Original Article

TLK2 promotes progression of hepatocellular carcinoma through Wnt/β-catenin signaling

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Background: Hepatocellular carcinoma is a widespread cancer worldwide, ranking as the fifth most frequent cancer and the fourth leading cause of cancer-related deaths. According to comprehensive research, *TLK2*, a phosphorylated kinase, has been discovered to play a crucial role in promoting tumor development. However, the prognostic significance and influence of *TLK2* on hepatocellular carcinoma tumor cells and the immune microenvironment remain unexplored, warranting further investigation. The aim of this study was to investigate the role of *TLK2* in promoting the development of hepatocellular carcinoma.

Methods: The present study utilized The Cancer Genome Atlas (TCGA) database and other databases as training sets to examine the expression of *TLK2* and its prognostic significance. The findings were subsequently validated through cell proliferation assays and cell colony assays. Gene set enrichment analysis (GSEA) was employed to investigate the tumor-related biological processes associated with *TLK2* in hepatocellular carcinoma, while the relationship between *TLK2* expression and Wnt/β-catenin signaling pathway was analyzed via TCGA dataset analysis. Western blotting and immunofluorescence assays were used to confirm the experimental results.

Results: *TLK2* showed higher expression levels in tumor tissues than in normal tissues. Alpha fetoprotein (AFP), T stage, pathological stage, and histological grade were significantly associated with *TLK2* expression. High *TLK2* expression correlated with worse overall survival (OS) [hazard ratio (HR) =1.62, 95% confidence interval (CI): 1.14–2.29, P=0.007], progression-free survival (PFS) (HR =1.88, 95% CI: 1.40–2.52, P<0.001) and disease specific survival (DSS) (HR =1.86, 95% CI: 1.18–2.93, P=0.007) in the training and validation sets. Both univariate and multivariate analyses showed that *TLK2* was an independent prognostic factor. GSEA showed that *TLK2* was significantly enriched in tumor-related biological processes. *TLK2* induced the activation of β-catenin signaling, resulting in sustained tumor growth. Methyl thiazolyl tetrazolium (MTT) and colony formation assays demonstrated that *TLK2* could promote hepatocellular carcinoma progression. Furthermore, *TLK2* showed a significant association with β-catenin in the Wnt pathway.

Conclusions: *TLK2* represents an independent prognostic factor in hepatocellular carcinoma and can promote cancer progression via the β-catenin signaling pathway.

Keywords: *TLK2*; Wnt; β-catenin; hepatocellular carcinoma; proliferation

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Introduction

Hepatocellular carcinoma is one of the leading causes of cancer-related deaths worldwide and has a high incidence (1), and the incidence rates of hepatocellular carcinoma in Hispanics have surpassed those of Asians in the United States (2). Chronic liver disease is caused by a vicious cycle of liver damage, inflammation, and regeneration that can last decades, leading to hepatocellular carcinoma (3). The main causes of chronic liver disease and hepatocellular carcinoma include hepatitis B and hepatitis C virus infections, alcoholic liver disease, fatty liver, and schistosome infection (4,5). Although the etiology and pathogenesis of hepatocellular carcinoma are multifaceted, the exact molecular mechanisms underlying its development are not yet completely understood.

Prior research has indicated that the activation of β-catenin, a key signaling pathway involved in cell proliferation and survival, is highly prevalent in hepatocellular carcinoma (6). A comprehensive genomic analysis of hepatocellular carcinoma revealed the presence of gain-of-function mutations in CTNNB1, which encodes β-catenin in approximately 35% of human hepatocellular carcinoma samples (7). Wnt/β-catenin plays a central role in human embryonic development and regulates liver development during the embryonic period via Wnt signaling. During adulthood, the Wnt/β-catenin pathway is typically inactive in the liver. However, abnormal expression of this pathway can occur in certain instances, such as cell renewal, regeneration, pathological conditions, diseases, and various types of cancers (8). In recent years, numerous studies have shown that Wnt/β-catenin protein signaling plays a major role in not only enhancing cancer cell stemness but also maintaining sorafenib resistance in hepatocellular carcinoma (9,10), and also mediates immune escape in hepatocellular carcinoma (11), suggesting that it may play an essential role in drug resistance.

Tousled-like kinases (*TLKs*) are an evolutionarily conserved family of serine-threonine kinases that are mainly located in the nucleus (12). In mammals, there are two distinct TLK genes, *TLK1* and *TLK2*. *TLK2* is an extensively studied protein that is aberrantly expressed in various types of cancer, including breast cancer, lung cancer, ovarian cancer, hepatocellular carcinoma, and melanoma (13). This protein is closely associated with DNA repair, DNA replication, chromatin structure, transcription, viral latency, cell cycle checkpoint control, and chromosome stability in a wide range of organisms (14). *TLK2* primarily functions in the cell division cycle, and its expression typically peaks during the S phase (15). *TLK2* amplification has been found to induce genomic instability, leading to the development of breast cancer through the impairment of the G2–M checkpoint (16). In particular, ectopic expression of *TLK2* has been shown to lead to increased aggressiveness in breast cancer and glioblastoma (17,18). The *TLK-ASF1* axis can promote leukemogenesis by affecting cell cycle and DNA damage pathways (19). These studies support that *TLK2* may be an important target for the development of novel cancer therapies.

Although the stimulatory effect of β-catenin on hepatocellular carcinoma progression has been established, the relationship between *TLK2* and hepatocellular carcinoma is not understood. Herein, we present a new mechanism for promoting hepatocellular carcinoma by regulating β-catenin. The Cancer Genome Atlas (TCGA) database analysis revealed high *TLK2* expression in hepatocellular carcinoma. Patients with elevated *TLK2* levels had a poorer prognosis. Moreover, *TLK2* was found to activate the classical Wnt pathway and was closely

Highlight box

Key findings

- *TLK2* stimulates hepatocellular carcinoma growth through the activation of the Wnt/β-catenin pathway.

What is known and what is new?

- *TLK2* is highly expressed in tumor tissue has been studied.
- *TLK2* promotes hepatocellular carcinoma proliferation through β-catenin is a new finding from our study.

What is the implication, and what should change now?

- Our study revealed that *TLK2* may act as a new potential factor to promote the proliferation of hepatocellular carcinoma. Previous studies have indicated that *TLK2* is associated with tumor proliferation, but no specific mechanism has been proposed. Our study is the first demonstration that *TLK2* promotes hepatocellular carcinoma proliferation in association with β-catenin activation.

