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TET1 Suppresses Hepatocellular Carcinoma Progression by Modulating the PI3K/Akt Signaling Pathways

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Abstract： Hepatocellular carcinoma (HCC) is a highly aggressive malignancy with a poor prognosis, underscoring the urgent need to identify novel therapeutic targets. The epigenetic regulator TET1, a key enzyme involved in active DNA demethylation, has been implicated in various cancers, however, its precise role in HCC remains controversial and poorly defined. This study demonstrates that TET1 is significantly upregulated in HCC tissues, and elevated TET1 expression is associated with advanced tumor stage, shorter overall survival and reduced disease-free survival in patients. Functional assays revealed that TET1 knockdown significantly suppressed HCC cell proliferation and induced apoptosis; it also triggered G1-phase cell cycle arrest. Mechanistically, we found that the oncogenic effects of TET1 are mediated through activation of the PI3K/Akt signaling pathway. In summary, our results establish TET1 as a critical promoter of HCC progression and elucidate its role in regulating the PI3K/Akt pathway. These findings highlight its value as both a prognostic biomarker and a potential therapeutic target in HCC.

Keywords: TET1；Hepatocellular Carcinoma；PI3K/Akt; Prognosis

Introduction

Among all malignancies, HCC ranks third in terms of mortality and sixth in terms of incidence worldwide[1]. Eighty to ninety percent of cases of primary liver cancer are HCC, the main histologic subtype[2]. With over 72.5% of new cases and 72.7% of deaths happening in the Asia-Pacific region, there are significant geographic disparities in the distribution of HCC, making it a significant public health concern in this region[3]. The majority of HCC patients are discovered at advanced stages because to its sneaky onset, and only a small percentage are eligible for curative therapy, which contributes to an overall dismal prognosis[4]. The complicated, multi-step process of hepatocarcinogenesis is fueled by the gradual accumulation of genetic and epigenetic changes. Together, these molecular mechanisms result in the activation of oncogenes, the inactivation of tumor suppressor genes, and eventually broad disruption of basic cellular functions[5]. HCC is a unique clinical problem due to its high rates of recurrence and mortality despite improvements in clinical care[6]. Finding useful molecular biomarkers is crucial for directing customized treatment plans because HCC is associated with significant biological heterogeneity and uncertain clinical outcomes. A recent clinicopathological study involving 96 HCC patients detected high expression of Vav1 protein in tumor tissues through immunohistochemistry, and its high expression was significantly associated with poor tumor differentiation, advanced TNM stage, and high recurrence rates [7]. Cox regression analysis further confirmed that Vav1 is an independent adverse prognostic factor affecting the 5-year overall survival rate, suggesting that Vav1 could serve as a potential prognostic biomarker for HCC and provide new evidence for postoperative recurrence risk assessment. Beyond clinicopathological indicators, multi-omics studies have revealed the association between metabolic abnormalities and prognosis in HCC. An integrated proteomic and transcriptomic analysis found that KIF5B is closely related to metabolic reprogramming in HCC, and both its protein and mRNA upregulation are significantly associated with poor prognosis[8]. Mechanistic studies showed that KIF5B activates the AKT/mTOR pathway and reprograms triglyceride metabolism (upregulating FASN and SCD1), promoting HCC cell

proliferation and migration. In vitro and in vivo knockdown of KIF5B significantly inhibited tumor growth, indicating its dual role as both a novel prognostic marker and a potential therapeutic target. In terms of treatment strategies, immune checkpoint inhibitors (ICI) combined with anti-angiogenic drugs have become the first-line standard treatment for advanced HCC. For example, the IMbrave150 trial demonstrated that atezolizumab + bevacizumab significantly prolonged median overall survival compared to sorafenib (19.2 vs. 13.4 months). Additionally, the HIMALAYA trial confirmed the survival benefit of durvalumab + tremelimumab. However, in the context of liver transplantation, although ICIs can be used for downstaging or postoperative recurrence control, they carry a risk of graft rejection, and their safety must be carefully evaluated with strategies such as drug washout and PD-L1 testing. Given that non-alcoholic fatty liver disease (NAFLD) has become a major cause of HCC in developed countries, a bioinformatics and machine learning study based on GEO and TCGA data identified CDT1 as a core gene in the transition from NAFLD to HCC[9]. Single-cell sequencing revealed significant differential expression of CDT1 during disease progression[9]. As a DNA replication licensing regulator, its abnormal expression can induce replication stress and malignant transformation. The study also found that the small-molecule compound AF615 targets the CDT1/Geminin complex to inhibit the tumor cell cycle, highlighting the broad prospects of CDT1 in early diagnosis and targeted therapy. In summary, the diagnosis and treatment of HCC are moving toward an era of molecular typing and precision intervention: Vav1 aids in prognostic stratification, KIF5B reveals mechanisms driven by metabolic dysregulation, CDT1 bridges the molecular link between NAFLD and HCC, and innovations in immunotherapy have significantly improved survival in advanced patients. These findings provide multidimensional solutions to overcome the high heterogeneity and prognostic uncertainty of HCC and lay a solid foundation for individualized treatment decisions.

DNA methylation is a major form of epigenetic modification that can significantly influence genomic stability, transcriptional activity, and developmental processes by

regulating gene expression without altering the DNA base sequence[10,11]. This process is primarily catalyzed by DNA methyltransferases (DNMTs), which use S-adenosylmethionine as the methyl donor to add a methyl group to the carbon-5 position of cytosine within CpG dinucleotides, forming 5-methylcytosine (5-mC). A key biological feature of DNA methylation is its reversibility, and the dynamic regulation of methylation status plays a critical role in cell differentiation, genomic imprinting, and environmental responses[12]. Cells maintain genomic methylation homeostasis through the coordinated actions of DNA methyltransferases and demethylases. Disruption of this balance can lead to various diseases, including cancer. DNA demethylation is a complex biological process, broadly categorized into active and passive mechanisms. Active demethylation involves the enzymatic removal or chemical modification of the methyl group from 5-mC, while passive demethylation occurs when the DNA methylation maintenance machinery fails, leading to gradual dilution of 5-mC through DNA replication over cell divisions. The TET family of dioxygenases, which comprise TET1, TET2, and TET3, plays a central catalytic role in DNA demethylation. TET proteins iteratively oxidize 5-mC to generate 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Both 5fC and 5caC can be recognized and excised by thymine DNA glycosylase (TDG), leading to active demethylation via the base excision repair (BER) pathway. Notably, dysregulated expression or function of TET1, one of the key 5-mC hydroxylases, is closely associated with the development and progression of multiple cancers[13-17]. In recent years, research on TET1 has increased, particularly regarding its role in stem cell maintenance[18-22]. However, the function of aberrantly expressed TET1 in human cancers remains poorly understood[23-26].

