was used to compare differences between more than two groups. A p-value < 0.05 was considered statistically significant.

Results

Abnormally Overexpressed ZDHHC20 is Associated with Poor Prognosis in HCC

We analyzed the TCGA-LIHC, GSE214846, GSE144269, and GSE105130 datasets and found that the expression levels of ZDHHC20 were significantly higher in HCC tumor samples than normal liver tissues (Figure 1A–D). Immunostaining using the anti-ZDHHC20 antibody in the HCC tissue microarray showed that ZDHHC20 was upregulated in HCC tissue samples compared with normal liver tissues (Figure 1E). Additionally, analysis using the GEPIA web tool (http://gepia.cancer-pku.cn/) revealed that lower expression of ZDHHC20 was associated with favorable prognosis, while higher expression of ZDHHC20 was correlated with poor prognosis in patients with HCC (Figure 1F). We analyzed ZDHHC20 expression in various HCC cell lines (Figure 1G). Together, these findings suggest that ZDHHC20 is abnormally overexpressed and associated with poor prognosis in HCC patients.

Knockdown of ZDHHC20 Inhibits Proliferation of HCC Cells

We investigated the cancer-related function of ZDHHC20 in liver cancer cells. Specifically, we assessed the effect of downregulating ZDHHC20 expression in SK-Hep1 and Huh7 cells using two different short hairpin RNAs (shRNA) that targeted ZDHHC20 (Figure 2A and B). Our findings showed a significant decrease in cell proliferation capability in these

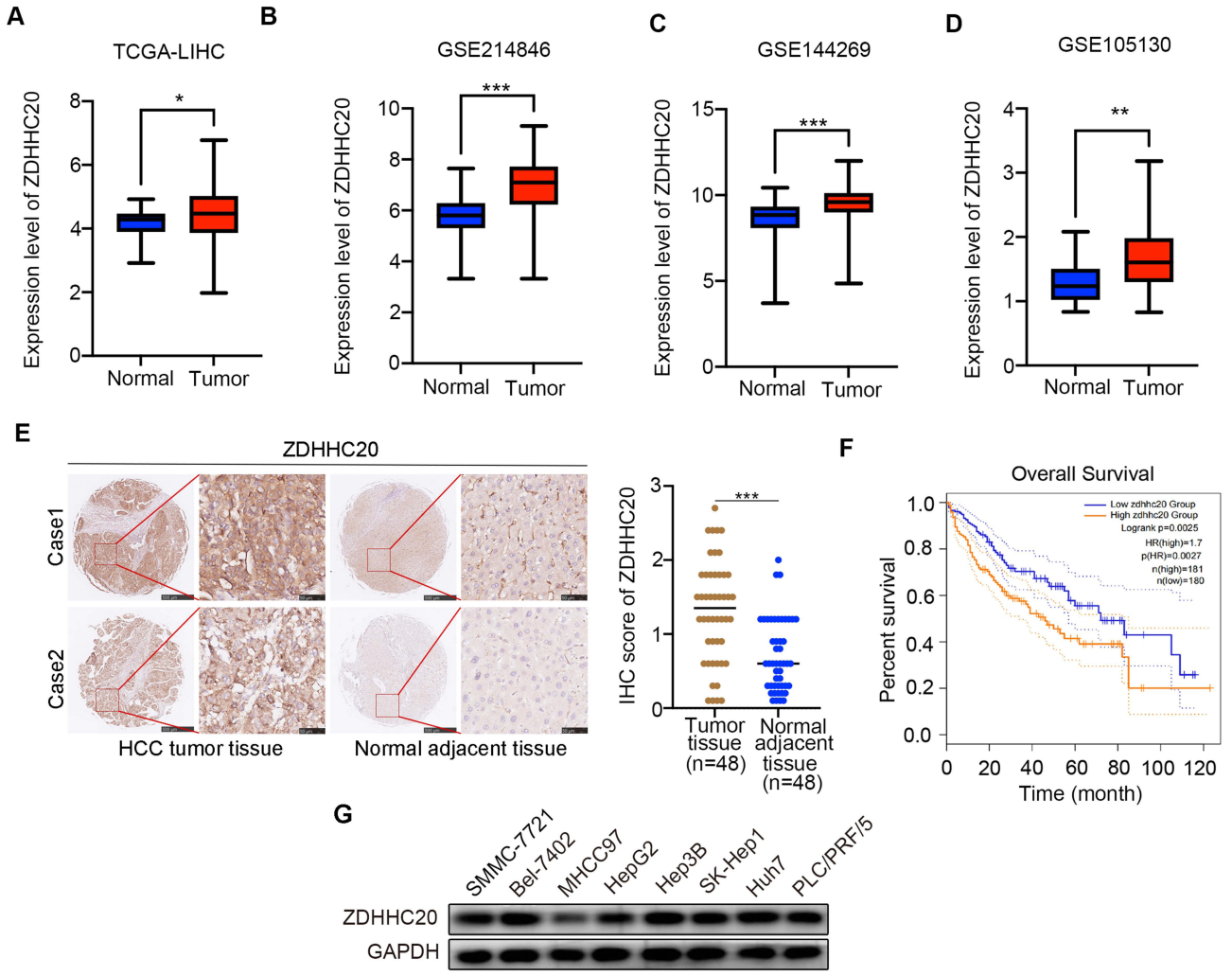

Figure 1 Abnormally overexpressed ZDHHC20 is associated with poor prognosis in HCC. (**A**–**D**), The expression of ZDHHC20 in HCC tumor samples and normal liver tissues was analyzed using the TCGA-LIHC (A), GSE214846 (**B**), GSE144269 (**C**) and GSE105130 (**D**) datasets. *P < 0.05; **P < 0.01, ***P < 0.001. (**E**), HCC tissues (adjacent non-tumor tissue n=48, liver cancer n=48) were stained with anti-ZDHHC20 antibody; representative images are shown. ***P < 0.001. (**F**), Overall survival in patients with HCC with different levels of ZDHHC20 as determined by GEPIA web tool. (**G**), Western blotting analysis was used to determine the expression level of ZDHHC20 in various hepatocellular carcinoma cell lines.

