CCT4 suppression inhibits tumor growth in hepatocellular carcinoma by interacting with Cdc20

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Abstract

Background: The chaperonin containing t-complex (CCT) proteins play an important role in cell cycle-related protein degradation in yeast and mammals. The role of the chaperonin containing t-complex 4 (CCT4), one subtype of CCT proteins, in the progress of hepatocellular carcinoma (HCC) was not fully elucidated. Here, we aimed to explore the mechanisms of CCT4 in HCC.

Methods: In this study, we used the UALCAN platform to analyze the relationship between CCT4 and HCC, and the association of CCT4 with the overall survival (OS) of HCC patients was also analyzed. CCT4 expression in HCC tumor tissues and normal tissues was also determined by western blot (WB) assay. Lentivirus vector was used to knock down the CCT4 expression, and quantitative polymerase chain reaction and WB were used to determine the level of CCT4 in HCC cell lines. Cell counting kit-8 (CCK-8) and 5-ethynyl-2′-deoxyuridine (EdU) assays were used to detect the cell proliferation, and flow cytometry (FCM) was performed to evaluate the effect of CCT4 on the apoptosis of HCC cells. Co-immunoprecipitation (co-IP) assay and WB were used to explore the mechanisms of CCT4 regulating the growth of HCC. Data were calculated from at least three replicate experiments and expressed as mean ± standard deviation. Student's t test, paired t test, and Kaplan–Meier analysis were used to compare across different groups.

Results: We found CCT4 was upregulated in HCC tissues compared with normal tissues, and its high expression was associated with poor prognosis ($P < 0.001$). CCT4 was significantly increased in HCC tumor tissues compared with normal tissues (0.98 ± 0.12 $vs.$ 0.23 ± 0.05, $t = 7.73$, $P < 0.001$). After being transfected with CCT4 short-hairpin RNA (shRNA), CCT4 was decreased in mRNA level and protein level in both Huh7 (mRNA level: 0.41 ± 0.07 $vs.$ 1.01 ± 0.11, $t = 8.09$, $P = 0.001$; protein level: 0.61 ± 0.03 $vs.$ 0.93 ± 0.07, $t = 7.19$, $P = 0.002$) and Hep3b cells (mRNA level: 0.55 ± 0.11 $vs.$ 1.04 ± 0.15, $t = 4.51$, $P = 0.011$; protein level: 0.64 ± 0.10 $vs.$ 0.95 ± 0.08, $t = 4.32$, $P = 0.012$). CCK8 assay indicated that CCT4 knockdown inhibited cell proliferation in both Huh7 (OD value of 3 days: 0.60 ± 0.14 $vs.$ 0.97 ± 0.16, $t = 3.13$, $P = 0.036$; OD value of 4 days: 1.03 ± 0.07 $vs.$ 1.50 ± 0.12, $t = 5.97$, $P = 0.004$) and Hep3b (OD value of 3 days: 0.69 ± 0.14 $vs.$ 1.10 ± 0.11, $t = 3.91$, $P = 0.017$; OD value of 4 days: 1.12 ± 0.12 $vs.$ 1.48 ± 0.13, $t = 3.55$, $P = 0.024$) cells. EdU assay showed that CCT4 knockdown inhibited the cell proliferation in both Huh7 (EdU positive rate: $[31.25 \pm 3.41]\%$ $vs.$ $[58.72 \pm 3.78]\%$, $t = 9.34$, $P = 0.001$) and Hep3b cells (EdU positive rate: $[44.13 \pm 7.02]\%$ $vs.$ $[61.79 \pm 3.96]\%$, $t = 3.79$, $P = 0.019$). FCM assay suggested that CCT4 knockdown induced apoptosis in HCC cells (apoptosis rate of Huh7: $[9.10 \pm 0.80]\%$ $vs.$ $[3.66 \pm 0.64]\%$, $t = -9.18$, $P = 0.001$; apoptosis rate of Hep3b: $[6.69 \pm 0.72]\%$ $vs.$ $[4.20 \pm 0.86]\%$, $t = -3.84$, $P = 0.018$). We also found that CCT4 could regulate anaphase-promoting complex (APC)^{Cdc20} activity via interacting with Cdc20. Furthermore, CCT4 knockdown induced securin (0.65 ± 0.06 $vs.$ 0.44 ± 0.05, $t = -4.69$, $P = 0.009$) and B-cell lymphoma-2 (Bcl-2) interacting mediator of cell death (Bim; 0.96 ± 0.06 $vs.$ 0.61 ± 0.09, $t = -5.65$, $P = 0.005$) accumulation. The upregulation of securin inhibited cell growth by downregulating cyclin D1 (0.65 ± 0.05 $vs.$ 1.04 ± 0.07, $t = 8.12$, $P = 0.001$), and the accumulation of Bim inhibited Bcl-2 (0.77 ± 0.04 $vs.$ 0.87 ± 0.04, $t = 3.00$, $P = 0.040$) and activated caspase 9 (caspase 9: 0.77 ± 0.04 $vs.$ 0.84 ± 0.05, $t = 1.81$, $P = 0.145$; cleaved caspase 9: 0.64 ± 0.06 $vs.$ 0.16 ± 0.07, $t = 1.81$, $P = 0.001$), which led to elevated apoptosis.

Conclusions: Overall, these results showed that CCT4 played an important role in HCC pathogenesis through, at least partly, interacting with Cdc20.

Keywords: Hepatocellular carcinoma; Chaperonin containing t-complex 4 (CCT4); Cdc20; Securin; Bim

Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality and morbidity worldwide. It is the fourth most common cause of cancer-related death.[1] Moreover, HCC has become one of the primary causes of cancer-related deaths in the United States (US); and according to the estimation from the American Cancer Society, it may become the third leading cause of cancer-

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related mortality by 2030.[2,3] Liver cancer is a highly heterogeneous disease: it varies by race, region, genders, and ages, exhibiting highly specific epidemiological characteristics. Pinheiro et al[4] showed that HCC was the first leading cause of cancer-related death among Mexican American males, the second among Puerto Rican males, and the fifth for white males in the US.