This study highlights the upregulation of TET1 in HCC cells and tissues, and delineates its critical role in promoting proliferation by modulating the PI3K/Akt pathway to suppress apoptosis. These findings establish TET1 as a promising molecular target worthy of further investigation for HCC treatment.

Materials and methods

Patients and specimens

A total of 8 pairs of HCC tissue samples and their adjacent non-tumor liver tissues were collected at Wanbei Coal Electric Group General Hospital. None of the enrolled patients had received chemotherapy or radiotherapy prior to surgery. Immediately after surgical resection, each tissue sample was divided into two portions: one was snap-frozen in liquid nitrogen and stored at -80 °C for subsequent RNA and protein extraction, and the other was fixed in 10% neutral-buffered formalin within 30 minutes and embedded in paraffin for histological analysis. The study was approved by the Ethics Committee of the General Hospital of Wanbei Coal-Electricity Group (Suzhou, China). All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee, the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients with HCC before sample collection.

Cell culture

The human HCC cell lines HepG2 and Huh-7, along with a normal human hepatocyte line, were obtained from the Central Laboratory of the Wanbei Coal-Electricity Group General Hospital. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL, Australia) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Australia), and cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Bioinformatics Analysis

TET1 expression was assessed using RNA-seq data via the TIMER 2.0 database （http://timer.cistrome.org/）. We investigated the differential expression of the UFM1 gene between tumor and adjacent normal tissues across all TCGA cancers, as well as its expression variations across different tumor stages. The gene expression profiles GSE29057 and GSE36376 for HCC were downloaded from the NCBI GEO database. Differentially expressed genes (DEGs) were identified from these two datasets using the "limma" package in R, with a significance threshold of $p < 0.05$ and $|\log FC| > 1$.

Survival curves for TET1-high and TET1-low groups were generated using the Gene Expression Profiling Interactive Analysis (GEPIA) database, with the median TET1 expression level as the cutoff. The distribution of gene expression levels is presented in box plots.

Cell Transfection and Treatment

Human TET1-specific siRNAs and a negative control siRNA (siNC) were purchased from Shanghai Gemma Gene. The targeting sequences were as follows:

TET1-si1: sense 5′-CCCACCUCCAGUCUUAAUATT-3′, antisense 5′-UAUUAAGACUGGAGGUGGGTT-3′

TET1-si2: sense 5′-GGGUGUUAUUCCUCAAGAUTT-3′, antisense 5′-AUCUUGAGGAAUAACACCCTT-3′

TET1-si3: sense 5′-GGGUGUCCAAUUGCUAAGUTT-3′, antisense 5′-ACUUAGCAAUUGGACACCCTT-3′

shNC: sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′-ACGUGACACGUUCGGAGAATT-3′

To knock down endogenous TET1, HuH-7 and HepG2 cells were transfected with 20 nM of the respective siRNA using Lipofectamine 2000 (Life Technologies, #11668-019), following the manufacturer's instructions. Subsequent experiments were conducted 48 hours post-transfection.

Western Blot Analysis

Proteins were extracted from cells or tissues using RIPA lysis buffer. The extracted protein samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with specific primary antibodies targeting the proteins of interest to form antigen-antibody complexes. After extensive washing to remove unbound antibodies, target proteins were detected using an enhanced chemiluminescence reagent. The resulting protein bands were visualized and analyzed using a Tanon 5200 system to determine the presence and relative abundance of the target proteins. The following primary antibodies were used: TET1

(ab191698, Abcam, 1:1000), PI3K (40225-1-Ig, Proteintech, 1:1000), p-PI3K (#4228, Cell Signaling Technology, 1:1000), Akt (#9272, Cell Signaling Technology, 1:1000), p-Akt (#4060, Cell Signaling Technology, 1:1000), and GAPDH (60004-1-Ig, Proteintech, 1:5000).

Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from samples using TRIzol reagent and subsequently reverse-transcribed into complementary DNA (cDNA). The resulting cDNA was combined with PCR primers, SYBR Green fluorescent dye, and other PCR reaction components. Amplification was performed under specific thermal cycling conditions, which involved repeated replication of the target DNA sequences through controlled temperature fluctuations. Fluorescence signals were detected and recorded during each PCR cycle. The relative quantity of the target RNA in the samples was analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) based on the obtained cycle threshold (Ct) values. The primer sequences used were as follows:

TET1: Forward 5'-ATCCTTGCTGTGTAGAGTTC-3',

Reverse 5'-AGTATTGGTGATATGCTTCCTAA-3'

GAPDH: Forward 5'-AACGGATTTGGTCGTATTGGG-3',

Reverse 5'-AGGGGCCATCCACACAGTCTTC-3'

All experiments were performed in triplicate to ensure reproducibility and statistical significance.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue sections were subjected to antigen retrieval using sodium citrate buffer to dissociate proteins, retrieve antigens, and enhance epitope exposure for efficient antibody binding. The sections were then blocked with bovine serum albumin (BSA) to prevent nonspecific antibody binding. Subsequently, the samples were incubated overnight with a primary antibody against TET1 (ab191698, Abcam; 1:1000 dilution). After thorough washing to remove unbound primary antibody, a fluorophore- or enzyme-conjugated secondary antibody was applied to amplify the signal. Finally, the stained tissue sections were examined

under a microscope, and the expression of the target protein was determined based on the staining intensity and cellular localization.

Cell Proliferation Assay

Control and transfected hepatocellular carcinoma cells were seeded in 96-well plates at a density of 1,000 cells per well. Cell viability was assessed at 1, 3, and 5 days post-transfection using a Cell Counting Kit-8 (CCK-8; Solarbio, CA1210). For the colony formation assay, 3,000 cells were plated in 6-well plates. After approximately 7 days of incubation, the formed colonies were fixed with 4% paraformaldehyde and subsequently stained with 0.01% crystal violet solution (Solarbio, China). The stained colonies were then quantified using ImageJ software. All experiments were performed in triplicate to ensure reliability and consistency.