HCC cells as measured by CCK-8 assay and colony formation assay (Figure 2C and D). Flow cytometry results showed that knockdown of ZDHHC20 significantly increased the apoptosis rate of SK-Hep1 and Huh7 cells compared with the control group (Figure 2E). The distribution of cells in the G1 phase was significantly increased, while the distribution of cells in the S phase was significantly decreased (Figure 2F). The results of the EDU experiments indicated that cell proliferation in the ZDHHC20-knockdown group was significantly lower than in the control group, whereas in the ZDHHC20-overexpression group, it was significantly higher than in the control group (Figure 2G). A nude mouse xenograft assay demonstrated that overexpression of ZDHHC20 promoted tumor growth, whereas knockdown of ZDHHC20 inhibited tumor growth compared with the control group (Figure 2H). These data suggest that ZDHHC20 knockdown can inhibit the proliferation of HCC cells.

Overexpression of ZDHHC20 Can Promote the Proliferation of HCC Cells

We overexpressed ZDHHC20 in SK-Hep1 and Huh7 cells (Figure 3A and B) which led to an increase in cancer cell proliferation capacity in HCC cells, as shown by a CCK-8 assay (Figure 3C) and colony formation assay (Figure 3D). Flow cytometry results showed that overexpression of ZDHHC20 significantly inhibited the apoptosis rate of SK-Hep1 and Huh7 cells (Figure 3E). The distribution of SK-Hep1 and Huh7 cells in the G1 phase was significantly decreased and