The T-complex protein (TCP) 1-ring complex (TRiC), also known as cytosolic chaperonin containing t-complex (CCT) polypeptide 1 or CCT, is a type of adenosine triphosphate (ATP)-dependent molecular chaperonins responsible for the efficient folding of nascent polypeptides.[5] The CCT protein family consists of eight paralogous subunits (TCP1, CCT2, CCT3, CCT4, CCT5, CCT6A, or CCT6B, CCT7, and CCT8) and plays an essential role in maintaining cellular homeostasis.[6] The dysfunction of CCT protein is associated with cancer and neurodegenerative diseases, such as Alzheimer disease and Parkinson disease.[7] Zhang et al[8] reported that CCT3 depletion could suppress cell proliferation and induce apoptosis in HCC cell lines, which was mediated by its essential role in proper kinetochore-microtubule attachment during mitosis; moreover, CCT3 knockdown could increase the sensitivity of HCC cells to chemotherapy drugs. Guest et al[9] showed that TCP1 was necessary for the growth and survival of breast cancer cells and was associated with the overall survival (OS) of breast cancer patients. Gao et al[10] used proteomics to identify the biomarkers of non-small cell lung cancer (NSCLC) and found that CCT5 was overexpressed in both the serum and tumor tissues of NSCLC patients which supported the conclusion that CCT5 might be a potential tumor marker for an early stage of NSCLC. Another study identified CCT8 as an oncogene of HCC and found that CCT8 could promote the cell cycle progression of HCC cells.[11] CCT4 was also found overexpressed in HCC tissues compared with normal tissues,[12] yet the mechanisms of CCT4 regulating tumor growth are still unclear. In this study, we analyzed the data from The Cancer Genome Atlas (TCGA) and studied the role of CCT4 in HCC.

Methods

Extraction and analysis of datasets

Survival analysis was performed using the available CCT4 expression data and clinical information. TCGA database (https://www.cancer.gov/tcga) was used to extract data, which were analyzed on the UALCAN platform (http://ualcan.path.uab.edu).[13]

HCC tissues

The tumor tissues and adjacent normal tissues of 15 HCC patients were obtained from the First People's Hospital of Anqing. The patients received surgery from January 2020 to December 2020, including nine males and seven females, with an age of 34 to 68 years. The Medical Ethics Committee of the First People's Hospital of Anqing approved this study, and the informed consent was signed by all the patients in this study.

Cells and cell culture

Four HCC cell lines (HepG2, SMMC-7721, Huh7, and Hep3b) were purchased from the Shanghai Institutes of Biological Sciences (Shanghai, China). HCC cells were cultured in a humidified incubator at 37°C and 5% CO_2. The culture medium was Dulbecco's modified eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium).

Cell transfection

To knock down CCT4, HCC cells were infected with lentivirus containing short-hairpin RNA (shRNA) against CCT4 or control shRNA. The CCT4 shRNA vector was constructed by GeneChem Company (Shanghai, China). CCT4 knockdown efficiency was evaluated by real-time polymerase chain reaction (PCR) and western blot (WB). The shRNA sequences were CCT4 shRNA: 5′-CCGGGC-TTTCTCCAATGAGTGTAAACTCGAGTTTACACTCA-TTGGAGAAAGCTTTTTTG-3′; nontargeting control shRNA: 5′-CCGGCAACAAGATGAAGAGCACCAACT-CGAGTTGGTGCTCTTCATCTTGTTGTTTTTG-3′. The lentivirus transfection procedure followed the manufacturer's instructions.

Cell counting kit-8 (CCK-8) assay

HCC cells were seeded into 96-well plates at a density of 2000 cells/well and transfected with CCT4 shRNA and control shRNA. CCK-8 kit (Key GEN, Nanjing, China) was used to measure cell proliferation. At 1 day, 2 days, 3 days, and 4 days after transfection, the medium was removed from the plate and replaced with 10 μL CCK-8 reagent + 90 μL complete medium. After incubation for 1.5 hours, the absorbance at 490 nm wavelength was measured using a microplate reader (Tecan, Männedorf, Zurich, Switzerland). The values were calculated after background subtraction. All the CCK-8 experiments were repeated at least three times.

5-Ethynyl-2′-deoxyuridine (EdU) assay

EdU incorporation assay was used to determine cell proliferation rate. The rate of proliferation was calculated according to the manufacturer's instructions (BeyoClick[TM] EdU-555 EdU Kit, Beyotime, Nantong, China). Images were taken under a fluorescence microscope (Leica, Wetzlar, Hesse-Darmstadt, Germany) from five randomly selected areas of each group. The EdU incorporation experiments were repeated at least three times.

Flow cytometry (FCM)

The HCC cells transfected with CCT4 shRNA and control shRNA were harvested and washed twice with cold phosphate buffer saline (PBS). The cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (eBioscience Inc., San Diego, CA, USA) for 15 minutes at room temperature and then evaluated using a BD FACSCalibur Flow Cytometer (BD Biosciences,

Franklin Lakes, NJ, USA). FlowJo software (v10.0; TreeStar, Palo Alto, CA, USA) was used to analyze the data. This experiment was repeated at least three times.

