ORIGINAL ARTICLE

CDCA2 triggers in vivo and in vitro proliferation of hepatocellular carcinoma by activating the AKT/CCND1 signaling

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Summary

Purpose: This study aims to elucidate the biological functions of CDCA2 (cell division cycle associated 2) in hepatocellular carcinoma (HCC) progression and the potential mechanism.

Methods: CDCA2 levels in HCC tissues and cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between CDCA2 and clinical characteristics in HCC patients was analyzed. Cox proportional-hazards model was applied for assessing the potential factors influencing overall survival in HCC. Three CDCA2 siRNAs were generated and the most effective one was used in the following experiments. After knockdown of CDCA2 in HCC-LM3 cells, clonality and viability were examined. Meanwhile, cell cycle progression was detected by flow cytometry. Relative levels of CDCA2, p21, p27, CDK2, CCND1, CCNE1 and CCNB1 in HCC-LM3 cells were determined by *qRT-PCR*. The activation of the protein kinase B (Akt) signaling was examined by Western blot. Subsequently, we con-

structed HCC xenograft model in nude mice. Tumor volume and tumor weight of xenografted HCC were recorded.

Results: CDCA2 was upregulated in HCC tissues than that of para-tumor ones, especially HCC tissues with larger than 5 cm in tumor size or vascular invasion. CDCA2 level was related to tumor size, vascular invasion and tumor differentiation in HCC. Knockdown of CDCA2 inhibited clonality and viability in HCC-LM3 cells, and arrested cell cycle progression in G1 phase via downregulating CCND1. The phosphatidilinositol 3-kinase (PI3K)/Akt was activated by CDCA2 during the progression of HCC. Tumor volume and tumor weight of xenografted HCC decreased in nude mice with in vivo knockdown of CDCA2.

Conclusions: CDCA2 triggers proliferative potential in HCC by targeting CCND1 via activating the PI3K/Akt signaling.

Key words: CDCA2, CCND1, PI3K/Akt signaling, hepatocellular carcinoma

Introduction

malignant tumor. Its mortality and incidence rank 4th and 6th, respectively. The incidence of primary liver cancer in China is relatively high, and liver cancer cases in our country account for 55% of global cases. Hepatocellular carcinoma (HCC) is the major histological subtype of liver cancer, involving 75-85% of liver cancer cases [1,2]. The occurrence and progression of HCC involve angiogenesis, signaling transduction and gene mutations. Targeted therapy has been highlighted in fluenced [9,10].

Primary liver cancer is a globally prevalent HCC research nowadays. Well-ordered cell cycle progression maintains the normal cell functions. Disordered mitotic cyclin is likely to cause carcinogenesis [3,4]. The regulation on cell cycle proteins is a potential strategy for cancer treatment [5,6]. Cell cycle progression in tumors is usually out of control, thus leading to uncontrolled tumor growth [7,8]. Importantly, regulating cell cycle proteins affect tumor cell apoptosis and necrosis, whereas the normal cells and tissues are not in-

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CDCA2 (cell division cycle associated 2), also known as Repo-Man (recruits PP1 onto mitotic chromatin at anaphase), is a binding subunit of PP1 (protein phosphatase 1), which is involved in mitosis by assisting the binding between PP1 and chromatin [11,12]. It is reported that CDCA2 may prepare mitotic chromatin for intersegment transition and control PP1_Y-dependent DNA damage response [13,14]. Recent studies have demonstrated that CDCA2 is upregulated in many types of tumors, including oral squamous cell carcinoma and lung adenocarcinoma, and it is linked to the malignant level [15-17]. The potential role of CDCA2 in HCC remains unclear. This study aimed to explore the involvement of CDCA2 in regulating *in vivo* and in vitro proliferation of HCC, and the underlying mechanism.

Methods

Sample collection

Sixty pairs of HCC and para-tumor tissues were surgically resected in Chengmai People's Hospital from June 2017 to June 2019. Their clinical data were completely recorded. None of HCC patients had preoperative chemotherapy or radiotherapy. Samples were frozen at -80°C for RNA and protein extraction. This study got approval by Ethics Committee of Chengmai People's Hospital and informed consent was obtained from each patient.

Cell culture and transfection

HCC cell lines (SMMC-7721, SK-hep1, HCC-LM3 and HepG2) and the immortalized hepatocytes (L-02) were provided by China Center for Type Culture Collection (Shanghai, China). They were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, South Logan, UT, USA) or Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA).

CDCA2 siRNAs were constructed by GenePharma (Shanghai, China) and their sequences were: siR-NA1: 5'-CACCUGCCUUUCUAAAUAUTT-3'; siRNA2: 5'-GGGCAAAGGAUCAAGUGAUTT-3'; siRNA3: 5'-CUGC-CUUGGAAAGGAUUGATT-3'. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was evaluated at 48 h.