Flow Cytometry Analysis

For apoptosis analysis, control and transfected hepatocellular carcinoma cells were collected and stained using an Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions. The stained cells were then analyzed using a flow cytometer (CytoFLEX, Beckman Coulter) to distinguish between viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cell populations.

For cell cycle analysis, control and transfected cells were fixed overnight in 70% ice-cold ethanol at 4°C. The fixed cells were then stained with a propidium iodide (PI) solution containing RNase, and the DNA content was quantified by flow cytometry.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software. Continuous data are presented as the mean ± standard deviation (SD), while categorical data are expressed as frequency counts and percentages. Comparisons between two groups were conducted using Student's t-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was applied, followed by the Student-Newman-Keuls test for post-hoc multiple comparisons. Transcriptomic and bioinformatic analyses were carried out using R software (version 4.5.1).

Results

Elevated TET1 Expression Is Associated with Clinicopathological Features in HCC.

This study systematically analyzed the expression patterns of the TET1 gene across multiple human cancers using public databases and experimental validation. Analysis of the TIMER database revealed that TET1 mRNA levels were significantly upregulated in various cancer tissues, including cholangiocarcinoma (CHOL), head and neck squamous cell carcinoma (HNSC), hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), and lung squamous cell carcinoma (LUSC). In contrast, TET1 expression was markedly downregulated in breast cancer (BRCA), kidney chromophobe carcinoma (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), and thyroid carcinoma (THCA) (Figure 1a).

Further analysis focused on LIHC demonstrated that TET1 mRNA expression was significantly higher in tumor tissues compared to normal adjacent tissues, as validated by TIMER database profiling (Figure 1b). Consistent with this, two independent GEO datasets (GSE12452 and GSE61228) confirmed the upregulation of TET1 mRNA in LIHC (Figure 1c, d). Notably, no significant change in TET1 expression was observed in cirrhotic tissues (Figure 1e). To validate the above findings at the protein level, we performed Western blot and immunohistochemistry experiments. The results showed that TET1 protein expression was also significantly upregulated in LIHC tumor tissues (Figure 1f, g), which is consistent with the findings at the mRNA level. Together, these results confirm the dysregulated expression of TET1 in HCC.

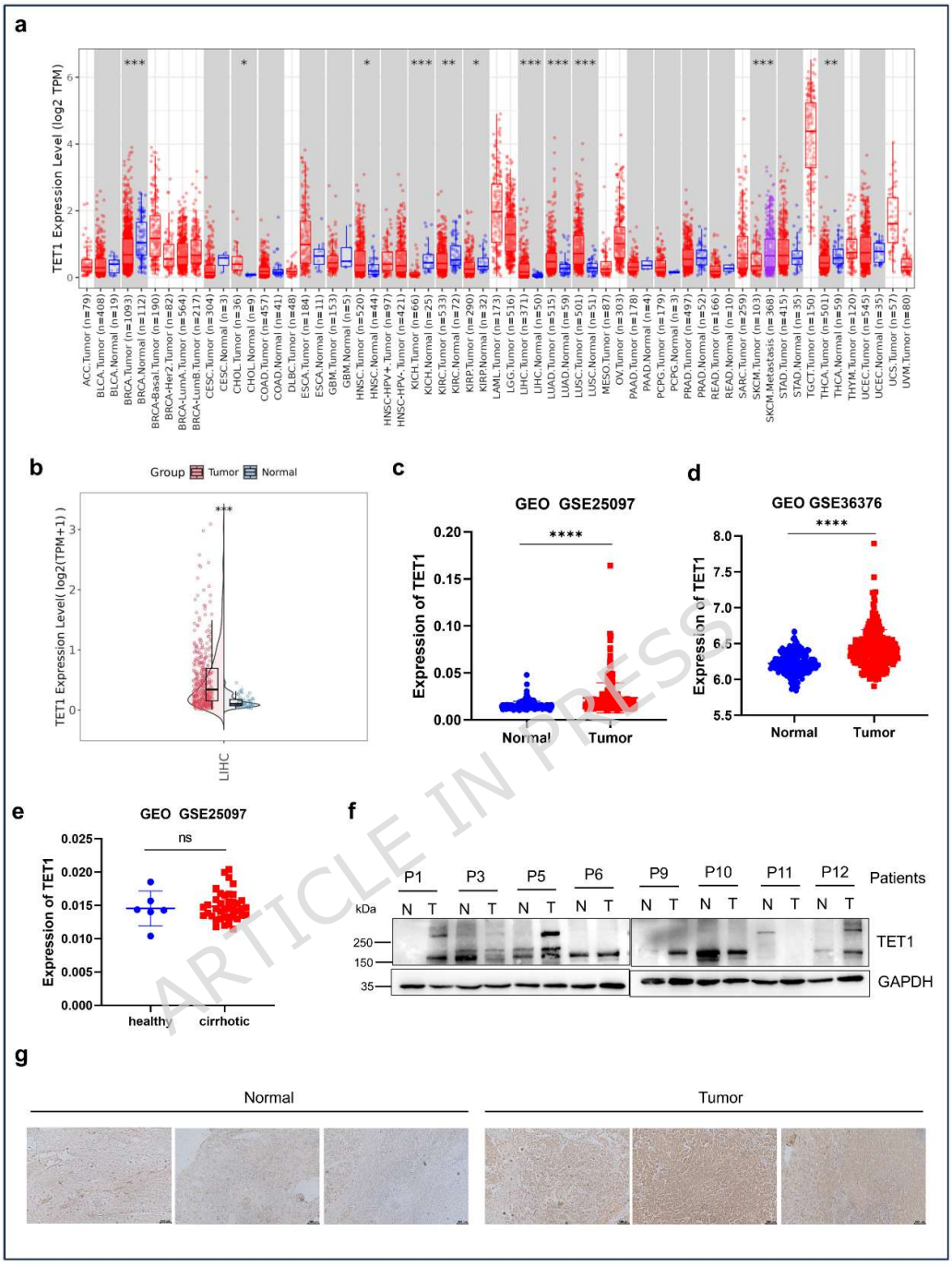

Figure 1 Elevated TET1 expression is associated with clinicopathological features in HCC.