Quantitative real-time PCR (RT-qPCR)

The total RNA was extracted from HCC cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. RT-qPCR was performed as previously described.[14] Total RNA was reversely transcribed into complementary DNA (cDNA) using the PrimeScript[TM] RT Reagent Kit (Takara Bio Inc., Shiga, Japan). Then, real-time polymerase chain reaction (RT-PCR) was performed using the SYBR® Premix Ex Taq[TM] II (Takara) with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). The following primers were used to detect the expression of CCT4 (forward: 5′- CCTATCAGGACCGC-GACAAG-3′ and reverse: 5′- AGCTCCACCAGCATTTT-ATCCA-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5′-TGACTTCAACAGCGACACCCA-3′ and reverse: 5′-CACCCTGTTGCTGTAGCCAAA–3′). GAPDH was selected as the reference gene and the relative gene expression was determined by the comparative Ct method. This experiment was repeated at least three times.

WB analysis

Cells transfected with CCT4 shRNA and control shRNA were harvested, and the total protein was extracted using radio-immunoprecipitation assay (RIPA) buffer supplemented with fresh protease and phosphatase inhibitors. The protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of proteins (50 μg) were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 3% bovine serum albumin (BSA) in 10 mmol/L Tris-hydrochloride (HCl) (pH 7.4, with 0.05% Tween-20) and incubated with primary antibody at 4°C

for 12 hours. After washing with Tris-HCl buffer three times, the membranes were incubated with a corresponding peroxidase-conjugated secondary antibody (Abcam, Cambridge, UK). The membranes were developed using Super-Signal West Pico Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.). The densitometry of the protein bands was quantified by ImageJ software (https://imagej.nih.gov/ij/). This experiment was repeated at least three times. The information about the antibodies is listed in Table 1.

Co-immunoprecipitation (co-IP)

Cell lysate (∼1.5 mg total protein) was harvested from Huh7 cells using ice-cold non-denaturing lysis buffer (Thermo Scientific, Rockford, IL, USA). co-IP was performed using the Thermo Scientific Pierce co-IP kit following the manufacturer's protocol as previously described.[15] Samples were analyzed by WB.

Statistical analysis

All statistical analysis was performed using IBM SPSS 20 statistics software (SPSS Inc., Chicago, IL, USA). Data were calculated from at least three replicate experiments and expressed as mean ± standard deviation. Student's t test and Kaplan-Meier analysis were used to compare across different groups. $P < 0.05$ was defined as statistically significant, and $P < 0.01$ was defined as statistically very significant.

Results

CCT4 expression level was associated with HCC progression and prognosis

To explore the association between CCT4 expression and HCC, we analyzed the datasets from TCGA on the UALCAN platform. We found that CCT4 expression was significantly higher in HCC tumor tissues compared with

Table 1: Antibodies used in WB and IP analysis.

Antibody	Catalog No.	Company	Application	Dilution
Cdc20	ab183479	Abcam	WB	1:2000
Cdc20	ab183479	Abcam	IP	1:100
CCT4	ab264353	Abcam	WB	1:1000
CCT4	ab264353	Abcam	IP	1:100
Securin	ab79546	Abcam	WB	1:5000
Cyclin D1	ab226977	Abcam	WB	1:1000
Bim	ab32158	Abcam	WB	1:1000
Bcl-2	ab182858	Abcam	WB	1:2000
Caspase9	ab185719	Abcam	WB	1:1000
Cleaved caspase 9	ab2324	Abcam	WB	1:1000
GAPDH	ab181602	Abcam	WB	1:10,000
Alpha tubulin	ab7291	Abcam	WB	1:5000
Goat anti-rabbit IgG H&L (HRP)	ab205718	Abcam	WB	1:10,000
Goat anti-mouse IgG H&L (HRP)	ab205719	Abcam	WB	1:10,000

Bcl-2: B-cell lymphoma-2; Bim: Bcl-2 interacting mediator of cell death; CCT4: Chaperonin containing t-complex 4; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; H&L: Heavy chain and light chain; HRP: Horseradish peroxidase; IgG: Immunoglobulin G; IP: Immunoprecipitation; WB: Western blot.

normal tissues [Figure 1A], and we also analyzed the association of CCT4 with the survival rate of HCC patients. According to the results from the UALCAN platform, high CCT4 expression could predict a poor prognosis [Figure 1B; $P < 0.01$].

CCT4 was significantly increased in tumor tissues compared with normal tissues

We detected the CCT4 expression in tumor tissues and adjacent normal tissues in 15 HCC patients using WB assay and found that the CCT4 expression was lower in normal tissues than in HCC tumor tissues. The representative image is shown in Figure 2A, and the statistical analysis also indicated that CCT4 was significantly higher in tumor tissues compared with adjacent normal tissues [Figure 2B] $(0.98 \pm 0.12$ vs. 0.23 ± 0.05, $t = 7.73$, $P < 0.001)$.

Downregulation of CCT4 suppressed tumor cell proliferation and increased the apoptosis of HCC cells in vitro

To investigate the role of CCT4 in HCC tumorigenesis *in vitro*, we determined the mRNA level of CCT4 by qPCR and found that CCT4 was significantly higher in Huh7 and Hep3b cells compared with HepG2 and SMMC-7721 cells [Supplementary Figure 1, http://links.lww.com/CM9/A824]. Furthermore, we used shRNA targeting CCT4 to suppress CCT4 expression in Huh7 and Hep3b cell lines.[14,16] After being transfected with CCT4 shRNA, both mRNA and protein levels of CCT4 were decreased in Huh7 and Hep3B cell lines (mRNA level of Huh7: 0.41 ± 0.07 vs. 1.01 ± 0.11, $t = 8.09$, $P = 0.001$; protein level of Huh7: 0.61 ± 0.03 vs. 0.93 ± 0.07, $t = 7.19$, $P = 0.002$; mRNA level of Hep3b: 0.55 ± 0.11 vs. 1.04 ± 0.15, $t = 4.51$, $P = 0.011$; and protein level of Hep3b: 0.64 ± 0.10 vs. 0.95 ± 0.08, $t = 4.32$, $P = 0.012$) [Figure 2C–F].