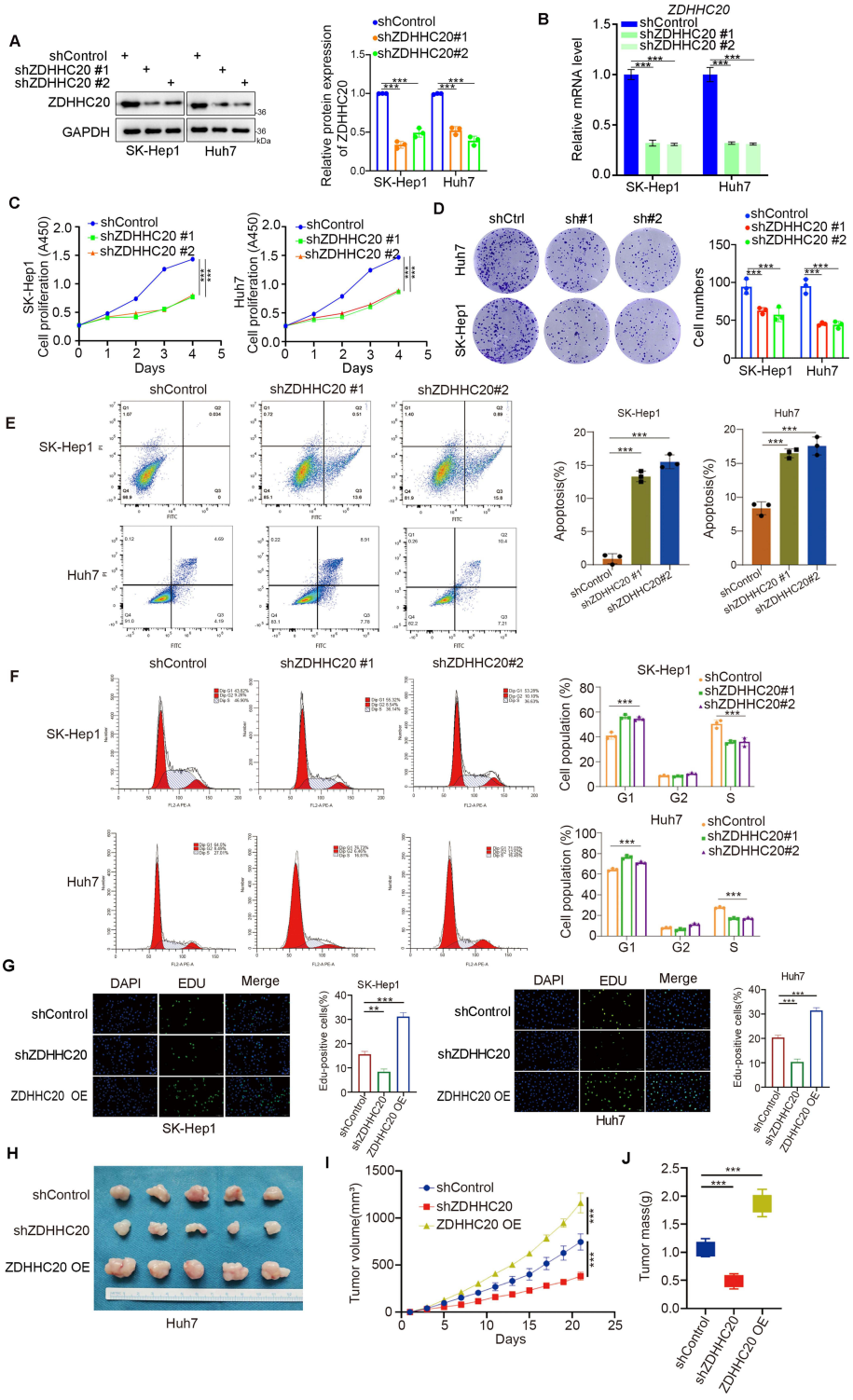

Figure 2 Knockdown of ZDHHC20 inhibits proliferation of HCC cells. (**A**–**D**), Huh7 and SK-Hep1 cells were transfected with shZDHHC20 for 72 hours. After transfection, cells were collected for Western blotting analysis (**A**), RT-qPCR analysis (**B**), CCK-8 assay (**C**) and colony formation assay (**D**). For panel (**A**–**D**), data are presented as mean ± SEM with three replicates. ***P < 0.001. (**E**), The cell apoptosis rate was measured by flow cytometry. Q1–Q4 corresponds to necrotic cells, late apoptotic cells, early apoptotic cells, and non-apoptotic cells, respectively. The results are presented as mean ± SEM with three replicates. ***P<0.001. (**F**), Cell cycle progression was measured by flow cytometry. G1 represents the prophase of DNA synthesis, S represents DNA synthesis, and G2 represents the late phase of DNA synthesis. The results are presented as mean ± SEM with three replicates. ***, P < 0.001. (**G**), The proliferation of Huh7 and SK-Hep1 cells after ZDHHC20 knockdown or overexpression was determined using EDU assay. The results are presented as mean ± SEM with three replicates. **P < 0.01, ***P<0.001. (**H**–**J**), ZDHHC20 overexpression plasmid or shZDHHC20 was transfected into Huh7 cells and stably transfected cell lines were constructed. Images depicting xenograft tumor tissue (**H**), tumor growth curves (**I**) and the weight distribution of excised tumors (**J**). Data are presented as mean ± SEM with five replicates. ***P < 0.001.

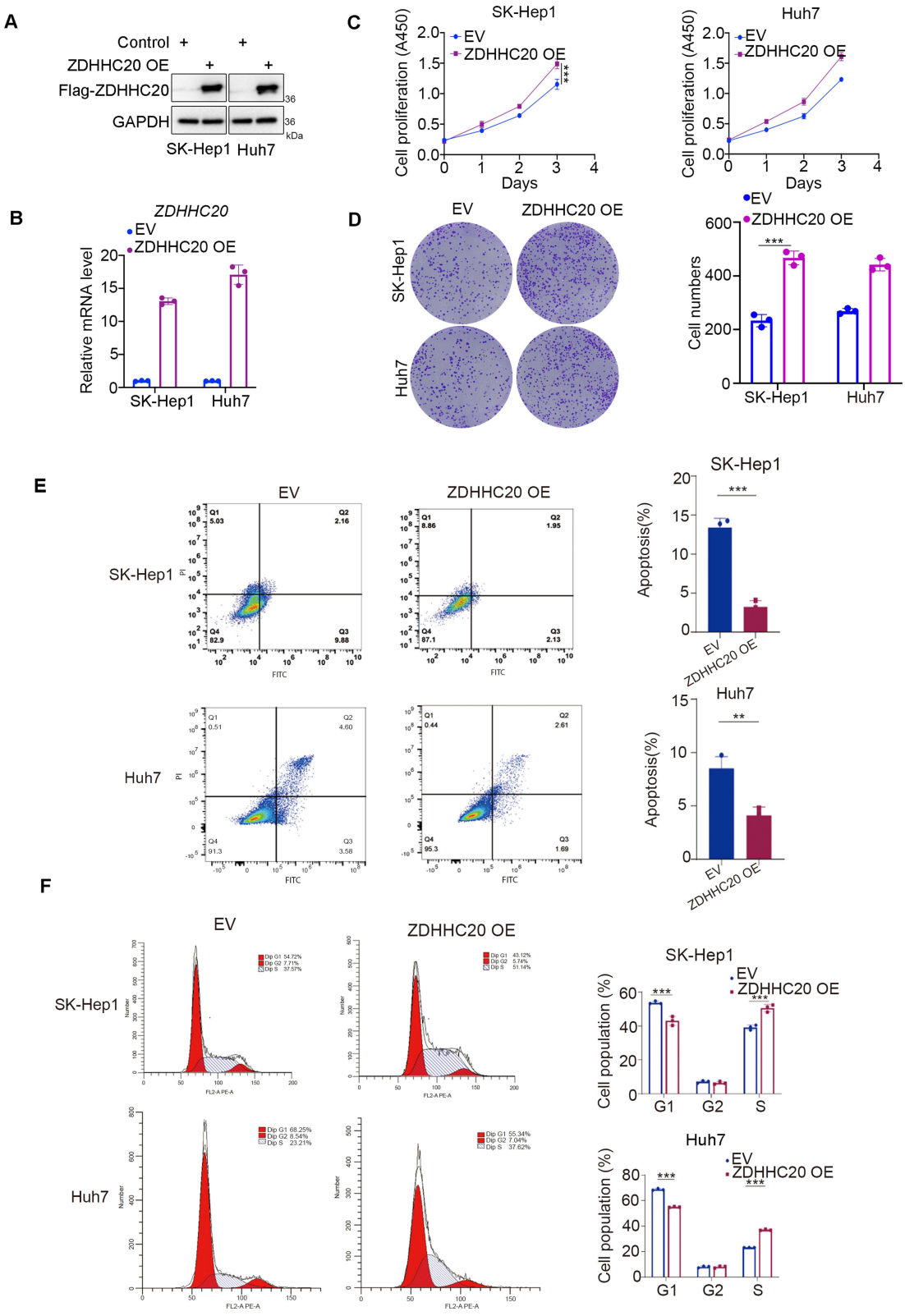

Figure 3 Overexpression of ZDHHC20 can promote the proliferation of HCC cells. **(A–D)**, Huh7 and SK-Hep1 cells were transfected with ZDHHC20 plasmid for 72 hours. After transfection, cells were harvested for Western blotting analysis (**A**), RT-qPCR analysis (**B**), CCK-8 assay (**C**) and colony formation assay (**D**). For panel (**A–D**) data are presented as mean ± SEM with three replicates. ***P < 0.001. (**E**), The cell apoptosis rate was measured by flow cytometry. The histogram shows the percentage of apoptotic cells. Q1–Q4 corresponds to necrotic cells, late apoptotic cells, early apoptotic cells, and non-apoptotic cells, respectively. The results presents as mean ± SEM with three replicates. **P <0.01; ***P<0.001. (**F**), The cell cycling progression were measured by flow cytometry assays. G1 represents the prophase of DNA synthesis, S represents DNA synthesis, and G2 represents the late phase of DNA synthesis. The results presents as mean ± SEM with three replicates. ***P<0.001.

the distribution of cells in the S phase was significantly increased (Figure 3F). Taken together, these data indicate that ZDHHC20 is responsible for promoting cancer cell proliferation in HCC.

