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Original Article

# GTF3C2 Promotes the Proliferation of Hepatocellular Carcinoma Cells through the USP21/MEK2/ERK1/2 Pathway

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## Abstract

***Background and Aims:*** General transcription factor IIIC subunit 2 (GTF3C2) is one of the polymerase III transcription-related factors. Previous studies have revealed that GTF3C2 is involved in regulating cell proliferation. However, the role of GTF3C2 in hepatocellular carcinoma (HCC) remains unclear. This study aimed to determine its expression, biological function, and mechanism in HCC. ***Methods:*** The expression of GTF3C2 in HCC and non-tumor tissues, along with its clinical significance, was investigated using public databases and clinical samples. Reverse transcription-quantitative polymerase chain reaction and Western blot assays were performed to detect the expression of GTF3C2, ubiquitin specific peptidase 21 (USP21), mitogen-activated protein kinase 2 (MEK2), extracellular signal-regulated kinase 1/2 (ERK1/2), and p-ERK1/2 in cells. A luciferase reporter assay was conducted to explore the regulatory effect of GTF3C2 on USP21 transcription. Cell Counting Kit-8, 5-ethynyl-2′-deoxyuridine, and colony formation assays were performed to assess HCC cell proliferation. Subcutaneous injection of HCC cells into nude mice was used to evaluate tumor growth *in vivo*. ***Results:*** GTF3C2 expression was upregulated in HCC tissues and was positively correlated with advanced tumor stages and high tumor grades. HCC patients with high GTF3C2 expression had significantly worse survival outcomes. Knockdown of GTF3C2 suppressed the proliferation of Hep3B and HCCLM3 cells, while overexpression of GTF3C2 facilitated the proliferation of SNU449 and Huh7 cells. GTF3C2 promoted USP21 expression by activating its transcription, which subsequently increased the levels of MEK2 and p-ERK1/2 in HCC cells. Overexpression of both USP21 and MEK2 counteracted the GTF3C2 knockdown-induced inactivation of the ERK1/2 pathway. Moreover, GTF3C2 promoted HCC cell proliferation *in vitro* and tumor growth *in vivo* by regulating the USP21/MEK2/ERK1/2 pathway. ***Conclusions:*** Upregulation of GTF3C2 is frequently observed in HCC tissues and predicts poor prognosis. GTF3C2 promotes HCC cell proliferation via the USP21/MEK2/ERK1/2 pathway.

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## Introduction

Primary liver cancer, 75–85% of which is hepatocellular carcinoma (HCC), is one of the most common malignant tumors worldwide.[1] According to the latest statistics, there are 410,000 new cases of liver cancer and 390,000 deaths in China, ranking fifth and second among all types of cancer, respectively, and seriously threatening the lives and health of the population.[2] Due to the characteristics of HCC, such as insidious onset, rapid progression, high recurrence rates, and drug resistance,[3,4] the overall prognosis remains poor, with a five-year relative survival rate of 21.7% in the SEER database (https://seer.cancer.gov/) and 14.4% in China.[5] The specific mechanisms underlying HCC progression remain to be further explored. Identifying key genes involved in HCC will help in discovering new targets and strategies for treatments.

The general transcription factor III (GTF3) family members function as RNA polymerase III (Pol III) transcription-related factors, inducing 5S ribosomal RNA gene transcription and participating in the biogenesis of the large ribosomal subunit.[6] The GTF3 family includes GTF3A, GTF3B, GTF3C1, and GTF3C2. GTF3A is highly expressed in colorectal cancer and is associated with tumor metastasis and poor prognosis.[7,8] It activates cystatin A transcription by directly binding to its gene promoter, promoting colorectal cancer cell proliferation, invasion, and epithelial-mesenchymal transition.[7] GTF3C1 functions as a protein partner of sirtuin 7 and mechanistic target of rapamycin kinase to regulate Pol III transcription

**Keywords:** Hepatocellular carcinoma; GTF3C2; USP21; MEK2; ERK signalling pathway; Cell proliferation.

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and the autophagy pathway, thereby affecting cell growth and proliferation.[9] GTF3C2 can form a fusion with the ALK receptor tyrosine kinase in the Spitz tumor.[10] The serum level of GTF3C2 is downregulated following acute myocardial infarction and may serve as a promising biomarker for acute myocardial infarction diagnosis and treatment.[11] A previous study reported that GTF3C2 is one of the critical regulators of differentially expressed genes in mouse-derived single spermatogonial stem cells.[12] GTF3C2 is transcriptionally regulated by Sp1 and implicated in Sp1-mediated Pol III transcription and cancer cell proliferation.[13,14] Additionally, transcription factor AP2 alpha has been identified as a positive regulator of GTF3C2, Pol III-directed transcription, and the proliferation of 293T and HeLa cells.[15] A recent study shows that GTF3C2 functions as a transcription factor to increase the expression of claudin-4 and claudin-8 in pulmonary microvascular endothelial cells.[16] Prior studies have found that GTF3C2 participates in the risk prognostic model in HCC.[17,18] However, the expression and biological function of GTF3C2 in HCC remain unclear.

In this study, we first determined the expression of GTF3C2 in HCC based on public databases and our clinical samples. We confirmed the impact of GTF3C2 alteration on HCC cell proliferation *in vitro* and tumor growth *in vivo*. We then investigated the regulatory effects and mechanisms of GTF3C2 on the ubiquitin specific peptidase 21 (USP21)/mitogen-activated protein kinase 2 (MEK2)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. Our data demonstrate that GTF3C2 is a potential biomarker for poor prognosis and facilitates cell proliferation by activating USP21 transcription and the MEK2/ERK1/2 pathway in HCC.

## Methods

### *Public data analysis*

The University of Alabama at Birmingham Cancer Data Analysis portal (hereinafter referred to as UALCAN)[19] and the R2 platform (https://r2.amc.nl/) were used to analyze the expression of GTF3C2 and USP21 in HCC and normal liver tissues from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus, the Clinical Proteomic Tumor Analysis Consortium, and the International Cancer Proteogenome Consortium databases. Additionally, the correlation between GTF3C2 expression and tumor stages and grades was examined in the TCGA database. The GepLiver platform (http://gepliver.org/) was used to investigate the overall survival (OS), disease-specific survival (DSS), disease-free survival (DFS), and progression-free survival (PFS) of HCC patients with high or low GTF3C2 and USP21 expression in the TCGA database.

### *Clinical specimens*

Thirty-eight pairs of tumor and matched tumor-adjacent tissues (2 cm from the tumor edge) were collected from patients who underwent liver resection at the Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China) between January 2023 and December 2023. The diagnosis of each patient was confirmed by postoperative pathology. Patients who received preoperative treatment or had other concurrent malignant tumors were excluded from this study. All specimens were quickly frozen in liquid nitrogen and stored at −80°C. All patients signed informed consent forms, and the research protocol was approved by The Research Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University (No: XJTU1AF2020LSY-08).

