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Formiminotransferase Cyclodeaminase Suppresses Hepatocellular Carcinoma by Modulating Cell Apoptosis, DNA Damage, and Phosphatidylinositol 3-Kinases (PI3K)/Akt Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1,2,3 **Jiajia Chen***
BCDEF 4 **Zemian Chen***
BCD 2 **Zhentian Huang**
BCD 5 **Hongrong Yu**
AEF 1,3 **Yanbing Li**
AEFG 1,3 **Wenhua Huang**

1 National Key Discipline of Human Anatomy, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, P.R. China
2 Department of General Surgery, Affiliated Chaozhou Central Hospital, Southern Medical University, Chaozhou, Guangdong, P.R. China
3 Guangdong Engineering Research Center for Translation of Medical 3D Printing Application, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, P.R. China
4 Department of Medical Oncology, Affiliated Chaozhou Central Hospital, Southern Medical University, Chaozhou, Guangdong, P.R. China
5 Department of Human Anatomy, School of Preclinical Medicine, Guangxi Medical University, Nanning, Guangxi, P.R. China

* Jiajia Chen and Zemian Chen contributed equally to this work

Corresponding Author: Wenhua Huang, e-mail: huangwenhua2009@139.com, Yanbing Li, e-mail: hnlybup001@163.com

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Background: Formiminotransferase cyclodeaminase (FTCD) is a candidate tumor suppressor gene in hepatocellular carcinoma (HCC). However, the mechanism for reduced expression of FTCD and its functional role in HCC remains unclear. In this study, we explored the biological functions of FTCD in HCC.

Material/Methods: The expression and clinical correlation of FTCD in HCC tissue were analyzed using TCGA (The Cancer Genome Atlas) and a cohort of 60 HCC patients. The MEXPRESS platform was accessed to identify the methylation level in promoter region FTCD. CCK-8 assay and flow cytometry analysis were used to explore the proliferation, cell apoptosis proportion, and DNA damage in HCC cells with FTCD overexpression. Western blot analysis was performed to identify the downstream target of FTCD.

Results: FTCD is significantly downregulated in HCC tissues and cell lines. Low FTCD expression is correlated with a poor prognosis ($P<0.001$) and an aggressive tumor phenotype, including AFP levels ($P=0.009$), tumor size ($P=0.013$), vascular invasion ($P=0.001$), BCLC stage ($P=0.024$), and pTNM stage ($P<0.001$). Bioinformatics analysis indicated promoter hypermethylation can result in decreased expression of FTCD. FTCD overexpression suppressed cell proliferation by promoting DNA damage and inducing cell apoptosis in HCC cells. FTCD overexpression resulted in increased level of PTEN protein, but a decrease in PI3K, total Akt, and phosphorylated Akt protein in HCC cells, suggesting involvement of the PI3K/Akt pathway.

Conclusions: FTCD acts as a tumor suppressor gene in HCC pathogenesis and progression and is a candidate prognostic marker and a possible therapeutic target for this disease.

MeSH Keywords: **Apoptosis • Carcinoma, Hepatocellular • DNA Damage • Glutamate Formimidoyltransferase • Phosphatidylinositol 3-Kinases**

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Background

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide [1]. Epidemiological data indicate that there are approximately 750 000 new cases of liver cancer diagnosed globally per year [2].The vast majority of HCC occurs from chronic liver disease due to type B or C hepatitis virus infection, alcohol abuse, and nonalcoholic steatohepatitis [3]. Thus, the poor parenchymal reserve of patients increases the challenge of treating patients with HCC. Although nonsurgical treatment platforms and standardization of liver transplant have improved, the 5-year survival rate is only about 26% in the United States [4]. Recent studies suggested that the loss of certain tumor suppressors and the deregulation of numerous signaling modules such as cell differentiation (WNT, Hedgehog, Notch), cell growth factor signaling (IGF, EGF, PDGF), and angiogenesis (VEGF) may play critical roles in HCC development and progression [5]. Therefore, identifying new molecules involved in the development and progression of HCC might accelerate the understanding of the mechanism involved in the development and progression of HCC, as well as providing new drug targets for HCC treatment.

Formiminotransferase cyclodeaminase (FTCD) is expressed in every mammalian cell type, but, interestingly, is most highly accumulated in the liver [6]. FTCD contains 2 active sites (FT and CD) at different positions on the protein structure, and catalyzes histidine degradation during the folate metabolism process [7]. Furthermore, in addition to participating in the metabolic process, FTCD is also associated with Golgi complex [8]. Recently, using a CRISPR-Cas9-based screen, FTCD was found to be associated with chemotherapeutic drug sensitivity. The deletion of the genes in the histidine degradation pathway markedly decreased sensitivity to methotrexate, meaning that dietary supplementation of histidine can increase flu x through the histidine degradation pathway and could enhance sensitivity to methotrexate [9]. In HCC, FTCD is significantly downregulated in tumor tissues and can serve as a useful diagnostic biomarker to distinguish early HCC from benign tumors [10]. However, the detailed molecular function of FTCD in tumors remains unclear and its downstream pathways still must be comprehensively determined.

In the present study, we determined via the Cancer Genome Atlas (TCGA) data set and our case cohort that FTCD expression was decreased in HCC and negatively correlated with disease progression. Notably, we found the abnormal expression of FTCD was due to the high methylation level in the promoter region. *In vitro* experiments showed a tumor-suppressive role of FTCD. The overexpression of FTCD inhibited the proliferation of HCC cells and promoted cell apoptosis and DNA damage. Furthermore, activation of the PI3K/Akt pathway was decreased after FTCD overexpression. These findings indicate that FTCD suppresses tumor progression in HCC and could be a potential new target for therapeutic interventions against HCC.