ZDHHC20 Activates the AKT Signaling Pathway in Liver Cancer Cells

Next, we divided patients in the TCGA-LIHC dataset into two subgroups based on ZDHHC20 expression levels. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) of the TCGA-

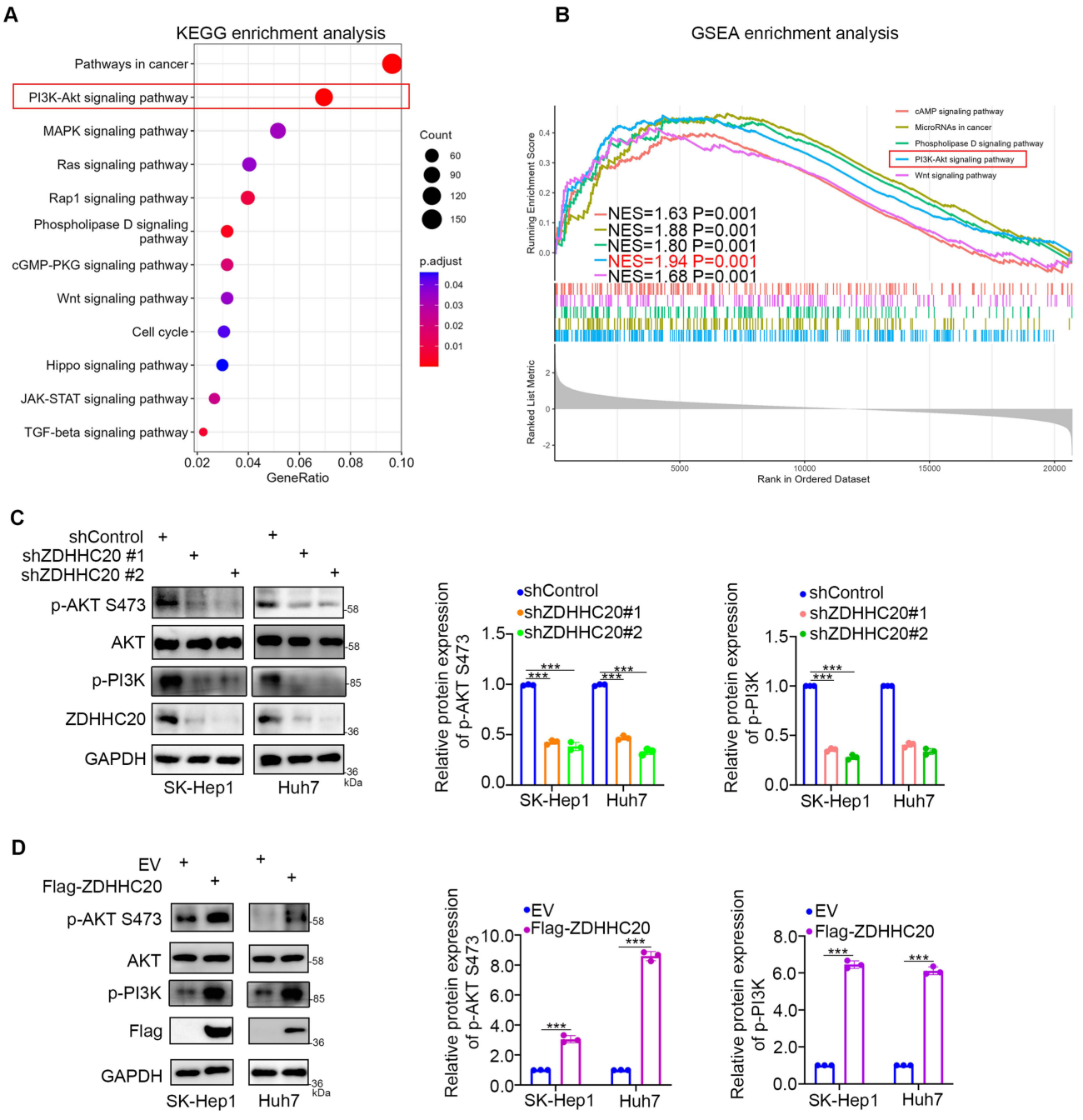

Figure 4 ZDHHC20 activates the AKT signaling pathway in liver cancer cells. **(A** and **B)**, Kyoto Encyclopedia of Genes and Genomes (KEGG) (**A**) and Gene Set Enrichment Analysis (GSEA) GSEA Enrichment analyses (**B**) were used to analyze two subgroups of HCC patients with different expression of ZDHHC20. (**C**), Huh7 and SK-Hep1 cells were transfected with shZDHHC20 for 72 hours. After transfection, cells were collected for Western blotting analysis. (**D**), Huh7 and SK-Hep1 cells were transfected with ZDHHC20 plasmid for 72 hours. After transfection, cells were harvested for Western blotting analysis. For panel (**C** and **D**), datas are presented as mean ± SEM with three replicates. ***P<0.001.

LIHC dataset, we found that ZDHHC20 was closely involved in the activation of multiple oncogenic signaling pathways, especially the PI3K-AKT signaling pathway (Figure 4A and B). We then demonstrated that knockdown of ZDHHC20 decreased the phosphorylation of PI3K and AKT in SK-Hep1 and Huh7 cells (Figure 4C), while overexpression of ZDHHC20 increased the phosphorylation of PI3K and AKT in these cells (Figure 4D). These results suggest that ZDHHC20 is involved in activating the PI3K-AKT signaling pathway in liver cancer cells.