### *Cell culture*

The human normal hepatocyte cell line (THLE-2) was purchased from Shanghai Jinyuan Biotechnology Co., Ltd. (Shanghai, China) and cultured in THLE-2 cell culture medium (Shanghai Jinyuan Biotechnology Co., Ltd.). HCC cell lines HCCLM3, SK-Hep-1, HepG2, Huh7, and Hep3B were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and SNU449 cells were obtained from ATCC. HCC cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA). All cells were maintained in a 5% $CO_2$ atmosphere at 37°C. The USP21 inhibitor (BAY-805) and the ERK1/2 inhibitor (LY3214996) were obtained from MedChemExpress (Monmouth Junction, NJ, USA) and used to treat HCC cells at the corresponding concentrations (10 μM and 5 μM).

### *Plasmids, lentivirus packaging and infection*

Plasmids containing non-targeting shRNA, GTF3C2 shRNA (shGTF3C2-1 and shGTF3C2-2), empty vector, GTF3C2 cDNA (OE-GTF3C2), USP21 cDNA (OE-USP21), or MEK2 cDNA (OE-MEK2) were purchased from Xi'an GeneCarer Biotech Co., Ltd (Xi'an, China). The corresponding plasmids were transfected into HEK293T cells along with the psPAX2 packaging plasmid and the pMD2.G envelope plasmid to produce lentivirus. Lentivirus infection of HCC cells was performed by incubating cells overnight with virus-containing supernatant (a 1:1 dilution of complete Dulbecco's modified Eagle's medium supplemented with 8 mg/mL polybrene). Seventy-two hours after infection, HCC cells were used for gain- and loss-of-function experiments.

### *Cell Counting Kit-8 (CCK-8) assay*

Ten μL of CCK-8 solution was added to each well of a 96-well plate, and HCC cells were cultured for the designated time. The optical density was measured at 450 nm using a multifunctional microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The CCK-8 kit was obtained from Yeasen (Shanghai, China).

### *5-ethynyl-2′-deoxyuridine (EdU) assay*

HCC cells were cultured in a 24-well plate ($5 \times 10^3$ cells/well). The Cell-Light EdU Apollo567 In Vitro Kit (Ribobio, Guangzhou, China) was used to assess HCC cell proliferation as previously described.[20] Representative images were captured using a microscope (Carl Zeiss AG, Jena, Germany) with appropriate excitation and emission spectra at 200× magnification, and the data were analyzed using ImageJ software (NIH, USA).

### *Colony formation assay*

HCC cells were inoculated in six-well plates (500 cells/well). After 14 days of culture, the cells were fixed with 4% paraformaldehyde for 15 m, washed with PBS three times, and stained with 0.1% crystal violet for 30 m. After staining, the colonies were counted using ImageJ software (NIH).

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

Reverse transcription-qPCR was performed using RNA isolated from HCC cells following the designated intervention. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). Specifically, 500 ng of RNA was reverse transcribed using the RevertAid First Strand cDNA Synthe-

sis Kit (Thermo Fisher Scientific). qPCR was performed using SYBR GreenER™ qPCR SuperMix Universal (Thermo Fisher Scientific), as previously described.[21] GAPDH was used as a control, and results were calculated using the $2^{-\Delta\Delta Ct}$ method. USP21 forward 5′-AGA AGA GCT AGA GTC GGA GAA T-3′ and reverse 5′-GAG GAT TCG AGG GAA TCT TTG T-3′; GTF3C2 forward 5′-CAG GTG GAG AAG AGG TGG-3′ and reverse 5′-CTA CAG GCT CAG GTT CAG-3′; GAPDH forward 5′-GGT GTG AAC CAT GAG AAG TAT GA-3′ and reverse 5′- GAG TCC TTC CAC GAT ACC AAA G-3′.

### *Western blotting*

Proteins were extracted from HCC tissues and cells using RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with a phosphatase inhibitor cocktail and protease inhibitors. Protein concentration was determined using the BCA protein detection kit (ZHHC, Xi'an, China). After quantification, 15–30 μg of protein per sample were loaded onto a sodium dodecyl-sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane (Millipore, Hong Kong, China), and incubated sequentially with primary and horseradish peroxidase-conjugated secondary antibodies (Beyotime). The signal was detected using enhanced chemiluminescence reagents (Millipore), and densitometry was performed using ImageJ software (NIH). Primary antibodies for GTF3C2 (27494-1-AP, Proteintech), USP21 (17856-1-AP, Proteintech), MEK2 (67410-1-Ig, Proteintech), p-ERK1/2 (28733-1-AP, Proteintech), ERK1/2 (66192-1-Ig, Proteintech), and GAPDH (60004-1-Ig, Proteintech) were used.

### *Luciferase reporter assay*

The pEZX-PG02 vector containing the USP21 promoter was purchased from GeneCopoeia (Guangzhou, China). The luciferase reporter assay was used to identify the transcription of USP21 regulated by GTF3C2 in HCC cells, as previously described.[22] The relative activity of firefly luciferase was determined and normalized to the constitutively expressed Renilla luciferase (Promega, Madison, WI, USA).

### *In vivo tumorigenesis assay*

Animal experiments were conducted according to protocols approved by the Ethics Review Committee of Xi'an Jiaotong University. Four-week-old male nude mice were purchased and housed at the Laboratory Animal Center of Xi'an Jiaotong University. For the subcutaneous tumor model, $1 \times 10^6$ HCCLM3 cells infected with lentivirus (non-targeting shRNA, shGTF3C2-1, or shGTF3C2-1+OE-MEK2) were suspended in 100 μL PBS and subcutaneously injected into the left flanks of nude mice (n = 7 per group). Tumor size was monitored every three days, and tumor volume was calculated using the formula: V (tumor volume in $mm^3$) = $0.5 \times L \times W^2$ (L: longer diameter; W: width diameter). After three weeks, mice were sacrificed, and tumors were collected for photography and subjected to hematoxylin-eosin staining and immunohistochemistry of Ki-67.[22]

### *Statistical analysis*

All experiments were performed in triplicate. Data are presented as mean ± standard deviation and analyzed using GraphPad Prism software (GraphPad Inc., San Diego, CA, USA). Student's t-test, Mann-Whitney test, or one-way analysis of variance followed by the LSD post hoc test were used to compare differences between two groups or more than two groups, respectively. A $p$-value <0.05 was considered statistically significant.