Material and Methods

Human tissue samples

This study was reviewed and approved by the Medical Ethics Committee of Nanfang Hospital of Southern Medical University, and the tissue samples were used with the informed written consent of the patients, according to the Declaration of Helsinki. The study comprised 60 pairs of randomly selected paraffin-embedded HCC tissue samples and paired non-tumor samples from patients who underwent resection of liver cancer without preoperative chemotherapy or radiotherapy. All samples were histologically confirmed by 2 independent pathologists.

Cell lines

HL-7702, Huh7, BEL-7402, SNU449, and SK-Hep1 cell lines were purchased from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). HL-7702, BEL-7402, Huh7, and SK-Hep1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco), and SNU449 cells were cultured in RPIM1640 medium (Gibco). Cells were incubated with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Immunohistochemical staining

Paraffin-embedded tissue samples were sliced into 4-μm sections, which were then routinely processed for immunohistochemistry. After incubation with an antibody against FTCD (1: 100; Proteintech, Rosemont, IL, USA), the sections were stained with diaminobenzidine in the Envision System (Dako, Carpinteria, CA, USA). FTCD protein expression in 60 paired tissue samples was classified into a positive group and a negative group based on staining intensity and extent, as previously described [11].

Gene expression datasets

A cohort from the TCGA Liver Hepatocellular Carcinoma (TCGA-LIHC) dataset (*https://portal.gdc.cancer.gov*) was used to analyze the expression pattern of FTCD and its correlation with clinical features. The database includes 442 HCC patients with FTCD expression based on RNA-Seq. It also contains 51 pairs of HCC and normal liver tissues. The fold change of FTCD expression in the HCC samples compared with that in the paired normal samples were calculated as previously described [12]. In addition, FTCD DNA methylation profile was analyzed using the MEXPRESS (*http://mexpress.be*) platform.

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De-methylation treatment

Cells were treated with growth medium containing 10 μM of 5-Aza (Sigma, St Louis, MO, USA) for 72 h. The growth medium and drug were replaced daily. Control cells were treated in parallel with DMSO agent.

Cell transfection

Cells were seeded into 6-well plates at a density of 3×10^5 cells per well and transfected with the pc-DNA3.1(+)-FTCD plasmid (GenePharma, Shanghai, People's Republic of China) or vector control when they reached 70–80% confluence using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Cells were cultured for 48 h before further experiments.

Real-time RT-PCR assay

Total RNA was extracted with RNAiso (TaKaRa, Tokyo, Japan) and then reverse transcribed to cDNA with PrimeScript RT Master Mix (TaKaRa) according to the manufacturer's instructions. The relative FTCD expression, normalized to expression of the endogenous control GAPDH, was determined by qPCR assay using the SYBR Premix Ex Taq II Kit (TaKaRa). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were: FTCD forward: 5'-GCG TGT TTG GCG CAT ATT-3' and reverse: 5'-GAT GGT GGA TCT GGT CCT TAA A-3'; GAPDH forward: 5'-AAG AAG AUG CGG CUG ACU GUC-3' and reverse: 5'-CAG UCA GCC GCA UCU UCU UUU-3'.

Western blot analysis

Total protein was extracted using RAPI (Beyotime, Shanghai, People's Republic of China) buffer mixed with the protease inhibitor phenylmethanesulfonyl fluoride (Beyotime). Equivalent protein was separated by 10% sodium-dodecyl-sulfate-polyacrylamide-gel electrophoresis (Fdbio Science, Hangzhou, People's Republic of China) and electro-transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% bovine serum albumin and incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish-peroxidase-labeled secondary antibody (1: 10 000; Proteintech). Immunodetection was performed using an ECL substrate kit (Millipore). The primary antibodies were: anti-FTCD rabbit polyclonal antibody (1: 5000; Proteintech), anti-PTEN rabbit polyclonal antibody (1: 1000; Cell Signaling Technology, Boston, MA, USA), anti-Akt rabbit polyclonal antibody (1: 1000; Cell Signaling Technology), anti-phospho-Akt-^{Ser473} (pAkt) rabbit polyclonal antibody (1: 1000; Cell Signaling Technology), anti-PI3K rabbit polyclonal antibody (1: 2000; Cell Signaling Technology), and anti-GAPDH rabbit polyclonal antibody (1: 10 000; Proteintech).

CCK-8 assay

Cells were seeded into 96-well plates at a density of 3×10^3 cells per well. CCK-8 solution (Dojindo Laboratories, Osaka, Japan) (10 μl/well) was added at 0, 24, 48, 72, and 96 h after adherence by using CCK-8 (Solarbio, Beijing, People's Republic of China) according to the manufacturer's protocol. Absorbance was recorded at 450 nm with a microplate reader.

Flow cytometric analysis

Cells (1×10^6) were trypsinized and resuspended into single-cell suspensions. They were separately treated with BrdU In-Situ Detection Kit (BD Biosciences, San Jose, CA, USA), Cell Apoptosis Detection Kit (KeyGEN, Nanjing, China) and Alexa Fluor® 647 Mouse anti-H2AX reagent (BD Biosciences) according to manufacturer's instructions. Stained cells were analyzed with a flow cytometer, and the data were analyzed and plotted using FlowJo software (Tree Star, Inc, Ashland, OR, USA).