Table 1 The siRNA sequences of *TLK2*

Genes primers (5'-3')	Sequences
SI-*TLK2*-1 forward	GCAAGAAACUCCUUAUAGA
SI-*TLK2*-1 reverse	UCUAUAAGGAGUUUCUUGC
SI-*TLK2*-2 forward	GUCCGACCUCACAAUAGAA
SI-*TLK2*-2 reverse	UUCUAUUGUGAGGUCGGAC

siRNA, small interfering RNA.

associated with β-catenin, thus promoting hepatocellular carcinoma. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2264/rc).

Methods

Acquisition of single gene expression data from TCGA

Gene expression profile matrix and clinical records of hepatocellular carcinoma were downloaded from TCGA (https://tcga-data.nci.nih.gov/tcga/). We conducted transcriptome profiling on 374 tumor and 50 normal tissue samples, with the primary site being the liver. The gene expression profile matrix was standardized using $\log_2$(value +1) (version 3.6.3; http://www.r-project.org). Additionally, *TLK2* expression in hepatocellular carcinoma was analyzed using the Integrative Molecular Database for Hepatocellular Carcinoma (HCCDB) (https://ngdc.cncb.ac.cn/). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Univariate/multivariate Cox hazard regression analyses and nomogram construction

We utilized univariate and multivariate Cox regression analyses to explore the impact of the *TLK2* gene and clinical characteristics, such as age, sex, alpha fetoprotein (AFP), pathological stage, and histological grade, on the prognosis of hepatocellular carcinoma. The R package "forestplot" was employed to display the P value, hazard ratio (HR), and 95% confidence interval (CI) of each variable. Additionally, *TLK2* expression in hepatocellular carcinoma was evaluated using the HCCDB database. To standardize the gene expression profile matrix, we employed R software (version 3.6.3; http://www.r-project.org). Kaplan-Meier Plotter was used to generate survival curves, including overall survival (OS), progression-free survival (PFS), and disease-specific survival (DSS) using the log-rank test.

Cell lines

The PLC/PRF/5 and HepG2 cell lines used in this study were purchased from the Cell Bank of the Chinese Academy of Sciences in Shanghai, China. These cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and maintained at 37 ℃ in a 5% CO_2 incubator. PLC/PRF/5 and HepG2 cells were authenticated by short tandem repeats (STRs).

Plasmids and siRNA transfection

To investigate the role of *TLK2*, we designed three small interfering RNA (siRNA) sequences to suppress its expression. The primers are listed in *Table 1*. The siRNAs were obtained from Tsingke Biotechnology Co., Ltd. (Beijing, China). The cells used for the transfection experiments were PLC/PRF/5 and HepG2 cells, and Lipofectamine 2000 was used at a concentration of 50 nmol according to the manufacturer's instructions. The pCDH-GFP-Puro-3xFlag plasmid encoding *TLK2* and a control plasmid were transfected into the cells with Lipofectamine 3000 (Invitrogen, Waltham, USA).

Western blot analysis

Thirty micrograms of protein was separated on a 10% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a 0.45 μm polyvinylidene difluoride (PVDF) membrane in a wet electron transfer device. Tris buffered saline (TBS) and Tween 20 were used to prepare 0.05% tris buffered saline with Tween 20 (TBST). Five percent skim milk was prepared with TBST to block the membrane for 2 hours at room temperature. After transferring the proteins onto the membrane, the membrane was incubated with primary antibodies overnight at 4 ℃. The primary antibodies used were *TLK2* (1:1,000, 13979-1-AP, Proteintech, Wuhan, China) and β-catenin (1:1,000, 51067-2-AP, Proteintech). The membranes were then incubated with horse radish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies for 1.5 hours at room temperature.

Methyl thiazolyl tetrazolium (MTT) analysis and colony formation assay

Hepatocellular carcinoma cells (4,000 per well) were seeded into 96-well plates. Cells were collected at 0, 24, 48, and 72 h, and the relative viable cell numbers were quantified using the MTT assay, with absorbance was measured at 490 nm. For the colony formation assay, hepatocellular carcinoma cells were seeded at a density of 500 cells per well on 6-well plates. After 2 weeks, each group of cells was fixed with 4% paraformaldehyde (PFA) for 20 minutes and then stained with 0.1% crystal violet solution for imaging and counting.

Immunofluorescence assay

Cells were grown in confocal dishes at a density of 1,000/well and washed three times with phosphate buffer saline (PBS). The cells were then fixed with 4% PFA for 15 min and washed three times with PBS. The cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature and washed three times with PBS. Fresh containment solution (1 mg/mL BSA, 10% normal goat serum, 0.1% Tween) was incubated for at least 30 min. The primary antibody (1:200) was diluted in the containment solution and incubated with the cells at room temperature for approximately 2 hours or overnight at 4 ℃. The cells were washed three times with PBS for 5 minutes each and incubated with goat anti-rabbit Alexa-coupled secondary antibody (1:500, 594 nm) obtained from Life Technologies at room temperature for 1 to 2 hours. The cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (5 μg/mL) by adding the stain dropwise for 5 min. After washing the cells twice with PBS, they were observed by confocal microscopy.

Statistical analysis

The results of the experiment were expressed as mean ± standard deviation. SPSS 21.0 and Graphpad Prism 9 were used for statistical analysis. Comparisons between two groups were made using *t*-test, and one-way analysis of variance (ANOVA) was used for groups of three or more. Kaplan-Meier curve and log-rank test were used to compare OS, PFS, and disease-free survival (DFS) among different patient groups. Differences were considered statistically significant when $P<0.05$.