## Results

### *GTF3C2 upregulation correlates with an unfavorable prognosis in patients with HCC*

Analysis of the TCGA and Gene Expression Omnibus datasets using UALCAN[19] and the R2 platform (https://r2.amc.nl/) indicated that GTF3C2 mRNA expression in HCC was significantly higher than in normal liver tissues ($p < 0.0001$, Fig. 1A and B). Moreover, the Clinical Proteomic Tumor Analysis Consortium and International Cancer Proteogenome Consortium data revealed upregulation of GTF3C2 protein in HCC tissues compared to normal liver tissues ($p < 0.001$, Fig. 1C). Detection of our clinical samples further confirmed that GTF3C2 was highly expressed in HCC compared to tumor-adjacent tissues ($p < 0.05$, Fig. 1D and E). GTF3C2 mRNA overexpression positively correlated with advanced tumor stages and high tumor grades ($p < 0.05$, Fig. 2A and B). Our data further confirmed the correlation of GTF3C2 mRNA expression with tumor stage and grade ($p < 0.05$, Supplementary Fig. 1). Importantly, TCGA data analyzed by the GepLiver platform (http://gepliver.org/) confirmed that HCC patients with high GTF3C2 mRNA expression had worse OS, DSS, DFS, and PFS ($p < 0.05$, Fig. 2C–F). These results indicate that GTF3C2 is a potential biomarker for predicting poor prognosis in HCC.

### *GTF3C2 facilitates HCC cell proliferation*

We found that the levels of GTF3C2 in HCC cell lines were prominently elevated compared to normal hepatocytes ($p < 0.05$, Supplementary Fig. 2). GTF3C2 expression was then downregulated by shRNAs in Hep3B and HCCLM3 cells ($p < 0.05$, Fig. 3A). CCK-8, EdU, and colony formation assays suggested that GTF3C2 knockdown markedly reduced the proliferation of HCC cells ($p < 0.05$, Fig. 3B–D). GTF3C2 overexpression was performed in SNU449 and Huh7 cells ($p < 0.05$, Fig. 4A). As expected, overexpression of GTF3C2 prominently enhanced HCC cell proliferation ($p < 0.05$, Fig. 4B–D). Therefore, these data suggest that GTF3C2 is an oncogene in HCC.

### *GTF3C2 participates in USP21 transcription*

TCGA data analyzed by the GEPIA 2 platform[23] revealed that USP21 was one of the top twenty genes most associated with GTF3C2 in HCC ($p < 0.05$, Supplementary Fig. 3A). USP21 expression was upregulated in HCC, and its overexpression was associated with poor prognosis in patients ($p < 0.05$, Supplementary Fig. 3B and C). Interestingly, GTF3C2 knockdown markedly reduced the levels of USP21 mRNA and protein in Hep3B and HCCLM3 cells ($p < 0.05$, Fig. 5A and B). GTF3C2 overexpression significantly increased USP21 protein levels in HCC cells ($p < 0.05$, Fig. 5C). As expected, we found that GTF3C2 knockdown reduced and GTF3C2 overexpression enhanced the luciferase activity of a reporter plasmid containing the USP21 promoter ($p < 0.05$, Fig. 5D and E). Thus, GTF3C2 promotes USP21 expression by activating its transcription.

### *GTF3C2 regulates the USP21/MEK2/ERK1/2 pathway*

A previous study reported that USP21 mediates the stability of MEK2 to activate the ERK1/2 pathway in HCC.[24] Next, we determined the regulatory effect of GTF3C2 on the ERK1/2 signaling pathway. As expected, the levels of MEK2 and p-ERK1/2 were downregulated by GTF3C2 knockdown but upregulated by GTF3C2 overexpression in HCC cells ($p < 0.05$, Fig. 6A and B). Importantly, USP21 overexpression markedly attenuated the GTF3C2 knockdown-induced decrease in