(a) Differential TET1 expression across 33 human cancer types based on TIMER database analysis. (b) TET1 mRNA expression levels are significantly higher in LIHC tumor tissues compared to normal tissues. (c, d) Validation of TET1 mRNA upregulation in LIHC using the (c) GSE25097 and (d) GSE36376 datasets from the GEO database. (e) Analysis of the GSE36376 dataset shows no

significant difference in TET1 expression between cirrhotic and normal liver tissues. (f) Representative Western blot analysis of TET1 protein levels in tumor (T) and adjacent normal (N) tissues from eight HCC patients. (g) Immunohistochemical detection of expression in normal and cancerous tissues of liver cancer patients. Data are presented as mean ± SD;* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

Prognostic Value of TET1 Expression in HCC

This study systematically analyzed the expression characteristics and clinical significance of TET1 in LIHC by integrating data from the GEPIA, Kaplan-Meier Plotter, and TCGA databases. The results showed that the promoter methylation level of TET1 in LIHC tumor tissues was significantly higher than that in normal tissues (Figure 2a), and its expression level varied among patients with different TNM stages (Figure 2b). Survival analysis indicated that patients in the low TET1 expression group had significantly lower overall survival rates (Figures 2c, e, g) and disease-free survival rates (Figures 2d, f, h) compared to the high expression group, suggesting that low TET1 expression is closely associated with poor prognosis. Furthermore, ROC curve analysis demonstrated that TET1 has a certain diagnostic discriminative ability for LIHC (Figure i). In summary, the expression level of TET1 is significantly correlated with the progression and prognosis of LIHC and holds potential as a diagnostic and prognostic biomarker.

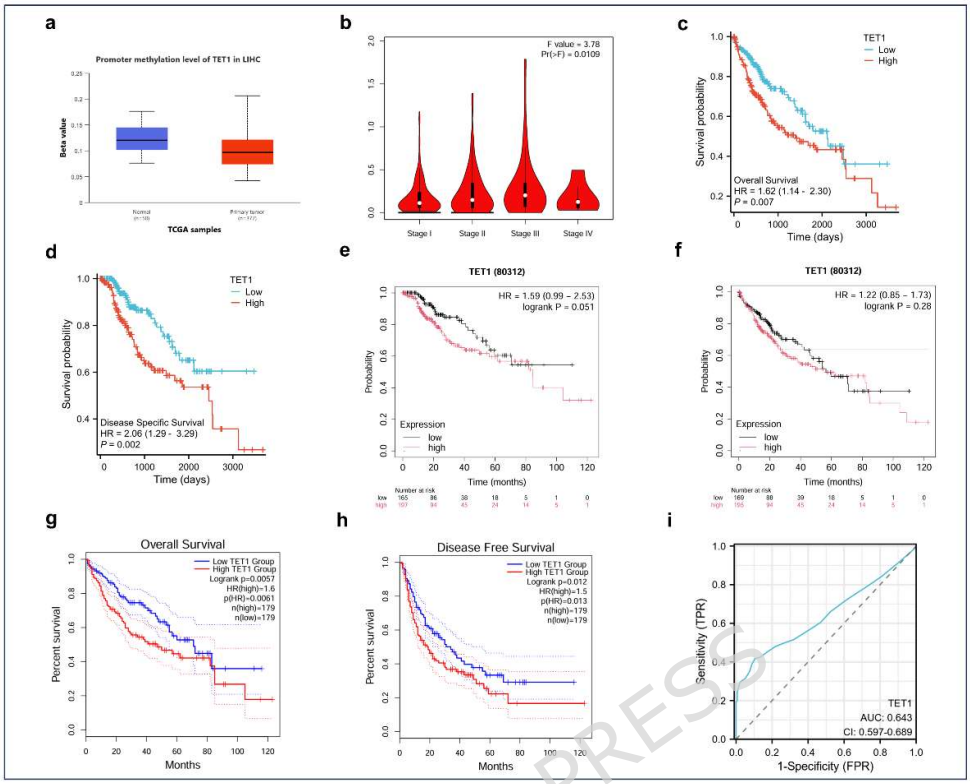

Figure 2 Prognostic value of TET1 expression in HCC. (a) The box plot shows the difference in promoter methylation levels of TET1 between LIHC tumor tissues and normal tissues. The association between TET1 expression and tumor stage. (b) The violin plot displays the expression levels of TET1 in LIHC patients across different TNM stages. Kaplan-Meier overall survival analysis of liver cancer patients stratified by high and low TET1 expression, based on TCGA data analyzed using GEPIA. (c, d) Survival curves based on the TCGA database show the differences in overall survival (c) and disease-free survival (d) between patients with low and high TET1 expression. (e, f) Kaplan-Meier Plotter survival curves illustrate the association between TET1 expression and patients' overall survival (e) and disease-free survival (f). (g, h) Survival curves based on the GEPIA database show the differences in overall survival (g) and disease-free survival (h) between patients with low and high TET1 expression. (i) The ROC curve evaluates the diagnostic efficacy of TET1 as a biomarker for LIHC, showing the area under the curve (AUC).

TET1 Modulates HCC Cell Proliferation

Based on the expression characteristics of TET1 in HCC, this study selected HuH-7 and HepG2 cell lines as experimental models and constructed TET1-knockdown

models using RNA interference technology. qPCR and Western blot analyses confirmed significant reductions in both TET1 mRNA and protein levels, validating the successful establishment of the gene-silencing model (Figure 3a-d). Functional assays demonstrated that TET1 silencing markedly impaired cell proliferation: CCK-8 assays revealed a decreased OD value in the experimental group compared to the control group at 72 hours post-transfection ($p < 0.001$) (Figure 3i, j). Colony formation assays further supported these findings, showing a significant reduction in clone formation efficiency in TET1-knockdown cells relative to controls (Figure 3e-h). These results collectively indicate that TET1 promotes tumor progression by regulating the proliferative capacity of HCC cells, providing experimental evidence for its role as a potential oncogenic factor. Notably, these findings align with TCGA-LIHC data showing TET1 upregulation in HCC patients but contrast with reports of TET1' s tumor-suppressive functions in other cancers, suggesting context-dependent roles of TET1 that warrant further investigation using animal models and epigenomic profiling.