Results

TLK2 was highly expressed in hepatocellular carcinoma

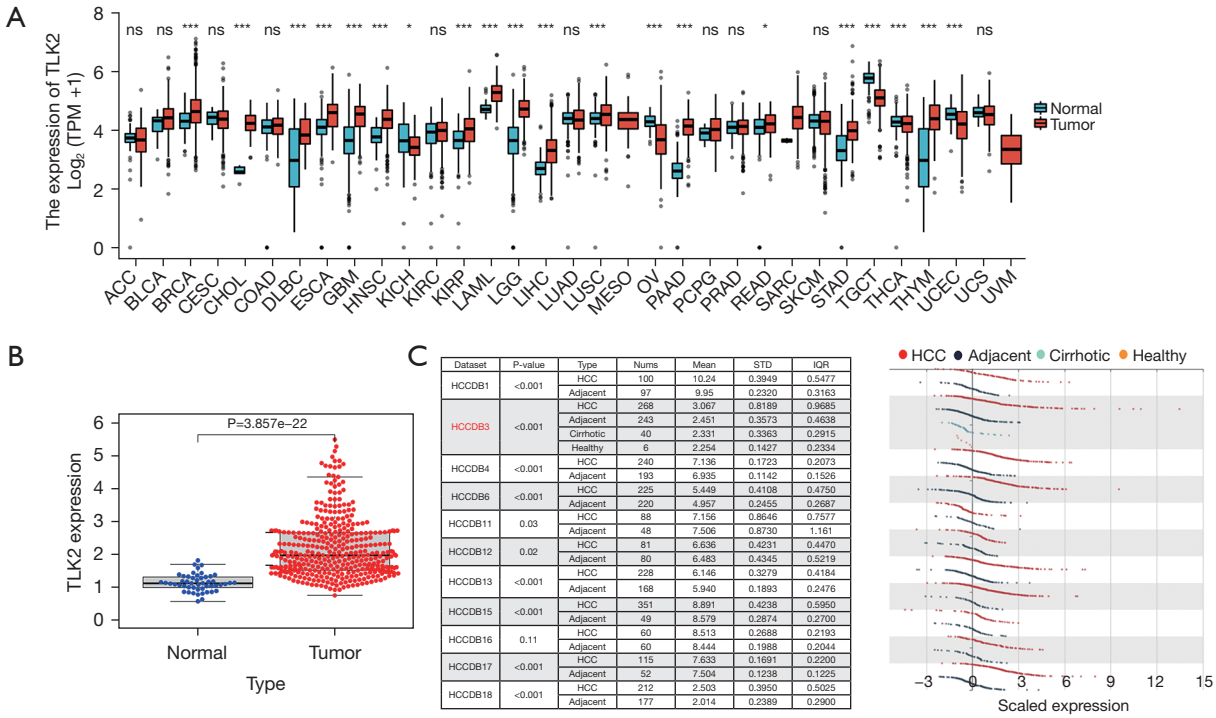
We compared the expression levels of *TLK2* in cancer samples and normal samples from different cancers using TCGA data. We found that the expression level of *TLK2* in hepatocellular carcinoma cells was significantly higher than that in normal samples (*Figure 1A*). We collected relevant patient clinical information from the TCGA database (*Table 2*). A boxplot was generated showing that the expression level of *TLK2* in hepatocellular carcinoma tissues (n=374) was higher than that in normal tissues (n=50) (*Figure 1B*). We also analyzed 11 hepatocellular carcinoma study cohorts in the HCCDB database and found that the mRNA expression levels of *TLK2* were significantly higher in hepatocellular carcinoma tissues than in adjacent normal tissues (*Figure 1C*). The above results suggested that *TLK2* expression was significantly higher in hepatocellular carcinoma tissues than in normal tissues.

High TLK2 expression was correlated with poor prognosis in hepatocellular carcinoma

To investigate the clinical significance of *TLK2*, we used logistic regression analysis to assess the relationship between *TLK2* expression and age, sex, AFP, T stage, pathological stage, and histological grade. Our analysis showed that high expression of *TLK2* was not associated with age (*Figure 2A*), but significantly correlated with sex (P=0.03) (*Figure 2B*), AFP (P<0.001) (*Figure 2C*), T-stage (P=0.03) (*Figure 2D*), pathological stage (P=0.01) (*Figure 2E*) and histological grade (P<0.001) (*Figure 2F*). These results substantiated that high expression of *TLK2* expression was significantly correlated with the prognosis of hepatocellular carcinoma patients.

TLK2 was an independent prognostic factor for hepatocellular carcinoma

We examined the prognostic relevance of *TLK2* in hepatocellular carcinoma to determine whether it consistently correlates with hepatocellular carcinoma patient outcomes. We performed univariate and multivariate Cox regression analyses to analyze the relationship between *TLK2* expression, clinical factors (such as age, sex, pT



Dataset	P-value	Type	Nums	Mean	STD	IQR
HCCDB1	<0.001	HCC	100	10.24	0.3949	0.5477
		Adjacent	97	9.95	0.2320	0.3163
HCCDB3	<0.001	HCC	268	3.067	0.8189	0.9685
		Adjacent	243	2.451	0.3573	0.4638
		Cirrhotic	40	2.331	0.3363	0.2915
		Healthy	6	2.254	0.1427	0.2334
HCCDB4	<0.001	HCC	240	7.136	0.1723	0.2073
		Adjacent	193	6.935	0.1142	0.1526
HCCDB6	<0.001	HCC	225	5.449	0.4108	0.4750
		Adjacent	220	4.957	0.2455	0.2687
HCCDB11	0.03	HCC	88	7.156	0.8646	0.7577
		Adjacent	48	7.506	0.8730	1.161
HCCDB12	0.02	HCC	81	6.636	0.4231	0.4470
		Adjacent	80	6.483	0.4345	0.5219
HCCDB13	<0.001	HCC	228	6.146	0.3279	0.4184
		Adjacent	168	5.940	0.1893	0.2476
HCCDB15	<0.001	HCC	351	8.891	0.4238	0.5950
		Adjacent	49	8.579	0.2874	0.2700
HCCDB16	0.11	HCC	60	8.513	0.2688	0.2193
		Adjacent	60	8.444	0.1988	0.2044
HCCDB17	<0.001	HCC	115	7.633	0.1691	0.2200
		Adjacent	52	7.504	0.1238	0.1225
HCCDB18	<0.001	HCC	212	2.503	0.3950	0.5025
		Adjacent	177	2.014	0.2389	0.2900

Figure 1 *TLK2* is highly expressed in hepatocellular carcinoma. (A) The expression distribution of *TLK2* in tumor tissues and normal tissues in 33 cancer types. (B) The expression distribution of *TLK2* in tumor tissues and normal tissues from TCGA dataset. (C) *TLK2* transcription was significantly elevated in HCC tissues compared with adjacent normal tissues in HCCDB. *, P<0.05; ***, P<0.001; ns, no significance. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower-grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; TPM, transcripts per million; TCGA, The Cancer Genome Atlas; HCC, hepatocellular carcinoma; HCCDB, Integrative Molecular Database of Hepatocellular Carcinoma; STD, standard deviation; IQR, interquartile range.