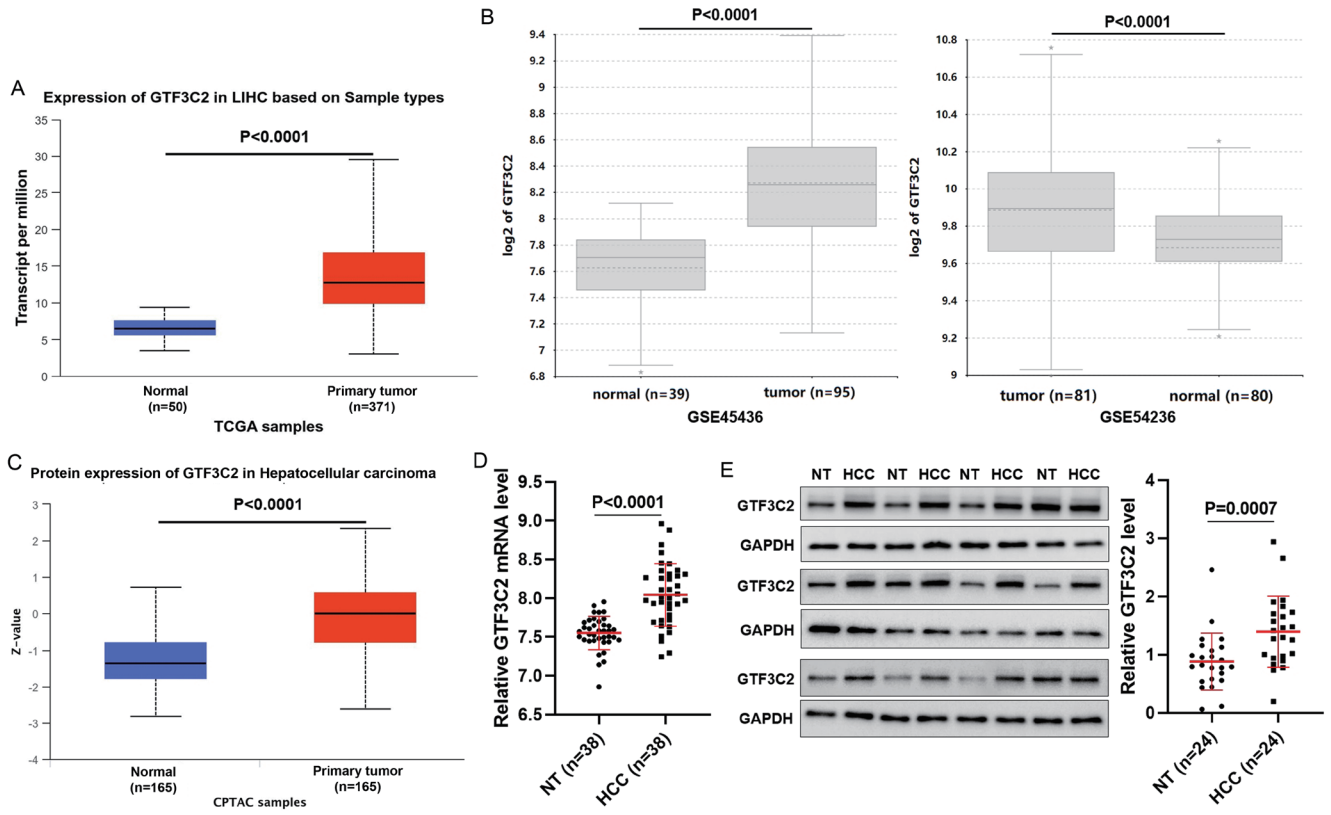


**Fig. 1. The expression of GTF3C2 in HCC.** (A) The expression difference of GTF3C2 mRNA between HCC and normal liver tissues in the TCGA database was analyzed using the UALCAN platform. (B) The expression difference of GTF3C2 mRNA between HCC and normal liver tissues in GEO database was analyzed using the R2 platform. (C) The expression difference of GTF3C2 protein between HCC and normal liver tissues in CPTAC database was analyzed using the UALCAN platform. (D) The expression of GTF3C2 mRNA was detected by RT-qPCR in thirty-eight pairs of HCC and NT tissues. (E) The expression of GTF3C2 protein was detected by Western blotting in twenty-four pairs of HCC and NT tissues. TCGA, The Cancer Genome Atlas; LIHC, Liver Hepatocellular Carcinoma; CPTAC, Clinical Proteomic Tumor Analysis Consortium; HCC, Hepatocellular Carcinoma; GTF3C2, General transcription factor IIIC subunit 2; NT, non-tumor.

MEK2 and p-ERK1/2 levels ($p < 0.05$, Fig. 6A). MEK2 overexpression prominently abrogated the GTF3C2 knockdown-induced reduction in p-ERK1/2 levels ($p < 0.05$, Fig. 6A). The USP21 inhibitor significantly reversed the GTF3C2 overexpression-induced increase in MEK2 and p-ERK1/2 levels in Huh7 cells ($p < 0.05$, Fig. 4A). Thus, our data confirm that GTF3C2 activates the USP21/MEK2/ERK1/2 pathway in HCC cells.

### *MEK2 mediates the oncogenic role of GTF3C2 in HCC*

Rescue experiments were performed to determine the role of MEK2 in GTF3C2-induced HCC progression. We confirmed that MEK2 overexpression prominently attenuated the GTF3C2 knockdown-induced decrease in HCC cell proliferation ($p < 0.05$, Fig. 7A–7C). Further experiments confirmed that either the USP21 inhibitor or the ERK1/2 inhibitor attenuated GTF3C2-induced Huh7 cell proliferation ($p < 0.05$, Supplementary Fig. 4B,C). Moreover, *in vivo* experiments further demonstrated that GTF3C2 knockdown markedly reduced the growth of HCC cells, which was significantly abolished by MEK2 overexpression ($p < 0.05$, Fig. 7D). IHC data revealed that GTF3C2 knockdown reduced the intensity of Ki-67 staining in xenografted tumor tissues, which was increased by MEK2 overexpression ($p < 0.05$, Supplementary Fig. 5). These results suggest that GTF3C2 enhances HCC growth by activating the USP21/MEK2/ERK1/2 pathway.

## Discussion

In this study, we found that GTF3C2 was overexpressed in HCC tissues and cell lines. GTF3C2 overexpression was closely associated with unfavorable clinical features, including high tumor stages and tumor grades. Importantly, HCC patients with high GTF3C2 expression had significantly poorer OS, DSS, DFS, and PFS. GTF3C2, BRF1, and c-MYC are Pol III transcription-related factors.[25] The dysregulation of Pol III products is implicated in tumor initiation and progression.[26] BRF1 and c-MYC are overexpressed and function as prognostic biomarkers in HCC.[27–30] Previous studies have found that GTF3C2 participates in the risk prognostic model in HCC.[17,18] These results indicate that GTF3C2 is a potential biomarker for predicting poor prognosis of HCC. Detection of GTF3C2 expression in HCC tissues may be used to assess patient survival. However, this study did not reveal the molecular mechanism underlying GTF3C2 upregulation in HCC. A recent study has identified Sp1 as a transcription factor of GTF3C2, which upregulates its expression.[13,14] Therefore, further investigation into the transcription factors that activate GTF3C2 expression in HCC is needed.

Here, we confirmed that GTF3C2 knockdown inhibited HCC cell proliferation, while GTF3C2 overexpression facilitated the proliferation of HCC cells. Previous studies have shown that GTF3C2 is implicated in regulating the proliferation of HeLa and SaOS2 cells.[13,14] Our data suggest that GTF3C2 functions