Inhibition of the AKT Signaling Pathway Impedes the Function of ZDHHC20 in the Proliferation of Liver Cancer Cells

The PI3K-AKT signaling pathway is well known to promote liver cancer cell growth. The above data suggested that ZDHHC20 enhances liver cancer cell proliferation, and ZDHHC20 increases AKT phosphorylation in liver cancer cells. We speculated that ZDHHC20 regulates cancer cell growth through the PI3K-AKT signaling pathway. To test this, we used

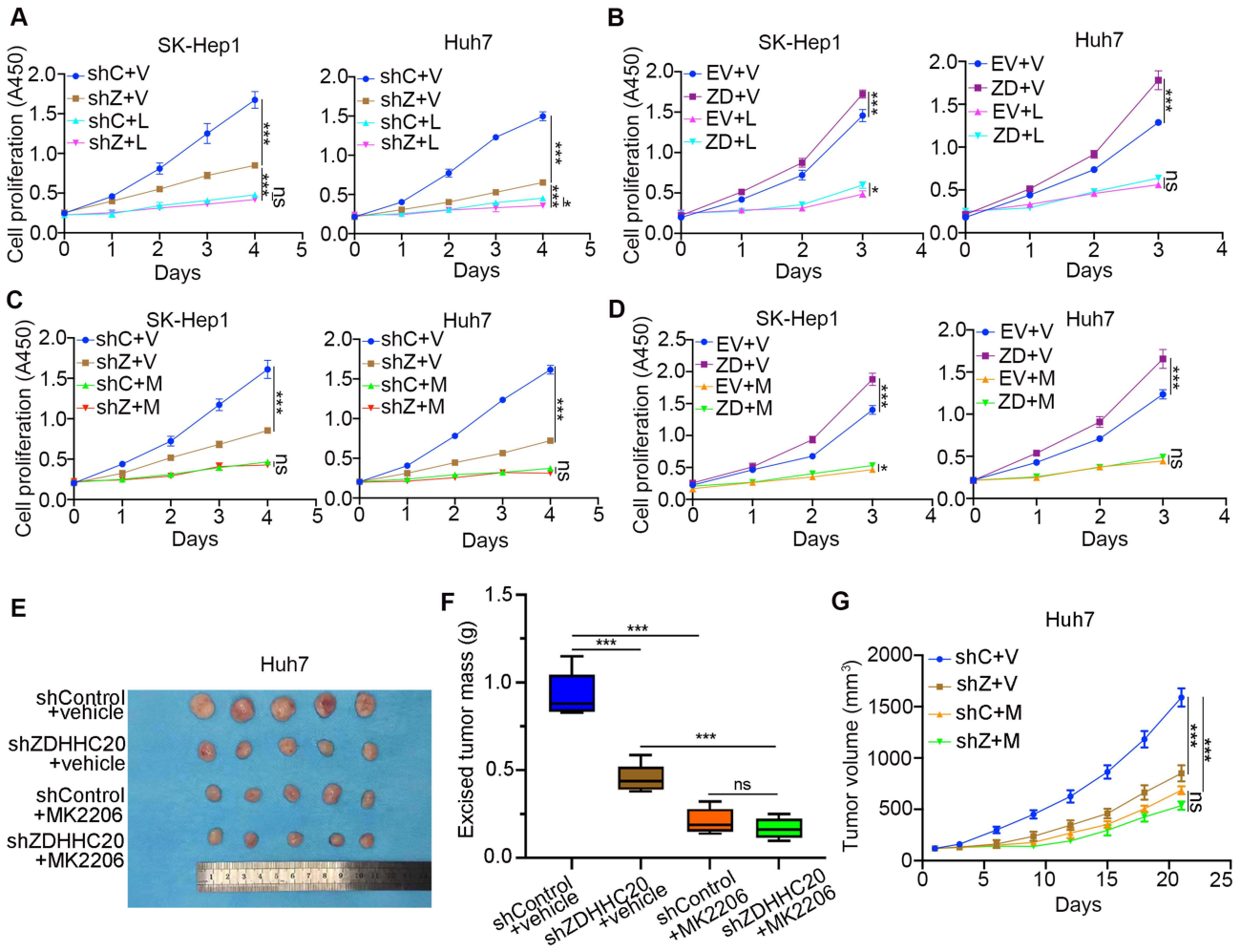

Figure 5 Inhibition of the AKT signaling pathway impedes the function of ZDHHC20 in the proliferation of liver cancer cells. (**A**) Huh7 and SK-Hep1 cells were transfected with indicated shRNA for 72 hours. CCK-8 assay was performed to determine the viability of cells treated with or without LY294002. shC means shControl, shZ means shZDHHC20, V means vehicle, L means LY294002 (50 μM). Data presents as mean ± SEM with three replicates. Ns, not significant; *P < 0.05; ***P < 0.001. (**B**) Huh7 and SK-Hep1 cells were transfected with ZDHHC20 plasmid for 72 hours. CCK-8 assay was performed to determine the viability of cells treated with or without LY294002 (50 μM). EV means empty vector, ZD means ZDHHC20 plasmids, V means vehicle, L means LY294002. Data presents as mean ± SEM with three replicates. Ns, not significant; *P < 0.05; ***P < 0.001. (**C**) Huh7 and SK-Hep1 cells were transfected with indicated shRNA for 72 hours. CCK-8 assay was performed to determine the viability of cells treated with or without MK2206 (50 μM). M means MK2206. Data presents as mean ± SEM with three replicates. Ns, not significant; ***P < 0.001. (**D**) Huh7 and SK-Hep1 cells were transfected with ZDHHC20 plasmid for 72 hours. CCK-8 assay was performed to determine the viability of cells treated with or without MK2206 (50 μM). Data presents as mean ± SEM with three replicates. Ns, not significant; *P < 0.05; ***P < 0.001. (**E**–**G**), The Huh7 cells were infected with indicated shRNAs. Stable expression cell lines were constructed after puromycin selection. And the cells with different treatment were injected subcutaneously into nude mice for xenografts. The MK2206 (120 mg/Kg, p. o.) was administrated once every three days. Images depicting xenograft tumor tissue (**E**) the weight distribution of excised tumors (**F**) and tumor growth curves (**G**). Data presents as mean ± SEM with five replicates. Ns, not significant; ***P < 0.001.

two specific inhibitors targeting PI3K (LY294002) (ref) or AKT (MK2206) (ref) to block the PI3K-AKT pathway in liver cancer cells. Our findings showed that LY294002 and MK2206 effectively blocked the effect of ZDHHC20 in promoting cancer cell proliferation in both SK-Hep1 and Huh7 cells (Figure 5A–D). Furthermore, a nude mouse xenograft assay demonstrated that depletion of ZDHHC20 inhibited tumor growth. Further, knockdown of ZDHHC20 combined with MK2206 did not significantly inhibit tumor growth further (Figure 5E–G). Taken together, these data suggest that inhibition of the AKT signaling pathway blocks the function of ZDHHC20 in the cell proliferation of liver cancer cells.