To determine the effect of CCT4 on cell proliferation, we performed CCK-8 [Figure 3A and 3B] and EdU assays [Figure 3C–F] in HCC cells after CCT4 knockdown. Both assays showed that the proliferation of HCC cells was decreased following CCT4 suppression (shRNA) [Figure 3]. CCK8 assay indicated that CCT4 knockdown inhibited cell proliferation in both Huh7 (OD value of 3 days: 0.60 ± 0.14 vs. 0.97 ± 0.16, $t = 3.13$, $P = 0.036$; OD value of 4 days: 1.03 ± 0.07 vs. 1.50 ± 0.12, $t = 5.97$, $P = 0.004$) and Hep3b (OD value of 3 days: 0.69 ± 0.14 vs. 1.10 ± 0.11, $t = 3.91$, $P = 0.017$; OD value of 4 days: 1.12 ± 0.12 vs. 1.48 ± 0.13, $t = 3.55$, $P = 0.024$). EdU positive rates in Huh7 and Hep3b were reduced after CCT4 knockdown (Huh7: $[31.25 \pm 3.41]\%$ vs. $[58.72 \pm 3.78]\%$, $t = 9.34$, $P = 0.001$, Hep3b: $[44.13 \pm 7.02]\%$ vs. $[61.79 \pm 3.96]\%$, $t = 3.79$, $P = 0.019$).

To evaluate the effect of CCT4 on apoptosis, we performed FCM analysis using Annexin V-FITC/PI kit. The results showed that CCT4 suppression could increase the apoptosis rate in Huh7 cells $([9.10 \pm 0.80]\%$ vs. $[3.66 \pm 0.64]\%$, $t = -9.18$, $P = 0.001$). A similar effect was also observed in Hep3b cells $([6.69 \pm 0.72]\%$ vs. $[4.20 \pm 0.86]\%$, $t = -3.84$, $P = 0.018$) [Figure 4].

Mechanism of CCT4 regulating the tumor cells

We explored the mechanism of CCT4 by analyzing the CCT4 interaction partners in the human gene database (https://www.genecards.org). We found that CCT4 interacted with a key cell cycle factor, Cdc20, which plays an important role in cell proliferation and apoptosis. Therefore, we performed co-IP in Huh7 cells and validated the interaction between CCT4 and Cdc20 [Figure 5A]. Cdc20 is involved in regulating the anaphase-promoting complex/cyclosome (APC/C) activity and is inhibited by mitotic checkpoint complex (MCC).[17] It is possible that CCT4 could promote the MCC dissociation from APC/C and affect APC/C^{Cdc20} activity.[17] Therefore, we determined the levels of two substrates of APC/C, securin and Bim, as well as their downstream factors. The results showed that securin was upregulated after CCT4 knockdown $(0.65 \pm 0.06$ vs. 0.44 ± 0.05, $t = -4.69$, $P = 0.009$) [Figure 5B and 5C], and cyclin D1 $(0.65 \pm 0.05$ vs. 1.04 ± 0.07, $t = 8.12$, $P = 0.001$), which is negatively regulated by securin,[18] was significantly decreased after CCT4 knockdown [Figure 5B and 5C]. The pro-apoptosis factor Bim $(0.96 \pm 0.06$ vs. 0.61 ± 0.09, $t = -5.65$, $P = 0.005$) was upregulated after CCT4 knockdown [Figure 5D and 5E], and we also detected the level of
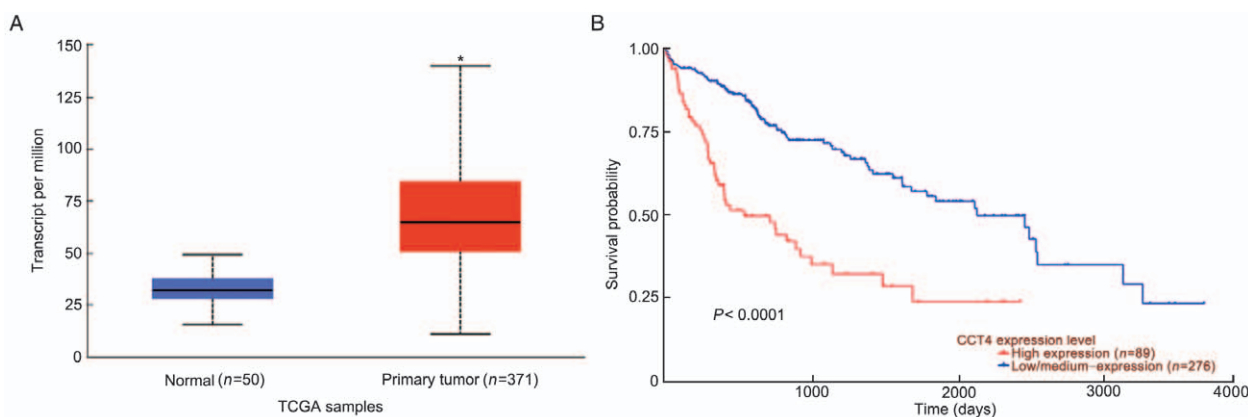