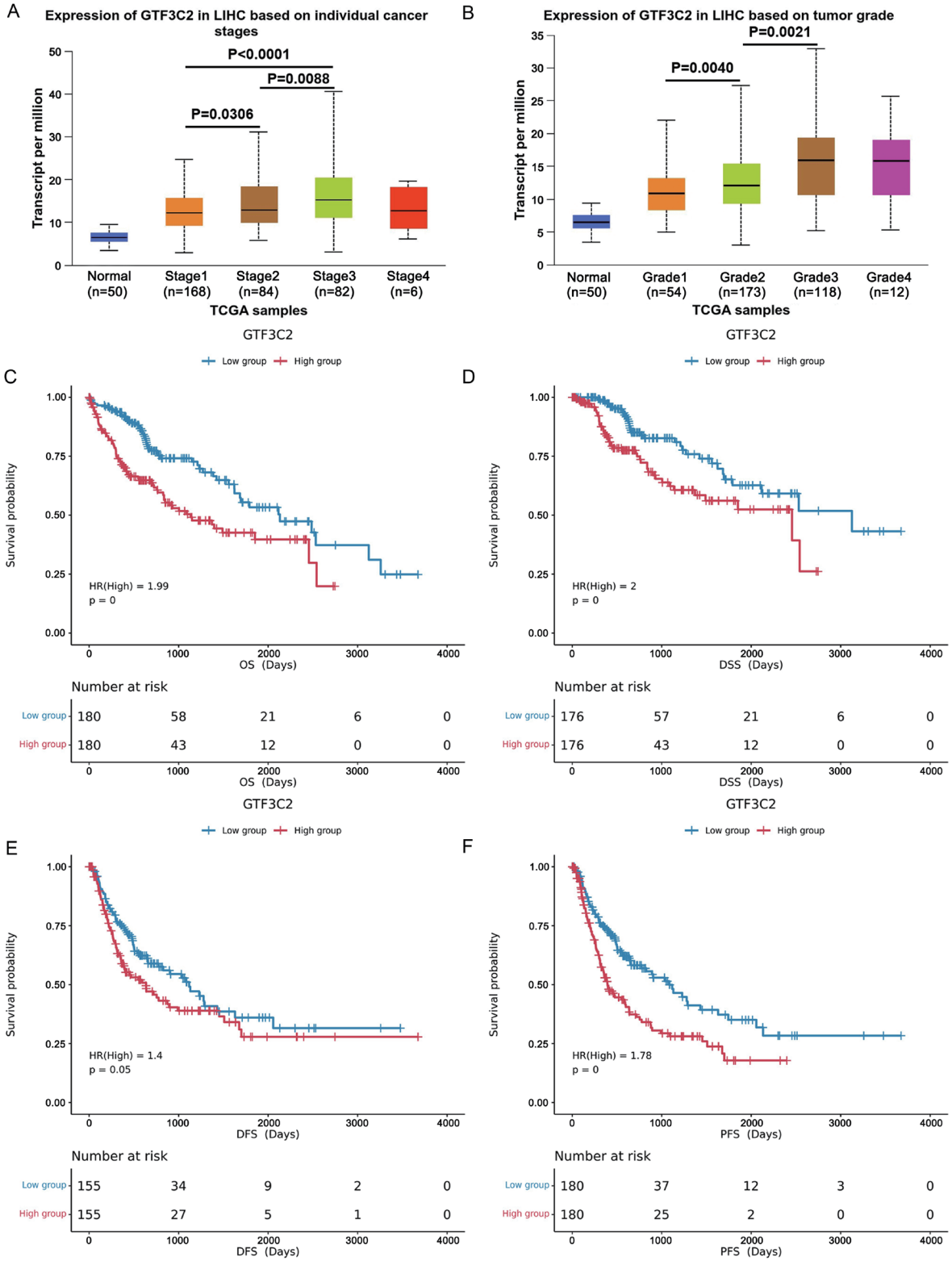


**Fig. 2. The clinical significance of GTF3C2 expression in HCC.** (A) The UALCAN platform analyzed the expression of GTF3C2 mRNA in HCC with different tumor stages in the TCGA database. (B) The UALCAN platform analyzed the expression of GTF3C2 mRNA in HCC with different tumor grades in the TCGA database. (C-F) Survival analysis based on GTF3C2 expression in the TCGA database was performed using the GepLiver platform. TCGA, The Cancer Genome Atlas; LIHC, Liver Hepatocellular Carcinoma; OS, overall survival; DSS, disease-specific survival; DFS, disease-free survival; PFS, progression-free survival; HCC, Hepatocellular Carcinoma; GTF3C2, General transcription factor IIIC subunit 2.

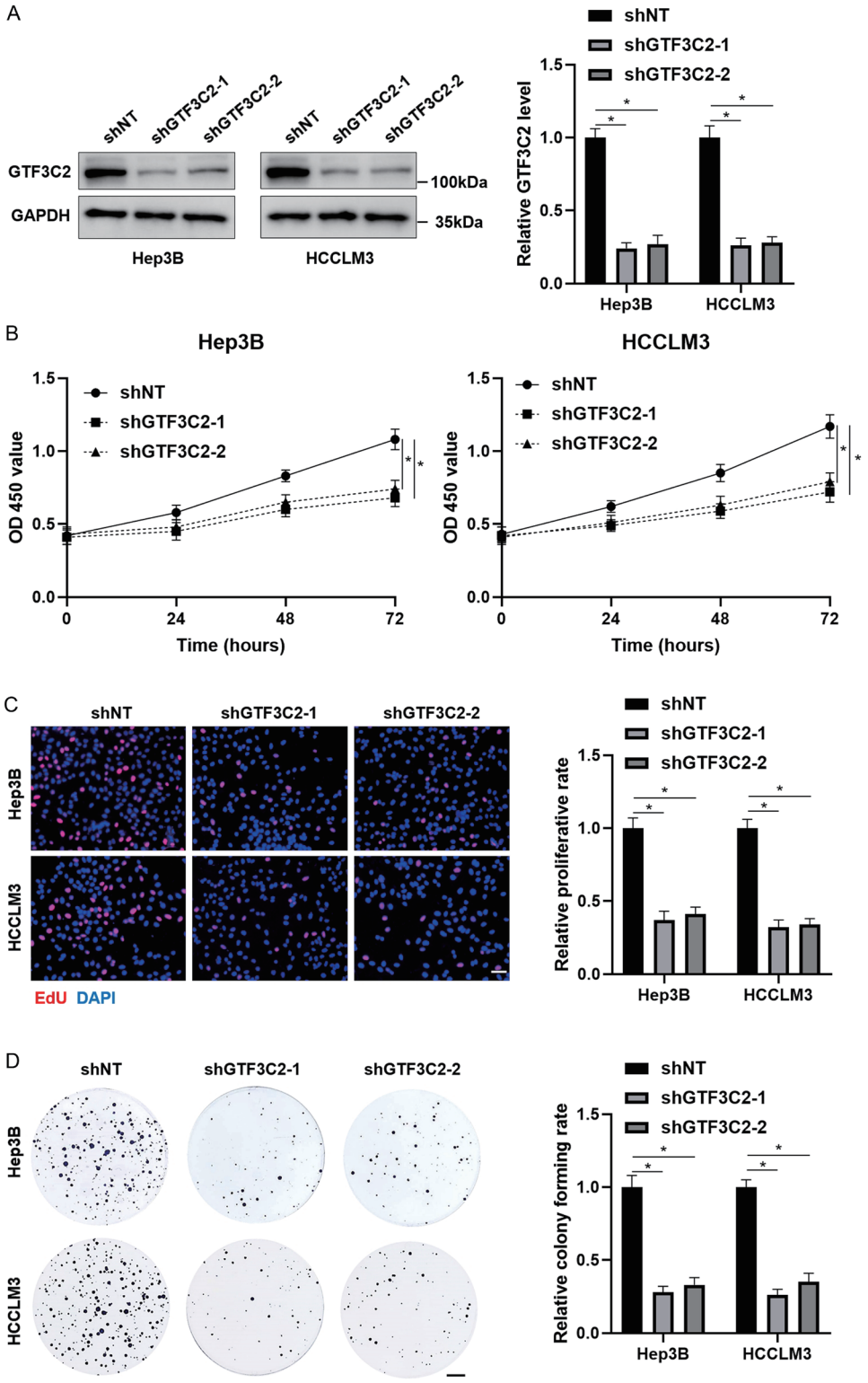


**Fig. 3. The effects of GTF3C2 knockdown on HCC cell proliferation.** (A) Lentiviruses containing shRNA against GTF3C2 (shGTF3C2-1 and GTF3C2-2) or non-targeting (shNT) sequences were transduced into Hep3B and HCCLM3 cells. The GTF3C2 level was detected by Western blotting. (B) The CCK-8 assay determined the viability of HCC cells with or without GTF3C2 knockdown. (C) The EdU assay investigated the proliferation of HCC cells with or without GTF3C2 knockdown. Scale bar: 25μm. (D) The colony formation assay was used to detect the proliferation of HCC cells in the GTF3C2 knockdown and control groups. Scale bar: 5mm. *$p < 0.05$. OD, optical density; EdU, 5-ethynyl-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; HCC, Hepatocellular Carcinoma; GTF3C2, General transcription factor IIIC subunit 2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