Cell counting kit-8 (CCK-8)

Cells were inoculated in 96-well plates with 1×10^4 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNAs from cells or tissues were isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and they were reversely transcribed using the PrimeScript RT reagent kit (gDNA Eraser TaKaRa Code: DRR047A, Tokyo, Japan). PCR was conducted by the SYBR Green I method (TaKaRa, Tokyo, Japan). Relative level was calculated by 2^{-ΔΔCt}. Primer sequences of CDCA2 were 5'-TGCCGAAT-TACCTCCTAATCCT-3' (forward) and 5'-TGCTCTACGGT-TACTGTGGAAA-3' (reverse).

Colony formation assay

Cells were inoculated in well plates with 500 cells per well and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Visible colonies were washed in phosphate buffered saline (PBS), fixed in methanol for 20 min and dyed in 0.1% crystal violet for 20 min, which were finally captured and calculated.

Western blot

Cells were lysed for extracting protein samples, and protein concentration was detected by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples were electrophoresed in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary antibody for 24 h and secondary antibody for 1 h. Band exposure and analyses of grey values were finally conducted.

Flow cytometry

Transfected cells were collected, and re-suspended in 70% ethanol for at least 10 h. They were washed and incubated with 300-500 µL of propidium iodide (PI) and RNase for 30 min. Cell ratio in each phase of cell cycle progression was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Xenograft model generation

Twelve male nude mice (3-5 weeks old, 12-16 g) were purchased from the Experimental Animal Center. They were randomly classified into two groups, and subcutaneously implanted with 2×10⁶ HCC-LM3 cells transfected with sh-CDCA2 or sh-NC, respectively. Tumor size was recorded with an interval of four days. Twenty-eight days later, mice were sacrificed for collecting xenografted tumors. Tumor volume=(width²×length)/2. This study was approved by the Animal Ethics Committee of Chengmai People's Hospital Animal Center.

Statistics

Statistical analyses were conducted by SPSS 20.0 software package (IBM, Armonk, NY, USA) and Graphpad (Version X; La Jolla, CA, USA). The relationship between CDCA2 and clinical characteristics in HCC patients was analyzed by Chi-square test. To identify potential risk factors influencing the overall survival in HCC patients, Cox proportional-hazards model was adopted. P<0.05 was considered as statistically significant.

| Clinicopathologic features | Number of cases | CDCA2 expression | | p value |
|----------------------------|-----------------|------------------|-------------|---------|
| | - | Low (n=30) | High (n=30) | |
| Age (years) | | | | 0.606 |
| ≤60 | 30 | 16 | 14 | |
| >60 | 30 | 14 | 16 | |
| Gender | | | | 0.426 |
| Male | 37 | 17 | 20 | |
| Female | 23 | 13 | 10 | |
| AFP (ng/mL) | | | | |
| ≤400 | 29 | 18 | 11 | 0.071 |
| >400 | 31 | 12 | 19 | |
| Tumor size (cm) | | | | 0.002 |
| ≤5 | 28 | 20 | 8 | |
| >5 | 32 | 10 | 22 | |
| HBV | | | | 0.791 |
| Absent | 37 | 18 | 19 | |
| Present | 23 | 12 | 11 | |
| Vascular invasion | | | | 0.015 |
| Negative | 21 | 15 | 6 | |
| Positive | 39 | 15 | 24 | |
| Tumor differentiation | | | | |
| Poor | 29 | 13 | 16 | 0.438 |
| Moderate-Well | 31 | 17 | 14 | |

Table 1. Correlation analysis between CDCA2 expression and clinicopathological parameters of HCC patients

HBV: hepatitis B virus.



Figure 1. CDCA2 was upregulated in HCC. **A:** CDCA2 was upregulated in HCC tissues than para-tumor ones. **B:** CDCA2 was upregulated in HCC tissues with large tumor size (> 5 cm) than those with small tumor size (\leq 5 cm). **C:** CDCA2 was upregulated in HCC tissues with vascular invasion than those without. **D:** CDCA2 was upregulated in HCC cell lines than the normal hepatocytes. **p<0.01, ***p<0.001.