Figure 1: Expression of CCT4 in normal and LIHC tissues and survival analysis of LIHC patients based on the UALCAN platform. (A) CCT4 expression was significantly higher in tumor tissues compared with normal tissues. *Compared with the normal tissues, $P < 0.01$. (B) Association of CCT4 expression with LIHC patients' survival rate. CCT4: Chaperonin containing *t*-complex 4; LIHC: Liver hepatocellular carcinoma; TCGA: The Cancer Genome Atlas.
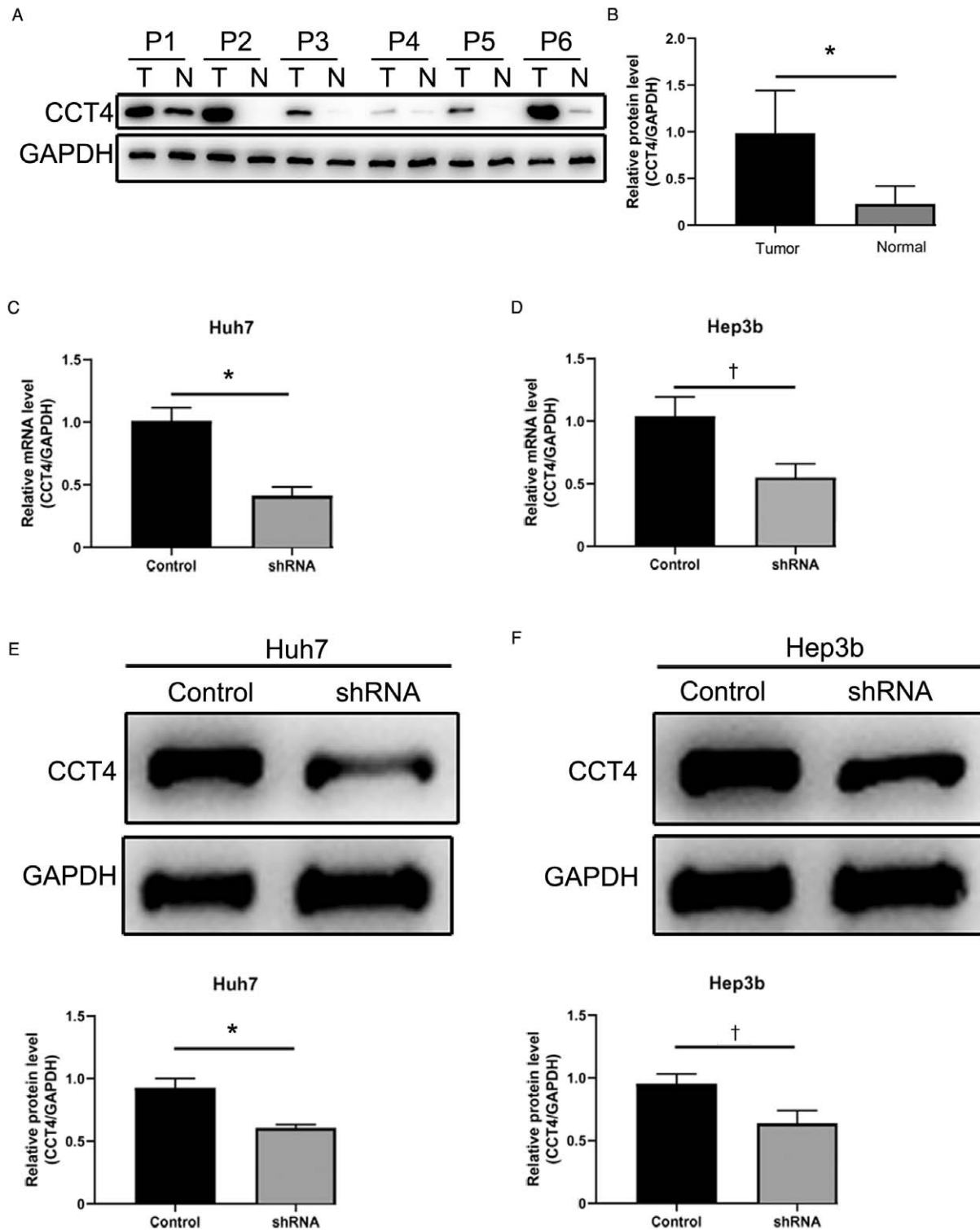
Figure 2: Determination of CCT4 in HCC tissues and efficiency of CCT4 knockdown in HCC cell lines. (A) Representative image of CCT4 expression in HCC tissues (T) and adjacent normal tissues (N). (B) Protein level of CCT4 was significantly increased in tumor tissues compared with adjacent normal tissues ($n = 15$). (C, D) mRNA level of CCT4 was significantly decreased in shRNA group compared with control in Huh7 cells (C) and Hep3b cells (D). (E, F) Protein level of CCT4 was significantly decreased in shRNA group compared with control in Huh7 cells (E) and Hep3b cells (F). $^{*}P < 0.01$, $^{†}P < 0.05$. CCT4: Chaperonin containing t-complex 4; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HCC: Hepatocellular carcinoma; mRNA: Messenger RNA; shRNA: Short-hairpin RNA.

Bcl-2 $(0.77 \pm 0.04$ vs. 0.87 ± 0.04, $t = 3.00$, $P = 0.040)$, caspase 9 $(0.77 \pm 0.04$ vs. 0.84 ± 0.05, $t = 1.81$, $P = 0.145)$, and cleaved caspase 9 $(0.64 \pm 0.06$ vs. 0.16 ± 0.07, $t = 1.81$, $P = 0.001)$ and found that the Bcl-2 was significantly