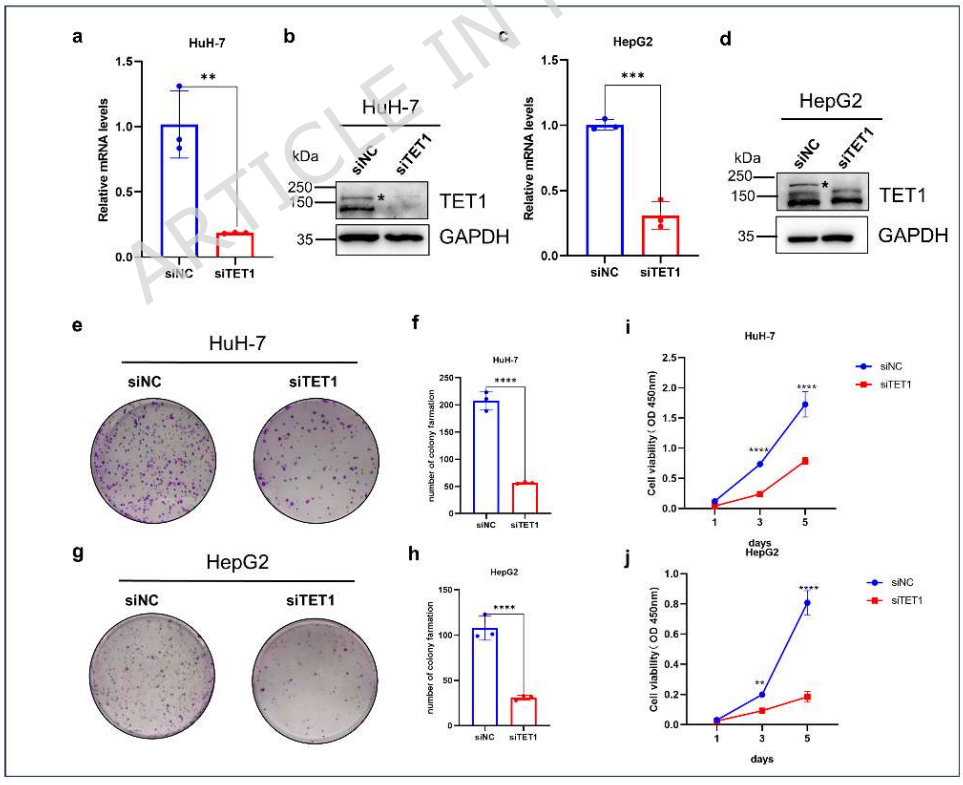

Figure 3 TET1 modulates HCC cell proliferation. (a-d) Efficiency of TET1 knockdown was

confirmed at both the mRNA and protein levels by (a, c) qRT-PCR and (b, d) Western blot analysis in HuH-7 (a, b) and HepG2 (c, d) cells, respectively. (e-h) Colony formation assays demonstrating the suppressive effect of TET1 silencing on the proliferative capacity of (e, f) HuH-7 and (g, h) HepG2 cells. Representative images (e, g) and corresponding quantitative analyses (f, h) are shown. (i, j) CCK-8 assays further confirming the inhibitory effect of TET1 knockdown on the proliferation of (i) HuH-7 and (j) HepG2 cells. Data are presented as mean ± SD; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

TET1 Modulates Cell Cycle and Apoptosis in HCC

Previous studies have suggested the potential impact of cell death on proliferation[27,28]. In this study, we further investigated the effects of TET1 knockdown on the biological behavior of HCC cells using flow cytometry and cell cycle analysis. The results demonstrated that TET1 silencing significantly increased the apoptosis rate in both HuH-7 and HepG2 cells compared to control groups (Figure 4a-d), indicating that TET1 may promote HCC cell survival by suppressing apoptosis. Concurrently, cell cycle analysis revealed that TET1 knockdown induced pronounced G1-phase arrest in both cell lines (Figure 4e-h). These findings suggest that TET1 depletion not only enhances apoptosis but also impedes cell cycle progression by inducing G1-phase arrest, further validating the oncogenic role of TET1 in HCC and providing a theoretical basis for its potential as a therapeutic target.

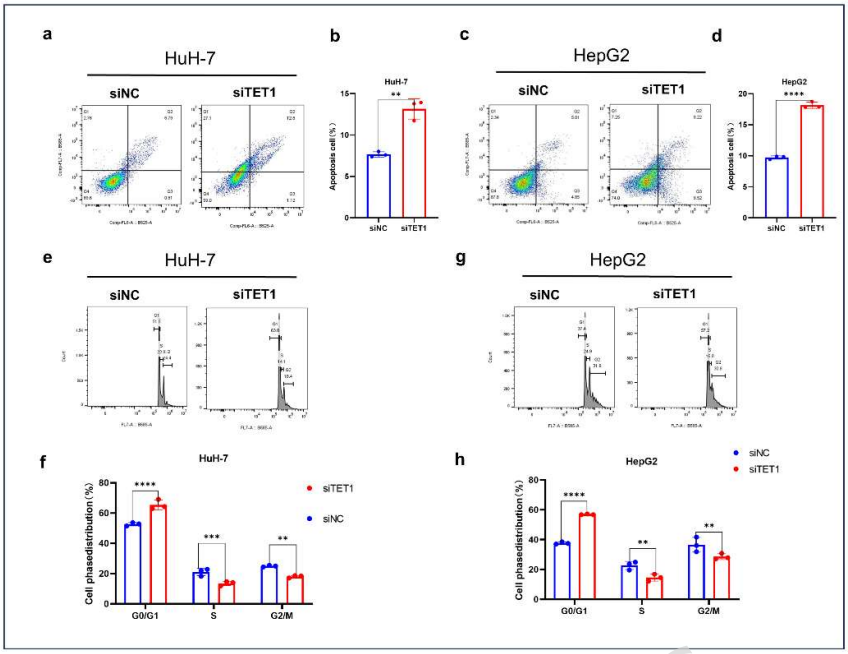

Figure 4 TET1 modulates cell cycle and apoptosis in HCC. (a–d) Apoptosis analysis by flow cytometry showing the effect of TET1 silencing on apoptosis rates in (a, b) HuH-7 and (c, d) HepG2 cells. Representative flow cytometry dot plots (a, c) and the corresponding quantitative analysis (b, d) are presented. (e-h) Cell cycle analysis by flow cytometry demonstrating the impact of TET1 silencing on the cell cycle progression of (e, f) HuH-7 and (g, h) HepG2 cells. Representative histograms (e, g) and the statistical summary (f, h) are shown. Data are presented as mean ± SD;$^{**}p < 0.01$; $^{****}p < 0.0001$.