Results

CDCA2 was upregulated in HCC

We collected 60 pairs of HCC and para-tumor tissues. QRT-PCR data showed higher level of CDCA2 in HCC tissues than para-tumor ones (Figure 1A). Furthermore, the relationship between CDCA2 and clinical characteristics of HCC patients was analyzed. Recruited HCC patients were classified into CDCA2 high level group and low level group. The analysis uncovered that CDCA2 was remarkably linked to tumor size and vascular invasion in HCC (Table 1). Consistently, higher abundance of CDCA2 was detected in HCC tissues with a large tumor size (> 5 cm) and those with vascular invasion (Figure 1B,1C). *In vitro* expression of CDCA2 was identically upregulated in HCC cell lines (Figure 1D). Among the four tested HCC cell lines, HCC-LM3 cells were used for the following experiments because they expressed the highest abundance of CDCA2. The above data indicated that upregulated CDCA2 may be related to advanced clinical characteristics of HCC.

Table 2. Univariate and multivariate analysis of clinicopathological parameters related to overall survival in HCC patients

| Variables | Univariate | | Multivariate | |
|--|----------------------|---------|----------------------|---------|
| _ | HR (95% CI) | p value | HR (95% CI) | p value |
| Age (≤60/>60) | 1.258 (0.678, 2.112) | 0.367 | 1.112 (0.701, 2.012) | 0.342 |
| Gender (Female/Male) | 0.769 (0.345, 2.001) | 0.678 | 0.698 (0.256, 2.342) | 0.603 |
| AFP (≤400/>400) | 3.889 (2.497, 6.691) | < 0.001 | 3.234 (1.423, 5.921) | < 0.001 |
| HBV (Absent/Present) | 0.897 (0.445, 1.567) | 0.701 | 0.821 (0.521, 1.672) | 0.687 |
| Tumor size (>5cm/≤5cm) | 1.886 (1.112, 3.321) | 0.034 | 1.623 (0.891, 2.121) | 0.125 |
| Vascular invasion (Positive/Negative) | 2.678 (1.345, 3.980) | < 0.001 | 2.123 (0.789, 4.012) | 0.231 |
| Tumor differentiation (Moderate-Well/Poor) | 0.385 (0.198, 0.702) | 0.003 | 0.462 (0.232, 0.887) | 0.012 |
| CDCA2 expression (High/Low) | 3.342 (1.784,7.892) | < 0.001 | 3.541 (2.034, 8.765) | < 0.001 |



Figure 2. CDCA2 stimulated HCC cells to proliferate. **A:** Transfection efficacy of siRNA1-CDCA2, siRNA2-CDCA2 and siRNA3-CDCA2 in HCC-LM3 cells; **B:** Protein levels of CDCA2 and Ki67 were downregulated in HCC-LM3 cells transfected with siRNA-CDCA2; **C:** Clonality was attenuated in HCC-LM3 cells transfected with siRNA-CDCA2 (magnification: 40×). **D:** Viability was attenuated in HCC-LM3 cells transfected with siRNA-CDCA2. *p<0.05, **p<0.01, ***p<0.001.

Independent risk factors for overall survival in HCC

To identify potential risk factors influencing the overall survival in HCC, Cox proportional-hazards model was adopted. Univariate analysis revealed that AFP, tumor size, vascular invasion, tumor differentiation level and CDCA2 were potential factors influencing HCC prognosis (HR was 3.889, 1.886, 2.678, 0.385 and 3.342, respectively; p<0.05). Subsequent multivariate analysis suggested that CDCA2 (HR=3.541, 95%CI: 2.034-8.765), AFP and tumor differentiation level were independent predictive indicators for the overall survival in HCC (Table 2).

CDCA2 stimulated HCC cells to proliferate

To elucidate the biological functions of CDCA2 in HCC, we generated three CDCA2 siRNAs and tested their efficacy. Transfection of siRNA1-CDCA2 displayed the best outcome of CDCA2 knockdown in HCC-LM3 cells, which was utilized in the fol-



Figure 3. Knockdown of CDCA2 slowed down HCC growth *in vivo*. HCC-LM3 cells transfected with sh-NC or sh-CDCA2 were subcutaneously implanted in nude mice for generating xenograft model. **A:** Tumor volume was smaller in sh-CDCA2 group than that of sh-NC group; **B:** Tumor weight was lower in sh-CDCA2 group than that of sh-NC group. *p<0.05, **p<0.01, ***p<0.001.



Figure 4. CDCA2 regulated G1/S phase transition in HCC by upregulating CCND1. **A:** HCC-LM3 cells were arrested in G1 phase by transfection of siRNA-CDCA2. **B:** Relative mRNA levels of CDCA2, CCND1, CCNE1 and CCNB1 were down-regulated, whereas those of p21, p27 and CDK2 were not changed by transfection of siRNA-CDCA2 in HCC-LM3 cells. **C:** Protein level of p-Akt was downregulated in HCC-LM3 cells transfected with siRNA-CDCA2. **D:** The rate of p-Akt/Akt decreased in HCC-LM3 cells transfected with siRNA-CDCA2. *****p<0.001.

lowing experiments (Figure 2A). In HCC-LM3 cells with CDCA2 knockdown, protein level of Ki67 was markedly downregulated (Figure 2B). Both clonality and viability in HCC-LM3 cells were attenuated by transfection of siRNA-CDCA2, suggesting the inhibited proliferative potential (Figure 2C, 2D).