stage, pTNM stage, and histological grade) (*Tables 3*,*4*), and OS in hepatocellular carcinoma patients. Univariate cox analysis showed that *TLK2* expression (P=0.002), pT stage (P<0.001), and pTNM stage (P<0.001) were significantly correlated with OS in hepatocellular carcinoma (*Figure 3A*). Multivariate cox regression analysis results suggested that *TLK2* expression (P=0.02) was an independent prognostic factor for hepatocellular carcinoma (*Figure 3B*). The median value of *TLK2* expression was used as a cutoff value to divide patients into low-expression and high-expression groups. According to the Kaplan-Meier survival curve illustrated in *Figure 3C*, patients in the high-expression group had significantly poorer OS, PFS, and DSS than patients in the low-expression group (OS, HR =1.62, 95% CI: 1.14–2.29, P=0.007; PFS, HR =1.88, 95% CI: 1.40–2.52, P<0.001; DSS, HR =1.86, 95% CI: 1.18–2.93, P=0.007) (*Figure 3C-3E*). These findings indicated that *TLK2* expression was an independent predictor of adverse outcomes in patients with

Table 2 Clinical characteristics of HCC patients in the TCGA database

Characteristics	Values (n=418), n (%)
Age (years)	
<60	172 (41.15)
≥60	204 (48.80)
Unknown	42 (10.05)
Gender	
Male	272 (65.07)
Female	146 (34.93)
T	
T1	204 (48.80)
T2	107 (25.60)
T3	90 (21.53)
T4	14 (3.35)
Unknown	3 (0.72)
N	
N0	290 (69.38)
N1	8 (1.91)
Unknown	120 (28.71)
M	
M0	303 (72.49)
M1	8 (1.91)
Unknown	107 (25.60)
Stage	
Stage I–II	292 (69.86)
Stage III–IV	102 (24.40)
Unknown	24 (5.74)
Histologic grade	
G1	55 (13.16)
G2	180 (43.06)
G3	124 (29.67)
G4	13 (3.11)
Unknown	46 (11.00)
OS event	
Alive	271 (64.83)
Dead	147 (35.17)

HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; T, tumor; N, node; M, metastasis; OS, overall survival.

hepatocellular carcinoma.

TLK2 expression was upregulated in hepatocellular carcinoma cell lines and promoted hepatocellular carcinoma progression

To investigate the role of *TLK2* in hepatocellular carcinoma tumor progression, we selected PLC/PRF/5 and HepG2 cells for functional validation. To further investigate the effect of *TLK2* on hepatocellular carcinoma proliferation, we designed two types of siRNAs (SI-*TLK2*-1 and SI-*TLK2*-2) based on the *TLK2* sequence, and SI-*TLK2*-1 significantly knocked down *TLK2* expression (*Figure 4A*). *TLK2* was significantly overexpressed (*Figure 4B*). MTT assays revealed that knockdown of *TLK2* could significantly reduce the proliferation of PLC/PRF/5 and HepG2 cells (*Figure 4C*). Colony formation assays confirmed that knockdown of *TLK2* also inhibited the growth of PLC/PRF/5 and HepG2 cells (*Figure 4D*). Furthermore, overexpression of *TLK2* increased cell proliferation and colony formation ability in PLC/PRF/5 and HepG2 cells (*Figure 4E,4F*). Taken together, these data indicated that *TLK2* was highly expressed in hepatocellular carcinoma and promoted hepatocellular carcinoma proliferation *in vitro*.

TLK2 promoted hepatocellular carcinoma proliferation through the Wnt/β-catenin pathway

To comprehend the biological importance of *TLK2* in hepatocellular carcinoma, gene set enrichment analysis (GSEA) was carried out. The results revealed that *TLK2* was predominantly linked to oocyte-meiosis, cell cycle, and ubiquitin-mediated proteolysis pathways in hepatocellular carcinoma. In contrast, activities related to fatty acid metabolism, retinol metabolism, and primary bile acid biosynthesis were significantly suppressed (Figure S1). *TLK2* was significantly enriched in pathways linked to cell proliferation, revealing a clear association between *TLK2* and the Wnt signaling pathway. Our results showed that *TLK2* was involved in the cell proliferation pathway, and among them, the Wnt pathway, which is associated with cell proliferation, was significantly enriched (*Figure 5A*). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that *TLK2* was significantly enriched in the Wnt pathway. We then assessed the correlation between CTNNB1 and *TLK2* and found that *TLK2* was significantly correlated with CTNNB1, a key factor in the Wnt pathway (*Figure 5B*).