Statistical analysis

Data from 3 independent experiments were presented as mean ±SEM. GraphPad Prism 7.0 (GraphPad Software, Inc, La Jolla, CA, USA) or IBM SPSS Statistics 20.0 (IBM, Armonk, IL, USA) was utilized to perform statistical analysis. All statistical analyses were performed using the Pearson chi-squared test, 2-tailed *t* test, or analysis of variance (ANOVA), as appropriate. $P<0.05$ (2-tailed) was considered statistically significant.

Results

Decreased expression of FTCD correlates with the progression of HCC

To identify the role of FTCD in HCC, we searched the transcriptomic data from the TCGA-LIHC data set and found that the mRNA levels of FTCD in patients with HCC were significantly downregulated in the tumor samples relative to the levels in normal liver tissues. We found that 92.0% (46/50) of the paired normal samples had an average 2.88-fold increased expression of FTCD compared with tumor samples (Figure 1A, 1C). In addition, the distribution of FTCD mRNA levels was significantly decreased in the patients with advanced T stage ($P<0.001$, Figure 1D) or poor survival time ($P<0.001$, Figure 1E). We also found that FTCD was downregulated in HCC cells compared to normal liver cell line HL-7702 (Figure 1F, 1G).

To validate our bioinformatic analysis findings, we analyzed FTCD expression by IHC in a cohort containing 50 pairs of HCC/non-tumor tissues. Results showed that the FTCD protein located in the cytoplasm of HCC tissues and its expression

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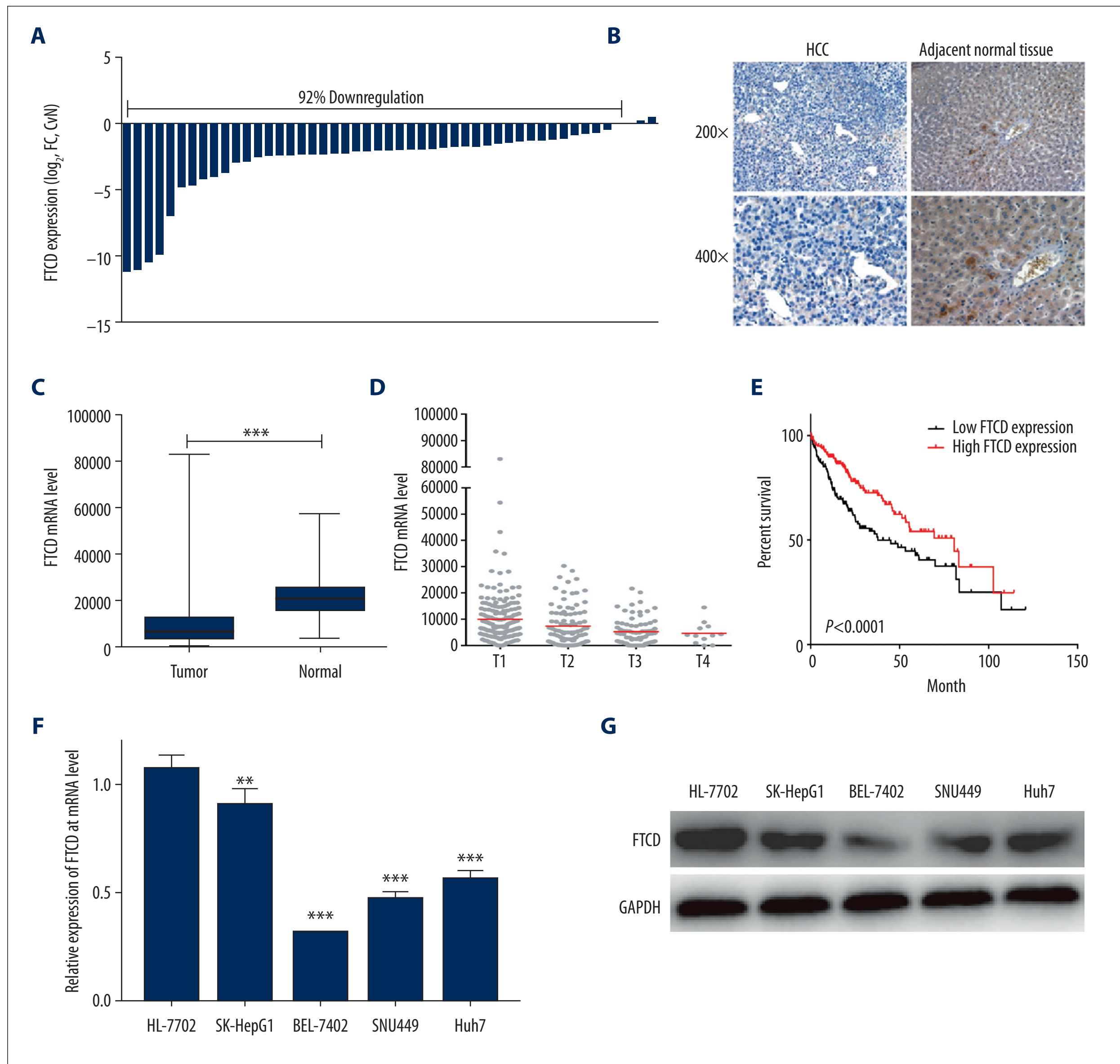