Discussion

Hepatocellular carcinoma is a significant global health issue, with its incidence increasing annually.[19] HCC has a stealthy onset and rapid progression, with most patients being diagnosed at an advanced stage.[20] Patients with advanced liver cancer have very limited treatment options, and their overall prognosis is poor.[21] Despite numerous breakthroughs in the treatment of advanced HCC, high metastasis rates, high recurrence rates, and targeted drug resistance remain major challenges.[22] Our study preliminarily explored the role of ZDHHC20 in HCC. The results showed that ZDHHC20 is highly expressed in HCC tissues and positively correlated with poor prognosis. We also found that high expression of ZDHHC20 promoted HCC cell proliferation, and bioinformatics analysis revealed a close association between high expression of ZDHHC20 and the PI3K-AKT signaling pathway. Through cell and animal experiments, we showed that inhibitors of the PI3K-AKT signaling pathway could block the promoting effect of ZDHHC20 on the proliferation and tumor growth of liver cancer cells. However, the specific regulatory mechanism between ZDHHC20 and the PI3K-AKT pathway remains unclear. In recent years, numerous studies have focused on the role of PI3K-AKT signaling in tumor progression. Abnormal activation of the PI3K-AKT pathway plays a vital role in the development and progression of liver cancer and is known to promote proliferation and invasion of HCC.[23,24] Evidence also suggests the involvement of the PI3K-AKT pathway in the anti-apoptotic regulation of HCC cells.[25,26] Moreover, aberrant activation of the PI3K-AKT pathway is a key factor in sorafenib resistance in HCC.[27–30] We showed that ZDHHC20 promotes HCC cell proliferation and tumor growth through the PI3K-AKT pathway. For HCC patients with high ZDHHC20 expression, the use of PI3K-AKT inhibitors to inhibit the ZDHHC20-activated PI3K-AKT signaling pathway may present a promising therapeutic strategy.

The regulation of palmitoylation plays a bidirectional role in tumor progression, and establishing a direct mechanistic link between palmitoylation of a specific protein and its function in tumors remains challenging. Palmitoylated proteins play a crucial role in various tumor cell signal transduction pathways,[31] and their involvement in extracellular signal reception and intracellular signal transmission has been extensively investigated. Proteins that bind to the plasma membrane, such as receptors, ligands, or secreted proteins, are among the most frequently identified palmitoylated proteins. Protein palmitoylation has been implicated in a wide range of cellular processes, including cell proliferation, migration, and apoptosis, and it has been shown to influence tumor growth and metastasis.[32–34] Moreover, recent studies have demonstrated that palmitoylation can also play essential roles in regulating the immune response[35] and inflammation.[36]

In recent years, studies have shown that AKT can be regulated by ubiquitination,[37] methylation,[38] acetylation,[39] and other post-translational modifications of proteins. However, further research is needed to explore the regulatory effects of these post-translational modifications on AKT. The fact that palmitoylation-modified genes can regulate phosphorylation indicates that there may be complex interactions among various post-translational modifications (PTMs). The regulatory role of protein palmitoylation-related genes in tumor cell signal transduction underscores their significant clinical potential. Therefore, further investigation into the role of protein palmitoylation in signal transduction pathways can provide insight into the mechanisms underlying tumor occurrence as well as the development of targeted therapies. Studies have shown that serine protease PCSK9 can undergo palmitoylation and promote tumor growth by inducing AKT phosphorylation, suggesting that AKT phosphorylation may be influenced by palmitoylation.[30] A greater understanding of the ZDHHC20-palmitoylation substrate-AKT axis may provide opportunities to improve the treatment of HCC.

To date, no specific inhibitors against ZDHHC have been developed, and our study suggests that targeting ZDHHC20 could be a promising strategy for monotherapy or combination approaches. Although 2-Bromopalmitate (2-BP) is one of the most commonly used ZDHHC inhibitor, its low specificity, high cytotoxicity, and limited clinical transformation are major limitations. The development of new ZDHHC inhibitors, such as cyanomyracrylamide (CMA)[40] and palmitoyl transferase probes (PTPs),[41] has improved the effectiveness of ZDHHC recognition, but no specific inhibitor has been

developed for a single ZDHHC. Importantly, targeting a single ZDHHC may not be the best option for palmitoylation-based therapies since a single protein may be modified by multiple palmitoylases, and a single palmitoylase may catalyze the modification of multiple proteins. Limited knowledge of the exact recognition mechanisms of ZDHHC-substrates and lack of a stable high-throughput screening system for palmitoylated small molecule inhibitors are key challenges for the development of specific ZDHHC-targeted small molecule inhibitors. However, a recent report on the three-dimensional structure of ZDHHC20[7] provides a strong basis for understanding the catalytic mechanisms of ZDHHC recognition. Moreover, studies have found that ZDHHC20-mediated palmitoylation enhances the antiviral activity of interferon-induced transmembrane protein 3 (IFITM3),[42] and ZDHHC20 is involved in the entry of the SARS-CoV-2 virus into cells.[43] These findings highlight the additional potential of ZDHHC20 inhibitors as antiviral therapeutics. Future research will explore the mechanisms of ZDHHC20 and its relationship with the PI3K-AKT pathway in liver cancer, as well as accelerating the screening and clinical transformation of ZDHHC20-specific inhibitors.