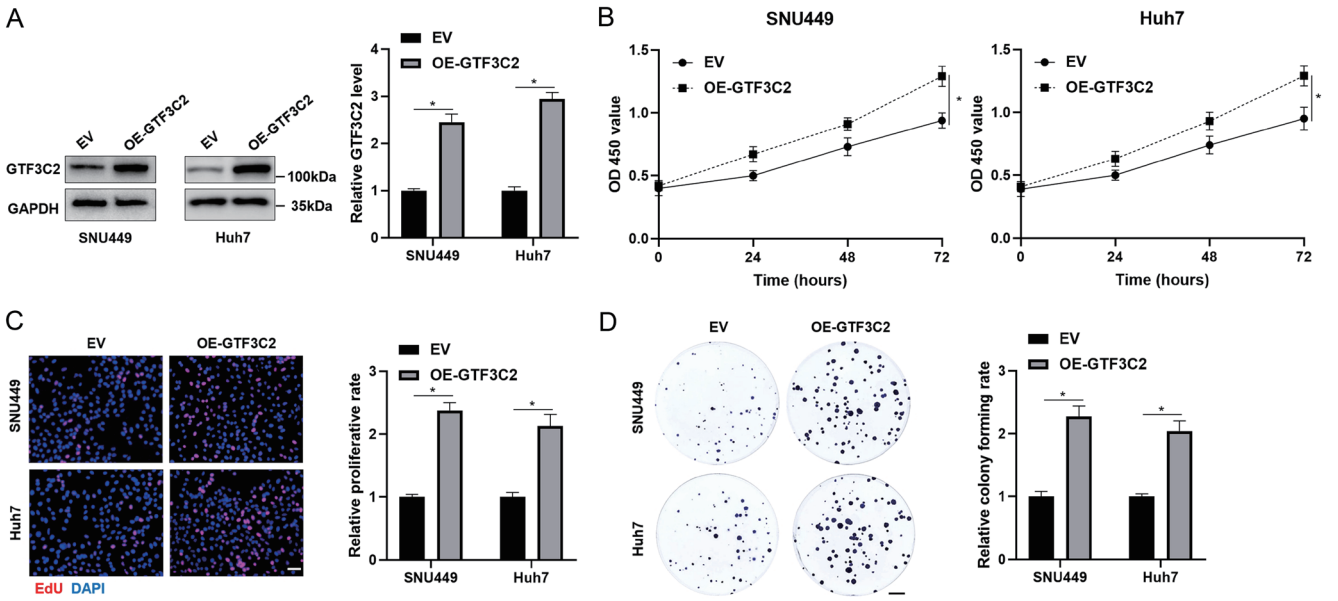


**Fig. 4. The effects of GTF3C2 overexpression on HCC cell proliferation.** (A) Lentiviruses OE-GTF3C2 or an EV were transduced into SNU449 and Huh7 cells, and the GTF3C2 level was confirmed by Western blotting. (B) The CCK-8 assay determined the viability of HCC cells with or without GTF3C2 overexpression. (C) The EdU assay investigated the proliferation of HCC cells with or without GTF3C2 overexpression. Scale bar: 25μm. (D) The colony formation assay was used to detect the proliferation of HCC cells in the GTF3C2 overexpression and control groups. Scale bar: 5mm. *$p < 0.05$. OD, optical density; EdU, 5-ethynyl-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; HCC, Hepatocellular Carcinoma; GTF3C2, General transcription factor IIIC subunit 2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; OE-GTF3C2, overexpressing GTF3C2; EV, empty vector.

as an oncogene in HCC. RNA sequencing data from the TCGA database indicated a significant positive correlation between GTF3C2 and USP21 in HCC. USP21 was highly expressed in HCC, and its overexpression was closely correlated with poor patient survival. GTF3C2 knockdown downregulated, while GTF3C2 overexpression upregulated USP21 mRNA and protein levels in HCC cells. A recent study reported that GTF3C2 functions as a transcription factor to increase the expression of claudin-4 and claudin-8 in pulmonary microvascular endothelial cells.[16] Our study revealed that GTF3C2 promoted USP21 expression by activating transcription. Previous studies have found that GTF3C2 is a forkhead box P3-associated