Figure 1. Decreased expression of FTCD contributes to the progression of HCC. (**A**) FTCD mRNA relative expression in HCC and normal samples from the TCGA-HCC data set. Data presented as $\log_2$ (cancer/non-cancer). (**B**) FTCD mRNA expression in HCC and normal samples from the TCGA-HCC data set. (**C**) Representative images of immunohistochemical staining against HCC in 60 tumor/normal matched sample pairs. (**D**) Correlation analysis of FTCD mRNA expressing and T stage based on the TCGA-HCC data set. (**E**) Kaplan-Meier survival analysis of patients with HCC based on the TCGA-HCC data set. (**F**) qRT-PCR analysis of FTCD mRNA expression in normal liver cells and 4 HCC cells. (**G**) Western blot analysis of FTCD protein expression in normal liver cells and 4 HCC cells. All experiments were performed in triplicate and results are expressed as means ±SD. ** *P*<0.01, *** *P*<0.001.

levels was significantly lower in HCC tissues than in adjacent normal liver tissues (*P*<0.05, Figure 1B).

To clarify the clinical significance of FTCD in HCC development, we assessed the correlation between FTCD expression and clinicopathological parameters of patients with HCC. As shown in Table 1, positive FTCD expression was strongly associated with low AFP levels (*P*=0.009), smaller tumor size (*P*=0.013), absence of vascular invasion (*P*=0.001), early BCLC stage (*P*=0.024), and early pTNM stage (*P*<0.001). Collectively, these findings strongly suggest that FTCD is downregulated in HCC, and its abnormal expression might contribute to disease progression.

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Table 1. Correlation between the FTCD protein expression and the clinicopathological parameters.

Variable	Cases	FTCD protein expression		χ^2 value	*P* value
		Positive	Negative		
Sex				0.080	0.778
Male	49	29	20		
Female	11	6	5		
Age				0.017	0.896
<60 years	48	21	27		
≥60 years	12	5	7		
Serum HBsAg				3.135	0.077
Negative	11	7	4		
Positive	49	17	32		
AFP				6.898	**0.009**
<400 μg/L	22	14	8		
≥400 μg/L	38	11	27		
Tumor size				6.193	**0.013**
≤5 cm	26	16	10		
>5 cm	34	10	24		
Tumor number				2.891	0.089
Solitary	51	27	24		
Multiple	9	2	7		
Histological grade				4.470	0.093
Well	16	10	6		
Moderate	31	16	15		
Poor	13	3	10		
Vascular invasion				11.760	**0.001**
Absent	50	38	12		
Present	10	2	8		
BCLC stage				7.493	**0.024**
0–A	41	26	15		
B	9	3	6		
C	10	2	8		
pTNM stage				14.689	**0.000**
I/II	45	34	11		
III/IV	15	3	12		

HBsAg – hepatitis B surface antigen; AFP – α-fetoprotein; pTNM – pathological Tumor Node Metastasis; BCLC – Barcelona Clinic Liver Cancer.

Promoter methylation contributes to the decreased expression of FTCD in HCC

To uncover the mechanism involved in the decreased FTCD expression in HCC, we analyzed the methylation status of FTCD in 374 tumor tissues and 40 normal tissues via MEXPRESS, a web tool for visualization of the DNA methylation based on the TCGA data on a single-gene level. Figure 2A shows the whole methylation profile of FTCD. Significantly, 5 CpG sites had greater hypermethylation in the HCC samples than in the normal tissues ($P<0.001$, Figure 2A). A notable negative correlation between DNA methylation and FTCD transcription was also observed ($r=-0.394$, $P<0.001$, Figure 2B). To evaluate the methylation status of FTCD *in vitro*, BEL-7402 and SNU449 cells