Conclusion

In summary, our study suggests that ZDHHC20 is aberrantly overexpressed in HCC tissue samples, and HCC patients with higher ZDHHC20 expression have poor prognosis. ZDHHC20 promotes cancer cell proliferation in HCC and activates the PI3K-AKT signaling pathway. Inhibition of the PI3K-AKT signaling pathway impedes the tumor-growth promoting effect of ZDHHC20. Thus, our study provides an initial understanding of ZDHHC20 in HCC cells and clarifies its role in promoting HCC growth, suggesting that ZDHHC20 may be a therapeutic target for the treatment of HCC.

Data Sharing Statement

The datasets used and/or analyzed during the present study are available from the corresponding authors on reasonable request.

Ethics Approval and Consent to Participate

All animal experiments were approved by The Institutional Animal Care and Use Committee at Tongji Medical College, HUST (3385).

Consent for Publication

All subjects provided written informed consent.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted and agree to be accountable for all aspects of the work.

Funding

The study was supported by National Natural Science Foundation of China (Grant No. 81772800 (P.L.) and 82072945 (P.L.)).

Disclosure

The authors declare that there is no competing interest.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209–249. doi:10.3322/caac.21660
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394–424. doi:10.3322/caac.21492
3. Jin J, Zhi X, Wang X, Meng D. Protein palmitoylation and its pathophysiological relevance. *J Cell Physiol*. 2021;236(5):3220–3233. doi:10.1002/jcp.30122