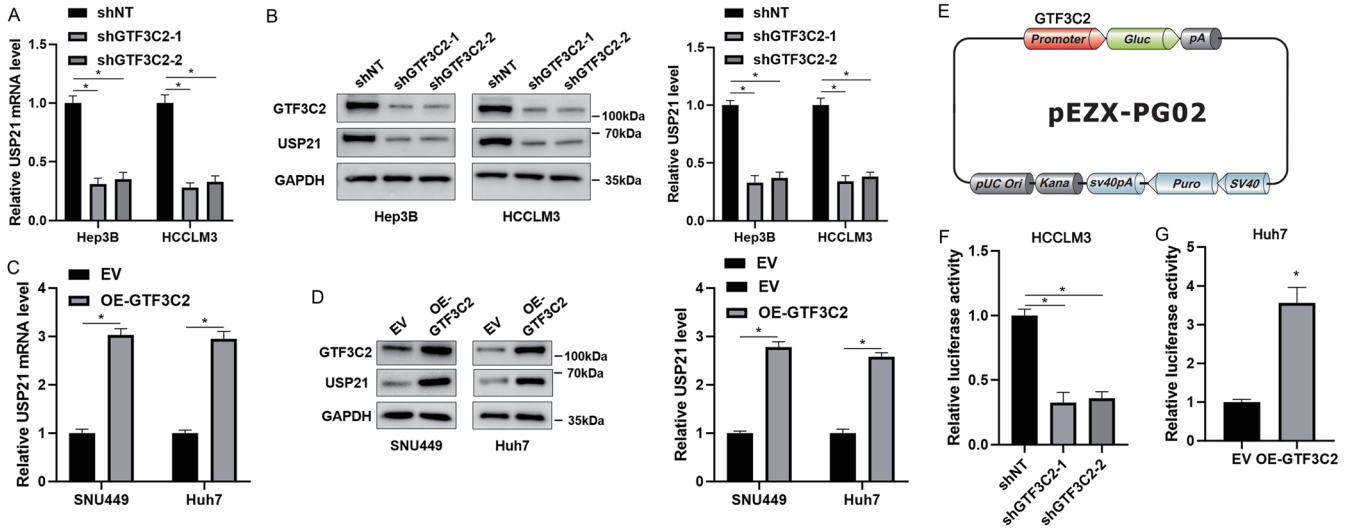


**Fig. 5. The role of GTF3C2 in regulating USP21 transcription in HCC cells.** (A) Lentiviruses containing shRNA against GTF3C2 (shGTF3C2-1 and GTF3C2-2) or non-targeting (shNT) sequences were transduced into Hep3B and HCCLM3 cells. The USP21 mRNA level was detected by RT-qPCR. (B) Western blotting confirmed the levels of GTF3C2 and USP21 in HCC cells with or without GTF3C2 knockdown. (C) Lentiviruses OE-GTF3C2 or an EV were transduced into SNU449 and Huh7 cells, and the USP21 mRNA level was confirmed by RT-qPCR. (D) Western blotting confirmed the levels of GTF3C2 and USP21 in the GTF3C2 overexpression and control groups. (E) Schematic diagram of the luciferase reporter vector. (F) HCCLM3 cells were co-transfected with the luciferase reporter vector and shGTF3C2 or shNT, and the luciferase activity was measured. (G) Huh7 cells were co-transfected with the luciferase reporter vector and OE-GTF3C2 or EV, and the luciferase activity was measured. *$p < 0.05$. HCC, Hepatocellular Carcinoma; GTF3C2, General transcription factor IIIC subunit 2; OE-GTF3C2, overexpressing GTF3C2; EV, empty vector; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

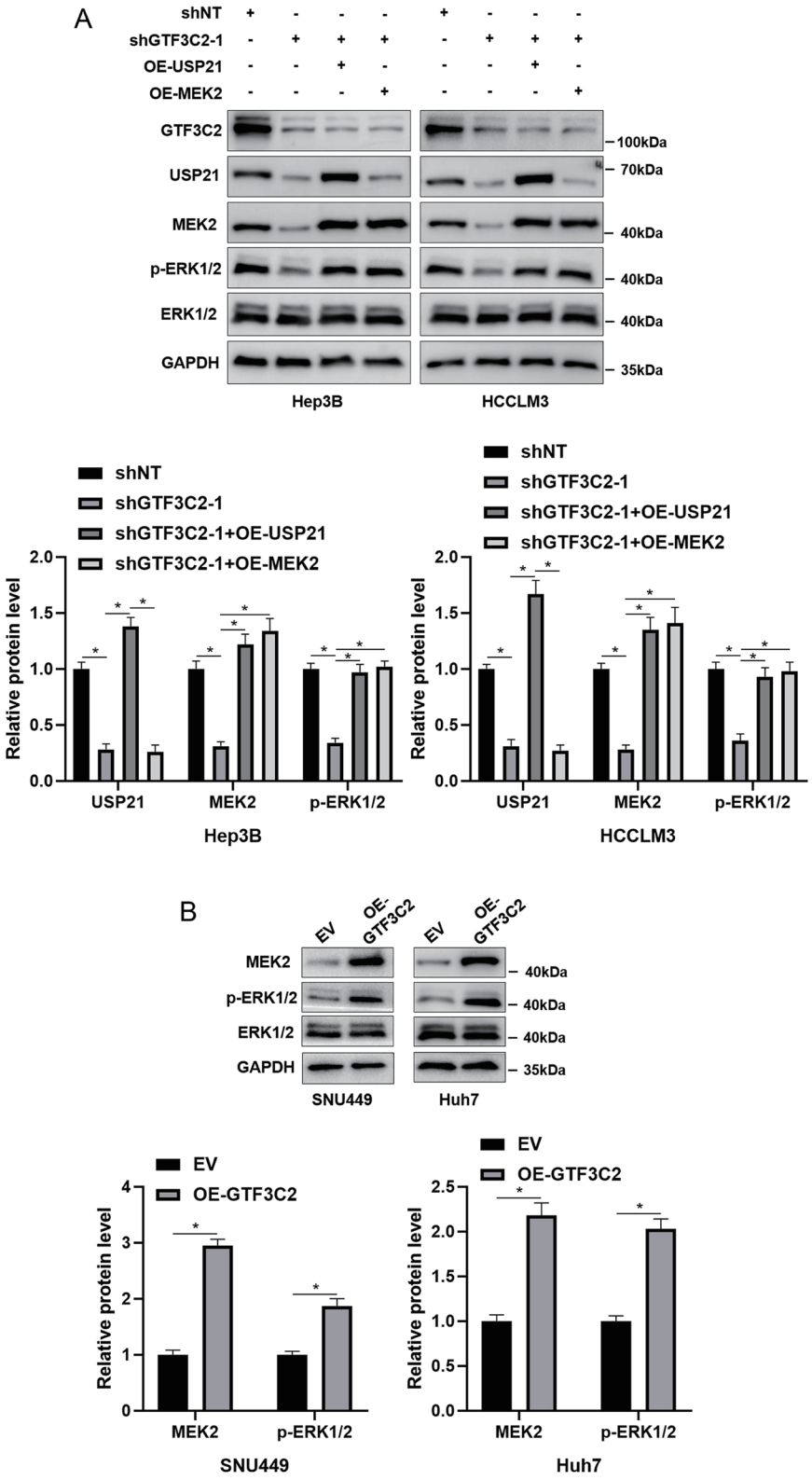


**Fig. 6. The role of GTF3C2 in regulating the ERK1/2 pathway in HCC cells.** (A) Hep3B and HCCLM3 cells transduced with the corresponding vectors were subjected to Western blotting for GTF3C2, USP21, MEK2, p-ERK1/2, and ERK1/2 expression. (B) Lentiviruses OE-GTF3C2 or an EV were transduced into SNU449 and Huh7 cells, and the MEK2, p-ERK1/2, and ERK1/2 levels were confirmed by Western blotting. *$p < 0.05$. +, Present; -, Absent; HCC, Hepatocellular Carcinoma; GTF3C2, General transcription factor IIIC subunit 2; OE-GTF3C2, overexpressing GTF3C2; EV, empty vector; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