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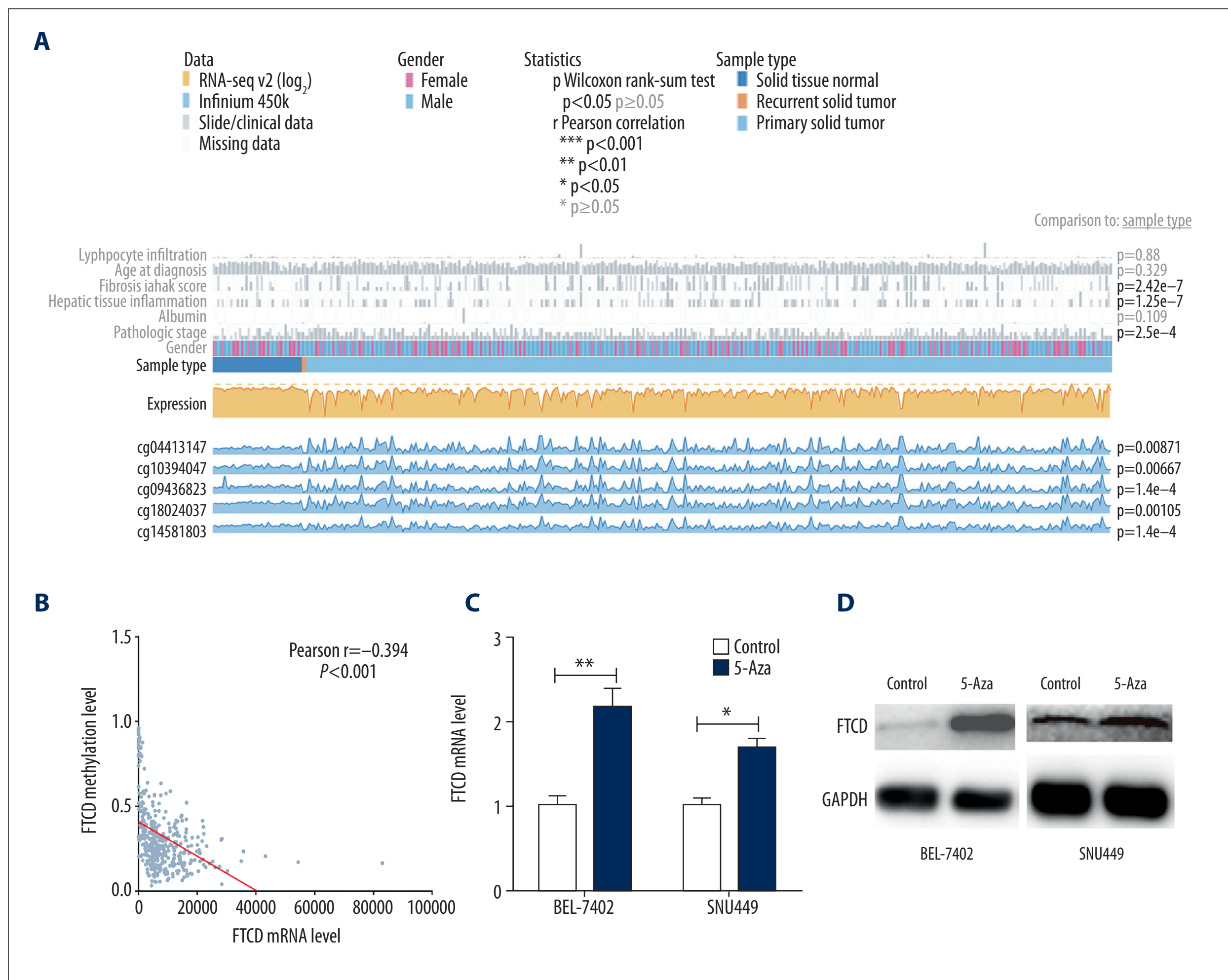

Figure 2. Promoter hypermethylation is correlated with the decreased expression of FTCD. (**A**) Visualization of the TCGA-HCC data for FTCD in HCC using MEXPRESS. Data were reordered by sample type. (**B**) FTCD mRNA levels were negatively correlated with DNA methylation in the TCGA-HCC data set. (**C, D**) qRT-PCR and Western blot analysis of FTCD expression in BEL-7402 and SNU449 cells treated with 10 μM 5-Aza for 72 h. All experiments were performed in triplicate and results are expressed as means ±SD. * *P*<0.05, ** *P*<0.01.

were treated with 5-Aza, an inhibitor of DNA methyltransferase, and the results indicated that both the FTCD mRNA and protein levels were significantly increased in the 5-Aza-treated group compared with the levels in the control group (*P*<0.001, Figure 2C, 2D). Therefore, the results indicated that the promoter region of FTCD was hypermethylated, which might, in part, lead to the decrease in FTCD expression in HCC.

Overexpression of FTCD inhibits HCC cell proliferation

To investigate the potential biological role of FTCD expression in the development and progression of HCC, we next performed cell function studies in HCC cells. After detecting FTCD expression in HCC cell lines (Figure 3A, 3B, 3E, 3F), we developed BEL-7402 and SNU499 cell lines with FTCD overexpression. By performing CCK-8 assay during a consecutive time segment, we discovered that FTCD overexpression reduced the proliferative capacity of HCC cells compared with that of parallel cell lines containing the empty vector (Figure 3C, 3G). BrdU assay with flow cytometry analysis was also performed to evaluate the proportion of proliferating cells and revealed that the proportion of BrdU-positive cells decreased in FTCD-overexpressing cells (Figure 3D, 3H).

Overexpression of FTCD induces cell apoptosis and promotes DNA damage in HCC

To discover the underlying molecular mechanism by which FTCD inhibits HCC cell proliferation, we analyzed differences in cell apoptosis rates after FTCD overexpression by flow cytometry analysis. The results showed that FTCD overexpression induced more apoptotic BEL-7402 and SNU499 cells compared to the control cells (Figure 4A, 4C).