downregulated and caspase 9 was activated after CCT4 depletion [Figure 5D and 5E]. These results suggested that CCT4 interacted with Cdc20 to promote the MCC dissociation, which promoted the APC^{Cdc20} activity. Thus,
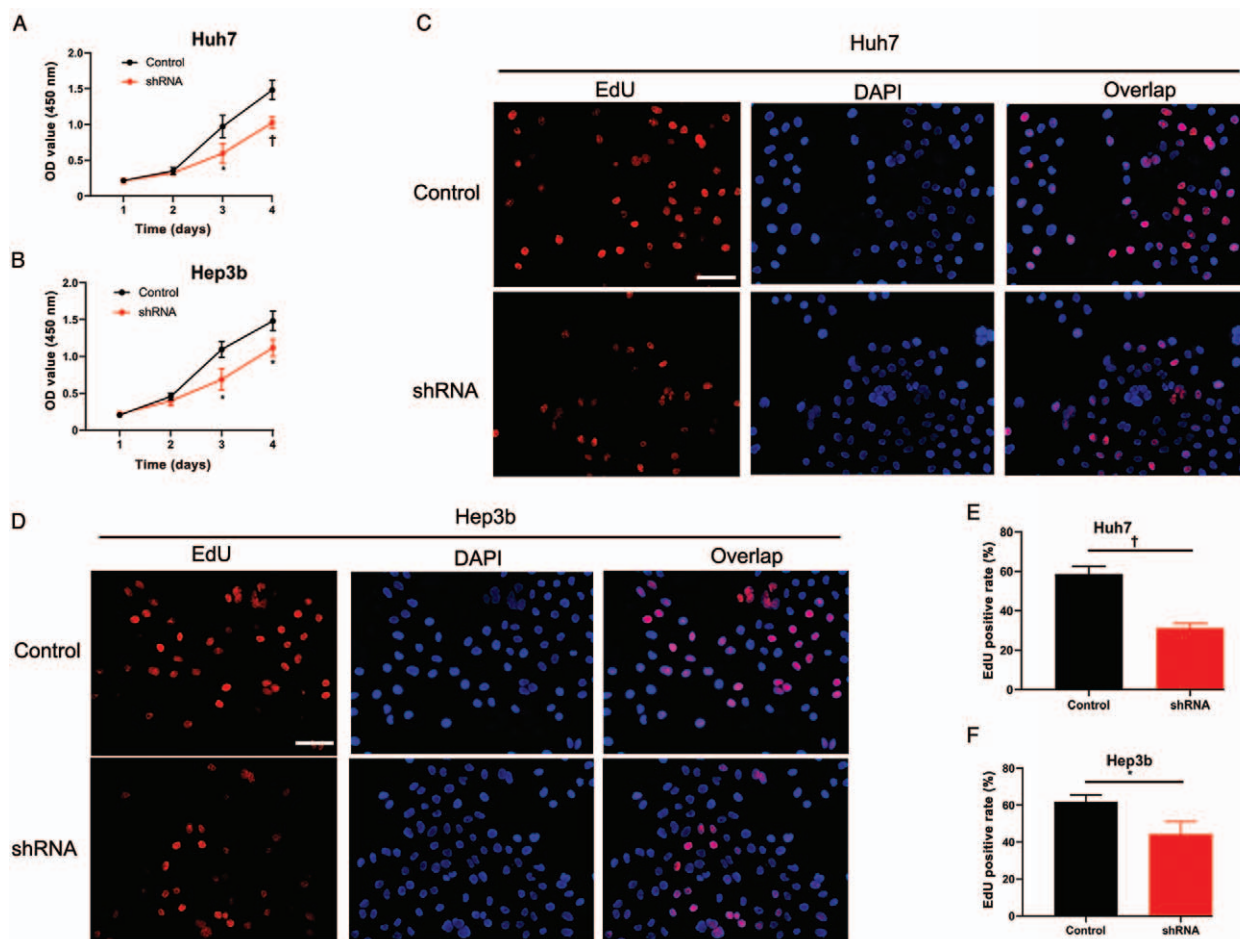
Figure 3: CCT4 depletion could restrain the growth of HCC cells. (A, B) CCK8 assay indicated that CCT4 knockdown could inhibit the proliferation of Huh7 cells (A) and Hep3b cells (B). (C, D) EdU assay revealed that CCT4 depletion could inhibit the proliferation in Huh7 cells (C) and Hep3b cells (D). (E, F) Statistical analysis indicated that CCT4 knockdown could inhibit the EdU positive rate in Huh7 cells (E) and Hep3b cells (F). $^*P < 0.05$, $^†P < 0.01$. Scale bar, 100 μm. CCK8: Cell counting kit-8; CCT4: Chaperonin containing *t*-complex 4; DAPI: 4',6-Diamidino-2-phenylindole, dihydrochloride; EdU: 5-Ethynyl-2′-deoxyuridine; HCC: Hepatocellular carcinoma; OD: Optical density.

CCT4 knockdown inhibited cell growth and promoted apoptosis through interacting with Cdc20 [Figure 5F].

Discussion

Evading growth suppressors and resisting cell death are two important hallmarks of cancer.[19] Cell cycle control plays an important role in cell growth. There are four ordered phases composing the cell cycle, namely, G1 (Gap 1), S (DNA synthesis), G2 (Gap 2), and M (Mitosis). To ensure the correct replication and segregation of chromosomes, cell cycle phase transitions are regulated by multiple checkpoints.[20] Cell cycle progression is orchestrated by cyclins and cyclin-dependent kinases (CDKs), of which the expression levels and activities fluctuate across different phases.[21] Another important complex that controls cell cycle progression is the APC; it degrades specific substrates at M and G1 phases to promote cell-cycle progression.[22] The substrates specificity of APC/C is controlled by distinct activator subunits, Cdh1 and Cdc20, which bind to APC/C at different cell cycle phases.[16] During the M phase, improper attachment between kinetochore and microtubule can activate the spindle assembly checkpoint (SAC), which inhibits APC/C activity by forming the MCC composed of

Mad2, BubR1, and the APC/C activator Cdc20; the MCC can stably bind to APC/C and inhibit its activity.[23] Except for the cell cycle regulation, Cdc20 is very important in the progression from cirrhosis to HCC.[24]

CCT/TRiC chaperonins can recognize substrates by identifying specific structures such as hydrophobic surfaces.[25] Proteins with high β-sheet propensity and WD-repeats are more likely to be the substrates for CCT/TRiC chaperonin. It has been shown that CCT chaperonin can bind to the WD-repeat domain of Cdc20 in yeast.[26] Moreover, CCT5 can bind to Cdc20 in insect cells, which is necessary for the complete disassembly of MCC and the inactivation of the mitotic checkpoint.[27] However, few research was focused on CCT4 and HCC, Yao *et al*[12] found that increased CCT proteins including CCT4 could predict poor prognosis and were associated with tumor progression in HCC. In this study, we verified that CCT4 could interact with Cdc20 in Huh7 cells and may play an important role in tumor growth and apoptosis.