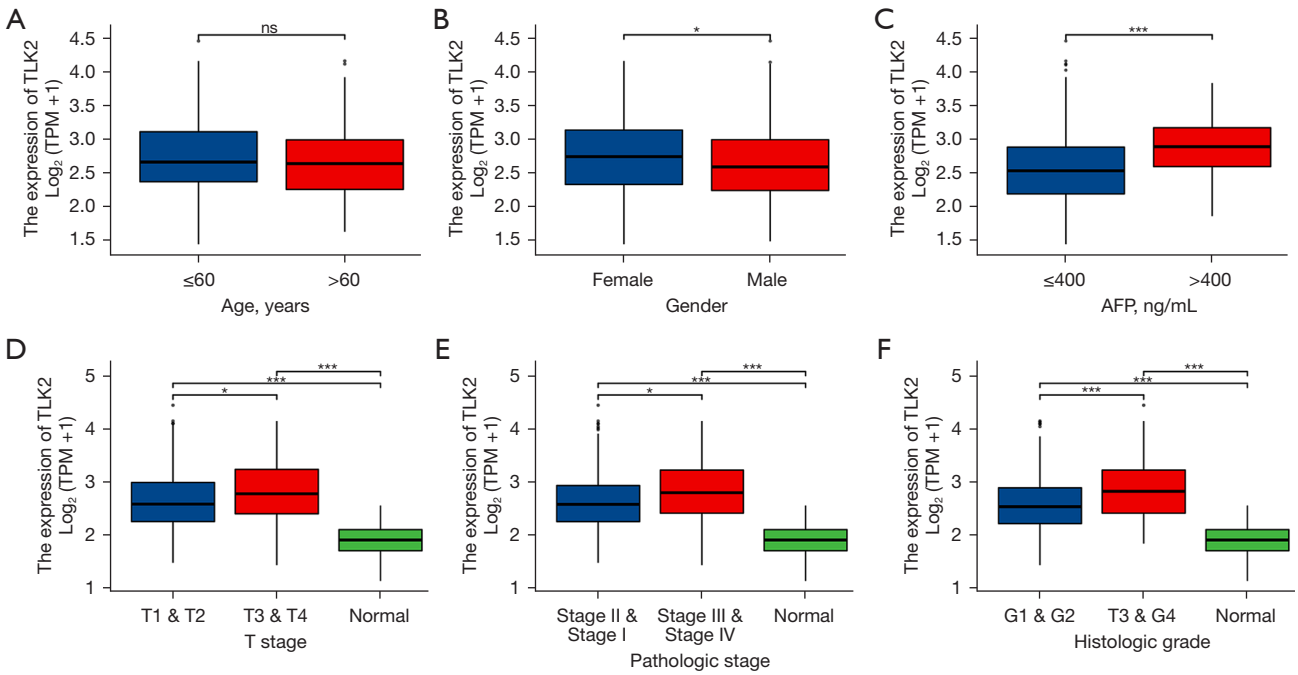


Figure 2 Relationship between *TLK2* expression and clinicopathologic factors. (A) *TLK2* expression is not associated with age. (B) *TLK2* expression was significantly positively correlated with sex (P=0.03). (C) *TLK2* expression was significantly positively correlated with AFP (P<0.001). (D) *TLK2* expression was higher in T3 and T4 than in T1 and T2 (P=0.03). (E) *TLK2* expression was higher in stage III & stage IV than in stage I & stage II (P=0.01). (F) *TLK2* expression was significantly positively correlated with histological grade (P<0.001). *, P<0.05; ***, P<0.001; ns, no significance. AFP, alpha fetoprotein; TPM, transcripts per million.

Table 3 Univariate Cox analysis of the relationship between *TLK2* and survival

ID	HR	HR.95L	HR.95H	P value
Age	1.007	0.988	1.026	0.48
Gender	0.765	0.474	1.234	0.27
Grade	1.060	0.773	1.453	0.72
Stage	1.861	1.449	2.389	<0.001
T	1.825	1.440	2.313	<0.001
M	3.914	1.226	12.499	0.02
N	2.044	0.499	8.374	0.32
TLK2	1.350	1.115	1.635	0.002

HR, hazard ratio; HR.95L, hazard ratio, low 95% CI; HR.95H, hazard ratio, high 95% CI; CI, confidence interval; T, tumor; N, node; M, metastasis.

Table 4 Multivariate Cox analysis of the relationship between *TLK2* and survival

ID	HR	HR.95L	HR.95H	P value
Age	1.012	0.992	1.031	0.24
Gender	1.028	0.611	1.729	0.92
Grade	1.112	0.786	1.571	0.55
Stage	0.952	0.319	2.845	0.93
T	1.872	0.682	5.138	0.22
M	1.358	0.362	5.094	0.65
N	1.508	0.231	9.856	0.67
TLK2	1.331	1.057	1.677	0.02

HR, hazard ratio; HR.95L, hazard ratio, low 95% CI; HR.95H, hazard ratio, high 95% CI; CI, confidence interval; T, tumor; N, node; M, metastasis.

Moreover, we verified the relationship between *TLK2* and β-catenin in hepatocellular carcinoma cells by Western blot and immunofluorescence assays. As shown in *Figure 5C*, β-catenin expression was significantly decreased in SI-*TLK2* cells compared to control cells (*Figure 5C*), consistent with the results of the immunofluorescence assay (*Figure 5D*).

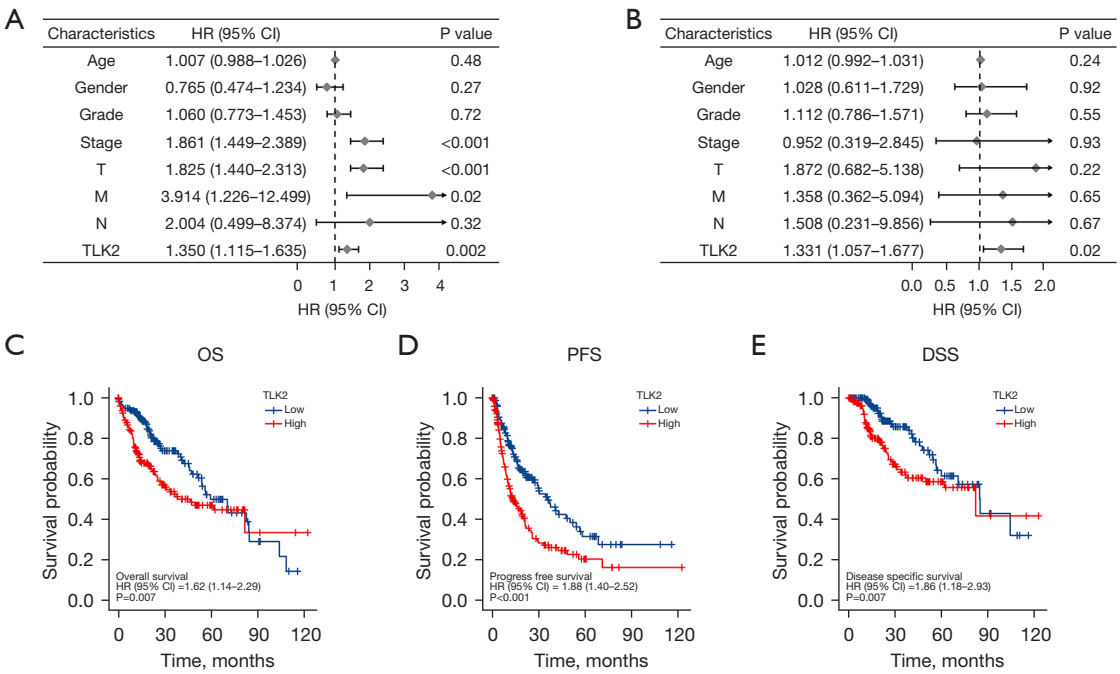


Figure 3 *TLK2* could be an independent prognostic factor for hepatocellular carcinoma. (A) Univariate Cox analysis showed that *TLK2* expression, pT stage and pTNM stage were significantly correlated with OS in hepatocellular carcinoma. (B) Multivariate Cox analysis showed that *TLK2* expression was significantly correlated with OS in hepatocellular carcinoma. (C-E) Association between *TLK2* expression and OS, PFS, DSS in patients with hepatocellular carcinoma assessed by Kaplan-Meier mapper. HR, hazard ratio; CI, confidence interval; T, tumor; N, node; M, metastasis; OS, overall survival; PFS, progression-free survival; DSS, disease specific survival.