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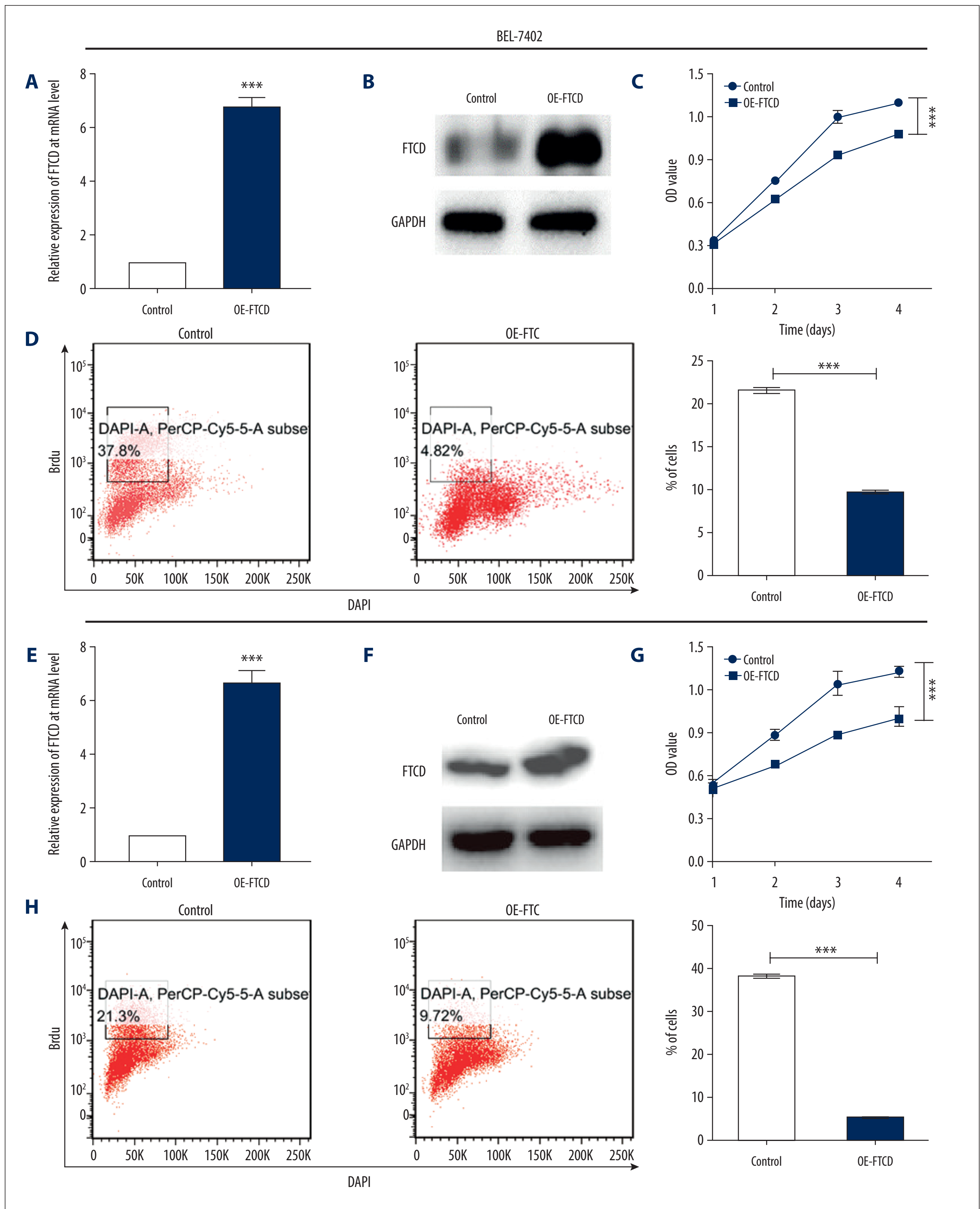

Figure 3. Overexpression of FTCD inhibits cell proliferation in HCC. (**A, E**) qRT-PCR verified the overexpression of FTCD in BEL-7402 and SNU449 cells. (**B, F**) Western blot analysis verified the overexpression of FTCD in BEL-7402 and SNU449 cells. (**C, G**) Cell viability analysis of BEL-7402 and SNU449 cells with FTCD overexpression. (**D, H**) Flow cytometric analysis of BrdU-labeled BEL-7401 and SNU449 cells with FTCD overexpression. All experiments were performed in triplicate and results are expressed as means ±SD. *** *P*<0.001.

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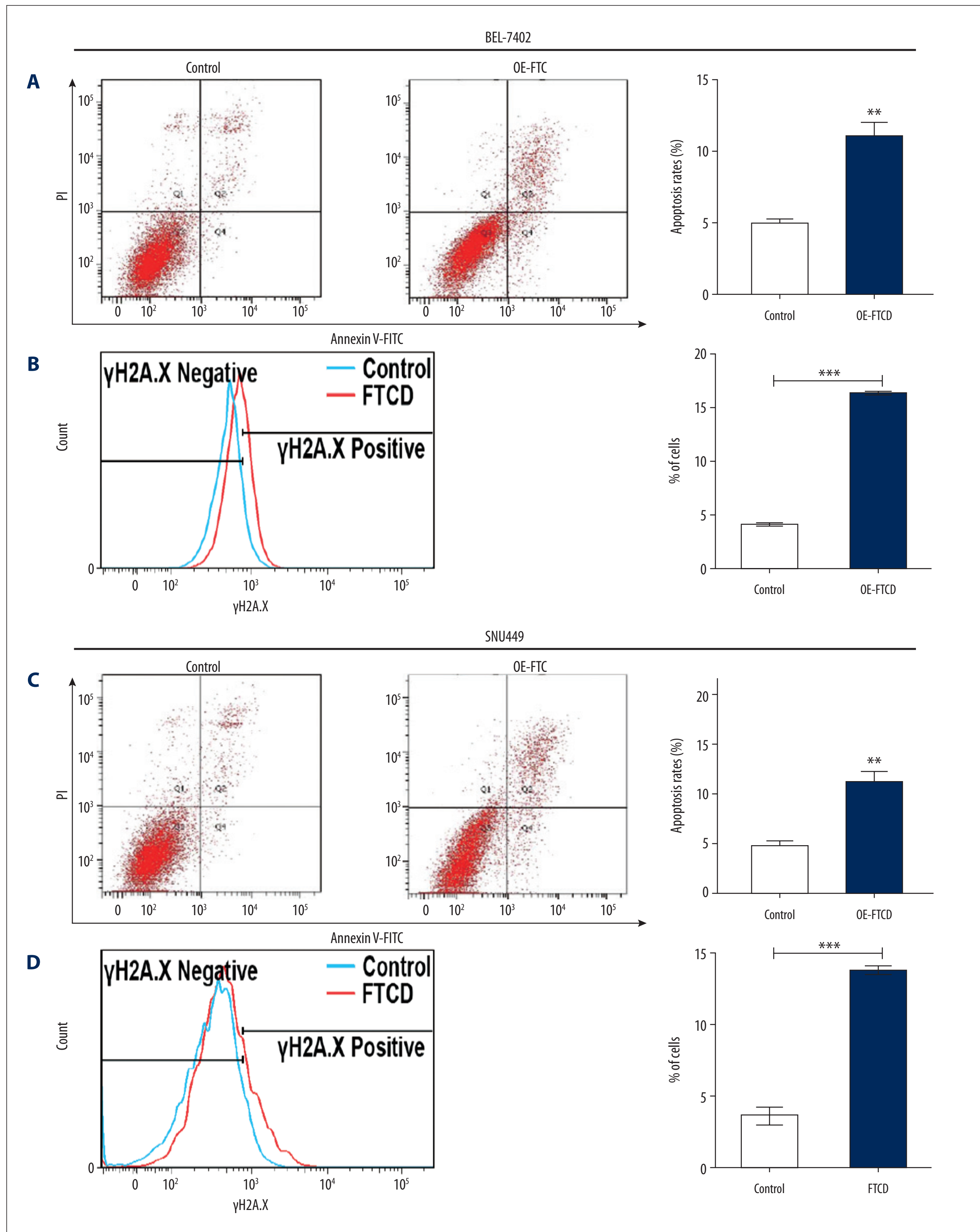