Securin is an inhibitor of separase – a protease that is required for the separation of sister chromatids in mitosis and meiosis.[18] Securin is also an important substrate of
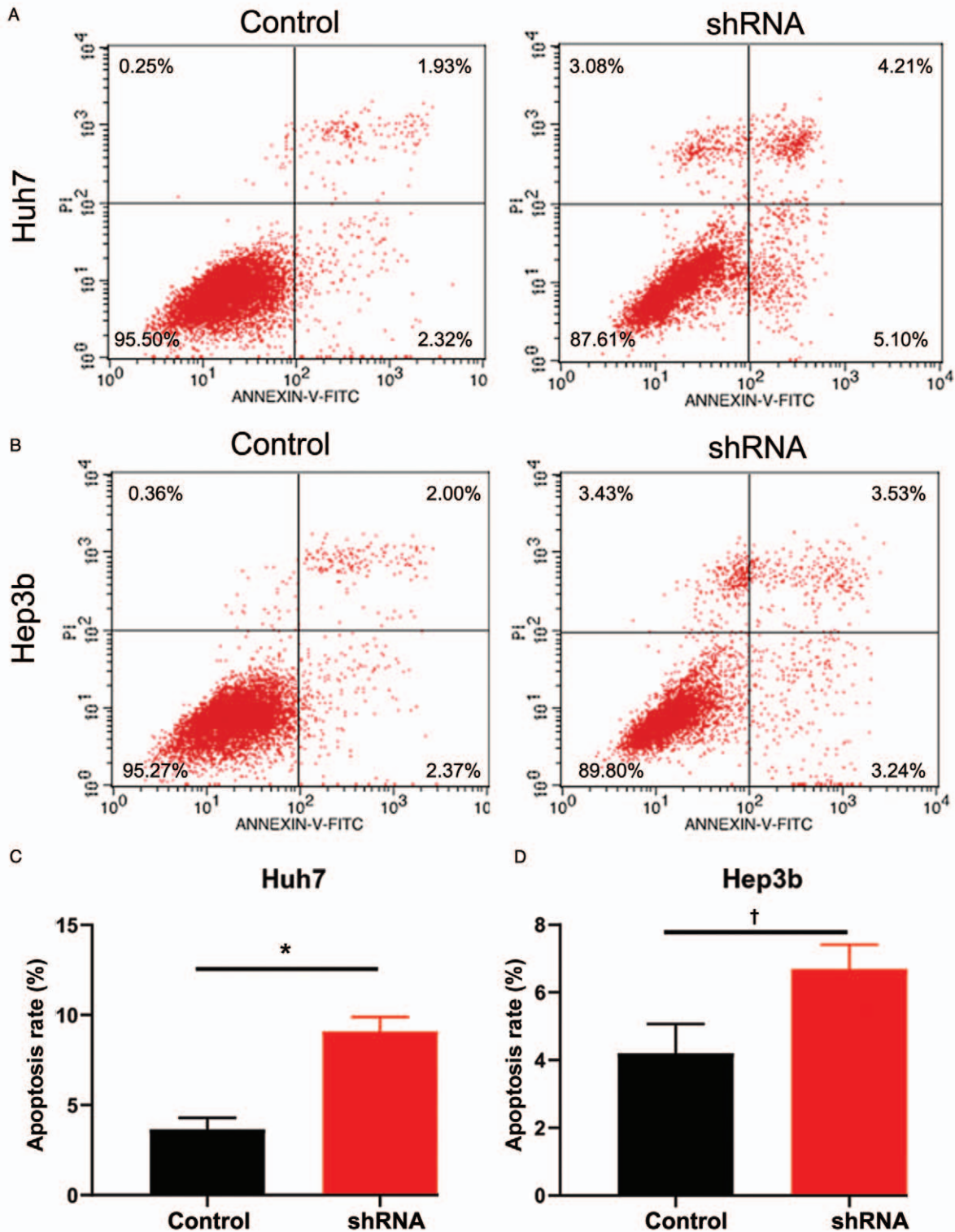
Figure 4: CCT4 deficiency could promote the apoptosis of HCC cells. (A) FCM analysis showed that CCT4 knockdown could promote apoptosis in Huh7 cells. (B) FCM analysis showed that CCT4 knockdown could promote apoptosis in Hep3b cells. Statistical analysis of FCM assay results in Huh7 cells (C) and Hep3b cells (D). $^*P < 0.01$, $^†P < 0.05$. CCT4: Chaperonin containing t-complex 4; FCM: Flow cytometry; FITC: Fluorescein isothiocyanate; HCC: Hepatocellular carcinoma; shRNA: Short-hairpin RNA.