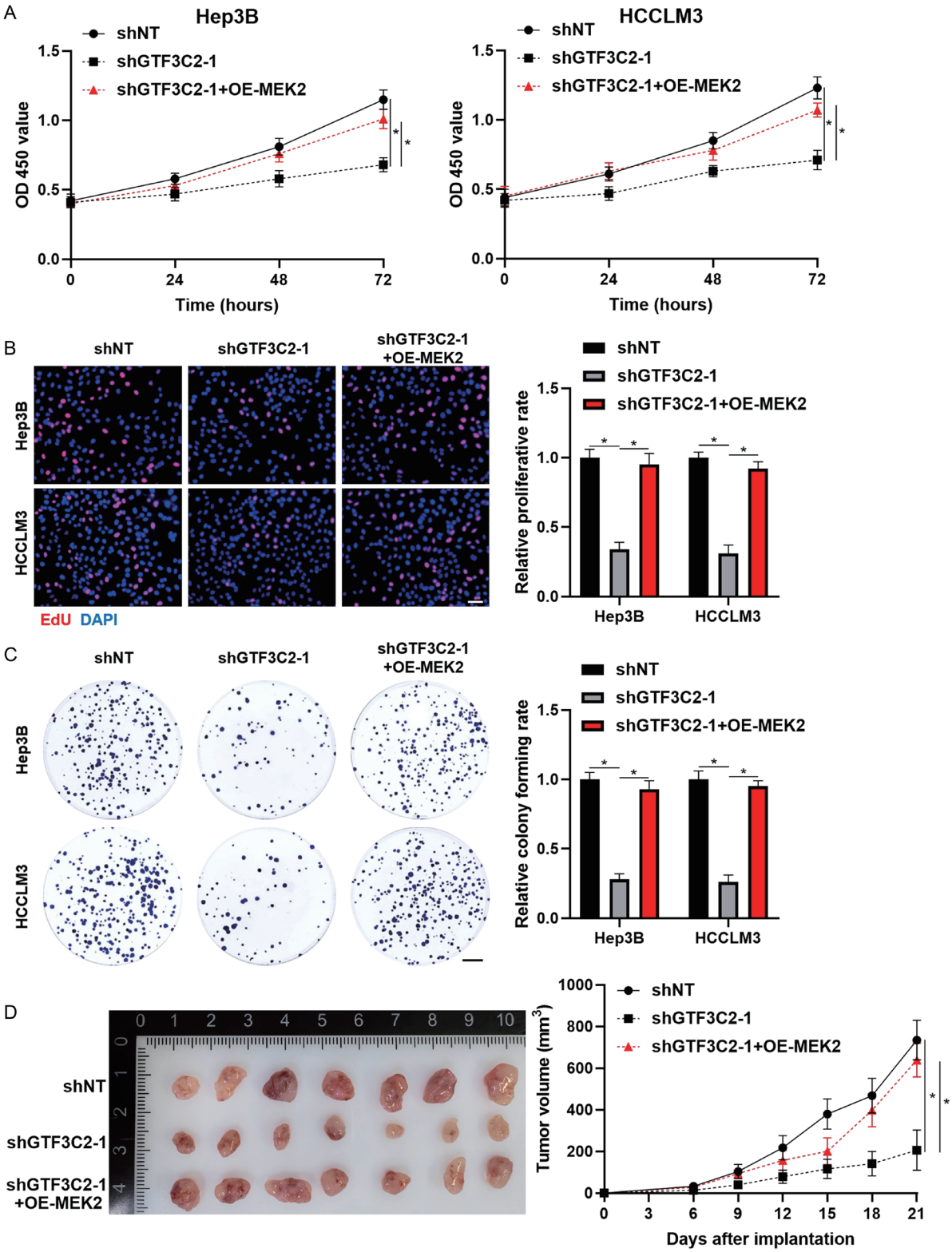


**Fig. 7. The role of MEK2 in GTF3C2-enhanced HCC cell proliferation and tumor growth.** Hep3B and HCCLM3 cells transduced with the corresponding vectors were subjected to (A) CCK-8, (B) EdU, and (C) colony formation assays to detect HCC proliferation. Scale bar: 25μm for EdU assay and 5mm for colony formation assay. (D) HCCLM3 cells transduced with the corresponding vectors were injected subcutaneously into the flanks of nude mice (n = 7). Subcutaneous tumor tissues from different groups were collected and photographed. The tumor growth curves were drawn based on tumor size measured every three days. *$p < 0.05$. OD, optical density; EdU, 5-ethynyl-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; HCC, Hepatocellular Carcinoma; GTF3C2, General transcription factor IIIC subunit 2.

factor implicated in transcription regulation, and forkhead box P3 activates USP21 transcription by directly binding to the gene promoter in regulatory T cells.[31,32] Therefore, further studies are needed to clarify whether GTF3C2 regulates USP21 expression as a transcription factor or a transcriptional coactivator in HCC.

Furthermore, we demonstrated that GTF3C2 knockdown reduced, and GTF3C2 overexpression increased, the levels of MEK2 and p-ERK1/2 in HCC cells. USP21 overexpression attenuated the GTF3C2 knockdown-induced decrease in MEK2 and p-ERK1/2 levels, while USP21 inhibition abolished the GTF3C2 overexpression-induced increase in the levels of MEK2 and p-ERK1/2. MEK2 overexpression abrogated the GTF3C2 knockdown-induced reduction in p-ERK1/2 levels. Importantly, MEK2 overexpression attenuated the GTF3C2 knockdown-induced decrease in HCC cell proliferation *in vitro* and tumor growth in nude mice. USP21 has been reported as a tumor-promoting factor in HCC.[24,33,34] USP21 targets MEK2 for deubiquitination and stabilization, thereby activating the ERK1/2 pathway and promoting the growth of HCC cells.[24] The ERK1/2 pathway is frequently activated in HCC and plays an essential role in tumor initiation and progression.[35,36] Notably, either USP21 inhibitor or ERK1/2 inhibitor attenuated GTF3C2 overexpression-enhanced HCC cell proliferation. These data suggest that GTF3C2 promotes tumor growth by activating the USP21/MEK2/ERK1/2 pathway in HCC.

## Conclusions

In conclusion, this study finds that GTF3C2 is overexpressed in HCC. GTF3C2 promotes HCC cell proliferation *in vitro* and tumor growth *in vivo*. Notably, GTF3C2 participates in USP21 transcription and increases MEK2 expression, thereby activating the ERK1/2 pathway in HCC. Our findings reveal a new molecular mechanism in HCC progression and provide a potential new target for anti-HCC therapy.

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## Conflict of interest

KT has been an Editorial Board Member of *Journal of Clinical and Translational Hepatology* since 2021. The other authors have no conflict of interests related to this publication.

## Author contributions

Study concept and design (DH, KT), acquisition of data (YW, YY, YZ), analysis and interpretation of data (YW, YY), drafting of the manuscript (YW, YY, KT), critical revision of the manuscript for important intellectual content (DH, KT), administrative, technical, or material support (QX), and study supervision (KT). All authors have made significant contributions to this study and have approved the final manuscript.

## Ethical statement

The research protocol was approved by The Research Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University (No: XJTU1AF2020LSY-08). All patients signed informed consent forms.

## Data sharing statement

No additional data are available.

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