Figure 4. Overexpression of FTCD induces cell apoptosis and leads to DNA damage in HCC. (**A, C**) Flow cytometric analysis of cell apoptosis rates of BEL-7401 and SNU449 cells with FTCD overexpression. (**B, D**) Flow cytometric analysis of γ-H2AX-labeled BEL-7402 and SNU449 cells with FTCD overexpression. All experiments were performed in triplicate and results are expressed as means ±SD. ** *P*<0.01, *** *P*<0.001

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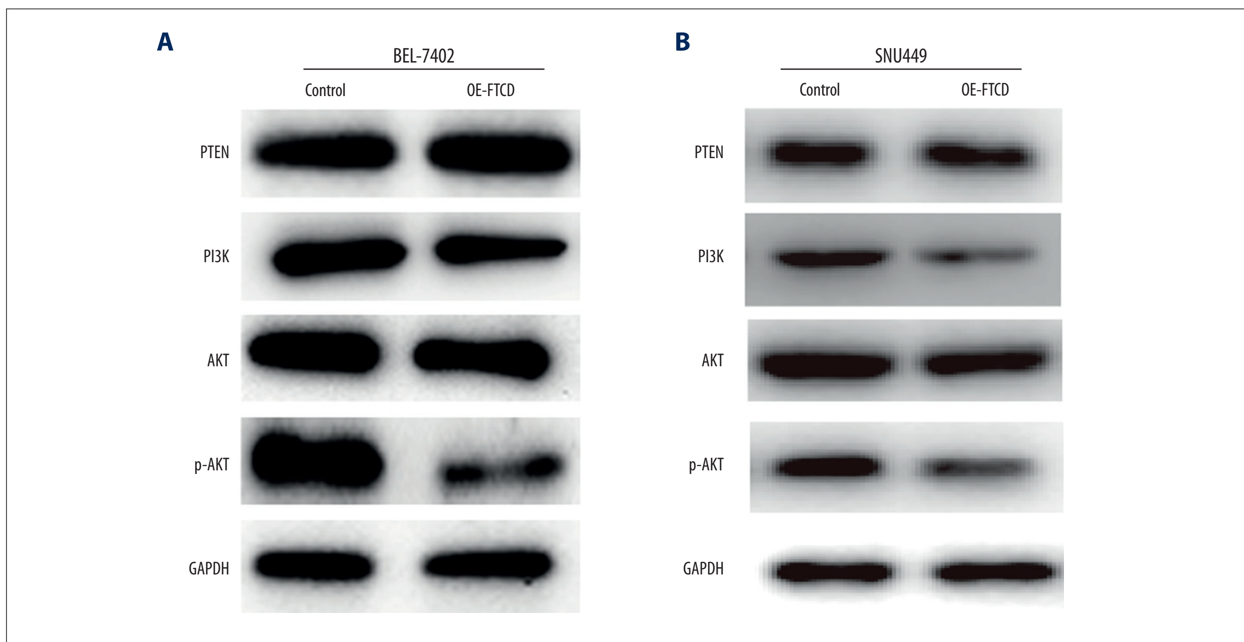

Figure 5. Overexpression of FTCD suppressed the activity of PI3K/Akt pathway signaling. (**A, B**) Western blot analysis of PI3K/Akt pathway-related proteins PTEN, PI3K, total Akt, and phosphorylated Akt expression in BEL-7402 and SNU449 cells with FTCD overexpression. (**C, D**) Relative expression of PI3K/Akt pathway-related proteins was quantified relative to the density of GAPDH. All experiments were performed in triplicate and results are expressed as means ±SD. ** $P<0.05$, ** $P<0.01$, *** $P<0.001$

Because cell apoptosis might be induced by DNA damage [13], flow cytometry analysis was performed on HCC cells with γ-H2AX-staining. The results showed that FTCD-overexpressing HCC cells had more γ-H2AX-positive cells than control cells (Figure 4B, 4D). Taken together, these data clearly show that FTCD inhibits cell proliferation by eliciting cell apoptosis and DNA damage.