APC^{Cdc20}, and we found that CCT4 knockdown could inhibit APC/C and induce securin accumulation. Securin depletion could promote cell proliferation through its downstream factor cyclin D1. Consistently, we found cyclin D1 was downregulated after CCT4 knockdown.[18]

The Bcl-2 family is one of the critical determinants for cell death. In mammalian cells, there are pro-survival proteins (Bcl-2, B-cell lymphoma-extra large [Bcl-xL], Bcl-w, Mcl-1 [myeloid cell leukemia-1], and A1) and pro-apoptotic groups (Bcl-2-associated X protein [Bax] group and
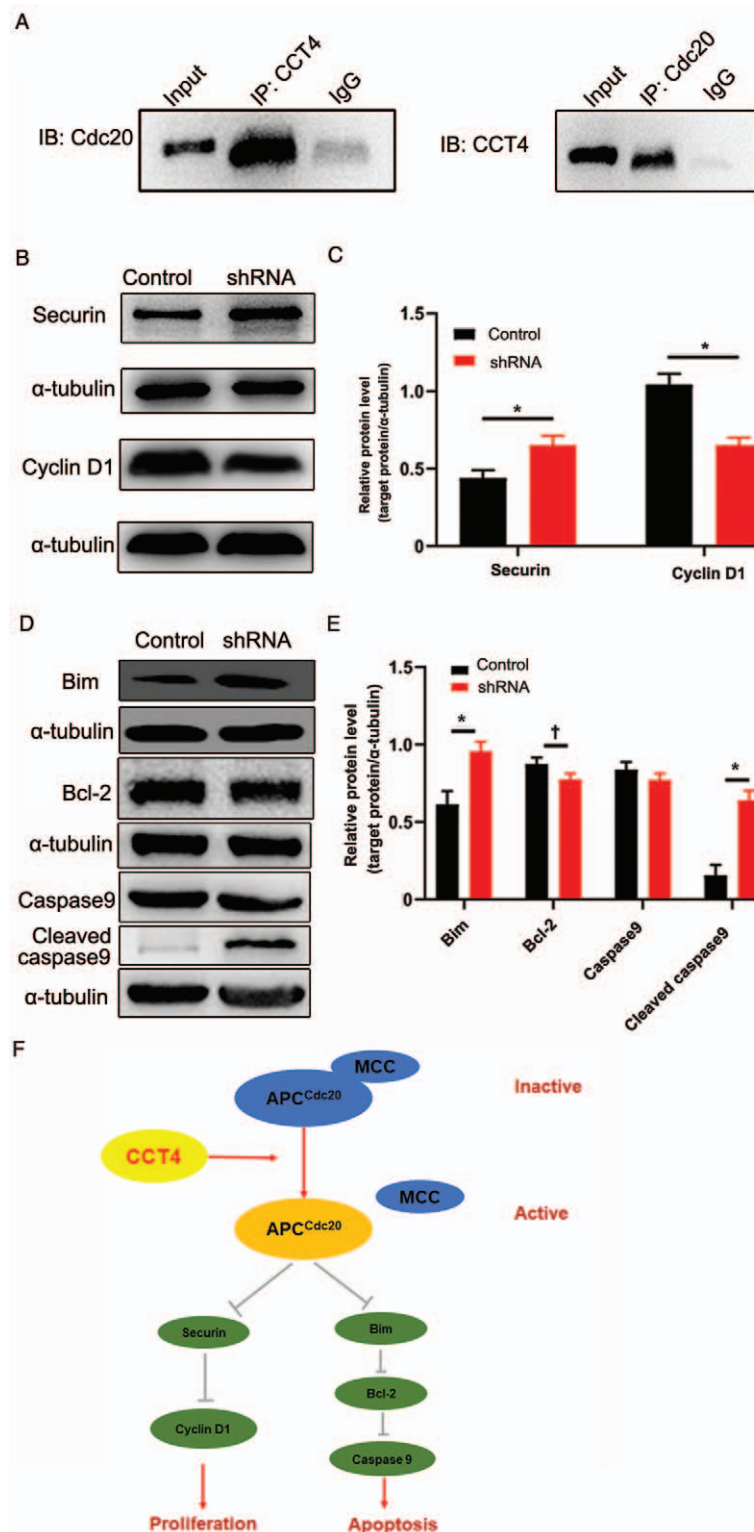
Figure 5: Mechanisms of CCT4 regulating the growth of HCC. (A) Cdc20 could be determined in IP lysate of CCT4 protein, and CCT4 could be detected in IP lysate of Cdc20 protein. (B) Securin and cyclin D1 were determined by WB in Huh7 cells containing shRNA of CCT4 and control. (C) Statistical analysis of securin and cyclin D1 expression. (D) Bim, Bcl-2, caspase 9, and cleaved caspase 9 were detected by WB in Huh7 cells containing shRNA of CCT4 and control. (E) Statistical analysis of Bim, Bcl-2, caspase 9, and cleaved caspase 9 expression. (F) Model of CCT4 influencing the cell proliferation and apoptosis. $^{*}P < 0.01$, $^{†}P < 0.05$. APC: Anaphase-promoting complex; Bcl-2: B-cell lymphoma-2; Bim: Bcl-2 interacting mediator of cell death; CCT4: Chaperonin containing t-complex 4; HCC: Hepatocellular carcinoma; IgG: Immunoglobulin G; IP: Immunoprecipitation; MCC: Mitotic checkpoint complex; shRNA: Short-hairpin RNA; WB: Western blot.

BH3-only proteins).[28] Studies have shown that BH3-only protein could promote cell apoptosis by facilitating the release of cytochrome C and activation of apoptotic protease activating factor-1 (Apaf-1) and caspase 9, whereas Bcl-2 inhibits the progress.[29] Moreover, Bim is an important target of APC^{Cdc20}, and thereby, APC can inhibit apoptosis by degrading Bim.[30] The expression level of Bim decreases more significantly during the M

phase when APC^{Cdc20} is active, and Cdc20 knockout causes Bim accumulation in tumor cells.[30] In our study, CCT4 knockdown inhibited APC^{Cdc20} activity, which led to the increased level of Bim and cell apoptosis.

Overall, in this study, we showed that CCT4 could regulate tumor cell growth in two ways. Although lack of clinical information is a limitation to our research, our data demonstrated that CCT4 could promote the progression of HCC mainly through interacting with Cdc20, which affected both cell cycle and cell apoptosis. Therefore, CCT4 could be a potential therapeutic target for treating HCC.

Funding

Conflicts of interest

None.

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