These findings suggested that *TLK2* can promote the proliferation of hepatocellular carcinoma cells by increasing the expression of β-catenin. We further validated that *TLK2* promoted the proliferation of hepatocellular carcinoma cells through β-catenin by MTT and colony formation assays. As shown in the results, *TLK2* was able to promote the proliferation of hepatocellular carcinoma cells, and the β-catenin inhibitor Foscenvivint significantly inhibited the proliferation of hepatocellular carcinoma cells (*Figure 6A,6B*). However, inhibition of β-catenin after *TLK2* overexpression rescued the inhibitory effect of β-catenin on hepatocellular carcinoma cells (*Figure 6A,6B*).

Discussion

Hepatocellular carcinoma is a highly aggressive type of hepatocellular carcinoma (20). Several molecular pathways have been implicated in hepatocellular carcinoma development and progression, but the role of *TLK2* in hepatocellular carcinoma is not fully understood. Current evidence suggests that the Wnt/β-catenin pathway is dysregulated in many types of cancer, including hepatocellular carcinoma, and has been shown to play a critical role in tumor initiation and progression (21). It is widely thought that β-catenin is a crucial driver of tumorigenesis in hepatocellular carcinoma, with aberrant activation of the β-catenin signaling pathway being a common feature of this type of cancer (22). Study revealed that GINS1 promotes ZEB1-mediated tumor metastasis in hepatocellular carcinoma through β-catenin signaling (23). MiR-557 blocked HCC progression through the Wnt/β-catenin pathway by targeting RAB10 (24). These studies have demonstrated the importance of β-catenin in the development of hepatocellular carcinoma. It has been shown that frequent upregulation of *TLK2* is associated with poor patient prognosis in several types of cancer and that depletion of *TLK2* activity leads to increased replication stress and DNA damage in cancer cells (25). Overwhelming evidence substantiates that the absence or abnormal expression of *TLK2* in DNA repair can lead to the accumulation of DNA damage and reduced DNA repair capacity, which increases the risk of cancer development (26). Meanwhile, aberrant expression of *TLK2* in cancer cells leads to cell cycle disruption and chromosomal instability, increasing the risk

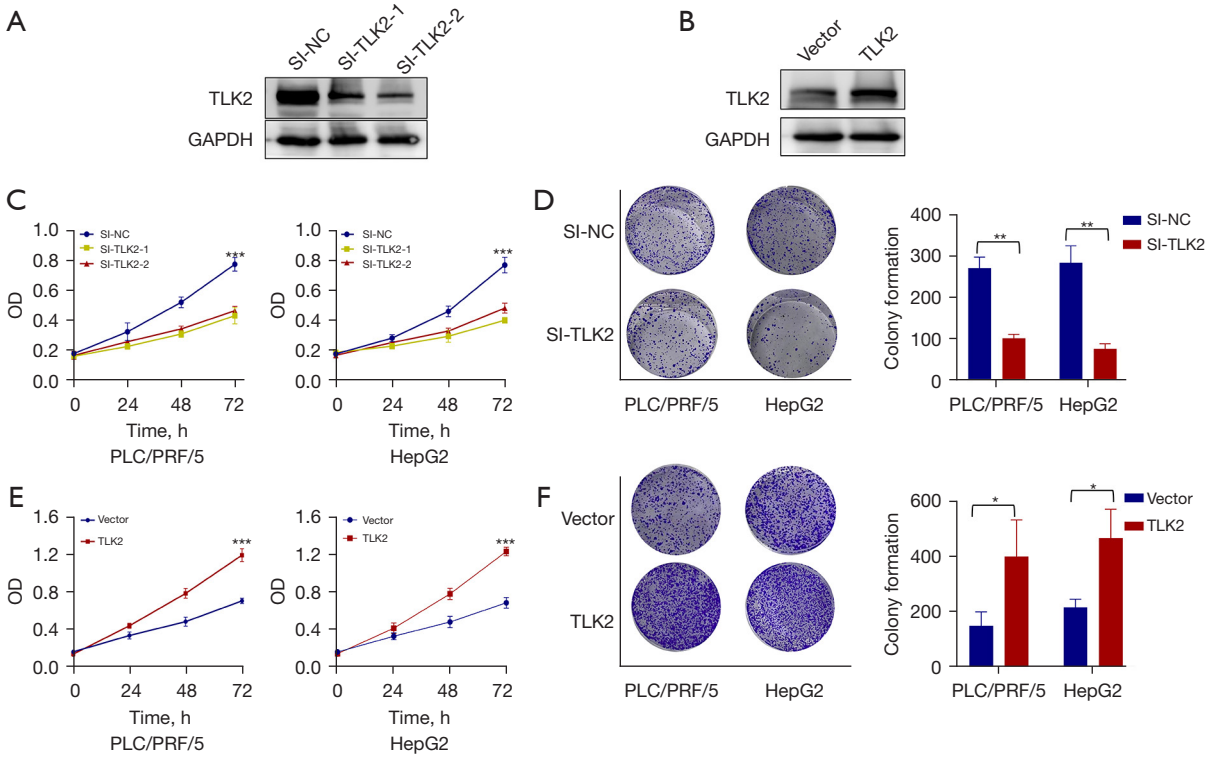


Figure 4 *TLK2* expression was upregulated in hepatocellular carcinoma cell lines and promoted hepatocellular carcinoma progression. (A) Silencing efficiency of *TLK2*. (B) Overexpression efficiency of *TLK2*. (C) Cell growth was measured in PLC/PRF/5 and HepG2 cell lines. Cells were transfected with SI-NC/SI-*TLK2* for 24 hours, and cell viability was measured by MTT assay every 24 h. Mean ± SD, n=3, P<0.001, *t*-test. (D) Colony formation assays were performed in PLC/PRF/5 and HepG2 cells transfected with SI-NC/SI-*TLK2*, 500 cells per well after 24 hours of transfection, and stained with crystal violet after 14 days (×0.5). Mean ± SD, n=3, PLC/PRF/5: P=0.005, HepG2: P=0.001, *t*-test. (E) Cell growth was measured in PLC/PRF/5 and HepG2 cell lines transfected with Vector/*TLK2* for 24 h, and cell viability was measured by MTT assay every 24 hours. Mean ± SD, n=3, P<0.001, *t*-test. (F) Colony formation assays were performed in PLC/PRF/5 and HepG2 cells transfected with Vector/*TLK2* at 500 cells per well after 24 hours of transfection and stained with crystal violet after 14 days (×0.5). Mean ± SD, n=3, PLC/PRF/5: P=0.04, HepG2: P=0.02, *t*-test. *, P<0.05; **, P<0.01; ***, P<0.001. MTT, methyl thiazolyl tetrazolium; SD, standard deviation; OD, optical density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

of cancer development and immune evasion (27). Therefore, *TLK2* may play a crucial role in cancer development and progression via β-catenin.