Overexpression of FTCD reduces PI3K/Akt pathway signaling

The signaling pathway by which FTCD may promote cell proliferation has not been shown. Previous studies have shown that the PI3K/Akt pathway can maintain the function of Golgi apparatus [14]; therefore, we assessed whether there was a link between FTCD and the PI3K/Akt pathway. We found that FTCD overexpression resulted in an increase in expression of PTEN protein, but a marked loss of PI3K, total Akt, and phosphorylated Akt protein in BEL-7402 and SNU499 cells (Figure 5A, 5B). These data suggest that the anti-tumorigenic effects of FTCD in HCC occur via the PI3K-Akt pathway.

Discussion

Hepatocellular carcinoma (HCC) is a common malignant tumor worldwide [1,2]. Due to lack of typical symptoms, people with HCC are rarely diagnosed at the early stage, and most of them lose the opportunity for surgery [15]. Therefore, early diagnosis would significantly prolong the survival time and improve the quality of life of patients with HCC, and many researches have sought new molecular biological markers and therapeutic methods for HCC. In recent years, with the development of high-throughput sequencing technology, the technology of proteome analysis has become widely used in scientific research. Previous studies found that the expression level of FTCD was downregulated in HCC via proteome analysis [16,17]. In addition, Yu et al. found that FTCD was a downstream target of HIF-1α and the crosstalk between FTCD and HIF signaling promotes the progression of HCC [18]. FTCD had been shown to have an important role in the occurrence and development of HCC.

However, the exact mechanism by which FTCD expression is decreased in HCC remains unclear. Therefore, in this study, we conceived and designed *in vivo* and *in vitro* experiments to investigate the mechanism of action of FTCD in HCC. After analyzing the relationship between the FTCD expression and the clinicopathologic characteristics of HCC, we discovered that the expression levels of FTCD were negatively correlated with AFP levels, tumor size, vascular invasion, and pTNM stage, and higher expression of FTCD is associated with higher differentiation of HCC. Consistent with previous results, the IHC results indicated that FTCD expression was higher in the non-tumor tissues than in HCC tissues. This means that HCC patients

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with overexpression of FTCD have better prognosis and longer survival time. Consequently, FTCD could be used as a biological marker for HCC prognosis, which was in line with previous reports [10,19].

DNA methylation is one of the common modification methods that occur during genetic replication and transcription, contributing to gene expression, cell proliferation, and cell division, as well as the development of some gene-related diseases and cancers [20,21]. Fei et al. revealed that the methylation of p53 induced by SETDB1 promotes HCC cancer cell growth [22]. There is substantial evidence that DNA methylation plays an important role in the progression of HCC [23,24]; therefore, we further explored the relationship between the expression level of FTCD and DNA methylation. After researching and analyzing the TCGA-LIHC data set, we discovered higher methylation levels in the promoter region of FTCD for HCC tissues than in the non-tumor tissues. Then, a demethylation drug (5-Aza) [25] was used to knock-down the methylation levels in the BEL-7402 and SNU449 cells. Consistent with the TCGA-LIHC data set, the methylation levels were negatively associated with the expression level of FTCD. Furthermore, the results of CCK-8 and flow cytometry showed that FTCD overexpression could significantly inhibit cell proliferation and induce cell apoptosis. These results show that the low methylation levels in the promoter region of FTCD directly lead to FTCD overexpression, which can remarkably inhibit tumor growth.

Accordingly, overexpressed FTCD could be a potential therapy method for HCC treatment, but its specific anti-tumor mechanisms are not clear. Previous studies have shown that FTCD and the PI3K/Akt signaling pathway are closely related to the Golgi apparatus function [8,14], so we speculated that the antitumor mechanisms of FTCD occur via suppressing the PI3K/Akt signaling pathway. PI3K, an intracellular phosphatidylinositol kinase, is involved in the development of many malignant tumors, including nasopharyngeal cancer [26], breast cancer [27], and liver cancer [28,29]. There is increasing evidence that PI3K downregulates the expression of cell apoptosis regulatory proteins via the phosphorylation of Akt, which functions as a vital regulator of cell proliferation [30,31]. In addition, Jin et al. indicated that the PI3K/Akt/GSK3 pathway could inhibit cell apoptosis via enhancing Chk1-dependent G2/M checkpoint activation by etoposide [32]. Consequently, the suppression of PI3K/Akt signaling pathway can arrest more cells in the G2/M phase related to DNA damage [13], then lead to cell apoptosis. In this study, Western blot analysis was applied to investigate the expression level of PI3K/Akt signaling pathway-related proteins. Interestingly, we identified that both the expression level of PI3K and phosphorylated Akt protein were downregulated in HCC cells overexpressing FTCD. Moreover, the results of flow cytometric analysis showed that FTCD overexpression could induce more apoptotic cells and more γ-H2AX-positive cells, and both of these were associated with DNA damage. In fact, DNA damage is one of the most common factors leading to cell growth disorders [33,34]. Overexpression of FTCD in HCC cell lines can further suppress the PI3K/Akt pathway, which can contribute to DNA damage and cell apoptosis.

Conclusions

Our study clarifies the role of FTCD in the procession of HCC and its possible mechanisms, showing that DNA methylation can cause FTCD downregulation, then suppress the PI3K/Akt signaling pathway, followed by inhibiting tumor growth. Therefore, FTCD could be a promising biological marker and potential target to treat HCC.

Conflict of interests

None.

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