Knockdown of CDCA2 slowed down HCC growth in vivo

HCC-LM3 cells transfected with sh-NC or sh-CDCA2 were subcutaneously implanted in nude mice for generating xenograft model. Tumor size was recorded with an interval of four days. Twentyeight days later, mice were sacrificed for collecting xenografted tumors. Compared with mice in sh-NC group, tumor volume and tumor weight were lower in sh-CDCA2 group (Figure 3A,3B). As a result, knockdown of CDCA2 slowed down HCC growth *in vivo*.

CDCA2 regulated G1/S phase transition in HCC by upregulating CCND1

Flow cytometry results depicted that HCC-LM3 cells were markedly arrested in G1 phase after knockdown of CDCA2 (Figure 4A). We thereafter examined mRNA levels of key checkpoints in G1/S phase. Knockdown of CDCA2 downregulated CCND1, CCNE1 and CCNB1, whereas p21, p27 and CDK2 levels were not influenced (Figure 4B). In particular, CCND1 was the most downregulated gene responded to CDCA2 knockdown. It is indicated that CDCA2 changed G1/S phase in HCC by upregulating CCND1. Previous studies have highlighted the involvement of the PI3K/Akt signaling in tumor cell proliferation [18,19]. Here, protein level of p-Akt was markedly downregulated by CDCA2 knockdown in HCC-LM3, as well as the rate of p-Akt/Akt (Figure 4C, 4D). Therefore, the PI3K/ Akt signaling participated in CDCA2-induced HCC progression.

Discussion

HCC is an urgent public health issue. Globally, the mortality of HCC is in the third place in malignancies [20]. Owing to the insidious onset and atypical symptoms of early stage HCC, the detection rate of advanced HCC is relatively high. These patients are insensitive to chemotherapy and radiotherapy. As a result, novel therapeutic strategies for HCC are urgently required. At present, gene therapy has emerged. The highly orderly cell cycle progression is maintained by various proteins and pathways. Uncontrolled proliferation and apoptosis caused by the disordered cell cycle are responsible for tumorigenesis and tumor development [21]. Our findings uncovered that CDCA2 was upregulated in HCC tissues and cell lines. Its level was positively linked to tumor size and vascular invasion in HCC, indicating the vital function of CDCA2 during HCC progression. Univariate and multivariate analyses yielded the conclusion that CDCA2, AFP and tumor differentiation level were independent risk factors for overall survival in HCC. Through a series of *in* vitro experiments, it was found that knockdown of CDCA2 markedly weakened clonality and viability in HCC cells. Notably, CDCA2 was of significance in cell cycle progression from G1 to S phase. Moreover, we generated xenograft model in nude mice by subcutaneous administration with HCC-LM3 cells. In vivo knockdown of CDCA2 in xenografted mice slowed down HCC growth.

Abnormally expressed cell cycle proteins induce tumor growth. Chen et al [22] discovered that CDCA5 drives HCC progression by stimulating proliferative rate and inhibiting apoptosis via the Akt signaling. Uchida et al [23] demonstrated that overexpression of CDCA3 triggers oral cancer aggravation through preventing G1 phase blockage. Feng et al [24] indicated that colorectal cancer proliferation is stimulated by CDCA2-induced activation of the PI3K/Akt signaling. Consistently, our findings revealed that CDCA2 stimulated HCC cells to proliferate. Many cancer-associated regulators are involved in G1/S phase transition [25,26]. Here, CCND1 was markedly downregulated by transfection of siRNA-CDCA2. CCND1 / CDK4 complex serves as a positive regulator of G1/S phase transition, thereby participating in the regulation of tumor cell behaviors [27,28]. The PI3K/Akt signaling is extensively involved in tumor progression by interacting with CCND1 [29-31]. Our results showed that knockdown of CDCA2 downregulated p-Akt in HCC cells, suggesting the activated PI3K/Akt signaling. To sum up, CDCA2 activated the PI3K/ Akt signaling and triggered HCC proliferation by targeting CCND1.

Conclusions

CDCA2 triggers proliferative potential in HCC by targeting CCND1 via the activation of the PI3K/ Akt signaling. CDCA2 may be utilized as a therapeutic target for HCC.

Conflict of interests

The authors declare no conflict of interests.

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