In this study, we first evaluated the expression levels of *TLK2* in cancer tissues and adjacent cells in each tissue and found that *TLK2* was highly expressed in hepatocellular carcinoma. We further analyzed *TLK2* expression levels in hepatocellular carcinoma and adjacent tissues and found that *TLK2* was abnormally highly expressed in hepatocellular carcinoma. Western blotting also confirmed that *TLK2* expression was higher in hepatocellular carcinoma than in normal hepatocytes. In recent study, it has been shown that inhibition of *TLK2* expression suppresses the progression of gastric cancer by inducing changes in the mTOR/ASNS axis (28). Our results implied that *TLK2* may represent a promising target for diagnosing and treating hepatocellular carcinoma. To verify the clinical relevance of *TLK2* high expression, we collected 374 tumor tissues and 50 normal tissues from TCGA. Our results showed that *TLK2* was significantly associated with sex, AFP, and clinical tumor stage. The correlation between *TLK2* and the progression of hepatocellular carcinoma was further strengthened.

On the basis of the clinical relevance of *TLK2*, we conducted multivariate and univariate analyses and found that *TLK2* could serve as an independent prognostic factor to predict the prognosis of hepatocellular carcinoma. The survival curve also indicated that patients with high *TLK2* expression had a significantly lower five-year survival rate

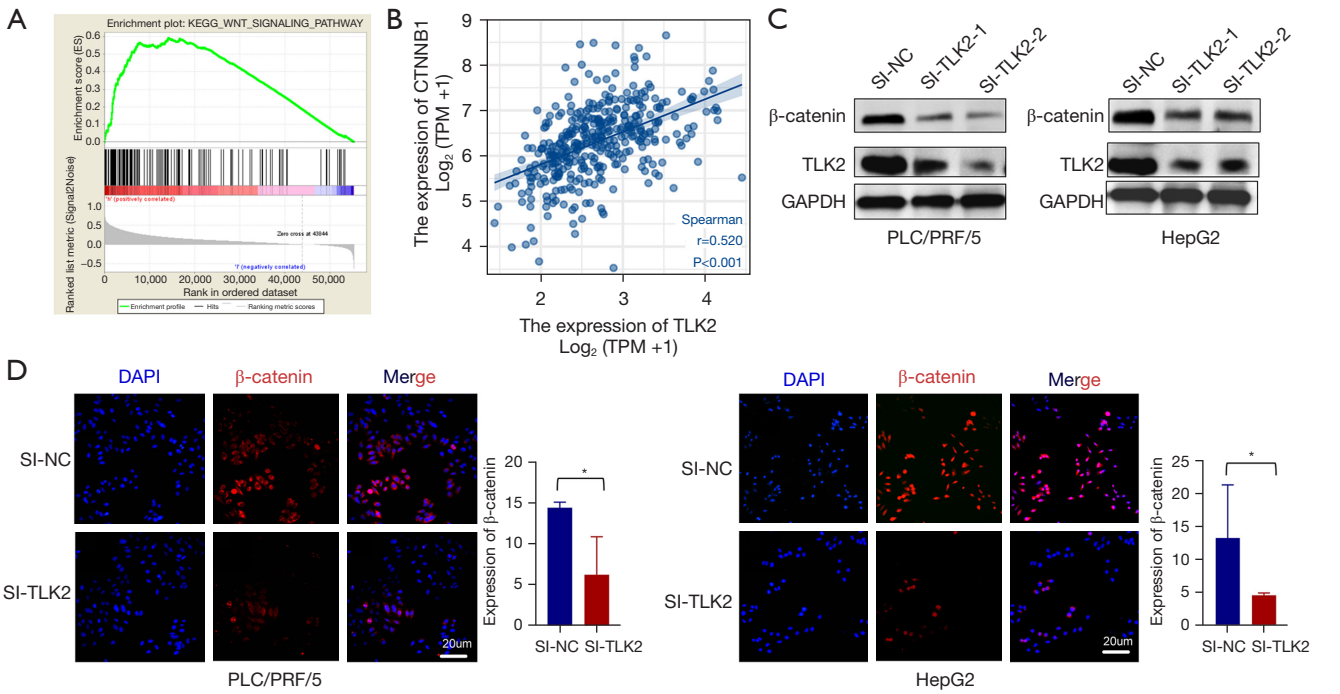


Figure 5 *TLK2* pathway enrichment analysis. (A) *TLK2* was enriched in the Wnt pathway. (B) *TLK2* showed a positive relationship with CTNNB1. (C) Western blotting was used to detect the relationship between *TLK2* and β-catenin. (D) Relationship between *TLK2* and β-catenin detected by immunofluorescence. Mean ± SD, n=3, PLC/PRF/5: P=0.04, HepG2: P=0.04. *, P<0.05. SD, standard deviation; KEGG, Kyoto Encyclopedia of Genes and Genomes; TPM, transcripts per million; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole.

than those with low *TLK2* expression, further highlighting the potential of *TLK2* as an independent prognostic factor for predicting hepatocellular carcinoma prognosis. Recent research has shown that *TLK2* plays a crucial role in promoting the development of breast cancer and glioblastoma. Kim *et al.* reported that *TLK2* amplifies and damages the G2-M checkpoint, leading to a defective G2-M checkpoint, delayed DNA repair process, and accumulated damage, promoting breast cancer development (16). Lin *et al.* found that *TLK2* overexpression via the SRC signaling pathway is a critical driver of glioblastoma (18). To investigate the role of *TLK2* in hepatocellular carcinoma progression, we carried out *TLK2* silencing and overexpression experiments and demonstrated that *TLK2* knockdown significantly suppressed hepatocellular carcinoma cell proliferation *in vitro*.