4. Chen JJ, Fan Y, Boehning D. Regulation of Dynamic Protein S-Acylation. *Front Mol Biosci*. 2021;8:656440. doi:10.3389/fmolb.2021.656440
5. Lawrence DS, Zilfou JT, Smith CD. Structure-activity studies of cerulenin analogues as protein palmitoylation inhibitors. *J Med Chem*. 1999;42 (24):4932–4941. doi:10.1021/jm980591s
6. Mitchell DA, Vasudevan A, Linder ME, Deschenes RJ. Protein palmitoylation by a family of DHHC protein S-acyltransferases. *J Lipid Res*. 2006;47(6):1118–1127. doi:10.1194/jlr.R600007-JLR200
7. Rana MS, Kumar P, Lee CJ, Verardi R, Rajashankar KR, Banerjee A. Fatty acyl recognition and transfer by an integral membrane S-acyltransferase. *Science*. 2018;359(6372). doi:10.1126/science.aao6326
8. Bailey MH, Tokheim C, Porta-Pardo E, et al. Comprehensive characterization of cancer driver genes and mutations. *Cell*. 2018;173(2):371–385e18. doi:10.1016/j.cell.2018.02.060
9. Ko PJ, Dixon SJ. Protein palmitoylation and cancer. *EMBO Rep*. 2018;19(10). doi:10.15252/embr.201846666
10. Zhou B, Hao Q, Liang Y, Kong E. Protein palmitoylation in cancer: molecular functions and therapeutic potential. *Mol Oncol*. 2023;17(1):3–26. doi:10.1002/1878-0261.13308
11. De I, Sadhukhan S. Emerging Roles of DHHC-mediated protein s-palmitoylation in physiological and pathophysiological context. *Eur J Cell Biol*. 2018;97(5):319–338. doi:10.1016/j.ejcb.2018.03.005
12. Resh MD. Palmitoylation of proteins in cancer. *Biochem Soc Trans*. 2017;45(2):409–416. doi:10.1042/BST20160233
13. Ducker CE, Stettler EM, French KJ, Upson JJ, Smith CD. Huntingtin interacting protein 14 is an oncogenic human protein: palmitoyl acyltransferase. *Oncogene*. 2004;23(57):9230–9237. doi:10.1038/sj.onc.1208171
14. Li SX, Tang GS, Zhou DX, et al. Prognostic significance of cytoskeleton-associated membrane protein 4 and its palmitoyl acyltransferase DHHC2 in hepatocellular carcinoma. *Cancer*. 2014;120(10):1520–1531. doi:10.1002/cncr.28593
15. Rocks O, Gerauer M, Vartak N, et al. The palmitoylation machinery is a spatially organizing system for peripheral membrane proteins. *Cell*. 2010;141(3):458–471. doi:10.1016/j.cell.2010.04.007
16. Omerovic J, Hammond DE, Clague MJ, Prior IA. Ras isoform abundance and signalling in human cancer cell lines. *Oncogene*. 2008;27 (19):2754–2762. doi:10.1038/sj.onc.1210925
17. Lan T, Delalande C, Dickinson BC. Inhibitors of DHHC family proteins. *Curr Opin Chem Biol*. 2021;65:118–125. doi:10.1016/j.cbpa.2021.07.002
18. Yang Z, Qin W, Chen Y, et al. Cholesterol inhibits hepatocellular carcinoma invasion and metastasis by promoting CD44 localization in lipid rafts. *Cancer Lett*. 2018;429:66–77. doi:10.1016/j.canlet.2018.04.038
19. Wang Y, Deng B. Hepatocellular carcinoma: molecular mechanism, targeted therapy, and biomarkers. *Cancer Metastasis Rev*. 2023;42(3):629–652. doi:10.1007/s10555-023-10084-4
20. Wang MD, Li C, Liang L, et al. Early and late recurrence of hepatitis b virus-associated hepatocellular carcinoma. *Oncologist*. 2020;25(10):e1541–e1551. doi:10.1634/theoncologist.2019-0944
21. Tabrizian P, Jibara G, Shrager B, Schwartz M, Roayaie S. Recurrence of hepatocellular cancer after resection: patterns, treatments, and prognosis. *Ann Surg*. 2015;261(5):947–955. doi:10.1097/SLA.0000000000000710
22. Kim E, Viatour P. Hepatocellular carcinoma: old friends and new tricks. *Exp Mol Med*. 2020;52(12):1898–1907. doi:10.1038/s12276-020-00527-1
23. Paskeh MDA, Ghadyani F, Hashemi M, et al. Biological impact and therapeutic perspective of targeting PI3K/Akt signaling in hepatocellular carcinoma: promises and challenges. *Pharmacol Res*. 2023;187:106553. doi:10.1016/j.phrs.2022.106553
24. Wu Y, Zhang Y, Qin X, Geng H, Zuo D, Zhao Q. PI3K/AKT/mTOR pathway-related long non-coding RNAs: roles and mechanisms in hepatocellular carcinoma. *Pharmacol Res*. 2020;160:105195. doi:10.1016/j.phrs.2020.105195
25. Mehdi Uremis M, Uremis N, Tosun E, et al. Cucurbitacin D inhibits the proliferation of HepG2 cells and induces apoptosis by modulating JAK/STAT3, PI3K/Akt/mTOR and MAPK signaling pathways. *Curr Cancer Drug Targets*. 2022;22(11):931–944. doi:10.2174/1568009622666220623141158
26. Wu Q, Liu TY, Hu BC, et al. CK-3, A novel methsulfonyl pyridine derivative, suppresses hepatocellular carcinoma proliferation and invasion by blocking the PI3K/AKT/mTOR and MAPK/ERK pathways. *Front Oncol*. 2021;11:717626. doi:10.3389/fonc.2021.717626
27. Chen KF, Chen HL, Tai WT, et al. Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. *J Pharmacol Exp Ther Apr*. 2011;337(1):155–161. doi:10.1124/jpet.110.175786
28. Zhou X, Li TM, Luo JZ, et al. CYP2C8 suppress proliferation, migration, invasion and sorafenib resistance of hepatocellular carcinoma via PI3K/Akt/p27(kip1) axis. *J Hepatocell Carcinoma*. 2021;8:1323–1338. doi:10.2147/JHC.S335425
29. Liu T, Xie XL, Zhou X, et al. Y-box binding protein 1 augments sorafenib resistance via the PI3K/Akt signaling pathway in hepatocellular carcinoma. *World J Gastroenterol*. 2021;27(28):4667–4686. doi:10.3748/wjg.v27.i28.4667
30. Sun Y, Zhang H, Meng J, et al. S-palmitoylation of PCSK9 induces sorafenib resistance in liver cancer by activating the PI3K/AKT pathway. *Cell Rep*. 2022;40(7):111194. doi:10.1016/j.celrep.2022.111194
31. Liu Z, Xiao M, Mo Y, et al. Emerging roles of protein palmitoylation and its modifying enzymes in cancer cell signal transduction and cancer therapy. *Int J Biol Sci*. 2022;18(8):3447–3457. doi:10.7150/ijbs.72244
32. Pei X, Li KY, Shen Y, et al. Palmitoylation of MDH2 by ZDHHC18 activates mitochondrial respiration and accelerates ovarian cancer growth. *Sci China Life Sci*. 2022;65(10):2017–2030. doi:10.1007/s11427-021-2048-2
33. Babina IS, McSherry EA, Donatello S, Hill AD, Hopkins AM. A novel mechanism of regulating breast cancer cell migration via palmitoylation-dependent alterations in the lipid raft affiliation of CD44. *Breast Cancer Res*. 2014;16(1):R19. doi:10.1186/bcr3614
34. Li P, Gong X, Yuan L, et al. Palmitoylation in apoptosis. *J Cell Physiol*. 2023;238(8):1641–1650. doi:10.1002/jcp.31047
35. Yao H, Lan J, Li C, et al. Inhibiting PD-L1 palmitoylation enhances T-cell immune responses against tumours. *Nat Biomed Eng*. 2019;3 (4):306–317. doi:10.1038/s41551-019-0375-6
36. Wang L, Cai J, Zhao X, et al. Palmitoylation prevents sustained inflammation by limiting NLRP3 inflammasome activation through chaperone-mediated autophagy. *Mol Cell*. 2023;83(2):281–297e10. doi:10.1016/j.molcel.2022.12.002
37. Zhu Z, Cao C, Zhang D, et al. UBE2T-mediated Akt ubiquitination and Akt/beta-catenin activation promotes hepatocellular carcinoma development by increasing pyrimidine metabolism. *Cell Death Dis*. 2022;13(2):154. doi:10.1038/s41419-022-04596-0
38. Wang G, Long J, Gao Y, et al. SETDB1-mediated methylation of Akt promotes its K63-linked ubiquitination and activation leading to tumorigenesis. *Nat Cell Biol*. 2019;21(2):214–225. doi:10.1038/s41556-018-0266-1

39. Sundaresan NR, Pillai VB, Wolfgeher D, et al. The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. *Sci Signal*. 2011;4(182):ra46. doi:10.1126/scisignal.2001465
40. Azizi SA, Delalande C, Lan T, Qiu T, Dickinson BC. Charting the chemical space of acrylamide-based inhibitors of zDHHC20. *ACS Med Chem Lett*. 2022;13(10):1648–1654. doi:10.1021/acsmedchemlett.2c00336
41. Qiu T, Azizi SA, Brookes N, Lan T, Dickinson BC. A high-throughput fluorescent turn-on assay for inhibitors of DHHC family proteins. *ACS Chem Biol*. 2022;17(8):2018–2023. doi:10.1021/acschembio.2c00193
42. McMichael TM, Zhang L, Chemudupati M, et al. The palmitoyltransferase ZDHHC20 enhances interferon-induced transmembrane protein 3 (IFITM3) palmitoylation and antiviral activity. *J Biol Chem*. 2017;292(52):21517–21526. doi:10.1074/jbc.M117.800482
43. Li D, Liu Y, Lu Y, Gao S, Zhang L. Palmitoylation of SARS-CoV-2 S protein is critical for S-mediated syncytia formation and virus entry. *J Med Virol*. 2022;94(1):342–348. doi:10.1002/jmv.27339