TET1 regulates the proliferative activity of HBV-positive hepatocellular carcinoma Hep3B cells.

To validate the specific role of TET1 in HBV-associated HCC, this study conducted a series of experiments in the HBV-positive hepatocellular carcinoma cell line Hep3B. First, Western blot analysis revealed that compared to the normal hepatocyte line L02, TET1 protein expression was significantly upregulated in Hep3B cells (Figure 5a). Subsequently, a TET1 knockdown Hep3B cell model was successfully established by transfecting specific siRNA. Validation via qPCR and Western blot confirmed that both mRNA and protein expression levels of TET1 were significantly reduced in the siTET1 transfection group compared to the negative control group (siNC) (Figures 5b, c). To

investigate the function of TET1 in HBV-positive HCC cells, cell proliferation assays were performed. CCK-8 results showed that after 72 hours of transfection, the proliferative activity of cells in the TET1-silenced group was significantly lower than that of the control group ($p < 0.01$) (Figure 5d). This suggests that TET1 plays an important role in maintaining the survival of HBV-positive hepatocellular carcinoma cells.

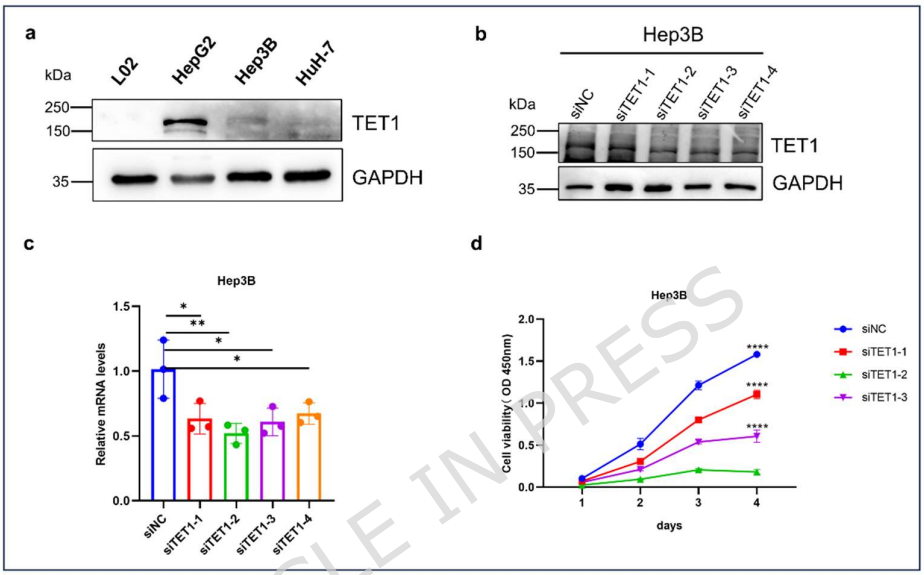

Figure 5 TET1 Promotes HBV-Associated Hepatocellular Carcinoma Proliferation. (a) Western blot analysis of TET1 expression levels in L02 (normal hepatocyte line) and Hep3B (HBV-positive hepatocellular carcinoma cell line), with GAPDH used as an internal reference control. (b, c) Knockdown efficiency of TET1 in Hep3B cells was validated by qPCR and Western blot. Compared with the negative control group (siNC), both mRNA and protein expression levels of TET1 were significantly reduced in the siTET1-transfected group. (d) CCK-8 assay results showed that the proliferative capacity of Hep3B cells was significantly inhibited after TET1 knockdown. Data are presented as mean ± SD; *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

TET1 Silencing Suppresses HCC Growth In Vivo by Activating the PI3K/Akt/mTOR Signaling Pathway

At both clinical and cellular levels, this study has confirmed that silencing TET1 significantly inhibits the growth of HCC cells. To further investigate the molecular

mechanisms underlying HCC, based on previous sequencing results of hepatocytes and HCC cells, volcano plot analysis revealed 4887 upregulated genes and 4578 downregulated genes (Figure 6a). We performed functional enrichment analysis on these differentially expressed genes (DEGs) to identify the biological processes and pathways potentially affected in HCC. GO analysis indicated that these DEGs were primarily enriched in pathways related to cell growth and the Wnt signaling pathway (Figure 6b). To verify whether TET1 functions through the Wnt pathway, we knocked down TET1 expression in HCC cells and examined the expression changes of key Wnt signaling molecules, β-catenin and c-Myc. The results showed that β-catenin protein levels remained largely unchanged, while c-Myc expression significantly decreased, suggesting that TET1 regulation of HCC cell growth may not depend on the canonical Wnt signaling pathway (Figures 6c-d). Furthermore, KEGG pathway analysis revealed that HCC-related DEGs were significantly enriched in important signaling pathways such as PI3K/Akt (Figure 6e). We first validated the correlation between TET1 and key molecules of the PI3K/Akt pathway (PI3K, Akt, PTEN, mTOR) using TCGA database analysis. The results showed that TET1 expression was positively correlated with PI3K, Akt, and mTOR, but negatively correlated with PTEN (Figures 6f-i). Subsequently, we knocked down TET1 expression in HCC cells and examined changes in the expression of key PI3K/Akt pathway proteins and their downstream molecules mTOR and phosphorylated mTOR (p-mTOR). The results demonstrated that TET1 knockdown significantly reduced mTOR phosphorylation levels and inhibited the activation of the PI3K/Akt signaling pathway (Figures 6j-k), suggesting that TET1 may regulate the PI3K/Akt pathway by modulating mTOR phosphorylation status. To further clarify whether TET1 regulation of the PI3K/Akt pathway depends on its DNA demethylase activity, we treated HCC cells with the TET enzyme activity-specific inhibitor Bobcat339 (an α-ketoglutarate competitive inhibitor) to inhibit the catalytic activity of endogenous TET family proteins, including TET1, and observed its effects on the PI3K/Akt pathway. Western blot analysis revealed that phosphorylation levels of p-Akt and its downstream key protein p-mTOR were significantly reduced in the inhibitor-

treated group (Figures 6l-m). These findings suggest that TET1 may participate in regulating the PI3K/Akt/mTOR signaling pathway in an enzyme activity-dependent manner, thereby influencing the growth of HCC cells.