Numerous studies have shown that certain genetic mutations, such as TP53 (29), PTEN (30) and CTNNB1 (31) can disrupt cell growth and lead to uncontrolled growth tumor formation in hepatocellular carcinoma. Additionally, epigenetic alterations, including dysregulation of DNA methylation and histone modifications, may be involved in tumor suppression, cell cycle regulation, and expression of DNA repair genes that contribute to the development of hepatocellular carcinoma (32). To elucidate the molecular mechanism of *TLK2* knockdown-mediated hepatoma cell suppression, we first performed pathway enrichment analysis using TCGA data. We observed strong correlation between *TLK2* and cell cycle regulation, endocytosis, and deceleration. Notably, our analysis revealed a significant correlation between *TLK2* and the Wnt pathway, a crucial pathway involved in cell proliferation. It has been established that in healthy liver tissue, the Wnt/β-catenin pathway is typically inactive but can be reactivated during cell regeneration and in the context of various diseases and cancerous conditions, including hepatocellular carcinoma (8). Studies in recent years have identified the roles of target genes in promoting the development of hepatocellular carcinoma through the Wnt signaling pathway (33,34). Our study further revealed a strong correlation between *TLK2* and β-catenin. MTT and colony formation assays further validated that *TLK2* promotes the proliferation of

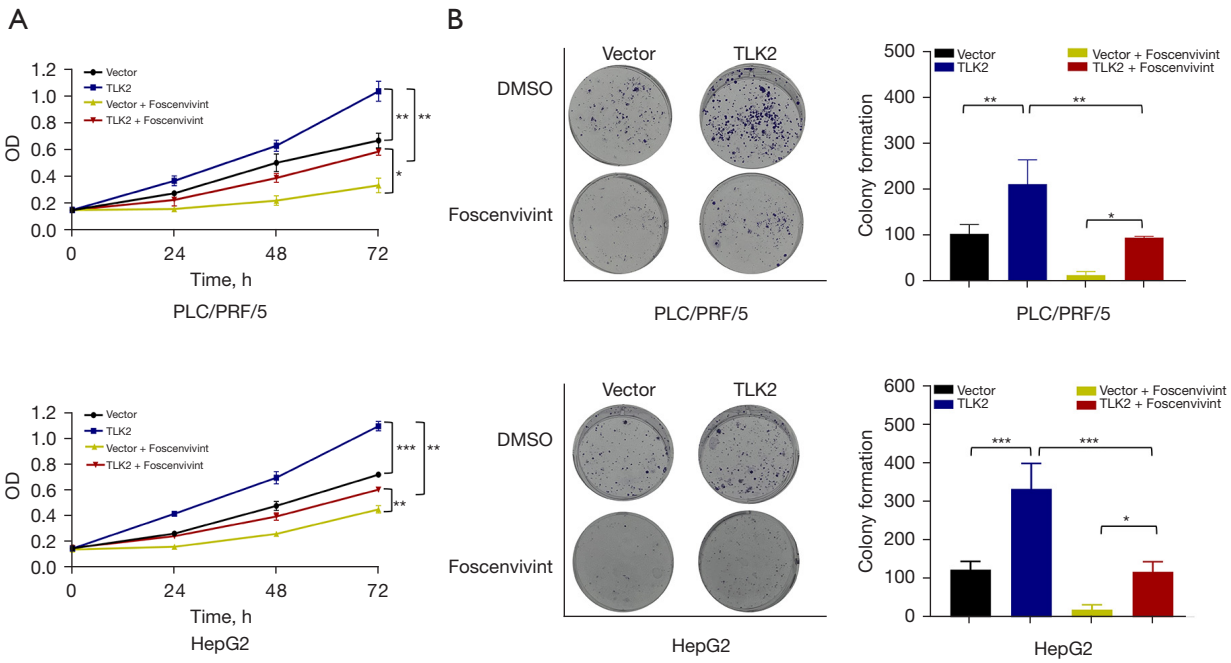


Figure 6 *TLK2* promotes hepatocellular carcinoma proliferation via β-catenin. (A) MTT assays were performed in PLC/PRF/5 and HepG2 cells transfected with Vector/*TLK2* and treated with the β-catenin inhibitor Foscenvivint for 24 hours. Mean ± SD, n=3, two-way ANOVA, PLC/PRF/5 Vector *vs. TLK2*: P=0.003, Vector + Foscenvivint *vs. TLK2* + Foscenvivint: P=0.03; HepG2 Vector *vs. TLK2*: P<0.001, Vector + Foscenvivint *vs. TLK2* + Foscenvivint: P=0.009. (B) Colony formation assays were performed in PLC/PRF/5 and HepG2 cells transfected with Vector/*TLK2*. After treatment with β-catenin inhibitor Foscenvivint for 24 hours, cells were stained with crystal violet for 30 min (×0.6). Mean ± SD, n=3, one-way ANOVA, PLC/PLF/5 vector *vs. TLK2*: P=0.007, vector + Foscenvivint *vs. TLK2* + Foscenvivint: P=0.03; HepG2 vector *vs. TLK2*: P<0.001, vector + Foscenvivint *vs. TLK2* + Foscenvivint: P=0.049. *, P<0.05; **, P<0.01; ***, P<0.001. MTT, methyl thiazolyl tetrazolium; SD, standard deviation; ANOVA, analysis of variance; OD, optical density; DMSO, dimethyl sulfoxide.

hepatocellular carcinoma through β-catenin.

This study's findings have several implications for hepatocellular carcinoma research and therapy. First, *TLK2* and the Wnt/β-catenin pathway represent promising targets for the development of new hepatocellular carcinoma treatments. Inhibition of *TLK2* or the Wnt/β-catenin pathway could slow down or halt hepatocellular carcinoma growth, providing a new avenue for drug development. Second, the study highlights the importance of understanding the molecular mechanisms underlying hepatocellular carcinoma growth, which could help identify new biomarkers for early diagnosis and prognosis. However, there are some limitations to the study that should be acknowledged. For example, the experiments were performed using only two hepatocellular carcinoma cell lines, and it is not clear if *TLK2* plays the same role in other hepatocellular carcinoma subtypes. Additionally, the study did not investigate the potential role of *TLK2* in hepatocellular carcinoma metastasis, which is a major contributor to hepatocellular carcinoma mortality.

Conclusions

In summary, the study provides compelling evidence that *TLK2* stimulates hepatocellular carcinoma growth through the activation of the Wnt/β-catenin pathway. These findings have important implications for hepatocellular carcinoma research and therapy and underscore the need for further investigations into the molecular mechanisms underlying hepatocellular carcinoma development and progression.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2264/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2264/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2264/coif). 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. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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