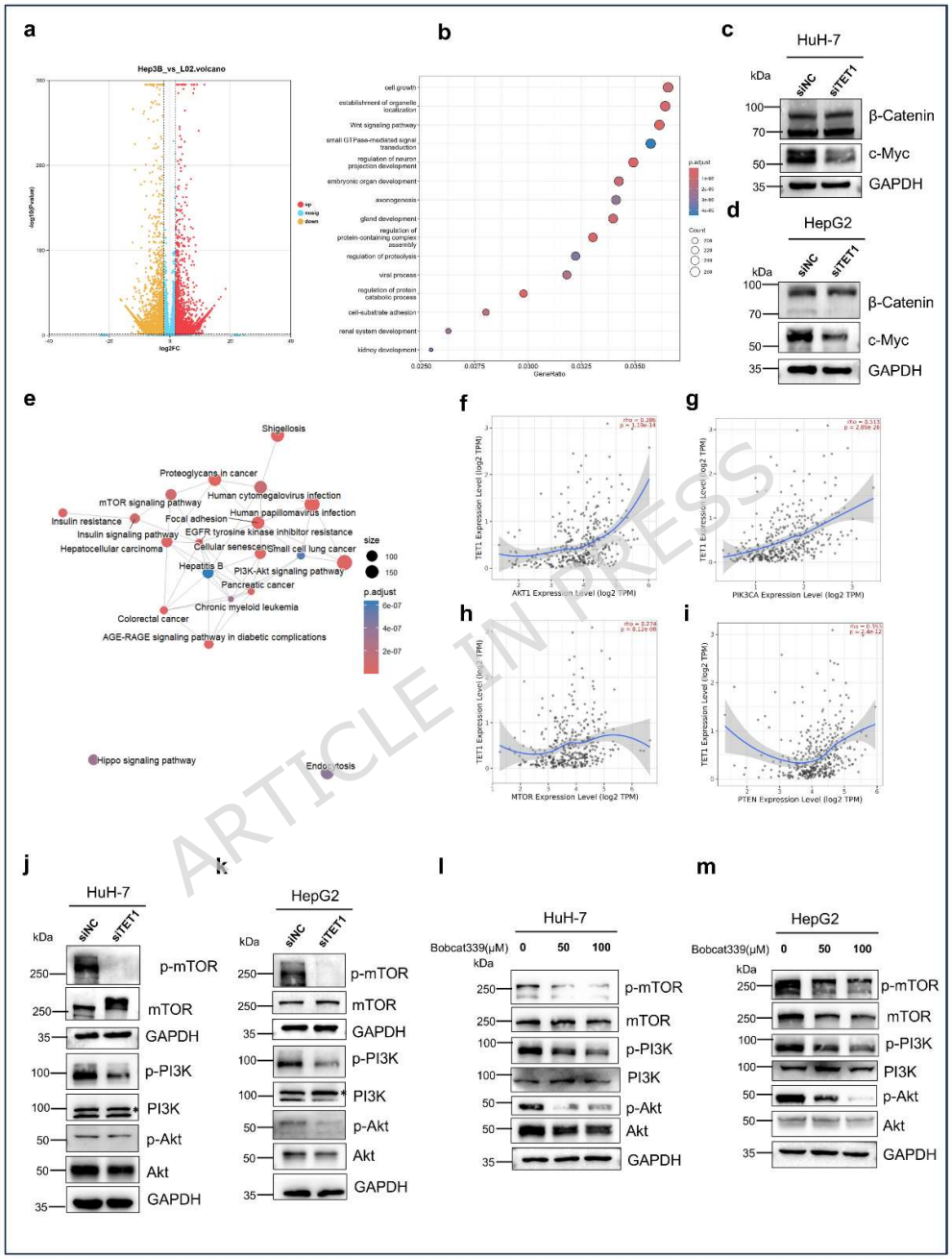

Figure 6 TET1 silencing suppresses HCC growth by activating the PI3K/Akt/mTOR signaling pathway. (a) RNA sequencing (RNA-seq) analysis comparing human hepatocyte cells and HCC cell

lines. Blue dots represent downregulated genes, red dots represent upregulated genes. (b) Functional enrichment analysis of differentially expressed genes from the RNA-seq data using Gene Ontology (GO) databases. (c, d) Western blot analysis of key components of the Wnt signaling pathway in (c) HuH-7 and (d) HepG2 cells following TET1 knockdown. (e) Functional enrichment analysis of differentially expressed genes from the RNA-seq data using Kyoto Encyclopedia of Genes and Genomes (KEGG) databases[29-31], revealing the biological functions associated with TET1. (f-i) Based on TCGA database analysis of the correlation between TET1 and AKT1, PIK3CA, MTOR, and PTEN. (j, k) Western blot analysis of key components of the PI3K/Akt/mTOR signaling pathway in (j) HuH-7 and (k) HepG2 cells following TET1 knockdown. (l, m) Western blot analysis of key components of the PI3K/Akt/mTOR signaling pathway in HuH-7 (l) and HepG2 (m) cells treated with the TET enzyme activity-specific inhibitor Bobcat339 to inhibit endogenous TET family proteins.

Discussion

Through integrated bioinformatic analysis, clinical sample validation, and in vitro functional experiments, this study systematically elucidated the biological functions and molecular mechanisms of TET1 in HCC progression. We found that TET1 was significantly upregulated in HCC tissues and cell lines, and its high expression correlated with advanced tumor stage and poor patient prognosis. Functionally, silencing TET1 effectively suppressed HCC cell proliferation, induced apoptosis, and triggered G1-phase cell cycle arrest. Mechanistically, the oncogenic role of TET1 depended on its activation of the PI3K/Akt signaling pathway. Additionally, our study suggested that TET1 might influence other oncogenes through epigenetic regulation, potentially playing a distinct role in HBV-associated HCC[32,33]. These findings not only reveal the critical role of TET1 in HCC but also provide a theoretical basis for its potential as a therapeutic target.

Analyses of independent databases consistently demonstrated that TET1 mRNA and protein levels were significantly higher in HCC tissues than in adjacent normal tissues, corroborating our Western blot and immunohistochemistry data. Notably, TET1 expression progressively increased from stage I to stage III but declined slightly at stage

IV, suggesting functional complexity in advanced or metastatic HCC. Survival analysis further confirmed that high TET1 expression was associated with shorter overall and disease-free survival, indicating its potential as an independent prognostic biomarker in HCC.

However, TET1's expression patterns and functions exhibit notable tissue specificity across cancer types. For instance, TET1 has been reported to act as a tumor suppressor in ovarian and cervical cancers[25,34], whereas it demonstrates clear oncogenic properties in HCC[16,23,35]. Such discrepancies may arise from differences in tissue microenvironments, TET1-interacting proteins, or specificity in downstream signaling pathways. Therefore, the context-dependent nature of TET1 must be carefully considered when evaluating its clinical utility for prognosis or targeted therapy.

Gene set enrichment analysis revealed that the differentially expressed genes in hepatocellular carcinoma cells are significantly enriched in the PI3K/Akt signaling pathway. Western blot experiments further confirmed that TET1 silencing reduced phosphorylation levels of PI3K and Akt, identifying TET1 as a key upstream activator of this pathway. As a central regulator of cell survival, proliferation, and metabolism, aberrant PI3K/Akt activation is closely linked to chemotherapy resistance, invasion, metastasis, and poor prognosis in HCC[36-39]. Our study is the first to directly connect the epigenetic modifier TET1 with the PI3K/Akt signaling axis in HCC, offering new insights into the epigenomic-signaling network cross-talk in this malignancy.

The regulatory mechanism of TET1 on the PI3K/Akt pathway may involve multiple indirect processes. On one hand, TET1, as a DNA hydroxymethylase, might directly activate transcription of positive regulators (or suppress negative regulators like PTEN) via demethylation. On the other hand, non-enzymatic functions or unidentified protein interactions could contribute to this regulation. These possibilities represent important directions for future research. In HBV-associated HCC, TET1's role is particularly noteworthy. Studies suggest that the hepatitis B virus X protein (HBx) may interact with TET1, indirectly upregulating oncogenes and driving HCC pathogenesis. This implies that in viral hepatitis-related HCC, TET1 might be "hijacked" by viral

proteins, serving as an epigenetic bridge to carcinogenesis. Furthermore, vitamin C, a potential enhancer of TET family activity, has been shown to activate TET2 (a TET1 homolog) to induce cGAS expression and vascular normalization, thereby enhancing anti-tumor immunity. Whether vitamin C supplementation or similar interventions could modulate HCC progression by regulating TET1 activity is an intriguing hypothesis worthy of further exploration[40].

TET1' s functional influence likely extends beyond a single pathway. For example, in nasopharyngeal carcinoma, TET1 has been reported to inhibit the Wnt/β-catenin signaling pathway and cancer cell invasion by promoting demethylation of Wnt antagonist promoters[41-43]. To investigate whether TET1 also functions through this pathway in HCC, we performed TET1 gene silencing experiments in hepatocellular carcinoma cells HuH-7 and HepG2. Western blot analysis showed that TET1 knockdown did not significantly alter β-catenin protein levels. This finding contrasts with the observed inhibitory effect of TET1 on the Wnt/β-catenin pathway in nasopharyngeal carcinoma. Combined with our previous results — that TET1 knockdown significantly suppressed the activation of the PI3K/Akt/mTOR pathway while β-catenin levels remained unchanged—this further confirms that the tumor-promoting function of TET1 in hepatocellular carcinoma cells primarily relies on the PI3K/Akt signaling axis, rather than the canonical Wnt/β-catenin pathway. This result highlights the complexity and tissue specificity of TET1's biological functions, indicating that in different cancer microenvironments, TET1 may regulate malignant phenotypes through distinct downstream pathways.

Our findings hold clear clinical translational potential. The overexpression of TET1 in HCC and its regulatory effect on key oncogenic pathways position it as a promising therapeutic target. Developing specific TET1 inhibitors, or combining them with existing PI3K/Akt-targeted agents, could offer new strategies for patients with advanced or drug-resistant HCC. Simultaneously, monitoring serum levels of TET1-related markers might aid in early diagnosis or treatment response assessment.

However, this study also has certain limitations. While we have comprehensively

validated the prognostic value of TET1 through multiple public cohorts, prospective validation within institutional clinical cohorts will further enhance its clinical applicability. We have planned future collaborations with clinical partners to verify these findings in both retrospective and prospective cohorts of hepatocellular carcinoma patients. The specific molecular details of how TET1 regulates the PI3K/Akt pathway-whether through direct binding or via intermediate molecules-have not yet been fully elucidated. Finally, we observed potential differences in the effects of TET1 between in vitro and in vivo experiments, suggesting that TET1 may indirectly exert its influence by affecting the tumor microenvironment (e.g., immune cell infiltration), which represents an important direction for future research.

In summary, this study confirms that TET1 acts as a significant oncogenic factor in hepatocellular carcinoma. It promotes tumor cell proliferation and inhibits apoptosis by activating the PI3K/Akt/mTOR signaling pathway, and its high expression is associated with poor patient prognosis. Additionally, TET1 may play a special role in HBV-associated HCC. This discovery not only deepens the understanding of the epigenetic regulatory mechanisms in HCC but also lays a solid theoretical foundation for developing novel targeted therapeutic strategies against TET1 and its downstream pathways. Future research should focus on elucidating the specific details of the TET1 regulatory network and exploring its potential applications in clinical diagnosis and treatment.

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Availability of data and materials

All relevant data supporting the findings of this study are included within the manuscript and its Supplementary material File. Other raw data generated during this study that are reasonably requested are available from the corresponding author upon request.

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Ethics declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

This study was conducted in strict accordance with the Declaration of Helsinki and relevant Chinese ethical guidelines. The research protocol involving human samples was reviewed and approved by the Ethics Committee of Wanbei Coal-Electricity Group General Hospital (Approval No.: [WBZY-LLWYH-2022-001]).

Consent for publication

The authors confirm that they have obtained consent for publication from all individuals whose personal data or images are included in this manuscript, and that these individuals are aware that their data will be made publicly available. The authors declare that there are no known financial or personal conflicts of interest that could have influenced the research reported in this manuscript.